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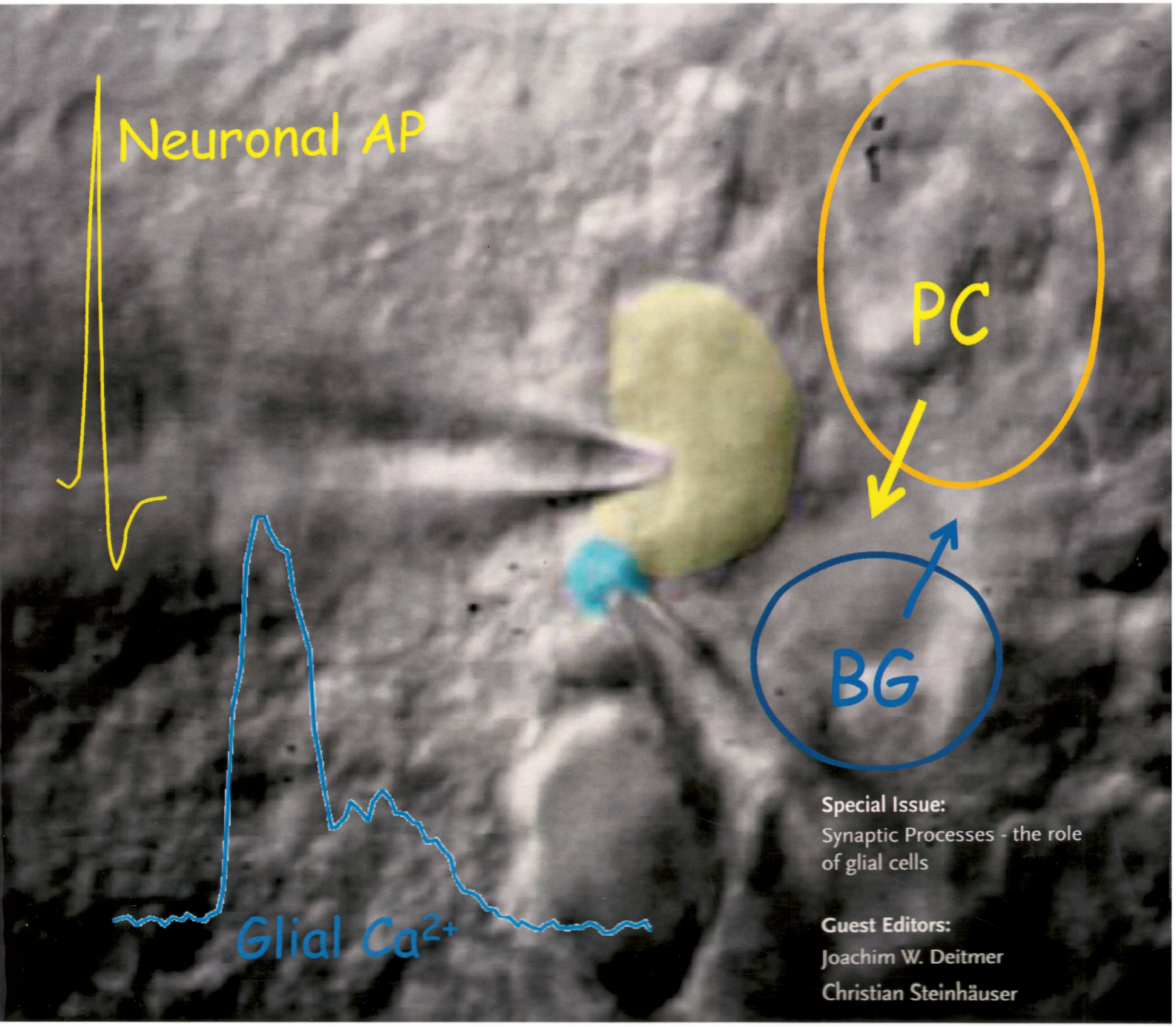

Special Issue:
Synaptic Processes - the role of glial cells

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Preface

Synaptic processes—The role of glial cells

The understanding of our brain has made enormous progress in the last few decades; therefore new concepts are required to take the vast amount of data currently being generated in the neurosciences into account. These new concepts should integrate the contribution of glial cells to information processing in central and peripheral nervous systems. It has been recognized for mammalian brains that glial cells play major roles in various physiological and pathological processes, including neurogenesis, neurodevelopment, synaptogenesis and synaptic transmission, cerebral blood flow, myelination, epilepsy, neuroinflammation, and neurodegenerative diseases, only to name few.

Synaptic processes in the brain, such as the formation and maintenance of synapses, or fast communication modes between neurons, have been known for decades to involve presynaptic terminals, from which neurotransmitters are released, and postsynaptic compartments, onto which neurotransmitters act via transmembrane receptors. In the past 10–15 years, evidence has rapidly accumulated suggesting that not only neuronal elements are involved in these synaptic processes, but also glial cells ("tripartite synapse"). This new view considers glial cells, in particular astrocytes in the vertebrate nervous system, and also glial cells in invertebrate nervous systems, as direct communication partners of neurons at synapses. Astroglial processes are often localized very close to pre- and postsynaptic neuronal elements, and are hence called "perisynaptic glia." This enables these perisynaptic glia processes to sense neurotransmitters, through the activation of various glial receptors and transporters, and to respond to neuronal activity by releasing gliotransmitters, which in turn influence synaptic signaling and synchronize neuronal activity. Moreover, astrocytic endfeet wrap the vasculature and release substances modulating the tone of blood capillaries, thereby contributing to the regulation of cerebral blood flow.

The present collection of reviews highlights essential roles of glial cells, in particular astrocytes, for the formation, function, and the plasticity of synapses. Ambient topics, such as glial precursor cells, astrocyte variety and metabolic demands, are also covered in some articles. The idea for this issue was born in a priority program funded by the Deutsche Forschungsgemeinschaft (German Research Council), in which about 20 groups have contributed over the past 6 years to this topic in a concerted program. This issue, written by selected international experts, should not only give state-of-the art overviews and discussions on the topic, but also help inseminate other initiatives and programs to boost research in this exciting and proliferating field of the neurosciences.

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BRAIN RESEARCH REVIEWS

Review

Heterogeneity in astrocyte morphology and physiology

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ABSTRACT

Astrocytes as a cell population are not well defined and comprise a heterogeneous population of cells. There are at least 9 different morphological variants which can coexist within one given brain region. Human astrocytes have a considerably more complex morphology as their rodent counterparts. There are also a number of functional differences depending on brain region and developmental stage in the normal (not pathologic) brain. Astrocytes can differ in functional gap junctional coupling, expression of transmitter receptors, membrane currents, and glutamate transporters. We feel that astrocyte heterogeneity has not yet been thoroughly explored and what we report here will just be a beginning of a new field of research.

Contents

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1. Introduction

Lenhossek (1895) coined the term astrocytes in 1895 in the second edition of his textbook on the nervous system and thought this as a replacement for the term glial cells in higher vertebrates. His intention was to get away from the term glia=glue which implies a passive function for these cells. With a new name, he intended to indicate that these cells are equivalent to the nerve cells. The name was based on morphology, in particular, on the presence of multiple processes which reminded him on a cartoon of a star.

Today, it is difficult to define what we include into the cellular class of astrocytes. Oligodendrocytes are characterized by their expression of myelin proteins, microglia by their macrophage-related markers, and neurons by their electrical properties and ability to make synapses, and thus, these cells can be well defined on a molecular level. For astrocytes, there is no unique marker which would label all astrocytes and not other cell types in the brain. As we will elaborate in this review, astrocytes are a very heterogeneous population of cells with distinct morphologies and properties. We will, however, only review astrocytes under non-pathological conditions. The reactive astrocyte will, in addition, represent a distinct, potentially also heterogeneous population of cells.

2. Morphological heterogeneity in different brain regions

In the 19th century, it was already recognized that astrocytes are a morphologically heterogeneous population (Fig. 1). They were already divided into two classes termed protoplasmic and fibrous astrocytes. Protoplasmic astrocytes are found in gray matter and are characterized by highly complex processes which occupy a large volume. In the light microscope, the fine branches of this process network cannot be resolved. In contrast, fibrous astrocytes, found in white matter, have (in the light microscope) clearly distinguishable processes with little to moderate branching (for review, see Reichenbach and Wolburg, 2005). These two cell types belong to distinct lineages since the transcription factor Olig2, which plays a crucial role in the formation of motoneurons and oligodendrocytes, is also important for the formation of white matter astrocytes but not cortical grey matter astrocytes (Cai et al., 2007). A number of morphologically distinct forms of astrocytes have been recognized early and were termed with a special name, such as Müller cells or Bergmann glia (for review, see Reichenbach and Wolburg, 2005).

Emsley and Macklis (2006) divided astrocytes into 9 classes based on three complementary astrocyte labeling methods—GFAP–GFP expressing mice and GFAP and S100β immunostaining. They termed these cell populations tanycytes, 'radial' cells, Bergmann glia, protoplasmic astrocytes, fibrous astrocytes velate glia, marginal glia, perivascular glia, and ependymal glia. They emphasized the point that within a given brain region, several types of astrocyte populations can coexist. They also noted that the density of astrocytes can vary by a factor of 1000, being very low in the core of the nucleus accumbens or very high in the in the subventricular zone.

The morphology of astrocytes is determined by the cytoarchitecture of a given brain region. In white matter, processes of the fibrous astrocytes are oriented along the fiber tracts. In the molecular layer of the dentate gyrus, protoplasmic astrocyte distribution is influenced by the boundary separating the associational/commissural and perforant path afferents. Astrocytes in this region are polarized, sending longer processes toward hippocampal fissure (Bushong et al., 2003). Another prominent example

Fig. 1 – Morphological diversity of glial cells. Golgi-stained glial cells from human cortex (Retzius, 1894). Vertical slice of one of the gyros of the frontal lobe obtained from a 42-year-old woman.

is the Bergmann glial cell which has its soma in the Purkinje cell layer and sends numerous processes through the molecular layer of the cerebellum terminating with endfeet at the pial surface.

3. Morphological differences between human and rodent astrocytes

Primate astrocytes are more complex and diverse as compared to rodent astrocytes (for review see Oberheim et al., 2006; Fig. 2). Protoplasmic astrocytes in human neocortex are 2.6-fold larger in diameter and extend 10-fold more primary processes than their rodent counterparts (Oberheim et al., 2009). The primate cerebral cortex possesses a distinct morphological subtype of astrocytes, the interlaminal astrocytes (Colombo et al., 1995; Colombo and Reisin, 2004). The cell bodies as well as a number of relatively short processes are located in layer 1 of the cortex, and these cells have one or two very long (up to 1 mm) processes toward deeper layers of cerebral cortex up to layers 2–4, where they terminate at the neuropile forming a small bulb or occasionally on vasculature. A remarkable feature of these long processes is their tortuosity —they often can be seen having spiriform or corkscrew appearance. Their shorter processes contribute to the pial glia limitans (Oberheim et al., 2009). In a recent study, Oberheim et al. (2009) described a novel, human-specific subtype of the astroglia designated as varicose projection astrocyte. These GFAP-positive cells reside in layers 5–6 of the cortex, their processes are straighter than in protoplasmic astrocytes and, most remarkably, they extend 1 to 5 long processes up to 1 mm in length, which can terminate in the neuropile or in the blood vessels. The processes of these cells possess varicosities spaced approximately 10 μm apart. Similar, but smaller and less complex cells were also seen in chimpanzee cortex but not in other primates or rodents. The protoplasmic astrocytes in the human brain are much more complex and larger than their rodent or primate counterparts. They have 10 times more (GFAP-positive) processes and show increased GFAP expression in their endfeet. This implies that they contact much more synapses an estimated 20,000–120,000 for rodents and an estimated 270,000–2 million synapses for a human astrocyte. Also, fibrous astrocytes found in white matter are significantly larger when comparing human and rodents (Oberheim et al., 2009).

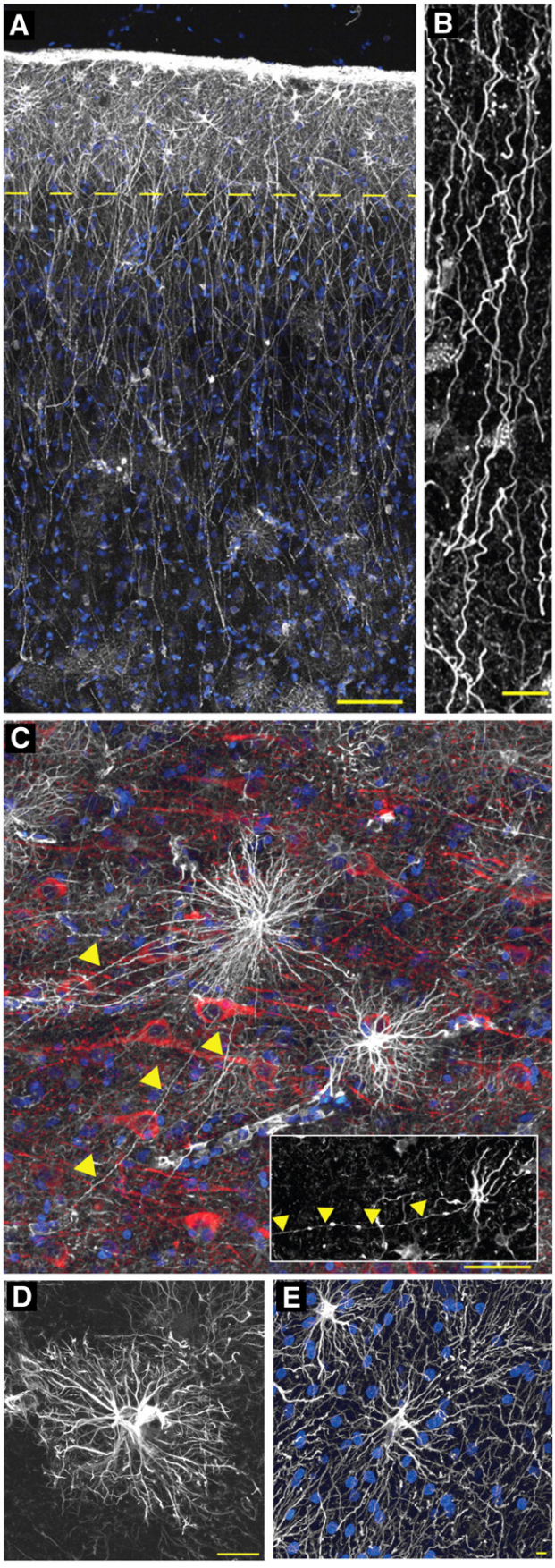

Fig. 2 – Morphological heterogeneity of primate cortical astrocytes. (A) Pial surface and layers 1–2 of human cortex. GFAP, white; DAPI, blue. Scale bar, 100 μm. Yellow line indicates border between layer 1 and 2. (B) Interlaminar astrocyte processes characterized by their tortuosity. Scale bar, 10 μm. (C) Varicose projection astrocytes reside in layers 5–6 and extend long processes characterized by evenly spaced varicosities. Inset: Varicose projection astrocyte from chimpanzee cortex. GFAP, white; MAP2, red; and DAPI, blue. Yellow arrowheads indicate varicose projections. Scale bar, 50 μm. (D) Typical human protoplasmic astrocyte. Scale bar, 20 μm. (E) Human fibrous astrocytes in white matter. Scale bar, 10 μm. (Modified from Oberheim et al., 2009.)

4. Heterogeneity of membrane currents

The membrane conductance of astrocytes is dominated by passive currents as reported for different brain regions including hippocampus (Steinhäuser et al., 1992), cerebellum (Müller et al., 1994), or spinal cord (Pastor et al., 1995; Chvatal et al., 1995). In a recent study, Kir4.1 and TREK channels were identified as the pivotal K^+ channel subunits of hippocampal astrocytes, and it was shown that superposition of these currents underlies the "passive" current pattern of hippocampal astrocytes (Seifert et al., 2009). In the spinal cord, the expression of the Kir4.1 channel is region-specific. In the ventral horn, Kir4.1 channel expression is highest as compared to very low levels of Kir4.1 in the apex of the dorsal horn as revealed with immunohistochemical staining. This was confirmed by Western blots from tissue of these two regions and by whole-cell patch-clamp recordings from astrocytes in rat spinal cord slices. Astrocytes from the dorsal horn showed significantly smaller K^+ uptake currents than astrocytes from the ventral horn, indicating that this differential expression also has functional consequences for potassium clearance rates in these two regions of the spinal cord (Olsen et al., 2007).

The expression of membrane currents also changes during development. In Bergmann glial cells from postnatal day 5 to 7, delayed outward- and inward-rectifying K^+ currents were recorded, while older Bergmann glial cells were characterized by large, voltage- and time-independent K^+ currents (Müller et al., 1994). Similarly, the upregulation of Kir4.1 transcripts and protein was observed during the first 10 postnatal days in astrocytes from the hippocampus; this was accompanied by a 4-fold increase in astrocyte inward current density (Seifert et al., 2009).

Cultured astrocytes express functional Ca^{2+} channels (MacVicar, 1984). The cultured cells express transcripts for the alpha(1B) (N-type), alpha(1C) (L-type), alpha(1D) (L-type), alpha(1E) (R-type), and alpha(1G) (T-type), but not alpha(1A) (P/Q-type), voltage-gated Ca^{2+} channels. These results in combining Western blot analysis and immunocytochemistry indicate that cultured astrocytes express several high- and low-threshold Ca^{2+} channels (Latour et al., 2003). In isolated hippocampal astrocytes, membrane depolarization by high potassium or GABA did activate Ca^{2+} channels (Duffy and MacVicar, 1994; Fraser et al., 1995). In Bergmann glial cells, however, there was no evidence for the expression of Ca^{2+} channels and also other groups failed to identify Ca^{2+} channels in astrocytes in situ.

5. Heterogeneity in glutamate receptor expression

The expression of AMPA type glutamate receptors is considered to be a general property of astrocytes. In Bergmann glial cells, the AMPA receptors are highly Ca^{2+} permeable due to the lack of the GluRB (or GluR2) subunit in the receptor complex. These AMPA currents are also characterized by a doubly rectifying current–voltage relation in contrast to the outward-rectifying pattern typical for AMPA receptors containing the GluRB subunit (Müller et al., 1992, Burnashev et al., 1992).

A large population of astrocytes in the supraoptic nucleus of the rat hypothalamus, however, lacks functional glutamate receptors as well as glutamate uptake current. Instead, these cells express $GABA_A$ receptors. These glial fibrillary acidic protein-positive astrocyte cells had a radial glia-like morphology, with cell bodies lined up along the base of the brain and long processes traversing the nucleus. There was, however, also a second population of astrocytes in the supraoptic nucleus: these cells were located next to the subarachnoid space, had a round morphology and few processes, and had functional glutamate transporter expression (Israel et al., 2003).

Functional NMDA receptors are not expressed by astrocytes in hippocampal slices (Seifert and Steinhäuser, 1995) or by astrocytes in cell culture (Kettenmann and Schachner, 1985). A lack of NMDA responses was also found for astrocytes in the medial nucleus of the trapezoid body associated with the Calyx of Held (unpublished observation). In mouse cortex, functional NMDA receptors have been identified in astrocytes. The NMDA-evoked membrane currents reversed at 0 mV, were blocked by the NMDA receptor antagonist MK-801 and were sensitive to Mg^{2+}. Moreover, NMDA triggered a focal Ca^{2+} elevation (Schipke et al., 2001). Also, spinal cord astrocytes (Ziak et al., 1998) and Bergmann glial cells respond to NMDA (Müller et al., 1993), but in those early experiments, an indirect effect via activation of neuronal receptors could not be excluded.

6. Heterogeneity in expression of other transmitter receptors

The expression level of $GABA_A$ receptors is developmentally regulated in Bergmann glial cells. GABA triggered two types of responses in Bergmann glia, a rapid activation of a Cl^- conductance and a longer-lasting decrease in the (resting) K^+ conductance and both were mediated by benzodiazepine-insensitive $GABA_A$ receptors. Responses in cells of postnatal days 5–7 were larger compared to the small or even undetectable responses in cells from postnatal day 20 to 30. Studies with subunit-specific antibodies revealed that Bergmann glial cells exhibit a distinct but transient labeling for $GABA_A$ receptor alpha 2-, alpha 3-, and delta-subunits. The labeling peaked between postnatal day 7 and P10 (Müller et al., 1994).

In contrast, in the hippocampus, prominent GABA responses can be recorded in astrocytes isolated from the adult rat hippocampus. A GABA-activated Cl^- conductance was mimicked by the $GABA_A$ receptor agonist muscimol and depressed by the $GABA_A$ antagonists bicuculline and picrotoxin and potentiated by the barbiturate pentobarbital. In contrast to Bergmann glia, the benzodiazepine diazepam also potentiated the response. The benzodiazepine inverse agonist DMCM either enhanced or depressed $GABA_A$-mediated responses, suggesting receptor heterogeneity. GABA induced membrane depolarization and caused Ca^{2+} influx through voltage-gated Ca^{2+} channels (Fraser et al., 1995). In Bergmann glial cells, in contrast, activation of Ca^{2+} channels was not observed.

Glycine receptors are another transmitter receptor which can be differentially expressed in astrocytes. Bergmann glial cells respond to application of glycine with an inward current, but this current was insensitive to strychnine, a glycine

receptor antagonist, but due to the activity of glycine transporter activity. Thus, Bergmann glial cells express no functional glycine receptors (Huang et al., 2004). In contrast, spinal cord astrocytes express functional glycine receptors since the glycine induced current response was blocked by strychnine and reversed close to the Cl^- equilibrium potential (Pastor et al., 1995). Analysis of cytoplasmic RNA harvested by using the patch-clamp technique in the whole-cell configuration, transcripts of the alpha-1 subunit, but not of alpha-2 or alpha-3 subunits of the glycine receptor was detected. In addition, about one-half of the astrocytes contained beta-subunit mRNA (Kirchhoff et al., 1996). Moreover, astrocytes can express an atypical variant of the glycine receptor, namely a splice variant of the glycine receptor beta-subunit. This subunit betaDelta-7 lacks amino acid positions 251–302 encoded by exon 7 of the Glrb gene resulting in a profoundly altered prediction of transmembrane topology. Despite these topological alterations, this variant forms receptor complexes with the alpha1 subunit and gephyrin. The functional consequence of this expression is not known (Oertel et al., 2007).

While almost all cultured astrocytes express functional beta-adrenergic and metabotropic purinergic (P2Y) receptors, only a subpopulation of astrocytes (less than half) acutely isolated from postnatal day 8–12 rat hippocampus coexpress beta-adrenergic receptor mRNA subtypes with metabotropic ATP receptor mRNA subtypes (P2Y1, P2Y2, or P2Y4). Immunocytochemical characterization of beta-adrenergic or P2Y1 receptor distribution revealed that numerous astrocytes in the CA1 region of P9–P10 rat hippocampus stained positive for either of these receptors (Zhu and Kimelberg, 2004). While cultured astrocytes and astrocytes from cortical slices express functional P2X receptors (Walz et al., 1994; Lalo et al., 2008), no detection of P2X receptors in astrocytes in the hippocampus (Jabs et al., 2007) or Bergmann glial cells (Kirischuk et al., 1995) was reported.

Fibroblast growth factor (FGF)-2 is an abundant astroglial cytokine. FGF-2 downregulates gap junctions in primary astroglial cultures (Reuss et al., 1998) and induces astroglial dopamine sensitivity and dopamine 1-receptor expression in cortical and striatal astroglial cultures.

Dopamine triggered transient increases in $[Ca^{2+}]_i$ in cortical and striatal cultured astrocytes indicating that astrocytes express functional dopamine receptors. The expression level of these receptors is upregulated by fibroblast growth factor-2. This could be uncovered by blocking gap junctions which isolated a smaller population of astrocytes intrinsically sensitive to dopamine and this population was significantly increased by fibroblast growth factor-2 (Reuss et al., 2000).

7. Heterogeneity in glutamate transporter expression

Glutamate transporters expressed by astrocytes control excitatory neurotransmission and prevent glutamate-mediated excitotoxicity. By generating transgenic mice which coexpress DsRed under control of GLAST promoter and GFP under the control of GLT-1 promoter, the expression pattern of these two important glutamate transporter molecules could be studied and compared within different brain regions. GLT-1 promoter activity is almost completely restricted to astrocytes. GLT-1 expression in spinal cord astrocytes was about 10-fold lower than in brain pointing to a difference between astrocytes in brain and spinal cord. Early in development, the GLAST promoter was active in radial glia and many astrocytes while it was downregulated in most astrocytes of adult mice. Thus, in the adult CNS, the highest GLAST promoter activity was observed in stem cells such as radial glia in the subgranular layer of the dentate gyrus (Regan et al., 2007).

8. Heterogeneity in gap junction coupling

Astrocytes show remarkable differences in the extent of coupling via gap junction. In grey matter, astrocytes commonly form large syncytia (Fig. 3). In contrast, in white matter as studied in the corpus callosum, astrocytes coupling is rather at low level and single, obviously uncoupled astrocytes were observed after biocytin injection while even in the same slice preparation cortical astrocytes show a wide range biocytin spread (Haas et al., 2006). Even in grey matter, astrocyte coupling can vary. Astrocytes in the layers 1 and 2/3 of the cortex formed networks elongated in the transverse direction (parallel to the surface of the cortex), whereas in layers 4 and 5, networks were circular. A similar heterogeneity of astrocyte coupling was observed in the hippocampus—in the center of the stratum radiatum the astrocyte networks were circular, while astrocytes located close to pyramidal cell layer formed networks elongated in the direction parallel to the pyramidal cell layer (Houades et al., 2006). This correlates with differences in the morphology and orientation of individual astrocytes in different layers of CA1 region of the hippocampus. While astrocytes in stratum lacunosum moleculare are small, possess uniform, radially oriented processes, astrocytes in stratum radiatum are much bigger, polarized, often showing bipolar shape and show a preferred orientation, which is perpendicular to the pyramidal cell layer (Nixdorf-Bergweiler et al., 1994). Interestingly, potassium buffering is not affected by disruption of astrocytic coupling in stratum radiatum, whereas it is affected in the stratum lacunosum moleculare (Wallraff et al., 2006).

In the barrel cortex, the expression of the major astrocyte connexins, Cx43 and Cx30, is higher within the barrel fields as compared to the septal region and other areas of the cortex. Coupling in astrocytes was preferentially oriented along the extension of the barrel fields and astrocytes located between two barrels were either weakly or not coupled (Houades et al., 2008). There was, however, no apparent difference in astrocyte morphology throughout that region.

While astrocyte networks in general extent in a three-dimensional fashion, Bergmann glial cells form a layered network in the cerebellar cortex. The cells are coupled only in the direction orthogonal to the parallel fibers, resulting in planar astrocytic networks, which form layers which are in the same orientation than the dendritic tree of Purkinje neurons (Muller et al., 1996).

In aged mice, the extent of coupling was significantly lower than in postnatal mice. In this study slices of the cortex or hippocampus of 21- to 28-month-old mice were prepared and

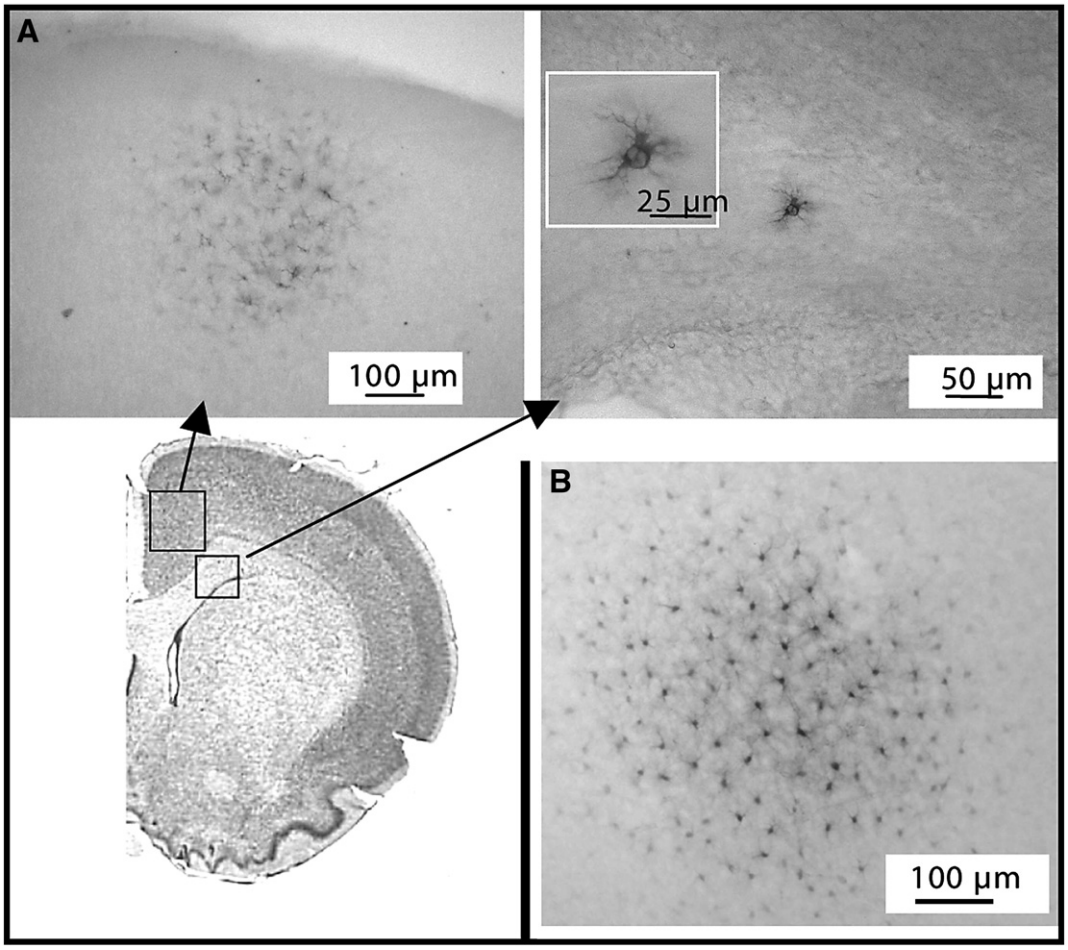

Fig. 3 – Astrocytic coupling in the neocortex and in the corpus callosum. (A) Biocytin was injected into a cell of the neocortex and a cell in the corpus callosum within the same slice from a GFAP/EGFP transgenic mouse. Astrocytes were selected using their fluorescence at 488 nm. Left: In the neocortex, the biocytin injection resulted in the labeling of a large population of astrocytes. Right: In the corpus callosum, only a single cell is stained with biocytin. The insert on the right is a magnified image of the single injected cell. Images are from a 150-µm-thick slice that was processed as a whole after injection of the two cells. (B) One cell in the cortex of a slice from a GFAP/EGFP transgenic mouse was injected with biocytin. After fixation 50-µm cryosections were obtained from this 300-µm-thick slice. This procedure resulted in improved biocytin visualization as compared to processing the entire 150-µm-thick slices (Haas et al., 2006).

biocytin injection into astrocytes indicated that there was either no dye spread or the dye labeled a network which was much smaller as compared to early postnatal mice injected in parallel experiments. Interestingly, in aged mice which over-expressed the β-amyloid precursor protein leading to the formation of amyloid plaques, biocytin injection revealed much stronger coupling among astrocytes (Peters et al., 2009). There is also evidence for populations of astrocytes which lack functional coupling: a large population of astrocytes in the supraoptic nucleus of the rat hypothalamus with a radial glia-like morphology was not dye-coupled (Israel et al., 2003). Also, in dentate gyrus, only half of the radial glia-like cells are coupled as small (2–16 cells) networks and about the same portion of cells express connexins (Kunze et al., 2009).

In conclusion, astrocyte networks can vary considerably in extent depending on brain region and age. Given the fact that astrocyte coupling can be modulated on a short-term basis by membrane receptor activation, e.g., by glutamate, cannabinoids, or endothelin (Müller et al., 1996; Blomstrand et al., 2004; Venance et al., 1995), communication between astrocytes via gap junctions can be highly flexible and dynamic.

9. Heterogeneity in Ca^{2+} signaling

Astrocytes show two distinct forms of calcium signaling, namely spontaneous calcium oscillations in individual cells and a coordinated form as an intercellular calcium wave. Both activities can occur independently of neuronal activity as they are insensitive to blockers of action potentials (by tetrodotoxin) or blockers of glutamatergic, purinergic, and cholinergic receptors. Astrocytes in somatosensory cortex of anesthetized mature rats show layer-specific differences in spontaneous oscillation patterns. In cortical layer 2/3, astrocyte calcium oscillations are highly synchronized between different cells, whereas in layer 1, calcium fluctuations are asynchronous and

have frequency 2-fold higher than in layer 2/3 (Takata and Hirase, 2008).

Intercellular calcium waves propagate differently in astrocytes in white and grey matter: In the cortex, functional gap junctions are a prerequisite for the wave to propagate. In contrast, in the corpus callosum where gap junctional coupling is low or absent, calcium waves can still be elicited, which depend on release of ATP. Gap junction blockers do not affect the propagation of the wave (Schipke et al., 2001). In contrast, in the neocortex, propagation of the calcium waves requires intact gap junctional conductance (Haas et al., 2006).

The astrocyte Ca^{2+} wave in rodents propagates with a speed of 20 μm/s both in culture and in brain slices (Schipke et al., 2001). In human astrocytes, the wave propagates with a higher speed, namely at 40 μm/sec (Oberheim et al., 2009). The difference between mouse and human might even be higher, since in this study the authors evoked the wave by photolysis of caged calcium which in the mouse evoked a wave with a slow velocity (4–5 μm/sec) compared to the 10–20 μm/sec waves evoked by mechanic or electrical stimulation (Oberheim et al., 2009, M. Nedergaard, personal communication). Whether this is due to the difference in morphology of human and mouse astrocytes remains open.

Ca^{2+} response due to metabotropic receptor activation leads to a release of Ca^{2+} from internal stores and subsequently to a capacitative Ca^{2+} entry. Comparing astrocytes in culture and in hippocampal slices revealed a significant difference between these two preparations. While the endoplasmic reticulum was usually located in close apposition to the plasma membrane in astrocytes in situ, the endoplasmic reticulum in cultured astrocytes was close to the nuclear membrane. This leads to a different physiologic behavior. In culture, Ca^{2+} signals were commonly first recorded close to the nucleus and with a delay at peripheral regions of the cells. Store-operated Ca^{2+} entry could be easily identified in cultured astrocytes as the Zn^{2+}-sensitive component of the Ca^{2+} signal. In contrast, such a Zn^{2+}-sensitive component was not recorded in astrocytes from hippocampal slices despite of evidence for store-operated Ca^{2+} entry. Our data indicate that astrocytes in hippocampal slices and in culture are different on their ultrastructural level which results in a distinct physiological phenotype (Pivneva et al., 2008). Whether the cultured astrocytes have an in situ counterpart anywhere in the brain or whether this behavior is a mere cell culture artefacts remains open.

10. Heterogeneity in volume regulation

Subjecting cortical slices to ischemic conditions (oxygen and glucose deprivation) revealed two groups of astrocytes which respond differently to this challenge. Both types of cells were identified as astrocytes by using a mouse line in which EGFP is expressed under the GFAP promoter (Nolte et al., 2001). The first type of astrocyte show large volume increase 20 minutes after the challenge as revealed with two-photon microscopy. In addition, membrane potential depolarised. The second type of astrocyte showed only small volume increase and small membrane potential change toward more negative values. Acidification led to a uniform volume increase in both types of astrocytes. The authors speculated that this different cellular behaviour is due to different expression levels of the potassium channel Kir4.1 and the intermediate filament protein GFAP, as well as in their ability to accumulate taurine (Benesova et al., 2009; Chvatal et al., 2007).

Bergmann glia, retinal Müller cells, and hippocampal astrocytes show differences in volume response to a hypoosmotic shock. The hypoosmotic challenge induced immediate swelling in somata of Bergmann glia and hippocampal astrocytes, whereas swelling of Müller cell somata was delayed, and processes of hippocampal astrocytes remained unaffected. Pharmacological blockade of K_{ir} channels caused swelling in brain astroglial somata and processes, but had no effect on Müller cells. The blockade of K_{ir} channels in hypoosmotic conditions induced immediate swelling of Müller cell somata but induced no additional swelling in somata of brain astrocytes (Hirrlinger et al., 2008).

11. The complex glial cell—A specialized form of astrocyte

A distinct group of glial cells has been identified in the mature brain, which is distinct in morphology and physiological features from what is considered the classical astrocyte. Due to the presence of voltage-gated channels, they were named complex cells in contrast to the classical astrocytes which were termed passive cells (Steinhäuser et al., 1994). These cells were originally considered as oligodendrocyte precursor cells (Sontheimer et al., 1989). Now they are considered as a distinct population of glial cells. They can be identified by the expression of the proteoglycan NG2. Whether they can be incorporated into the larger class of astrocytes remains a discussion.

In the hippocampus, these cells were identified by the use of a transgenic mouse line which expresses the fluorescent protein EGFP under the GFAP promoter (Nolte et al., 2001). The NG2-positive cells express low levels of EGFP, have clearly visible processes, express AMPA receptors, and lack glutamate transporter currents and gap junctional coupling. They are thus distinct from the classical astrocytes which are characterized by high EGFP levels, fuzzy processes, lack of AMPA receptors, and expression of functional glutamate transporter currents and gap junctional coupling (Jabs et al., 2005; Wallraff et al., 2004). Interestingly, the cells receive synaptic input, both glutamatergic and GABAergic (Bergles et al., 2000; Jabs et al., 2005). Cells with similar properties have been described in the cerebellum (Lin et al., 2005) and hypoglossal nucleus (XII) of brainstem (Grass et al., 2004). In contrast to hippocampus, the NG-2 cells in the medial nucleus of the trapezoid body receive only glutamatergic input (Müller et al., 2009). These cells have been given various names like complex glial cells (Steinhäuser et al., 1994), GluR cells (Matthias et al., 2003), polydendrocyte, and synantocyte (Butt et al., 2002).

12. Conclusion

The examples of astrocyte heterogeneity listed in this review are most likely just a beginning of a long list. One can

distinguish at least nine types of astrocytes on a morphological basis, but there is no thorough comparison of the physiological behaviour of these cells. Moreover, morphologically undistinguishable astrocytes can exhibit a different physiological behaviour as observed with respect to coupling in the barrel cortex. Thus, astrocyte heterogeneity is just emerging as a topic in glial biology and will be an important issue for future studies.

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BRAIN RESEARCH REVIEWS

Review

Morphology and dynamics of perisynaptic glia

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ABSTRACT

The major glial population of the brain is constituted by astroglia. Highly branched and ramified protoplasmic astrocytes are the predominant form in grey matter and are found in almost all regions of the central nervous system. In cerebellum and retina, there two forms of elongated radial glia exist (Bergmann glia and Müller cells, respectively) that share many features with the protoplasmic astrocytes in respect to their perisynaptic association. Although these three astroglial cell types are different in their gross morphology, they are characterized by a polarized orientation of their processes. While one or only few processes have contacts with CNS boundaries such as capillaries and pia, an overwhelming number of thin filopodia- and lamellipodia-like process terminals contact and enwrap synapses, the sites of neuronal communication. The perisynaptic glial processes are the primary compartments that sense neuronal activity. After signal integration, they can also modulate synaptic transmission, thereby contributing to neural plasticity. Despite their importance, the mechanisms that (1) target astroglial processes toward pre- and postsynaptic compartments and (2) control the interaction during plastic events of the brain such as learning or injury are poorly understood. This review will summarize our current knowledge and highlight some open questions.

Contents

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1. Introduction

Astrocytes are polarized glial cells, known as integral entities interacting with blood vessels and neighbouring neuronal compartments. In this review, we will focus on the synaptic association of glial cell processes. Historically, it became obvious when electron microscopy was introduced into brain research that the neuropile consists not only of neuronal cell processes and synapses but also of glial cell processes (Luse, 1956). Soon after, it was realized that the glial processes are in close contact to the pre- and postsynaptic compartments (Schultz et al., 1957). These observations supported earlier speculations that astrocytes contribute to neurotransmission (Lugaro, 1907; quoted in Somjen, 1988).

However, it was a long way from there to the concept of the 'tripartite synapse' (Araque et al., 1999) that added the perisynaptic glial element to the pre- and postsynapse as a functional unit. Here, we will use the term 'tripartite synapse' in a broad sense with the astrocyte being an equitable component. The astroglial compartment is not only mechanically associated, it fulfils homeostatic (e.g., ion and transmitter regulation; Kimelberg, 2007), metabolic (e.g., energy supply; Pellerin et al., 2007) and regulatory (sensing and responding to neuronal activity; Perea et al., 2009; Halassa et al., 2009) functions.

Synapse-associated glial processes arise from typical protoplasmic astrocytes as found in the cortex. But, they are also formed by other astroglial cell types such as cerebellar Bergmann glia or Müller cells of the retina. Since the (ultra-) structural and functional properties of their glial processes appear astonishingly similar, they will be discussed together. However, it has to be stated that the definition of a synapse-associated glial process and its discrimination from other adjacent glial cell processes without synaptic contact provides a challenging task that we try to illustrate in the following sections.

2. Basic ultrastructure of perisynaptic glia

The most striking feature of glial cell morphology is the enormous complexity and irregularity of process arborisation and ramification (Fig. 1). The terminals of the highly branched glial process are the structural elements that are associated with pre- and/or postsynaptic compartments (Figs. 2 and 3). These finest process terminals can appear as filipodia- or lamellipodia-like structures (Fig. 4). However, it has to be stressed that only a fraction of a pre- or postsynapse is covered by glia (Figs. 2B, 3A, G). In addition, synaptic elements can be covered by glial cell processes arising from different cells. A part of a glial process with contacts to the synapse can be simultaneously associated to other glial processes. It is tempting to speculate that these glio-glial contact areas, which are often coupled via gap junctions directly or indirectly modulate the synapse–glia association (Houades et al., 2008). This complex situation makes it not only difficult to define 'normal' (functionally relevant) glio-neuronal contacts including perisynaptic sheaths, it also raises a series of questions such as (i) if there is a perisynaptic contact, does it result from specific 'attraction' or from stochastic glial process growth; (ii) if a (part of a) synapse is devoid of glial coverage, does this result from specific 'repellance' or is this pure accident; and, (iii) is it possible to extract rules for the occurrence and shape of perisynaptic glial processes in different animal species, brain areas, or types of synapses? Due to the lack of reliable models of stochastic glial cell process growth, these questions cannot be solved by 'clear-cut' mathematical analysis; rather, we have to rely on a guess of probability. For example, if a given part of a well-defined synapse is virtually always found to be covered by a glial sheath in brain sections from animals of a given species, age and gender, this should be indicative of a non-stochastic relationship. To mention only one of the additional problems, it becomes clear after a glance on Fig. 3 that three-dimensional (3D) reconstructions of high spatial resolution rather than single 2D microphotographs are required to unequivocally reveal the topographic relations between given synaptic and glial cell processes. Such data, however, are very rare because they require technically difficult and time-consuming procedures (Grosche et al., 1999; Grosche et al., 2002; Chao et al., 2002). With all these constraints in mind, we will try to extract basic conclusions from the available data.

The main or stem processes of glial cells and their side branches traverse the neuropile, but usually do not contact specific neuronal compartments such as synapses. Rather, they give rise to numerous thin, convoluted cytoplasmic tongues. These are highly variable in shape, as reflected by a variety of different terms that were used to describe them, including lamellar processes (Wolff, 1968), lamellipodia and filopodia (Chao et al., 2002) or velate processes (Palay and Chan-Palay, 1974) each addressing particular aspects of their sometimes region dependent appearance. In general, they have no round or oval profile as most neuronal structures and do not follow any apparent specific track. Rather, they appear to meander and to branch 'randomly' in the neuropile. The terms peripheral astrocytic process (PAP) (Derouiche and Frotscher, 2001; Derouiche et al., 2002) or peripheral glial process (PGP) (Reichenbach et al., 2004) were introduced to avoid any pre-assumptions regarding their shape. These processes can be defined by (1) the presence of glia-specific cytoplasmic or membrane markers such as glutamine synthetase, the glutamate transporters EAAT1 (GLAST) and EAAT2 (Glt-1) and (2) the absence of glial filaments (seen as absence of GFAP immunoreactivity) (Chao et al., 2002; Derouiche, 2004; Reichenbach et al., 2004). A general classification of astroglial processes (primary and secondary

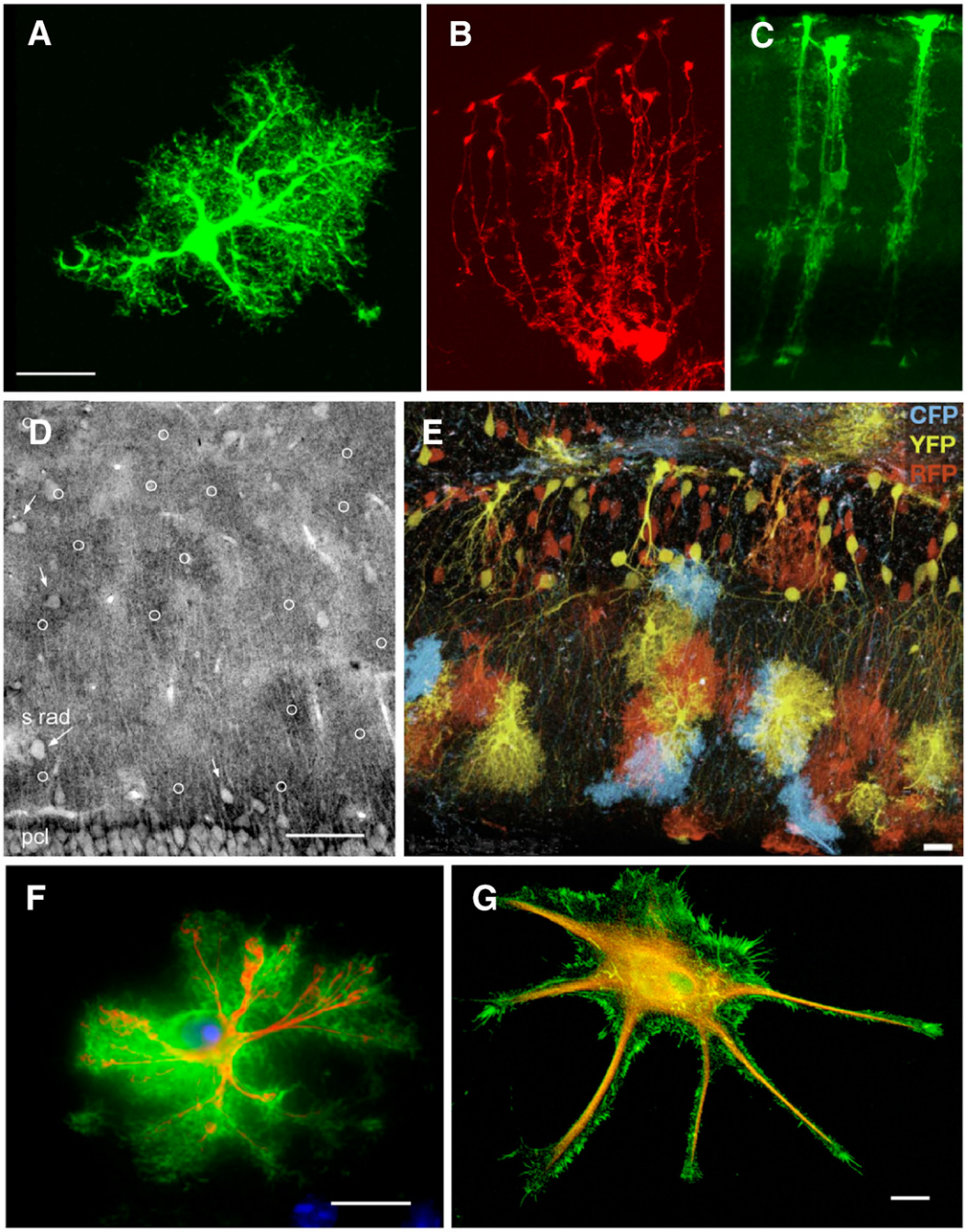

Fig. 1 – Astroglial cell populations represent morphologically diverse classes of polarized and highly arborized cells of the central nervous system. A: Single astrocyte of the mouse neocortex with numerous processes one of which contacting a blood vessel (left). The other processes end in thin lamellipodia- and filopodia-like terminals that enwrap synapses of the neuropile. B: Bergmann glial cells of the mouse cerebellum. While the cell bodies are localized at one end, distal process endings contact the pia mater. C: Müller glial cells of the mouse retina span the entire distance between the vitrous body and pigment epithelium. D: Immunoperoxidase-staining of ezrin, an actin-binding protein of the peripheral process tips, visualizes the territorial distribution of astrocytes (seen as darker grey shadows, somata labelled by white circles) in the stratum radiatum of the rat hippocampus. Note the 'negative staining' of pyramical cells and interneurons (arrows), as well as the proximal dendrites. E: Random and combinatorial Cre/loxP mediated DNA excision in Brainbow transgenic mice reveals a similar "tiling" of astrocytes. F–G: Co-immunostaining for the glial fibrillary acidic protein (red) and ezrin (green) distinguishes between the major stem and the peripheral astroglial processes (PAP) in acutely isolated (F) and in cultured (G) astrocytes (F). Scale bars in μm: 20 (A), 100 (D), 20 (E), 15 (F), 10 (G). With permission Livet et al., 2007 (E); Derouiche and Frotscher, 2001 (G).

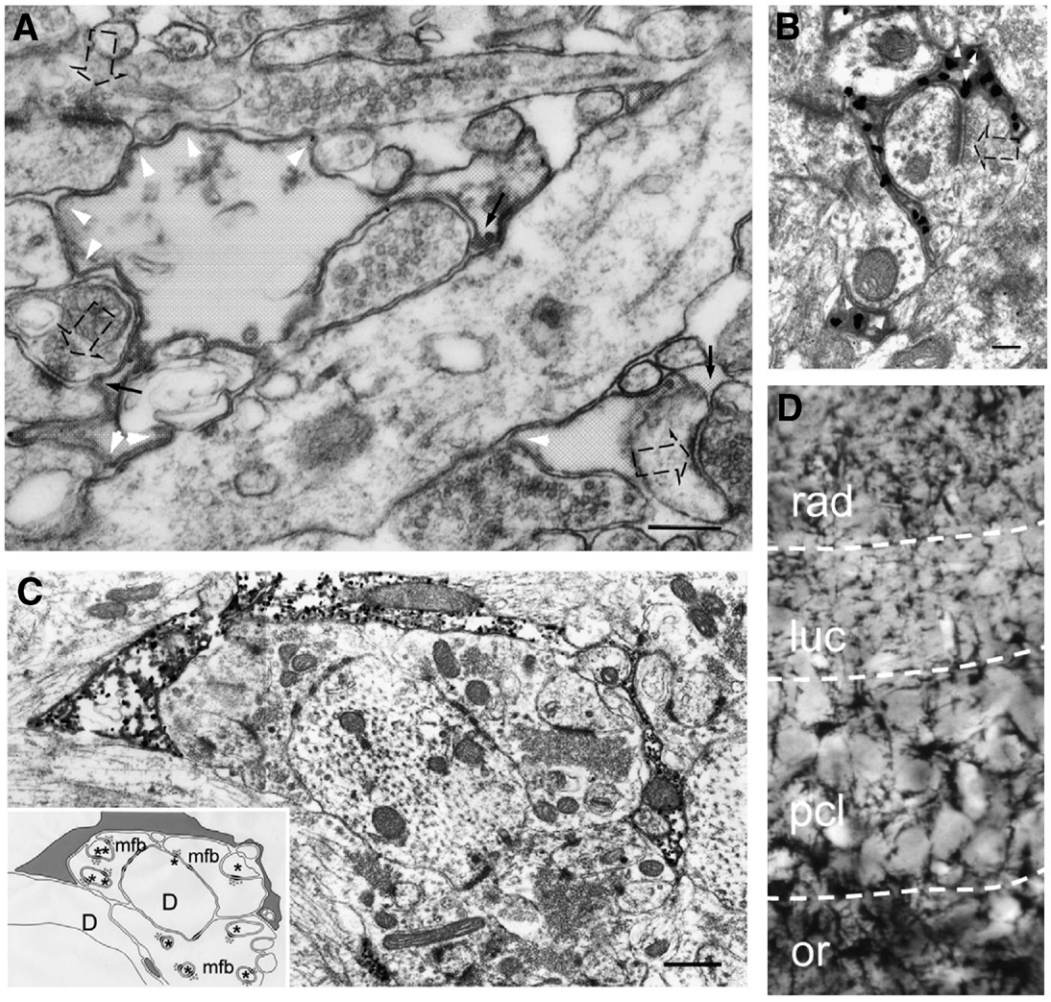

Fig. 2 – Glutamine synthetase immunostaining of peripheral astroglial processes in the hippocampal CA1 region of the adult rat. A: The PAP (hatched area) has a central, bulging portion devoid of glial filaments with two narrow extensions. Spine synapses (open arrows) display glial contact only at the spine (top left and bottom right), or around the axon terminal and the spine (bottom left). The glial adaptation to the round neuronal structures often produces the typical glial 'fingers' between curvatures (white arrows in A and B), which also approach the synaptic cleft (small black arrows in A, bottom arrowhead in B). B: The glial process marked by the black silver grains ensheaths a synapse (open arrow) and is thinner than 100 nm, clearly below light microscopic resolution. C–D: Peripheral astrocyte processes in the stratum lucidum of the rat hippocampus at the electron (C) and light microscopic level (D). The PAP in (grey in inset in C) embraces three giant mossy fibre boutons (mfb in inset). They form multiple synapses with spines (asterisks). Most of the spines are fully embedded in the bouton and emerge from the two dendrites (indicated as D) seen in longitudinal or cross section. Note that there is no PAP approximating the synaptic cleft or intervening between the mossy fibre boutons. D: The reduced fraction of glial processes in the neuropile is also evident in light microscopy, where the stratum lucidum is typically more lucid than the neuropile in the overlying stratum radiatum, which does not contain the giant boutons. or, stratum oriens; pcl, pyramidal cell layer; luc, s. lucidum; rad, s. radiatum. Scale bars, 0.5 μm (A, B, C).

processes, surface extensions) based on cell biological and developmental aspects has been described previously (Wolff and Chao, 2004). The perisynaptic glial sheaths are formed by – and are part of – these processes.

Typically, on transmission electron micrographs the cytoplasm of PAPs appears clear and poor in organelles, while glial filaments are restricted to the main processes. Although PAPs can be very thin they do not taper towards their ends; rather, they can have bulging sections interposed between very narrow ones (Fig. 2A). Such bulged sections will typically contain larger organelles such as a mitochondrium, which can also be found occasionally in PAPs. PAPs contain glycogen granules and proteinacious aggregates ("fluffy material") (Peters et al., 1991). The nature of organelles in PAPs has not been studied in sufficient detail yet. Although individual vesicles or small groups of them can be observed, they are rarely mentioned in the classical accounts of glial ultrastructure (Peters et al., 1991), in accordance with the classical view that astrocytes are not capable of vesicular exocytosis. However, there is now accumulated evidence for vesicular

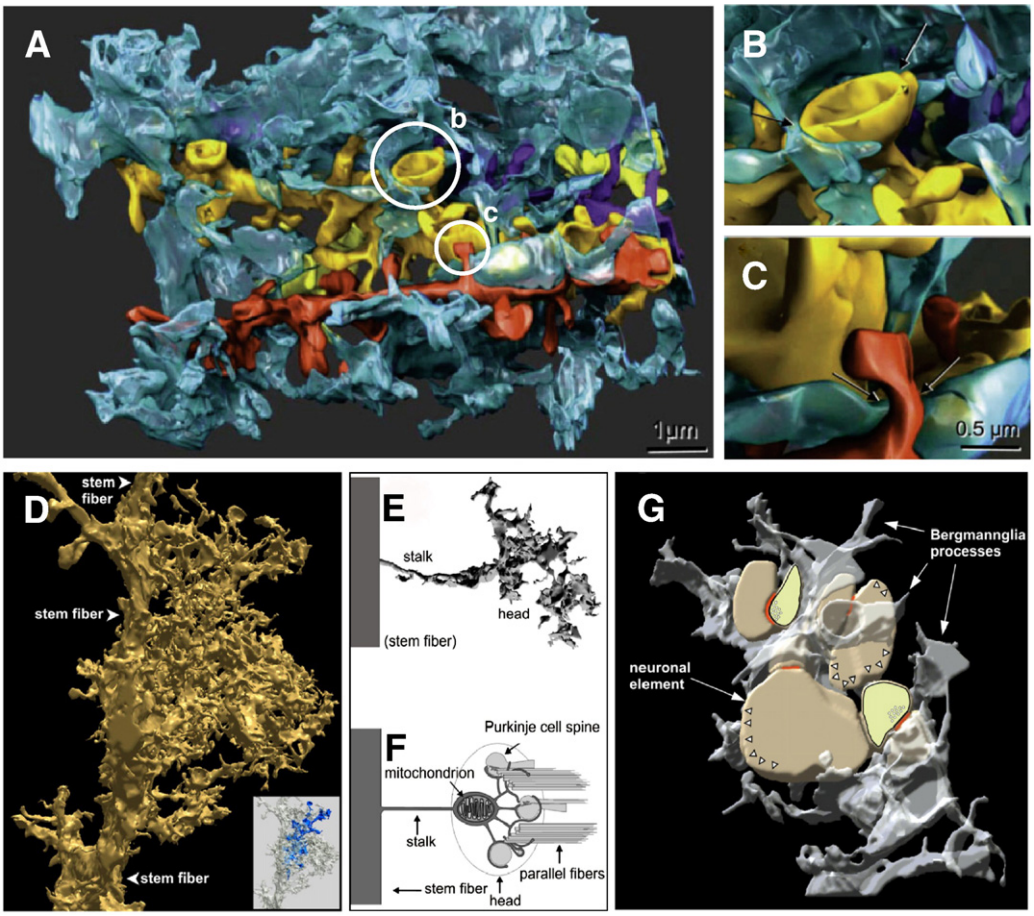

Fig. 3 – Reconstruction of dendrites, synapses and associated peripheral astroglial processes demonstrate the tight interactions at the tripartite synapse. A: 3D reconstruction of a single astroglial process (blue) interdigitating several dendrites within the rat hippocampus, four of which are reconstructed (gold, yellow, red, and purple). Presynaptic axonal boutons are not displayed. B: At the synaptic cleft of mushroom spine approximately 50% of the postsynaptic surface was apposed by astroglia (arrows). C: Only the neck of this thin dendritic spine was apposed by astroglia (arrows). D: 3D reconstruction of a mouse Bergmann glial cell process. The living cell was dye-injected, photo-converted and electron-micrographed. 600 consecutive serial ultrathin sections were used for 3D-reconstruction. The inset shows a substructure labelled in blue; this part was quantitatively analyzed (see E, F). E: Glial microdomain as part of the 3D reconstruction shown in D. Note that a microdomain is composed of several PAPs with filopodial as well as lamellipodial morphology. F: Schematic drawing of such a glial microdomain and its relationships to the neuronal elements. G: 3D reconstruction of a group of neighbouring cerebellar synapses (yellow; synaptic clefts: orange) together with the surrounding leaflets of the Bergmann glial cell (ochre). The arrowheads point to neuronal surfaces not covered by glial sheaths from the labelled cell. Modified from A–C: Witcher et al. (2007); with permission D–G: Reichenbach et al. (2004).

release of 'gliotransmitters' (reviewed by Bergersen and Gundersen, 2009). In cultured astrocytes, particularly in their filopodia, exocytotic vesicles containing glutamate or ATP were demonstrated (Coco et al., 2003; Anlauf and Derouiche, 2005). In rat hippocampal sections, vesicles immunoreactive for vesicular glutamate transporters were found within perisynaptic glial processes (Bezzi et al., 2004) however conspicuous vesicle clusters could not be detected in the PAPs. Although the studies performed in acutely isolated brain slices indicate a bidirectional communication between neurons and astrocytes, serious scepticism has been raised in respect to the Ca^{2+}-dependent gliotransmitter release *in vivo* (Kimelberg, 2007; Agulhon et al., 2008; Fiacco et al., 2009).

A quantification of astrocytic processes revealed a considerable variation in different brain regions. Astrocytic processes (including PAPs) account for not more than 4% of the cross sectional tissue area/volume in the stratum radiatum (CA1, rat hippocampus; Ventura and Harris, 1999), for 10% in the complete CA1 region of hippocampus or 33% in the cerebellar cortex (Lehre and Rusakov, 2002). In ultrathin sections, profiles of such processes may exceed 1 µm in diameter, but are typically smaller than 200 nm. Frequently they are as narrow as 50–100 nm, representing membrane appositions virtually without cytoplasm (Figs. 2B and C). Consequently, they contain only a minor portion of the glial cytoplasm volume. However, they display the major part of the cell surface area (70–80% of the astrocytic cell membrane; Chao et al., 2002) giving an extremely high surface-to-volume ratio (up to 25 μm^{-1}; (Grosche et al., 2002), which is also reflected in selective staining of PAPs in freshly dissociated or cultured

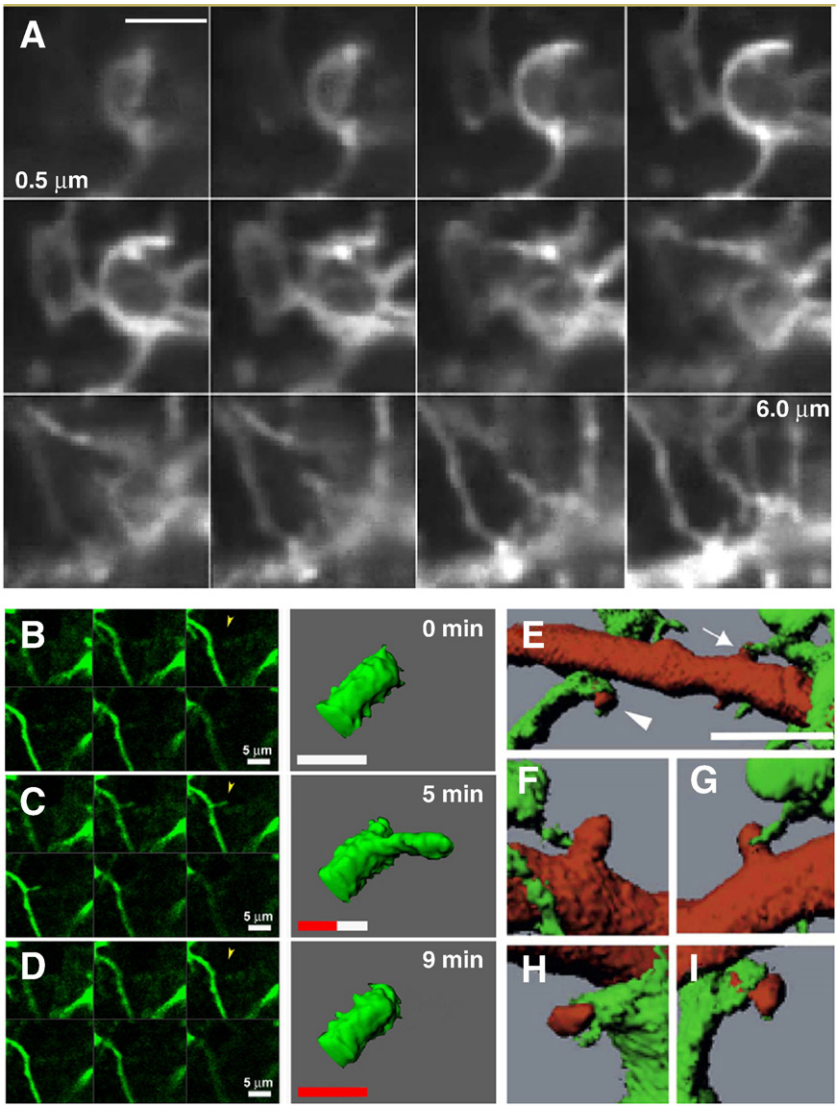

Fig. 4 – Lamellipodia- and filipodia-like astroglial processes are highly motile structures that stabilize synapses. A: Two modes of astroglial motility are revealed by three-dimensional laser-scanning time-lapse microscopy of an acutely isolated brain stem slice prepared from a TgN(hGFAP-EGFP) transgenic mouse with fluorescently tagged astrocytes. Image stack (*z*-separation 0.5 μm) was recorded with a time interval of 30 s for 25 min (see Supplementary Movie 1). Displayed are the optical sections of a single time point (0–6 μm in *z*, 12 image frames). Note the thin velate processes in the upper left region of the stack and filopodial processes in the lower right. The astroglial somata as well as the large processes are stationary, whereas small processes are highly motile observed as (i) nondirected gliding of lamellipodia-like membrane structures and (ii) filopodial process extension. B–D: Spontaneous transient extension of astroglial filopodia in the brainstem. The left panel shows a stack of 6 images recorded at time 0, 5 and 10 min in an acutely isolated brainstem slice. The right panel displays the 3D-reconstruction and surface rendering of the complete dataset and reveals the transient extension of a peripheral astroglial process. See also Supplementary Movie 2. E–I: Variable morphology of contact sites revealed by three-dimensional modelling of two-photon image stacks. Contact sites are either large enough to encircle the neck of dendritic protrusions (arrowhead in E; enlarged in H, I) or confined to a small area (arrow in E; enlarged in F, G). Higher-magnification views are from different angles (G and I are from the similar direction as in A, and views from different angles are in F and H). Scale bars: A, 2 μm, E, 5 μm. With permission from A–D: Hirrlinger et al. (2004); E–I: Nishida and Okabe (2007).

astrocytes (Figs. 1F and G). The large membrane area – placed within small volume compartments of CNS tissue – provides the space for the plethora of ion channels, ligand receptors, and uptake carrier proteins necessary to maintain the variety of glia–neuron interactions. Even the narrowest PAPs are endowed with uptake and degradation systems for neurotransmitters released at the synapse (Fig. 2) (Derouiche and Frotscher, 1991).

As compared to their supposed impact for brain functioning (Araque et al., 1999), it appears striking that no structural specializations have been reported for PAPs in perisynaptic position. Throughout the neuropile, not even molecular

differences are known between synapse-associated PAPs and those without synaptic contact. As there is no structural or molecular marker for perisynaptic processes, the only criterion for a perisynaptic astrocytic process thus remains its immediate spatial relation to the synapse. Strictly speaking, such processes may seal the synaptic cleft laterally, and/or cover the pre- and/or postsynaptic element (Figs. 2 and 3). This suggests that the synapse-associated PAPs do not differ functionally from PAPs elsewhere in the neuropile. Their specific functional relevance may arise from their localization next to the synaptic cleft, resulting in short diffusion distances suitable to maintain the variety of glia–synaptic interactions. However, since ultrastructural and functional specializations are already known for astroglial endfeet contacting pial membranes or capillaries, the existence of molecular specializations in synapse-associated PAPs are likely and their identification could reveal novel mechanisms of neuron–glia interactions.

Although we cannot yet distinguish PAPs with or without synaptic contacts molecularly, the association of glial processes with synapses is by no means a purely stochastic phenomenon based on their chance of encountering a synapse. The percentage of lateral synaptic clefts sealed by PAPs was found to be 56% (rat neocortex; Wolff, 1970) or 64–90% (rat hippocampus; Witcher et al., 2007), which is significantly more than expected, given that 4–33% of the tissue consists of glial cell processes. By contrast, an ultrastructural analysis of the cerebellar cortex revealed that although profusely permeating the neuropile, the peripheral processes of Bergmann glial cells strictly spare the axon (i.e. parallel fibre) bundles (Eccles et al., 1967). Correspondingly, the density of PAPs in the neocortex was found to decrease with increasing numbers of unmyelinated fibres (Wolff, 1968). These data suggest that indeed (at least, some) synapses attract PAPs.

3. Regional heterogeneity in glial coverage

There is now ample evidence for a regional heterogeneity in the glial coverage of synapses (for a detailed review, see Chao et al., 2002). Whereas individual synapses may be completely enveloped by PAPs, large groups of synapses (so-called glomeruli) may be largely devoid of them. Such glomeruli are found in the sensory thalamus, in the olfactory bulb or as rosettes in the cerebellar cortex. The glial relation to glomeruli has been described (Spacek and Lieberman, 1974) and reviewed in detail (Chao et al., 2002). In general, PAPs completely ensheath the complex axodendritic arrangement of a glomerulus, sometimes even with multiple lamellae, thus delimiting and defining it. However, PAPs are completely absent within the glomerulus proper. The multiple synaptic contacts within, and the neuronal cell types contributing to, a glomerulus are repetitive in a given brain region, establishing the glomerulus as a higher-scale signal processing unit. Instead of diffusely encountering synapses, the PAPs may thus structurally separate functional units and establish distinct compartments within the glomerular (glia-free) and extraglomerular neuropile (Spacek and Lieberman, 1974; Wolff and Chao, 2004).

Regarding coverage by glial processes, synapses fall in a continuum of two extremes: glomerular (glia-free) synapses and, for example, synapses on neuronal somata that are always invested by perineuronal glial and extracellular matrix nets (Bruckner et al., 1993). 'Quasi-glomerular' synapses, with a single type of large axon terminal invaginated by the postsynaptic element, are glia-free but display an overall glial ensheathment, such as for example the retinal rod spherule or the hippocampal mossy fibre synapses (Fig. 2C). Similarly, multiple synaptic contacts, involving reciprocal synapses and more than one postsynaptic element, as seen in the retina (diad synapses) or olfactory bulb, are not separated by a PAP but frequently ensheathed at their outer circumference. Further on, neighbouring axon terminals synapsing on a dendritic shaft or a soma may either lack intervening PAPs, or may be separated from one another by PAPs to varying degrees. Complete separation, resulting in a configuration of axon terminals alternating with PAPs, often occur in perisomatic (inhibitory) baskets, such as on hippocampal pyramidal cells, cerebellar Purkinje cells, inhibitory interneurones, and on output neurones in deep cerebellar nuclei.

Considerable differences in neuron–glial contacts can be found, even if the same brain region or related types of synapses are investigated. While 64% of hippocampal synapses are contacted by PAPs at the synaptic cleft, only 7% of have contacts at the postsynaptic spine and 8% at the presynaptic terminal (Witcher et al., 2007). A preference for post- vs. presynaptic contacts was observed on hippocampal PAPs (Lehre and Rusakov, 2002). When synapses were classified for type and spine structure, a PAP was found at the synaptic cleft in 90% of hippocampal mushroom spines and perforated synapses, i.e. at synapses of a high degree of differentiation and functional activity (Witcher et al., 2007). Similarly, the extent of glial synaptic cleft contact depends on the type of synapse, 64% for CA1 spine synapses (Witcher et al., 2007) but only 25% for all synapses. This variability may also depend on age or maturation, since lower values were found for hippocampal spine synapses in slightly younger animals (Lehre and Rusakov, 2002). Perisynaptic glia association also depends on the origin of the axon terminal, even if synapses on the same cell are compared; a significant difference in synaptic cleft ensheathment was found between terminals of parallel (65% ensheathed) and climbing fibres (87% ensheathed) on Purkinje cells (Xu-Friedman et al., 2001).

In summary, although many data have been accumulated, still we cannot answer the basic question why differences in perisynaptic glial ensheathment exist. We do not know any common type of neurotransmitter, neuron, synaptic compartment or common functional or developmental denominator that would predict whether a synapse will become associated with perisynaptic glia and to what extent.

4. Structural and functional domains

The complex interaction of astroglial processes and its neuronal neighbourhood can only be fully appreciated after 3D reconstructions of serial electron microscopic sections (Fig. 3). Unfortunately, there are still only few studies in which this cumbersome task has been undertaken (Ventura and

Harris, 1999; Grosche et al., 1999; Grosche et al., 2002; Genoud et al., 2006; Witcher et al., 2007).

Attributed to its involvement in learning and memory processes, the hippocampus is the best studied brain region in respect to synaptic plasticity. Several recent studies also demonstrated the role of astrocytes in modulating hippocampal neurotransmission (reviewed by Perea et al., 2009; but see Fiacco et al., 2009). Dye-filling of single cells, immunostaining against PAPs or genetic labelling by mosaic Cre/loxP mediated recombination in Brainbow mice reveals distinct territories of single astrocytes (Bushong et al., 2002; Derouiche et al., 2002; Livet et al., 2007). One astrocyte is thought to enwrap more than 100,000 synapses in rodents and probably more than a million in the human (Oberheim et al., 2006). This relation is depicted in Fig. 3A, where a single astroglial process of the stratum radiatum enwraps numerous synapses of several reconstructed dendrites at the CA3–CA1 synapse. The astroglial process can be decomposed into a number of subcompartments emanating from main processes. Magnified views demonstrate that the processes do not form a sponge-like net, but rather distinct arborizations and ramification with the terminals forming a morphological continuum varying between filopodia- and lamellipodia-like endings. The whole range of process structures can form tight contacts to the pre- and/or postsynapse (Figs. 3B and C). It has been shown recently that rising intracellular Ca^{2+} in single astrocytes enhances synaptic efficacy (Perea and Araque, 2007). However, we are still lacking data addressing the impact of Ca^{2+} changes in single subcompartments, i.e. in PAPs.

The case of glomerular synapses demonstrates that PAPs themselves can structurally separate functional units and establish neuropilar modules (Chao et al., 2002). In the cerebellum Bergmann glial cells enwrap synapses of the parallel fibres with the Purkinje cell dendrites. Here, several PAPs together constitute a common subcellular structure called a 'microdomain' (Fig. 3D–G) (Grosche et al., 1999; Grosche et al., 2002). Such a glial microdomain consists of a thin stalk and a small, cabbage-like head to which the synapse-associated PAPs belong. A microdomain may contain one or a few mitochondria and is thought to be capable of autonomous interaction with the ensheathed group of synapses. Computational modelling of microdomains indicates that each may be electrotonically independent of the stem process from which it arises, as well as of neighbouring domains (Grosche et al., 2002). These calculations were substantiated by monitoring intracellular Ca^{2+} rises in the Bergmann glial cells. When parallel fibres were weakly stimulated, Ca^{2+} transients in distinct glial cytoplasmic regions could be observed that corresponded in size to the morphologically defined microdomains. At increasing stimulus intensities, however, groups of microdomains, entire processes or even complete Bergmann glial cells displayed Ca^{2+} rises (Grosche et al., 1999). Thus, the microdomains – as well as higher hierarchical entities such as cell processes or entire cells – may vary in their activity or functional autonomy. This concept is underlined when progressing from microdomains to higher hierarchical structures of the adult cerebellum. It has been estimated for the rat that one of the 4 to 5 Bergmann glial cell processes contributes to glial ensheathing of 500 to more than 1000 synapses, a single cell forms the sheaths of 2000–6000 synapses and the eight Bergmann glial cells surrounding a single Purkinje cell can provide perisynaptic coverage of all its 17,000–51,500 dendritic spine synapses (Reichenbach et al., 1995). Thus, depending on the strength of neuronal activation and on the metabolic state of the tissue, glio-synaptic interactions may involve a wide range from individual synapses to larger functional domains (Reichenbach and Wolburg, 2008). However, it is conceivable that the transition between the hierarchical levels is not a continuum but rather occurs stepwise, due to morphologically (or functionally) organized 'gates'. Such 'gates' may be provided by the electrotonic resistance of the thin stalks of the microdomains (Grosche et al., 1999; Grosche et al., 2002) or by the cellular boundaries of individual glial cells. Glial cells are usually coupled by gap junctions with an open probability that varies, and may even be zero (Houades et al., 2008). It is intuitively evident from the regular spacing of telencephalic astrocytes that there is a spherical or ellipsoidal tissue compartment around every individual astrocyte soma which is occupied by only this astrocyte (Bushong et al., 2004; Livet et al., 2007). In immunocytochemical stainings for astrocytic proteins preferentially labelling PAPs such as ezrin (Derouiche and Frotscher, 2001), the approximate territorial boundaries and the 'tiling' become obvious (Fig. 1D–E). Indeed, when the extension of Golgi-stained astrocyte processes is arithmetically related to the mean inter-astrocytic distance (30–50 μm in rat cortex, depending on region), the non-overlapping center of an astrocyte territory has a diameter of 57 μm, that of the entire territory being 78 μm (rat cortex, Wolff and Chao, 2004). When the overlap is directly visualized by injecting two neighbouring astrocytes with different dyes, similar values can be obtained, resulting in only 4–6% territorial volume overlap (Ogata and Kosaka, 2002; Bushong et al., 2002). These cellular domains appear to develop early postnatally by a competitive interaction between neighbouring astrocytes (Bushong et al., 2004). Noteworthy, however, the overlap of neighbouring astrocyte territories may be considerably larger as seen in the cerebellum (Grosche et al., 1999; Grosche et al., 2002). It has been shown that within the tissue volume occupied by a microdomain, not all synapses are ensheathed by it. This prompted to the hypothesis that the two different functional populations of synapses on the Purkinje cell dendrite (Eilers et al., 1995; Denk et al., 1995) are interacting with different glial microdomains (Grosche et al., 1999). These findings were confirmed recently for the mouse cerebellum. Using a mosaic Cre/loxP approach (Brainbow transgenics) it was found that parallel fibre synapses at the same Purkinje cell were enwrapped by membrane appendages originating from different Bergmann glial cells (Livet et al., 2007).

While the idea of – hierarchically variable but morphologically defined – domains is appealing, there are recent data which may challenge it. Endogenous, spontaneous Ca^{2+} signals of Bergmann glial cells were analyzed by two-photon imaging in vivo (Hoogland et al., 2009; Nimmerjahn et al., 2009). These Ca^{2+} signals did not encompass single Bergmann glial processes, but they spread as a 3D ellipsoidal wave, clearly extending beyond a microdomain and involving PAPs from several Bergmann glial process. Such transglial waves are of a unitary size of approximately 50 μm. They are not further propagated and do not rely on local gap junctional

coupling but rather on the local diffusion of ATP which is released from a Bergmann glial process (or microdomain?). Thus, a functional readout of a glial network (i.e. the spontaneous Ca^{2+} signals) shows that at least glia–glia communication is not restricted to structural boundaries (cells, stem processes and/or PAPs). But, it is the dense and almost isotropic meshwork of PAPs within a defined spatial arrangement that represents the matrix for Ca^{2+}-signalling.

5. Dynamics and plasticity of perisynaptic glia

Astrocyte motility involves cell migration, the outgrowth of new stem processes and the transient extension and retraction of the peripheral filopodia and lamellipodia. Here, we focus on perisynaptic glial plasticity, i.e. the locally restricted and rapid changes in the shape of PAPs. Cell migration and outgrowth of stem processes are more general motility phenomena that occur preferentially during ontogenetic development and glioma formation, and will not be considered.

Still much of our knowledge about the plasticity of glial processes relies upon electron microscopic studies performed on fixed tissue samples. So far, the small size of the perisynaptic glial processes and the limited spatial resolution of light microscopy prevented the analysis of structural changes in real-time. Although it has long been assumed that glial morphology may undergo dynamic changes related to functional activity, this has been difficult to demonstrate directly. Quantitative comparison of electron micrographs of the visual cortex before and after optic nerve stimulation indicated an activity-induced swelling of the perisynaptic glial processes (Güldner and Wolff, 1973). The first unequivocal demonstration of function-related glial plasticity was not achieved at a typical synapse but rather at the neuron–blood interface of the hypothalamo-neurohypophysial system, i.e. the supraoptic nuclei of lactating rats (Theodosis and Poulain, 1984). The magnocellular neurones in this nucleus are 'normally' separated by glial processes. During lactation, however, more than 40% of neurosecretory somata and dendrites are in direct contact without glial separation. This ultrastructural change could modify extracellular ion homeostasis and glutamate neurotransmission. Therefore, it has been hypothesized that the direct neuronal contact could facilitate the synchronization of neuronal firing found during suckling-induced reflexes evoking milk ejections. Two months after weaning the glial separation is re-established demonstrating its plasticity.

Later, similar morphological changes of perisynaptic glial cell processes related to the activity of glutamatergic synapses in the hippocampal dentate gyrus were described after induction of long-term potentiation in rats (Wenzel et al., 1991) and at higher spatial and temporal resolution in organotypic slice cultures (Lushnikova et al., 2009). 3D reconstructions of synapses revealed significant increases of spine head volume and the area of the postsynaptic density (PSD) already 30 to 60 min after theta burst stimulation. Even more striking, the elevated level of synaptic activity augmented the glial coverage at both pre- and postsynaptic structures (Lushnikova et al., 2009).

Unfortunately, EM studies are hampered by their 'snapshot character'. Sophisticated and exhaustive quantitative analysis is required to compare brain sections from 'control' (e.g., untreated) animals with sections of the identical brain region from animals subjected to a defined stimulation and to demonstrate statistically significant differences. Apart from the methodological effort, there is a basic problem. The brain of a live animal is never a 'control' in the sense of a zero baseline. At the moment of the experiment, the brain has been the site of a multitude of information processing during its entire life. Every morphological 'snapshot' displays the result of both long-lasting and recent plasticity, even in control animals. The investigator sees only a presumed average of various previous activities at the individual synapses.

New staining procedures, the application of fluorescent proteins and the use of two-photon laser-scanning microscopy (2P-LSM) fostered the analysis of structural neuron–glia interactions at the time scale of minutes and faster (Fig. 4) (Feng et al., 2000; Nolte et al., 2001; Nimmerjahn et al., 2004; Helmchen and Denk, 2005). In brain stem sections of transgenic TgN(GFAP-EGFP) mice with fluorescently labelled astrocytes and active synaptic terminals, two distinct forms of spontaneous process motility were found: (i) gliding of thin lamellipodia-like membrane protrusions along neuronal surfaces and (ii) transient extensions of filopodia-like processes into the neuronal environment (Hirrlinger et al., 2004). Using organotypic slice cultures, the dynamics of astroglial processes were confirmed for the hippocampus and cerebellum (Haber et al., 2006; Nishida and Okabe, 2007; Lippman et al., 2008). In addition, it could be shown that the glial PAPs contacted also motile postsynaptic dendritic spines. Interestingly, the astroglial motility was higher than the neuronal (Haber et al., 2006). In the cerebellum motile Bergmann glial processes were stabilized during spine ensheathment (Lippman et al., 2008).

So far all of the above experiments were performed in acutely isolated or cultured brain slices derived from young postnatal brains. When such experiments were repeated in live, anaesthetized mice and older than 4 weeks, the dimensions of PAPs, in particular the thin lamellipodia, were found to be too small for light microscopy. As it has already been pointed out, with dimensions of 100 nm and less, the astroglial PAPs are below the resolution limits of in vivo 2P-LSM. Imaging in vivo is, so far, restricted to recordings from somata, main processes and bulging portions of PAPs. However, imaging of Ca^{2+} transients in live and awake mice revealed heterogeneous functional signals (Nimmerjahn et al., 2009). In the mouse cerebellum, Bergmann glia exhibit three forms of Ca^{2+} excitation: sparkles, bursts and flares. Sparkles occur highly localized and spontaneously. They are small and probably represent Ca^{2+} signals of microdomains. Burst signals are initiated in a single process, but their propagation involves several cells. Flares are Ca^{2+} waves that can spread over several hundreds of Bergmann glial cells, thereby extending across several hundred microns and are induced by locomotion.

The structural imaging studies show that PAPs can change their shape at a time scale of minutes. Since most investigations used tissue of young mice or even cultured tissue, it is tempting to speculate that such morphological changes are

more related to developmental aspects of the growing brain. This does not rule out structural changes in the adult animal, e.g. during a learning paradigm. However, cognitive brain function with expeditious signal integration and comparison will probably occur too fast to involve structural transformations of the neighbouring glia. Here, functional imaging of Ca^{2+} transients or other second messengers such as cyclic nucleotides comes into play. During neurotransmission such signals could be initiated in single PAPs and lead to extended waves sweeping through the complete brain region such as the cerebellar cortex.

6. Molecular mechanisms of perisynaptic glial dynamics and plasticity

Astrocytes and their processes are generally viewed as 'passively' adapting to their surrounding structures which might be intuitively apparent by looking at the highly complex morphology of PAPs (Fig. 3). To understand this striking morphology, it is helpful to consider that generation and differentiation of most vertebrate CNS glial cells occur relatively late in ontogeny, when most of their prospective contact structures (the pia mater, blood vessels, and neurons including many of their synapses) are already established. Thus, the young glial cells and their processes would have to integrate into this preexisting environment. In accordance with this scenario, it has been recently shown that glial cells are softer than neurons (Lu et al., 2006). The morphology of glia has, thus, been compared to a fluid concrete mass poured on a bed of pebbles. Indeed, the extremely thin PAPs fill in virtually all inter-neuronal spaces by forming typical concave curvatures as if pressed into these spaces (Figs. 2B, C and 3A, D). As illustrative as this scenario may be, it is only half the truth. Not every neuronal synapse is covered by a glial process, not to mention that the synaptic clefts proper are devoid of glia.

Thus, when addressing the mechanisms of perisynaptic glial plasticity experimentally, the two key questions are: (i) what is the cellular (biomechanical) machinery providing astroglial motility, and (ii) what are the signalling mechanisms triggering an outgrowth of fine astrocytic processes, and directing them (or not) towards a given synaptic element?

6.1. *Intracellular mechanism of motility*

Biomechanically, astroglial motility may be based upon either 'active' forces involving the actin cytoskeleton or on 'passive' mechanisms such as cell swelling (or both). Unfortunately, the available data do not allow for a clear-cut identification of the (main) mechanism(s). The complex morphology of PAPs is compatible with a role for rather passive mechanisms, such as process swelling (i.e. an osmotic pressure-mediated transformation). It is conceivable that the pathways leading to astrocyte swelling under pathological conditions (Mongin and Kimelberg, 2005) might also be physiologically effective, in a locally restricted and more subtle manner. This might preferentially modify the shape of the PAPs (rather than of the stem processes and somata). Indeed the PAPs, in contrast to the stem processes, appear to be well-suited for swelling, as they display an enormous membrane surface enclosing a negligible volume (Chao et al., 2002). Thus, insertion of lipids into the membrane and water entry could lead to instantaneous changes in shape that could account for the 'adaptive' morphology of PAPs.

Astroglial swelling is suggested to be mediated by aquaporins (Manley et al., 2000; Kimelberg, 2004). Aquaporin 4 (AQP-4), an astrocyte-specific water channel protein, has immunohistochemically been localized predominantly to perivascular endfeet rather than PAPs. However, this could be a matter of spatial resolution and sensitivity. A pronounced AQP-4 staining on PAP membranes around synapses has been ultrastructurally demonstrated (Nagelhus et al., 2004) which appears only as a diffuse background in fluorescence microscopy. Perisynaptic AQP-4 localization, thus, fulfils the structural prerequisite for mediating PAP plasticity. Its mode of action may involve the colocalization with the major astrocyte-specific subunit of inwardly rectifying K^+ channels, Kir 4.1 (Nagelhus et al., 2004). Kir 4.1 and AQP-4 may co-operate to induce PAP re-shaping by mediating K^+ and water entry from extracellular space. Furthermore, the involvement of elevated extracellular K^+ might also provide a link between increased neuronal activity and PAP remodeling. Astrocytic swelling has also been elicited by glutamate (Koyama et al., 1991; O'Connor et al., 1993) although these experiments appear less relevant for PAP plasticity since glutamate was applied in vitro, at very high concentrations (0.5–1 mM). Another potential player, Cl^- channels – beyond their role in astrocytic swelling (Mongin and Kimelberg, 2005) – have been functionally associated with glial re-shaping. For instance, inhibition of (volume-activated) Cl^- currents results in a reduced migration of glioma cells (Ransom and Sontheimer, 2001). A similar mechanism has been suggested for the dynamic process motility of microglia (Hines et al., 2009). In astrocytes, the subcellular distribution of Cl^- channels has been established for some channel types. In hippocampus, the chloride channel-2 (ClC-2), a predominant astroglial Cl^- channel, is restricted to the perivascular endfeet and to some glial processes covering pyramidal cell somata and proximal dendrites, but is absent from perisynaptic processes (Sik et al., 2000).

'Active' intracellular motility mechanisms of perisynaptic PAPs proper have been addressed by only very few studies. From live imaging in hippocampal slice culture, it has been concluded that the motility of perisynaptic PAPs is dependent on actin mechanisms since it is reduced after treatment with cytochalasin D (Haber et al., 2006). Confirming this conclusion, the PAP motility in relation to outgrowing dendritic spines was diminished by a dominant-negative molecular approach in slice cultures, suppressing astrocytic Rac1 signalling (Nishida and Okabe, 2007). Rac1 is known to organize actin-based motility. But likely, this finding also applies to PAPs in any perisynaptic position. Structural support for a role of actin mechanisms in perisynaptic PAPs comes from the preferential localization of actin-binding proteins in PAPs (as opposed to stem processes). These proteins include ezrin (Fig. 1D (Derouiche and Frotscher, 2001) and α-adducin (Seidel et al., 1995). Ezrin is a plasma-to-cytoskeleton linker which enables rapid re-shaping (in less than a few min) by phosphorylation/dephosphorylation. Thus, changes in protein expression or cytoskeleton breakdown are not required (Gautreau et al., 2002). The adducins are actin-capping proteins and participate

in forming submembraneous protein complexes with spectrin and actin. They may also be involved in the organization and rapid plasticity of the actin cytoskeleton in PAPs. These observations strongly indicate that active, actin-related mechanisms are active within the PAPs. Thus, the current concept of the ultrastructurally 'empty' PAP as a rather passive structure prone to swelling, has to be complemented by the view of an active element, equipped with a highly regulated biomechanical machinery.

The relative contribution of swelling- versus actin-based mechanisms for perisynaptic glial plasticity remains to be elucidated. However, the two mechanisms must not be considered as mutually exclusive; rather, there appears to be some interplay. The application of actin-depolymerizing agents – such as cytochalasin B or D – to primary astrocyte cultures leads to morphological changes and to concurrent activation of outwardly rectifying Cl^- currents. These Cl^- currents depend on cytoskeletal alterations (Lascola et al., 1998). A comparable effect on Cl^- currents is seen upon hypo-osmotic challenge, brief trypsinization, or exposure of astrocytic membrane patches to actin. This indicates that cytoskeletal mechanisms lead to activation of outwardly rectifying Cl^- channels and that actin interacts with the channel protein (Lascola et al., 1998). Similar results have been obtained with the ClC-2 protein, which can bind actin directly (Ahmed et al., 2000). Vice versa, mechanical stimulation of glial cells was shown to induce rises in the intracellular Ca^{2+} concentration (Newman, 2001). Swelling of the PAPs may cause similar Ca^{2+} transients which then may trigger active biomechanical responses involving the cytoskeleton.

Perisynaptic PAP dynamics involve process outgrowth, comparable to the postnatal formation of glial processes, which leads to regional PAP patterns related to glutamatergic terminal areas (Derouiche et al., 1996). Thus, the mechanisms of postnatal PAP formation and of life-long, activity-dependent PAP plasticity might be similar. Also, the dynamics of peripheral glial processes in situ and of astroglial filopodia in tissue culture are comparable in time, as both can elongate within a few minutes in acute slice preparations (Hirrlinger et al., 2004) or in cultured astrocytes, respectively (Cornell-Bell et al., 1990). Thereby, the formation and motility of postnatal glial processes may represent a functional adaptation also in the adult brain.

6.2. *Extracellular regulation of astroglial process motility*

Still sparse is our knowledge of the signalling mechanisms triggering and directing astroglial process growth. Primary astrocytes rapidly react to bath application of glutamate by filopodia formation (Cornell-Bell et al., 1990). However, there are hardly any further reports on stimuli for PAP or filopodia formation. In cultured Müller cells it was shown that elevated levels of extracellular potassium ions stimulate the formation of new branches (Reichelt et al., 1989). These scarce observations support the idea that synaptic activity (known to cause extracellular K^+ ion rises and glutamate release, at glutamatergic synapses) may directly deliver signalling molecules which are 'sensed' by adjacent glial cell membranes, via their numerous metabotropic and more limited amount of ionotropic receptors.

And indeed, structural plasticity of PAPs is mediated by a bidirectional neuron–glia pathway with exchange of neuropeptides and glia-derived neurotransmitters ("gliotransmitters"). In the supraoptic nucleus of lactating rats neuronal oxytocin release reduces the glial coverage resulting in lower levels of the gliotransmitter D-serine and attenuated activity of neighbouring neuronal NMDA receptors (due to reduced occupancy of the glycine binding site by D-serine) (Langle et al., 2002; Panatier et al., 2006). These findings strongly suggest that defined neuron–glia interactions are a prerequisite for structural and functional cooperativity in the CNS.

While a direct adhesive mechanism of PAPs and synapses has not yet been shown, a repulsive interaction that regulates postsynaptic morphology has been described recently (Murai et al., 2003). The EphA4 receptor tyrosine kinase is enriched on dendritic spines of pyramidal neurons in the adult mouse hippocampus, while its ligand ephrin-A3 is localized on the perisynaptic astroglial processes that enwrap spines. The astroglial ephrin-A3 activates the neuronal EphA4 and induces spine retraction. However, inhibiting ephrin-A3/EphA4 interactions reduces spine stability resulting in shape distortion and improper organization. The transient interactions between the ephrin-A3 ligand and the EphA4 receptor are essential components of the astroglia-dependent stabilization and structure of excitatory synapses (Murai et al., 2003; Nishida and Okabe, 2007; Nestor et al., 2007). In addition, the interaction of EphA4/ephrin-A3 determines the perisynaptic expression of astroglial glutamate transporters and, thereby, modulates synaptic long-term potentiation (Filosa et al., 2009).

So far, we have seen only a small subset of molecules modulating perisynaptic neuron–glia interactions. Other factors for cell adhesion and cell recognition are integrins and extracellular matrix molecules (Hama et al., 2004; Dityatev et al., 2006). It is obvious that the mechanisms controlling the formation and plasticity of perisynaptic PAPs largely remain to be elucidated and will require the development of novel approaches.

7. Limits of observation

Time-lapse recordings using confocal and two-photon laser-scanning microscopy have been a major step forward to our understanding of structural neuron–astroglia interaction. Studies could be performed in acutely isolated or cultured brain slices under rather defined conditions. Simultaneously, interfering substances can easily be applied. However, such tissue slices come with several drawbacks: First, for technical reasons (tissue scattering and survival) one is mostly confined to slices obtained from rather young animals. Second, not all slice preparations are suitable for long-term culture due to neuronal degeneration. Third (and rather obvious), a slice cannot replace the living animal.

Genetically modified mice have become an important model to study neuron–glia interaction in vivo. On one hand, there is the possibility to directly target glial or neuronal genes; on the other hand, fluorescent protein labelling allows the direct visualization of structural interactions. However, limitations remain. Although we can ablate genes-of-interest in astrocytes or neurons inducibly and selectively, we are restricted to a global deletion. Yet, we do not have tools in our hand to reliably

manipulate only cortical or hippocampal astrocytes, for example. The transgenic expression of the split-Cre system under two different astroglial promoters could provide a solution to this problem (Hirrlinger et al., 2009a,b).

In vivo two-photon imaging has generated a wealth of novel information on brain function. But, we are limited to a spatial resolution of about 1 μm. While this dimension is sufficient to study pre- and postsynaptic compartments, it is not suitable to visualize the tiny perisynaptic processes. New promises are provided by sub-micrometer super-resolution imaging such as stimulated-emission depletion (STED; Willig et al., 2006; Nagerl et al., 2008; Wildanger et al., 2009), photoactivation localization (PALM; Shroff et al., 2008), stochastic optical reconstruction (STORM; Huang et al., 2008) and total internal reflection (TIRF; Axelrod et al., 1983; Li et al., 2008) microscopy. Unfortunately, these techniques are still limited to the analysis of cultured cells or to fixed material attributed to their limited depth penetration or long sampling times. The development of novel genetic targeting approaches and super-resolution imaging techniques for in vivo-use will be an essential requirement to advance our understanding of brain function in general and neuron–glia interactions in particular.

8. Conclusions and open questions

So far, perisynaptic glial elements can be identified only topologically, based on their close association with neuronal compartments of the synapse. Yet, we do not know any specific molecular or ultrastructural features. Simultaneously, only a subset of all synapses (dependent on region, type of synapse or activity) is contacted by glial processes. Importantly, there is no common principle for perisynaptic glial ensheathment. The molecular and developmental mechanisms determining the absence or presence of glial cell processes at a given synapse remain to be elucidated. The majority of the peripheral astrocyte processes, including those in perisynaptic position are below light microscopic resolution.

The tripartite synapse is a central entity of the brain. However, we still have only a limited knowledge on the mechanism leading to its formation, maintenance and turnover, both in situ and in vivo.

Future studies have to address the following open questions:

- What is the molecular repertoire of the perisynaptic astroglial process, in the cytoplasm as well as in the membrane?
- How is the glial coverage of pre- and postsynaptic terminals controlled?
- What kind of specific differences in glia coverage or molecular repertoire can be identified at different types of synapses such as excitatory or inhibitory ones?
- Can super-resolution imaging techniques be optimized to visualize the dynamic interactions of the extremely thin glial processes with their adjacent neuronal surface?
- Can we functionally perturb the glial elements with super-resolution at nanometer scale to reveal its impact on synaptic transmission?

By answering these questions we will experience a fascinating journey into the most complex organ of our body.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainresrev.2010.02.003.

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BRAIN RESEARCH REVIEWS

Review

Contributions of astrocytes to synapse formation and maturation — Potential functions of the perisynaptic extracellular matrix

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ABSTRACT

The concept of the tripartite synapse proposes that in addition to the presynapse and the postsynaptic membrane closely apposed processes of astrocytes constitute an integral part of the synapse. Accordingly, astrocytes may influence synaptic activity by various ways. Thus glia- and neuron-derived neurotrophins, cytokines and metabolites influence neuronal survival, synaptic activity and plasticity. Beyond these facts, the past years have shown that astrocytes are required for synaptogenesis, the structural maintenance and proper functioning of synapses. In particular, astrocytes seem to play a key role in the organization of the brain's extracellular matrix (ECM) — most prominently the so-called perineuronal nets (PNNs), complex macromolecular assemblies of ECM components. Due to progress in cellular and molecular neurosciences, it has been possible to decipher the composition of ECM structures and to obtain insight into their function(s) and underlying mechanisms. It appears that PNN-related structures are involved in regulating the sprouting and pruning of synapses, which represents an important morphological correlate of synaptic plasticity in the adult nervous system. Perturbation assays and gene elimination by recombinant techniques have provided clear indications that astrocyte-derived ECM components, e.g. the tenascins and chondroitinsulfate proteoglycans (CSPGs) of the lectican family participate in these biological functions. The present review will discuss the glia-derived glycoproteins and CSPGs of the perisynaptic ECM, their neuronal and glial receptors, and in vitro assays to test their physiological functions in the framework of the synapse, the pivotal element of communication in the central nervous system.

Contents

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1. The concept of the tripartite synapse

The synapse as central information relay of the nervous system consists of the presynapse and the postsynapse separated by the synaptic cleft. It represents the central functional element of the nervous system. (Choquet and Triller, 2003; Garner et al., 2002; Sanes and Lichtman, 1999, 2001). This functional unit is formed during development of the nervous system and is subject to malleability in the adult nervous system (Araque et al., 2001). Until recently, our knowledge about the machinery involved in the assembly of the central nervous system (CNS) synapse was sparse. Clear evidence has accumulated that, similar to the peripheral nervous system, the CNS synapse is composed of three elements, namely the presynapse, the postsynaptic membrane and the nearby astrocyte and its processes that make intimate contact with the neuronal synaptic structures (Fig. 1) (Haydon, 2001; Sanes and Lichtman, 2001; Slezak and Pfrieger, 2003). Functional participation of astrocytes in stability and integrity of synapses had originally been inferred on the basis of morphological studies, for example the role of astroglia in the generation of the neuronal networks and the observation of astroglial processes in the proximity of CNS synapses by transmission electron microscopy (TEM) (Araque et al., 1999a,b). With the help of TEM, the phenomenon of synaptic stripping could be documented in the facial nucleus after peripheral lesion of the facial nerve, where astrocytes seal the neuronal cell bodies and withdraw afferent synapses (Scheiffele, 2003; Theodosis et al., 2008). Regulation of the proportion of neuronal surface covered by astrocytes has also been reported for hypothalamic nuclei, where the extent of astroglial-derived appositions varies with physiological parameters (Langle et al., 2002; Theodosis et al., 2008). The maintenance of plasticity in this brain region correlates with the expression of extracellular matrix (ECM) glycoproteins that are otherwise down-regulated in the CNS, namely tenascin-C, which is mainly expressed by astrocytes (Theodosis et al., 1997). Following this concept, co-culture experiments clearly indicated that astrocytes release molecules that are required to sustain the structural maturation as well as the functional activation of synapses (Beattie et al., 2002; Nagler et al., 2001; Pfrieger and Barres, 1997; Slezak and Pfrieger, 2003; Song et al., 2002; Ullian et al., 2001, 2004b). Some of these are released into the medium of astrocyte cultures and enriched in the high molecular weight fraction, displaying characteristic biochemical properties of

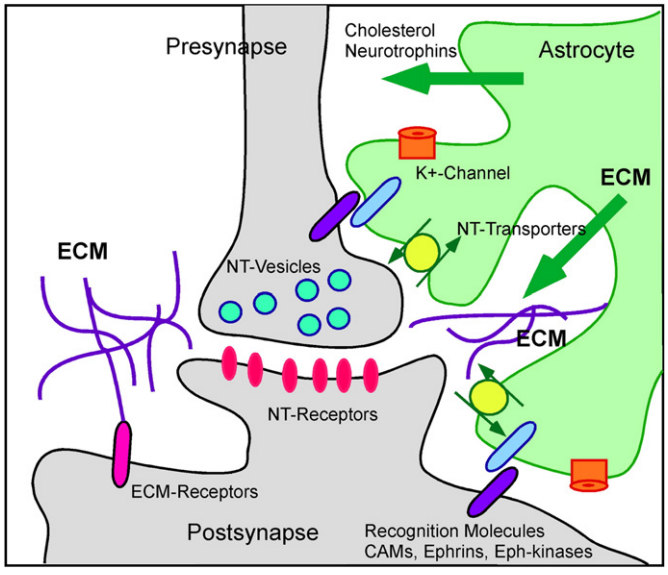

Fig. 1 – Concept of the tripartite synapse. The cartoon illustrates the concept of the tripartite synapse. Astrocyte processes are in close neighborhood of the pre- and postsynapse and interact with these functional elements in various multiple functional pathways. Thus, astrocytes release factors including ECM, cytokines and neurotransmitters (so-called gliotransmitters), metabolites such as cholesterol and interact with neurons via cell–cell and cell–ECM based adhesion mechanisms. For details see text.

ECM. Meanwhile, cholesterin and ApoE have been identified as components of conditioned media involved, but the high M_r fraction possesses synapse maturation-inducing effects in its own right (Mauch et al., 2001; Slezak and Pfrieger, 2003).

The present review will discuss the functional role(s) of glial-derived ECM molecules on synapse formation and plasticity. In particular, we will briefly survey some aspects form the vertebrate neuromuscular junction, where the functional role of the synaptic ECM is well understood, and introduce perineuronal nets (PNN) and PNN-like structures of the CNS, which serve as major ECM structures in the adult brain, before we discuss glia-derived ECM molecules and molecular mechanisms involved in synaptogenesis and synaptic plasticity.

2. Lessons from the vertebrate neuromuscular junction

Analysis of the peripheral synapse at the vertebrate neuromuscular junction, a structure that is much larger than a CNS synapse, revealed initial information about the intimate structural and functional interaction of neurons and glial cells at synapses (Burden, 1998; Sanes and Lichtman, 1999). The corresponding motor unit comprises a motor neuron in the anterior horn of the spinal cord, a motor nerve, the terminal on the myotube, and a non-myelinating Schwann cell in the vicinity of the synapse. A basal lamina surrounds the motor unit, and the subsynaptic space contains ECM components that are locally specialized. This ECM encodes significant information with regard to the site of synapse formation on the myotube, as illustrated by the findings that regenerating axons form a synapse precisely at the former site of the junction in dependence of the local ECM. Likewise, the ECM dictates the local accumulation of nicotinic acetylcholine receptors in the myotube membrane at the site of the former neuromuscular junction. Moreover, specific synaptic laminins seem to organize the exact glia-synapse geometry by preventing Schwann cell processes to enter the synaptic cleft (Patton et al., 1998).

Systematic analysis has shown that a pivotal component of the ECM in this process is the molecule agrin, a heparan sulfate proteoglycan of the ECM that occurs in several isoforms. It interacts with a receptor complex in the myotube membrane that contains the tyrosine protein kinase MuSK and dystroglycan. Downstream of MuSK, the molecule rapsyn is required for clustering the acetylcholine receptors (Burden, 1998). The synthesis of these neurotransmitter receptors is under the control of neuregulin (formerly ARIA, for "acetylcholine receptor inducing activity"), which is a ligand of erbB receptor tyrosine kinases. Activation of this pathway contributes to the induction of receptor message in the nuclei beneath the neuromuscular junction (Sanes and Lichtman, 1999). In parallel, the expression of the acetylcholine receptor genes is suppressed in extrajunctional nuclei by a pathway involving the activation of protein kinase C and the influx of calcium. Downstream, repression is mediated by inactivation of myogenic factors such as myoD and myf 5 that bind to the E-box-containing sequences in the promoter of the acetylcholine receptor gene. Interestingly, ARIA/neuregulin is anterogradely transported down the axon and incorporated into the junctional ECM, again highlighting the functional significance of this extracellular compartment for synaptic function (Burden, 1998; Sanes and Lichtman, 1999). Schwann cells were shown to express active agrin isoforms (Yang et al., 2001), but they also secrete TGF-ß1 as a key promoting factor for synaptogenesis, possibly via upregulation of agrin in the spinal neurons (Feng and Ko, 2008b). The multisided role of perisynaptic Schwann cells in the development, transmission and maintenance of NMJs is comprehensively reviewed in (Feng and Ko, 2008a).

3. The ECM at CNS synapses

While the vertebrate neuromuscular junction is characterized by a prominent ECM structure, the basal lamina, which is visible in the electron microscope and serves important functions in synaptogenesis and synaptic transmission (Sanes and Lichtman, 1999), much less is known about the ECM of CNS synapses, which do not form a basal lamina and have a less wide synaptic cleft (Sykova, 2001). Several lines of evidence suggest, however, that nonetheless it is at least in part composed of ECM components (Chavis and Westbrook, 2001; Dityatev and Schachner, 2003). Thus by cryo-electron microscopy of vitreous sections periodically organized high-density 8.2 nm complexes have been observed (Zuber et al., 2005), the molecular identity of which is currently unclear.

The most prominent ECM structures in the CNS are the so-called perineuronal nets (PNN). Originally they have been discovered by the pioneers of brain cell biology including Camillo Golgi and Santiago Ramon y Cajal (Celio et al., 1998). PNNs rich in specific chondroitin sulfate epitopes reside in particular on GABAergic, parvalbumin-positive neurons that frequently also express potassium channels containing Kv3.1b subunits (Hartig et al., 1999). They have been detected in the vicinity of glial processes, which abut onto the CNS synaptic unit and are thought to represent a molecular scaffold deposited in the interface between the astrocyte and subsets of neurons (Bruckner et al., 2004; Celio et al., 1998; Matthews et al., 2002). With staining techniques not based on lectin immunohistochemistry but on immunofluorescence, PNN-like ECM structures have nowadays also been observed covering a wide range of other brain neurons (Fig. 2) (Alpar et al., 2006; Dityatev et al., 2007; John et al., 2006; Wegner et al., 2003).

A number of different functions have been attributed to PNNs. These include the generation of polyanionic ion-buffering local environments, trapping and local concentration of growth factors and trophic factors, generation of permissive or non-permissive substrates for growth cones during development or regeneration, and linkage of intracellular cytoskeletal elements of neighboring cells (Blumcke et al., 1994; Bruckner et al., 1993; Celio et al., 1998; Hartig et al., 1999). Moreover, PNN have been attributed a protective role against oxidative stress (Morawski et al., 2004) and are documented to be modified in response to cerebral ischemia (Hobohm et al., 2005). At synapses, PNN may act as diffusion barriers for extracellular signalling molecules including neurotransmitters (thus preventing transmitter spillover), they may have a role in stabilizing synapses and in synaptic plasticity as well as in the compartmentalization of the neuronal surface (Frischknecht and Seidenbecher, 2008; Frischknecht et al., 2009, for details see below).

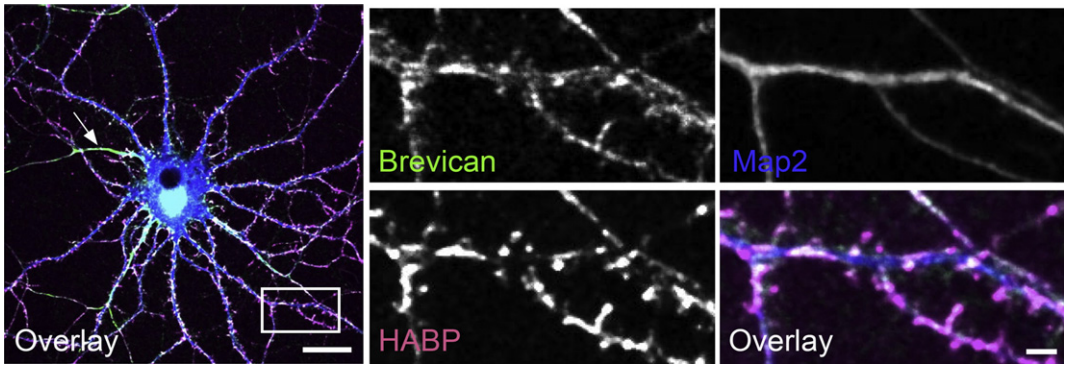

Fig. 2 – PNN-like structures on mature neurons in culture. The CSPG brevican (green) co-localises with hyaluronic acid, as visualized by binding to hyaluronic acid binding protein (HABP, red) on soma (left) and dendrites (high magnification images of the boxed area on the right) of an excitatory hippocampal neuron after 4 weeks in culture. The somato-dendritic marker protein MAP2 is stained in blue. The brevican-containing ECM forms a net-like structure on the surface of neurons (for details see John et al., 2006). Note, that brevican is also found on the axon initial segment (arrow) where it constitutes a hyaluronic acid-free ECM specialization. Scale bars: left 10 μm, right 2 μm.

The functional significance of these PNN-like structures has been highlighted by the discovery that their destruction by injection of chondroitinase ABC into the CNS restores ocular dominance column plasticity in the adult visual cortex (Pizzorusso et al., 2002; Pizzorusso et al., 2006). This enzyme destroys chondroitin sulfate chains, which are a constituent of a subclass of CNS proteoglycans, the chondroitin sulfate proteoglycans (Bandtlow and Zimmermann, 2000; Rauch, 2004), which will be referred to as CSPGs in this review. It should be noted, that depending on pH conditions chondroitinase ABC also has hyaluronidase activity and degrades hyaluronic acid (HA) in addition to chondroitin sulfate glycosaminoglycan (CS-GAG) chains (Yamagata et al., 1968). CSPGs with distinct core proteins have been identified in the CNS, including neurocan, brevican, versican, aggrecan, NG2 and phosphacan, several of which are produced by glial cells, primarily astrocytes, or oligodendroglial precursors (OPCs) (Bandtlow and Zimmermann, 2000; Harroch et al., 2002). Thus CSPGs are prime candidates to mediate astroglial influences on synapse formation and stability (Dityatev and Schachner, 2003; Dityatev and Schachner, 2006; Kawachi et al., 1999). Moreover, CSPGs are also highly concentrated in PNNs and PNN-like structures that are closely associated with the interface between glial processes and the synapse (Celio et al., 1998; Langle et al., 2002; Matthews et al., 2002).

Purification and analysis of synaptic junctions from the mammalian brain, the so-called postsynaptic density (PSD) protein fraction, has revealed ECM components closely associated with CNS synapses. One of these synapse-associated proteins is brevican (e.g. Li et al., 2004; Seidenbecher et al., 1995), a member of the lectican/hyalectan family of CSPGs (Bandtlow and Zimmermann, 2000; Yamaguchi, 2000). Brevican is primarily synthesized by astrocytes (John et al., 2006; Yamada et al., 1994) and constitutes as a major component the hyaluronan-based adult ECM of the brain (Rauch, 2004; Yamaguchi, 2000). Hyaluronan (HA) expression in the brain is a long proven fact (Aquino et al., 1984a,b; Margolis et al., 1975). HA interacts with various CSPGs of the ECM in the CNS (Bignami et al., 1993), in particular within PNNs (Carulli et al., 2006; Galtrey et al., 2008). The knowledge that HA is not present within the synaptic cleft of CNS synapses dates back to the 1960s, when the resistance of synaptic cleft macromolecules to hyaluronidase treatment (Bloom and Aghajanian, 1968) and the unaffected morphology of synaptic profiles in hyaluronidase-digested synaptosomes were demonstrated (Bondareff and Sjostrand, 1969). However, clear functional implications of HA expression at synaptic sites have emerged only recently.

4. Cell-interaction molecules and ECM components in synaptogenesis

With regard to cell interactions involved, several types of cellular recognition molecules are being discussed. For example, the homophilic cadherins of the calcium-dependent superfamily of adhesion molecules have been implicated in recognition events between pre- and postsynaptic membranes (Tanaka et al., 2000; Wu and Maniatis, 1999). Similarly, the neurexins and neuroligins, trans-interacting cell adhesion molecules bridging the synaptic cleft, which occur in large numbers of isoforms, have been attributed a role in synapse stabilization (Choquet and Triller, 2003). Altogether a whole range of cell adhesion molecules has been implicated in synaptogenesis and synaptic maturation (for review see (Dalva et al., 2007). Thus, several gene families including cytokines (Beattie et al., 2002), Eph-kinases and ephrin ligands (Aoto and Chen, 2007; Aoto et al., 2007; Contractor et al., 2002; Klein, 2009), cell adhesion molecules, ECM glycoproteins (Patton et al., 2001) including agrin (Matsumoto-Miyai et al., 2009), complementary integrin receptors (Chavis and Westbrook, 2001), NARP/NP2, wnt7A, FGF22 (Sanes and Lichtman, 2001) and several intracellular scaffolding molecules that anchor subsynaptic receptors (Choquet and Triller, 2003; El-Husseini et al., 2000) are implicated in membrane-mediated interactions between the pre- and postsynaptic membranes (Fig. 1) (Chavis and Westbrook, 2001; Contractor et al., 2002).

Strikingly not only cell adhesion between pre- and postsynaptic terminals is important for synapse formation. Thus

neuron–glia interactions between glial ephrin-A3 and neuronally expressed EphA4 regulate hippocampal dendritic spine morphology and long-term potentiation via glutamate transport (Carmona et al., 2009; Filosa et al., 2009). Another study has demonstrated that local astrocytic contact facilitated excitatory synaptogenesis through integrin signalling (Hama et al., 2004).

Furthermore astrocytes release CSPGs into the culture medium (Carulli et al., 2005; Siddiqui et al., 2009; Slezak and Pfrieger, 2003). Interestingly, the high Mr fraction of astrocyte-conditioned culture medium possesses synapse maturation-inducing properties on its own right (Christopherson et al., 2005; Ullian et al., 2004a). In the case of retinal ganglion cells, some of the molecular species involved could be identified. These include members of the thrombospondin (TSP) gene family, which were revealed as one important constituent of astrocyte-conditioned medium that impacts on synapse formation in vitro. Five TSPs exist, each encoded by a separate gene. TSP1 and TSP2 are closely related trimeric proteins that share the same set of structural and functional domains. TSP4 is a pentameric protein and localises to some synapses of the adult CNS and to the neuromuscular junction. Although necessary for the emergence of structurally intact synapses, additional astroglial-derived signals are required to obtain a fully functional synaptic structure (Christopherson et al., 2005). Indeed, synapses formed in the presence of TSPs release neurotransmitters from their presynapses, but remain silent on the postsynaptic site because AMPA-type glutamate receptor responses are missing. Recently, the hitherto elusive TSP receptor implicated in synaptogenesis was identified as the alpha2delta1 Gabapentin receptor that is part of a voltage-gated calcium channel expressed by neurons (Eroglu et al., 2009). Restitution of the correct postsynaptic current requires additional, as yet unidentified signals. Along these lines, it has recently been shown that the reorganization of synaptic connections requires the complement cascade and that astrocytes induce expression of the complement factor C1q in neurons (Stevens et al., 2007).

5. Role of tenascins and lecticans for synaptic function

Tenascin-C as well as tenascin-R are present in perineuronal nets (Celio et al., 1998), which in addition regulate the formation of myelin membranes in the CNS (Czopka et al., 2009). The importance of tenascin-C for the function of CNS synapses is underscored by studies in knock-out mice which display dramatic modifications of synaptic activity in the hippocampus (Evers et al., 2002). A role for tenascin-C in the regeneration of the neuromuscular junction has been demonstrated in amphibians by antibody perturbation and in mice using the knock-out strategy (Cifuentes-Diaz et al., 1998; Cifuentes-Diaz et al., 2002; Irintchev et al., 1993). The latter result, however, is controversial. Summarizing these findings, it appears that morphological analysis, the expression patterns of ECM components, selective perturbation approaches and gene elimination studies concur to support the notion that astrocytes contribute to synapse formation and maintenance, and that at least part of these influences are exerted by astroglial-derived ECM (Dityatev and Schachner, 2006). In this regard, it is noteworthy that some ECM glycoproteins directly modulate the activity of membrane channels. Thus, tenascin-C and tenascin-R affect the L-type calcium channel and the voltage-gated sodium channel beta subunit, respectively (Evers et al., 2002; Xiao et al., 1999).

Along these lines, studies in the gene elimination models of tenascin-R and tenascin-C have revealed modification of synaptic structures and activity. Thus, the generation of LTD is impaired in tenascin-C-deficient mice (Cifuentes-Diaz et al., 2002; Dityatev and Schachner, 2003; Evers et al., 2002; Xiao et al., 1999). Phenotypes of knock-out animals for ECM components are summarized in Table 1.

Less is known about the molecular mechanisms involved in the tenascin-dependent effects on synapse function. Recent results of our laboratories suggest that tenascin-C might be implicated in the modulation of intracellular Ca^{2+}-levels and regulates fiber outgrowth of embryonic hippocampal neurons, thus gearing neuronal functions (Michele and Faissner, 2009).

Tenascins are known to interact extracellularly with CSPGs of the lectican type like neurocan or brevican (Aspberg et al., 1997; Grumet et al., 1994; Hagihara et al., 1999). While neurocan is a constituent mainly of the ECM of the juvenile brain, the expression of brevican is up-regulated only late during brain development, i.e. after the major period of synapse formation (Milev et al., 1998). Mice deficient for either brevican or neurocan have been analyzed for the role of these proteins in brain development and plasticity (Table 1). Neurocan knock-out mice display mild deficits in synaptic plasticity, i.e. the maintenance of long-term potentiation (LTP) at CA1 hippocampal synapses is impaired, but appear otherwise normal (Zhou et al., 2001). In particular the PNN seem to be not affected by the mutation. Brevican mutants, in contrast, display a more severe impairment of CA1 LTP maintenance and display fuzzy PNN (Brakebusch et al., 2002). Interestingly, the LTP phenotype can be mimicked acutely by applying anti-brevican antibodies to rat hippocampal slice preparations. Brevican–neurocan double-mutants are also viable. They display impairments of synaptic plasticity (Seidenbecher et al., unpublished data).

The fact that components of the hyaluronan-based ECM do have no vital functions in the vertebrate brain but rather serve more subtle tasks in brain development and plasticity is underscored by the generation of quadruple knock-out mice for brevican, neurocan, tenascin-C and tenascin-R (Rauch et al., 2005). These mice seem to synthesize an alternative matrix in the brain, which incorporates fibulins. Altogether, Tenascins as well as lecticans/hyalectins have a large variety of functions in classical and homeostatic synaptic plasticity as well as in metaplasticity of synaptic networks (Dityatev and Fellin, 2008).

6. Signalling downstream of ECM and CAM-mediated interactions

After having reached their respective targets, the neurons differentiate and extend axons to build connections with other neurons and dendrites to receive information from different regions of the nervous system (da Silva and Dotti, 2002).

Table 1 – Functions of glia-derived ECM molecules in the CNS.

Protein	Phenotype	Reference
Tenascin-C	Tenascin-C knock-out mice (TNC ko) show ...	
	Reduced plasticity after vibrissectomy	Cybulska-Klosowicz et al. (2004)
	Reduced LTP after TBS in CA1 and reduction of L-type VDCCs	Evers et al. (2002)
	Hyperlocomotion, stereotyped turning behavior, poor swimming, reduced dopamine transmission in hippocampus and striatum	Fukamauchi and Kusakabe (1997)
	Delayed habituation to new environments, poor appetite, abnormal circadian rhythms, low pregnancy rate probably due to reduced neuropeptide Y expression in the limbic area	Fukamauchi et al. (1998)
	Increased rate of OPC migration along the optic nerve, reduced OPC proliferation in different CNS regions	Garcion et al. (2001)
	Accelerated rate of maturation, earlier morphological maturation, precocious MBP expression, higher apoptosis of OPCs in vitro, earlier loss of PDGF response, enhanced myelin membrane formation in OPCs generated from TNC knock-out neurospheres	Garwood et al. (2004)
	Increased oligodendrocyte differentiation on TNC deficient astroglial ECM matrix	Czopka et al. (2009)
	Worse functional recovery after facial nerve transaction	Guntinas-Lichius et al. (2005)
	Abnormal spontaneous local field potentials (LFP) in freely moving TNC knock-out mice, enhanced theta and gamma oscillations in CA1, subfield specific reduction in volume of CA1 and number of somatostatin-positive interneurons in the hippocampus	Gurevicius et al. (2009)
	Abnormal high neuronal density, astrogliosis, low density of parvalbumin-positive neurons and reduced ratio of inhibitory to excitatory neurons, aberrant dendrite tortuosity and redistribution of stubby spines within first- to third-order dendritic arbors, enhanced responses upon whisker stimulation	Irintchev et al. (2005)
	Delayed onset of odor detection in neonatal mice	de Chevigny et al. (2006)
	A transiently increased neurogenesis at E 12.5 and a delayed acquisition of the EGFR by neural stem cells, which is required for maturation	Garcion et al. (2004)
	Minor structural differences in and around the SEZ, but normal numbers of stem cells and their progeny and unaffected regeneration of the SEZ after cytosine-b-D-arabinofurasonide treatment	Kazanis et al. (2007)
Tenascin-R	Tenascin-R knock-out (TNR ko) mice show....	Bruckner et al. (2000)
	PNNS with granular component within their lattice-like structure, reduced staining of hyaluronan, brevican, neurocan and nearly no phosphacan, changes in the synaptic structure of net-bearing Neurons	
	Two fold reduction of LTP after thetaburst stimulation in the Schaffer-Collaterals in Stratum radiatum in the CA1 region of the hippocampus, increased basal excitatory synaptic transmission, reduced potentiation immediately after TBS, reduced LTP, reduced short-term depression	Bukalo et al. (2001)
	Reduced perisomatic GABAergic inhibition	Saghatelyan et al. (2000)
	Metaplastic increase in the threshold for the induction of LTP	Bukalo et al. (2007)
	Alterations in WFA labelled nets around cortical interneurons, phosphacan and neurocan lack in PNNs	Haunso et al. (2000)
	Rota-rod compromised (deficits in cerebellar functions)	Montag-Sallaz and Montag (2003)
	Impaired GABA release at perisomatic inhibitory synapses in CA1 pyramidal cell layer; diffuse and disordered arrangement of neuronal cell bodies in CA1 pyramidal cell layer; reduced coverage of the plasma membrane by symmetric synapses, reduced synaptic activity	Nikonenko et al. (2003)
	Impaired NMDA-receptor-dependent LTP, increased basal excitatory synaptic transmission in CA1 pyramidal neurons	Saghatelyan et al. (2001)
	Fewer newborn cells in the olfactory bulb, altered cell migration	Saghatelyan et al. (2004)
	TNR over expressing ESCs transplanted into the striatum attracted host-derived neuroblasts from the rostral migratory stream and promoted stem cell-mediated recruitment of host-derived newborn neurons within the grafted area	Hargus et al. (2008)
Neurocan	Neurocan knock-out mice show ...	Zhou et al. (2001)
	Mild deficits in synaptic plasticity (late-phase hippocampal LTP reduced)	
Brevican	Brevican knock-out mice show ...	Bekku et al. (2009)
	Reorganization of the matrix at the nodes of ranvier; tenascin R and phosphacan upregulation	
	Altered neurocan expression, less prominent PNNs, hippocampal LTP defects	Brakebusch et al. (2002)

These processes also rely on the activation of cell recognition molecules of the Ig-, cadherin- and integrin-superfamilies and their reciprocal interactions, or interactions with the ECM environment (Takeichi, 2007; Tessier-Lavigne and Goodman, 1996; Zaidel-Bar et al., 2007). These membrane-receptor based systems elicit signal transduction pathways that eventually regulate the cytoskeleton and determine the pathway choices of the axonal growth cone. The signalling pathways converge on the Rho-family of GTPases that play roles both in the activation of growth cone motility and, likewise, in processes

of growth cone inhibition (Dickson, 2002; Filbin, 2003). Thus, the GTPases are of decisive importance for the generation of dendritic spines (Chen and Firestein, 2007). Human mutations in this pathway have been reported that are characterized by malformations of spines and associated with reduced intellectual abilities (Gripp et al., 2006; Yang and Mattingly, 2006). Moreover, it has been shown that the inhibition of axonal regeneration in the CNS partly depends on the activation of Rho-type GTPases by inhibitory molecules, including extracellular matrix constituents (Filbin, 2003). The reorganization of the cytoskeleton in response to the activation of cell-interaction molecules in responsive cells critically depends on GEFs. For example, Vav2 proteins trigger the endocytosis of an EphB-ephrin ligand–receptor pair, which results in the conversion of an initially adhesive interaction to repulsion (Cowan et al., 2005).

7. Various neuron astrocyte co-culture systems are suitable for the study of synaptogenesis

It is now a well-established concept that astrocytes intervene in the development, functional validation, reorganization and plasticity of CNS synapses (see above). In vitro cell culture systems have been implemented that allow the analysis of synapse formation. The micro-island co-culture assay permits the establishment of small arrays of glia, on top of which singular or small subsets of neurons can be cultivated. This set-up allows for the analysis of cell–cell contact-dependent influences of glial cells on neurons. Synapses can be visualized at the morphological level or assayed by electrophysiological methods as autapses.

An independent strategy has been developed that scans for the role of soluble astrocyte-derived factors in synaptogenesis and synaptic maturation. The establishment of synapses was followed on the basis of appearance of Bassoon or synaptophysin and ProSAP/Shank or PSD-95 as pre-synaptic and post-synaptic markers, respectively (Garner et al., 2002). Cultures were fixed and analyzed for these markers, and the co-localisation of pre- and postsynaptic markers was taken as evidence that a structurally intact synapse had formed. In order to monitor the efficiency of synapse formation the total number of co-localising pre- and postsynaptic puncta was counted using a computer-based automated programme for the analysis of confocal microscopy pictures in order to avoid individual bias effects in the evaluation.

In this type of culture system using embryonic day 18 (E18) rat hippocampal neurons synapse development begins after about 7 days in vitro (DIV) when grown in defined medium and in the presence of primary astrocytes kept in cell culture inserts (Fig. 3). Primary astrocytes proved by far the most efficient partners for the long-term co-culture. Other cell types including fibroblasts or various glial-derived cell lines were not suited to maintain the hippocampal primary cultures for a longer period (M. Pyka, A. Faissner, unpublished observations). Perturbation of CS-GAG synthesis or integrity significantly enhanced the number of synapses that are formed in the cultures (M. Pyka, A. Faissner et al., submitted).

To gain insight into potential mechanisms, the role of the DSD-1-epitope, a CS-GAG discovered by the Faissner laboratory, was investigated in more detail. This carbohydrate structure overlaps with a midkine/pleiotrophin-binding site, depends on sulfation in CS A and D units, comprises iduronic acid residues and stimulates neurite outgrowth (Clement et al., 1998; Ito et al., 2005; Nadanaka et al., 1998). The proteoglycans released by astrocytes contain the DSD-1-epitope expressed on phosphacan (Dobbertin et al., 2003), which binds to neuronal surfaces in the co-culture assay. This opens an avenue to the investigation of the role of the RPTPβ-related isoforms, because phosphacan/DSD-1-PG represents a released variant of this gene that occurs as CSPG (Faissner et al., 1994; Garwood et al., 1999).

The integrity of the synapses formed in the assay is a central criterium for the validation of the assay systems used in our study. In order to examine synaptic activity in vitro and evaluate the potential influence of defined ECM constituents on the modulation of synaptic currents, whole cell patch-clamp studies were performed. The synapses generated EPSCs and IPSCs and the currents generated could be characterized using standard methods (M. Pyka, A. Faissner et al., submitted).

8. Molecular functions of the hyaluronan-based ECM in regulating synaptic plasticity

The experiments performed by Pizzorusso et al. (2002, 2006) suggest a crucial role for the brain's ECM in restricting synaptic plasticity and the rewiring of local neuronal circuits in the adult brain. Indeed, excitatory activity seems to be crucially involved in proper PNN formation as indicated by both mutant models of Munc13-deficient mice brain slices that are synaptically silent and by pharmacological intervention with excitatory activity (Reimers et al., 2007). Another striking example for the function of the brain's extracellular matrix has been reported by Gogolla et al. (2009). These authors could demonstrate that the ECM is responsible for life-long establishment of fear memories. Removal of the ECM with chondroitinase ABC in the amygdala allowed the rapid extinction of fear memories in these experiments. Based on these findings one can hypothesize that an important function of the adult, hyaluronan-based ECM is to preserve the synaptic circuitry that has been established during CNS development and to manifest the switch from juvenile to adult synaptic plasticity mechanisms.

In order to elucidate putative mechanisms contributing to this plasticity-regulating function of perisynaptic ECM, we analyzed molecular physiological consequences of its removal in culture systems. In glutamatergic brain synapses, the availability of functional AMPA-type glutamate receptors is an important factor for the fidelity and strength of synaptic transmission. The synaptic content of AMPA receptors is controlled by the number of receptor molecules docked in the postsynaptic density and the exchange of synaptic and extrasynaptic receptors due to lateral diffusion (Choquet and Triller, 2003; Triller and Choquet, 2005). Actually, the exchange rate between synaptic and extrasynaptic receptor pools has been shown to tune synaptic short-term plasticity (Heine et al., 2008). Thus, limitation of lateral mobility of AMPA receptors by antibody cross-linking impairs the replacement of desensitized receptors

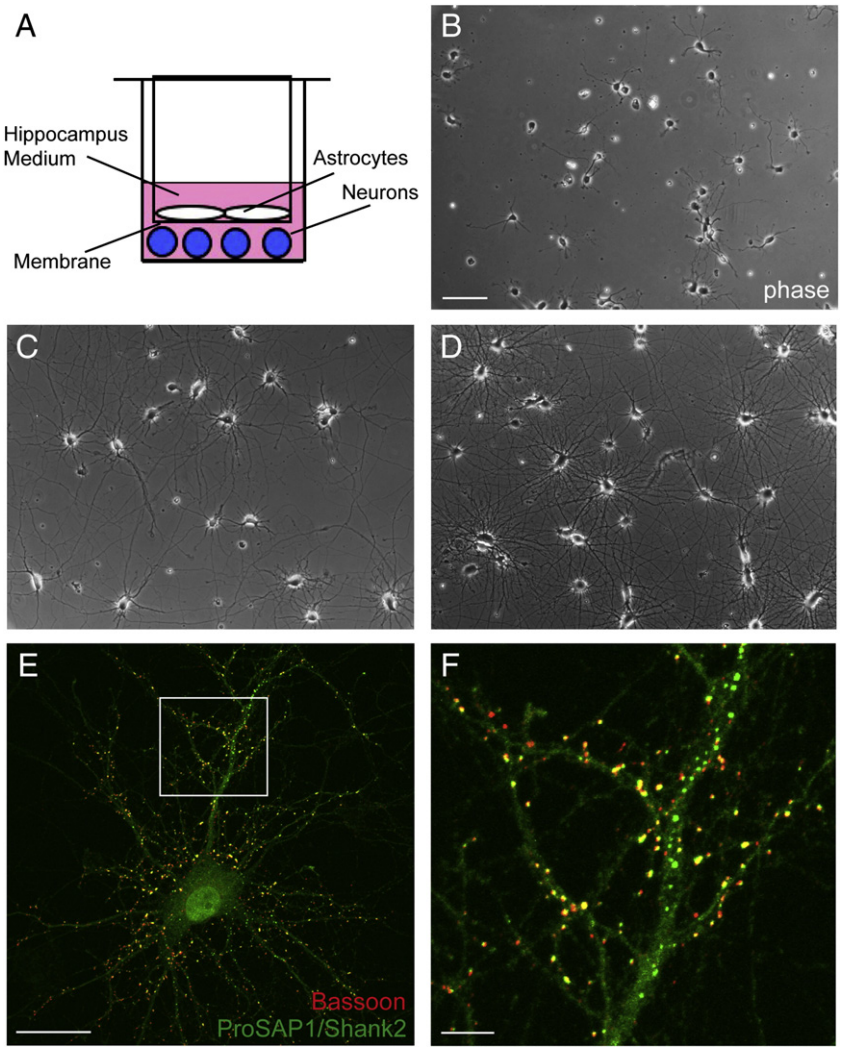

Fig. 3 – Synapse formation in vitro. E18 hippocampal neurons survive for long time periods in the cell-insert co-culture system and furthermore express synaptic proteins. (A) Neurons and glial cells were cultured without direct contact to each other to investigate effects of soluble astroglial-derived components on synaptogenesis. Astrocytes or glial cells were cultivated on a membrane with 0.4 μm pore size. The hippocampal neurons were plated on a glass coverslip coated with 15 μg/ml Poly-L-Ornithin. Cells were cultivated in 24 well plates. (B)–(D) Phase contrast images of hippocampal neurons in co-culture with primary astrocytes for 3 d (B), 6 d (C) and 13 d (D). Note that the hippocampal neurons survived more than two weeks and developed complex morphologies in completely defined medium in the presence of primary astrocytes. Scale bar: 80 μm (E) Expression of the pre-synaptic protein Bassoon and the postsynaptic protein ProSAP1/Shank2 after 13 d in culture. Scale bar: 10 μm (F) Higher magnification of boxed area in E. Note that co-localizations between pre- and postsynaptic proteins indicate the existence of structurally integral synapses. Scale bar: 3 μm.

by naïve ones and thus enhances paired-pulse synaptic depression. The gel-like ECM wrapping CNS synapses might be an ideal regulator for the lateral diffusion of AMPA receptors (and membrane proteins in general) in the cell membrane, thus controlling the accessibility of these molecules to the synaptic area.

To study the role of the ECM in this context and to test this hypothesis, we investigated first the appearance of hyaluronan-based, brevican-containing ECM in primary hippocampal cultures (John et al., 2006). PNN-like structures wrapping both excitatory and inhibitory synapses are found on virtually all neurons after two to three weeks in culture or longer. Utilizing this system, we analyzed the possibility that these PNN-like structures might affect lateral movement of AMPA receptors (Frischknecht et al., 2009). To this end the hyaluronan-based nets were labelled on living cells with fluorescently labelled hyaluronic acid binding protein (HABP; Fig. 4) and AMPA receptor mobility was monitored either utilizing single particle tracking with quantum dot-coupled GluR1-antibodies or by studying bulk movement of pHluorin-tagged GluR1 and GluR2 AMPA receptor subunits by FRAP (fluorescence recovery after photobleaching) or FLIP (fluorescence loss in photobleaching). Treatment of these cultures either with the hyaluronic acid-degrading enzyme hyaluronidase (Fig. 4) or with chondroitinase

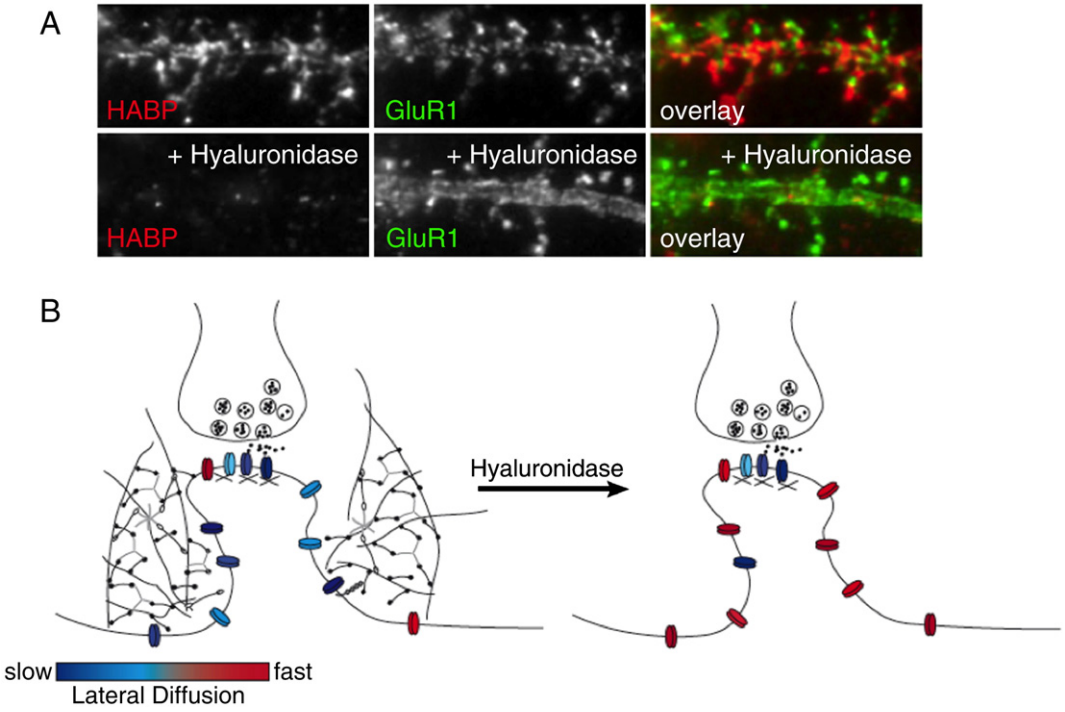

Fig. 4 – Regulation of lateral receptor diffusion by neural ECM. Lateral diffusion of AMPA receptors is accelerated after removal of the hyaluronan-based ECM. A) ECM stained with hyaluronic acid binding protein (HABP, red) surrounds dendrites and many synapses, stained here for GluR1 (green). After hyaluronidase treatment most of the HABP staining vanished (lower panel). B) Cartoon illustrating the changes in lateral mobility of AMPA receptors after hyaluronidase treatment. Receptors in the neuronal membrane diffuse laterally with non-uniform velocities (blue slow – red fast). After ECM removal using hyaluronidase (or chondroitinase ABC) lateral diffusion of AMPA receptors is accelerated and consequently the exchange of synaptic and extrasynaptic receptors is increased (see text for details). Note that diffusion within the synapse is not altered.

ABC removed the ECM and increased the mobility of extrasynaptic AMPA receptors. Synaptic receptors are not affected in their mobility, presumably because they are interacting with the intracellular PSD scaffold, however, also the exchange rate between synaptic and extrasynaptic receptors was enhanced significantly (Fig. 4). While ECM removal did not affect synaptic structures and basic synaptic transmission, the short-term synaptic plasticity, i.e. paired-pulse characteristics, was affected by hyaluronidase treatment. After ECM removal, synapses displayed less synaptic depression and in this respect resembled juvenile synapses, as they are found around DIV10 in these cultures, i.e. before the PNN-like structures are established (Frischknecht et al., 2009; John et al., 2006).

From these data we conclude that the hyaluronan/brevican-containing ECM, which at least in part is synthesized by astrocytes and appears late during CNS development, compartmentalizes neuronal surfaces and restricts lateral mobility of neurotransmitter receptors, thus affecting synaptic plasticity. Our data revealed that this ECM function might be a passive one, as artificial cell surface anchored molecules like GPI-anchored green-fluorescent protein (GFP) or GFP-tagged NrCAM lacking the cytoplasmic domain also change their lateral mobility upon ECM removal (Frischknecht et al., 2009). Besides many other proven or postulated functions for the adult brain ECM (see above — for a comprehensive review see (Celio and Blumcke, 1994; Dityatev and Schachner, 2003; Rauch, 2004) these recent studies provide a potential mechanism how the PNN-like structures can change the physiological state of CNS synapses.

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BRAIN RESEARCH REVIEWS

Review

Role of glial cells in the formation and maintenance of synapses

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ABSTRACT

Synaptogenesis is a decisive process for the development of the brain, its plasticity during adulthood and its regeneration after injury and disease. Despite tremendous progress during the last decades, it remains unclear, whether neurons can form synapses autonomously. In this review, I will summarize recent evidence that this is probably not the case and that distinct phases of synapse development depend on help from glial cells. The results supporting this view come from studies on the central and peripheral nervous system and on different experimental models including cultured cells as well as living flies, worms and mice. Our understanding of synapse–glia interactions in the developing, adult and diseased brain is likely to advance more rapidly as new experimental approaches to identify, visualize and manipulate glial cells in vivo become available.

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Abbreviations: CEPsh, cephalic sheath; NMJs, neuromuscular junctions; PCs, Purkinje cells; PSCs, perisynaptic Schwann cells; RGCs, retinal ganglion cells; Tgfb1, transforming growth factor beta 1

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1. Introduction

Synapses are key elements for brain function, and their formation is a decisive process throughout life. Neurons establish synaptic connections not only during development but also in the adult brain to implement their natural turnover and plasticity-related changes in the neuronal circuitry (Chklovskii et al., 2004; De Roo et al., 2008). The assembly of a synaptic connection requires a cascade of precisely coordinated processes in two partner neurons. Once synaptic partners have found each other, they assemble complex pre- and postsynaptic structures that mediate transmitter-based communication. Subsequently, connections acquire their specific transmission properties. In a last break-up phase, superfluous connections are eliminated. Research within the last decade has greatly advanced our understanding of molecules and mechanisms that mediate synapse development (Waites et al., 2005; Akins and Biederer, 2006; Craig et al., 2006; Fox and Umemori, 2006; Gerrow and El Husseini, 2006; Paukert and Bergles, 2006; Prokop and Meinertzhagen, 2006; Chen et al., 2007b; Dalva et al., 2007; McAllister, 2007; Polleux et al., 2007; Arikkath and Reichardt, 2008; Bicker and Schratt, 2008; Biederer and Stagi, 2008; Greer and Greenberg, 2008; Hanus and Ehlers, 2008; Huang and Scheiffele, 2008; Margeta et al., 2008; Salinas and Zou, 2008; Chao et al., 2009). However, a key question remains unanswered: Can neurons form synaptic connections by themselves, or do they require help from neighboring glial cells? In this review, I will focus on recent experimental evidence for the latter. Complementary overviews on this subject can be found elsewhere (Slezak et al., 2006; Barker and Ullian, 2008; Feng and Ko, 2008b; Stevens, 2008; Bolton and Eroglu, 2010). Reviews of glial contributions to axon guidance and growth (Learte and Hidalgo, 2007; Pfrieger, 2009) and global views on astrocyte function (Wang and Bordey, 2008; Barres, 2008) have been published recently.

2. Glial cells in touch with synapses

The general term “glial cells” is used here for simplicity. In the mammalian nervous system, glial neighbors of synaptic connections are astrocytes and perisynaptic (or terminal) Schwann cells (PSCs). The glial coverage of synapses was first described more than 30 years ago in pioneering ultrastructural studies (Spacek, 1971) and has now been shown in 3D in the cerebellum (Grosche et al., 1999) and the hippocampus, where synapse size appears to correlate with the extent of astrocytic contacts (Witcher et al., 2007). A recent study of fly glia detected astrocyte-like cells near synapses of the antennal lobe indicating that synapse-surrounding glial cells are not limited to the mammalian CNS (Doherty et al., 2009). The intimate vicinity of synapses and astrocytes has led to speculations about the functional implications and inspired the concept of “tripartite synapses”, which regards glial cells as integral elements of synaptic connections (Araque et al., 1999). Information on the steady progress in this field is summarized elsewhere in this issue.

3. Timing of synaptogenesis and glial development

During brain development, most synapses are generated between the first and third postnatal weeks. This happens after the formation of astrocytes (Miller and Gauthier, 2007), which suggests that synaptogenesis requires glia (Pfrieger and Barres, 1996). Fluorescent labeling of individual astrocytes by dye injection followed by immunohistochemical staining revealed morphological changes in astrocytes during postnatal synapse development (Bushong et al., 2004). Notably, the temporal coincidence of synapse development and astrocyte differentiation applies only to glutamatergic connections. GABAergic neurons establish a functional network in the embryonic brain well before astrocytes are generated (Ben-Ari, 2002; Huang and Scheiffele, 2008). Therefore, the formation of GABAergic contacts is likely to proceed independently from the presence of astrocytes, but it may require help from other types of non-neuronal cells.

4. Formation of glutamatergic synapses in the absence of glia

Glia-free cultures allow to test directly whether neurons can form synapses without glia. Such cultures can be prepared from embryonic rodent brains before glial cells are generated or from postnatal brains after active separation from glial cells. This can be accomplished by immunopanning (Barres et al., 1988; Ullian et al., 2004; Steinmetz et al., 2006; Buard et al., 2010) or fluorescence-activated cell sorting (Calof and Reichardt, 1984; Tomomura et al., 2001; Pennartz et al., 2004). Finally, neurons can be generated from stem cells by manipulating their differentiation (Jungling et al., 2003; Berninger et al., 2007; Johnson et al., 2007). These methods reach purities of up to 99.5% and thereby establish virtually glia-free conditions. Studies on such neuronal preparations suggest that there is no absolute requirement for glia: in the absence of glia, some neurons form numerous synaptic connections (Fig. 1A) (Steinmetz et al., 2006), whereas others including retinal ganglion cells (RGCs; Nagler et al., 2001; Ullian et al., 2001), motoneurons (Ullian et al., 2004) and cerebellar Purkinje cells (PCs; Buard et al., 2010) form only very few connections (Fig. 1B). In some of these preparations, synapses may be regarded as artificial, as neurons lack their natural partners. However, few synapses were also observed in glia-free cultures of subplate neurons from embryonic rats or mice, which normally form synapses among each other (McKellar and Shatz, 2009). On the other hand, strong glutamatergic synaptic activity was found in glia- (and serum-) free cultures of neurons from superior cervical ganglia prepared from newborn rats (Perez-Gonzalez et al., 2008), from spinal cords of embryonic mice (Cuevas et al., 2005) and from hippocampi and cerebella of postnatal mice (Steinmetz et al., 2006). Together, these results suggest that the requirement for glia varies with the neuronal cell type and its state of differentiation.

Notably, the competence of neurons to form and to receive synapses may develop independently. RGCs immunoisolated from embryonic rats were shown to form, but not to receive, synapses and the latter required contact to astrocytes (Barker

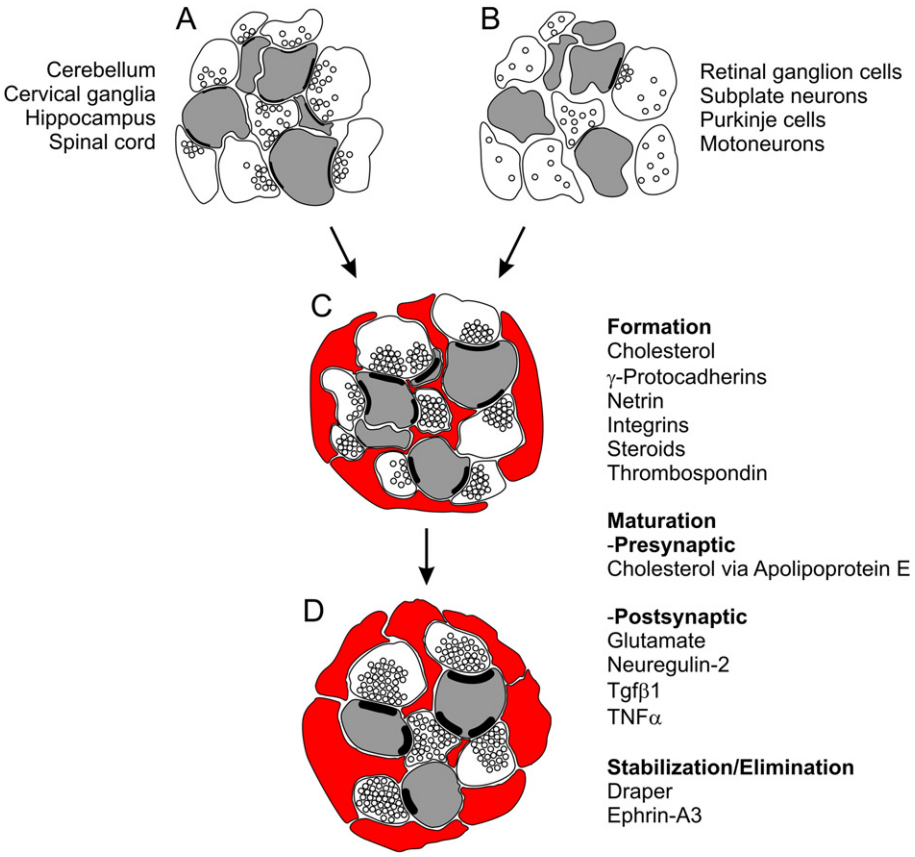

Fig. 1 – Influence of glial cells on synapse formation and maturation. The indicated types of neurons form (A) numerous or (B) only few synapses in the absence of glial cells. Depending on the neuronal cell type, glial cells promote the formation, (C) maturation and (D) elimination of synapses via the indicated molecules. Schematic drawings of synapses derived from electron microscopic images with pre- (white) and postsynaptic elements (gray) and processes of perisynaptic glia (red).

et al., 2008). A reverse sequence was observed in neurons that were generated by genetic re-programming of glial fibrillary acid protein-positive cells from cerebellar cortex of postnatal mice. These neurons could receive glutamatergic inputs, but they could not form presynaptic contacts (Berninger et al., 2007). The discrepancy may be due to differences among the neurons under study.

Does synaptogenesis require glial cells in living animals? This can only be addressed in selected invertebrate and vertebrate species, but not in mammals, because genetic ablation of astrocytes in developing and adult mice damages neurons massively (McCall et al., 1996; Cui et al., 2001) thus precluding conclusions about their relevance for synaptogenesis. In *Caenorhabditis elegans*, whose cells are inventoried, four of its 50 glia-like cells, the so-called cephalic sheath (CEPsh) glia, are in contact with dendrites of sensory neurons and with synaptic connections in the nerve ring (Shaham, 2006). Their ablation by optical and genetic methods perturbs the morphology of associated sensory neurons (Yoshimura et al., 2008) and impairs chemotactic behavior (Bacaj et al., 2008) without affecting neuronal survival. Still, it remains unclear, whether these changes are due to reduced synapse formation. Selective and efficient ablation of PSCs in developing and adult frogs has been accomplished by a PSC-specific antibody combined with complement-mediated cell lysis. These experiments revealed that PSCs are required for the formation and growth of neuromuscular junctions (NMJs) in developing frogs in vivo (Reddy et al., 2003).

5. Glial cells enhance the number of synapses

The aforementioned coincidence of synaptogenesis and glia development implies a causal relation. Direct evidence that glial cells increase the number of glutamatergic synapses was obtained in cocultures of frog spinal cord neurons and myocytes (Peng et al., 2003; Feng and Ko, 2008a) and in cultured rat and mouse RGCs (Pfrieger and Barres, 1997; Ullian et al., 2001; Nagler et al., 2001; Steinmetz et al., 2006), PCs (Buard et al., 2010), motoneurons (Ullian et al., 2004; Feng and Ko, 2008a), neurons derived from human embryonic stem cells (Johnson et al., 2007) as well as cortical (Hu et al., 2007; McKellar and Shatz, 2009) and hippocampal neurons (Tournell et al., 2006; Boehler et al., 2007) from embryonic rats (Fig. 1C). Previous studies showed that astrocytes enhance the number of inhibitory connections among cultured hippocampal neurons from embryonic rats by enhancing axon length and branching (Elmariah et al., 2005) via secreted proteins (Hughes et al., 2010). However, this effect may depend on the specific culture preparation, since the formation of inhibitory synapses in cultures of hippocampal neurons purified from

postnatal mice was not affected by glia (Steinmetz et al., 2006).

It should be noted that glial cells can enhance synaptic connectivity indirectly, by promoting neuronal survival. A glia-induced increase in the number of surviving neurons can explain the enhanced level of synaptic activity (Boehler et al., 2007). Other studies have excluded this possibility (Pfrieger and Barres, 1997; Ullian et al., 2004; Elmariah et al., 2005; Steinmetz et al., 2006; McKellar and Shatz, 2009). Moreover, glial signals may enhance the neuronal ability to receive synaptic inputs by promoting the growth or differentiation of axons and dendrites. Effects on dendrites have been observed in stem cell-derived neurons (Johnson et al., 2007), in RGCs (Goritz et al., 2005) and in PCs (Buard et al., 2010).

Finally, glial cells have been shown to impede synapse formation, for example by preventing physical contact between neurons. A shortening of Bergmann glia processes by expression of dominant negative forms of Rac1 or RhoG reduced the glial ensheathment of PCs and enhanced the number of parallel fiber inputs (Lippman et al., 2008). A similar effect was observed after experimentally induced retraction of Bergmann glia processes due to overexpression of a specific glutamate receptor subunit: this caused an increase in the number of climbing fiber inputs to PCs (Iino et al., 2001).

6. Glial cells promote synapse maturation

Once a synaptic contact is established, it probably undergoes a maturation phase, during which it attains its characteristic transmission properties (Fig. 1C). So far, the mechanisms of this process are largely unknown, but there is evidence that it is influenced by glial cells. In single-cell microcultures of neurons from rat superior cervical ganglia, Schwann cells were dispensable for the formation of cholinergic connections, but they enhanced the frequency of action potential-independent release and paired-pulse depression (Perez-Gonzalez et al., 2008). This points to a glial enhancement of presynaptic release efficacy, which has also been observed in cultured RGCs (Nagler et al., 2001; Christopherson et al., 2005; Goritz et al., 2005) and PCs (Buard et al., 2010). In addition, glial cells appear to augment the postsynaptic sensitivity of neurons. They increased the size of miniature excitatory postsynaptic currents in cultures of purified hippocampal neurons (Steinmetz et al., 2006), cortical subplate neurons (McKellar and Shatz, 2009), RGCs (Nagler et al., 2001; Ullian et al., 2001; Steinmetz et al., 2006) and spinal cord motoneurons (Ullian et al., 2004). How can glia affect the postsynaptic sensitivity to glutamate? Recent studies start to provide answers: Transcript profiling of cultured neurons revealed that glial factors upregulate mRNAs encoding for glutamate receptor subunits and for components that regulate their clustering and stability (Goritz et al., 2007; McKellar and Shatz, 2009).

7. Signals and mechanisms mediating the glial influence on synaptogenesis

Within the last years, we have learnt more about the molecules and mechanisms that mediate the influence of glial cells on synapse development (Fig. 1).

So far, several molecules have been identified that mediate the glial influence on synapse development. A genetic screen for abnormal synapse distribution in *C. elegans* revealed that contact establishment between interneurons requires the netrin receptor/UNC-40 on axons of the presynaptic cell and its ligand netrin/UNC-6 on CEPsh glia (Colon-Ramos et al., 2007). A netrin-dependent function of CEPsh glia as guideposts may explain why growth and branching of axons and dendrites fail following the elimination of these cells (Yoshimura et al., 2008).

A candidate approach uncovered transforming growth factor beta 1 (Tgfb1) as one of the factors by which Schwann cells promote the formation of NMJs in vitro (Feng and Ko, 2008a), albeit indirectly. The authors showed that Tgfb1 mediates the previously reported increase in the neuronal agrin level (Peng et al., 2003), which in turn controls the expression and clustering of nicotinic acetylcholine receptors. Tgfb1 did not mimic the potentiation of spontaneous transmitter release from motoneuron terminals observed after acute application of Schwann cell-conditioned medium (Cao and Ko, 2007) indicating the involvement of additional factors. Schwann cell-derived neuregulin 2 has been show to stimulate transcription of nicotinic acetylcholine receptors by activation of the ErbB4 receptor (Rimer et al., 2004; Ponomareva et al., 2006).

In the CNS, thrombospondin was identified as an astrocyte-derived matrix component that promotes the formation of ultrastructurally normal, but postsynaptically silent synapses in cultured RGCs. Transgenic mice lacking thrombospondins showed a reduced density of immunohistochemically identified synapses in the cortex (Christopherson et al., 2005). Recently, the Barres group identified the receptor mediating the synaptogenic effect of thrombospondin as alpha2/delta 1 protein (Cacna2d1), an accessory subunit of voltage-activated calcium channels (Eroglu et al., 2009). The involvement of this protein in synapse development is further confirmed by recent data on Drosophila, where its absence disturbs the formation of synaptic boutons by motoneurons (Kurshan et al., 2009). Recent studies also suggest that thrombospondins promote neurite outgrowth in cultured RGCs and enhance their expression of synaptic and dendritic components (Wang et al., 2009), possibly by acting as matrix organizers (Dunkle et al., 2007). Parallel studies on cultured RGCs identified glia-derived cholesterol as a component that promotes different aspects of synapse development (Mauch et al., 2001) including the differentiation of dendrites and the efficacy and stability of presynaptic transmitter release (Goritz et al., 2005). Our finding that neurons produce cholesterol less efficiently than glial cells (Nieweg et al., 2009) reinforces the idea that they depend on import of cholesterol via lipoproteins (Pfrieger, 2003). Neurons may convert glia-derived cholesterol to steroids, which then promotes synapse formation. A recent study on organotypic hippocampal cultures shows that cholesterol or testosterone, but not estradiol enhanced the density of spine synapses by 40%, whereas pharmacologic inhibition of steroid synthesis reduced synapse numbers (Fester et al., 2009). Other studies on cultured cells imply that astrocytes produce and release estradiol, which in turn enhances neurite growth and promotes synapse number and function (Chen et al., 2007a; Hu et al., 2007). The discovery of

other synaptogenic signals from glia may be facilitated by a recent study, which identified proteins secreted by astrocytes using shotgun proteomics (Dowell et al., 2009).

There is also evidence that contact between neurons and astrocytes promotes synapse development. A recent study revealed that γ-protocadherins are located in perisynaptic processes of astrocytes in the spinal cord of neonatal and adult mice. Absence of γ-protocadherins in astrocytes led to a transient decrease in synapse density among spinal cord neurons in vitro and in vivo without affecting neuronal survival (Garrett and Weiner, 2009). Moreover, contact of cultured hippocampal neurons to astrocytes increased the number of synapses possibly by activation of integrins and protein kinase C (Hama et al., 2004). As described above, RGCs from embryonic day 17 rats were shown to receive synaptic inputs, when contacted by astrocytes, possibly by changes in the subcellular distribution of neurexins (Barker et al., 2008). In both cases, however, the astrocytic signals that initiate these changes remain unknown.

Unexpected mechanisms of glia-induced synapse development have been uncovered by transcriptional profiling of purified neurons. Three recent reports have proven the validity of this approach (Goritz et al., 2007; Stevens et al., 2007; McKellar and Shatz, 2009). Studies on cultured RGCs revealed glia-induced upregulation of components that had not been discussed in a neuronal context. This included a component of the complement system, which contributes to synapse elimination (see below; Stevens et al., 2007) and matrix gla protein (Mgp; Goritz et al., 2007), a component of the extracellular matrix, which may regulate signaling by members of the Tgf family (Moon and Birren, 2008). So far, the glial signals that mediate transcriptional changes remain unknown.

An exciting idea is that the ability of astrocytes to promote synapse development is controlled by neurons. Although the evidence supporting this view comes mainly from studies on the growth-promoting activity of astrocytes, similar mechanisms may apply to their synaptogenic action. Activation of purinergic receptors in cultured astrocytes appeared to control the expression and release of synapse-promoting thrombospondin (Tran and Neary, 2006) and the release of protease nexin-1, which stimulates neurite growth, was regulated by G protein-coupled receptors (Giau et al., 2005). Progesterone treatment enhanced the astrocytic production of agrin (Tournell et al., 2006) and lysophosphatidic acid (Guizzetti et al., 2008), whereas acetylcholine (de Sampaio et al., 2008) enhanced the ability of astrocytes to promote neuronal differentiation and growth. A study on hippocampal cultures from embryonic rats reported that blocking spontaneous calcium transients in astrocytes reduced axonal and dendritic growth, possibly by reducing astrocytic levels of the cell adhesion molecule N-cadherin (Kanemaru et al., 2007). Notably, cultured astrocytes lacking MeCP2 failed to support neuronal differentiation compared to wild-type cells (Ballas et al., 2009) suggesting that transcription factors control the growth-promoting effects of astrocytes.

Neurons may harbor signals that modulate astrocyte properties, but non-neuronal cells may also participate in this regulation. An example has been reported recently in vitro (Au et al., 2007). The authors showed that olfactory ensheathing glia prepared from embryonic mice released osteonectin/secreted protein acidic rich in cysteine. This factor in turn enhanced the ability of Schwann cells to promote neurite outgrowth from explants of dorsal root ganglia from mouse embryos via secretion of Tgfb. These data provide a first glimpse that the influence of astrocytes on neuronal differentiation probably requires cooperative actions of multiple signals, whose cellular sources and molecular targets remain to be clarified.

8. Glial cells affect the turnover of synapses

Once formed, synapses last for a limited time period. In vivo two-photon imaging of fluorescently labeled neurons revealed that the lifetime of spines, and probably their associated synapses, ranges from minutes to hours during postnatal development and from days to months in the adult brain (Bhatt et al., 2009). Whereas controlled synapse turnover is required for brain development and plasticity, uncontrolled synapse loss may spearhead pathological changes in brain diseases (Walsh and Selkoe, 2007; Knobloch and Mansuy, 2008).

Recent studies suggest that glial cells influence the stability of synaptic connections (Fig. 1D). Simultaneous time-lapse imaging of fluorescently labeled astrocytes and dendritic spines in organotypic hippocampal cultures revealed that contact to astrocytes enhances the lifetime of dendritic protrusions and promotes their conversion to spines. This effect was possibly mediated by astrocytic ephrin-A3 and dendritic EphA4 receptors (Nishida and Okabe, 2007). Along this line, ablation of PSCs in adult frogs in vivo caused long-term loss of NMJs (Reddy et al., 2003) indicating that the maintenance of these connections also depends on the glial sheath. There is also evidence for the opposite effect, namely that glial cells promote synapse elimination (Fig. 1D). A striking mechanism has been uncovered in cultures of purified RGCs (Stevens et al., 2007). Coculture with glial cells strongly enhanced the neuronal level of C1q, a component of the complement cascade (Perry and O'Connor, 2008), which appeared to be present at synapses. Mice lacking C1q or C3, another complement factor, showed defects in the eye-specific segregation of synaptic inputs to the lateral geniculate nucleus (Stevens et al., 2007). These observations suggest that astrocytes command the onset and extent of synapse execution.

Synapse destruction involves removal of axonal branches that form obsolete connections. In the peripheral nervous system, motoneurons form supernumerary NMJs, most of which are destroyed postnatally (Sanes and Lichtman, 1999). Repeated imaging of NMJs in living transgenic mice combined with electron microscopy revealed that axon remnants, so-called axosomes, end up in PSCs (Bishop et al., 2004). Moreover, elimination of unwanted NMJs and climbing fiber inputs to cerebellar PCs was accompanied by enhanced lysosomal activity in glial cells (Song et al., 2008). Whether glial cells play an active or passive role in this process remains unclear.

Direct evidence for a glial contribution to axon—and thereby synapse—removal comes from studies on the

olfactory system of flies, which undergoes substantial remodeling during metamorphosis (Jefferis et al., 2002). Selective labeling of neurons and glial cells in the mushroom body and genetic disturbance of glial membrane function revealed that the pruning of axons is mediated by glial cells expressing draper and related receptors (Awasaki et al., 2006; Hoopfer et al., 2006; MacDonald et al., 2006; Kurant et al., 2008; Doherty et al., 2009; Fig. 1D).

9. Summary and Outlook

The experimental results summarized above support the idea that neurons require help from glia to form and maintain proper synaptic connections. The evidence still relies largely on in vitro studies, and it remains important to determine whether and how glial cells instruct synapse development in vivo. This will require the identification of the signaling pathways that mediate the glial influence on synapses and their cell-specific manipulation in vivo. In this review, I have focused on how glial cells influence synapses. A key question to be addressed by future studies is whether the reverse is true, i.e., whether synapses instruct the differentiation of the glial processes surrounding them. Given recent technological advances research on glia–synapse interactions will progress on three "techno-tracks" (Pfrieger, 2009). First, advanced genetic and proteomic approaches will divulge the molecular setup of glial cells in contact with synapses. Second, new imaging techniques enable the visualization of glia–synapse interactions and third, new transgenic animals allow for temporally controlled manipulation of glia in vivo. Armed with this arsenal of new tools, we should now be able to find out whether the coincidence of glia development and synaptogenesis is mere chance or bare necessity.

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BRAIN RESEARCH REVIEWS

Review

What determines neurogenic competence in glia?

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ABSTRACT

One of the most intriguing discoveries during the last decade of developmental neurobiology is the fact that both in the developing and adult nervous system neural stem cells often turn out to have a glial identity: Radial glia generates neurons in the developing telencephalon of fish, birds and mammals and astro/radial glial stem cells in specialized neurogenic zones give rise to new neurons throughout life. What are the extrinsic signals acting on and the intrinsic signals acting within these glial populations endowing these with a neurogenic potential, whilst most other glia seemingly lack it? Studies on postnatal astroglia shed interesting light on this question as they are the intermediate between neurogenic radial glia and mature parenchymal astrocytes. At least in vitro their decision to acquire a glial fate is not yet irrevocable as forced expression of a single neurogenic transcription factor enables them to transgress their lineage and to give rise to fully functional neurons acquiring specific subtype characteristics. But even bona fide non-neurogenic glia in the adult nervous system can regain some of their radial glial heritage following injury as exemplified by reactive astroglia in the cerebral cortex and Müller glia in the retina. In this review first we will follow the direction of the physiological times' arrow, along which radial glia become transformed on one side into mature astrocytes gradually losing their neurogenic potential, while some of them seem to escape this dire destiny to settle in the few neurogenic oases of the adult brain where they generate neurons and glia throughout life. But we will also see how pathophysiological conditions partially can reverse the arrow of time reactivating the parenchymal astroglia to re-acquire some of the hallmarks of neural stem cells or progenitors. We will close this review with some thoughts on the surprising compatibility of the co-existence of a neural stem cell and glial identity within the very same cell from the perspective of the concept of transcriptional core networks.

Contents

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1. Introduction

The last ten years have seen the rise of specialized glial cells to the rank of neuronal precursors both in the developing and adult nervous system. In the developing telencephalon neuroepithelial cells differentiate into radial glia which in turn generate either directly or indirectly via intermediate progenitors most forebrain neurons (Anthony et al., 2004; Kriegstein and Alvarez-Buylla, 2009; Malatesta et al., 2003; Malatesta et al., 2000; Pinto and Gotz, 2007). While most of the radial glia (RG) eventually transform into parenchymal astrocytes at the end of neurogenesis (Alves et al., 2002; Voigt, 1989), some of them give rise to astroglial stem cells in the adult subependymal zone (SEZ) or ependymal cells (Merkle et al., 2007; Spassky et al., 2005). Both, radial glia and astroglial stem cells display hallmark features of classical astrocytes [for a detailed review on the glial nature of radial glia see (Pinto and Gotz, 2007)]. Thus the question arises as to what endows these glial cells with a neurogenic competence so noticeably lacking in astroglia in most other regions of the brain, such as e.g. the cerebral cortex. What signals are required for the neurogenic endowment of glia and what processes take place in radial glia while they transform into non-neurogenic astrocytes? Does the loss of neurogenic potential occur abruptly or gradually? Is it in fact irreversible? Our studies during the last few years have led to the recognition that astroglial cells at an early stage of postnatal development are not irrevocably fixed in their lineage, but forced expression of single neurogenic transcription factors can render these cells capable of transgressing their own lineage and generating diverse types of neurons, at least in vitro (Berninger et al., 2007a; Heins et al., 2002). However, similar processes might be evoked in the early postnatal brain by damage such as caused by hypoxia (Fagel et al., 2009; Fagel et al., 2006). Finally, partial neurogenic competence can be regained by glial cells in the adult nervous system following injury as they de-differentiate and resume proliferation, as shown in the retina and the cerebral cortex (Buffo et al., 2008; Fischer and Reh, 2001; Karl et al., 2008). Thus it appears that not all the radial glial heritage is spent during early life, but some of it is latently preserved stimulating the hope that it might be possible to unearth this potential for the development of novel strategies for brain repair.

2. Radial glia: the founding fathers of the telencephalon and the adult neurogenic zones

Radial glial cells (RGCs) comprise a specialized cellular population in most regions of the vertebrate brain during restricted developmental periods, the functions of which have been highly disputed since their first description at late 19th century [reviewed in (Rakic, 2003)]. Nowadays, there is a general consensus that RGCs differentiate from neuroepithelial cells acquiring typical astroglial features, as for instance the presence of glycogen storage granules and the expression of astroglial markers, such as e.g. the astrocyte-specific glutamate and aspartate transporter (GLAST), brain lipid-binding protein (BLBP) and tenascin-C [for review see (Pinto and Gotz, 2007)]. In addition, it is now well established that RGCs function both as neuronal and glial progenitors at least in the developing telencephalon (Anthony and Heintz, 2008; Anthony et al., 2004; Malatesta et al., 2003; Malatesta et al., 2000; Miyata et al., 2001; Noctor et al., 2001; Tamamaki et al., 2001). Finally, with completion of most neuro- and gliogenesis they sign responsible for generating ependymal cells as well as particular astro/radial glial cells that function as neural stem cells (NSCs) in the adult brain (Chojnacki et al., 2009; Kriegstein and Alvarez-Buylla, 2009; Merkle et al., 2004). The fact that at least some RGCs are multipotent, i.e. can generate both neuronal and glial progeny, and are capable of self-renewing cell divisions generating either two new RGCs (symmetric division) or one RGC and a fate-restricted progenitor (asymmetric division) (Miyata et al., 2001; Noctor et al., 2004) indicate that RGCs exhibit defining stem cell hallmarks and are thus often considered as embryonic NSCs (Kriegstein and Alvarez-Buylla, 2009). The generation of adult NSC from RGCs may be considered as a particular case of RGC self-renewal raising interesting points about the precise lineage relationships between RGCs and adult NSCs. For example, given the evidence that there are virtually no quiescent RGCs during embryonic development (Hartfuss et al., 2001), consequently all adult NSCs must be derived from RGCs that have been previously contributing to neuro- or gliogenesis. Moreover, given the notion that NSCs in the adult SEZ are quite heterogeneous with respect to the distinct progenies they give rise to (Brill et al., 2009; Brill et al., 2008; Hack et al., 2005;

Merkle et al., 2007), it can be argued that each class of adult generated neuron and glia may likely descend from a different type of RGC. Indeed there is compelling evidence that the adult SEZ exhibits a similar dorsoventral organization as the developing telencephalon [for review see (Kriegstein and Alvarez-Buylla, 2009)]: for instance, transcription factors involved in interneuron specification in the developing ventral telencephalon such as Dlx2, (Petryniak et al., 2007) are highly expressed in the ventral SEZ (Brill et al., 2008), while transcription factors expressed dorsally such as Pax6 and Neurogenin2 (Neurog2) (Brill et al., 2009; Brill et al., 2008) are well known for their important role in the developing cerebral cortex (Bertrand et al., 2002). This regionalisation of the SEZ has been taken as sign for the construction of the adult SEZ from "building blocks" of different origins (Kohwi et al., 2007), not unlike a mosaic (Merkle et al., 2007; Young et al., 2007). Finally, while the majority of adult NSCs generate neurons, some of them give rise to oligodendrocytes and possibly also non-stem cell astroglia (Hack et al., 2005) leading to two interrelated questions: (i) Do all adult NSCs in vivo generate neuronal and glial progeny, possibly even in a sequential manner, or are we dealing here with distinct sets of neuro- and gliogenic progenitors (which would no longer justify the use of the term "stem cell")? (ii) In the latter case are different adult progenitors derived from different types of RGCs, i.e. adult neurogenic progenitors from neurogenic RGCs and adult gliogenic progenitors from gliogenic RGCs?

3. Lineage relationships between adult NSC astroglia and embryonic radial glia

Astro/radial glial cells functioning as NSCs in the adult brain have been long suggested to derive from embryonic radial glia (Alvarez-Buylla et al., 2001; Chojnacki et al., 2009). However, embryonic RGCs are heterogeneous (Malatesta et al., 2000) and it is not clear whether adult NSCs would belong to the lineage of multipotent (Fig. 1, scenario I), lineage-restricted (Fig. 1, scenarios II and III) or even of a very small and hence previously missed set of quiescent RGCs (Fig. 1, scenario IV). Recently, we have provided additional evidence indicating that the early developing cerebral cortex is devoid of glia-restricted progenitors (Costa et al., 2009). By using retrovirally-based transduction and video time-lapse microscopy to follow the progeny of single cortical progenitors in vitro and in vivo, we showed that the majority of early cortical progenitors generate exclusively neurons, a pool which becomes depleted with time, whereas a subset of cortical progenitors generate neurons before giving rise to glia-restricted progenitors observed after mid-neurogenesis. Therefore, every actively dividing cortical progenitor seems to generate neurons at early developmental stages and only a subset of progenitors undergoes the alleged sequence of transitions from neurogenesis to gliogenesis during cortical development (Qian et al., 2000). As RGCs have been shown to constitute the majority of progenitor cells in the developing cerebral cortex at the developmental stages when most of these lineage studies were performed (Hartfuss et al., 2001; Malatesta et al., 2000; Noctor et al., 2002, 2004), most of the cortical progenitors studied in previous work [see (Costa et al., 2009) and references herein] are RGCs and, therefore, cell lineage data would indicate that early RGCs comprise two major populations, namely a neuron-restricted and a multipotent population. Since the former seem to become depleted over time as they generate neurons, this may suggest that adult NSCs are encompassed in the lineage of multipotent RGCs contributing both neurons and macroglial cells during development (Alvarez-Buylla et al., 2001) (Fig. 1, scenario I). Although this may be considered to be the most likely scenario, there is no direct experimental support to dismiss the possibility that adult NSCs are derived from symmetrically dividing RGCs generating only daughter RGCs (Fig. 1, scenario III) or from a small, and therefore difficult to be detected, subpopulation of RGCs that did not divide at all during development (Fig. 1, scenario I). None of these alternatives has been directly tested, yet the fact that several signalling pathways involved in the transition from neurogenesis to gliogenesis in the developing cerebral cortex also play a role in the specification of adult NSCs (see below) may lend some support to the view that adult NSCs are indeed encompassed in the lineage of multipotent radial glia (Fig. 1, scenario I).

4. The role of intercellular signalling pathways for the endowment of glia with neurogenic potential

Irrespective of the exact lineage relationship between RGCs and adult NSCs, there is good experimental support for the notion that embryonic RGCs transform into adult SEZ astroglia at postnatal stages (Merkle et al., 2007, 2004). These two cell populations share an intriguing property: they exhibit astroglial features and at the same time have the potential to generate neurons. In contrast, other astroglial population directly derived from RGCs, such as parenchymal astroglia in the cerebral cortex (Alves et al., 2002; Voigt, 1989) and ependymal cells (Spassky et al., 2005) do not generate neurons in the adult brain under physiological conditions. What are the factors endowing RGCs and SEZ astro/radial glia with neurogenic potential and what restricts parenchymal astroglia and ependymal cells to their lineage and prevents them from generating neurons notwithstanding their close ties to RGCs and SEZ astro/radial glia? To gain some more insights into the differences between stem cell and parenchymal glia we need to have a closer look at the signalling pathways activated in these cells. We will focus here on the discussion of Notch and sonic hedgehog (Shh) signalling as several lines of evidence suggest that these pathways play a major role in stem cell fate determination and may hence shed light on the question how astroglial cells are endowed with stem cell properties.

4.1. *Notch signalling*

On first sight, the canonical Notch pathway seems to be gliogenic: Forced expression of Hes1, a downstream target and effector of Notch, impairs neurogenesis in the embryonic cortex (Ishibashi et al., 1994), whereas expression of an active form of Notch1 (NICD1) or Notch 3 (NICD3) promotes increased generation of parenchymal astrocytes and ependymal cells (Dang et al., 2006; Gaiano et al., 2000). These effects of Notch

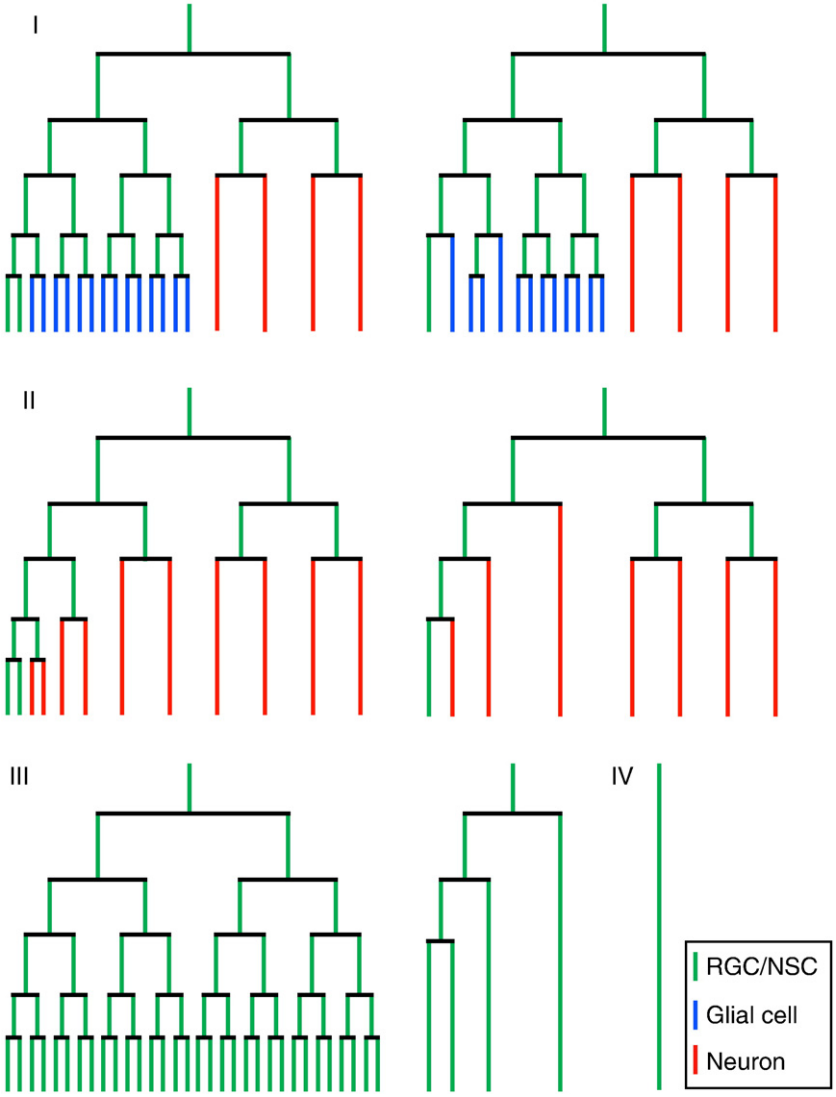

Fig. 1 – Possible lineage relationships between RGCs and adult NSCs. In the first scenario (I) RGCs generate neurons and macroglial cells during development before becoming adult NSCs. In the second scenario (II), RGCs that give rise to adult NSCs generate only neurons during development. The third scenario (III) suggests that adult NSCs are derived from a separate population of RGCs that do not contribute neurons or macroglial cells during development. The two lineage trees depicted in each of these 3 scenarios are fictious and have the purpose to illustrate that RGCs may progress to adult NSCs via symmetric and asymmetric cell divisions. The last scenario (IV) suggests the existence of quiescent RGCs that do not divide during development and directly become adult NSCs.

signalling in RGCs have been thought to depend on the repression of proneural genes such as Neurogenin1 (Neurog1), Neurog2 and the mouse homologue of achaete–scute 1(Mash1) by the Notch effectors Hes1 and Hes5 (Hatakeyama et al., 2004). However, it has also been shown that most RGCs express Notch target genes at early embryogenesis (Mizutani et al., 2007), indicating that Notch signalling occurs at stages when RGCs are actively engaged in the generation of neuronal progeny. Moreover, expression of NICD1 or NICD3 significantly increases the frequency of embryonic telencephalic progenitors that form neurospheres (Dang et al., 2006; Yoon et al., 2004), suggesting that Notch signalling might be involved in the maintenance of a stem cell state in RGCs. Accordingly, active Notch 1 and 3 promote the generation of subependymal astrocytes (Dang et al., 2006; Yoon et al., 2004). Although these data seem at first glance contradictory with previous findings pointing to a gliogenic role for Notch, it might only highlight a limitation inherent to studies based on forced expression or deletion of genes, namely their "all or none" effect that is blind to the temporal fine tuning of physiological expression levels. In fact, it has been recently demonstrated that the levels of Hes1 and Neurog2 oscillate out of phase to each other within RGCs (Shimojo et al., 2008), indicating that the susceptibility of RGCs for fate decisions is dynamically regulated and may thus depend on temporally coincident signalling events impinging on them rather than on the static activation of isolated signalling pathways, such as mimicked by forced expression of Notch effectors or their counter-players. Indeed, promotion

of gliogenesis by Notch depends on the concomitant activation of the janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathway (Ge et al., 2002). When these two pathways are not simultaneously activated, the Notch signalling mediator CBF1 binds to a repressive cofactor protein, NCoR, which functions to repress gliogenic genes (Hermanson et al., 2002). Therefore, Notch signalling in embryonic RGCs may prevent neurogenesis by two different means: i) by maintaining RGCs in an undifferentiated, proliferative state and ii) by promoting gliogenesis. In the adult SEZ, Notch signalling functions to maintain ependymal cells in a quiescent state and inhibition of this signalling allows these cells to produce olfactory bulb neurons (Carlen et al., 2009). In contrast, activation of Notch signalling has also been shown to be important for survival and proliferation of adult NSCs (Alexson et al., 2006; Androutsellis-Theotokis et al., 2006), suggesting that similarly to the embryonic brain, the role of Notch signalling in the adult neurogenic niches may also depend on dynamic interactions with other signalling pathways. Interestingly, Notch signalling becomes activated in the cerebral cortex after stroke (Arumugam et al., 2006), a condition in which parenchymal astroglia de-differentiate and resume proliferation and can generate multipotent neurospheres (Buffo et al., 2008, 2005) (see discussion below).

4.2. *Sonic hedgehog (Shh) signalling*

The Shh member of the Hedgehog protein family plays critical roles in the patterning of the developing neural tube and in the induction of ventral forebrain structures [for a recent review see: (Dessaud et al., 2008)]. Recently, it has been shown that conditional knockout of the *Shh* and *Smo* genes in the early developing dorsal telencephalon resulted in a smaller dorsal telencephalon by prolonging the cell cycle of RGCs (Komada et al., 2008). Moreover, Shh signalling has been shown to be crucial for the generation of NSCs in the postnatal brain (Palma et al., 2005). Consistent with a role in neural stem cell maintenance, Shh signalling in the adult neurogenic regions is important to maintain normal levels of NSC proliferation and neurogenesis (Balordi and Fishell, 2007; Han et al., 2008; Lai et al., 2003). Furthermore, in an elegant study, Ahn and Joyner provided unequivocal evidence that a small population of cells (probably RGCs) in the developing brain respond to Shh and establish the two major neurogenic compartments in the adult brain (Ahn and Joyner, 2005). Using a genetic fate-mapping strategy based on the expression of the Shh target Gli1 in vivo, they showed that Shh responding cells are established at late embryogenesis and comprise NSCs capable of self-renewal and generation of multiple cell types in the adult brain. More recently, it has also been shown that NSCs fail to develop in animals with defective Shh signalling caused by embryonic ablation of ciliary genes in RGCs (Han et al., 2008). Collectively, these data suggest a pivotal role of Shh for the establishment of NSCs within the neurogenic germinative zones of the adult brain. Surprisingly, yet consistent with such role, Shh can stimulate differentiated parenchymal astroglia from the adult cerebral cortex to initiate the formation of multipotent neurospheres in vitro (Jiao and Chen, 2008). The latter data suggest that this factor may not only be important for the establishment and maintenance of astroglial NSCs within the stem cell niche, but rather may exert a stem cell identity inducing activity over other astroglial cell populations.

Other molecular determinants may also contribute to the neurogenic potential of stem cell astroglia. For example, members of the bone morphogenetic protein family (BMPs) promote neuronal differentiation at early embryogenesis (Li et al., 1998) and BMP signalling is also active in GFAP-positive cells in the adult SEZ where it is required for neurogenesis (Colak et al., 2008). Likewise, Wnt signalling has also been shown to be involved in the control of cortical progenitors/RGCs proliferation (Chenn and Walsh, 2002; Woodhead et al., 2006) and adult neurogenesis (Kuwabara et al., 2009; Lie et al., 2005). The precise role of these factors in maintaining the pool of NSCs in developing and in the adult neurogenic germinative zones is currently under active investigation and lessons learned from these studies may help to reactivate neurogenic programs in classically non-neurogenic areas of the brain, as illustrated by the case of Shh.

5. Postnatal astroglia: a transition state between neurogenic radial glia and mature parenchymal astrocytes?

As previously discussed, while some RGCs give rise to astroglial stem cells of the adult neurogenic zones, ependymal cells or late oligodendroglial progenitor cells (Kessaris et al., 2006), the majority is thought to transform directly or indirectly into parenchymal astroglia (Kriegstein and Alvarez-Buylla, 2009). Eventually many of these astroglial cells will take up functions quite different from generating neurons such as regulation of blood flow, ion homeostasis, energy metabolism and regulation of synaptic function (for review see articles within this issue of Brain Research Reviews), i.e. intricate functions which require a high degree of specialization (Wang and Bordey, 2008). Very little is known about the molecular mechanisms underlying the specialization of astroglia, but evidence from the spinal cord white matter points to the possibility that transcription factors involved in neuronal specification also contribute to astrocyte specification, with Pax6 being a prime example for a transcription factor not only regulating neuronal, but also astroglial specification (Hochstim et al., 2008). For instance, astroglia in the ventral and dorsal spinal cord white matter can be distinguished by their mutually exclusive expression of reelin and Slit1, respectively. Intriguingly, reelin requires expression of Pax6, while Nkx 6.1 promotes expression of Slit1 (Hochstim et al., 2008). This data suggest a transcriptional code for astrocyte positional identity in the white matter of the spinal cord. Does expression of neurogenic fate determinants play a similar role in the specification of cortical astroglia? Indeed Pax6 has been detected in postnatal astrocytes and their progenitors (Sakurai and Osumi, 2008) (S. Gascón and M. Götz, unpublished observation) suggesting that continued expression of neurogenic fate determinants – most likely at drastically reduced levels – by cortical astrocytes at early postnatal stages ensures a gradual change from neurogenic RGCs to gliogenic RGCs and finally astrocytes. Indeed several lines of evidence point to such a gradual change: for instance, postnatal astroglia can be induced to initiate the formation of

clonal aggregates called neurospheres when cultured in the presence of epidermal growth factor (EGF) and fibroblast growth factor 2(FGF2) (Laywell et al., 2000) (Heinrich et al., unpublished observation). These neurospheres are characterized by their ability to self-renew (i.e. can give rise to secondary, tertiary etc. neurospheres) and are multipotent, i.e. can generate all three major neural lineages, thus exhibiting the hallmarks of neurospheres generated by NSCs. The neurosphere-forming capacity of astroglia rapidly declines during the second postnatal week (Laywell et al., 2000). Thus, under the influence of EGF/FGF2 early postnatal astroglia can sufficiently de-differentiate to revert to a multipotent lineage. But by postnatal days 14–18 apparently changes take place within astroglia that disable the neurosphere-forming capacity of these cells. Conceptually, one may assume that these changes correspond with the in parallel increasing involvement of astroglia in their above mentioned regulatory functions. For instance, by about 2 weeks astroglia acquire a mature expression of inwardly rectifying potassium channels (K_{ir}), thought to be involved in buffering of potassium (Bordey and Sontheimer, 1997; Seifert et al., 2009). Interestingly, up-regulation of $K_{ir}4.1$ has been associated with cell cycle exit, while loss of functional K_{ir} channels is associated with re-entry into cell cycle and gliosis (Olsen and Sontheimer, 2008). Indeed, functional specialization of astroglia is likely to require these cells to cease proliferating and to acquire a postmitotic status thereby rendering these cells refractory to the mitogenic action of EGF and FGF2. However, as we will see below, astroglia can de-differentiate and resume proliferation following injury, an effect which is accompanied by the regaining of neurosphere-forming capacity (Buffo et al., 2008, 2005).

5.1. *Stable reprogramming of postnatal astroglia*

Another striking example for the plasticity of early postnatal astroglia can be seen in their high susceptibility for neuronal reprogramming following forced expression of neurogenic fate determinants. Based on the fact that neurogenic RGCs in the developing cortex express high levels of Pax6 (Gotz et al., 1998), our laboratory has demonstrated that forced re-expression of Pax6 in culture via retroviral vectors can drive some mouse astroglia towards the neuronal lineage (Heins et al., 2002). Subsequently, we found that retrovirally-mediated expression of the Pax6 target gene Neurog2 or the related proneural gene Mash1 are even more efficient in driving early cultured postnatal astroglia towards neurogenesis (Berninger et al., 2007a). Intriguingly, forced expression of these transcription factors does not only induce expression of neuronal markers, but also results in the gradual acquisition of neuronal conductances and the ability to generate repetitive action potentials discharges suggesting that forced expression of these transcription factors had caused early postnatal astroglia to undergo a stable lineage transgression.

Single cell tracking of astroglia derived from transgenic mice expressing GFP under the human GFAP promoter revealed interesting details about the reprogramming process. First of all, consistent with the viral integration, cells undergoing reprogramming typically divided before undergoing neuronal metamorphosis with many cells apparently dying from apoptosis (Berninger et al., 2007a). It is currently not clear whether this apoptosis is a specific mechanism for aborting failed reprogramming, but it is conceivable that forced expression of neurogenic fate determinants may induce a transcriptional program that enters in catastrophic conflict with the intrinsic astroglial differentiation. Another lesson learned from these single cell tracking experiments is the fact that cells undergoing successful reprogramming exhibit morphological changes surprisingly similar to early cortical progenitors. For instance, like early cortical precursors (LoTurco and Bai, 2006), astroglial cells expressing Neurog2 seem to pass through distinct morphological stages, such as a multipolar followed by a bipolar phase (Fig. 2), and eventually enter a migratory stage (Berninger et al., 2007a). These data are consistent with the recent finding that one of Neurog2's direct transcriptional targets is the small GTP binding protein Rnd2, the activity of which is constitutive and plays a fundamental role in regulating the morphology and migration of early cortical precursors (Heng et al., 2008).

5.2. *Synapse formation by reprogrammed postnatal astroglia*

Notably, astroglia-derived neurons were capable of receiving functional synapses from co-cultured embryonic cortical neurons suggesting a remarkable ability for functional integration. Somewhat surprisingly, however, astroglia-derived neurons failed to form functional presynaptic outputs. Yet, consistent with Neurog2 playing a fundamental role in specifying a glutamatergic identity in telencephalic precursors (Bertrand et al., 2002), a substantial proportion of astroglia expressing Neurog2, but not Mash1 or Pax6, up-regulated the T-box transcription factor Tbr1 (Berninger et al., 2007a), a hallmark in glutamatergic neurogenesis (Hevner et al., 2006). However, the failure of forming functional presynaptic specializations argued that neuronal reprogramming remained only partial. In an effort to overcome these limitations by using viral vectors providing higher levels of expression and less susceptible to silencing we have been recently able to show that early postnatal astroglia can be directed towards fully functional synapse forming neurons, at least in vitro (Heinrich et al., unpublished observation). Importantly, this study showed that forced expression of distinct fate determinants such as Neurog2 and Dlx2, known for their critical roles in specifying glutamatergic and GABAergic neurons, respectively, within the developing telencephalon (Bertrand et al., 2002; Petryniak et al., 2007) directs astroglial cells towards distinct neuronal transmitter subtypes (Heinrich et al., unpublished observation), consistent with their developmental role. Thus, forced expression of neurogenic fate determinants can result in the stable and complete lineage reprogramming of early postnatal astroglia. Notably, in parallel to the decline in neurosphere-forming capacity, the susceptibility of undergoing reprogramming gradually decreases with the age of the cultured astroglia (Berninger, unpublished observation) again arguing for a gradual change in the internal milieu of astroglia undergoing maturation. Future studies will have to show whether these changes are accounted for by epigenetic modifications and whether and under what conditions they are reversible.

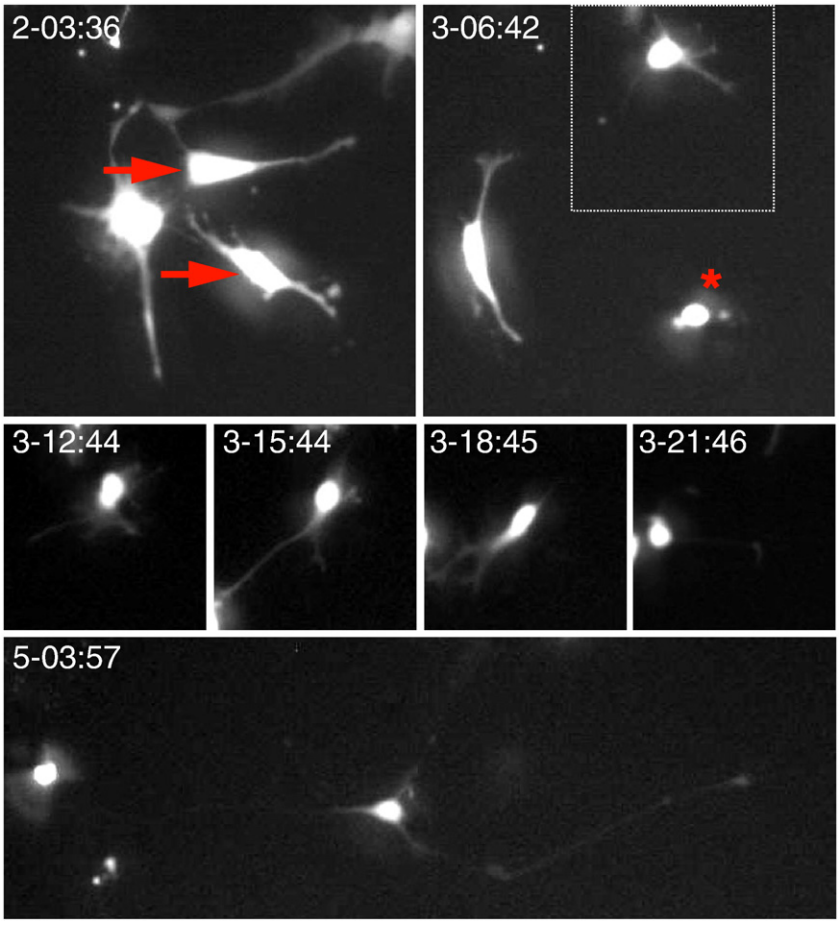

Fig. 2 – Metamorphosis of an astrocyte from the postnatal cerebral cortex into neuron upon forced expression of Neurog2. Panels depict different stages of cellular siblings (red arrows) derived from a hGFAP-GFP positive postnatal astroglia transduced with Neurog2. Time is shown as "days-hours:minutes". One of the daughter cells dies at time point 3-06:42 (red asterisk), whereas the second cell (dashed box) undergoes progressive morphological changes finally acquiring a neuronal morphology and TuJ1 expression (not shown) after 5 days.

5.3. *Recruitment of local or SEZ astroglia following injury of the postnatal cortex?*

While the present data argue for a high degree of plasticity of early postnatal astroglia in culture, astroglia fate-mapping studies from the Vaccarino lab suggest that to some low degree (<1%) astroglia can spontaneously generate neurons even in the postnatal cortex in vivo (Ganat et al., 2006). Moreover, following hypoxic insult, which causes a marked reduction in cortical thickness and neuron number at early postnatal stages, a much more substantial incorporation of new neurons can be observed (Fagel et al., 2009, 2006) which may eventually result in total recovery of cortical size and cell number. However, it is currently not known whether these newly incorporated neurons are derived from astroglia endogenous to the injured cortex or are rather from astroglial cells residing within the emerging adult neurogenic SEZ, which responds to hypoxic injury with a marked increase in proliferation (Fagel et al., 2009, 2006; Sundholm-Peters et al., 2005; Szele and Chesselet, 1996). The fact that many of the newly generated neurons were found to express Tbr1 (Fagel et al., 2006), as mentioned above a hallmark of glutamatergic neurogenesis, may on first sight argue for local generation, as postnatally generated neurons in the SEZ comprise largely GABAergic neurons. However, a recent study has demonstrated that the adult SEZ also contains a pool of glutamatergic progenitors which can be recruited from the SEZ upon injury (Brill et al., 2009).

6. Re-acquisition of a stem or progenitor-like state by mature glia following injury

6.1. *Reactivation of astroglia following injury of the cerebral cortex*

Thus, several lines of evidence suggest that the abysm between RGCs and early postnatal astroglia is not yet unbridgeable, but becomes wider and wider with development. Upon reaching adulthood at the latest, however, astrocytes resident to the cortical parenchyma seem to have lost all neurogenic potential and accordingly the adult cortex is devoid of newly generated neurons. Recently, Buffo and et al. re-assessed the question whether astroglia proliferates in

the mature cerebral cortex by genetic fate-mapping, using a mouse line expressing a tamoxifen-inducible Cre recombinase driven by the astrocyte-specific GLAST promoter crossed to a reporter line (Buffo et al., 2008). Notably, at that stage astroglia was found to become largely quiescent and hardly any genetically fate-mapped astroglia incorporated the thymidine analogue BrdU. Similar data were also obtained by double-labelling immunohistochemical makers for astrocytes with BrdU (Buffo et al., 2008) (C. Simon and M. Götz, unpublished observation). This situation changes dramatically following injury. When the cerebral cortex was locally injured by a stab wound, genetically fate-mapped astroglia was found to resume proliferation along side with the up-regulation of GFAP and other classical markers of reactive glia. Despite the observation that some injury paradigms appear to be inductive for low degree neurogenesis in the cerebral cortex (Magavi et al., 2000), no new neurons could be found in the stab wound model (Buffo et al., 2008, 2005). In fact, virtually all fate-mapped astroglia were found to give rise to new astrocytes (Buffo et al., 2008). Does this mean that these proliferating astroglia are intrinsically restricted to the astroglial lineage? In fact, in vivo the answer to this question seems to be yes. Surprisingly, however, when these astroglial cells were placed in vitro, these, but not their counterparts of the intact cortical hemisphere, could give rise to neurospheres characterized by the ability to generate secondary and tertiary neurospheres, suggestive of self-renewal capacity, and by multipotency, i.e. the ability to generate not only new astroglia, but also oligodendrocytes and neurons (Buffo et al., 2008). Intriguingly, ectopic grafting of committed neuronal precursors derived from the SEZ into a non-neurogenic milieu such as the adult striatum causes their glial conversion (Seidenfaden et al., 2006) suggesting that the lack of neurogenesis from reactivated astroglia in vivo may not be due to cell-intrinsic shortcomings of these cells compared to astro/radial glial stem cells of the SEZ, but their exposure to a highly non-neurogenic microenvironment.

Notably, data from our laboratory show that these neurosphere cells can be efficiently reprogrammed in vitro to generate glutamatergic and GABAergic neurons by forced expression of Neurog2 and Dlx2, respectively (Heinrich et al., unpublished observation). Can these cells also be reprogrammed in vivo? The answer to this question is a partial yes. Retrovirally-mediated forced expression of Pax6 was found to induce doublecortin (DCX) in some of the transduced progeny suggesting early stages of neurogenesis, but this population of DCX positive cells eventually disappeared (Buffo et al., 2005). This abortive neurogenesis may have many reasons, such as only partial transdifferentiation leading to failure of functional integration and ultimately apoptosis, therein resembling the high incidence of cell death following recruitment of DCX positive cells from the SEZ into the ischemic striatum (Arvidsson et al., 2002). This data suggests that successful reprogramming of mature astroglia represents only one of the hurdles to be taken if aiming at functional neurological reconstitution of damaged brain circuits. At the same time the microenvironment must be made favourable or at least permissive. Suffice it to say that neurons generated from reprogrammed astroglia must be able to form functional synaptic connections between themselves and the adjacent intact tissue, a process which will require the help of astroglia, well known to play a fundamental role in regulating synapse formation (Christopherson et al., 2005; Slezak and Pfrieger, 2003; Song et al., 2002) and plasticity (Perea and Araque, 2007).

Studies performed in our laboratory clearly indicate that while some early stages of neurogenesis can be indeed attained by forced expression of single neurogenic transcription factors, the response falls short in comparison to the effect observed in early postnatal astroglia. Yet, hope for improvement originates from work in adult pancreas. The Melton laboratory could show that while forced expression of single transcription factors failed to induce an effective reprogramming of exocrine cells into insulin secreting beta cells, a quite substantial degree of transdifferentiation was observed in the adult pancreas in vivo following expression of three defined factors involved in distinct stages of beta cell specification and differentiation (Zhou et al., 2008). It is thus conceivable that joint expression of several neurogenic transcription factors may be more effective in achieving stable lineage transgression towards neuronal identity and thereby also increase the chances for functional integration which in case of incomplete neuronal reprogramming will remain highly compromised.

Although the neurosphere-forming capacity seems to originate from reactive astroglia, we do not know whether all types of parenchymal astroglia de-differentiate to the same degree as to re-acquire NSC like properties. Intriguingly, however there are some hints to which molecular pathways may account for this remarkable response. As mentioned above, the morphogen Shh can induce GFAP-positive cells from the adult cerebral cortex to generate neurospheres (Jiao and Chen, 2008). A recent study has now revealed that Shh expression is indeed induced within cortical astroglia following a local injury {Amankulor, 2009 #267}. Surprisingly, the induction of Shh expression was found to depend on astroglia–macrophage interactions. There is an obvious need to gain more insights into the molecular processes underlying reactive gliosis. Indeed, recent progress unravelled a key role of astroglia–basement membrane contact mediated by integrins in maintaining astroglia in a non-reactive state (Robel et al., 2009). Of greatest interest in regard to the theme of astroglial de-differentiation into stem cells, loss of β1-integrin-mediated signalling leads to the activation of all hallmarks of reactive astrocytes except proliferation and stem cell de-differentiation, thereby highlighting that specific aspects of reactive gliosis are elicited by distinct signalling pathways.

6.2. *Reactivation of Müller glia following retinal injury*

Intriguingly, a similar response to injury can be observed in the retina. In teleost fish, substantial regeneration of the neural retina can be spontaneously achieved from Müller glia which may be a pendant to astroglia within other central nervous tissues (Lamba et al., 2008a). Indeed, Müller glia in the teleost retina have a complex response to local injury that includes some features of reactive gliosis (up-regulation of glial fibrillary acidic protein, GFAP, and re-entry into the cell cycle) along with characteristics associated with radial glia (expression of BLBP) and re-acquisition of molecular

characteristics of multipotent retinal progenitors, such as activation of Notch–Delta signalling and expression of Pax6 (Raymond et al., 2006). The regenerative capacity of the neural retina appears to be progressively lost during evolution. In birds, following acute damage to the neural retina Müller glia still re-enter the cell cycle with many expressing transcription factors characteristic of embryonic retinal progenitors such as Pax6 (Fischer and Reh, 2001). Notably, some of these progenitors then give rise to new neurons, while others produce new Müller glia or remain progenitors. Thus, despite some regenerative capacity, the response remains limited. In mammals such as mice, the regenerative response to neurotoxic injury resulting in the selective death of amacrine and retinal ganglion cells is even more restricted as Müller glia does not spontaneously enter the cell cycle (Karl et al., 2008). However, following intraocular injection of growth factors such as EGF, FGF2 or FGF2 in combination with IGF, it is possible to stimulate Müller glia proliferation and de-differentiation, which now do express Pax6 (Karl et al., 2008). Of note, this up-regulation of Pax6 is in strong contrast to what can be observed in reactive astroglia in the cerebral cortex where instead the basic helix loop helix transcription factor Olig2 is up-regulated, persistent expression of which is anti-neurogenic in the telencephalon (Buffo et al., 2005; Colak et al., 2008; Hack et al., 2005). Turning back to the retina, consistent with the induction of Pax6, when stimulated with growth factors Müller glia even generate some amacrine neurons. Again, many of these newly generated neurons die (Karl et al., 2008). One may speculate that this could be in part due to the fact that no new retinal ganglion cells are regenerated and thus functional integration of newly generated amacrine neurons remains incomplete eventually leading to their apoptosis.

Using the retinal injury model the Reh laboratory has provided some intriguing evidence to what actually happens when reactive glia de-differentiate. They found that Müller glia start to re-express a component of the SWI/SNF core complex called BAF60c (Lamba et al., 2008b). The SWI/SNF complex is an ATP dependent chromatin remodelling complex that undergoes changes in its composition in parallel to the differentiation from neural stem cell to neuron or glia. Notably, BAF60c is normally expressed in retinal progenitors and re-expression of this factor by Müller glia following injury combined with growth factor treatment suggests that the target specificity and hence remodelling activity SWI/SNF complex becomes progenitor-alike. One particular interesting aspect of BAF60c is its role in stabilizing the interaction between activated Notch and its DNA-binding partner RBP-J (Takeuchi et al., 2007). Thus, re-expression of BAF60c may render Müller glial cells more susceptible to Notch signalling. Intriguingly, in birds Notch signalling seems to play a dual role, namely it contributes to the de-differentiation process, but when maintained at high levels counteracts neurogenesis (Hayes et al., 2007) not unlike to what has been stated above about the role of Notch signalling in RGCs of the developing cerebral cortex.

Taken together, reactive gliosis in the cerebral cortex and the retina results in the re-acquisition of stem cell or progenitor-like properties, but in order to promote a regeneration eventually leading to the reconstitution of neurological function, the details of the reactive response need to be much better understood.

7. The concept of a transcriptional network underlying neural stemcellness

The fact that neural stem and progenitor cells often come in the disguise of glia leads us to one particularly perplexing question: what are the common denominators of the transcriptional core network regulating neural stem cell and (astro)-glial identity? Under transcriptional core network we understand a set of transcription factors which mutually regulate each other, thereby stabilizing the differentiation status of a cell. The concept of a transcriptional core network has been extremely fruitful in the case of embryonic stem cells which eventually led to the discovery of a defined set of transcription factors that allow for the reprogramming of somatic cells (Jaenisch and Young, 2008; Takahashi and Yamanaka, 2006). Forced expression of Oct4, Sox2, Klf4 and c-myc eventually superimposes onto fibroblasts a transcriptional circuitry such that these stably acquire the status of pluripotency. Is there a similar transcriptional circuitry underlying the differentiation state of a "neural stem cell"? The observation that neural stem cells are fate-restricted in a region-specific manner, at least with respect to neuronal subtype specification (Merkle et al., 2007), may suggest that there is indeed not a single transcriptional core network conveying the status of a neural stem cell, but potentially more than one. Yet, SEZ stem cells can be easily forced to acquire other neuronal subtype identity following forced expression of appropriate neurogenic fate determinants (Berninger et al., 2007b; Brill et al., 2008; Hack et al., 2005) indicating that they possess the competence to correctly interpret these transcriptional cues. These may argue in favour of a neural stem cell core network that is not region-specific, but universal. Furthermore, some transcription factors such as Sox2 (Cavallaro et al., 2008; Favaro et al., 2009; Graham et al., 2003; Kessaris et al., 2006; Suh et al., 2007; Taranova et al., 2006), Tlx (Liu et al., 2008; Roy et al., 2004; Shi et al., 2004) and Bmi-1 (Fasano et al., 2009; Molofsky et al., 2005, 2003; Moon et al., 2008; Zencak et al., 2005) appear to play a critical role in the maintenance and self-renewal of NSCs of different regions and developmental stages, suggesting that these might be either constituents of a NSC transcriptional network or at least closely related to it. A functional proof for the existence of a NSC transcriptional core network would be provided if forced expression of transcription factors defining this network could superimpose a NSC fate on parenchymal astroglia or even other types of somatic cells that are ontogenetically more distant than astrocytes from NSCs.

Two aspects merit further consideration: first the surprising closeness of neural to pluripotent stem cells. While fibroblast require several transcription factors for reprogramming into induced pluripotent stem cells, sole expression of Oct4 is sufficient to reprogram mouse adult as well as human foetal neural stem cells into pluripotent cells (Kim et al., in press, 2009). These data suggest that the stable transition between a neural and a pluripotent stem cell core networks may be rather simple, and reflect the other side of the coin that neural determination often appears a default pathway of embryonic stem cell differentiation (Tropepe et al., 2001). Such

facile transition may reflect a high degree of commonalities between these core networks, as exemplified by the common expression of Sox2.

The second point deals with the question posed at the beginning of the paragraph: How can a NSC core network co-exist with a hypothetical transcriptional core network conveying "glianess" within a single cell? Obviously, a NSC transcriptional network is not active in all astroglia, as classical parenchyma astroglia are not functioning as NSCs. However, some candidate constituents like Sox2 are expressed not only in NSCs, but also in parenchymal astroglia (Komitova and Eriksson, 2004). Other candidate genes such as Tlx seem to be restricted to stem cell astroglia (Liu et al., 2008), and loss of Bmi-1 even increases the genesis of non-stem cell astroglia at the expense of NSCs (Zencak et al., 2005), while forced expression of Bmi-1 induces stem cell characteristics in cultured astroglia (Moon et al., 2008). Thus, there seems to be a clear partitioning between stem cell and parenchymal astroglia. Yet, as the studies in the injured cerebral cortex or retina have shown, parenchymal astroglia have the capacity to regain NSCs characteristics (Buffo et al., 2008; Karl et al., 2008). Possibly, this capacity is due to a partial overlap of the two otherwise distinct transcriptional circuits, as represented by the common expression of Sox2, which may enable astroglia in some circumstances to switch from one to the other mode of being, i.e. being stem or parenchymal glia.

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Review

Neurotransmitter signaling in postnatal neurogenesis: The first leg

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ABSTRACT

Like the liver or other peripheral organs, two regions of the adult brain possess the ability of self-renewal through a process called neurogenesis. This raises tremendous hope for repairing the damaged brain, and it has stimulated research on identifying signals controlling neurogenesis. Neurogenesis involves several stages from fate determination to synaptic integration via proliferation, migration, and maturation. While fate determination primarily depends on a genetic signature, other stages are controlled by the interplay between genes and microenvironmental signals. Here, we propose that neurotransmitters are master regulators of the different stages of neurogenesis. In favor of this idea, a description of selective neurotransmitter signaling and their functions in the largest neurogenic zone, the subventricular zone (SVZ), is provided. In particular, we emphasize the interactions between neuroblasts and astrocyte-like cells that release gamma-aminobutyric acid (GABA) and glutamate, respectively. However, we also raise several limitations to our knowledge on neurotransmitters in neurogenesis. The function of neurotransmitters *in vivo* remains largely unexplored. Neurotransmitter signaling has been viewed as uniform, which dramatically contrasts with the cellular and molecular mosaic nature of the SVZ. How neurotransmitters are integrated with other well-conserved molecules, such as sonic hedgehog, is poorly understood. In an effort to reconcile these differences, we discuss how specificity of neurotransmitter functions can be provided through their multitude of receptors and intracellular pathways in different cell types and their possible interactions with sonic hedgehog.

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Abbreviations: EGFR, epidermal growth factor receptor; FLRFa, phe-leu-arg-phe amide; GABA, gamma-aminobutyric acid; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; GLAST, glutamate–aspartate transporter; mGluR5, metabotropic glutamate receptor 5; MrgA1, Mas-related gene A1; NMDAR, NMDA receptors; RMS, rostral migratory stream; SHH, Sonic hedgehog; SGZ, subgranular zone; SVZ, subventricular zone; VGLUT1, vesicular glutamate transporter 1; YFP, yellow fluorescent protein

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Contents

1. Introduction

Until recently, the generation of neurons was thought to occur only during the embryonic period while the brain was considered relatively "stable" after birth. It is now clear that the mammalian brain is more plastic than previously perceived and has a remarkable ability to adapt to environmental stimuli or stress by modifying its structural and physiological characteristics. New neurons continue to be produced in two regions of the adult forebrain, the subgranular zone (SGZ) of the dentate gyrus and the subventricular zone (SVZ) lining the lateral wall of the lateral ventricle. This review focuses on the SVZ, the largest neurogenic zone, characterized by a well-defined migratory route, called the rostral migratory stream (RMS), taken by neuroblasts to reach the olfactory bulb where they integrate as interneurons.

The production of adult neurons is an ongoing process. While the extent of SVZ neurogenesis remains controversial in humans (Sanai et al., 2004; Curtis et al., 2007), in rodents, estimates suggest that 10,000 to 30,000 neurons are produced every day (Lledo et al., 2006). This high turnover rate requires profound homeostatic mechanisms that regulate and coordinate the different stages of neurogenesis to prevent neuron overproduction. Homeostasis is ensured through interplay between genetic (intrinsic) program and microenvironmental (extrinsic) signals. Here, we propose that among the microenvironmental signals neurotransmitters are master regulators of neurogenesis.

Neurotransmitters are classically released from synaptic terminals and are the basis of the chemical communication between neurons. Pioneering studies have suggested that these small diffusible molecules play important functions on cell development during embryonic life (Behar et al., 1994, 1998; LoTurco et al., 1995; Haydar et al., 2000; Platel et al., 2005) as well as adult SGZ neurogenesis (Gould et al., 1994; Cameron et al., 1995). More recently, a series of elegant studies reported that several neurotransmitters regulate postnatal neurogenesis in the SVZ. Findings from these studies are described here and divided into two groups; we first discuss the signaling of two monoamines, dopamine and serotonin, which are released by neuronal inputs in the SVZ. We next discuss the signaling by the metabolically related amino acids GABA and glutamate, which are locally released by neuroblasts and astrocyte-like cells in the SVZ, respectively. The SVZ is composed of different cell types including astrocyte-like cells, transit-amplifying cells, and neuroblasts. Some of these astrocyte-like cells act as neural stem cells. GABA and glutamate signaling have been described between astrocyte-like cells and neuroblasts providing a bidirectional communication between these two cell types. For each neurotransmitter, we describe the signaling mechanisms, including receptor, source(s) of release, and transporter. We then discuss the functions of each neurotransmitter on neurogenesis.

Despite a growing wealth of information on neurotransmitter signaling and function in adult neurogenesis, there are clear limitations to the present work. First, the *in vivo* impact of selectively perturbing neurotransmitter signaling remains poorly addressed. Addressing this issue has been limited for technical reasons as will be discussed here. Neurotransmitter signaling has been described as uniform across the SVZ, which dramatically contrasts with the mosaic cellular and molecular nature of the SVZ. It is unknown whether GABA and glutamate signaling differ among astrocyte-like cells considering that some of these cells act as neural progenitor cells. These neural progenitor cells located in different regions of the SVZ generate different types of interneurons (Merkle et al., 2007; Young et al., 2007). However, it remains unclear, whether and if so how, neurotransmitter signaling selectively regulates some subpopulations of neural progenitor cells. It is also hard to reconcile the uniformity of neurotransmitter signaling with their multiple actions such as regulators of migration and proliferation. One possible explanation is that each neurotransmitter activates different receptor subtypes that are developmentally regulated and composed of different subunits, and thus activate different intracellular pathways. Finally, our perhaps egocentric view of neurotransmitter signaling needs to be revisited in light of the presence of a multitude of other well-conserved and critical signals. We will

speculate how neurotransmitters and one crucial neurogenic signal, Sonic hedgehog (Shh), may interact, providing even more selectivity to neurotransmitter signaling as well as amplifying their diversity of actions. After addressing each limitation, we propose future directions and emerging concepts that may reconcile these different views.

2. Cellular and functional organization of the SVZ

Organization of the SVZ has been well-described and extensively reviewed (Doetsch et al., 1997; Mercier et al., 2002; Quinones-Hinojosa et al., 2006; Mirzadeh et al., 2008a) (for a few reviews, see Privat and Leblond, 1972; Privat, 1977; Alvarez-Buylla and Lim, 2004; Bordey, 2006). Neural progenitor cells (also called neural stem cells, type B cells, SVZ astrocytes) are scattered along the SVZ and RMS (Doetsch et al., 1999; Gritti et al., 2002). They self-renew and generate transit-amplifying cells (called type C cells) that also asymmetrically divide to give birth to neuroblasts (type A cells). Neuroblasts remain proliferative along the SVZ and migrate to the olfactory bulb via long distance, tangential migration throughout the SVZ and RMS (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994; Peretto et al., 1997) (Fig. 1a). Once in the olfactory bulb, they leave the RMS and migrate radially to the different neuronal layers. Neuroblasts will mature into two main interneuron types, granule and periglomerular cells that are located in the granule cell and glomerular layer, respectively (Fig. 1a).

Antigenically all SVZ cells express nestin, a marker of neural progenitor cells (Hockfield and McKay, 1985; Doetsch et al., 1997; Platel et al., 2009). Astrocyte-like cells of the SVZ inherited such a name from the expression of astrocytic markers such as glial fibrillary acidic protein (GFAP) and glutamate–aspartate transporter (GLAST, Braun et al., 2003; Bolteus and Bordey, 2004; Platel et al., 2009). In fact, they express other astrocytic markers including gap junction connexin 43, Aldh1L1 (in neonates, see www.gensat.org), Lex (Cd15), the other glial glutamate transporter GLT-1, glial GABA transporter GAT3/4, and glutamine synthase (Capela and Temple, 2002; Bolteus and Bordey, 2004; and see Platel JC and Bordey A, unpublished observations for glutamine synthase). Transit-amplifying cells express epidermal growth factor receptor (EGFR; Platel et al., 2009; Cesetti et al., 2009; Pastrana et al., 2009), dlx2, and mash1 (Aguirre et al., 2004; Parras et al., 2004). Astrocyte-like cells and some EGFR-expressing cells also share some markers such as brain lipid basic protein and GLAST (Platel et al., 2009). Neuroblasts express doublecortin (DCX), β-III tubulin, and PSA-NCAM (Bonfanti and Theodosis, 1994; Rousselot et al., 1995; Nacher et al., 2001; Brown et al., 2003). Findings regarding the astrocyte-like cells and transit-amplifying cells have raised several issues. First, it is unclear whether all astrocyte-like cells or only a subset of them can behave as neural progenitor cells during their lifetime. In other words, is there a unique population of astrocyte-like cells with stem cell characteristics or is there a turnover of cells that behave as neural progenitor cells? A related issue concerns the astrocytic nature of these neural progenitor cells. These SVZ astrocytes express properties of mature astrocytes that are fully differentiated cells. It thus remains unknown what makes them so different to behave as neural progenitor cells. It is even more confusing considering that based on a transcriptome analysis mature astrocytes have recently been shown to express transcription factors such as Sox2, Pax6, Id1, and Id3, thought to be enriched in neural progenitor cells (Cahoy et al., 2008). This is addressed in a detailed manner in the review by Berninger et al. in this special issue. SVZ astrocytes are derived from radial glia, the embryonic neural progenitor cells (Paterson et al., 1973; Malatesta et al., 2000; Noctor et al., 2001; Merkle et al., 2004), and perhaps the presence of embryonic extracellular matrix allows these astrocytes to retain their stemness (Gates et al.,

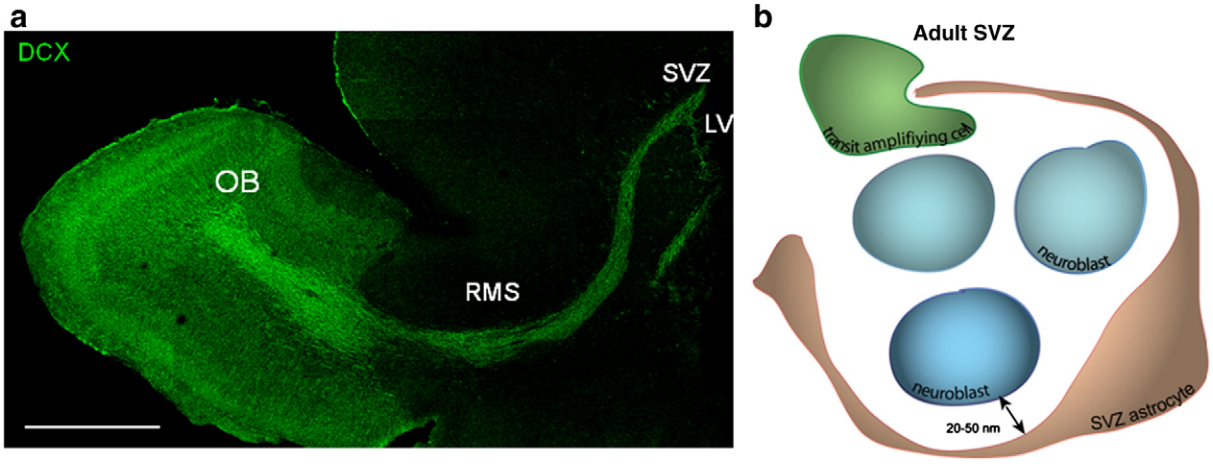

Fig. 1 – Structure of the neurogenic subventricular zone. (a) Reconstruction of a sagittal slice of a postnatal (P) 25 mouse brain immunostained for doublecortin (DCX). Chains of DCX-expressing neuroblasts from the subventricular zone (SVZ) converge to form the rostral migratory stream (RMS), which terminates in the olfactory bulb. Scale bar: 500 μm. (b) Simplified diagram illustrating the cellular composition of the SVZ. The processes of a SVZ astrocyte ensheath a cluster of neuroblasts (in a coronal plan).

1995). Finally, transit-amplifying cells may contain distinct populations with different fates, neuronal, glial, or both, that need to be further clarified.

On a structural level, the SVZ is a thin region that spans the whole lateral wall of the lateral ventricle and contains tubes filled with neuroblasts also called chains of neuroblasts. Each chain composed of 4–5 neuroblasts is tightly ensheathed by processes of astrocyte-like cells (Lois and Alvarez-Buylla, 1994; Doetsch and Alvarez-Buylla, 1996; Peretto et al., 1997) (Figs. 1a and b). Astrocyte-like cells are located between these chains and their processes form the wall of the "tubes" containing neuroblasts. Astrocyte-like cells also seem to isolate the neurogenic zone from the mature parenchyma (Platel et al., 2009). A subset of astrocyte-like cells also make contact with the ventricle on one side and the blood vessels on the other side and those SVZ astrocytes may be the neural progenitors (Shen et al., 2008; Tavazoie et al., 2008; Mirzadeh et al., 2008b; Lacar et al., 2009). Based on electron microscopic observations, the extracellular space between SVZ astrocytes and neuroblasts is between 20 and 50 nm (Privat and Leblond, 1972), which is comparable in distance to a synaptic cleft. This led us to hypothesize that signaling occurring in the SVZ may resemble that at the tripartite synapse. In particular, we described a novel signaling between astrocyte-like cells and neuroblasts in the glutamatergic signaling section below.

3. Functions of dopaminergic and serotoninergic inputs in the SVZ

Both dopamine and serotonin are monoamines implicated in the regulation of mood, motivation, and movement and, more recently, have been shown to regulate adult SVZ neurogenesis (for recent reviews on dopamine in neurogenesis, see Borta and Hoglinger, 2007; O'Keeffe et al., 2009).

3.1. *Dopaminergic and serotoninergic signaling mechanisms*

Dopamine receptors are G protein-coupled and classified as D_1-like (D_1 and D_5) and D_2-like (D_2, D_3, and D_4) receptors according to structural homologies (Callier et al., 2003). In the embryonic and adult SVZ, high levels of D_3 receptor expression have been shown by *in situ* hybridization and autoradiography (Diaz et al., 1997). D_2-like dopamine receptors have been found on transit-amplifying cells, and both D_1- and D_2-like receptors have been reported in neuroblasts (Hoglinger et al., 2004). Expression on SVZ astrocytes has not been reported so far. Although some SVZ neuroblasts differentiate into dopaminergic interneurons in the olfactory bulb, they do not express tyrosine hydroxylase, the enzyme involved in the synthesis of dopamine (Baker et al., 2001). This enzyme was not found in any SVZ cell type. Nevertheless, dopamine was shown to be released from dopaminergic afferents that directly contact transient amplifying cells in the SVZ (Hoglinger et al., 2004). In non-human primates, some of those fibers were shown to come from the substantia nigra (Freundlieb et al., 2006). Concerning dopamine uptake mechanisms, dopamine transporters were found on the dopaminergic fibers entering the SVZ but not on SVZ cells using immunostaining (Hoglinger et al., 2004; Shibui et al., 2009).

Serotonin (5-HT) receptors, which are encoded by 14 genes, are G protein-coupled receptors with the exception of the 5-HT_3 receptor (Barnes and Sharp, 1999; Pauwels, 2000). 5-HT_3 receptor is a ligand-gated ion channel that is mainly permeable to sodium and potassium ions. Using ligands for these receptors and cell proliferation assay *in vivo*, four of these receptors have been found to be involved in the regulation of neurogenesis: 5-HT_{1A}, 5-HT_{1B}, 5-HT_{2A}, and 5-HT_{2C} (Banasr et al., 2004). However, the cellular localization of these receptors in the SVZ remains unknown. Another study showed that transgenic mice expressing green fluorescent protein (GFP) under the 5-HT3 receptor promoter showed a high expression on SVZ neuroblasts that was confirmed by *in situ* hybridization (Inta et al., 2008). A dense plexus of 5-HT-immunoreactive fibers projects into the SVZ from the raphe nucleus and thus provides an extrinsic source of serotonin; the exact cell type targeted by these fibers remains to be examined (Simpson et al., 1998; Brezun and Daszuta, 1999; Banasr et al., 2004; Diaz et al., 2009). Serotonin transporters have been observed on either neuroblasts or 5-HT fibers entering the SVZ using immunostaining (Diaz et al., 2009; Shibui et al., 2009). Additional studies are required to further assess the pattern of serotonin transporter expression in the SVZ.

3.2. *Functions*

In individuals with Parkinson's disease where dopaminergic signaling is disturbed, proliferation in the SVZ is decreased (Hoglinger et al., 2004). Consistent with this finding, dopaminergic agonists increase neurogenesis in mouse models of Parkinson's disease (Yang et al., 2008) and removal of the dopaminergic projections decreases proliferation in the SVZ (Hoglinger et al., 2004; Baker et al., 2004) (for review, see Borta and Hoglinger, 2007; O'Keeffe et al., 2009). This effect is mediated by activation of D_2-like receptors on transit-amplifying cells most likely via the EGF receptor in conjunction with release of EGF in a PKC-dependent manner (Coronas et al., 2004) or via a ciliary neurotrophic factor-dependent mechanism (Yang et al., 2008). However, dopaminergic innervations of the SVZ and dopamine functions on cell proliferation depend on the strain of mice used, suggesting that caution is required when comparing from different studies and extrapolating from mice to humans (Baker et al., 2005). In addition, the physiological significance of these dopaminergic projections into the SVZ needs to be further explored. Similarly, the clinical relevance of changes in SVZ cell proliferation during the course of Parkinson's disease remains unclear (for review, see Borta and Hoglinger, 2007).

Regarding serotoninergic signaling, activation of 5-HT_{1A} and 5-HT_{2C} receptors *in vivo* increased proliferation in the SVZ, while activation of 5-HT_{1B} decreased cell proliferation (Banasr et al., 2004). Activation and inhibition of 5-HT_{1B} receptors decreased and increased cell proliferation, respectively, suggesting an opposite effect compared to the other receptors. The increase in cell proliferation with 5-HT_{1A} and 5-HT_{2C} agonists resulted in an increased number of adult born BrdU-labeled granule cells several weeks after a single dose of agonist injection (Banasr et al., 2004). Consistent with these findings,

inhibition of serotonin synthesis or lesions of the serotoninergic raphe neurons reduce neurogenesis by approximately 60% (Brezun and Daszuta, 1999). The effects of serotonin seem to be mediated in part by its stimulation of BDNF expression (Mattson et al., 2004). Overall, serotonin is considered as a positive regulator of adult neurogenesis in the SVZ.

4. GABAergic and glutamatergic signaling in the SVZ

The neurotransmitter GABA (gamma-aminobutyric acid) and glutamate have been extensively studied in the context of development and more recently neonatal/adult neurogenesis (for reviews see (Nguyen et al., 2001; Owens and Kriegstein, 2002; Schlett, 2006; Bordey, 2007; Manent and Represa, 2007; Henschel et al., 2008; Platel et al., 2008a)). In adult neurogenesis GABA has received particular attention compared to glutamate perhaps due to the ubiquitous expression of GABA receptors in immature cells.

4.1. *GABAergic signaling and functions*

GABA's action is mediated by at least three types of GABA receptors (GABARs) (Chebib and Johnston, 1999): the ligand-gated chloride (Cl^-) channels, $GABA_AR$ and $GABA_CR$, and the G protein-coupled receptor $GABA_BR$. $GABA_BRs$ are functionally expressed in SVZ cells based on calcium imaging (Platel JC and Bordey A, personal observation). It is unknown whether $GABA_CRs$ are expressed in the SVZ. $GABA_ARs$ are functionally expressed on both neuroblasts and astrocyte-like cells based on calcium imaging and patch clamp recordings (Wang et al., 2003; Nguyen et al., 2003; Bolteus and Bordey, 2004; Liu et al., 2005). The expression of these receptors on transient amplifying cells still needs to be investigated. One limitation in studying transit-amplifying cells has been the difficulty in identifying them in the live SVZ (e.g., acute slices). Using a viral vector encoding a green or red fluorescent protein under the EGFR promoter may provide one method to visualize them in live tissue. $GABA_ARs$ are composed of multiple subunits (16 total) conferring different properties (Henschel et al., 2008). However, very little is known about the expression of these subunits in SVZ cells. Neonatal neuroblasts expressed α2, 3, 4, β1, 2 and 3, and γ2S mRNA *in vitro* (Stewart et al., 2002) while neonatal PSA-NCAM-positive progenitors from neurospheres (which may include glioblasts) expressed α2, 4, 5, β1 and 3, and γ1, 2, 3, and δ subunit transcripts (Nguyen et al., 2003). As mentioned later, identifying the sequential acquisition of the different subunits in the different SVZ cell types is critical to better understand $GABA_AR$ specificity of action. In the SVZ and RMS, neuroblasts synthesize and release GABA as shown by immunostaining for GABA in slices (Wang et al., 2003; Bolteus and Bordey, 2004) and *in vitro* (Stewart et al., 2002), and by patch clamp recordings using $GABA_ARs$ as sensors for GABA (Liu et al., 2005). Liu et al. (2005) reported that electrical stimulation or high potassium application induced GABA release from SVZ cells. The release was independent of action potentials, external calcium, and the SNARE-dependent vesicular machinery, but it was dependent on intracellular calcium changes. Together with the absence of synapses in the SVZ and RMS at the electron microscopic levels and the lack of immunostaining for the synaptic marker synapsin 1, it was concluded that GABA signaling was nonsynaptic (i.e., paracrine). Recent data also showed that vesicular GABA transporters as well as a member of the SNARE complex synaptobrevin 2 (VAMP2) are not expressed in SVZ neuroblasts (Platel et al., 2007, 2010). The mechanism(s) of GABA release from neuroblasts remain unknown. Released GABA is taken up by the GABA transporters GAT3/4 in SVZ astrocytes (Bolteus and Bordey, 2004; Liu et al., 2005) while the presence of the neuronal GABA transporter GAT1 in neuroblasts remains unclear. It is unknown whether transit-amplifying cells have the ability to release or take up GABA.

GABA exerts a tonic inhibitory control on the proliferation of SVZ astrocytes in 30- to 40-day-old transgenic mice expressing GFP under the human *GFAP* promoter (Liu et al., 2005) and striatal neuroblasts from neonatal mice (Nguyen et al., 2003). These studies used the S-phase mitotic marker bromodeoxyuridine (BrdU) in both organotypic slices as well as neurospheres. Ambient GABA was found to reduce the speed of neuroblast migration in acute sagittal slices from both juvenile and adult mice (Bolteus and Bordey, 2004; Platel et al., 2008b). GABA also increased dendritic growth of neuroblasts, which were integrating in the olfactory bulb in acute slices (Gascon et al., 2006). These studies suggest that GABA controls some of the early and late stages of cell development via paracrine signaling. The function of GABA on transit-amplifying cells has not been explored, although changes in their proliferation could profoundly impact the number of adult-born neuroblasts.

4.2. *Glutamatergic signaling and functions*

Glutamatergic signals are conveyed by different glutamate receptor subtypes, namely ionotropic NMDA and AMPA/kainate receptors (Sommer and Seeburg, 1992; Hollman and Heinemann, 1994) and metabotropic glutamate receptors (mGluRs; groups I–III subtypes, mGluR1-8; Conn and Pin, 1997; Coutinho and Knopfel, 2002). The presence of mGluRs and NMDA, AMPA, and kainate receptors was observed in cultured SVZ cells derived from neonates (Brazel et al., 2005). Using calcium imaging and electrophysiological recordings in acute slices, we found that neuroblasts express AMPA (Platel et al., 2007), kainate (Platel et al., 2008b), and NMDA receptors (Platel et al., 2010) and mGluR5 (Platel et al., 2008b). mGluR5 immunoreactivity was also reported in SVZ cells in fixed tissue (Di Giorgi Gerevini et al., 2004). It is important to emphasize that neuroblasts express a mosaic of glutamate receptors, but an individual neuroblast does not express all four receptor types. It is unclear whether neuroblasts expressing different populations of receptors have different fates (e.g., periglomerular cells versus granule cells). In addition, at least for NMDA receptors, it was clearly shown that neuroblasts acquire an increasing number of NMDA receptors during their migration along the SVZ and RMS before entering the olfactory bulb synaptic network (Platel et al., 2010). The characterization of glutamate receptor expression in astrocyte-like cells and transit-amplifying cells is less clear. Astrocyte-like cells did not display any NMDA or AMPA-induced currents or calcium increases even in the presence of an inhibitor of AMPA receptor desensitization (Liu et al., 2006; Platel et al., 2010).

Whether transit-amplifying cells express glutamate receptors is unknown. Regarding the source of glutamate in the SVZ, we recently found that astrocyte-like cells immunostain for glutamate and vesicular glutamate transporter 1 (VGLUT1) (Platel et al., 2007, 2010). In addition, using post-embedding immnogold labeling for VGLUT1, we found gold particles on vesicles including fusing vesicles in astrocyte-like cells of the SVZ (Platel et al., 2010). Importantly, to test for calcium-dependent glutamate release from astrocyte-like cells, we used transgenic mice in which GFAP-expressing cells express a Gq-protein-coupled receptor (called Mas-related gene A1, MrgA1) that is not endogenous to the brain and has no endogenous ligands. These mice were generated by Dr. Ken McCarthy using the inducible tet-off system (Fiacco et al., 2007). Mice expressing the tetracycline transactivator (tTA) under the human *GFAP* promoter were crossed to mice in which the MrgA1 receptor was transcribed off the tet (tetO) minimal promoter. In the SVZ of *hGFAP*-tTA×tetO-MrgA1 mice (referred MrgA1+ mice), GFP fused to MrgA1 was found to be selectively expressed in SVZ astrocytes but not in neuroblasts (Fig. 2a diagram). Application of the MrgA1-selective peptide agonist (phe-leu-arg-phe amide, FLRFa) resulted in calcium increases in GFP fluorescent cells in the SVZ, i.e., astrocyte-like cells. In addition, FLRFa increased the frequency of NMDA receptor-mediated channel activity in neuroblasts recorded in the RMS of acute slices (Platel et al., 2010). It was concluded that astrocyte-like cells released glutamate in a calcium-dependent manner onto neuroblasts. It is noticeable that in acute hippocampal slices from MrgA1 mice, FLRFa application did not affect synaptic transmission (Fiacco et al., 2007). This emphasizes the

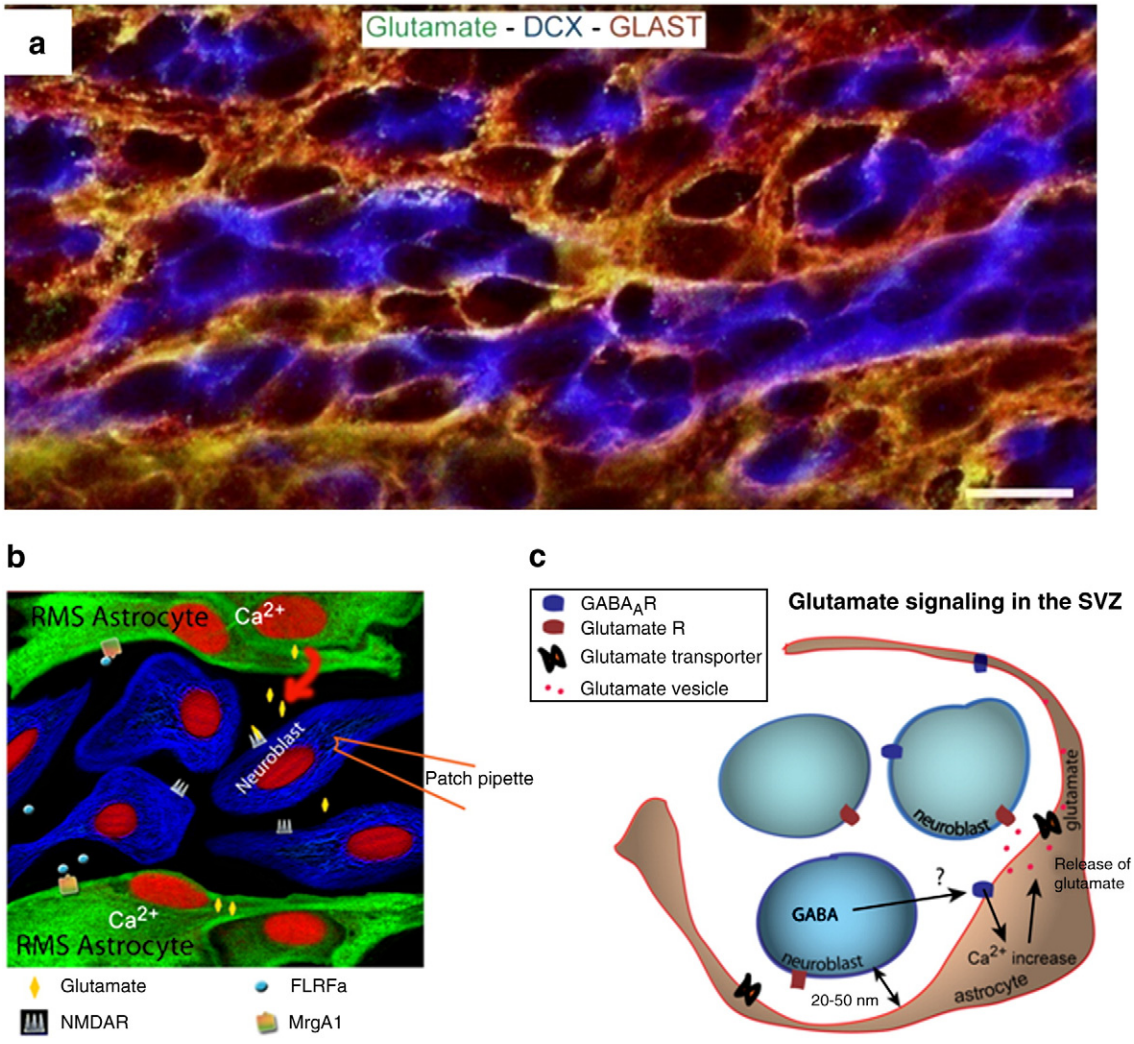

Fig. 2 – GABAergic and glutamatergic signaling in the SVZ. (a) Confocal photograph displaying coimmunostaining for glutamate (green), GLAST (glutamate–aspartate transporter) which labels astrocytes (red), and DCX (a marker of neuroblasts, blue) in the rostral extension of the SVZ. Scale bar: 10 μm. (b) Model illustrating that FLRFa peptide induces calcium increases in RMS astrocytes leading to glutamate release and NMDA receptor activation in adjacent neuroblasts recorded with a patch clamp pipette. (c) Simplified diagram illustrating the expression of GABA and glutamate signaling molecules in the SVZ. Neuroblasts can release GABA into the extracellular space. GABA activates $GABA_A$ receptors in SVZ astrocytes. Upon intracellular calcium increase, glutamate is released by SVZ astrocytes. Released glutamate diffuses and activates glutamate receptors on neuroblasts.

importance of the glutamate signal from astrocyte-like cells to neuroblasts in the neurogenic zone. We also found that astrocyte-like cells in the SVZ, like mature astrocytes, immunostained for glutamine synthase, which is an enzyme involved in the conversion of glutamate into glutamine (Platel JC and Bordey A, unpublished observation). Although this enzyme may decrease the concentration of glutamate in astrocyte-like cells, the amount of vesicular glutamate may not be affected. In addition, it is possible that glutamate transporters expressed in SVZ astrocytes are specifically localized to load vesicles. However, these issues remain to be addressed in future studies. Another attractive question is whether SVZ astrocytes release glycine or D-serine, which, when released from mature astrocytes, was shown to activate NMDA receptors in surrounding neurons and modulate neuronal transmission (for review, see Parpura and Zorec, this issue). Finally, SVZ astrocytes also contain high-affinity glutamate transporters, GLAST and GLT-1 (Bolteus and Bordey, 2004; Liu et al., 2005; Platel et al., 2009) (Fig. 2a). The neuronal glutamate transporter EAAT-3 (for excitatory amino acid transporter) was not found in the SVZ using immunostaining (Liu et al., 2005).

In the SVZ, we and others have recently observed that glutamate receptors carry out different functions. Using an acute whole mount of the lateral wall of the lateral ventricle, we found that inhibition of GLU_{K5} kainate receptors (previously called GluR5 and now called GluK2) significantly increased the speed of neuroblast migration while mGluR5 inhibition had no effect (Platel et al., 2008b). Regarding mGluRs, adult mice lacking mGluR5 or treated with mGluR5 antagonists showed a dramatic reduction in the number of proliferating cells in the SVZ (Di Giorgi-Gerevini et al., 2004, 2005). Recently, "single-cell" knockout of NR1, a critical NMDA receptor subunit for receptor functionality, led to a significant increase in neuroblast apoptosis resulting in a dramatic reduction of neurogenesis in the olfactory bulb (Platel et al., 2010; Lin et al., 2010). Neuroblast apoptosis occurred during their migration in the SVZ and RMS, which is much earlier than previously thought and to some extent during synaptic integration (Platel et al., 2010), as expected based on data in the SGZ (Tashiro et al., 2006). Genetic removal *in vivo* or pharmacological blockade of NMDA receptors did not affect the speed of neuroblast migration in the RMS (Platel et al., 2010). To genetically remove NR1, a Cre recombinase-containing vector was either transduced using a viral vector or electroporated into SVZ cells in adult and neonatal transgenic mice, respectively, where the NR1 subunit is flanked by loxP sites (i.e., floxed). Neonatal electroporation was recently described by two studies (Boutin et al., 2008; Chesler et al., 2008). In addition, for the electroporation experiments, these mice were crossed with Rosa26 reporter mice so that Cre recombinase expression led to excision of both NR1 and a Stop sequence in front of a yellow fluorescent protein (YFP) (Platel et al., 2010). As a result, a subset of neuroblasts were YFP-fluorescent and lost NR1 leading to loss of functional NMDA receptors, which was validated with both patch clamp recordings and calcium imaging. Data in the study of Platel et al. suggest that acquisition of NMDA receptors in neuroblasts is critical for their survival in an asynaptic environment where glutamate is provided from astrocyte-like cells. This study raises several questions; it was shown that astrocyte-like cells tonically release glutamate in a calcium-dependent manner. However, the signal(s) leading to intracellular calcium increases in SVZ astrocytes remain unclear. One possibility is the neurotransmitter GABA released from neuroblasts (Liu et al., 2005), but it is not known whether GABA increases calcium in SVZ astrocytes (Fig. 2b for diagram). The function of NMDA receptors on neuroblast proliferation remains to be examined. Finally, the *in vivo* function of glutamate release from astrocyte-like cells on neurogenesis remains to be explored. This would require a genetic conditional knockout of glutamate release from SVZ astrocytes, for example, using transgenic mice in which VGLUT1 is floxed.

5. Limitation 1: *In vivo* effects of manipulating signaling mechanisms on neurogenesis

Beside the studies on NMDA receptors in transgenic $NR1^{floxed}$ mice and on mGluR5 in knockout mice, the work on neurotransmitters in neurogenesis has been performed in acute slices or following drug injection *in vivo*. The work in slices is hard to extrapolate to the process of neurogenesis *in vivo*. The use of *in vivo* drugs may lack specificity and involves indirect pathways such as hormonal responses. Nevertheless, despite the lack of specificity and mechanistic insight, testing the effects of drugs on neurogenesis is important to eventually use these drugs for therapeutic interventions aimed at boosting neurogenesis.

To address this limitation, we clearly need to use genetic manipulations of receptors as shown for NMDA receptors. Knockout mice are helpful and they will provide valuable information, but compensation by other receptor subtypes is common and may bias the results and data interpretation. One of the best approaches in terms of selectivity and complete removal is using transgenic mice where a receptor or subunit flanked by *loxP* sites (i.e., floxed) is excised upon Cre recombinase expression. Fortunately, the Knockout Mouse Project (KOMP) will soon be making scores of *loxP* conditional strains available to the research. Another approach is to use RNA interference (RNAi) technology. RNAi is now easy to design and deliver using viral constructs or electroporated plasmids encoding short hairpin RNA (shRNA). RNAi is less selective than using "floxed" transgenic mice due to off-target effects. Nevertheless, together with rescue experiments, the use of RNAi is becoming a standard and relatively simple approach. These two approaches ("floxed" mice or RNAi) require knowing which receptors or subunits are expressed in each SVZ cell type both at the transcript and protein levels. This is an imperative next step to advance our knowledge of neurotransmitter function on neurogenesis.

6. Limitation 2: Mismatch between the uniform actions of neurotransmitters with the mosaic nature of the SVZ and the diversity of functions on cell development

Almost every study thus far has presented neurotransmitter signaling as uniform along the SVZ. This is particularly true for

GABAergic signaling, which seems ubiquitous in the SVZ; every neuroblast and astrocyte-like cell express $GABA_A$ receptors (Wang et al., 2003; Liu et al., 2005; Platel et al., 2008b). This is in drastic contrast with the mosaic cellular and molecular nature of the SVZ and the diversity of neurotransmitter functions.

The SVZ contains several populations of cells. The population of SVZ astrocytes contains non-proliferative astrocyte-like cells and self-renewing neural progenitor cells (see Section 2 on organization of the SVZ). At the molecular level, it has recently been reported that neural progenitor cells in the SVZ are heterogeneous with respect to their neurogenic fate and embryonic origin (Merkle et al., 2007; Young et al., 2007). This heterogeneity translates into subpopulations of neural progenitor cells each in a distinct location of the SVZ resulting in a mosaic appearance (Merkle et al., 2007). In addition, the same neurotransmitter may regulate every stage of cell development. The extreme example is for GABA, which regulates neuroblast proliferation, migration, and dendrite extension through the same type of receptor $GABA_A$ receptors. The question is thus: how is neurotransmitter selectivity provided for distinct cellular populations, subpopulations, and stages of cell development? We provide three possible explanations: (1) The presence of distinct receptor subtypes or subunits allow for receptor heterogeneity, which may lead to activation of different intracellular pathways, thus regulating distinct stages of cell development or cell populations. In addition, it is suggested that receptor subtypes are developmentally regulated by the sequential acquisition of different glutamate receptor subtypes (Platel et al., 2008a,b, 2010). Even for $GABA_A$ receptors, it is expected that different cell populations will express different subunits and that the subunit composition may change as cells mature. (2) The dopaminergic and serotoninergic inputs into the SVZ may provide regional selectivity. For example, it is conceivable that dopaminergic inputs may predominate in the rostral section of the SVZ while serotoninergic inputs may be more caudal. This, however, has not been investigated. (3) Neurotransmitters may interact with other signaling molecules that are critical for neurogenesis (e.g., Shh or Wnt signaling, see Section 7 for additional details) and may be differentially expressed along the SVZ. These types of interactions would amplify the diversity of neurotransmitter action and also would provide regional and cellular specificity of functions.

Because each receptor subtype or subunit is expected to play unique roles during neurogenesis, it is important to knockout or knockdown every receptor subtype or subunit one by one. This would help tease out their selective role on different stages of cell development and cell type. In addition, stage-dependent conditional removal of receptor subtypes may reveal their sequential function during cell development. For example, the same receptor subtype or subunit may play a role on cell proliferation and later on cell integration through activation of different intracellular cascades. This could be explored using a promoter selective to maturing postmitotic neuronal precursors to drive Cre recombinase or shRNA expression in these cells once they enter the olfactory bulb. Analyzing the effect on the different types of interneurons should reveal whether certain genetic manipulations affect certain subpopulations of neural progenitor cells.

7. Limitation 3: How does neurotransmitter signaling integrate with other crucial signals?

It is perhaps egocentric to view neurotransmitters as master regulators of neurogenesis in light of the diversity of signals implicated in regulating the different stages of neurogenesis (for reviews, see Pozniak and Pleasure, 2006; Pathania et al., 2010). However, neurotransmitters, such as GABA and glutamate, have conserved signaling functions across phyla. For example, GABA accumulates rapidly in plant tissues in response to biotic and abiotic stress. Recent evidence suggests that a gradient of GABA regulates plant growth possibly through regulation of intracellular calcium dynamics (for review, see Ma, 2003; Bouche and Fromm, 2004; Roberts, 2007). Although genes highly homologous to the mammalian GABA receptors are not present in the *Arabidopsis* genome, it has been proposed that GABA could interact with a family of proteins (designated atglrs for *Arabidopsis* glutamate receptors) that share sequence and structural homology with the mammalian ionotropic glutamate receptors (iGluRs) (for review and references, see Bouche and Fromm, 2004). Neurotransmitters may also interact with and perhaps control both the expression and function of other signals such as Sonic hedgehog (Shh), Wnt, and/or Notch signaling. We provide here a speculation on interactions between neurotransmitters and Shh. Shh homolog is one of three proteins in the signaling pathway family called hedgehog, the others being Desert and Indian hedgehog. Shh is well known to be critical for vertebrate organogenesis. More recently, Shh was found to be a crucial signal for adult neurogenesis, particularly in maintaining stem cell self-renewal and acting as a mitogen in the SVZ and SGZ (Palma et al., 2005; Ahn and Joyner, 2005; Wang et al., 2007; Han et al., 2008). Changes in monoamine levels *in vivo* through chemical depletion have been shown to regulate the expression of the Shh signaling cascade in the adult rodent brain measured with *in situ* hybridization (Rajendran et al., 2009). The same group also reported that electroconvulsive seizures upregulated Shh signaling pathways at the mRNA level in the SGZ (Banerjee et al., 2005). These data suggest that neurotransmitters released during seizures may impact the expression of Shh and its receptors. It is thus conceivable that neurotransmitters regulate the expression of Shh components in the SVZ. Shh acts through a receptor complex associating Patched and Smoothened (for review, see Philipp and Caron, 2009). Smoothened is structurally similar to G protein-coupled receptors and accumulating evidence suggests that Smoothened relies on heterotrimeric G proteins to transduce Shh signal. mGluRs, which are G protein coupled receptors could thus interact with Smoothened signaling cascade. Similarly, changes in intracellular calcium levels may affect kinase activity and G protein phosphorylation. Further studies are clearly required to examine such types of interactions and may help better understand the specificity of neurotransmitter action.

8. Conclusion

A better picture of neurotransmitter signaling in the largest postnatal neurogenic zone, the SVZ, is beginning to emerge. The signaling in this region is more complex that we anticipated with intricate communication between the different cell types. In particular, bidirectional communication between astrocyte-like cells and neuroblasts has just been reported. Neuroblasts release GABA, which activates $GABA_A$Rs on themselves and astrocyte-like cells while astrocyte-like cells release glutamate, which activates glutamate receptors on neuroblasts. How these two signaling interact remain to be investigated. In this asynaptic network, astrocyte-like cells are in charge of glutamatergic signaling that is critical for neuroblast survival and ultimately proper neurogenesis. Glutamate release from astrocytes appears to be a conserved function from immature to mature networks with distinct impacts on network activity.

Despite an increase in our understanding of neurotransmitters in neurogenesis regarding their signaling and function, this is clearly only the first leg of the research journey as discussed through the limitations above. In the coming years, it is critical to identify the role of every receptor subtype or subunit in neurogenesis using genetic manipulations and to address whether neurotransmitters interact with crucial neurogenic signals. In addition, there is increasing interest in studying the role of glutamatergic signaling on cell development because genetic studies suggest that mutations or epigenetic misregulation in receptors and their subunits (e.g., glutamate receptors) are associated with neurodevelopmental disorders such as autism spectrum disorder, bipolar disorders, epilepsy, and schizophrenia, to name a few (please see for example the following database: www.genecards.org, section disorders and mutations of the gene of interest). Studying the consequences of gain or loss-of-function of receptors and subunits will help us understand the etiology of developmental disorders. A better understanding of neurotransmitter signaling in neurogenesis will hopefully also help us design better strategies to promote brain repair from endogenous progenitor cells or improve transplant efficiency.

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BRAIN RESEARCH REVIEWS

Review

NG2 cells: Properties, progeny and origin

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ABSTRACT

The NG2 proteoglycan is a type 1-transmembrane protein expressed by a range of cell types within and outside the mammalian nervous system. NG2-expressing (NG2) cells are found in grey and white matter tracts of the developing and adult CNS and have previously been assumed to represent oligodendrocyte precursor cells: new work using transgenic mice has shown that NG2 cells generate oligodendrocytes, protoplasmic astrocytes and in some instances neurons *in vivo*. NG2 cells express GABAA receptors and the AMPA subtype of glutamate receptors. They make intimate contact to neurons prior to myelinating axons and also form electron-dense synaptic specialisations with axons in the cerebellum, cortex and hippocampus and with non-myelinated axons in the corpus callosum. These synaptic NG2 cells respond to neuronal release of glutamate and GABA. This neuron–glia interaction may thus regulate the differentiation and proliferation of NG2 cells. The C-terminal PDZ-binding motif of the NG2 protein binds several PDZ proteins including Mupp1, Syntenin and the *Glutamate Receptor Interacting Protein* (GRIP). Since GRIP can bind subunits of the AMPA receptors expressed by NG2 cells, the interaction between GRIP and NG2 may orientate the glial AMPA receptors towards sites of neuronal glutamate release. The origin, heterogeneity and function of NG2 cells as modulators of the neuronal network are important incompletely resolved questions.

Contents

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1. Introduction

Cells expressing the proteoglycan NG2 make up 5–10% of all glia in the developing and adult CNS. They are evenly distributed in white and grey matter and some cells proliferate even in the adult, implying a continual turnover of this cell population. NG2 protein expression is down-regulated upon maturation of the cells. Expression of NG2 has in the past been used as a marker for oligodendrocyte precursor cells (OPC; see Nishiyama et al., 2009) and recent evidence discussed below has demonstrated that indeed NG2 cells give rise to oligodendrocytes, but also to subpopulations of astrocytes during normal development. The discovery at the turn of the last century of the unusual synaptic association between NG2 cells and neurones in multiple regions of the developing and adult CNS (Bergles et al., 2000; Jabs et al., 2005) has spurred widespread interest in this cell population and suggested that in addition to acting as a plastic progenitor pool for more differentiated cells, NG2 glia may form a unique glial network in continual parlance with neurones. In addition to the expression on immature myelinating glia in the CNS, NG2 is expressed in the PNS by immature Schwann cells.

In this review we will focus on the properties, progeny and origin of NG2 cells but first summarise current knowledge regarding the structure and function of the NG2 protein, the defining feature of this class of cells.

2. Structure of NG2 and its partner molecules

The NG2 protein was originally defined by antibodies directed against surface proteins on a rat cell line with glial and neuronal properties (Stallcup, 1981). Independently NG2 homologues were discovered in human and mouse (Harper et al., 1984; Niehaus et al., 1999; Pluschke et al., 1996; Schneider et al., 2001; Stegmuller et al., 2002). The mammalian protein of 330 kDa (also termed CSPG-4) is encoded by a single gene with multiple exons coding for 2327 amino acids. To date no alternatively spliced variants have been described. The amino terminus exhibits two *Laminin G/Neurexin/Sex Hormone Binding Globulin* (LNS) domains (Fig. 1), thus placing NG2 firmly in the large family of the neurexins; cell adhesion molecules dictating synaptic specificity in neurones and exhibiting a large degree of alternative splice forms (Missler and Sudhof, 1998). The large extracellular domain of NG2 includes sites near the single transmembrane domain which are readily cleaved by a variety of proteases *in vitro* and *in vivo* (Nishiyama et al., 2009). This can result in the deposition of the ectodomain in the extracellular matrix, thus making the identification of NG2-expressing cells by antibodies, especially in lesions rich in proteases, particularly difficult. The biological relevance of this cleavage is unclear: one possibility is that it could be a mechanism to release NG2 from a putative receptor via regulated proteolysis. The intracellular domain is

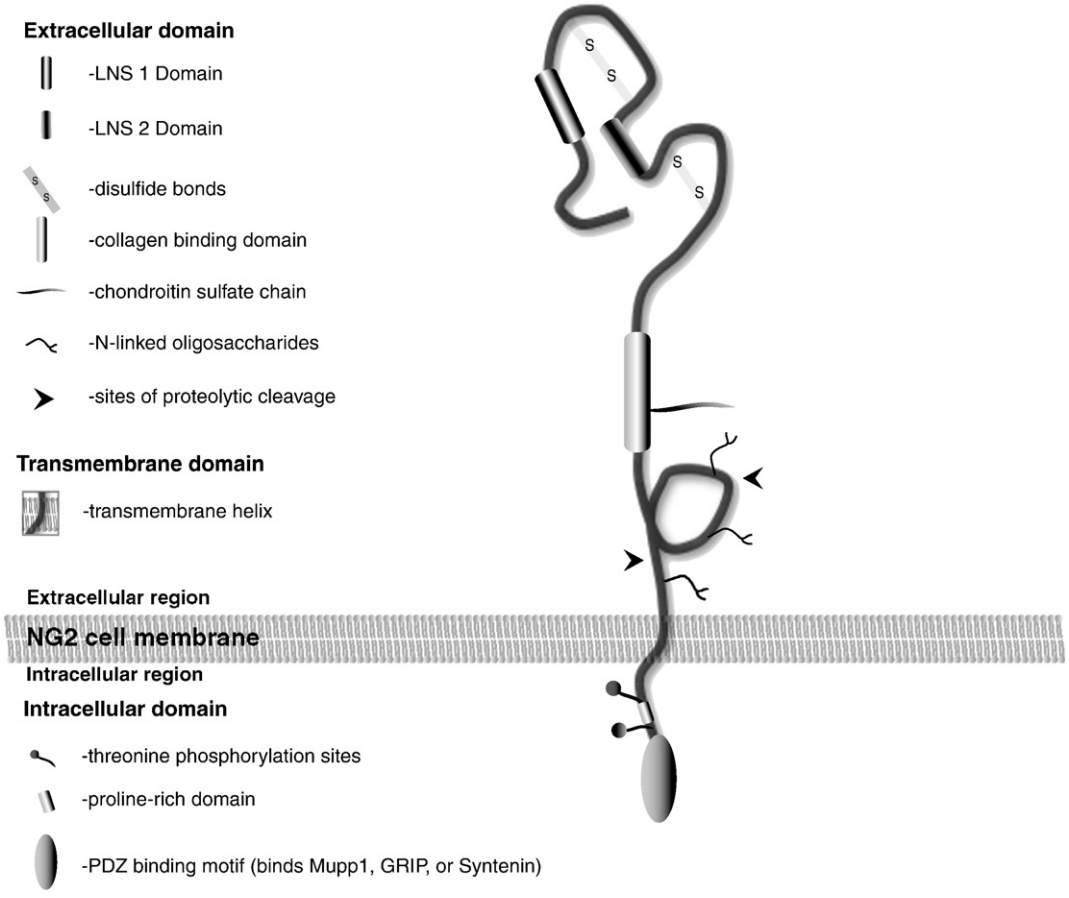
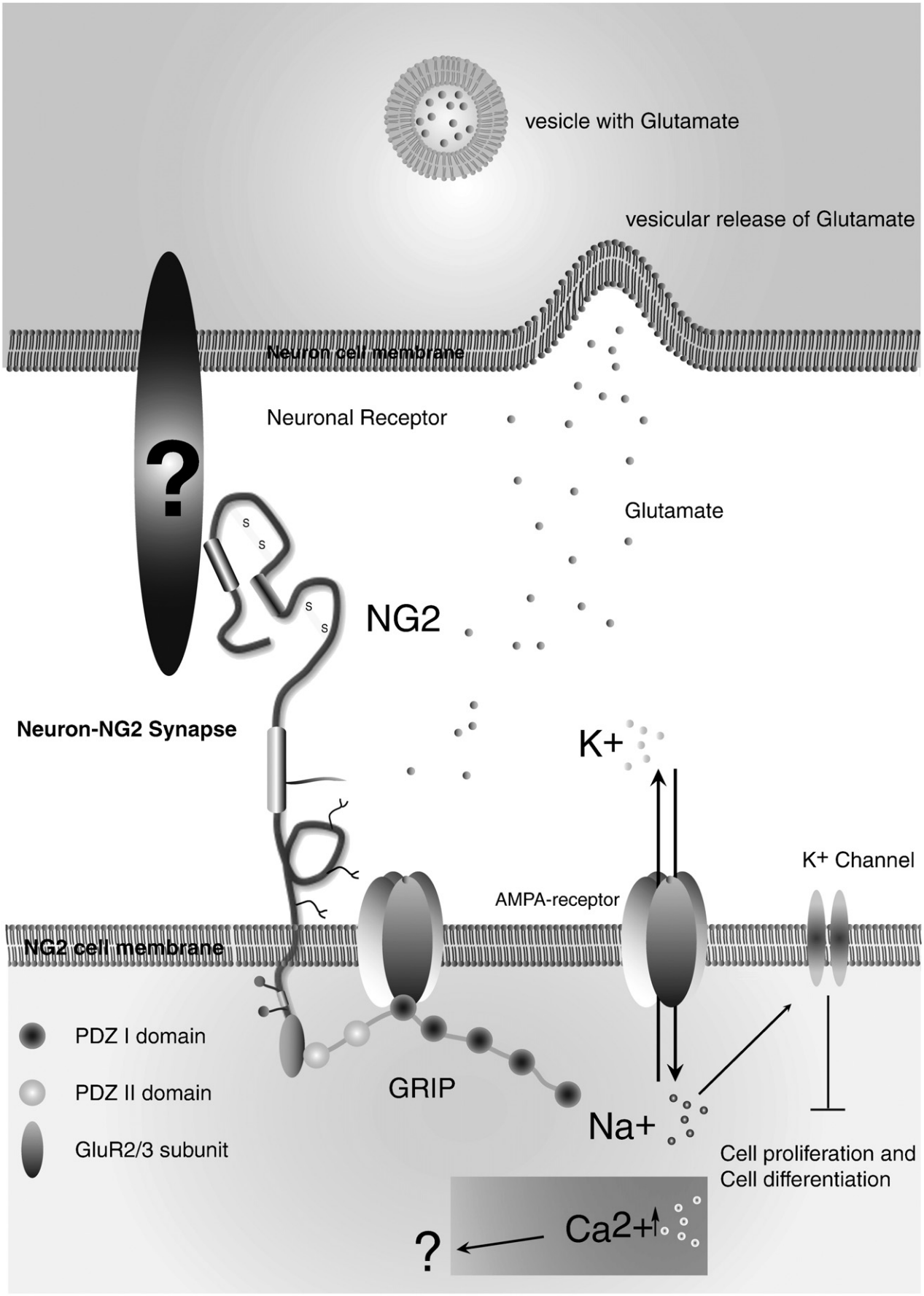


Fig. 1 – Schematic diagram of the NG2 protein modified from (Stallcup and Huang, 2008).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vesicular release of Glutamate
Neuron cell membrane
Neuronal Receptor
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NG2
Neuron-NG2 Synapse
K+
K+ Channel
AMPA-receptor
NG2 cell membrane
PDZ I domain
PDZ II domain
GluR2/3 subunit
GRIP
Na+
Cell proliferation and
Cell differentiation
Ca2+
?

rather short (76 Aa) and includes a C-terminal type I *Postsynaptic density 95/Discs Large/Zonula-occludens-1* (PDZ) domain recognition motif, as well as several threonines whose phosphorylation state regulates cell behaviour such as spreading and migration (Fang et al., 1999; Lin et al., 1996; Majumdar et al., 2003; Tillet et al., 2002). In addition, a type II PDZ domain binding motif, a *Src Homology type 2* (SH2) domain binding motif and a WW-domain binding motif all underline interactions of NG2 with intracellular signalling and structural proteins. The NG2 protein is a part-time proteoglycan; chondroitin sulfate gylcosaminoglycan (GAG) chains are linked to the extracellular domain (Nishiyama et al., 2009). The amount of GAG chains carried by the core protein varies with cell type and developmental stage (Schneider et al., 2001).

Several partner molecules have been identified for the NG2 protein. These include β1 integrins in melanoma and astrocytoma and the receptor for PDGFα: these associate in cis with NG2. PDGFAA, FGF2, Collagen V and VI, MT3MMP, Plasminogen, tPA and galectin 3 have all been described as binding directly to the large NG2 ectodomain (summarised in Nishiyama et al., 2009). Interestingly, no cell adhesion molecule ligands for the LNS domains have been described to date. In the light of the interaction of NG2 cells with axons during myelination and at synapses in development and in the adult, it is likely that neuronal receptors exist. Several binding partners for the C-terminal type I PDZ domain-binding motif have been identified. These are *Multi-PDZ Domain protein 1* (MUPP1), *Glutamate Receptor Interacting Protein* (GRIP) and Syntenin-1(Barritt et al., 2000; Chatterjee et al., 2008; Stegmuller et al., 2003). In particular, the latter two partners may be relevant for the function of NG2 cells at synapses and in wrapping axons at early stages of myelination. GRIP binds to the GluR2/3 subunits of the AMPA receptor, glutamate receptors expressed by NG2 cells which are activated by neuronally released glutamate at the neuron-glial synapse and also influence oligodendrocyte differentiation (see articles in this issue by Steinhauser et al., (Gallo et al., 1996) and also Fig. 2). Syntenin provides connections to the cell cytoskeleton, which may be relevant for migration of NG2 cells to axons prior to myelination as well as process movement of synaptic NG2 glia. Furthermore, NG2 has been shown to recruit the small GTPase cdc42 and p130cas (crk-associated substrate) in melanoma cells (Eisenmann et al., 1999), intracellular molecules regulating diverse processes in migration and cell polarisation.

3. NG2 in evolution

NG2-like proteins are found in non-vertebrates including *Caenorhabditis elegans* and *Drosophila melanogaster*. Interestingly, in both these species the C-terminal PDZ binding domain is conserved, furthermore in Drosophila NG2 two LNS domains are recognisable.

Two recent papers have reported that Drosophila NG2 (called Kon-tiki or Perdido) associates with Drosophila GRIP demonstrating the evolutionary conservation of this interaction (Estrada et al., 2007; Schnorrer et al., 2007). Both groups reported a role of NG2 expressed by immature muscle cells in attaching to tendons. In vertebrates NG2 expression outside the nervous system includes muscle progenitor cells and pericytes (Nishiyama et al., 2009). It remains to be seen whether Drosophila glia express NG2, similar to their mammalian counterparts.

4. Functions of NG2 cells

The study of NG2 cells *in situ* as well as their progeny has been facilitated by the generation of new mouse lines. The DsRed protein chromophore or the Cre recombinase with the NG2 promoter has been inserted using Bacterial Artificial Chromosome technology to generate transgenic mouse lines (Nishiyama et al., 2009). Alternatively the EYFP protein has been inserted after the start AUG of the first exon of the endogenous NG2 gene to yield a "knock-in" mouse line (Karram et al., 2008). Use of these mice to study the generation of oligodendrocytes and astrocytes from NG2 progenitors is discussed below.

Synaptic contacts formed by NG2 cells have been described with axons of neurones in the hippocampus and cerebellum (Bergles et al., 2000; Lin et al., 2005), as well as with unmyelinated axons in the corpus callosum (Kukley et al., 2007; Ziskin et al., 2007). In addition to these synapses which appear largely on the processes of NG2 cells, the cell bodies of NG2 cells are very closely apposed to neurons in many brain regions including the hippocampus, cerebellum and cortex (Fig. 3). Intimate contact between NG2 cells and neurons has been observed earlier in an electron microscopic analysis of cortex in rats, where NG2 glia were defined as beta glial cells and were considered a fourth glial cell type (Peters, 2004). In contrast to published literature where double-labelling of brain sections was carried with polyclonal antibodies to NG2 and neuronal markers (Belachew et al., 2003; Dayer et al., 2005) we have never observed expression of neuronal markers by EYFP cells in the EYFP-NG2 knockin mouse or by DsRed+ cells in the NG2-DsRed transgenic mice (Fig. 4).

In contrast to observations that the NG2 protein appeared to be a repulsive molecule for cerebellar neurons *in vitro* (Tan et al., 2005, 2006), axons are clearly not repulsed by NG2 cells but in fact appear to be actively contacting them (Butt et al., 2005; Nishiyama et al., 2005; Yang et al., 2006). We have observed that axons of rat hippocampal neurones readily grow over the plasma membrane of HEK cells expressing a truncated version of NG2 lacking a large part of the extracellular domain but containing the LNS domains (Chatterjee et al., 2008); (Griemsmann and Trotter, unpublished observations). In contrast, the chondroitin sulfate side chains of NG2 are likely to be inhibitory to axonal growth (Galtrey and Fawcett, 2007). Neurones exclusively form synapses *in vivo* with NG2 cells but not with neighbouring astrocytes, oligodendrocytes or microglia: NG2 glial cells are thus unique

Fig. 2 – The role of the NG2 protein at the neuron–glial synapse. The NG2 protein could play a role in clustering the glial AMPA receptors towards the site of neuronal glutamate release. Glutamate acting on NG2 cells may thus regulate proliferation and differentiation and also cause a rise in intracellular calcium.

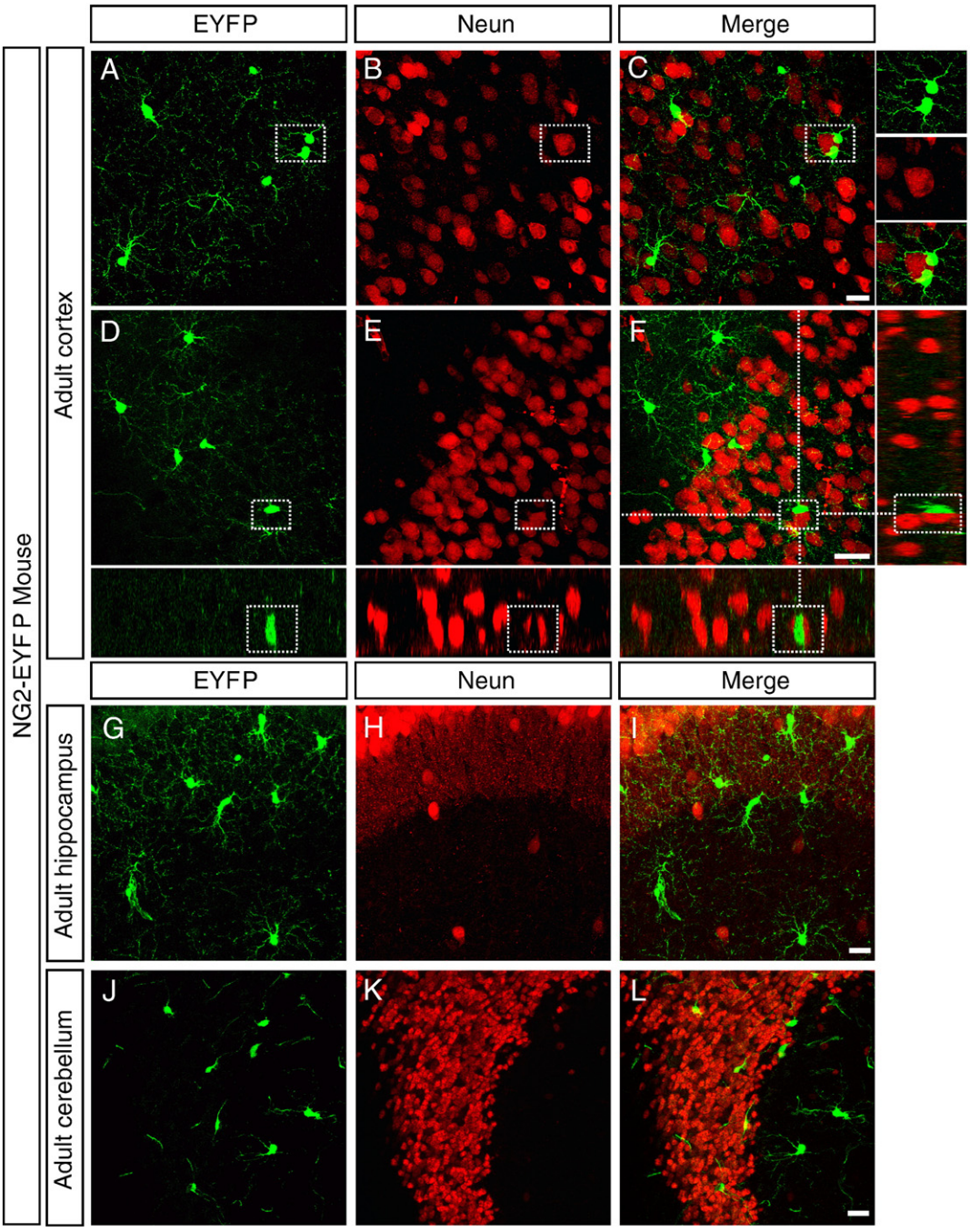

Fig. 3 – Intimate contact between neurons and NG2 cells in the CNS. Confocal image scan of cortex, hippocampus, and cerebellum of adult mice expressing EYFP (A, D, G, J) stained with an antibody that recognizes Neun (B, E, H, K). Merged images (C, F, I, L) shows no overlap, but close association between EYFP+ cell and Neun+ neurons. Inserts at high magnification show EYFP+ cells close to Neun+ neurons. Scale bars=20 μm.

in promoting presynaptic specialisation in neurones. Could the NG2 protein itself be a synapse-promoting molecule? Furthermore, it is likely that NG2 cells release synapse-modulatory substances such as *Brain Derived Neurotrophic Factor* (BDNF), as suggested by a recent publication (Tanaka et al., 2009) (Fig. 5).

Electrophysiological and imaging evidence has demonstrated that excitation of NG2 glia by neuronal release of GABA acting on the GABAA receptors of NG2 cells, or via glutamate acting on the AMPA receptors, invokes a calcium signal (Gallo et al., 2008; Hamilton et al., 2009; Paukert and Bergles, 2006). Recent work has linked the GABA-induced signals in OPC to activation of sodium channels prior to changes in calcium and a stimulation of migration (Tong et al., 2009). It will be important to define events subsequent to this rise in calcium, for example changes in gene expression in NG2 cells.

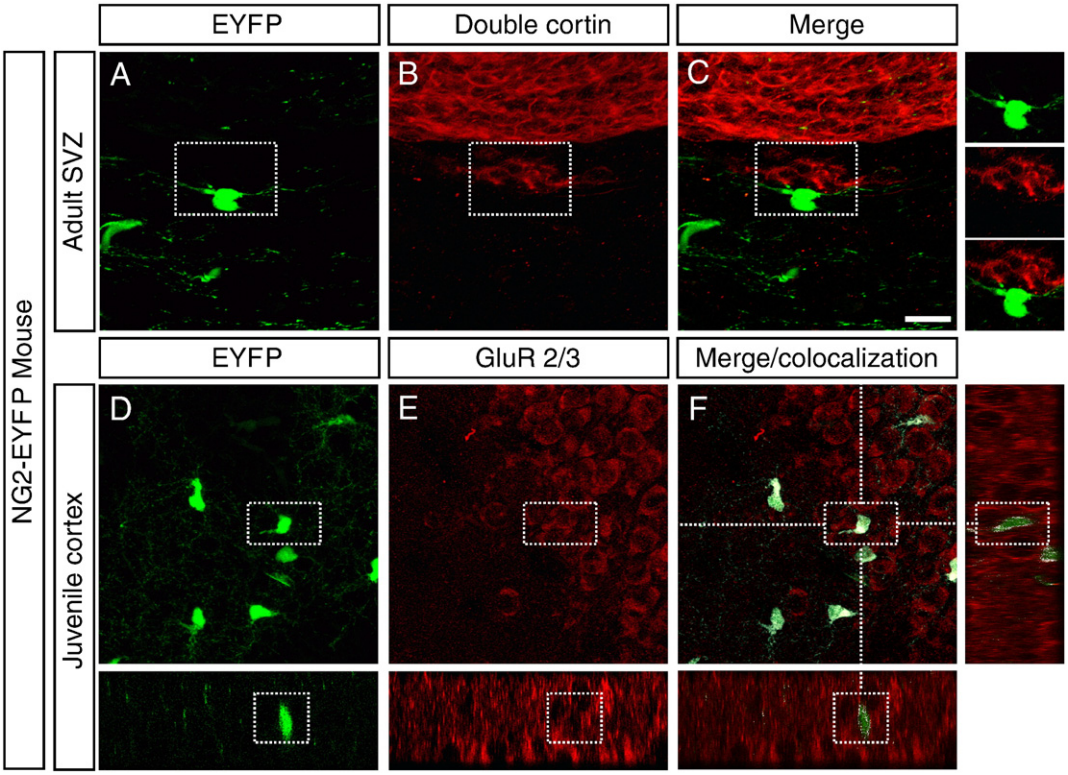

Fig. 4 – Intimate contact between neurons and NG2 cells in the SVZ and expression of GluR2/3 by NG2 cells *in vivo*. Confocal image scan of the SVZ of adult mice expressing EYFP (A), stained with an antibody that recognizes Doublecortin (B). Merged images (C) show no overlap but close association between EYFP+ cells and Doublecortin+ neurons. Hogh magnification inserts show EYFP+ cells very close to Doublecortin+ neurons. EYFP+ cells (D) in the juvenile cortex stain with an antibody recognising GluR2/3 (E). Merge and co-localization analysis shows expression of the GluR2/3 on the processes and cell body of the EYFP+ cells. Scale bars = 20 μm.

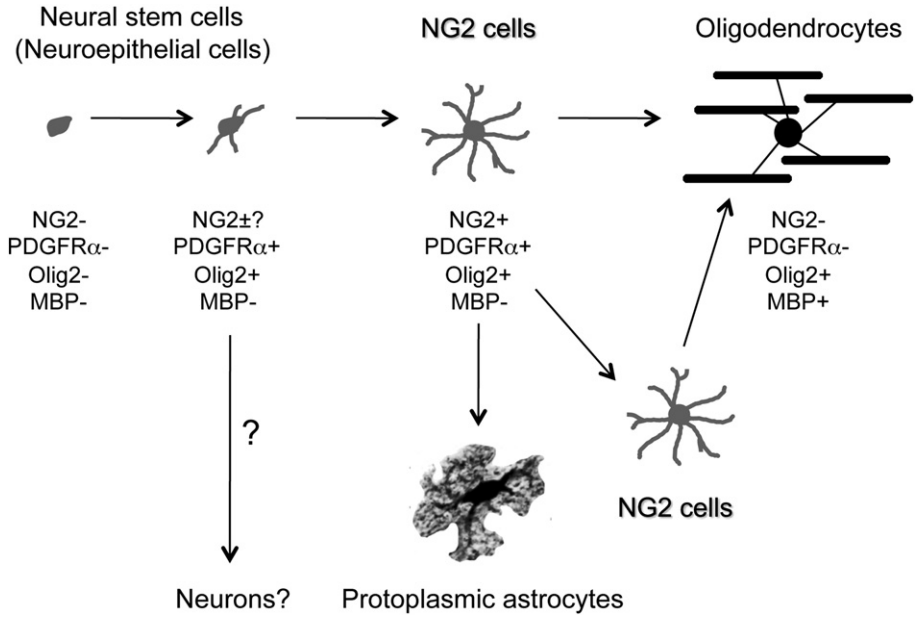

Fig. 5 – A schematic diagram showing the NG2 cell lineage. NG2 cells originate from NG2-/PDGFRα- cells in the germinal zone and acquire NG2 expression as they migrate to their destination. They have the ability to self-renew and generate oligodendrocytes at all ages. NG2 cells in the immature brain also generate a subpopulation of protoplasmic astrocytes. The neuronal fate of NG2 cells is still debated.

5. Heterogeneity of the NG2 population

Since NG2 cells can give rise to different cell types (see below), an obvious question is whether NG2 cells are a heterogeneous population. Are there regional or developmental differences in the functions and differentiation potential of NG2 cells? All NG2 cells appear to express the receptor for PDGF AA. Analysis of transcription factor expression by NG2 cells in different regions of the brain have shown that almost all NG2 cells express Olig 2 and Sox 10 (Karram et al., 2008; Kitada and Rowitch, 2006; Ligon et al., 2006a,b). The NG2 population is heterogeneous when examined for the expression of glutamine synthestase, at least in the hippocampus (Karram et al., 2008). Study of a transgenic mouse in which EGFP was fused to the 3′ UTR of the PLP gene, demonstrated two populations of NG2 cells in the developing subventricular zone: one population expressed EGFP and another lacked EGFP expression. The authors suggested that one population generated oligodendrocytes while the other population was more immature (Mallon et al., 2002). Several groups have shown heterogeneity in the NG2 cell population based on electrophysiological measurements. NG2 cells in the white and grey matter areas of the developing mouse were shown to differ in morphology and electrophysiological properties: furthermore, a few NG2+ cells in the grey matter elicited depolarization-induced spikes similar to immature action potentials (Chittajallu et al., 2004). In contrast, two other groups (Ge et al., 2009; Karadottir et al., 2008) reported subpopulations of NG2 cells in white matter which were able to generate action potentials; a finding which has generated controversy in the field as it would force a reclassification of these NG2 cells as bona-fide neurons. Within the hippocampus, a grey matter area, the EYFP+ cells are heterogeneous at a given developmental stage based on their electrophysiological properties (Karram et al., 2008). Unfortunately, neither the immunohistochemical nor the electrophysiological studies permit a distinction of functional diversity within the lineage from lineage heterogeneity. This question can better be addressed by studies using the transgenic mouse lines described below.

6. Progeny of NG2 cells

The fate of NG2 cells has been a subject of intense debate and has been studied using a variety of approaches. Here we will review primarily the recent literature on *in vivo* fate mapping of endogenous NG2 cell using the Cre-loxP technology. This method utilizes transgenic mouse lines that express the site-specific recombinase Cre driven by various promoters that are active in NG2 cells. When these mouse lines are crossed to Cre reporter mouse lines, the expression of the reporter gene is activated permanently in cells that express Cre, thereby allowing identification of their progeny by persistent reporter expression.

In NG2creBAC transgenic mice, constitutively active Cre is expressed from a large BAC (bacterial artificial chromosome) transgene in the context of 200 kb of sequence including all of the regulatory sequences of the 34 kb NG2 (CSPG4) gene. Using these mice, Zhu et al., (2008a,b) demonstrated that NG2 cells generate oligodendrocytes throughout the gray and white matter of the brain and spinal cord. Quantification of oligodendrocytes that expressed the reporter in these mice revealed that the percentage of oligodendrocytes that expressed the reporter gene was similar to the Cre recombination efficiency, indicating that all of the oligodendrocytes are derived from cells that express NG2 at some time in their life. In addition, a subpopulation of protoplasmic astrocytes in the gray matter of ventral forebrain and spinal cord appear to be derived from NG2 cells. Surprisingly, none of the GFAP+ astrocytes in the white matter were generated from NG2 cells under normal conditions, suggesting heterogeneity of the source of astrocytes.

Other studies have used inducible Cre lines in which Cre-mediated excision is activated by tamoxifen in transgenic lines that express a fusion protein consisting of Cre and various forms of the mutated ligand-binding domain of estrogen receptor engineered to bind tamoxifen with a higher affinity than endogenous estradiol (Metzger and Chambon, 2001). Rivers et al. (2008) generated Pdgfra-creER^{T2}BAC transgenic mice that express CreER^{T2} under the regulatory sequences of PDGFRA, which is expressed in all NG2 glial cells (Rivers et al., 2008). Induction of Cre in adult Pdgfra-creER^{T2}BAC transgenic mice revealed that the vast majority of the reporter+ cells were either NG2 cells or oligodendrocytes. No astrocytes expressed the reporter when Cre was activated in adult mice. In addition to oligodendrocyte lineage cells, a small number of cells with the morphology of projection neurons were detected in the piriform cortex, and these cells expressed the neuronal antigens NeuN and MAP2 but not markers of interneurons. The observation that the number of reporter+ neurons gradually increased after Cre induction suggests that they had been generated from NG2+/PDGRα+ cells. The identity of these PDGFRα+ neuronal precursors and whether they express NG2 remain unknown.

Using Olig2-creER™ transgenic mice that were generated by inserting CreER™ (Danielian et al., 1993) into the Olig2 gene (Takebayashi et al., 2002), Dimou et al., (2008) observed that when Cre was induced in adult mice, the reporter gene was expressed almost exclusively in either NG2 cells or mature oligodendrocytes and in a few protoplasmic astrocytes in gray matter but not in neurons (Dimou et al., 2008). The number of reporter+ oligodendrocytes in the white matter increased over time to >80% of the total induced cells. By contrast, those in the gray matter reached a plateau at <20% of total reporter+ cells. These findings differ slightly from the results obtained by Cre activation at similar ages in NG2creER™BAC transgenic mice, where the number of reporter+ oligodendrocytes continued to rise in both gray and white matter for 60 days after induction, although the rate of increase was smaller in the gray matter ((Zhu et al., 2008c); manuscript submitted).

Recently, another NG2 cell fate-mapping study was performed using PLP-creER^{T} transgenic mice, which express CreER^{T} under the control of the PLP promoter (Guo et al., 2009). Previous studies using PLP-EGFP transgenic mice had revealed that PLP transcriptional activity is detected in a subpopulation of NG2 cells in adult mice as described above (Mallon et al., 2002). When Cre was activated at postnatal day 7 (P7) in PLP-creER^{T} transgenic mice, 90–94% of the reporter+ cells in the forebrain were NG2+, while only 27% of the induced

cells were NG2+ in the spinal cord, presumably due to activation of Cre in oligodendrocytes that were more prevalent in the caudal CNS at this age. Eight days after Cre induction, the majority of the reporter+ cells in the white matter of the forebrain were oligodendrocytes, while those in the gray matter consisted of a mixture of oligodendrocytes and NG2 cells. In addition, reporter expression was detected in some protoplasmic astrocytes in the ventral forebrain and scattered neurons throughout the forebrain. However, a prior study using plp-cre transgenic mice had shown that PLP transcription is activated not only in oligodendrocyte lineage cells but also in neuronal progenitor cells prior to glial development (Delaunay et al., 2008).

While all of these studies consistently support the oligodendrocyte fate of NG2 cells in both white and gray matter, the findings related to astrocyte and neuronal fate of NG2 cells vary. The precise reason for the differences observed in the fate of NG2 cells among these studies remains unclear, but it is likely that differences in the specificity of Cre-targeting to NG2 cells and the efficacy of Cre induction contribute to the different results obtained in these studies.

7. Origin of NG2 cells

In the spinal cord, the majority of oligodendrocyte lineage cells arise from discrete ventral domains under the influence of Sonic hedgehog (Shh) (Lu et al., 2000; Orentas et al., 1999; Richardson et al., 2006; Takebayashi et al., 2000; Zhou et al., 2000). Cells that express Olig1 and Olig2 in the pMN domain and those that express Nkx2.2 in the ventrally adjacent P3 domain comprise the early committed oligodendrocyte lineage cells. NG2 becomes detectable after these cells migrate out of the ventricular zone and expand to occupy the entire spinal cord (Nishiyama et al., 1996a; Pringle and Richardson, 1993). While the majority of oligodendrocytes arise from the ventral sources, some PDGFRα+ oligodendrocyte precursors in the dorsal spinal cord arise from the dorsal structures independently of Shh in the absence of the homeodomain transcription factor Nkx6.1 (Cai et al., 2005; Vallstedt et al., 2005), and some arise from the dorsal domains defined by Dbx1 expression via radial glia (Fogarty et al., 2005).

The origin of NG2 cells in the forebrain is somewhat more complex, but as in the spinal cord, the ventral subpallial regions appear to be the major source of oligodendrocytes. The first PDGFRα+ cells appear in the anterior entopeduncular region (AEP) between the median ganglionic eminence (MGE) and anterior hypothalamus at E13.5 in the rat (Pringle et al., 1992; Tekki-Kessaris et al., 2001) and subsequently expand dorsolaterally. Their appearance is dependent on the homeodomain transcription factor Nkx2.1, which is necessary for the correct expression of Shh (Nery et al., 2001; Tekki-Kessaris et al., 2001).

Prior to E14 in the mouse or E15 in the rat, NG2 expression in the forebrain is confined to the vasculature. The first NG2+ parenchymal cells appear after E15, at least 2 days after the first appearance of PDGFRα+ cells, and all the non-vascular NG2+ cells also express PDGFRα (Nishiyama et al., 1996b). These cells are likely to be the earliest NG2 cells that appear in the forebrain. At this early stage a small number of PDGFRα+ cells without detectable NG2 are seen scattered throughout the parenchyma, along with a larger number of cells that co-express NG2 and PDGFRα. By the end of the embryonic development, there is an almost complete overlap in the expression of NG2 and PDGFRα, with the exception of the SVZ, where there are PDGFRα+NG2- cells (Nishiyama et al., 1996b). This is consistent with the finding that >99% of PDGFRα-immunopanned cells from E19 rat brains were NG2+ (Tekki-Kessaris et al., 2001).

Genetic fate mapping using three different Cre transgenic mouse lines revealed that oligodendrocytes are generated in three waves (Kessaris et al., 2006). The earliest PDGFRα+ cells are generated from Nkx2.1+ cells in the MGE and AEP beginning around E12. Using quail/chick transplantation, Olivier et al. (2001) demonstrated that cells in AEP supplies all the oligodendrocytes in the telencephalon. In the mouse, PDGFRα+ cells derived from Gsh2+ cells in the lateral ganglionic eminence (LGE) appear after E16.5 and eventually replace the earlier Nkx2.1-derived cells. A third wave of PDGFRα+ cells appears mainly postnatally from dorsal Emx1+ cells and generates oligodendrocytes in the pallium including the neocortex and the corpus callosum. The original Nkx2.1-derived cells disappear after birth. It has not been tested whether NG2 is expressed equally in the both the early PDGFRα+ cells derived from Nkx2.1+ cells and the later generated Gsh2-derived PDGFRα+ cells. Both Gsh2-derived and Emx1-derived cells are capable of generating oligodendrocytes, but it has not yet been tested whether NG2 cells derived from the different sources differ in their ability to interact with neurons or generate astrocytes.

The literature on the development of NG2 cells from ventral progenitor cells that express the DLX family of homeodomain transcription factors varies. Migration studies using DiI labeling and cross transplantation in slice cultures revealed that cells that are generated early from MGE migrate dorsally and become dispersed widely through the neocortex, while those that develop later from LGE and MGE migrate into the proliferative zones of the neocortex to form the future subventricular zone (Anderson et al., 2001. The DLX family of transcription factors that includes Dlx1, Dlx2, Dlx5, and Dlx6 is expressed in the embryonic ganglionic eminences. Short-term fate mapping of Dlx2+ cells using DLX2-tau-lacZ transgenic mice revealed that the location of the cluster of (-galactosidase+ cells representing the progeny of Dlx2+ cells shifts from LGE in late embryonic brain to the central core region of the dorsolateral SVZ in the early postnatal stage {Marshall, 2002 #463). Among the progeny of Dlx2+ cells found at P6-10 were GFAP+ astrocytes and CNPase+ oligodendrocytes in the neocortex and corpus callosum. In another study, cells from the corpus callosum and subcortical white matter from E18 mice were found to coexpress NG2 and Dlx1/2/4/5 (He et al., 2001). By contrast, a recent fate-mapping study using the enhancers URE2 and I12b from DLX1 and 2 genes revealed that DLX1/2+ cells give rise to GABAergic interneurons but very few oligodendrocytes and no astrocytes (Potter et al., 2009). It was also recently shown that Dlx1/2 negatively regulates oligodendrocyte development by repressing Olig2, and that lack of Dlx1/2 increased the number of PDGFRα+ cells in early MGE and LGE (Petryniak et al., 2007). Thus, it appears that Dlx1/2 is upregulated as the

progenitor cells become committed to a neuronal fate and is lost in cells that become committed to an oligodendrocyte lineage. The function of Dlx1/2 is in turn repressed by Mash1 (Petryniak et al., 2007) which has been shown to be required for the development of NG2 cells in some regions (Parras et al., 2004).

In the postnatal CNS throughout adulthood, the SVZ appears to play an important role in the generation of NG2 cells. Retroviral marking of perinatal SVZ showed that NG2 cells in the corpus callosum and neocortex are generated from neonatal SVZ (Levison et al., 1999). In adult mice, retroviral marking of GFAP+ SVZ type B cells resulted in generation of NG2 cells in the corpus callosum in normal and demyelinated states (Gonzalez-Perez et al., 2009; Menn et al., 2006). Although a small number of NG2 cells are found in the SVZ, and it has been reported that NG2 cells represent transit-amplifying progenitor cells in the SVZ (Aguirre et al., 2004), recent studies suggest that the majority of these progenitor cells in the SVZ do not express NG2, but NG2 is highly expressed in the parenchyma surrounding the SVZ (Cesetti et al., 2009; Diers-Fenger et al., 2001; Komitova et al., 2009; Platel et al., 2009). Besides the SVZ, local proliferation of NG2 cells also contributes to the maintenance of the NG2 cell population in the mature CNS.

In addition to the ventral sources of tangentially migrating cells in embryonic stages and the neocortical SVZ in postnatal rodents, radial glia have also been implicated as a source for NG2 cells. By using human GFAP-cre fate mapping, the predominant fate of radial glia in the striatum was found to be NG2 cells and oligodendrocytes, while neurons comprised the majority of the progeny of radial glial in the neocortex (Malatesta et al., 2000, 2003). In another study, when neonatal dorsal radial glia were marked with adenovirus expressing Cre, Cre reporter was detected in NG2 cells in the neocortex and subcortical white matter, indicating that at least some NG2 cells are derived from dorsal radial glia (Ventura and Goldman, 2007).

These observations from recent studies suggest that the general pattern of NG2 cell development in the forebrain is fundamentally similar to those in the spinal cord in that cells arising from the early ventral source become intermingled with cells that arise later from dorsal regions. Further studies are needed to determine whether the NG2 cells that originate from different regions are functionally equivalent in various aspects of NG2 cell function described above.

8. Conclusion

Recent insights discussed above address the function and lineage of NG2-expressing cells in the CNS. However, we are still far from a complete understanding of the functional roles they play in the CNS. Multiple questions remain to be answered concerning their heterogeneity, response to CNS damage and most importantly, mechanism of integration into and modulation of the neuronal network. NG2-expressing cells may vary between different CNS regions with regard to expression of proteins and mRNAs. During the next few years, we expect to unravel the mysteries surrounding these exciting NG2-expressing cells.

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BRAIN RESEARCH REVIEWS

Review

Gliotransmission: Exocytotic release from astrocytes

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ABSTRACT

Gliotransmitters are chemicals released from glial cells fulfilling a following set of criteria: (i) they are synthesized by and/or stored in glia; (ii) their regulated release is triggered by physiological and/or pathological stimuli; (iii) they activate rapid (milliseconds to seconds) responses in neighboring cells; and (iv) they play a role in (patho)physiological processes. Astrocytes can release a variety of gliotransmitters into the extracellular space using several different mechanisms. In this review, we focus on exocytotic mechanism(s) underlying the release of three classes of gliotransmitters: (i) amino acids, such as, glutamate and D-serine; (ii) nucleotides, like adenosine 5′-triphosphate; and (iii) peptides, such as, atrial natriuretic peptide and brain-derived neurotrophic factor. It is becoming clear that astrocytes are endowed with elements that qualify them as cells communicating with neurons and other cells within the central nervous system by employing regulated exocytosis.

Contents

Abbreviations: ANP, atrial natriuretic peptide; ATP, adenosine 5′-triphosphate; BAPTA, 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid; BDNF, brain-derived neurotrophic factor; EGFP, enhanced green fluorescent protein; EM, electron microscopy; ER, endoplasmic reticulum; IP_3, permeant inositol 1,4,5-trisphosphate; IEM, immuno-EM; NMDA, N-methyl-D-aspartate; PMA, phorbol 12-myristate 13-acetate; Sb2, synaptobrevin 2; SNAP-23, synaptosome-associated protein of 23 kDa; SNARE, the soluble N-ethyl maleimide-sensitive fusion protein attachment protein receptor; SOC, store-operated Ca^{2+}; TCA, tricarboxylic acid; TIRF, total internal refection fluorescence; TRPC, canonical transient receptor potential; UTP, uridine 5′-triphosphate; VGLUT, vesicular glutamate transporter; V-ATPase, the vacuolar type of proton ATPase; YFP, yellow fluorescent protein; 2-APB, 2-aminoethyl ester

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1. Introduction

The criteria for a chemical released from neurons to be classified as a neurotransmitter have been defined and frequently modified (Axelrod, 1974; Boehning and Snyder, 2003). Since transmitter release from glia was demonstrated at a much latter time than that from neurons (Bowery et al., 1976), only recently has a similar set of criteria been put forth (Do et al., 1997; Martin et al., 2007; Volterra and Meldolesi, 2005) to establish what compounds qualify as "gliotransmitters": (i) synthesis by and/or storage in glia; (ii) regulated release triggered by physiological and/or pathological stimuli; (iii) activation of rapid (milliseconds to seconds) responses in neighboring cells; and (iv) a role in (patho)physiological processes.

Astrocytes and other glial cells can release a variety of transmitters into the extracellular space using several different mechanisms: (i) through channels like anion channel opening, induced by cell swelling (Pasantes Morales and Schousboe, 1988), release through functional unpaired connexons/pannexons, "hemichannels", on the cell surface (Cotrina et al., 1998; Iglesias et al., 2009) and ionotropic purinergic receptors (Duan et al., 2003); (ii) through transporters, such as, reversal of uptake by plasma membrane excitatory amino acid transporters (Szatkowski et al., 1990), exchange via the cystine-glutamate antiporter (Warr et al., 1999) or organic anion transporters (Rosenberg et al., 1994); and (iii) through Ca^{2+}-dependent exocytosis (Parpura et al., 1994).

In this review, we focus on the exocytotic mechanism(s) underlying the release of three classes of gliotransmitters: (i) amino acids, such as glutamate and D-serine; (ii) nucleotides, like adenosine 5′-triphosphate (ATP); and (iii) peptides, such as, atrial natriuretic peptide (ANP) and brain-derived neurotrophic factor (BDNF). We only disclose the consequences of transmitter release from astrocytes onto neighboring cells when the effect of transmitter release from astrocytes is used as an assay for release.

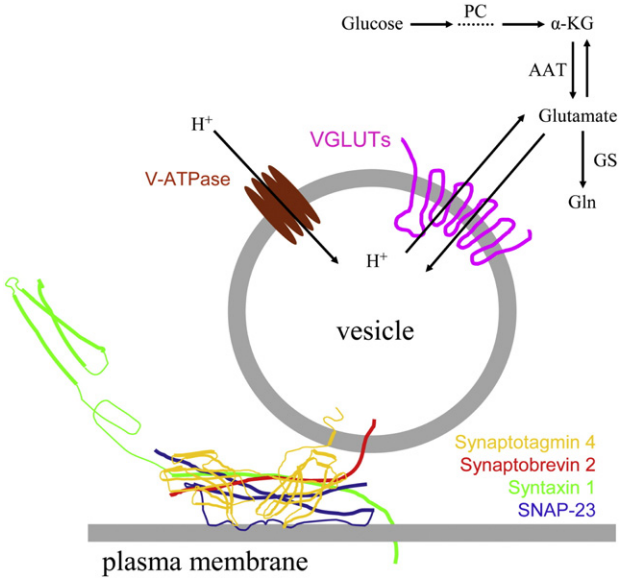

Fig. 1 – Glutamate release by Ca^{2+}-dependent exocytosis. Glutamate packaged in vesicles is released from the astrocyte when the vesicle fuses with the plasma membrane. This fusion process is mediated by synaptotagmin 4 and SNARE proteins: syntaxin 1, synaptobrevin 2 and synaptosome-associated protein of 23 kDa (SNAP-23). Glutamate can be synthesized in astrocytes *de novo* from glucose entry to the tricarboxylic acid cycle via pyruvate carboxylase (PC). Glutamate is converted from the cycle intermediate, α-ketoglutarate (α-KG), usually by transamination of aspartate *via* aspartate amino transferase (AAT). The synthesized glutamate once in the cytosol can then be converted to glutamine (Gln) by glutamine synthetase (GS) or transported into vesicles via proton-dependent vesicular glutamate transporters (VGLUTs), especially isoform 3. The proton gradient is generated by vacuolar type H^+-ATPases (V-ATPase).

2. Amino acids as astrocytic transmitters

Glutamate is synthesized within astrocytes as a by-product of the tricarboxylic acid (TCA) cycle. Since astrocytes possess the enzyme pyruvate carboxylase, they can synthesize glutamate *de novo* (Hertz et al., 1999). Glutamate is converted from the TCA cycle intermediate, α-ketoglutarate, usually via transamination of another amino acid, such as, aspartate (Westergaard et al., 1996) (Fig. 1). D-Serine is converted from L-serine by the action of serine racemase, an enzyme found predominately in astrocytes (Wolosker et al., 1999).

Evidence for Ca^{2+}-dependent glutamate release from astrocytes was first shown using high performance liquid chromatography to monitor the release of this transmitter from cultured astrocytes (Parpura et al., 1994). Astrocytes were equilibrated for prolonged periods of time (40–60 min) either in a solution containing normal external free Ca^{2+} (2. 4 mM) or in a solution depleted of external free Ca^{2+} (24 nM); the latter solution caused a depletion of internal Ca^{2+} stores and prevented Ca^{2+} entry from the extracellular space. Addition of the Ca^{2+} ionophore, ionomycin, in the presence of normal external Ca^{2+}, caused an increase in the release of glutamate from astrocytes. Stimulation of astrocytes, bathed in a solution depleted of free Ca^{2+}, failed to cause an increase in glutamate release. These data indicate that elevated intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) is sufficient and necessary to stimulate glutamate release. Consistent with the former finding, other stimuli that directly increased astrocytic $[Ca^{2+}]_i$, including mechanical stimulation (Araque et al.,1998a,b, Hua et al., 2004; Montana et al., 2004; Parpura et al., 1994), photostimulation (Parpura et al., 1994) and photolysis of Ca^{2+} cages (Araque et al., 1998b; Parpura and Haydon, 2000; Zhang et al., 2004), all caused release of glutamate. The notion that elevated $[Ca^{2+}]_i$ is necessary for glutamate release from astrocytes is further supported by the reduction of the evoked glutamate release from astrocytes when the astrocytic buffering capacity for cytosolic Ca^{2+} was augmented using the Ca^{2+} chelator 1,2-bis(o-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid (BAPTA), or when $[Ca^{2+}]_i$ increases where dampened by the depletion of internal Ca^{2+} stores due to pre-incubation of these cells with thapsigargin, a blocker of store specific Ca^{2+}-ATPase (Araque

et al.,1998a; Bezzi et al., 1998; Hua et al., 2004; Innocenti et al., 2000; Montana et al., 2004).

The majority of intracellular Ca^{2+} necessary for glutamate release originates from endoplasmic reticulum (ER) internal stores (Fig. 2), since Ca^{2+}-dependent glutamate release from astrocytes is most prominently reduced in the presence of thapsigargin (Hua et al., 2004). Diphenylboric acid 2-aminoethyl ester (2-APB) solution, a cell-permeant inositol 1,4,5-trisphosphate (IP_3) receptor antagonist, greatly reduced exocytotic glutamate release from astrocytes, implicating the role of IP_3-sensitive internal stores in mediating Ca^{2+}-dependent glutamate release from astrocytes. Similarly, ryanodine/caffeine-sensitive ER stores play a role, as well, since the treatment of astrocytes with ryanodine, at concentrations that blocked the release of Ca^{2+} from the ryanodine/caffeine-sensitive stores, also attenuated mechanically induced glutamate release. Furthermore, the sustained presence of caffeine, that depleted ryanodine/caffeine stores, also reduced mechanically induced glutamate release. Thus, Ca^{2+}-dependent glutamate release from astrocytes requires co-activation of IP_3- and ryanodine/caffeine-sensitive internal Ca^{2+} stores, which operate jointly (Hua et al., 2004). It should be noted, however, that the functionality of ryanodine receptors in astrocytes is still debated since the lack of their activity in astrocytes *in situ* had been reported (Beck et al., 2004). Nonetheless, increase in Ca^{2+} within subplama membrane cytosolic microdomains, delimited by ER and containing glutamatergic vesicles, controls exocytotic vesicular fusions (Marchaland et al., 2008).

Ca^{2+} entry from the extracellular space across the astrocytic plasma membrane is ultimately required for the (re) filling of ER Ca^{2+} stores (Fig. 2). This occurs via so-called store-operated Ca^{2+} (SOC) channels (Golovina, 2005; Takemura and Putney, 1989). Astrocytes express canonical transient receptor potential (TRPC) channels, implicated in SOC entry, which play a role in the regulation of Ca^{2+} homeostasis in these cells (Golovina, 2005; Grimaldi et al., 2003; Pizzo et al., 2001). Specifically, TRPC1 functionally contributes to Ca^{2+}-dependent glutamate release from astrocytes (Malarkey et al., 2008), since an antibody against TRPC1 protein, that was designed to bind to the pore forming region of TRPC1 protein and that blocks the functioning of the channel (Wang et al., 1999), caused a significant decrease in the measured SOC entry and mechanically induced glutamate release from these cells. This is consistent with the finding that the presence of extracellular Cd^{2+}, a blocker of Ca^{2+} entry from the extracellular space, reduces mechanically induced Ca^{2+}-dependent glutamate release from astrocytes (Hua et al., 2004). Voltage-gated Ca^{2+} channels (Parri et al., 2001) might mediate additional entry of Ca^{2+} from the extracellular space that is utilized to trigger exocytotic glutamate release from astrocytes of ventrobasal thalamus. The role of the ionotropic transmitter receptors, which represent an additional entry of Ca^{2+} in astrocytes (reviewed in Verkhratsky, 2009), in exocytotic glutamate release from astrocytes is intangible at the moment (reviewed in Reyes and Parpura, 2009).

Mitochondria can modulate intracellular Ca^{2+} handling and affect exocytosis in astrocytes (Reyes and Parpura, 2008).

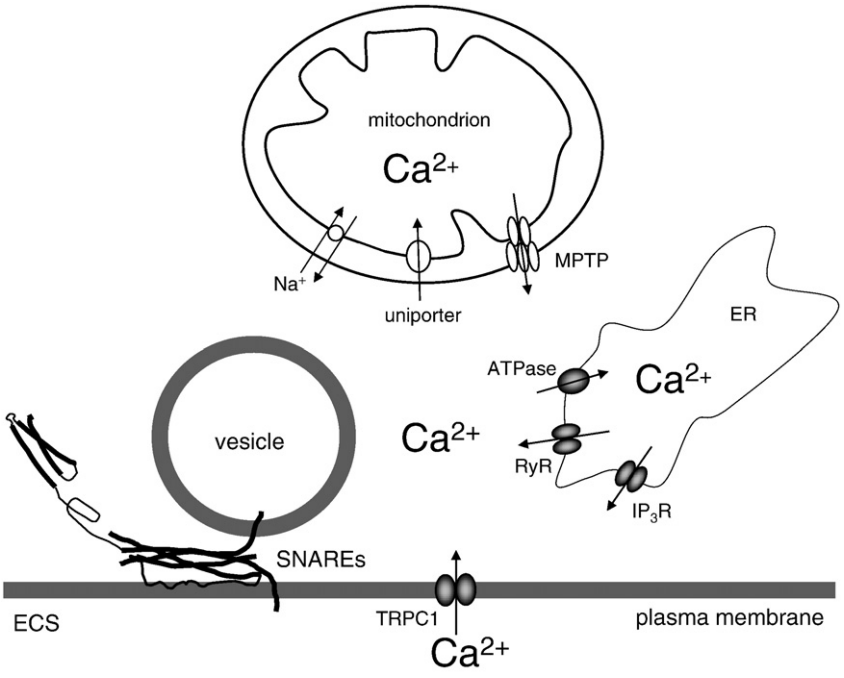

Fig. 2 – Sources of cytosolic Ca^{2+} in vesicular release from astrocytes. Increase of cytosolic Ca^{2+} is sufficient and necessary to cause vesicular fusions and release of gliotransmitters. This process of regulated exocytosis requires the action of the ternary SNARE complex. Cytosolic Ca^{2+} accumulation could be caused by the entry of Ca^{2+} from the ER internal stores via IP_3 and ryanodine receptors (IP_3R and RyR). Store specific Ca^{2+}-ATPase fills these stores, although ultimately this action requires Ca^{2+} entry from the extracellular space (ECS) through canonical type 1 transient receptor potential (TRPC1) channels. Mitochondrial Ca^{2+} uptake is mediated by the uniporter, while free Ca^{2+}within the mitochondrial matrix exits through the Na^+/ Ca^{2+} exchanger and the mitochondrial permeability transition pore (MPTP). Drawing is not to scale.

These organelles possess a mitochondrial Ca^{2+} uniporter that can transport Ca^{2+} into the mitochondrial matrix and it operates at cytosolic [Ca^{2+}] greater than ~0. 5 μM (Miyata et al., 1991; Simpson and Russell, 1998). Blocking this uniporter with ruthenium 360 increased mechanically induced cytosolic Ca^{2+} accumulation and glutamate release in cortical astrocytes. Conversely, decreasing mitochondrial Ca^{2+} efflux by blocking the mitochondrial Na^{+}/Ca^{2+} exchanger with 7-chloro-5-(2-chlorophenyl)-1,5-dihydro-4,1-benzothiazepin-2(3H)-one (CGP37157), or increasing mitochondrial Ca^{2+} load by inhibiting formation of the mitochondrial permeability transition pore with cyclosporin A, decreased cytosolic Ca^{2+} accumulation and glutamate release in cortical astrocytes. Taken together, these data suggest that mitochondria have the capacity to modulate the magnitude of Ca^{2+}-dependent glutamate release from astrocytes (Fig. 2).

Ca^{2+}-dependent release of transmitters depends on the presence of exocytotic secretory machinery. Indeed, astrocytes express the proteins of the soluble *N*-ethyl maleimide-sensitive fusion protein attachment protein receptor (SNARE) complex: synaptobrevin 2 [(Sb2), also referred to as vesicle–associated membrane protein 2 (VAMP 2)], syntaxin 1, synaptosome-associated protein of 23 kDa (SNAP-23) as well as several ancillary proteins to this complex, including synaptotagmin 4 (reviewed in Montana et al., 2006) (Fig. 1). The use of clostridial, tetanus and various types of botulium toxins, which cleave SNARE proteins necessary for exocytosis, caused a reduction in the level of Ca^{2+}-dependent glutamate release in astrocytes (reviewed in Montana et al., 2006). Additionally, the use of tetanus toxin, which cleaves astrocytic Sb2 and cellubrevin (Parpura et al., 1995a), caused a reduction in plasma membrane capacitance (C_m) increase (Kreft et al., 2004) and a reduction in the number of amperometric spikes (Chen et al., 2005), both reporting on exocytosis from astrocytes. The experimental manipulation of the SNARE complex by expressing the cytoplasmic domain of Sb2, which acted as a dominant-negative SNARE, resulted in the inhibition of glutamate release from astrocytes (Zhang et al., 2004b). It should be noted that Sb2 cytosolic domain contains the SNARE domain but lacks the ability to anchor to the vesicular membrane. Similarly, the expression of mutated synaptotagmin 4, acting in a dominant-negative manner, caused the reduction of Ca^{2+}-dependent glutamate release from astrocytes (Zhang et al., 2004a). Furthermore, α-latrotoxin, an active component of black widow spider venom, which binds to neurexins/latrophilins to induce release of transmitter by stimulating the secretory machinery (reviewed in Südhof and Jahn, 1991), has been shown to cause glutamate release from astrocytes (Jeftinija et al., 1996; Parpura et al., 1995b).

Proteins utilized for sequestering glutamate into vesicles have also been found in astrocytes (Fig. 1). Hence, the vacuolar type of proton ATPase (V-ATPase), which drives protons into the vesicular lumen creating the proton concentration gradient necessary for glutamate transport into vesicles, has been detected in astrocytes (Wilhelm et al., 2004). Its blockage with bafilomycin A_1 reduces glutamate release from astrocytes caused by various stimuli (Araque et al., 2000; Bezzi et al., 2001; Crippa et al., 2006; Montana et al., 2004; Pasti et al., 2001). The three known isoforms of vesicular glutamate transporters (VGLUTs) 1, 2 and 3, which use the proton gradient created by V-ATPases to package glutamate into vesicles, have been detected in astrocytes (Anlauf and Derouiche, 2005; Bezzi et al., 2004; Crippa et al., 2006; Fremeau et al., 2002; Kreft et al., 2004; Montana et al., 2004; Zhang et al., 2004b). These transporters are functional within astrocytes since Rose Bengal, an allosteric modulator of VGLUTs, greatly reduced glutamate release (Montana et al., 2004). VGLUT3 and the cytosolic concentration of glutamate appear to be key limiting factors in regulating the Ca^{2+}-dependent release of glutamate from astrocytes (Ni and Parpura, 2009) (Fig. 1). Selective over-expression of individual VGLUT proteins in astrocytes showed that VGLUT3, but neither VGLUT1 nor VGLUT2, enhanced mechanically induced Ca^{2+}-dependent glutamate release. Similarly, inhibition of glutamine synthetase activity by L-methionine sulfoximine in astrocytes, which leads to increased cytosolic glutamate concentration, greatly increased their mechanically induced Ca^{2+}-dependent glutamate release without affecting intracellular Ca^{2+} dynamics (Ni and Parpura, 2009).

Secretory vesicles are the essential morphological elements for regulated Ca^{2+}-dependent exocytosis. Hence, based on immunoelectron microscopy (IEM), Sb2 can be found located in vicinity of electron-lucent (clear) vesicular structures (Maienschein et al., 1999), while VGLUTs 1 or 2 in astrocytes *in situ* were found associated with small, clear vesicles with a mean diameter of ~30 nm (Bezzi et al., 2004). Similarly, immuno-isolated Sb2-containing vesicles that originated from cultured astrocytes (Crippa et al., 2006) were heterogeneous in size, ranging from 30 to over 100 nm, and predominately appeared as electron-lucent. Recycling glutamatergic vesicles which can capture the extracellular antibody against VGLUT1 in a Ca^{2+}-dependent manner are electron-lucent and have a diameter of ~50 nm (Stenovec et al., 2007). Furthermore, gliosomes (Stigliani et al., 2006), pinched off astrocytic processes, expressing Sb2 and VGLUT 1, contained clear vesicles with diameters of ~30 nm; some of them were clathrin-coated vesicles. Much larger vesicles, over 1 μm in diameter, have been observed to form within minutes of repeated stimulation with pharmacological dosages (5–50 mM) of glutamate (Kang et al., 2005; Xu et al., 2007); these vesicles can release glutamate, although it is highly likely that they represent a pharmacologically induced phenomenon or may play a role in pathological processes (see also the discussion in Bergersen and Gundersen, 2009).

The recycling of secretory vesicles at the plasma membrane has been investigated in astrocytes using fluorescence microscopy. Application of ionomycin in the presence of extracellular Ca^{2+}, but not in its absence, caused uptake of the membrane recycling dye, FM 4-64 (Krzan et al., 2003). Similarly, using a pre-loading technique that stimulated membrane recycling and the trapping of styryl dyes (FM 1-43 or FM 2-10) in secretory organelles, astrocytes displayed a punctate pattern of FM fluorescence (Chen et al., 2005). Trafficking of glutamatergic vesicles in astrocytes was assessed using an immunological approach. Hence, after increasing cytoplasmic Ca^{2+} levels in astrocytes in the presence of antibodies against VGLUT1 in extracellular space, presumably binding to luminal/intravesicluar epitope of this transporter, there was an increase in intracellular VGLUT1 labeling (Stenovec et al., 2007). The delivery of secretory vesicles and fusion to the plasma membrane was also studied in astrocytes. Crippa et al.

(2006) expressed a chimeric protein, where enhanced green fluorescent protein (EGFP) was fused to the C-terminus of Sb2 (Sb2-EGFP), in astrocytes. Since the C-termimus of Sb2 is located in the vesicular lumen, EGFP was targeted intravesicularly. When astrocytes were stimulated with Ca^{2+} ionophore, many fluorescent Sb2-EGFP puncta vanished with a simultaneous increase in plasma membrane fluorescence, consistent with regulated exocytosis and fusion of labeled vesicles to the plasma membrane. Net addition of vesicular membrane to the plasma membrane can be directly measured by monitoring changes in C_m. Indeed, an agonist-induced rise in astrocytic $[Ca^{2+}]_i$, causing regulated exocytosis, resulted in an increase of C_m, while concomitant measurements recorded a release of glutamate (Zhang et al., 2004b). Further evidence for vesicular exocytosis from astrocytes was provided by total internal reflection fluorescence (TIRF) microscopy (Bezzi et al., 2004; Bowser and Khakh, 2007; Domercq et al., 2006; Marchaland et al., 2008), where exocytosis of VGLUT1-, VGLUT2- or Sb2-positive vesicles were reported. As a consequence of vesicular fusions, quantal events of glutamate release, representing an exocytotic hallmark (Del Castillo and Katz, 1954), have been recorded from astrocytes. Such events were detected using reporter cells expressing *N*-methyl-D-aspartate (NMDA) receptors (Pasti et al., 2001) or by amperometric measurements used to detect the release of dopamine, acting as a "surrogate" transmitter for glutamate, from glutamatergic vesicles (Chen et al., 2005).

Astrocytes can also release the amino acid D-serine (Schell et al., 1995), a ligand to the glycine modulatory binding site of the NMDA receptor. Mothet et al. (2005) investigated the mechanism of this release using an enzyme-linked assay to measure extracellular D-serine concentration. Following glutamate receptor stimulation, astrocytes released D-serine in a Ca^{2+}-dependent manner; the release was augmented by Ca^{2+} ionophore and inhibited by application of thapsigargin or removal of extracellular Ca^{2+}. Furthermore, this release of D-serine was reduced by concanamycin A, a V-ATPase inhibitor and tetanus toxin, implicating the involvement of a vesicular mechanism. Consistent with this notion, D-serine was found to co-localize with Sb2 based on immunocytochemistry and fluorescence microscopy. The investigation of the mechanism underlying a Ca^{2+}-dependent release of D-serine from astrocytes was expanded in a subsequent study using confocal fluorescence microscopy (Martineau et al., 2008). Using pharmacological inhibition of vesicular budding indicated that D-serine was packaged in vesicles down stream of the Golgi apparatus. The molecular identity of the vesicular transporter for D-serine, however, remains undetermined. Nonetheless, the delivery of secretory vesicles and fusion to the plasma membrane showed the recruitment of Sb2 to the plasma membrane with related disappearance of intracellular D-serine punctate stain. Taken together, D-serine appears to be secreted from astrocytes using a regulated exocytosis/vesicular pathway.

3. ATP as an astrocytic transmitter

ATP is produced via glycolysis and oxidative phosphorylation. Intracellular ATP provides energy for a variety of processes, including vesicular recycling. Once released into extracellular space, ATP can be used in intercellular signaling acting directly onto purinergic receptors. Alternatively, upon its hydrolysis by membrane-bound ecto-nucleotidases, the extracellular degradation products, ADP and adenosine, can activate different plasma membrane receptors (reviewed in Fields and Burnstock, 2006).

As already outlined, astrocytes possess secretory vesicles and a variety of exocytotic proteins. Cultured astrocytes investigated under EM displayed large dense core granules with diameters of ~115 nm, containing the secretory peptide secretogranin II (Calegari et al., 1999) and ATP (Coco et al., 2003). Based on immunoblotting, subcellular fractions containing secretogranin II were mainly distinct from fractions containing Sb2 (Calegari et al., 1999). Consistent with this finding, dense core vesicles represented ~2% of the total number of immuno-isolated Sb2-containing vesicles (Crippa et al., 2006). Similarly, using IEM, it was demonstrated that Sb2 can be associated with some dense core vesicular structures, with diameters ranging from 100 to 700 nm (Maienschein et al., 1999). Following subcellular fractionations, immunoblotting for several exocytotic proteins, Sb2, syntaxin 1, cellubrevin and synaptotagmin 1, were found to co-localize with ATP-containing organelles (Maienschein et al., 1999). It should be noted, however, that the presence of synaptotagmin 1 was not detected in astrocytes by others (Crippa et al., 2006; Parpura et al., 1994; Zhang et al., 2004a). The protein responsible for the ATP accumulation in secretroy vesicles has recently been identified as SLC17A9 (Sawada et al., 2008). This vesicular nucleotide transporter (VNUT) was found present in astrocytes based on immunocytochemistry.

Morphological and biochemical evidence suggests that ATP as an astrocytic transmitter may be released by Ca^{2+}-dependent exocytosis. The first evidence in support of such a notion comes from experiments in which astrocytes exposed to nitric oxide responded with an increase in cytoplasmic Ca^{2+} and the release of ATP to the extracellular space (Bal-Price et al., 2002). Buffering of intracellular Ca^{2+} with BAPTA or preventing vesicular release with botulinum toxin C greatly reduced the release of ATP. Furthermore, Coco et al. (2003) demonstrated that mechanically stimulated astrocytes released ATP which could be inhibited by application of bafilomycin A_1 or tetanus toxin. Interestingly, the reduction of ATP release caused by tetanus toxin was less pronounced than the reduction in release of glutamate, indicating that ATP and glutamate release may be regulated in a different manner, perhaps through distinct vesicular pools.

ATP release from cultured astrocytes could be evoked by uridine 5′-triphosphate (UTP) via the likely activation of $P2Y_2$ receptors (Abdipranoto et al., 2003). This release was reduced by thapsigargin and lithium ions that can block the intracellular generation of IP_3. Further pharmacology on vesicular trafficking implicates that the exocytotic pathway is involved in UTP-induced ATP release from astrocytes: a blocker of transport vesicles budding off the Golgi apparatus, brefeldin A, a disruptor of actin microfilaments, cytochalasin D and the exocytosis inhibitor, botulinum toxin A, all blocked ATP release. However, the pre-incubation with a cell permeable form of BAPTA showed a trend in the reduction of release, although the effect was insignificant; this may be ascribed to an insufficient concentration of BAPTA within the cell.

To study the quantal nature of ATP release from astrocytes, Pangrsic et al. (2007) incubated astrocytes with quinacrine, a compound that fluorescently labels ATP-containing structures. Using TIRF microscopy, quinacrine showed punctate stain. The rapid loss of these puncta was evident upon receptor stimulation using glutamate or ATP and stimuli that directly raise intracellular Ca^{2+} levels, ionomycin or flash photolysis of caged Ca^{2+} (Pryazhnikov and Khiroug, 2008). Expressing a dominant-negative SNARE in astrocytes resulted in the inhibition of the Ca^{2+}-induced reduction in the quinacrine fluorescent puncta representing ATP-containing vesicle exocytosis (Pangrsic et al., 2007). Glutamate stimulation of astrocytes showed quantal release of ATP as recorded by ATP reporter cells (Pangrsic et al., 2007), human embryonic kidney cells expressing a mutated $P2X_3$ receptor with reduced desensitization (Fabbretti et al., 2004).

Under particular experimental conditions, the exocytotic release of ATP stored in astrocytic lysosomes could be detected (Zhang et al., 2007). Hence, prolonged (more than 1 hour) incubation with FM recycling dyes stained astrocytic lysosomes based on a co-localization of FM and various lysosomal markers under fluorescence microscopy. Agonist stimulation or metabolic blockade of astrocytes revealed regulated exocytosis of these lysosomes under TIRF microscopy that was blocked by intracellular Ca^{2+} buffering with BAPTA. These lysosomes could readily load with the fluorescent ATP analogue MANT-ATP that was also released upon stimulation. Indeed, the astrocytic lysosomal fraction in density gradient centrifugation contained abundant ATP. Two subsequent studies confirmed that in astrocytes lysosome-like organelles can assume the role of secretory vesicles and undergo Ca^{2+}-depenent exocytosis (Jaiswal et al., 2007; Li et al., 2008). Thus, it appears that ATP, and perhaps other gliotransmittes, in astrocytes could be stored in at least two distinct organelles, secretroy vesicles and lysosomes, from which it can be released by regulated exocytosis. It will be necessary to determine under which conditions these two distinct pools of organelles would be recruited. For example, do the same organelles deliver transmitter for release under physiological and pathological conditions or are there specific organelles that operate under particular conditions?

4. Peptides as astrocytic transmitters

In contrast to amino acids and ATP, which are loaded into vesicles by membrane transporters, peptidergic gliotransmitters enter vesicles via the synthetic secretory pathway (Fig. 3). Pro-peptides are made in the ER, transit Golgi compartments where they get concentrated and sorted into organelles, then they are processed to their final form before release (Dannies, 1999). Vesicles carrying peptidergic transmitters appear to have a distinct morphological appearance under EM; they exhibit electron dense cores and are termed dense-core vesicle, large dense-core vesicles or secretory granules. Their diameter is somewhat larger in comparison with the small synaptic-like clear-core vesicles and appear to contain secretogranins (Winkler and Fischer-Colbrie, 1992). Therefore, in the first studies where the presence of dense-core vesicles in astrocytes was considered, subcellular distribution and the

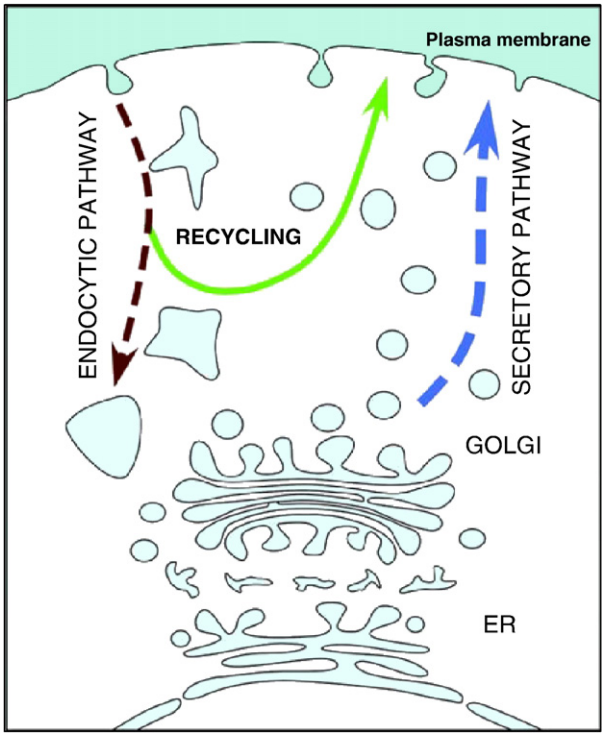

Fig. 3 – Secretory pathway of peptidergic transmitters. The pro-peptides are synthetized in the endoplasmic reticulum (ER) . They then enter the Golgi compartments from which vesicles bud off, containing concentrated and sorted peptides. Secretory vesicles traffic away from the Golgi compartment along the secretory pathway to the plasma membrane where they dock and fuse with the plasma membrane upon a stimulus delivery, typically an increase in cytosolic Ca^{2+} levels. Vesicles pinching off the plasma membrane via the endocytic pathway may be rerouted to the recycling pathway, where the substances captured from the extracellular space may be returned to the surface plasma membrane/extracellular space by entering regulated exocytosis of the secretory pathway.

secretory pathway of secretogranin II was studied (Calegari et al., 1999). The EM results of this study have shown that, in astrocytes, dense-core vesicles are present near the Golgi complex, typically have a diameter of approximately 100 nm, and that secretogranin II is released upon stimulation by different secretagogues, including bradykinin, adenosine 3′:5′ cyclic monophosphate (cAMP), ionomycin and phorbol 12-myristate 13-acetate (PMA). In Ca^{2+}-containing media, the Ca^{2+} ionophore ionomycin in combination with PMA produced large increases in cytosolic Ca^{2+} levels and appeared to be the most effective stimulus for secretogranin II release (Calegari et al., 1999). This study also reported that astrocytes contain fewer smaller and less dense secretory granules containing secretogranin II, indicating that peptidergic granules in astrocytes are not uniform in morphological appearance.

One of the first peptides studied for exocytotic release from astrocytes was ANP. This peptide is a diuretic vasorelaxant hormone typically stored in specific secretory vesicles and is secreted from the heart atrial myocytes in response to cardiac

overload and oxygen deficiency (Baertschi et al., 2001; Jiao and Baertschi, 1993). The function of ANP release from astrocytes, however, may play a role in cerebral blood regulation (McKenzie et al., 2001). To study the discharge of ANP, Krzan et al. (2003) transfected astrocytes with a construct to express pro-ANP fused with the emerald green fluorescent protein (ANP.emd). Transfection of astrocytes resulted in fluorescent puncta, representing vesicles. The number of puncta was reduced upon stimulating the cells by the Ca^{2+} ionophore ionomycin, which strictly depended on the extracellular Ca^{2+}. Concomitant with the Ca^{2+}-dependent decrease in fluorescent puncta, the fluorescence intensity of the FM 4-64 dye, a reporter of cumulative exocytosis, increased in a Ca^{2+}-dependent manner as well. Together these data strongly indicated that regulated exocytosis mediates the release of ANP from astrocytes. Interestingly, vesicles containing ANP also appear to contain ATP (Pangrsic et al., 2007), which is consistent with the report that ATP is stored in secretorgranin II-containing vesicles (Coco et al., 2003).

In atrial myocytes, EM indicates that pro-ANP is condensed in the *trans*-Golgi network, and because pro-ANP is cleaved only on release, secretory vesicles budding off the *trans*-Golgi network are already mature. Their shape and size (120 to 175 nm) appears to be determined by the aggregation of the pro-ANP in vesicles (Baertschi et al., 2001). In astrocytes the size of ANP recycling vesicles was studied by IEM after exposing live astrocytes to extracellular anti-ANP antibody, which sequestred within vesicles with diameters averaging 50 nm and ranging between 30 and 100 nm (Potokar et al., 2008). The mobility of these recycling ANP-containing vesicles was one order of magnitude smaller than that of ANP-containing vesicles trafficking to the plasma membrane vesicle docking site (Potokar et al., 2007; Potokar et al., 2005). The clear-core nature and the smaller size of anti-ANP capturing vesicles in astrocytes relative to the values reported in atrial myocytes (Baertschi et al., 2001) is consistent with the observation that the vesicular ANP content determines the size and the shape of ANP-containing secretory vesicles (Baertschi et al., 2001).

The mobility of anti-ANP antibody capturing vesicles is dramatically reduced upon the stimulation of cells (Potokar et al., 2008), which differs from the stimulation-increased mobility of anti-VGLUT1 antibody capturing vesicles in astrocytes (Stenovec et al., 2007). The functional significance of these observations is not clear, but the results clearly show that retrieving vesicle mobility is subject to the physiological state of the astrocyte (Potokar et al., 2008). This may play a role in the regulation of the vesicle cycle and vesicle cargo discharge (Potokar et al., 2008). It is possible to envision that arrested mobility of retrieving vesicles may affect the vesicle cargo discharge, at least by prolonging the interaction between the plasma membrane and the vesicle membrane. It was shown that the main mode of peptidergic vesicle exocytosis is the transient fusion (kiss-and-run), and that stimulation increases the frequency of occurrence of vesicle fusion as well as the dwell time of the established fusion pore and vesicle content discharge (Stenovec et al., 2004; Vardjan et al., 2007). This mode of vesicle fusion would be facilitated, if the vesicle interaction with the cytoskeleton during retrieval is prevented or attenuated. The stimulation-induced vesicle mobility arrest is consistent with this view of vesicle cycle regulation; it increases the probability of peptide hormone discharge. In contrast, while such a mechanism may be related to peptidergic vesicles, in glutamatergic vesicles capturing the anti-VGLUT1 antibody, stimulation-induced enhanced post-fusion vesicle mobility may have a different function (Stenovec et al., 2007). In this case, where the diffusional mobility of glutamate transmitter is orders of magnitude more mobile than the peptidergic hormones, the stimulation-increased vesicle mobility may terminate the glutamate discharge upon the reduction of interaction time between the vesicle and the plasma membrane. Furthermore, recycling vesicles may not only carry lumenal cargo but also membrane associated signalling molecules which participate in contact cell-to-cell interactions (Kopan and Ilagan, 2009; Soos et al., 1998) or in determining the density of plasma membrane transporters (Robinson, 2002) such as the glutamate transporter EAAT2 (Stenovec et al., 2008).

Astrocytes also contain recycling vesicles, specialized endocytic compartments, which may serve bidirectional communication between neurons and glia. These vesicles may take-up extracellular peptides, process them and recycle them back into the extracellular space via secretory pathway and regulated exocytosis (Fig. 3). When studying the activity-dependent secretion of BDNF and its extracellular availability, Bergami et al. (2008) conducted very interesting experiments and provided evidence that BDNF, which is de novo synthetized in neurons, gets secreted after theta-burst stimulation in its pro-form into the extracellular medium. Then the pro-BDNF is rapidly internalized via the pan-neurotrophin receptor $p75^{NTR}$ in perineuronal astrocytes via endocytosis, thereby restricting the availability of this neurotrophin at neuron–astrocyte contacts. After internalization, the pro-BDNF can undergo a recycling process, endowing astrocytes with the ability to resecrete this neurotrophin upon their stimulation. Ultrastructural IEM characterization revealed that BDNF fluorescently tagged with yellow fluorescent protein (BDNF-YFP) and labeled with gold particles localized in clear-core vesicles with 125 nm in diameter. The fluoresently labeled vesicles exposed their lumen to extracellular solution, presumably via a fusion pore, since the lumenal pH increased, detected as an increase in pH-dependent YFP fluorescence. To further investigate the entry of BDNF into the secretory pathway, astrocytes were preincubated with BDNF-YFP and their secretion was studied by stimulating cells with glutamate. The glutamate-evoked secretion of BDNF-YFP was inhibited, if cells were pretreated with tetanus toxin. Co-localization between pro-BDNF and Sb2 confirmed that endocytosed pro-BDNF was routed into Sb2-containing vesicles. Taken together, this study shows that endocytic vesicles expressing $p75^{NTR}$ represent the main storage/recycling compartment for endocytosed pro-BDNF before routing it to the SNARE-dependent secretory pathway (Bergami et al., 2008).

5. Concluding remarks

It is becoming clear that astrocytes are endowed with elements that qualify them as cells communicating with

neurons and other cells in brain by employing regulated exocytosis. Astrocytes can synthesize and store gliotransmitters, i.e., amino acids, ATP and peptides, in SNARE-associated vesicles. The vesicular cargo discharge from these cells via regulated release occurs upon a delivery of physiological/pathological stimulus. It should be noted that neighboring neurons and other cells rapidly sense and respond to the released gliotransmitters, albeit this subject was out of scope of the present review (reviewed in Ni et al., 2007). Consequently, astrocytes appear as key players in central nervous system (patho)physiological processes. Further understanding of vesicular traffic to and from the plasma membrane via secretory pathway and within endocytic routes/recycling as well as determining the location of exocytotic sites on astrocytes is of importance for astrocyte–neuron signaling. While astrocytic processes appear to be the ideal site for the location of vesicular fusions, exocytotic release can also occur on their bodies (reviewed in Montana et al., 2006). Although studies addressing these specific issues are only at the very beginning, there is palpable evidence that they will provide new insights into the understanding of how astrocytic membrane dynamics shape the signaling within the complex network of the brain tissue.

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BRAIN RESEARCH REVIEWS

Review

GLIA modulates synaptic transmission

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ABSTRACT

The classical view of glial cells as simple supportive cells for neurons is being replaced by a new vision in which glial cells are active elements involved in the physiology of the nervous system. This new vision is based on the fact that astrocytes, a subtype of glial cells in the CNS, are stimulated by synaptically released neurotransmitters, which increase the astrocyte Ca^{2+} levels and stimulate the release of gliotransmitters that regulate synaptic efficacy and plasticity. Consequently, our understanding of synaptic function, previously thought to exclusively result from signaling between neurons, has also changed to include the bidirectional signaling between neurons and astrocytes. Hence, astrocytes have been revealed as integral elements involved in the synaptic physiology, therefore contributing to the processing, transfer and storage of information by the nervous system. Reciprocal communication between astrocytes and neurons is therefore part of the intercellular signaling processes involved in brain function.

Contents

Accumulating evidence obtained by many different groups has demonstrated the existence of reciprocal communication between astrocytes and neurons, which has become one of the most stimulating topics on current neurobiology because its revolutionary implications in our understanding of the intercellular signaling processes involved in brain function. The cellular and molecular properties that govern the signaling between astrocytes and neurons as well as their functional implications were substantiated in the "tripartite synapse" concept (Araque et al., 1999). This concept is based on the ability of astrocytes to respond to neurotransmitters released during neuronal activity and to release neuroactive

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substances – called gliotransmitters – that influence neuronal excitability and synaptic transmission. Consequently, astrocytes are integral synaptic elements actively involved in the synaptic function. While the mechanisms and properties of the astrocytic responsiveness to neuronal activity have been reviewed elsewhere (Perea et al., 2009), this review will focus on the molecular mechanisms underlying astrocyte–neuron signaling and the functional consequences on synaptic physiology. In addition to modulation of synaptic function, gliotransmitters released from astrocytes play important roles in brain microcirculation (Metea and Newman, 2006; Mulligan and MacVicar, 2004; Takano et al., 2006; Zonta et al., 2003). This has been the topic of excellent recent reviews (Gordon et al., 2007; Haydon and Carmignoto, 2006; Iadecola and Nedergaard, 2007) and is discussed elsewhere in this special issue.

1. Gliotransmission

The intracellular Ca^{2+} signal represents the substrate of the cellular excitability in astrocytes (Deitmer and Araque, 2009; Deitmer et al., 2006; Perea and Araque, 2005a). The astrocyte Ca^{2+} signal plays a pivotal role in the bidirectional communication between astrocytes and neurons because it is triggered by neurotransmitters released during synaptic activity (Fellin and Carmignoto, 2004; Perea and Araque 2005b; Schipke and Kettenmann, 2004), and, in turn, it triggers the release of gliotransmitters (Volterra and Bezzi, 2002; Haydon and Araque, 2002) that lead to neuromodulation (Newman, 2003; Perea et al., 2009; Volterra and Steinhauser, 2004). Indeed, astrocytes have been shown to be capable of releasing a wide variety of gliotransmitters, such as glutamate, D-serine, ATP, adenosine, GABA, tumor necrosis factor alpha (TNFα), prostaglandins, atrial natriuretic peptide (ANP), brain-derived neurotrophic factor (BDNF), etc. (Araque et al., 1998a,b, 2000; Beattie et al., 2002; Bergami et al., 2008; Bezzi et al., 2004; Kozlov et al., 2006; Krzan et al., 2003; Martin et al., 2007; Mothet et al., 2005; Richard and Bourque, 1996; for reviews, see Montana et al., 2006; Volterra and Meldolesi, 2005), that can modulate neuronal activity and synaptic physiology (Perea et al., 2009).

The molecular mechanisms responsible for the release of gliotransmitters are still under debate. Compelling evidence indicate that gliotransmitter release is based on Ca^{2+}- and SNARE protein-dependent mechanisms (Araque et al., 1998a, b; 2000; Bezzi et al., 2004, Martineau et al., 2008; Zhang et al., 2004a), through vesicle (Bezzi et al., 2004; Jourdain et al., 2007; Martineau et al., 2008; Mothet et al., 2005; Pangrsic et al., 2007) and lysosome exocytosis (Jaiswal et al., 2007; Li et al., 2008; Zhang et al., 2007). In addition to data from cell physiology studies (Araque et al., 1998a,b, 2000; Jourdain et al., 2007; Perea and Araque, 2007), the identification of small vesicles in astrocytes, similar to synaptic vesicles in neurons (Bezzi et al., 2004; Jourdain et al., 2007), and the detection of vesicular release machinery-related proteins expressed by astrocytes (Araque et al., 2000; Bezzi et al., 2004; Martineau et al., 2008; Montana et al., 2006; Mothet et al., 2005; Zhang et al., 2004b) strongly support this notion (see Bergersen and Gundersen, 2009, for a discussion of the morphological evidence for vesicular release from astrocytes).

In spite of the amount of data supporting Ca^{2+}-dependent gliotransmitter release (see above), a recent study using transgenic mice failed to detect neuromodulation when astrocyte Ca^{2+} elevations were evoked by activation of transgenically expressed G-protein coupled receptors (Fiacco et al., 2007), which has questioned the Ca^{2+} dependence of gliotransmission (Agulhon et al., 2008). Further investigations are required to reconcile the controversy, but perhaps the negative results might be due to inherent problems of transgenic mice as experimental models (for discussions, see Agulhon et al., 2008; Perea et al., 2009).

Several Ca^{2+}-independent mechanisms for the release of gliotransmitters have also been proposed, including reversal of glutamate uptake (Anderson and Swanson, 2000), through connexin hemichannels (Ye et al., 2003), pannexin hemichannels (Iglesias et al., 2009), cysteine–glutamate exchanger (system Xc-; Chung et al., 2005), pore-forming P2X7 receptors (Duan et al., 2003; Fellin et al., 2006), and volume-regulated anion channels (Kimelberg et al., 2006). It is noteworthy that all these mechanisms are not mutually exclusive and they might coexist. While neurotransmitter release from synaptic terminals seems to be largely governed by very conserved cellular and molecular mechanisms that grant a high temporal and spatial definition of the intercellular signalling (Augustine et al., 2003), the possible existence of different mechanisms of gliotransmitter release from astrocytes is puzzling. A better understanding of astrocyte cell biology, the identification of the phenotypic changes occurring during different experimental conditions, and specially the identification of the physiological and pathological conditions under which the different mechanisms might act, will help to understand the actual role of each mechanism in the (patho) physiology of the brain.

2. Astrocytes modulate neuronal excitability

The ability of astrocytes to modulate neuronal excitability and synaptic transmission was originally described in cell culture preparations (for a review see Araque and Perea, 2004) and has been considerably expanded by later studies on acute brain slices (for reviews, see Haydon and Araque, 2002; Newman, 2003; Volterra and Steinhauser, 2004), More recently, the role of gliotransmitters on complex brain activity such as slow cortical oscillations (Fellin et al., 2009) as well as on animal behaviour has also been recently investigated (Halassa et al., 2009). However, studies aimed to identify the neuromodulatory role of astrocyte in vivo are still largely lacking.

Glutamate released from astrocytes has been reported to exert many effects on neuronal excitability (Fig. 1). Astrocytic glutamate evokes slow inward currents (SICs) in CA1 hippocampal pyramidal neurons through activation of postsynaptic NMDA receptors (NMDARs) (Angulo et al., 2004; Ding et al., 2007; Fellin et al., 2004; Kozlov et al., 2006; Navarrete and Araque, 2008; Parri et al., 2001; Perea and Araque, 2005b) (Figs. 1a, b). In addition to increase neuronal excitability, astrocytic glutamate has been shown to synchronously excite clusters of hippocampal pyramidal neurons, indicating that gliotransmission may contribute to neuronal synchronization (Angulo et al., 2004; Fellin et al., 2004). Furthermore, the astrocyte Ca^{2+}

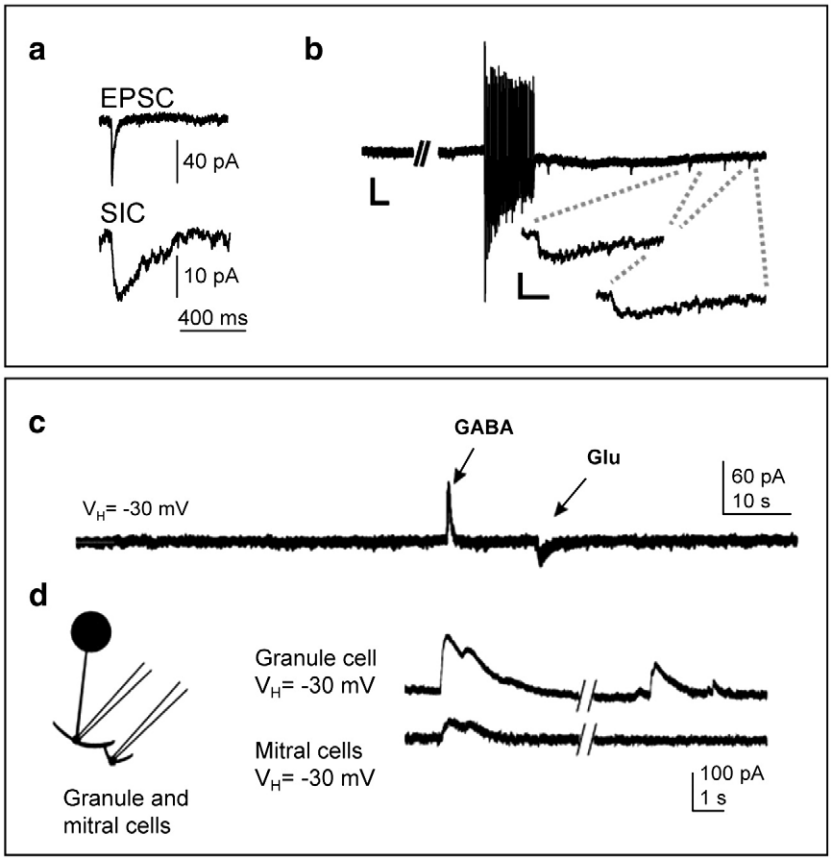


Fig. 1 – Astrocytes modulate neuronal excitability. (a) Representative spontaneous excitatory postsynaptic currents (EPSCs) and astrocyte-induced glutamate-mediated slow inward currents (SICs) from a hippocampal pyramidal neuron. Note the different amplitude and time courses. Adapted from Perea and Araque (2005b). (b) High-frequency Schaffer collateral (SC) stimulation increases the frequency of SICs. Two SICs have been expanded. Scale bars: 100 pA and 10 s (top), 50 pA and 200 ms (bottom). Adapted from Fellin et al. (2004). (c) Astrocytes in the olfactory bulb release glutamate and GABA, inducing glutamate-mediated SICs and GABA-mediated slow outward currents (SOCs) in granule cells. (d) Paired recordings from mitral and granule cells in the olfactory bulb show that some SOCs occur simultaneously in adjacent cells. (c, d) Adapted from Kozlov et al. (2006).

signal stimulated by endocannabinoids released from one pyramidal neuron triggers the release of glutamate that evokes NMDAR-dependent SICs in an adjacent neuron (Navarrete and Araque, 2008), indicating that this mechanism may serve to astrocytes to operate as a bridge for non-synaptic interneuronal communication.

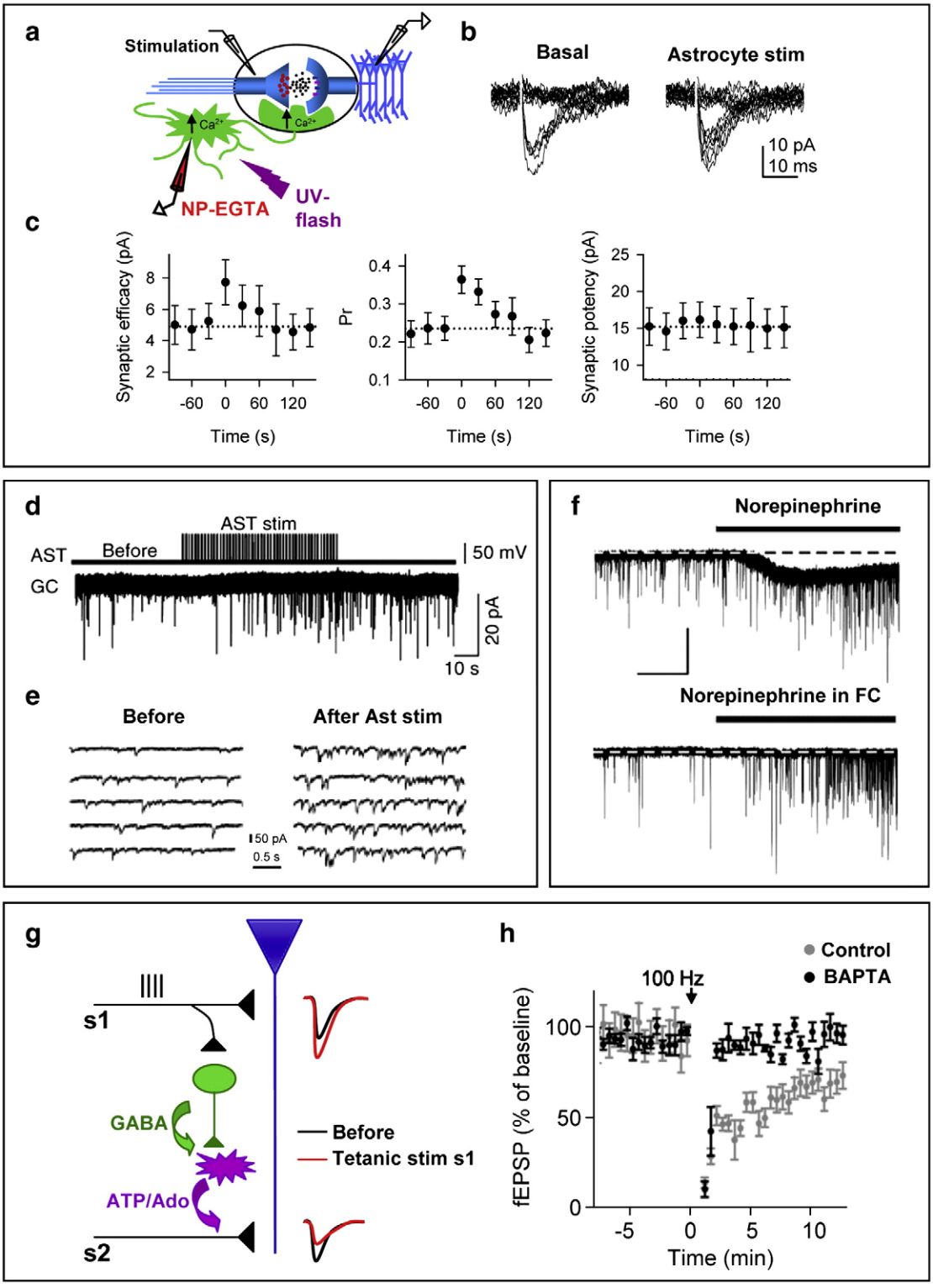
In addition to glutamate, ATP released by astrocytes stimulates the interneuron excitability acting on P2Y1 receptors and consequently potentiates GABAergic synaptic transmission (Bowser and Khakh, 2004). On the other hand, D-serine released by astrocytes contributes to activation of postsynaptic NMDA receptors, enhancing the neuronal excitability and modulating the NMDAR-mediated synaptic plasticity (Panatier et al., 2006; Stevens et al., 2003; Yang et al., 2003). These data suggest that astrocytes may differentially modulate the neuronal physiology on different cell types through the release of different gliotransmitters. Indeed, target cell-specific modulation of neuronal activity by astrocytes has been found in olfactory bulb, where the release of GABA from astrocytes evokes long-lasting and synchronous inhibition of mitral and granule cells, whereas the release of glutamate activates NMDARs in granule cells (Kozlov et al., 2006) (Figs. 1c, d).

3. Astrocytes regulate synaptic transmission

In addition to neuronal excitability, astrocytes can also modulate the synaptic activity through activation of pre and postsynaptic receptors by gliotransmitters released from adjacent astrocytes (Fig. 2). Astrocytic glutamate has been shown to enhance synaptic strength in different synapses through different mechanism. Activation of presynaptic group I metabotropic glutamate receptors (mGluRs) by astrocytic glutamate increased the frequency of spontaneous and evoked excitatory postsynaptic currents (EPSCs) (Fiacco and McCarthy, 2004; Perea and Araque, 2007), potentiating the probability of neurotransmitter release (Perea and Araque, 2007) in CA3–CA1 hippocampal synapses (Figs. 2a–c). Synaptic facilitation mediated by astrocytic glutamate through activation of presynaptic kainate receptors (Liu et al., 2004a) and NMDARs (Jourdain et al., 2007) has also been observed in

interneurons and in dentate granule cells of the hippocampus, respectively (Fig. 2d). Furthermore, by releasing glutamate astrocytes modulate inhibitory synaptic transmission increasing the synaptic strength between interneurons and CA1 pyramidal cells (Kang et al., 1998) (Fig. 2e). In contrast, glutamate released from astrocytes may also have opposite effects because depression of synaptic transmission has been reported when glutamate activates group II/III mGluRs of hippocampal inhibitory terminals (Liu et al., 2004b).

Other gliotransmitters, such as ATP or adenosine (a metabolic product of ATP), may also influence synaptic transmission. ATP released from astrocytes increases the amplitude of miniature EPSCs through activation of postsynaptic P2X7 receptors in the hypothalamic paraventricular nucleus (Gordon et al., 2005) (Fig. 2f). In contrast, astrocytic ATP/adenosine in the hippocampus contributes to heterosynaptic depression of synaptic transmission (Andersson et al., 2007; Serrano et al., 2006; Zhang et al., 2003), an effect



mediated by the coordinated activation of successive intercellular signaling events, i.e., excitatory Schaffer collateral axons stimulate interneuron activity that triggers GABA-mediated astrocyte Ca^{2+} signal that stimulates ATP release (Serrano et al., 2006) (Figs. 2g, h). This result represents an elegant example of the complex signalling that results from the coordinated neuron–glia network activity. Furthermore, because synaptically evoked ATP release by astrocytes acts as non-synaptic messenger that convey neuronal information beyond activated synapses, synapses may influence other relative distant synapses through stimulation of astrocytes. Likewise, as describe above, astrocytes provide a bridge for non-synaptic communication between neurons, because astrocytic glutamate released in response to endocannabinoid neuronal signalling can act as extracellular messenger to adjacent neurons (Navarrete and Araque, 2008).

Finally, adenosine itself has also been shown to be a gliotransmitter that may regulate excitatory synaptic transmission. Adenosine released from astrocytes has been proposed to serve as a protective mechanism by down regulating the synaptic activity level during demanding conditions such as transient hypoxia (Martin et al., 2007).

Data presented above indicate that a single gliotransmitter may have different effects depending on the target neurons and neuronal elements (pre or postsynaptic), and the activated receptor subtypes, which provides a high degree of complexity of the astrocytic effects on the neuron–glia network activity. This complexity becomes more intricate when considering that several different gliotransmitters might act cooperatively on the same neuron or act on different cell types evoking diverse responses. Furthermore, it must be taken into account that astrocyte Ca^{2+} signal evoked by activation of different receptors could not be equally competent to stimulate gliotransmitter release, as recently demonstrated by Shigetomi et al. (2008) in hippocampal slices, where astrocyte Ca^{2+} elevations induced by activation of PAR-1 receptors, but not P2Y1 receptors, evoke NMDAR-mediated SICs in pyramidal neurons.

Finally, the effects of the activity of single astrocytes on single synapses have been recently investigated in the hippocampus by performing paired recordings from pyramidal neurons and single astrocytes while stimulating SC single synapses – that is, experimentally isolating the tripartite synapse (Perea and Araque, 2007). Astrocyte Ca^{2+} elevations transiently increase the probability of neurotransmitter release from presynaptic terminals, thus enhancing the synaptic efficacy (Figs. 2a–c). This effect is mediated by Ca^{2+}- and SNARE-protein-dependent release of glutamate from astrocytes, which activates group I metabotropic glutamate receptors at the presynaptic terminal (Perea and Araque, 2007).

The experimental suitability as well as the morphological and functional properties of the hippocampus made it the brain area of choice to study synaptic physiology. Consequently, it has also been widely used to analyze the astrocyte effects on synaptic transmission. Nevertheless, our current knowledge of the role of astrocytes in neurotransmission is also gained from studies directed to other brain areas. Indeed, glia-mediated synaptic transmission modulation has been documented in retina, supraoptic nucleus and cerebellum, as well as at the neuromuscular junction in the peripheral nervous system (Brockhaus and Deitmer, 2002; Colomar and Robitaille, 2004; Newman, 2003; Theodosis et al., 2008).

In summary, current data provide a mere glimpse of the high level of complexity that the astrocyte–neuron communication can reach and the degrees of freedom that this communication may grant to the neuron–glia network. Therefore, to gain a better understanding of the specific effects of astrocytes on a defined neural network, several questions need to be addressed. It is crucial to define not only what type of gliotransmitter is released, but under what conditions is released and what are the specific modulatory effects on a particular neural network. It is also relevant to identify the intracellular regulatory mechanisms of gliotransmitter release as well as the spatially defined specific intercellular signaling pathways that grant a coherent

Fig. 2 – Astrocytes regulate synaptic transmission. (a–c) Astrocytes potentiate transmitter release at single hippocampal synapses. (a) Schematic drawing showing recordings from one CA1 pyramidal neuron and one astrocyte and the stimulation of a single synapse. (b) Synaptic responses (15 consecutive stimuli) obtained from paired whole-cell recordings before and after Ca^{2+} uncaging in a single astrocyte. Note the increase in the successful rate of synaptic responses after UV-flash astrocyte stimulation. (c) Astrocytes potentiate synaptic efficacy (i.e., mean amplitude of all responses including failures) by enhancing the probability of transmitter release (Pr, ratio between the numbers of successes versus the total number of stimuli) without changing the amplitude of synaptic responses (i.e., synaptic potency, defined as mean amplitude of the successful responses). Note the transient increase of synaptic efficacy and Pr after astrocyte stimulation (at time zero). Adapted from Perea and Araque (2007). (d) Electrical stimulation of one astrocyte (AST) potentiates miniature EPSCs in a hippocampal granule cell (GC). Adapted from Jourdain et al. (2007). (e) Astrocyte stimulation increases the frequency of miniature inhibitory postsynaptic currents in CA1 pyramidal neurons. Traces correspond to recordings before and after astrocyte stimulation. Adapted from Kang et al. (1998). (f) Norepinephrine increases the amplitude of AMPA-mediated miniature EPSCs in magnocellular neurosecretory cells by stimulating ATP release from astrocytes. Note that the inward current and the increase in miniature synaptic current amplitude evoked by Norepinephrine application (top) are abolished by the glial metabolic inhibitor fluorocitric acid (FC, bottom). Scale bar: 40 pA, 30 s. Adapted from Gordon et al. (2005). (g) Schematic drawing showing the proposed intercellular network involved in the hippocampal heterosynaptic depression. The sequential activation of tetanized excitatory input (s1), GABAergic interneurons (green) and astrocytes (violet) lead to the depression of a non-tetanized input (s2) by presynaptic inhibition of neurotransmitter release by adenosine (Ado). Black and red traces represent synaptic responses before and after tetanic stimulation of s1, respectively. (h) Graph showing changes in fEPSP amplitude in s2 before, during, and after tetanic stimulation of an independent pathway s1 (arrow, at 100 Hz) in control (grey) and after blockade of the astrocyte Ca^{2+} signal by BAPTA dialysis in glial cells (black). (g, h) Adapted from Serrano et al. (2006).

astrocyte–neuron communication. Whether different gliotransmitters are co-released or released by different astrocytes or by different astrocytic processes or domains need to be investigated. It is also important to elucidate the specific incoming inputs, the molecular mechanisms and the physiological conditions that govern the precise release of each gliotransmitter.

4. Astrocytes as a source for long-term synaptic plasticity

Astrocytes may impact neurotransmission in different time scales. As discussed above, astrocytes can transiently control the synaptic strength (during seconds or few minutes), but they

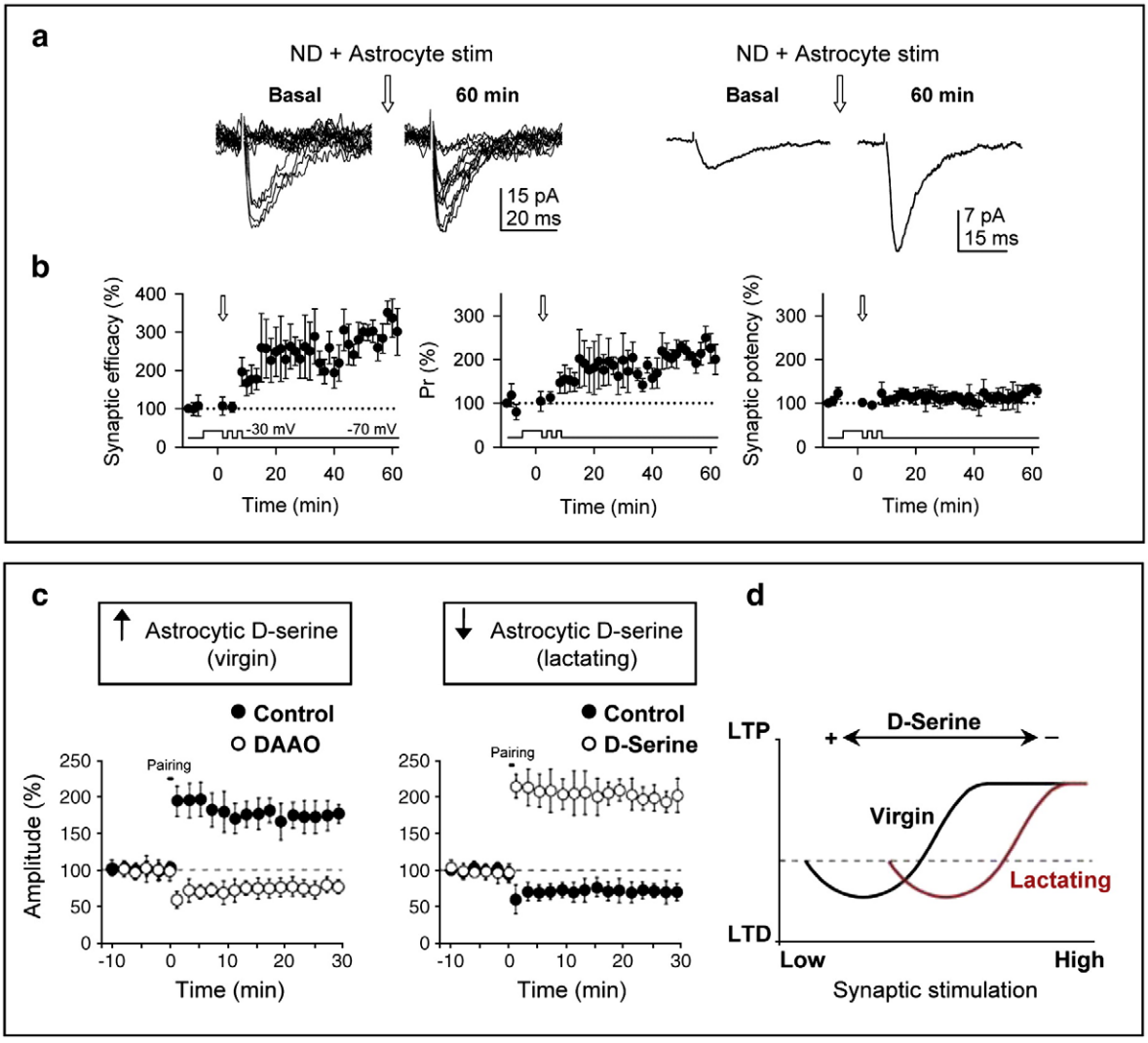


Fig. 3 – Astrocyte activity induces long-term changes in synaptic transmission. (a, b) Temporal coincidence of the astrocyte Ca^{2+} signal and postsynaptic neuronal depolarization induces long-term potentiation (LTP) of synaptic transmission in CA1 hippocampal pyramidal neurons. (a) Fifteen consecutive EPSCs (left) and average EPSCs (n = 50 trials including failures) (right) before and 60 min after transiently pairing mild neuronal depolarization (to –30 mV) and astrocyte Ca^{2+} elevations through Ca^{2+} uncaging. (b) Graphs showing relative changes in synaptic efficacy, Pr and synaptic potency parameters over time. Arrows indicate pairing of astrocyte Ca^{2+} signal and mild neuronal depolarization. Note the persistent potentiation of synaptic efficacy and Pr induced by the transient pairing (for 5 min) of neuronal and astrocyte stimulation (at time zero). Adapted from Perea and Araque (2007). (c, d) D-serine released by glia induces metaplasticity in the supraoptic nucleus. (c) While pairing synaptic stimulation with postsynaptic depolarization induces LTP in virgin rats (left panel; control), in lactating rats, which show lower levels of D-serine in the synaptic cleft, it causes LTD (right panel; control). Slices from virgin rats treated with DAAO to reduce endogenous levels of D-serine, LTP is transformed into LTD (left panel; DAAO). Exogenous supply of D-serine restores LTP in lactating animals (right panel; D-serine). (d) Model of rate of change in synaptic plasticity in virgin and lactating rats (black and red curves, respectively). Glial withdrawal around synapses in the supraoptic nucleus (red curve, lactating) causes lower availability of D-serine in the synaptic cleft and a rightward shift of the activity dependence of synaptic plasticity. As a consequence, an LTP-inducing protocol in virgin animals causes LTD in lactating rats. Adapted from Bains and Oliet (2007).

can also contribute to long-term changes of synaptic transmission, which are thought to be the mechanism of learning and memory at cellular level. Several mechanisms underlying the astrocyte effects on long-term potentiation (LTP) have been described. Some studies indicate a passive or tonic mode of action, in which astrocytes tonically depress or potentiate synaptic transmission (Beattie et al., 2002; Panatier et al., 2006; Pascual et al., 2005; Stellwagen and Malenka, 2006). Astrocytes through ATP/adenosine release control the strength of basal hippocampal synaptic activity by tonic depression of neurotransmission, which results in an increase of the dynamic range to induce LTP (Pascual et al., 2005). Furthermore, by releasing ATP (Gordon et al., 2005) or TNFα (Beattie et al., 2002; Stellwagen and Malenka, 2006) astrocytes promote the insertion of AMPA receptors in the membrane of the postsynaptic neurons, which increase the quantal efficacy of neurotransmission and provides a homeostatic control of the synaptic strength. Likewise, changes in the ambient levels of D-serine released by astrocytes influence NMDA receptor activation, playing a critical role in NMDAR-mediated synaptic responses (Fellin et al., 2009; Panatier et al., 2006; Yang et al., 2003) (Figs. 3c, d).

Astrocyte consequences on LTP can also be induced through phasic signaling processes. Indeed, the temporal coincidence of astrocytic glutamate signaling and postsynaptic neuronal activity induces long-term potentiation of transmitter release from presynaptic terminals (Figs. 3a, b) (Perea and Araque, 2007). The demonstration of this potentiation, which is independent of NMDARs and requires the activation of presynaptic type I mGluRs, has expanded our concept of the traditional Hebbian LTP because it includes the astrocytes as new sources of cellular signals involved in synaptic plasticity.

5. Plasticity at the tripartite synapses

The organization and activity of synapses are subject to changes elicited by their previous activity (Collingridge et al., 2004). Changes in synaptic transmission, i.e., synaptic plasticity, is the major mechanism thought to be the bases of learning and memory. While most of the studies of the cellular and molecular events underlying synaptic plasticity have been focused on changes on the neuronal elements, much scarce data exists regarding the plasticity of the astrocyte-synapse units, i.e., the plasticity at the tripartite synapses. Astrocytes are dynamic elements of synapses that display notable structural as well as functional modifications (Theodosis et al., 2008). Indeed, synaptically evoked astrocytic responses mediated by membrane expression of neurotransmitter receptors and transporters

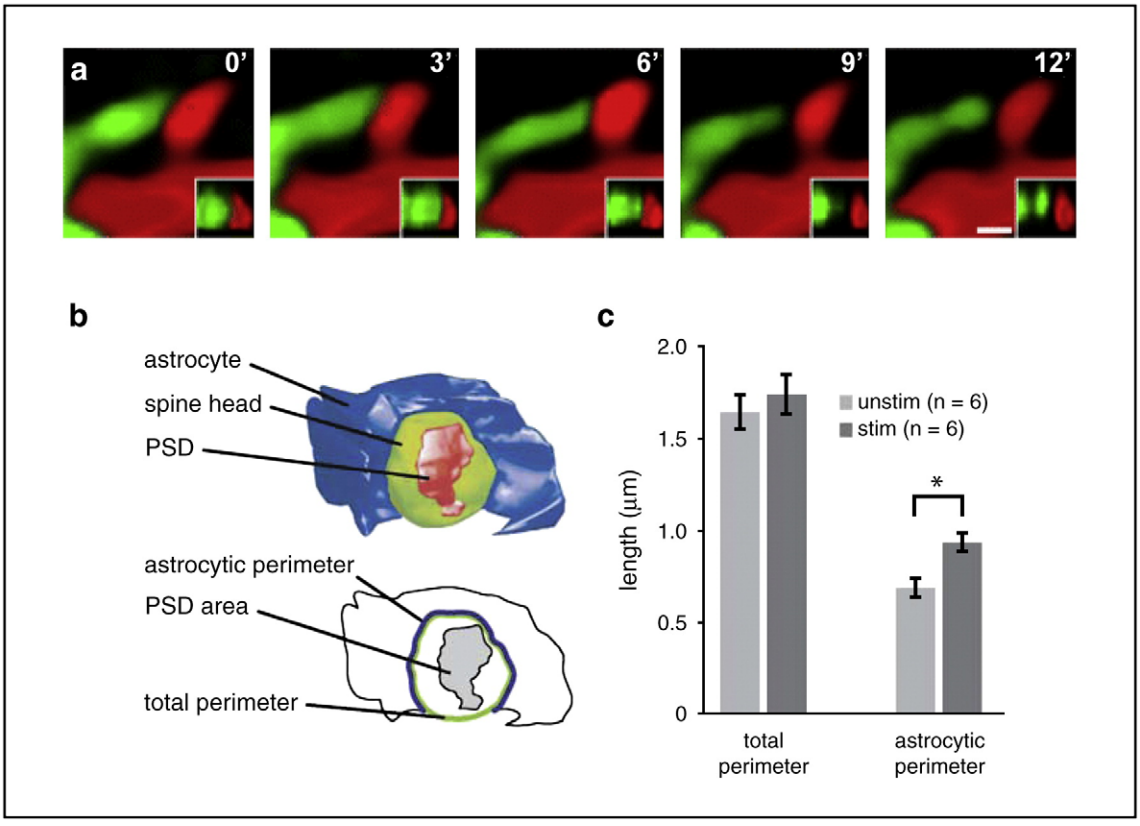


Fig. 4 – Activity-dependent plasticity of the tripartite synapses. (a) Astrocyte–spine interactions show dynamic structural interplay. Astrocytic process (green) extends toward a dendritic spine (red). Insets show 90° views of the astrocyte–spine interactions. Scale bar, 1 µm. Adapted from Haber et al. (2006). (b, c) Whisker stimulation increases the astrocytic coverage at the bouton–spine interface. (b) Three-dimensional reconstruction of a spine head (green), the postsynaptic density (PSD; red), and the associated astrocyte (blue) in the barrel cortex. (c) Whisker stimulation increases the astrocytic perimeter in stimulated (stim) versus unstimulated animals (unstim). Adapted from Genoud et al. (2006).

undergo activity-dependent modifications similar to short-term and long-term plasticity (Fig. 4) (Bellamy and Ogden, 2005; Ge et al., 2006; Yang et al., 2009). Oligodendrocyte precursor cells (OPCs), a glial cell subtype, that express Ca^{2+}-permeable AMPA receptors, show potentiation of the AMPA-mediated currents evoked by high frequencies of synaptic activity (Ge et al., 2006). Furthermore, Bergmann glial cells express short-term plasticity in response to paired-pulse stimulation of parallel fibers (Bellamy and Ogden, 2005).

Structural changes may also have strong impact on the synaptic function, because they influence neurotransmitter uptake from synaptic cleft and extrasynaptic receptor activation. The functional significance of the changes in the astrocytic coverage that occur under different physiological conditions (Theodosis et al., 2008) has been elegantly demonstrated in rat hypothalamic supraoptic nucleus, where a decrease of astrocytic ensheathing of synapses drastically reduces the effective levels of the gliotransmitter D-serine, which strongly influences NMDAR-mediated synaptic transmission (Panatier et al., 2006). Likewise, hippocampal astrocytes can rapidly extend and retract their processes in coordination with changes in dendritic spines, which may lead to the consolidation of dendritic spines (Haber et al., 2006) (Fig. 4a). Remarkably, after whisker stimulation astrocytes located in the barrel cortex increase their coverage of the bouton–spine interface and increase the expression levels of glutamate transporters in astrocytic processes (Genoud et al., 2006) (Figs. 4b, c).

6. Concluding remarks

Astrocytes have been revealed as key active elements of synapses, responding to neuronal activity and regulating synaptic transmission and plasticity. Accumulating data obtained during the last years indicate that astrocytes are actively involved in the processing, transfer and storage of information by the nervous system. Therefore, although key questions still need to be addressed and a more comprehensive characterization of the cellular events and the actual impact of astrocytes on the activity of the neuron–glia network is still required, current evidence challenges the accepted paradigm that brain function results exclusively from neuronal activity and proposes a new vision of the physiology of nervous system in which brain function actually arise from the concerted activity of the neuron–glia network.

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BRAIN RESEARCH REVIEWS

Review

Neurotransmitter transporters expressed in glial cells as regulators of synapse function

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ABSTRACT

Synaptic neurotransmission at high temporal and spatial resolutions requires efficient removal and/or inactivation of presynaptically released transmitter to prevent spatial spreading of transmitter by diffusion and allow for fast termination of the postsynaptic response. This action must be carefully regulated to result in the fine tuning of inhibitory and excitatory neurotransmission, necessary for the proper processing of information in the central nervous system. At many synapses, high-affinity neurotransmitter transporters are responsible for transmitter deactivation by removing it from the synaptic cleft. The most prevailing neurotransmitters, glutamate, which mediates excitatory neurotransmission, as well as GABA and glycine, which act as inhibitory neurotransmitters, use these uptake systems. Neurotransmitter transporters have been found in both neuronal and glial cells, thus suggesting high cooperativity between these cell types in the control of extracellular transmitter concentrations. The generation and analysis of animals carrying targeted disruptions of transporter genes together with the use of selective inhibitors have allowed examining the contribution of individual transporter subtypes to synaptic transmission. This revealed the predominant role of glial expressed transporters in maintaining low extrasynaptic neurotransmitter levels. Additionally, transport activity has been shown to be actively regulated on both transcriptional and post-translational levels, which has important implications for synapse function under physiological and pathophysiological conditions. The analysis of these mechanisms will enhance not only our understanding of synapse function but will reveal new therapeutic strategies for the treatment of human neurological diseases.

Contents

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1. Introduction

In the central nervous system (CNS), neurotransmission proceeds with high temporal and spatial resolutions. At chemical synapses, transmitters presynaptically released upon arrival of an action potential diffuse through the synaptic cleft where they bind and activate postsynaptic receptors. Termination of this signal requires the released neurotransmitters to be rapidly removed or inactivated on a millisecond timescale. At cholinergic synapses, e.g. at the neuromuscular junction, this is achieved by rapid degradation of acetylcholine by the enzyme acetylcholinesterase. Other transmitters, like glutamate, glycine and γ-amino butyric acid (GABA), are not degraded extracellularly, but termination of neurotransmission and prevention of neurotransmitter spreading into the extrasynaptic space are achieved by active transport of transmitter molecules into the surrounding neuronal and glial cells through complex transport systems.

Different studies have highlighted the important role of glial cells on the functional status of the CNS. Glia modulates clearance, availability and release of both excitatory and inhibitory neurotransmitters, therefore operating as key elements for controlling brain homeostasis. The necessity for involving glia in the clearance and/or recycling of transmitters released at high frequency is most likely due to the limited membrane surface present at the neuronal synaptic terminals. This physical constriction excludes the positioning of a high number of neuronal transporter molecules close to the transmitter release zone. Surrounding glial cells supply additional surface that allows the positioning of increased uptake capacity through high-affinity transporters in the perisynaptic region. This finally results in efficient recycling pathways through glia and prevents constant loss of neurotransmitters from the synapses. Thus, e.g. in the GABAergic system, synaptically released GABA is transported into astrocytes where it is either degraded to CO_2 or converted to glutamine in order to be subsequently transferred to GABAergic neurons and re-transformed to GABA. Moreover, several steps required for efficient synthesis of some neurotransmitters, e.g. glutamate, occur through enzymes exclusively located in the astrocytes. In addition to their contribution to recycling and synthesis of neurotransmitters, glial cells have been found to release neurotransmitters in specific CNS regions, e.g. GABA in rat dorsal root ganglia (Minchin and Iversen, 1974; Roberts, 1974). This action is possibly mediated by a non-vesicular transporter mediated release, as transporters are expected to reverse under circumstances such as local cell depolarization or low extracellular neurotransmitter concentrations (Richerson and Wu, 2003). Thus, glial cells may play a key role in directly regulating the basal transmitter concentration at extrasynaptic sites. Conversely, neurotransmitters known to have a relatively low turn-over rate, like dopamine, serotonin or norepinephrine, use exclusively neuronal transporters for reuptake and termination of neurotransmission. In summary, glial cells have been shown to play an active role in the maintenance and modulation of fast neurotransmission in tight cooperativity with neuronal cells.

The significance of glial transport in the regulation of neurotransmitter levels in the synaptic cleft is underscored by the fact that alterations in the expression of predominantly glial transporters cause severe perturbations of neurotransmission at respective synapses. This demonstrates that glial cells function as an efficient buffering system or sink for presynaptically released neurotransmitter. Additionally, it has become clear that transporter proteins expressed by glial cells play a crucial role in shaping synaptic transmission. In this review we discuss our present understanding of the function of these transporters.

2. *In vivo* functions of glial GABA transporters

GABA is the principal inhibitory neurotransmitter in the adult mammalian brain, where it activates $GABA_A$, $GABA_B$, and $GABA_C$ receptors. High-affinity GABA transport processes play a key role in controlling the levels of this amino acid in the extracellular space upon its release from the presynaptic terminal. Pioneering studies of GABA transport on bulk-prepared neuronal and glial cells (Henn and Hamberger, 1971), in addition to following studies on cell lines and primary cultures of astrocytes and neurons (Hertz and Schousboe, 1987), have clearly demonstrated that GABA transporter mediated uptake takes place in both cell types. This is thought to have crucial consequences in the regulation of the GABAergic system. First, GABA uptake is believed to be a key event in terminating phasic synaptic currents induced by GABA release via synaptic vesicle exocytosis from presynaptic terminals. Second, GABA transporters expressed by neurons may also play a role in replenishing the supply of presynaptic transmitter by transporting it back into the GABAergic nerve ending. Third, GABA transporters are expected to be involved in regulating the extracellular GABA concentration throughout the brain, thus modulating tonic activation of synaptic and extrasynaptic GABA receptors. This may also involve reverse activity of GABA transporters, thereby mediating non-vesicular release of GABA to the surrounding cerebrospinal fluid (CSF) (Richerson and Wu, 2003). Interestingly, the kinetic characterization of GABA uptake in GABAergic neurons and astrocytes in primary cultures showed that the capacity of GABA transport is much higher in neurons than in astrocytes (Hertz and Schousboe, 1987). Therefore, it was hypothesized that GABA neurotransmission is preferentially based on recycling of GABA in GABAergic neurons (Schousboe, 2003). In addition, a secondary fraction of released GABA would be taken up by astrocytes surrounding the synapse, where it can be metabolized. Thus, the activity of astrocytic GABA

transporters would inevitably lead to a loss of GABA from the overall neurotransmitter pool.

Cloning of GABA transporters has facilitated the elucidation of the precise functional roles of GABA transport processes in neurons and glial cells. To date, four GABA transporters have been identified in the central nervous system: GABA transporter subtype 1 (GAT1) (slc6a1), Betain-GABA transporter-1 (BGT-1) (slc6a12), GAT2 (slc6a13), and GAT3 (slc6a11) (Borden et al., 1992; Guastella et al., 1990; Yamauchi et al., 1992). Molecular characterization of these proteins has revealed that GAT1, GAT2 and GAT3 are high-affinity GABA transporter subtypes whereas BGT1 shows a lower affinity. GABA transporters are mainly energized by the sodium gradient across the membrane. Although Cl^- can significantly enhance the rate of transport, Cl^- alone cannot drive GABA uptake in the absence of Na^+. The stoichiometry for GAT1, GAT2 and GAT3 has been proposed to be 2 Na^+:1 Cl^-:1 GABA, whereas for BGT1 is 3 Na^+:2 Cl^-:1 GABA (Karakossian et al., 2005; Loo et al., 2000; Sacher et al., 2002). Immunohistochemical studies using antibodies raised against recombinant proteins confirmed GAT expression on both neurons and astrocytes. Notably, GABA transporters do not follow a cell type specific expression pattern. Thus, GAT-1 is not only localized to axon terminals forming symmetric synaptic contacts but also to distal astrocytic processes (Conti et al., 1998; Minelli et al., 1995; Ribak et al., 1996). GAT2 is weakly expressed throughout the brain, primarily in arachnoid and ependymal cells, where it has been proposed to have a nutritional role, and to a much lesser extent in neurons and astrocytes (Conti et al., 1999). GAT3 expression is found predominantly on glial cells. BGT-1 is not present at GABAergic synapses but seems to be located extrasynaptically on both neurons and astrocytes (Borden et al., 1995; Zhu and Ong, 2004). Therefore, glial cells mainly express GAT1, GAT3 and, to a lower extent, BGT1 and GAT2, located both extrasynaptically and on neighboring GABAergic synapses, thus raising several issues regarding the relative contribution of each transporter to overall GABA uptake by glial cells and their functional significance.

The functional importance of GABA transporters in the regulation of GABAergic neurotransmission has been initially highlighted by studies on the modulatory action of GAT inhibitors on GABA receptor activation. In different brain areas, GAT1 antagonists increase the decay time of evoked inhibitory postsynaptic currents (IPSCs) without modifying that of spontaneous IPSCs (sIPSCs) (Engel et al., 1998; Keros and Hablitz, 2005; Overstreet and Westbrook, 2003; Thompson and Gahwiler, 1992). In addition, they also enhance the amplitude of tonic GABA mediated currents (Keros and Hablitz, 2005; Marchionni et al., 2007; Nusser and Mody, 2002; Semyanov et al., 2003), thus indicating that GAT1 affects both phasic and tonic GABA receptor mediated inhibition. By using microdialysis techniques, GAT1 inhibitors have been also shown to increase extracellular GABA concentration in the brain (Fink-Jensen et al., 1992; Juhasz et al., 1997; Richards and Bowery, 1996). Altogether, these findings suggest that GAT1 is responsible for controlling extracellular levels of GABA at synapses. Because GAT1 is located both on neurons and glia, these studies do not provide direct information about the relative contribution of glial uptake in this context. In addition, inhibitors of GABA transporters have been shown to exert anti-convulsant activity. In different animal models of epilepsy, highly selective GAT1 blockers like Tiagabine, which is a clinically useful antiepileptic drug, EF1502, which exhibits inhibitory activity at BGT1 and GAT1 (Clausen et al., 2005), and SNAP-5114, a GAT2/3 selective compound (Borden et al., 1994), display a broad anti-convulsant profile following intraperitoneal administration (Madsen et al., 2009). Interestingly, EF1502 combined with tiagabine displayed a synergistic anti-convulsant action, suggesting that increased extracellular concentrations of GABA induced by GAT1 inhibition result in the spill-over of this transmitter into the extrasynaptic space where the subsequent inhibition of extrasynaptically BGT1 facilitates the activation of different populations of GABA receptors. This hints to an important role of BGT1 in regulating GABA levels at extrasynaptic sites. In contrast, combination of SNAP-5114 with tiagabine only produced an additive interaction (Madsen et al., 2009). A likely explanation to this is that inhibition of GAT1 in neurons and glia together with glial GAT3 ultimately causes an increased concentration of GABA at the synapse, which results in an anti-convulsant effect mediated by GABA receptors in close proximity to GAT1 and GAT3. This indicates that inhibitors of astroglial GAT3 are able to increase the availability of GABA in the extracellular neurotransmitter pool (Sarup et al., 2003a,b; Schousboe, 2003) thus suggesting that glial GABA uptake plays a functionally important role in regulating the availability of GABA in the synapse.

Genetic approaches have confirmed GABA transporter functions previously disclosed by pharmacological studies. GAT1 knockout (KO) mice also display increased GABA mediated tonic conductances (Chiu et al., 2005; Jensen et al., 2003), prolonged decay time of evoked IPSCs (Bragina et al., 2008) but no alterations in the frequency, amplitude and kinetics of sIPSCs (Jensen et al., 2003). No major compensatory changes in proteins or structures related to GABA transmission were observed in GAT1 KO mice (Chiu et al., 2005). Notably, no change in GABA content of synaptic vesicles was observed in GAT1 deficient mice (Bragina et al., 2008). Microdialysis studies also showed that spontaneous release of GABA was comparable in wild-type and GAT1 deficient mice, whereas KCl-evoked output of GABA was significantly increased in KO mice (Bragina et al., 2008). These findings demonstrate that ablation of GAT1 chronically elevates ambient GABA without affecting its intracellular levels. Thus, GAT1 mediated uptake plays an essential role *in vivo* controlling extracellular levels of GABA in the synapse with no obvious function on recycling of GABA in GABAergic neurons. In addition, prolongation of evoked IPSC, but not sIPSCs, decay time indicates that in GAT1 KO mice synchronous activity of several synaptic terminals results in the accumulation of extracellular GABA whereas after unitary release events the uptake capacity provided by other GABA transporters is sufficient for the fast clearance of synaptically released GABA. Thus, GAT-1 limits the interaction between closely spaced sites by restricting diffusion beyond the synaptic specializations (Overstreet and Westbrook, 2003). A second GAT KO mouse line has been generated for the glial GAT3 (slc6a11). These animals show early postnatal lethality (Mouse Genome Database (MGD), Mouse Genome Informatics, The Jackson Laboratory, Bar Harbor, Maine, (URL: http://www.

informatics.jax.org, June, 2009)) possibly due to GABA mediated overinhibition, although no analyses have been carried out to clarify this remarkable phenotype. Nevertheless, heterozygous mice carrying only one functional allele of GAT3 survive and appear grossly indistinguishable from wild-type animals. Interestingly, these heterozygous mice require a significantly higher dose of the $GABA_A$ receptor antagonist metrazol to reach various seizure stages when compared with wild-type control mice (Deltagen, Inc., NIH initiative supporting placement of Deltagen, Inc. mice into public repositories, MGI Direct Data Submission 2005). This increased resistance to pharmacologically induced seizures is in agreement with observations using GAT3 inhibitors, as mentioned above, and suggests that glial GAT mediated uptake plays an essential *in vivo* role controlling extracellular levels of GABA in the synapse, as hypothesized more than 3 decades ago (Schousboe, 1979).

3. Glycine transporters

The glycinergic system is the second important inhibitory transmitter system within the mammalian CNS. In contrast to GABA that is used throughout all major brain regions as an inhibitory neurotransmitter, glycinergic synapses are found predominantly in caudal regions of the CNS, i.e. brainstem and spinal cord. Upon vesicular release, glycine binds to the predominantly postsynaptically localized glycine receptors (GlyRs), resulting in the opening of an intrinsic chloride channel which causes inhibition of the postsynaptic cells via an influx of chloride ions causing a hyperpolarisation of the cell or alternatively shunting inhibition. In addition to its inhibitory function at glycinergic synapses, glycine is a high-affinity coagonist for ionotropic glutamate receptors of the N-methyl-D-aspartate (NMDA) subtype (Dingledine et al., 1990; Johnson and Ascher, 1987). This heterotetrameric receptor is assembled from two NR1 subunits that have been shown to contain the glycine binding side (Kuryatov et al., 1994) and two glutamate binding NR2 subunits (Schorge and Colquhoun, 2003). The binding of a ligand, glycine, or alternatively D-serine, to the NR1 subunit of the receptor is a prerequisite for the activation of the receptor by glutamate (Kleckner and Dingledine, 1988). The affinity of the NR1 subunit for its ligand, however, appeared to be determined by the coassembled NR2 subunit (Laurie and Seeburg, 1994). More recently, NMDA receptors that contain two different glycine binding subunits, NR1 and NR3, were identified (Chatterton et al., 2002). These heteroreceptors are activated by glycine alone and thus constitute a novel class of excitatory glycine receptor. Their *in vivo* functions, however, remain elusive.

The dual function of glycine at both inhibitory as well as excitatory synapses underscores the importance of a precise regulation of the extracellular glycine concentration. Similar to GABA, the CSF glycine concentration is regulated by both neuronal and glial transporters. Up to now, two different high-affinity transporters, GlyT1 (Slc6a9) and GlyT2 (Slc6a5), have been discovered (Guastella et al., 1992; Liu et al., 1992, 1993). Both transporters are encoded by single genes, although multiple splice variants have been described (Adams et al., 1995; Ebihara et al., 2004). Glycine transporters are structurally related to the previously discussed GABA transporters and use predominantly the sodium gradient across the membrane as an energy source for the intracellular accumulation of glycine (Gether et al., 2006). Interestingly, GlyT2 requires 3 Na^+ and 1 Cl^- for the import of one glycine molecule whereas GlyT1 takes only 2 Na^+ and 1 Cl^- per transport cycle (Roux and Supplisson, 2000; Supplisson and Roux, 2002). As a consequence of this stoichiometry difference, it is widely accepted that under physiological conditions GlyT2 can only mediate import of glycine into the cytosol maintaining millimolar intracellular versus submicromolar extracellular glycine levels, whereas GlyT1 might reverse transport directionality after cell depolarization or at low extracellular glycine concentrations, thus facilitating non-vesicular release of glycine from the cytosol into the surrounding extracellular space. Immunohistochemical analysis of glycine transporters has revealed that GlyT2 is exclusively expressed by glycinergic neurons in caudal regions of the CNS, representing the only known reliable marker for these neurons (Poyatos et al., 1997; Zeilhofer et al., 2005). In contrast to GlyT2, GlyT1 shows a broader expression pattern in the CNS. Although GlyT1 is predominantly expressed in glial cells of brain stem and spinal cord (Zafra et al., 1995), i.e. regions which are rich in glycinergic neurotransmission, it can be also found in brain regions like cortex or hippocampus (Jursky and Nelson, 1996; Zafra et al., 1995) both in glial cells and in a subset of presumptive glutamatergic neurons (Adams et al., 1995; Cubelos et al., 2005). Altogether, these differences both in their ionic stoichiometry and cellular location raise several issues regarding the relative contribution of each transporter to the regulation of the extracellular glycine concentration throughout the brain and their physiological function.

Genetic inactivation of GlyT expression revealed different but complementary functions of both transporters in the neonatal animal. Inactivation of each of the GlyT genes caused severe perturbation of glycinergic neurotransmission that finally resulted in premature death of the mutant animals (Gomeza et al., 2003a,b). Electrophysiological analysis of the glycinergic neurotransmission in the brainstem of GlyT2 deficient mice revealed that neuronal GlyT2 is essential for the replenishment of glycine within the presynaptic terminal for vesicular release. Thus, loss of GlyT2 from the presynaptic nerve terminal results in a decrease of the vesicular glycine content and thereby a reduction in glycinergic IPSC amplitude. The extracellular concentration of glycine within the CSF, more specifically at synaptic sites, however, appeared not to be controlled by this neuronal transport system, since no changes in GlyR mediated tonic conductances were observed in these animals. Therefore, the efficient removal of glycine from the synaptic cleft of glycinergic synapses in neonatal animals was expected to be mediated by glial GlyT1. Consistently, ablation of GlyT1 expression by gene inactivation resulted in a strong facilitation of GlyR activity in the brainstem and spinal cord and consequently overinhibition of the animal. Moreover, recordings from hypoglossal motoneurons of GlyT1 deficient mice revealed strong GlyR mediated tonic currents and prolonged mIPSC decay (Gomeza et al., 2003a) kinetics. This indicates that the removal of glycine from the synaptic cleft by predominantly glial expressed

GlyT1 is the rate limiting step in the off kinetic glycinergic neurotransmission.

More recently, these results have been verified and extended by pharmacological analyses. In agreement with observations on GlyT1-deficient mice, systemic inhibition of GlyT1 by ALX5407 or LY2365109 in adult rats resulted in respiratory depression and impaired motor performance (Perry et al., 2008). Likewise, recordings from lamina X neurons from spinal cord revealed that acute inhibition of GlyT1 resulted in an extracellular accumulation of glycine at synaptic sites (Bradaia et al., 2004). However, *in vivo* application of both the GlyT1 specific inhibitor Org24598 or the GlyT2 blocker Org25543 into the dorsal spinal cord of rats resulted in a significant increase in extracellular glycine levels (Whitehead et al., 2004), suggesting that in the mature spinal cord the extracellular glycine concentration is not exclusively controlled by GlyT1 but also by GlyT2. Similar analyses from lamina X neurons showed that inhibition of GlyT2 also caused an extracellular accumulation of glycine at synaptic sites (Bradaia et al., 2004). Moreover, inhibition of GlyT2 *in vivo* resulted in a significant impairment of motor performance as well as respiratory activity in adult rats (Hermanns et al., 2008), symptoms previously observed in GlyT1 deficient mice due to extracellular accumulation of glycine (Gomeza et al., 2003a). After long lasting inhibition of GlyT2, however, a rundown of presynaptically released glycine has been monitored (Bradaia et al., 2004) in agreement with the symptoms observed in GlyT2 KO mice. In summary, these observations suggest that in the mature nervous system, neuronal GlyT2 is not only essential for the replenishment of presynaptic glycine for vesicular release but also contributes to the regulation of the synaptic glycine concentration.

Together, these findings suggest that in caudal regions of the CNS, glial GlyT1 and neuronal GlyT2 closely cooperate in the regulation of extracellular glycine at inhibitory synaptic sites. After presynaptical release of glycine, both transporters contribute to the fast removal of glycine, thus ensuring the rapid termination of glycine dependent neurotransmission. In addition, glycine neurotransmission is based on recycling of glycine in glycinergic neurons by GlyT2. Thus, a significant extent of released glycine is subsequently accumulated into glycinergic nerve endings, enabling its incorporation into synaptic vesicles and subsequent release upon depolarization. A secondary fraction of the transmitter is incorporated into glial cells via GlyT1. At later timepoints, when glycine concentration at glycinergic synapses is too low for GlyR activation, GlyT1 might reverse transport directionality, releasing glycine from the cytoplasm of glial cells and thus providing additional substrate for neuronal accumulation of glycine by GlyT2.

In higher brain regions, like cortex or hippocampus, where the GlyT2 is only expressed sporadically, the availability of extracellular glycine is thought to be controlled exclusively by GlyT1. As mentioned above, binding of glycine to the NR1 subunit of the NMDA receptor is essential for the activation of the receptor by glutamate and allows thereby the modulation of synaptic plasticity. As expected, application of GlyT1 inhibitors to hippocampal slice preparations resulted in the facilitation of the NMDA receptor component of glutamatergic synaptic transmission. Systemic inhibition of GlyT1 by LY2365109 in rats caused a facilitation of the NMDA induced dopamine release in the prefrontal cortex and hyperactivity (Perry et al., 2008). Inhibition of GlyT1 by NFPS in rat hippocampus resulted in a partial inhibition of long term potentiation (LTP) elicited by tetanic high frequency stimulation (Manahan-Vaughan et al., 2008). Interestingly, the inhibition of GlyT1 ameliorated the effects of prior exposure to potent NMDA receptor inhibitors like MK801 (Manahan-Vaughan et al., 2008). In contrast, application of high concentrations of glycine to a similar slice preparation alone resulted in the induction of a robust GlyT1 dependent LTP that was independent of the NMDA receptor (Igartua et al., 2007). Whether these effects are mediated by glially or neuronally expressed GlyT1 is not clear at present, since both cell types have been shown to express the transporter in this brain region (Adams et al., 1995; Cubelos et al., 2005). First indication that effects caused by GlyT1 inhibitors were associated at least in part with forebrain neuronal GlyT1 comes from the analysis of mice carrying a neuron specific disruption of the GlyT1 gene (Singer et al., 2007; Yee et al., 2006). In these mice, an enhanced NMDA receptor component of glutamatergic neurotransmission was found. Consequently, they were more resistant in pharmacological model-systems for psychosis and showed improved recognition memory (Singer et al., 2007; Yee et al., 2006). This contrasts findings from mice that carried a general deletion of glial and neuronal GlyT1 in the forebrain. Here, no enhancement of the NMDA receptor component of glutamatergic neurotransmission was observed, although these mice display some behavioral changes (Singer et al., 2009) different from those observed in animals deficient for only forebrain neuronal GlyT1. Therefore, genetic approaches have suggested different functions of glial and neuronal GlyT1 in the modulation of glutamatergic neurotransmission that would result in additional mechanisms of GlyT1 mediated synaptic plasticity in the forebrain.

4. Glial expressed glutamate transporters

In contrast to glycine and GABA, which both have predominantly inhibitory functions, glutamate serves as the principal excitatory neurotransmitter in most regions of the CNS. After presynaptic release, glutamate binds to both ionotropic and metabotropic receptors which differ in their affinity and localization. There are two major classes of ionotropic glutamate receptors. First, the non-NMDA receptors, which include α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors and Kainate receptors. Although both receptor classes are structurally related, they differ in their functional role in glutamatergic neurotransmission. Whereas AMPA receptors are the major receptors mediating fast excitatory synaptic transmission, Kainate receptors appear to have predominantly modulatory functions. Due to the low affinity of these receptors to glutamate, their activation is closely coupled to their localization to synaptic sites. The second major class of ionotropic glutamate receptors is the NMDA receptor. It displays that higher affinity for glutamate and active NMDA receptors can be found both at synaptic sites as well as extrasynaptically. Although the activity of this receptor class is blocked at large by Mg^{2+} under resting

conditions, it has been shown to have important functions as an essential coincidence detector that is involved in a variety of effects like learning and memory. Additionally, glutamate activates metabotropic glutamate receptors (mGluRs), G-protein coupled receptors that modulate the activity of enzymes producing second messengers, such as phospholipase C or adenylyl cyclase, different channels and ionotropic glutamate receptors (Conn and Pin, 1997). They are localized at synaptic sites and within their periphery, and contribute to the regulation of glutamatergic neurotransmission in the brain.

High extracellular glutamate concentrations not only prevent efficient neurotransmission but additionally cause excitotoxicity. Thus, the extracellular concentration of glutamate must be precisely regulated at synaptic sites as well as at extrasynaptic locations. In addition, the high abundance of glutamatergic transmission requires an efficient recycling system for the neurotransmitter to ensure a constant transmitter supply for presynaptic release. It is generally accepted that major proportions of glutamate released by presynaptic terminals are taken up not by neurons but by surrounding astrocytes that encase the presynaptic terminal. In the plasma membrane of these cells, high-affinity glutamate transporters are located that play a key role in the control of glutamate clearance and its availability, thus modulating glutamatergic neurotransmission. Within the astrocytic cytoplasm, glutamate is efficiently converted by the enzyme glutamine synthetase (GS) to glutamine that does not bind to glutamate receptors. This "inactivated" metabolite of glutamate is subsequently returned to neurons to be reconverted back to glutamate by a phosphate activated glutaminase (PAG), being thus reused for vesicular release. Interestingly, PAG is mainly expressed in neurons whereas GS is exclusively localized in astrocytes (Hertz et al., 1999). Therefore, glutamate metabolism in the brain is cell compartmentalized, placing astrocytes in a key position with regard to the regulation of glutamate homeostasis and, consequently, glutamatergic neurotransmission (Hertz et al., 1999; Schousboe and Waagepetersen, 2006). The disadvantage of the high energy cost of this glutamate–glutamine cycle is greatly compensated by the prevention of excitotoxicity caused by elevated levels of extracellular glutamate in the synapses.

The efficient clearance of glutamate from the extracellular space is achieved by a whole family of high-affinity transporters, the excitatory amino acid transporters (EAATs). In contrast to the GATs and GlyTs, the EAATs do not belong to the Slc6 family of neurotransmitter transporters but form a family on their own. In total, 5 different EAATs have been characterized in the mammalian nervous system: EAAT1 (also named Glast), EAAT2 (Glt1), EAAT3 (EAAC1), EAAT4 and EAAT5 (Danbolt, 2001). In addition to their main substrate glutamate, these transporters mediate L-aspartate and D-aspartate uptakes. EAAT3 also accepts L-cysteine as a substrate. Similar to the transporters for glycine and GABA, the EAATs use predominantly the sodium gradient across the membrane as the main energy source for the intracellular accumulation of glutamate. The transport stoichiometry for this family of transporters has been proposed to be 3 Na^+ and one proton per transport cycle of one molecule of glutamate, while one potassium ion is concurrently extruded from the cell (Danbolt, 2001). Consequently, activity of these transporters results in an increase of the intracellular sodium concentration. In glial cells it has been reported that this augmentation causes increased activity of the Na^+/K^+ ATPase (Rose and Ransom, 1996; Voutsinos-Porche et al., 2003) and upregulation of the astrocytic glucose metabolism (Loaiza et al., 2003). This mechanism is considered to couple astrocytic energy metabolism to the synaptic activity in its surrounding.

Initially, Glast1 (EAAT1) and Glt1 (EAAT2) have been described as glial transporters, whereas EAAT4 and EAAC1 (EAAT3) were assumed to be exclusively expressed in neurons (Rothstein et al., 1994). More recently, however, it has been demonstrated that most transporters are expressed at different levels by both neuronal and glial cells (Chen et al., 2002, 2004; Hu et al., 2003). Thus, the contribution of individual transporters and/or cell types to the total glutamate transport activity depends on the expression of EAATs in the respective brain regions.

The major glutamate transporter present in regions that are rich in glutamatergic neurotransmission is the predominantly glially expressed transporter Glt1 (EAAT2). In the forebrain regions, Glt1 alone accounts for more than 95% of the total high-affinity glutamate uptake capacity (Tanaka et al., 1997). Mice carrying genetically inactivated Glt1 alleles displayed a marked neurological phenotype including spontaneous epileptic seizures and neuronal degeneration as well as an increased mortality of homozygous mutant mice. Consistently, the synaptic concentration of glutamate was found to be increased in these mice. These findings confirm that Glt1 is involved in the maintenance of a low extracellular glutamate concentration. There were, however, no differences in the decay kinetic of AMPA or NMDA receptor mediated postsynaptic responses, showing that this glutamate transporter does not determine the postsynaptic decay rates. Additionally, lowering of the extracellular glutamate concentration by Glt1 restricts mGluR activation within hippocampal interneurons and thus contributes to the control of their activity (Huang et al., 2004b). Whether the effects seen in Glt1 deficient mice are caused by the loss of neuronal or glial expressed Glt1 is not clear at present. The contribution of Glt1 to the regulation of glutamatergic transmission, however, appears to depend on a tight encasement of the synapse by glial cells (Oliet et al., 2001). At synapses tightly wrapped by glial membranes, as found in the supraoptic nucleus of rats, inhibition of Glt1 leads to a buildup of glutamate in the synaptic cleft that results in enhanced activation of presynaptic mGluRs. Loosening of the glial encasement of the synapse, as found in lactating animals, reduced this effect, thus demonstrating that control of the synaptic glutamate concentration depends on the intimate contact between glial cells and the respective synapse. In contrast to the strong phenotype seen in Glt1 deficient mice, mice deficient for the second predominantly glial expressed glutamate transporter Glast (EAAT1) display only a very subtle motor coordination phenotype (Watase et al., 1998), which is most likely caused by cerebellar dysfunction. Additionally, Glast seems to cooperate with Glt1 in controlling mGluR function in the interneurons of the hippocampus (Huang et al., 2004b). Deficiency for the predominantly neuronal expressed glutamate transporters EAAC1 (EAAT3) and EAAT4 causes only very moderate

phenotypes. Even animals deficient for both transporters (EAAC1$^{-/-}$/EAAT4$^{-/-}$) survived until adulthood, were fertile and did not show any obvious motor coordination phenotype (Huang et al., 2004a). Together, these data suggest that the predominant glial expressed transporters Glt1 and Glast are the major players in regulating extracellular glutamate concentration in most brain regions, although the loss of one specific transporter can be compensated by other transporters at least in case of low frequency activity.

It has to be stressed that the precise localization as well as the expression levels of these transporters, and consequently their contribution to the regulation of glutamate dependent neurotransmission, might not be the same in different brain regions. In cortical regions, i.e. regions where synapses are tightly surrounded by astrocytic processes, most likely glial glutamate uptake is responsible for controlling extracellular glutamate concentration. In other brain regions, like e.g. the hippocampus, where synapses are only partially encased by astrocytic processes, a major proportion of glutamate uptake might be accomplished by the neurons themselves. Up to now, the precise analysis of these questions has been hampered by the lack of suitable mouse models that allow the inactivation of transporter expression in specific cell types. More information, however, is available about the regulation of EAAT levels and their influence on overall glutamate uptake. Although some interacting proteins have been identified recently that stabilize EAAT localization at the plasma membrane (Jackson et al., 2001; Lin et al., 2001) or facilitate their internalization (and subsequent degradation) (Gonzalez et al., 2003, 2005), bulk EAAT activity is thought to be regulated predominantly on the level of expression. Several lines of evidence suggest that unknown secreted neuronal factors contribute to the regulation of the Glast and Glt1 gene expressions (Schlag et al., 1998; Zelenaia et al., 2000) in the brain. Interestingly, dysregulation of glutamate transporter expression is discussed to be causal and/or associated with several neurodegererative diseases like amyotrophic lateral sclerosis (ALS), ischemia/stroke and epilepsy (Maragakis and Rothstein, 2004). Moreover, hypoxia resulted in a massive downregulation of Glast and glial Glt1 expressions both *in vitro* (Dallas et al., 2007) and *in vivo* (Pow et al., 2004) whereas neuronal expression of Glt1 was upregulated (Pow et al., 2004). This differential regulation of EAAT expression in neurons and glial cells might enhance excitotoxicity induced by elevated glutamate concentration after strong hypoxia (Rossi et al., 2000). Similarly, reduced Glt1 expression is thought to be causal for glutamate induced neurodegeneration of motoneurons in genetic models for ALS (Howland et al., 2002). Recently, astrocytic Glt1 expression has been shown to be induced by the presence of axons, a process depending on the Kappa-B motif binding phosphoprotein (KBBP) (Yang et al., 2009). Notably, dysregulation of KBBP expression is found in genetic models of ALS, suggesting that this pathway contributes to the pathogenesis of this disease. The involvement of EAATs in the pathogenesis of neurodegenerative and/or neurological syndromes suggests that the manipulation of their expression might constitute a new strategy for the treatment of these diseases. Indeed, the partial replacement of astrocytes by transplantation of "healthy" precursor-cells into SOD^{G93A} rats that develop ALS, resulted in marked improvement of the disease progression (Lepore et al., 2008). Interestingly, the expression of Glt1 can be increased by antibiotics of the β-lactam family, and this upregulation appears to be neuroprotective both in neurotoxicity induced by oxygen/glucose deprivation and in animal models for ALS (Rothstein et al., 2005). The precise mechanism how β-lactam antibiotics regulate Glt1 expression however still remains elusive.

5. Summary and perspectives

In the last couple of years the analysis of transporter deficient mice together with pharmacological approaches has greatly enhanced our understanding on how glial cells and neurons cooperate in the fast removal of transmitter from the synaptic cleft of active synapses. This has resulted in new insights into the physiology and pathophysiology of the nervous system. Nevertheless, the complexity of the expression pattern of many of these transporters has prevented deeper insights in the destiny of neurotransmitters after presynaptic vesicular release. The development of new mouse models that allow specific inactivation of the respective transporter genes in individual cell types would greatly enhance the progress in this field. Findings from the glutamate transporters, but also from other neurotransmitter transporters, have suggested that transporter expression levels in neurons and glial cells might be differentially regulated. The precise analysis of these regulatory mechanisms will not only enhance our understanding of the function of neuron/glia interaction but might additionally result in the development of new therapeutic strategies for the treatment of human neurological diseases like epilepsy, ALS or even psychiatric diseases like schizophrenia.

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BRAIN RESEARCH REVIEWS

Review

Ion changes and signalling in perisynaptic glia

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ABSTRACT

The maintenance of ion gradients across plasma membranes is a prerequisite for the establishment of cellular membrane potentials, electrical signalling, and metabolite transport. At active synapses, pre- and postsynaptic ion gradients are constantly challenged and used for signalling purposes. Perisynaptic glia, mainly represented by fine processes of astrocytes which get into close vicinity to neuronal synapses, are required to normalize the extracellular ionic milieu and maintain ion gradients. On the other hand, perisynaptic glia itself is activated by synaptically released transmitters binding to plasma membrane receptors and transmitter carriers, and experiences significant ion changes as well. In this review we present an overview of dynamic changes of the major ion species in astrocytes in response to neuronal, especially synaptic, activity. We will focus on calcium, sodium, and proton/hydroxyl ions that play key roles in signalling processes, and will discuss the functional consequences of the glial ion signals and homeostatic processes for synaptic transmission.

Contents

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1. Introduction

Perisynaptic glia are cell processes, in general of astrocytes, which get into close vicinity to neuronal synapses. They have been functionally associated with the formation and maintenance of synaptic domains and are involved in the removal and recycling of transmitters. While some neurotransmitters, such as acetylcholine, are rapidly cleaved by extracellular enzymes within the synaptic domain, the inactivation of other transmitters, such as glutamate and GABA, is critically dependent on their binding by, and uptake into, surrounding glial cells. Transmitter uptake occurs at the expense of transmembrane ion gradients, above all the sodium gradient across cell membranes, but is also associated with transmembrane fluxes of other ions like potassium, H^+/OH^-, and chloride. In addition, astrocytes express ionotropic and metabotropic receptors and their activation by transmitters can result in intraglial ion transients, in particular of calcium, as well.

Ionic gradients across pre- and postsynaptic neuronal membranes are essential for electrical signalling and for maintenance of synaptic function, imposing a strong demand for efficient regulation of ion concentrations in the synaptic domain. The prime presynaptic function is activity-dependent release of neurotransmitters, which is initiated by an influx of calcium from the small extracellular, perisynaptic space through voltage-gated calcium channels in the presynaptic membrane. Major ion movements into and from the extracellular space are generated by postsynaptic ionotropic receptors and ion channels. While activation of glial transmitter carriers and receptors may add to these ionic changes in synaptic domains, perisynaptic astrocytes may also be challenged to counteract them and thus contribute to the maintenance of ionic gradients between intra- and extracellular compartments.

In this review, we shall consider the major ion species which are involved in neurotransmitter-induced glial signalling and in pre- and postsynaptic ionic processes, as well as in the removal of neurotransmitters from the synaptic cleft. We shall summarize the role of glial cells in this ionic signalling and the functional implication of glial regulation of intra- and extracellular ion concentrations, in particular with respect to excitatory neurotransmission.

2. Ion gradients, cellular compartments and activity-related ion signals in the extracellular space

2.1. *Extra- and intracellular ion concentrations*

The maintenance of ion gradients across plasma membranes is a prerequisite for the establishment of cellular membrane potentials, electrical signalling, and metabolite transport driven by ion gradients. Here, the activity of the Na^+/K^+-ATPase, which exchanges intracellular sodium for potassium with a ratio of 3:2, is of uttermost importance. A wealth of secondary and tertiary active transporters uses the inwardly directed sodium gradient to generate gradients for other ions such as potassium, magnesium, protons, bicarbonate and, at least partly, calcium and chloride. This results in distinct differences in the extra- and intracellular concentrations of these ions. While the potassium concentration in the extracellular space is around 2.5 mM, intracellular potassium amounts to about 130 mM (Kofuji and Newman, 2009). For sodium, extracellular and intracellular concentrations are around 145 and 8–15 mM, respectively (Rose, 1997). Despite the fact that intracellular pH is often close to extracellular pH in brain tissue (pH 7.1–7.3), there is an inwardly directed electrochemical gradient in neurons and glial cells due to their negative membrane potential, because the reversal potential for protons and bicarbonate is near 0 mV (Deitmer and Rose, 1996). The steepest electrochemical gradient exists for calcium ions. Its intracellular 'resting' concentration is near 100 nM or below, while its extracellular concentration is 1–2 mM (Deitmer et al., 1998). Intracellular chloride concentrations, finally, differ between mature CNS neurons and astrocytes, with profound functional consequences. While chloride concentration is maintained low (around 5 mM) in mature neurons, it is in the range of 30–60 mM in astrocytes, resulting in a significantly more positive chloride reversal potential for astrocytes as compared to neurons (Ben-Ari et al., 2007). However, many neurons in the earlier stages of development have a higher intracellular chloride concentration than mature neurons, with the consequence that an increase in the chloride conductance, as, e.g., evoked by classical inhibitory neurotransmitters like GABA and glycine, results in a membrane depolarization (Farrant and Kaila, 2007; Blässe et al., 2009). This chloride-mediated excitatory input is critical for the structural and functional establishment of neuronal networks in the developing brain.

At synapses, electrochemical gradients of different ion species between the extracellular space and the cytosol drive their fast diffusion across the plasma membrane following the opening of voltage- and ligand-gated ion channels, and thus provide the charge movements that are the basis of electrical signalling. While fast electrical signalling is a hallmark property of neurons, it seems to play a less important functional role for astrocytes, which do not fire action potentials. Instead, astrocytes are characterized by expression of a multitude of metabotropic receptors in their plasma membrane which are coupled to the PLC/IP_3 signalling cascade and subsequent calcium release from intracellular stores

(Verkhratsky, 2009). The ER calcium concentration is above 100 μM and thus several orders of magnitudes higher than cytosolic calcium concentration. Calcium ions represent intracellular second messengers and serve pivotal signalling functions in both neurons and glial cells (Berridge et al., 1998).

Ion movements across the plasma membrane in response to synaptic activity not only arise following activation of transmitter receptors and opening of voltage-gated ion channels. Another important pathway for ion movements across glial membranes is the activity of different ion-coupled membrane carriers. These can be transporters that are activated in response to changes in extracellular ion concentrations and/or membrane potential such as the electrogenic Na^+/K^+ pump or the electrogenic Na^+/HCO_3^- cotransporter. Furthermore, transporters for transmitters such as glutamate and GABA mediate ion transport in response to synaptic activity (see chapter by Eulenburg and Gomeza, 2010). Depending on the exact stoichiometry of the transporters, significant currents can be generated upon their activation. In neurons, this can lead to changes in excitability: activation of the Na^+/K^+-ATPase generates an outward current and hence counteracts excitation, while activation of Na^+/Ca^{2+} exchange generates an inward current which depolarizes the cell membrane and promotes excitation. Because of the very low electrical input resistance of astrocytes as compared to neurons, transporter currents have usually less influence on their membrane potential.

2.2. *Morphology of the involved compartments determines the properties of ion transients*

The effect of synaptically-induced ion movements on extra- and intracellular ion concentrations and their electrochemical gradients, respectively, strongly depends on the surface-to-volume-ratio of the involved compartments. Moreover, parameters influencing ion diffusion such as buffering, as well as spatial restrictions by a complex morphology or a dense extracellular perineuronal net, will have a profound influence on the amplitude, time course and spatial distribution of activity-related ion transients (Sykova and Nicholson, 2008). Finally, homeostatic mechanisms, provided by ion pumps and transporters will usually counteract activity-related ion changes and will also modulate ion transients.

Electrical signalling only requires small ion fluxes: the sodium flux necessary to evoke a single action potential in a 1000-μm squid axon yields a change of the electrochemical sodium gradient across the plasma membrane of only about 0.01% (Hille, 2001). This situation is, however, completely different at central synapses. Dendritic spines, protuberances of minute volume (<1 μm^3) which frequently represent the postsynaptic structures of excitatory synapses in the vertebrate CNS, host both ligand-gated and voltage-gated ion channels. They have a very high surface-to-volume ratio, and diffusion is limited by the spine necks. Thus even small ion fluxes during synaptic transmission cause significant ion transients in spines which can have important physiological consequences (Yuste et al., 2000).

While spines are compact structures that can be analysed by conventional light microscopy, the very fine and tortuous processes of astrocytes that reach close to synapses are below the resolution limit of conventional fluorescence-based dynamic imaging techniques (Derouiche and Frotscher, 2001; Bushong et al., 2004). 3-D reconstructions based on electron microscopy have revealed that Bergmann glial processes tightly enclose synapses of climbing and parallel fibers onto Purkinje cells (Grosche et al., 1999). In the hippocampus, glial coverage of synapses is less pronounced, indicating that glutamate released at approximately two thirds of hippocampal synapses might diffuse to other synapses (Ventura and Harris, 1999).

Because of the small size of perisynaptic astrocyte processes, most studies concerning synaptically-induced ion changes in astrocytes have reported ion changes from astrocyte cell bodies and/or primary processes. While such measurements only enable a distant, filtered look at what presumably happens in the very fine perisynaptic processes, considerable ion transients have been described in response to synaptic transmission even in these comparatively large volume structures. These will be discussed in the following chapters. Since the magnitude and kinetics of intracellular ion changes crucially depend on the surface-to-volume ratio, even larger ion changes are therefore expected in the smaller, sheet-like perisynaptic processes.

2.3. *Extracellular ion transients*

Glial ion homeostasis and signalling at synapses are closely related to processes in the extracellular spaces (ECS). Measurement of the exact magnitude and time course of extracellular ion transients are, however, technically difficult or not feasible. While the width of the ECS is only around 20–50 nm, the tip diameter of ion-selective microelectrodes, still routinely used to measure extracellular ion changes, is around 1 μM and thus will drastically disturb the tissue architecture. Despite these pitfalls, it is now firmly established that neuronal activity results in extracellular ion transients. If any, it seems safe to argue that the changes determined using ion-selective microelectrodes represent underestimates of the actual amplitudes of extracellular ion transients close to synapses.

Measurements with potassium-selective electrodes demonstrated that neuronal activity results in an increase in the extracellular potassium concentration in the low mM range due to the loss of potassium from activated neurons. Because increased extracellular potassium alters neuronal excitability, this excess potassium has to be removed quickly, a task taken over – at least in part – by astrocytes by mechanisms known as 'spatial buffering' and 'potassium siphoning' (Kofuji and Newman, 2009; Newman and Reichenbach, 1996). This includes net uptake of potassium at sites of elevated potassium, i.e., where neurons are firing action potentials, and release of potassium at sites where no or little neuronal activity is going on, resulting in a redistribution of extracellular potassium. Correspondingly, intraglial potassium concentration may rise slightly in response to synaptic activity (Ballanyi et al., 1987; Coles and Schneider-Picard, 1989; Karwoski et al., 1989).

Synaptic stimulation of afferent fibers evokes a decrease in the extracellular sodium concentration due to sodium influx into cellular compartments through voltage- and ligand-gated

ion channels (Dietzel et al., 1982). The same is true for the extracellular calcium concentration because calcium ions rapidly enter both pre- and postsynaptic structures (Zanotto and Heinemann, 1983). Synaptic activity also causes changes in extracellular pH that are partly buffered by concomitant changes in intraglial pH (Deitmer and Rose, 1996; Chesler, 2003). Chloride, finally, is subject to complex movements in response to synaptic activity (Dietzel et al., 1982). While it enters adult CNS neurons through $GABA_A$-receptor-coupled channels, chloride can leave glial cells via the same pathway (Ballanyi et al., 1987; Walz, 1995). In addition, chloride may cross plasma membranes through separate chloride channels or in response to activation of chloride-dependent transporters (Blaesse et al., 2009; Chen and Sun, 2005; Kimelberg et al., 2006). Chloride channels and chloride-dependent transporters have been associated with activity-induced changes in cell volume of neurons and astrocytes (Chen and Sun, 2005; Kimelberg et al., 2006). These processes are closely interrelated, as ion changes may cause, and be challenged, by water movements across cell membranes. Local volume changes may in particular also occur in synaptic domains, where ion flow across the postsynaptic membrane can be large during high frequency activity of neurons.

3. Calcium signalling in perisynaptic glia

Calcium is a prime intracellular second messenger in most cell types, and numerous processes in the nervous system are initiated or modulated by intracellular calcium transients in both neurons and astrocytes (Berridge, 2005; Verkhratsky et al., 2009). Astrocytes respond to neurotransmitters with Ca^{2+} elevations, and astrocytic Ca^{2+} signalling at synapses is a central element in reciprocal neuron-glia signalling (Hirase et al., 2004; Agulhon et al., 2008; Perea et al., 2009). One of the most consequential processes initiated by Ca^{2+} transients in astrocytes is the release of "glio"-transmitters that bind to neighbouring neurons and modulate their activity. Astrocyte-induced modulation of synaptic transmission has been observed in primary culture as well as in tissue slices acutely isolated from several brain areas. Some of these Ca^{2+}-dependent processes in astrocytes could also be elicited by photolysis of caged Ca^{2+} compounds by UV illumination (Parpura and Haydon, 2000; Fellin et al., 2004; Kreft et al., 2004; Fiacco and McCarthy, 2004).

Ca^{2+} signalling in astrocytes has also been associated with the control of cerebral blood flow. Ca^{2+} transients and Ca^{2+} oscillations propagate to the astrocytic endfeet, which ensheath the endothelial layer of blood capillaries, and can result in vasodilation and vasoconstriction by the release of lipid metabolites (Zonta et al., 2003; Parri and Crunelli, 2003; Mulligan and MacVicar, 2004; Takano et al., 2006; Metea and Newman, 2006; Gordon et al., 2008; Döngi et al., 2009). Inhibition of cyclooxygenase-1 activity blocked astrocyte-evoked vasodilation, supporting the notion that Ca^{2+}-dependent release of prostaglandins from astrocytes mediates the control of local blood flow. This modulation may be initiated by neuronal activity that evokes astrocyte Ca^{2+} signals which propagate towards blood vessels and in turn influence microcirculation. As this affects the supply of oxygen and glucose in the brain, these mechanisms are likely to represent an important glial contribution to the maintenance of brain energy supply (Iadecola and Nedergaard, 2007; Koehler et al., 2009).

Astrocytes can also communicate among each other by generating intercellular Ca^{2+} waves, which are propagated along cell processes into neighbouring astrocytes. Different stimuli, such as mechanical stimuli or exogenous application of neurotransmitters, may induce single or multiple intracellular Ca^{2+} transients that propagate as Ca^{2+} waves between astrocytes in cultured cells as well as organotypic and acute brain slices (Cornell-Bell et al., 1990; Parri et al., 2001; Fellin, 2009). These waves can propagate long distances (<500 μm) at relatively low speed (~14 μm/s) and seem to represent a unique form of slow, long-distance intercellular communication in the nervous system. Astrocyte Ca^{2+} signals that occur spontaneously or in response to synaptic activity can be initiated in spatially restricted areas, called "microdomains," of the astrocytic processes. From these discrete regions, Ca^{2+} signals can spread along the processes to other regions of the astrocyte generating a wave of intracellular Ca^{2+}, which may cross cell boundaries to become intercellular waves. Temporally coordinated astrocyte Ca^{2+} waves that are triggered by neuronal activity have recently been reported to attract outgrowing growth cones (Hung and Colicos, 2008). Because the spatial spread of astrocyte Ca^{2+} signals extends the area of activated synapses significantly, it was suggested to represent a form of brain information pathway that establishes a functional link between distant synaptic areas (Haydon, 2001; Fellin, 2009; Perea et al., 2009). A thorough characterization of intra- and intercellular astrocyte calcium waves is yet to be accomplished, but it is tempting to speculate that their spatial spread might be influenced or even controlled by synaptic activity.

3.1. *Ca^{2+} responses to transmitters and other signalling molecules*

Numerous reviews have focused on glial Ca^{2+} signalling in response to extracellular messengers in the last few years (Fiacco and McCarthy, 2006; Agulhon et al., 2008; Deitmer and Araque, 2009; Fellin, 2009; Verkhratsky et al., 2009). Cytosolic Ca^{2+} transients may be triggered by a variety of signalling molecules, including hormones, growth factors, prostaglandins, and neurotransmitters. Astrocytes express a wealth of neurotransmitter receptors, and many of them belong to the family of metabotropic receptors and are coupled to G proteins and second messenger pathways that lead to the IP_3-mediated Ca^{2+} release from intracellular stores. The glial Ca^{2+} signals can be linked to initiating cellular activity, such as transmitter release, a rise in the K^+ conductance of the cell membrane, mitochondrial energy production, or activation of enzymes. In general, communication between neurons and astrocytes is thought to be mediated by *spillover* of neurotransmitter from the synaptic cleft to initiate the metabotropic signalling cascade in glial cells leading to the release of Ca^{2+} from the endoplasmic reticulum These occur in general during neuronal activity and will be discussed below.

Astrocytes may also respond with intracellular Ca^{2+} elevations to other signalling molecules such as chemokines. Chemokines are a class of small proteins that bind to G protein-coupled receptors, which were originally identified as inflammatory

mediators of leukocyte chemotaxis, but were later shown also to be involved in functions beyond neuroinflammation. Various types of chemokine receptors are expressed in cells of the nervous system, including astrocytes. The chemokine stromal cell-derived factor 1 (SDF-1) has been demonstrated to increase astrocytic Ca^{2+} through activation of the receptor CXCR4 (Bajetto et al., 1999), which can result in glutamate release from astrocytes (Bezzi et al., 2001).

The list of ligands that trigger a release of Ca^{2+} from intracellular stores in astrocytes is steadily growing. Since some of these ligands, e.g., glutamate and ATP, may be released by astrocytes themselves in a Ca^{2+}-dependent and Ca^{2+}-independent way, autoactivation of astrocytes might be expected. However, the cellular distribution of metabotropic receptors is not yet known. At postsynaptic membranes, receptors are clustered in specialized regions and such clustering may also occur in astrocyte cell processes facing synaptic and extrasynaptic release sites. A more or less uniform distribution of astrocyte receptors on the cell surface, on the other hand, would enable them to sense ambient concentration of ligands.

3.2. *Ca^{2+} responses to neuronal activity*

The modulation or initiation of Ca^{2+} signals in astrocytes by synaptic activity has been reported from the retina, cerebellum, hippocampus, and cortex and has been shown to be mediated by glutamate, ATP, adenosine, GABA, acetylcholine, noradrenaline, histamine, nitric oxide and a variety of other signalling molecules (for reviews see Araque et al., 2001; Fiacco and McCarthy, 2006; Perea et al., 2009; Verkhratsky et al., 2009). The amplitude and duration of Ca^{2+} transients as well as the frequency of Ca^{2+} oscillations in astrocytes may vary depending on the level of synaptic activity. Moderate stimulation of parallel fibers in the cerebellum has been shown to evoke Ca^{2+} increases that were restricted to only a few small processes of Bergmann glial cells, closely associated with synaptic sites (Grosche et al., 1999). Calcium signals could spread between these glial "microdomains" (Beierlein and Regehr, 2006), indicating that glial cells are able to respond locally to neuronal activity. Strong stimulation of parallel fibers, in contrast, evoked widespread Ca^{2+} transients that included the soma. These were shown to be mediated by activation of metabotropic glutamate (mGluR1), purinergic ($P2Y_1$, adrenergic (alpha 1), and ionotropic AMPA-type glutamate receptors (Kulik et al., 1999; Matyash et al., 2001; Beierlein and Regehr, 2006; Piet and Jahr, 2007).

In the olfactory nerve and bulb, Ca^{2+} transients were recorded during nerve stimulation in both olfactory ensheathing cells and glomerular astrocytes (Fig. 1; Rieger et al., 2007; Döngi et al., 2008). These Ca^{2+} signals could be repeatedly evoked and were similar in shape and amplitude as those elicited by ATP or glutamate. This and further evidence suggested that the olfactory nerve releases glutamate and ATP both at its terminals as well as along the axons by vesicular exocytosis. It has been reported that astrocytes release GABA in the olfactory bulb, which caused long-lasting and synchronous inhibition of mitral and granule cells (Kozlov et al., 2006; Angulo et al., 2008). It needs to be shown, however, whether olfactory nerve stimulation initiates a reciprocal dialogue between neurons and glial cells.

Recent studies have demonstrated that astrocytes may display integrative properties for synaptic information processing, as the properties of astrocyte calcium signals differ if different axon pathways are stimulated (Perea and Araque, 2005, 2007). Synaptically-evoked Ca^{2+} signals in astrocytes can be modulated bidirectionally by the interaction of different synaptic inputs, either being potentiated or being depressed depending on the level of synaptic activity. This modulation may also influence the intracellular spread of the Ca^{2+} signal in form of waves, which may have important consequences on brain function by regulating the spatial extension of a single astrocyte's impact on different synapses.

Ca^{2+} responses in glial cells were involved in heterosynaptic depression in the hippocampus following high-frequency stimulation of Schaffer collaterals (Pascual et al., 2005; Serrano et al., 2006). Schaffer collateral stimulation caused an excitation of interneurons and release of GABA, which activated glial $GABA_B$ receptors and resulted in glial Ca^{2+} transients (Serrano et al., 2006). This triggered the release of ATP from these astrocytes, which was degraded to adenosine by ecto-nucleotidases. The subsequent activation of presynaptic adenosine A_1 receptors on adjacent, non-stimulated synapses resulted in heterosynaptic depression. In hippocampal astrocytes it was shown that Ca^{2+} signals following $GABA_B$ receptor activation were attributable to Ca^{2+} release from intracellular stores (Meier et al., 2008). In addition, GABA evoked glial calcium transients by activation of $GABA_A$ receptors, cellular depolarization and activation of voltage-dependent Ca^{2+} channels. Yet another mode of GABA-evoked Ca^{2+} responses has recently been found in olfactory bulb astrocytes. Here, Na^+ loading attributable to Na^+-dependent GABA uptake into astrocytes modified Na^+/Ca^{2+} exchange leading to Ca^{2+}-induced Ca^{2+} release (Döngi et al., 2009).

In the retina, a flickering light stimulus evoked Ca^{2+} transients in Müller glial cells (Newman, 2005; Metea and Newman, 2006). Addition of adenosine greatly potentiated this light-evoked Ca^{2+} response. The ATP hydrolysing enzyme apyrase blocked the glial Ca^{2+} responses, indicating that neuron-to-glia signalling in the retina is mediated by ATP release from neurons and subsequent activation of glial purinergic receptors. On the other hand, Müller cell stimulation can evoke a hyperpolarization in neighbouring ganglion cells, which is blocked by the A1 adenosine receptor antagonist DPCPX and is reduced by ecto-ATPase and ecto-nucleotidase inhibitors. This suggests that glial cells release ATP, which is converted to adenosine by ecto-enzymes, leading to the activation of neuronal adenosine receptors. It was concluded that there is reciprocal exchange of signals between neurons and glial cells, involving cytosolic Ca^{2+} transients and release of transmitters in both types of cells. In olfactory bulb astrocytes, both ATP and adenosine could elicit Ca^{2+} responses via P1 and P2Y receptors (Döngi et al., 2008). ATP and its metabolites seem to play a prominent and diverse role in mediating different kinds of Ca^{2+} signals and their propagation in astrocytes, and as neuro-/gliotransmitter or co-transmitter in general (Burnstock, 2007).

Ca^{2+} signalling in astrocytes was also found *in vivo* following stimulation of sensory neurons. In anaesthetized mice, whisker stimulation resulted in transient Ca^{2+} increases in astrocytes in the barrel cortex mediated by metabotropic

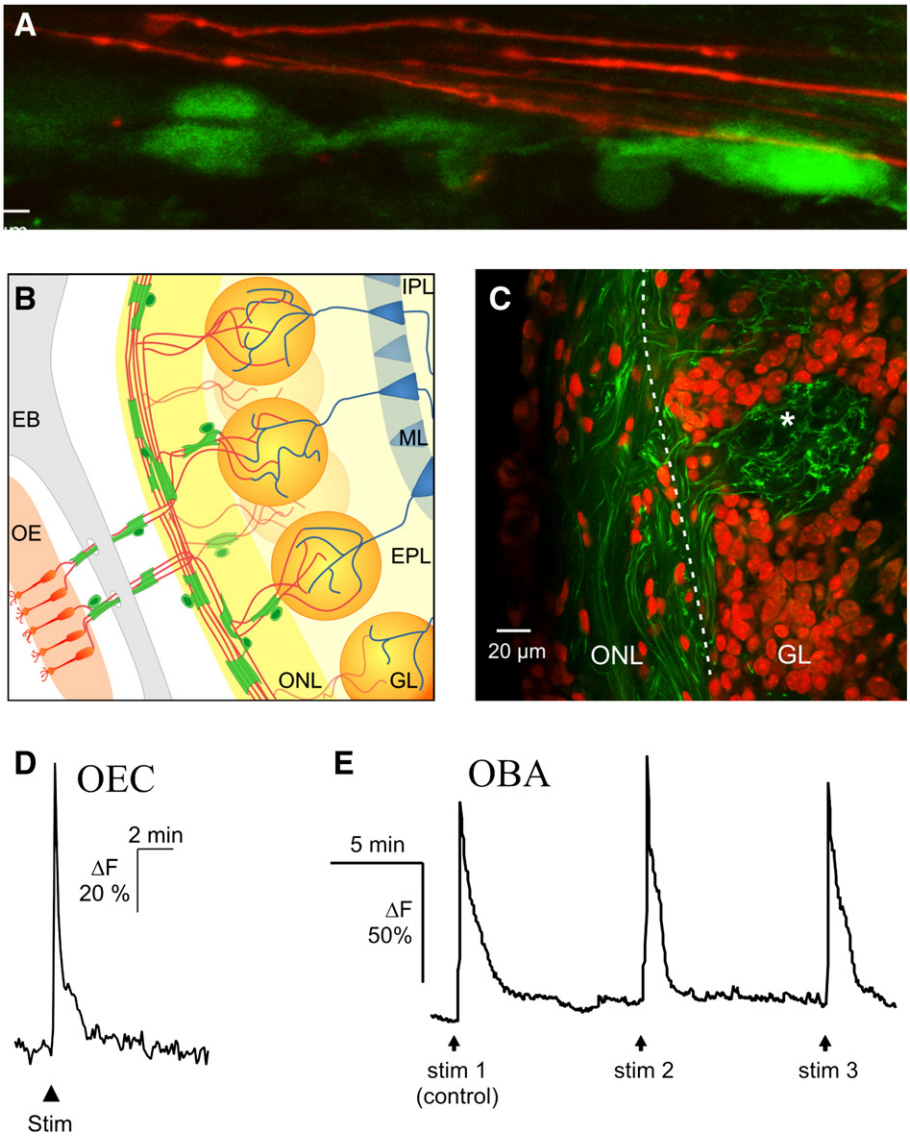

Fig. 1 – Calcium transients in olfactory glia following olfactory nerve stimulation. (A) DiI-tracing of olfactory receptor nerve axons (red) in an acute mouse brain slice with Fluo-4-stained olfactory ensheathing cells (OEC; green). (B) Schematic drawing of the olfactory bulb with the somata of the olfactory receptor neurons (red) located in the olfactory epithelium (OE), extending their axons to the olfactory bulb, thereby crossing the ethmoid bone (EB), and entering the glomeruli. Axons are accompanied by the OECs (green) and project into the olfactory nerve layer (ONL) until they reach the glomerular layer (GL) where they connect to mitral cells. The somata of the mitral cells in the mitral cell layer (ML) are separated from the GL by the external plexiform layer (EPL). (C) Anti-GFAP immunohistochemistry shows intense labelling in the glomeruli (asterisks) of the GL and in the ONL. (D, E) Fluorescence changes of Fluo-4-loaded OEC and olfactory bulb astrocyte (OBA) following electrical stimulation of the olfactory nerve. (A–D from Rieger et al., 2007, and E from Döngi, M., Deitmer, J.W. and Lohr, C., unpublished).

glutamate receptor activation. The astrocytic Ca^{2+} increases persisted in the presence of ionotropic glutamate receptor blockers, which reduced activation of postsynaptic neurons, suggesting that Ca^{2+} signalling during whisker stimulation was independent on the postsynaptic activation of neurons located in the barrel cortex but was directly evoked by glutamate released from nerve terminals of the afferents (Wang et al., 2006). Two-photon imaging of Ca^{2+} signals in the ferret visual cortex *in vivo* revealed that astrocytes, like neurons, respond to visual stimuli, where they play a key role in coupling neuronal organization to mapping signals critical for non-invasive brain imaging (Schummers et al., 2008). Robust peripheral stimulation, known to result in phasic *locus coeruleus* activity, evoked Ca^{2+} responses in astrocytes throughout the sensory cortex in living mice (Bekar et al., 2008). In cerebellar Bergmann glial cells, different forms of Ca^{2+}-based excitation were found in awake, behaving mice. During locomotor performance, concerted Ca^{2+} excitation arises in networks of hundreds of Bergmann glial cells extending across several hundred microns (Nimmerjahn et al., 2009). Furthermore, using fluorescent dyes or the Ca^{2+}-sensor protein G-CaMP2, spontaneous Ca^{2+} waves, propagating with

a speed of 4–11 μm/s were recorded in Bergmann glial processes extending 50 μm in the intact cerebellum (Hoogland et al., 2009). In the future, further *in vivo* recording of Ca^{2+} signalling will certainly consolidate, promote and inspire our understanding of information processing involving neuron-glia communication.

3.3. *Ca^{2+}-induced release of gliotransmitters*

There is growing evidence that elevations of cytosolic Ca^{2+} can initiate regulated release of a variety of molecules, including classical transmitters such as glutamate, GABA, and ATP, as well as co-transmitters like D-serine (Parpura et al., 1994; Coco et al., 2003; Newman, 2003; Mothet et al., 2005; Kozlov et al., 2006; Xu et al., 2007). Large rises of cytosolic Ca^{2+} as induced by flash photolysis of caged Ca^{2+} can trigger the release of these gliotransmitters (Oheim et al., 2006). Many studies indicate that release of gliotransmitters is calcium-dependent and mediated by exocytosis of transmitter-filled vesicles. Other mechanisms proposed for transmitter release from astrocytes include reversed neurotransmitter uptake (Szatkowski et al., 1990; Rossi et al., 2000) and transmitter efflux through pores such as connexin hemichannels (Ye et al., 2003), purinergic $P2X_7$ receptors (Duan and Neary, 2006) and anion channels (Kimelberg et al., 2006). Recently, Ca^{2+}-regulated exocytosis of lysosomes was reported in astrocytes (Jaiswail et al., 2007; Li et al., 2008), which may release ATP (Zhang et al., 2007). Clathrin- and dynamin-independent endocytosis was found in astrocytes, which could be enhanced by glutamate and ATP (Jiang and Chen, 2009).

Many proteins belonging to the exocytotic machinery, including the glutamate accumulating transporters VGLUT-1 and VGLUT-2, and the *n*-ethyl maleimide-sensitive fusion protein attachment protein receptor (SNARE) proteins cellubrevin, synaptobrevin II and SNAP-23 have been found in astrocytes (Zhang et al., 2004; Bezzi et al., 2004; Montana et al., 2004). Release of glutamate from cultured astrocytes was reported to be triggered by cytosolic Ca^{2+} elevations (Parpura et al., 1994; Araque et al., 1998; Bezzi et al., 2004) and was reduced when astrocytic Ca^{2+} rises were suppressed by the Ca^{2+} chelator BAPTA (Araque et al., 1998; Zhang et al., 2004). In addition, inhibition of the endogenous SNARE complex by introduction of an exogenous SNARE motif into astrocytes prevented the release of glutamate and ATP (Zhang et al., 2004; Pascual et al., 2005), emphasizing the involvement of the SNARE complex for the release of gliotransmitters. It was reported that glutamate release from hippocampal astrocytes, initiated by P2Y1 receptor-mediated Ca^{2+} signals, strengthens synaptic transmission and hence modulates synaptic tuning in neuronal circuits (Jourdain et al., 2007). Uncaging Ca^{2+} or inositoltrisphosphate (IP_3) to evoke a robust cytosolic Ca^{2+} response resulted in the release of glutamate, which affected presynaptic processes, such as the frequency of spontaneous excitatory currents in hippocampal neurons (Fiacco and McCarthy, 2004) or persistent potentiation of hippocampal synapses (Perea and Araque, 2007). Furthermore, Ca^{2+}-mediated release of ATP from astrocytes was shown to be attributable to recycled vesicles (Kreft et al., 2009).

Taken together, these findings suggest that transmitters such as glutamate and ATP can be released from astrocytes in a Ca^{2+}-dependent manner by vesicular exocytosis and possibly also by Ca^{2+}-independent mechanisms. While vesicular and/or lysosomal release of gliotransmitters may be the predominant mechanism employed by astrocytes as a response to cytosolic Ca^{2+} increases, other mechanisms may also mediate transmitter release during certain patho-physiological processes, such as cell swelling, which activates anion channels and therefore allows efflux of glutamate or ATP (Kimelberg et al., 2006), and P2X7 receptor activation by high extracellular ATP levels found during CNS injury (Duan and Neary, 2006).

Hence, present evidence leaves little doubt that glial cells *in situ* not only respond to neuronal activity, but also modulate this activity by the release of gliotransmitters, which act on neighbouring neurons and glial cells (see also chapter by Parpura and Zorec, 2010). Still, studies using *genetic* models with selective G protein activation or suppressed IP_3-mediated signalling failed to find any evidence for an involvement of astrocytic Ca^{2+} signals in glutamatergic transmission between neurons at the Schaffer collateral-CA1 synapse *in situ* (Fiacco et al., 2007; Petravicz et al., 2008). This suggests that calcium-mediated glia-neuron interaction may depend on the experimental situation and may only occur under certain conditions, which still need to be clearly defined.

4. Sodium changes in perisynaptic glia

4.1. *Sodium transients in astrocytes in culture*

As mentioned above, work with ion-selective microelectrodes has established more than 25 years ago that synaptic stimulation of Schaffer Collaterals is accompanied by a decrease in the sodium concentration in the extracellular space of the hippocampus (Dietzel et al., 1982). This decrease is partly due to influx of sodium into activated neurons (Rose and Konnerth, 2001; Rose et al., 1999). In addition, it has recently been shown that excitatory synaptic transmission also evokes transient sodium increases in the mM range in astrocytes' processes close to activated synapses. Earlier experiments had already demonstrated that bath application of glutamate causes a rapid elevation of intracellular sodium in cultured astrocytes (Chatton et al., 2000, Kimelberg et al., 1989, Rose and Ransom, 1996). In such primary cultures, intracellular sodium concentrations are equalized between neighbouring astrocytes by diffusion of sodium through gap junctions (Rose and Ransom, 1997). Moreover, intercellular waves of sodium, propagating with a speed of about 15 μm/s, can be evoked in response to mechanical or direct electrical stimulation of single cells (Bernardinelli et al., 2004). Interestingly, glutamate-evoked cytosolic sodium elevations are also transmitted into mitochondria (Bernardinelli et al., 2006). Mitochondria themselves exhibit spontaneous increases in the sodium concentration (Azarias et al., 2008), indicating that they efficiently sense cellular sodium signals and also dynamically regulate their sodium content.

In cultured astrocytes, cytosolic sodium rises upon exogenous application of glutamate, are primarily generated by activation of sodium-dependent, electrogenic glutamate uptake (Chatton et al., 2000, 2001;Rose and Ransom, 1996). Glial

cells express EAAT1 (GLAST) and EAAT2 (GLT-1) (Anderson and Swanson, 2000; see chapter by Eulenburg and Gomeza, 2010), which exhibit a stoichiometry of 3 Na^+/1 glutamate and are, thus, strongly dependent on the inwardly directed sodium gradient (Barbour et al., 1991; Szatkowski et al., 1990; Zerangue and Kavanaugh, 1996). Pharmacological inhibition with TBOA, a widely used competitive glutamate blocker (Marcaggi and Attwell, 2004; Schousboe et al., 2004; Tzingounis and Wadiche, 2007), results in a maximum reduction of glutamate-induced sodium transients by 70–80% in cultured astrocytes (Chatton et al., 2001). In addition, these signals are partly sensitive to CNQX, indicating that AMPA receptor activation also plays a role (Chatton et al., 2000; Chatton et al., 2001; Rose and Ransom, 1996). In the leech giant glial cell, the glutamate/aspartate-induced rise in cytosolic sodium mediated by the glutamate uptake carrier was counteracted by transport of sodium in the opposite direction via the sodium–bicarbonate cotransporter (NBC). This allowed a higher transport capacity of the glutamate uptake carrier as the dissipation of the sodium gradient following accumulation of intracellular sodium was mellowed by NBC activity (Deitmer and Schneider, 2000). The transport activity of NBC (together with the sodium/hydrogen exchanger, NHE) couples the sodium gradient with the H^+/OH^- gradient across the cell membrane (see chapter on acid/base changes), similar as the Na^+/Ca^{2+} exchange links sodium and calcium gradients across cell membranes.

In contrast to glutamate, the inhibitory transmitter GABA (γ-amino-butyric acid), which is transported into glial cells by the sodium-dependent transporter BGT-1, evokes only modest sodium influx into astrocytes (Chatton et al., 2003). The stoichiometry of GABA transport is presumed to be 2 Na^+/1 GABA (Gadea and Lopez-Colome, 2001), which partly explains its smaller capacity to alter intracellular sodium as compared to glutamate transport.

4.2. *Synaptically-induced sodium signals in astrocytes in situ*

Recently, two reports established the occurrence of activity-induced sodium signals in cell bodies (Kirischuk et al., 2007) and in processes (Bennay et al., 2008) of cerebellar Bergmann glial cells in acute brain slice preparations. Bergmann glial cells tightly enclose synapses of climbing and parallel fibers onto Purkinje cells (Grosche et al., 2002; Grosche et al., 1999). Stimulation of glutamatergic inputs to Purkinje neurons evokes inward currents in adjacent Bergmann cells that result from activation of both AMPA receptors and glutamate transporters GLAST and GLT-1 (e.g., Bergles et al., 1997). Combining somatic whole-cell patch-clamp recordings with quantitative sodium imaging with the sodium-sensitive fluorescent indicator dye SBFI (sodium-binding benzofuran isophthalate), it was shown that short bursts of synaptic activity resulted in glial sodium signals of up to 9 mM in cellular branches of cerebellar Bergmann glial cells that persisted for tens of seconds (Fig. 2A; Bennay et al., 2008). Whereas Bergmann glia sodium transients were partly mediated by AMPA receptor activation, most of the sodium influx was sensitive to blocking glutamate uptake by TBOA (Bennay et al., 2008; Kirischuk et al., 2007).

The spatial pattern of intracellular sodium transients depended on the specific input that was activated. Stimulation of parallel fibers resulted in local sodium transients that were largest in processes close to the stimulation pipette. Climbing fiber stimulation, in contrast, resulted in global intracellular sodium transients with similar kinetics, amplitudes and time courses throughout the entire tree of processes of Bergmann glial cells (Fig. 2A). Thus, the spatial distribution of sodium transients induced by stimulation of the two different inputs, parallel and climbing fibers, followed the profile expected from measurements of calcium transients in the dendritic tree of Purkinje neurons (Eilers et al., 1995a,b). This could point to a capacity of Bergmann glial cells to discriminate the input source.

Locally restricted sodium transients upon synaptic activity were also observed in hippocampal astrocytes *in situ* (Fig. 2B; Langer and Rose, in press). Weak electrical stimulation of Schaffer collaterals evoked sodium transients in astrocytes of the *stratum radiatum* which were confined to one to two primary branches and adjacent fine processes and only weakly invaded the soma. More intense stimulation, in contrast, elicited global signals, i.e., sodium transients throughout the entire cell. These results suggested the existence of microdomains for sodium signalling, in which sodium-dependent processes could be modulated independently. As observed in Bergmann glial cells (Bennay et al., 2008), sodium transients were partly mediated by activation of AMPA receptors, while TBOA-sensitive glutamate transport was the major pathway responsible for sodium influx into hippocampal astrocytes (Langer and Rose, in press).

4.3. *Possible functional consequences of astrocyte sodium signals*

The studies discussed above have established that glutamatergic synaptic transmission results in sodium transients in astrocytes which are mainly mediated by activation of glutamate uptake. The functional consequences of such activity-related sodium signals in perisynaptic glia are still largely unclear. Notwithstanding, intracellular sodium elevations will ultimately result in a reduction in the driving force for sodium-dependent transporters. Thus, sodium transients influence the recovery of calcium transients mediated by Na^+/Ca^{2+} exchange (Golovina et al., 2003; Kirischuk et al., 1997), and of cytosolic pH transients mediated by Na^+/H^+ exchange and/or Na^+–HCO_3^- cotransport (Deitmer, 2004, 2007). Together with an accompanying membrane depolarisation, sodium elevations will also reduce the capacity for glial glutamate uptake (Kelly and Rose, in press; Szatkowski et al., 1990; Zerangue and Kavanaugh, 1996). Even minor elevations in the sodium concentration might promote the release of GABA by transport reversal (Wu et al., 2007). In addition, a recent study has provided evidence that intracellular sodium elevations following prolonged activation of GLT-1 trigger a clustering and a decrease of the surface expression of this transporter (Nakagawa et al., 2008), suggesting a role of sodium ions in the regulation of transporter trafficking.

Another well documented consequence of glial sodium elevations is increased ATP hydrolysis by the Na^+/K^+-ATPase, followed by increased aerobic glycolysis, glucose uptake and glycogen breakdown (Chatton et al., 2000; Magistretti and Chatton, 2005; Pellerin and Magistretti, 1994; Voutsinos-Porche et al., 2003). Interestingly, activation of GABA uptake,

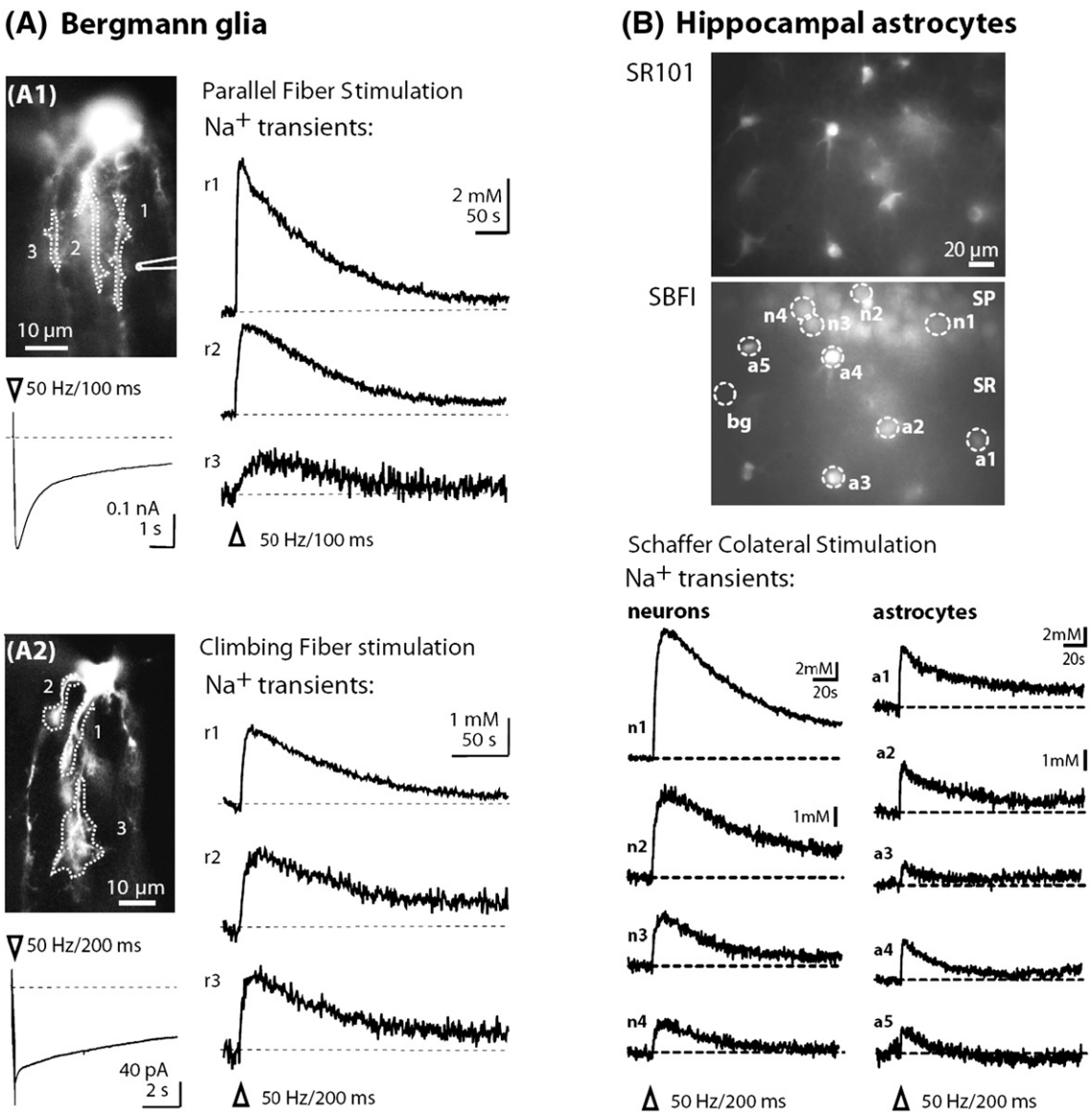

Fig. 2 – Synaptically-induced sodium transients. (A) Na^+ transients in different processes of Bergmann glial cells selectively loaded with the sodium-sensitive fluorescent dye SBFI during parallel versus climbing fibers stimulation. (A1) Na^+ signals following parallel fibers stimulation (50 Hz, 100 ms) were largest in processes that were located close to the stimulation pipette (r1 and r2). Note the change in kinetics of the Na^+ transients with increasing distance from the stimulation pipette. (A2) Na^+ transients following stimulation of climbing fibers (50 Hz, 200 ms) were not localized, but had comparable time courses and kinetics in different processes of a Bergmann glial cell (r1–r3). (B) Na^+ transients in somata of hippocampal neurons and astrocytes loaded with the membrane-permeable form of SBFI. Top: Images of the SR101 and of the SBFI fluorescence taken from the hippocampal CA1 area, double-labeled with SBFI and SR101. Dashed circles indicate the regions of interest analysed in the experiment depicted in *below* (SP: *stratum pyramidale*; SR: *stratum radiatum*). Bottom: Somatic sodium transients in different neurons (n1–n4) and astrocytes (a1–a5) upon short-burst electrical stimulation of afferent Schaffer collaterals as indicated by the arrowheads. Note the different scale for the amplitude of transients of regions n1 and a1 and all other regions. (A taken from Bennay et al., 2008 reprinted with permission, copyright John Wiley & Sons, Inc.; B taken from Langer and Rose, in press).

which is not associated with significant sodium loads, does not evoke a measurable metabolic response (Chatton et al., 2003). Furthermore, it was shown that stimulation of the glucose transporter GLUT1, responsible for glucose uptake into astrocytes, requires the concurrence of both sodium and calcium signals in astrocytes (Loaiza et al., 2003; Porras et al., 2008), suggesting an interaction between these two ions and glucose utilization (see chapter by Barros and Deitmer, 2010). The finding that long-lasting sodium signals are present in astrocytes following neuronal release of glutamate supports the proposed tight link between excitatory neuronal activity, glutamate transport, sodium homeostasis, pH and glucose utilization by astrocytes (Pellerin and Magistretti, 1994; Tsacopoulos and Magistretti, 1996).

5. Acid/base changes in perisynaptic glia

5.1. *The distribution of acid and base in the brain*

The intracellular pH (pH_i) of neurons and glial cells is actively maintained at a value between 7.0 and 7.4, which is similar to the extracellular pH (pH_o) in nervous tissue (7.1–7.3). This implies a free H^+ concentration of 40–100 nM in both the extracellular space and in the cytosol and results in reversal potentials around 0 mV for the movement of protons across the plasma membrane. While there is, consequently, only little chemical gradient for H^+ for "resting" neurons and glial cells, the negative cellular membrane potentials generate an electrical driving force for H^+ from outside to inside. Any pathway that allows the diffusion or mediates the transport of H^+ and/or related base equivalents such as HCO_3^-, will therefore result in a gain of acid or loss of base, and will hence promote an intracellular acidification.

Neurons are the main consumers of ATP and consequently the main producers of CO_2 (Attwell and Laughlin, 2001). CO_2 production and pH are tightly linked through the CO_2/HCO_3 buffer system, which plays a major role for the maintenance of both extra- and intracellular pH. The reversible conversion of CO_2 and H^+/HCO_3^- is catalysed by the carbonic anhydrase (CA), which hence contributes to the shaping of pH transients. There are several isoforms of CA in the nervous system, intracellular isoforms, such as CAII, and membrane-associated isoforms with their catalytic centre at the extracellular face, such as CAIV and CAXIV (Obara et al., 2008). CA can also enhance acid/base transport: some CA isoforms directly bind to a acid/base-coupled transporters, such as AE, NBC, NHE and MCT1 (Vince and Reithmeier, 1998, 2000; Li et al., 2002; Alvarez et al., 2003; Becker et al., 2005; Becker and Deitmer, 2007, 2008).

Metabolically generated acid must be actively extruded by the cells against the electrochemical H^+-gradient, which requires energy. In most cells, this energy is provided by the Na^+ gradient, which drives the sodium/hydrogen exchanger (NHE) and other acid/base transport systems (Fig. 3). The different isoforms of the NHE are the most prominent and ubiquitous transport proteins for acid extrusion in animal cells, and their malfunction is associated with a variety of diseases; these include general disturbance of acid/base status as well as a role in cancer, hypertension, and diabetes (Harguindey et al., 2005; Bobulescu et al., 2005; Orlowski and Gristein, 2004). The NHE helps the cytoplasm to maintain a more alkaline pH than the acid/base electrochemical equilibrium across the plasma membrane, which can be calculated to be around 6.4–6.8 in most animal cells. When cells become alkaline beyond their steady-state pH_i, base equivalents are extruded mainly by the Cl^-/HCO_3^- exchanger. If there is a predominant flux of acid or base into cells, then the extracellular pH shifts will be approximate mirror images of intracellular pH changes. Interestingly, net acid flux across glial and neuronal membranes can occur in opposite directions (Fig. 3). Thus, during neural activity, a cytosolic alkalization of glial cells, a cytosolic acidification of neurons, and an interstitial alkalization may occur simultaneously.

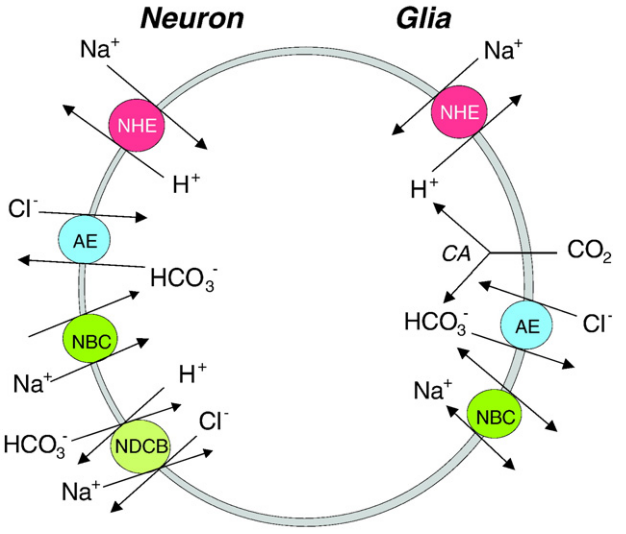

Fig. 3 – Acid/base transport in neurons and glial cells. Membrane transporters in neurons and glial cells which carry acid or base equivalents across the plasma membrane and which have been suggested to be involved in intracellular pH regulation. The sodium–proton exchanger (NHE) is expressed in both neurons and glial cells. An electrogenic, reversible Na^+-bicarbonate cotransporter (NBC) has been found in most types of glial, while neurons can be equipped with an electroneutral NBC and/or Na^+-dependent Cl^-/HCO_3^- exchanger (NDCB), which would carry acid equivalents out of the cell, and/or base equivalents into the cells. The Cl^-/HCO_3^- anion exchanger (AE), presumably present in most neurons, is activated by intracellular alkalinization; its presence in glial cells is not yet established. Higher carbonic anhydrase (CA) activity has been reported for glial cells as compared to neurons, which accelerates the equilibrium of the reversible conversion of CO_2 to H^+ and HCO_3^-, and which may render glial cells a sink for CO_2.

5.2. *pH regulation in glial cells*

While NHE is the major acid extruder from both glial cells and neurons, glial cells express another key pH regulating plasma membrane transporter, the sodium-bicarbonate cotransporter (NBC; Fig. 3). The NBC has been described in nearly all types of macroglial cells, including astrocytes, oligodendrocytes, Schwann cells and retinal Müller glial cells (cf. Deitmer and Rose, 1996; Deitmer and Chesler, 2009). In contrast to the NHE, the NBC is reversible and can shuttle base equivalents in both directions across the glial cell membrane (Deitmer, 1991; Brune et al., 1994). Thus, it will not only influence (and regulate) intraglial pH, but also pH_o. The glial NBC is electrogenic and cotransports 1 Na^+ with 2 HCO_3^- (Deitmer and Schlue, 1989; Deitmer and Schneider, 1995; Bevensee et al., 2000). When CO_2/HCO_3^- is introduced, e.g., when changing from a non-bicarbonate, HEPES-buffered saline to a CO_2/HCO_3^- buffered saline, NBC is activated in the inward direction, leading to a membrane hyperpolarization, an intracellular alkalinization and a rise in Na_i^+ (Deitmer, 1992a,b; Rose and Ransom, 1996).

The cotransporter could also be reversed in retinal Müller glial cells (Newman, 1991), i.e., operating inwardly *and*

outwardly, depending on the thermodynamic conditions. This is also reflected by the pH_i changes induced by slow voltage steps in voltage-clamped leech giant neuropile glial cells. De- and hyperpolarizing voltage steps resulted in an intraglial alkalinization and acidification, respectively, providing an extrapolated change of one pH unit/110 mV membrane potential change, supporting a stoichiometry of 2 HCO_3^-:1 Na^+ (Deitmer and Schneider, 1995). The cotransporter appears to have a remarkably high affinity for HCO_3^-, since it was found to be active even in the nominal absence of CO_2/HCO_3^-, when the extracellular bicarbonate concentration is usually less than 0.3 mM due to dissolved air-CO_2 (Deitmer and Schneider, 1998). Expressed in frog oocytes, the NBC significantly contributes to the apparent cytosolic buffer capacity (Becker and Deitmer, 2004) and may thus enhance the efficacy of other acid/base transport systems. This is supported by the binding of CA to the NBC, rendering the transport activity of the NBC significantly more efficient by forming a 'transport metabolon' with CA (Alvarez et al., 2003). The NBC from rat brain has recently been cloned, and expressed in frog oocytes (Giffard et al., 2000). *In situ* hybridization revealed NBC mRNA expression throughout the rat central nervous system, with particularly high levels in the olfactory bulb, the hippocampus dentate gyrus, and the cerebellum (Bevensee et al., 2000; Schmitt et al., 2000).

Glial cells have been ascribed a special role for acid/base regulation in the brain, in particular of the extracellular spaces (Deitmer and Rose, 1996; Chesler and Kaila, 1992; Chesler, 2003; Deitmer, 2004). On the one hand, extracellular pH is influenced by the acid/base-coupled plasma membrane transporters, which are, however, not only regulated by pH itself, but also by concentration gradients of other ions involved (often sodium), as well as by metabolites transported by these carrier systems. On the other hand, extracellular pH shifts are not necessarily be suppressed by active pH regulation but may be part of H^+ signalling (Deitmer and Rose, 1996; Deitmer, 2000, 2002). Thus, acid/base-coupled transporters modulate each other (Becker et al., 2004; Wendel et al., 2008), when activated together. Export of H^+ and lactate from glial cells via the monocarboxylate transporter 1 (MCT1), e.g., would thus activate the sodium-bicarbonate cotransporter (NBC), resulting in a net loss of Na^+ and lactate from the glial cell.

5.3. *pH changes induced by neuronal activity*

Neuronal activity leads to defined changes of intra- and extracellular pH in central nervous systems of both vertebrates and invertebrates (Deitmer and Rose, 1996). These consist of mono- or multiphasic pH shifts, indicating that they originate from multiple sources and/or via multiple processes. Activation of neurons usually results in an intracellular acidification. The loss of potassium from activated neurons as well as direct binding of synaptically released neurotransmitters may induce a substantial depolarization of astrocytes. In the rat cortex, stimulus-evoked glial depolarization is accompanied by an intracellular alkalinization, the amplitude of which is dependent on the extent of the depolarization (Chesler and Kraig, 1989). In parallel to the intraglial alkalinization, the extracellular space acidifies. Interestingly, while the intraglial pH shift was unaffected by the carbonic anhydrase (CA) inhibitor ethoxyzolamide, the extracellular pH transient was converted or enhanced to a large alkaline transient following inhibition of CA activity (Chen and Chesler, 1992; Rose and Deitmer, 1995b). This suggests that these pH_o changes are greatly affected by the activity of extracellular and/or intracellular CA.

Several lines of evidence suggest that the depolarization-induced alkalinization of glial cells in both vertebrate and invertebrate preparations is due to inward transport of bicarbonate via the electrogenic Na^+–HCO_3^- cotransporter (NBC), activated by the K^+-induced membrane depolarization (Deitmer and Szatkowski, 1990; Grichtenko and Chesler, 1994; Pappas and Ransom, 1994; Bevensee et al., 1997). In the cortex, the glial alkaline shift was partly inhibited in Na^+-free saline and turned into a small acidification when the K^+-induced depolarization was reduced by the application of Ba^{2+} (Chesler and Kraig, 1989; Grichtenko and Chesler, 1994). The stimulus-induced alkalinization of the leech giant glial cell was turned into an acidification by all experimental protocols suppressing the activation of the NBC: (1) by voltage-clamping the glial cell (Rose and Deitmer, 1994), (2) in the presence of the stilbene, DIDS and (3) in CO_2, HCO_3^- free saline (Rose and Deitmer, 1995a,b). Suppressing the glial depolarization during nerve root stimulation not only reversed the intraglial pH change, but also uncovered an alkaline pH_o transient, which preceded the extracellular acidification (Rose and Deitmer, 1994).

A significant acidosis likely occurs in the synaptic cleft during the release of acid vesicles containing a given neurotransmitter. Neurotransmitters are accumulated in presynaptic vesicles using a vesicular proton gradient, which itself is created by a ATP-consuming proton pump (Chaudhry et al., 2008; Marshansky and Futai, 2008). Using pH-sensitive mutants of green fluorescent protein linked to a vesicular membrane protein, a pH of 5.7 and 5.2 was determined in synaptic vesicles and secretory granules, respectively (Miesenböck et al., 1998). Confocal imaging of the pH-dependent fluorescence from the immobile extracellular SNARF dye showed transient acidification around the cell bodies and neurites of activated PC12 cells. The local acidification was abolished when extracellular solution was devoid of Ca^{2+} or pH buffering was increased (Shuba et al., 2008). Thus, protons are co-released with neurotransmitters, and may target both postsynaptic and perisynaptic glial sites. The acid-sensing ion channel ASIC1a has been suggested to function as a postsynaptic proton receptor that influences intracellular Ca^{2+} concentration and CaMKII phosphorylation and thereby the density of dendritic spines (Zha et al., 2006).

Activity-induced pH transients can also arise following activation of ligand-gated ionotropic receptors (Chesler and Kaila, 1992; Munsch and Deitmer, 1994). The majority of available data relates to pH changes induced by GABA and glutamate, while the effects of other transmitters on pH_o or pH_i in the nervous system have been investigated in only a few systems. It has become clear, however, that complex interactions between transmitter systems exist that effect both intra- and extracellular pH. Substantial pH shifts are also generated by transmitter and metabolite carriers, which are activated in response to transmitter release and contribute to acid/base fluxes (see below).

5.4. *Consequences of extra- and intracellular pH transients*

Active maintenance of the cytosolic pH is required for the efficient operation of many enzymatic processes in cells. Most of these processes and reactions have their optimum at a pH of 7.0 or higher. Changes in pH_i can therefore critically modulate biochemical processes and cellular activity. Moreover, there is a variety of pH-sensitive voltage- and ligand-gated ion channels. Protons can either block these channels, as is the case for NMDA-sensitive, ionotropic glutamate receptors, or they may modulate gating, inactivation and/or deactivation, as found for some voltage-dependent channels. Consequently, changes in intra- and extracellular pH can modulate the excitability of neurons and will do so with the temporal dynamics of these pH changes. Thus, H^+ may serve as signalling molecules, both in the cytosol and in the extracellular spaces.

Often, pH-dependent processes are linked to, or might induce, a cascade of H^+-induced signals in nervous systems. For example, pH modulation of gap junctions might result in a change in synchronous activity in neurons. In glial cells, transmitter release through hemi-channels in the glial cell membrane, as suggested for ATP and glutamate (Cotrina et al., 2000; Ye et al., 2003) may be pH dependent. In addition, pH-dependent Cl^- regulation via the chloride–bicarbonate anion exchanger (AE) in neurons and glial cells, and HCO_3^--permeable receptor channels activated by GABA or glycine, affects synaptic inhibition in nervous systems (Kaila, 1994). In addition, pH changes are often accompanied by changes in calcium, sodium and other ions, by coupling with one of these ions on carriers.

Some pathological processes are related to the impairment or the failure of acid–base regulation in the brain; in particular, hypoxia, anoxia, glucose deprivation and conditions associated with ischemia, can severely interfere with the normal acid–base homeostasis in the brain as in other tissues. Alkalosis in nervous tissue can contribute to the hyperexcitability of neurons and hence trigger epileptiform activity in the brain. Oxidative stress, e.g., decreases the activity of NHE and hence the intracellular pH as shown in rat solitary complex neurons. Hyperosmotic changes in the brain, which lead to shrinkage of cells, can result in an intracellular alkalinization due to activation of NHE. Changes in cellular and tissue pH are also associated with hyperammonemia: increased ammonia/ammonium concentrations caused by liver failure or urea cycle enzyme deficiencies, may lead to brain edema and hepatic encephalopathy (Häussinger and Schliers, 2008).

Extracellular H^+ can also gate channels, such as acid-sensing ion channels (ASICs). Blocking these ASICs or deleting the ASIC1a gene protects the brain from ischemic injury, suggesting that ASICs are responsible for glutamate-independent, acidosis-mediated brain injury during ischemia (Xiong et al., 2004). Thus, acidosis can injure the brain via membrane receptor-mediated mechanisms with subsequent Ca^{2+} overload. Extracellular acidosis, however, has also been associated with neuroprotective mechanisms. It may contribute to the survival of neurons during ischemia, by turning down excitatory voltage- and ligand-gated membrane channels, such as Na^+ and Ca^{2+} channels, NMDA-sensitive glutamate channels and other ionotropic receptor channels.

5.5. *Acid/base-coupled metabolite and neurotransmitter transporters*

Protons and bicarbonate are coupled to a variety of metabolite carrier systems in glial cells and neurons. They are either inorganic ion transporters, such as NHE, AE or NBC, which are related to intra- and extracellular pH regulation (see above), or they might be employed to drive acid/base-coupled metabolite transporters, such as the monocarboxylate transporter (MCT) or some amino acid transporters. In addition, the coupling of H^+, OH^- and/or HCO_3^- renders the pH a regulator of these membrane transporters (Becker and Deitmer, 2004). Indeed, though the Na^+ gradient plays the dominant role for many carrier systems in animal cells, some transporters are driven by, or coupled to, the H^+/HCO_3^- gradient. The MCT proteins belong to a family of transporters including multiple isoforms, which carry monocarboxylate anions, such as lactate, pyruvate, acetoacetate and ketone bodies, in cotransport with a proton in an electroneutral manner across the cell membrane. The activity of MCT isoforms 1 and 4 in astrocytes can result in the release of lactate or other monocarboxylates into the extracellular spaces. According to the lactate shuttle hypothesis, the monocarboxylic acids are then taken up by neurons via the neuronal MCT isoform 2, converted to pyruvate and consumed to generate ATP (Magistretti et al., 1999; Deitmer, 2002; Aubert et al., 2005). In particular in synaptic domains, where energy requirements are high, glial lactate may substitute or complement the supply of glucose in the neuronal synaptic elements (Schurr et al., 1988; Schurr, 2008; Rouach et al., 2008; see also chapter by Barros and Deitmer, 2010).

6. Conclusions

Ionic gradients and changes in neurons, astrocytes and extracellular spaces are essential elements in processes initiating, accompanying and following synaptic transmission. As in neurons, these ion changes in astrocytes are partly homeostatic, i.e., involved in the regulation and maintenance of ionic gradients, and are partly involved in signalling themselves, initiating subsequent cellular processes and functions. In particular, synaptically-evoked calcium transients in perisynaptic astrocytes are viewed as key signals in the glial modulation of synaptic properties, e.g., by the modified uptake of neurotransmitters and/or the release of gliotransmitters. Sodium loading of astrocytes during sodium-dependent uptake of neurotransmitters may challenge metabolic responses and sodium-dependent transport systems and was suggested to mediate the coupling between neuronal activity and glial metabolism. Acid transients in the synaptic cleft following release of synaptic vesicles as well as the activation of acid/base-coupled transporters in neuronal and glial cell membranes will modulate pH-dependent processes related to synaptic transmission and electrical excitation. Thus, ion gradients across cell membranes are energetic wheels for both homeostatic processes and signalling pathways in synaptic and perisynaptic domains.

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BRAIN RESEARCH REVIEWS

Review

Neuron-glia synapses in the brain

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$GABA_A$ receptor

ABSTRACT

The ability to investigate the electrophysiological properties of individual cells in acute brain tissue led to the discovery that many glial cells have the capacity to respond rapidly to neuronal activity. In particular, a distinct class of neuroglial cells known as NG2 cells, which exhibit many of the properties that have been described for glial subtypes such as complex cells, polydendrocytes, synantocytes and GluR cells, express ionotropic receptors for glutamate and GABA. In both gray and white matter, NG2 cells form direct synaptic junctions with axons, which enable transient activation of these receptors. Electrophysiological analyses have shown that these neuron-glia synapses exhibit all the hallmarks of 'classical' neuron-neuron synapses, including rapid activation, quantized responses, facilitation and depression, and presynaptic inhibition. Electron microscopy indicates that axons form morphologically distinct junctions at discrete sites along processes of NG2 cells, suggesting that NG2 cells are an overt target of axonal projections. AMPA receptors expressed by NG2 cells exhibit varying degrees of Ca^{2+} permeability, depending on the brain region and stage of development, and in white matter NG2 cells have also been shown to express functional NMDA receptors. Ca^{2+} influx through AMPA receptors following repetitive stimulation can trigger long term potentiation of synaptic currents in NG2 cells. The expression of receptors with significant Ca^{2+} permeability may increase the susceptibility of NG2 cells to excitotoxic injury. Future studies using transgenic mice in which expression of receptors can be manipulated selectively in NG2 cells have to define the functions of this enigmatic neuron-glia signaling in the normal and diseased CNS.

Contents

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Abbreviations: EGFP, enhanced green fluorescent protein; EM, electron micrograph; $[Cl^-]_i$, intracellular Cl^- concentration; OPC, oligodendrocyte precursor cell; VGLUT, vesicular glutamate transporter

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1. Introduction

Ionotropic neurotransmitter receptors play well-defined roles in neurons—they regulate excitability by allowing current flow and induce structural and functional plasticity by enabling Ca^{2+} influx. Despite the abundant evidence that these receptors are expressed by all major classes of glia, it is less clear what functions these receptors subserve in these diverse cell types. We are only beginning to understand the context under which these receptors are activated, what signals they produce and what consequences these responses have for glia and their environment. This is particularly true for a newly recognized distinct class of glial cells known as NG2 cells, as the roles of these abundant, widely distributed glial cells in the adult CNS remain largely undefined.

The application of the patch-clamp technique to cells in acute brain slices in the early 1990s revealed that glial cells display a much greater physiological diversity then anticipated from previous electrophysiological analyses using "blind" recording with sharp microelectrodes or by analysis of glia in cell culture (Berger et al., 1991; Steinhäuser et al., 1992). Based on their distinct whole-cell current patterns, the terms "complex" and "passive" cells were introduced to qualitatively distinguish glial subtypes in grey matter (Steinhäuser et al., 1994). At this time, passive cells were classified as *bona fide* astrocytes because they expressed GFAP, exhibited dye coupling and possessed glutamate and GABA transporters. However, the identity of the complex cells remained nebulous. The observation that the resting conductance of complex cells appeared to increase during postnatal maturation, and the finding that they typically lacked GFAP, but often expressed S100β, led to the conclusion that complex cells represented immature astrocytes (Bordey and Sontheimer, 1997; Kressin et al., 1995). Subsequent analyses of transgenic mice in which enhanced green fluorescent protein EGFP) is expressed under control of the GFAP promoter suggested that this assumption was incorrect, revealing that the hippocampus contained a distinct group of glial cells that shared some properties with astrocytes. Specifically, these and other studies provided evidence that the vast majority of cells with complex current patterns, which were later termed GluR cells because they express ionotropic glutamate receptors, do not represent immature astrocytes (Matthias et al., 2003; Wallraff et al., 2004), and are also found in the adult brain (Zhou et al., 2006).

In 2000, Bergles and colleagues demonstrated the existence of functional neuron-glia synapses in the brain (Bergles et al., 2000). The authors regarded the postsynaptic glia as oligodendrocyte precursor cells (OPCs), as they exhibited immunoreactivity to NG2, a chondroitin sulphate proteoglycan expressed by oligodendrocyte progenitors. These innervated glial cells shared many properties with GluR cells, including the expression of AMPA and $GABA_A$ receptors, lack of dye coupling and absence of glutamate transporters. Others have termed cells with similar morphological and functional properties synantocytes (Butt et al., 2002) or polydendrocytes (Nishiyama et al., 2002), as lineage tracing experiments suggest that not all NG2 cells develop into oligodendrocytes. There is now an emerging consensus in the field that (i) NG2 cells and GluR/complex cells represent overlapping populations of glia (although the degree of overlap is still unclear) and (ii) that NG2 cells represent a fourth class of neuroglial cells in the adult CNS (Nishiyama et al., 2009; Peters, 2004). Here, we will refer to these distinct glial cells as NG2 cells, although unpublished studies suggest the existence of complex cells that do not express this protein (Jenniβen and Steinhäuser, unpublished observation). In this review, we discuss the morphological and functional properties of NG2 cells, with a special emphasis on their intriguing capability to form synapses with neurons.

2. Receptor expression by complex cells/NG2 glia

2.1. *Ionotropic glutamate receptors*

Earlier findings using acute hippocampal slices suggested expression of Ca^{2+} permeable kainate receptors by complex cells (Jabs et al., 1994; Steinhäuser et al., 1994). However indirect effects, through activation of neighbouring cells, could not be excluded in these and several subsequent *in situ* experiments. Under more defined experimental conditions, in which whole-cell patch-clamp recording of freshly isolated cells was combined with single-cell transcript analyses, data indicated that in the hippocampus these cells express functional AMPA receptors, but not kainate or NMDA receptors. The AMPA receptors expressed by these glial cells displayed an intermediate Ca^{2+} permeability which was conserved during postnatal development, although the receptor composition varied early after birth (Seifert et al., 1997b; Seifert and Steinhäuser, 1995). By postnatal day 5, a mosaic of Ca^{2+}-permeable and less Ca^{2+}-permeable AMPA receptors coexist in individual complex cells, while receptors with a more uniform, low divalent cationic permeability dominate in older cells (Seifert et al., 2003). In juvenile mouse hippocampus, the most thoroughly investigated brain region, these cells predominantly express the AMPA receptor subunits GluR2 and GluR4, while GluR3 is least abundant (Seifert et al., 1997a). Furthermore, combined pharmacological and molecular analyses demonstrated enhanced relative expression of GluR2 flip

splice versions of the receptors and decreased inward rectification of AMPA receptor currents during early postnatal maturation of the hippocampus (Seifert et al., 2003). Similar properties have been reported for complex cells in human hippocampus where an alteration in GluR1 splicing was identified in epilepsy patients presenting with hippocampal sclerosis (Seifert et al., 2004). Together, these studies indicate that NG2 cells express a complex repertoire of AMPA receptor subunits that assemble to form glutamate receptors that exhibit rapid kinetics and an intermediate Ca^{2+} permeability.

Physiological studies indicate that Ca^{2+} permeable AMPA receptors are also present in NG2 cells of the cerebellum (Lin et al., 2005), which show even stronger inward rectification than in the hippocampus. In the corpus callosum, an opposite age-dependence of rectification of AMPA receptors has been found compared to that observed in the hippocampus: the current-to-voltage relationship of the responses shifted from linear at young ages to inwardly rectifying in adults (Ziskin et al., 2007). As inward rectification is an indication of AMPA receptors assembled without the GluR2 subunit, these findings suggest that there are age-and region-specific differences in Ca^{2+} permeability of AMPA receptors at NG2 cell synapses (cf. 3.2).

The Ca^{2+} permeability of AMPA receptors in NG2 cells could enable activation of intracellular signalling cascades in response to axonal firing. In the hippocampus, these receptors were reported to mediate long-term potentiation of neuron-NG2 cell synapses and to affect AMPA receptor trafficking (Ge et al., 2006) (cf. 3.2). However, whether synaptic activation of these receptors induces glial Ca^{2+} transients, which might potentially lead to the release of signalling molecules from NG2 cells, remains unknown. Sustained activation of glial AMPA receptors can lead to inhibition of Kir channels, due to receptor-mediated influx of Na^+, which occludes the channels from the intracellular side (Schröder et al., 2002). This transient inhibition of I_{Kir} can combine with the cationic receptor current to boost depolarization of the cell during glutamate exposure.

2.2. *Ionotropic GABA receptors*

The analysis of GABA receptors in NG2 cells has attracted less attention. Several years ago slice recordings qualitatively identified functional GABA receptors in complex glial cells of the hippocampus and spinal cord (Bekar et al., 1999; Pastor et al., 1995; Steinhäuser et al., 1994). In addition to activation of a Cl^- conductance, these reports provided evidence of a block of K^+ outward currents upon GABA application, with the mechanism linking receptor activation and K^+ channel inhibition remaining unknown. Recent findings indicate that in hippocampus, activation of $GABA_A$ receptors in NG2 cells leads to depolarization, due to the relatively high glial intracellular Cl^- concentration ($[Cl^-]_i$) maintained by these cells (Lin and Bergles, 2004). Interestingly, by increasing the membrane conductance and by altering $[Cl^-]_i$, GABA application concomitantly decreased AMPA receptor currents, which may serve to fine tune the effects of these two excitatory inputs. Recently, Tong et al. (2009) described that $GABA_A$ receptor induced depolarization may increase $[Ca^{2+}]_i$ in hippocampal NG2 cells. This process seems to require voltage gated Na^+ channels and was mediated by activation of the Na^+/Ca^{2+} exchanger, operating in the reversed mode. The subunit composition of $GABA_A$ receptors in NG2 cells is still largely unknown, although the slow kinetics and pharmacological properties of GABA responses in hippocampal NG2 cells suggest that these receptors contain the α5 subunit (Lin and Bergles, 2004). Whether NG2 cells in the corpus callosum possess $GABA_A$ receptors is unclear, as neither spontaneous or evoked GABA receptor mediated responses have been reported (Kukley et al., 2007; Ziskin et al., 2007).

3. Neuron-glia synaptic signalling

3.1. *Evidence for direct synapses*

It was generally assumed that receptors in glial cells are present to detect increases in ambient transmitter levels, such as those induced during periods of intense activity or trauma, or indirectly through spillover of transmitter from neuronal synapses, in cases where glial membranes are positioned adjacent to the synaptic cleft. While such mechanisms may contribute to receptor activation in cells such as astroglia (astrocytes and Bergmann glia) (Bergles et al., 1997; Clark and Barbour, 1997) that extend fine lamellae that surround synapses, biophysical studies of transmitter dynamics at excitatory synapses indicate that glutamate levels decline rapidly through diffusion and dilution after fusion of transmitter-laden vesicles at synapses containing single active zones (Clements et al., 1992; Diamond and Jahr, 1997), suggesting that activation of low-affinity AMPA receptors would be minimal in NG2 cells if the source of glutamate was from neighbouring synapses. Indeed, AMPA receptors are more likely to be desensitized by chronic exposure to glutamate than activated (Trussell and Fischbach, 1989). Nevertheless, whole-cell recordings from NG2 cells in hippocampal slices isolated acutely from rodent brain revealed that both AMPA and $GABA_A$ receptors are subject to transient rather than tonic activation in these cells (Fig. 1) (Bergles et al., 2000; Jabs et al., 2005; Lin and Bergles, 2004). The duration of these brief receptor-mediated currents induced by stimulation of Schaffer collateral-commissural axons was comparable to those produced at neuronal synapses through vesicular fusion, reaching a peak in the case of AMPA receptor currents in a few hundred microseconds and decaying with a time constant of slightly more than one millisecond at room temperature (Fig. 1). As the rate of receptor activation is dependent on ligand concentration, the rapid activation of these low affinity receptors suggests that they were exposed to a high concentration of glutamate. The only mechanism that has been described to produce such transients in brain is through fusion of vesicles loaded with glutamate with the plasma membrane. Subsequent studies have shown that these glial responses exhibit all the hallmarks of events produced at synapses. They occur with minimal delay after action potentials are triggered in surrounding axons; they can be reliably evoked by a single action potential; spontaneous events are visible when action potentials are prevented with tetrodotoxin; the smallest evoked responses have time courses similar to the spontaneous events; they facilitate in response to repetitive stimulation; and event amplitudes

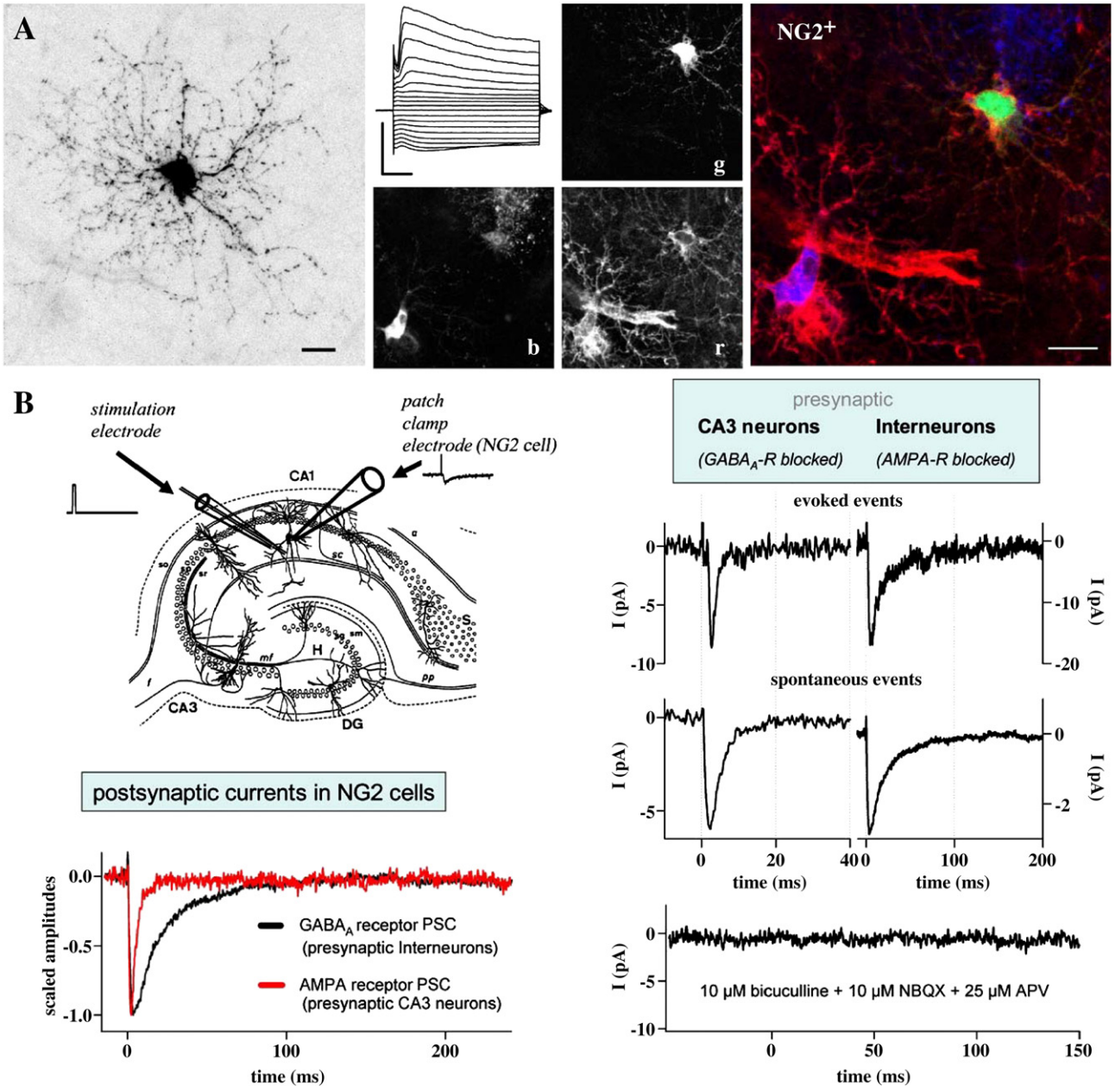

Fig. 1 – Properties of synaptic inputs to NG2 cells in acute slices of hippocampus from juvenile transgenic hGFAP/EGFP mice. (A) The morphology of NG2 cells was visualized by Texas Red dextran-filling during whole-cell recording. Subsequent confocal analysis and 2D maximum projection shows the extensive arborization of their processes. Note the typical nodules appearing as dots all along the fine processes (bar 10 µm). A typical current pattern is given in the middle panel. Current responses were evoked by de-and hyperpolarizing the membrane between +20 and −160 mV (holding potential −80 mV, 10 mV steps, bars 1 nA, 10 ms). Post-recording immunostaining and triple fluorescence confocal analysis were applied to test for NG2 immunoreactivity. The middle panel shows three separated colour channels of one confocal plane. Texas Red dextran labelling is given in green (g), NG2 immunoreactivity in red (r), and EGFP expression in blue (b). The superimposed RGB picture (right) shows the membrane-associated distribution of NG2 immunoreactivity of the recorded cell (yellow details). (B) Postsynaptic currents were recorded from an NG2 cell upon short single pulse stimulation. At least two types of presynaptic neurons, glutamatergic CA3 pyramidal neurons and GABAergic interneurons, innervate hippocampal NG2 cells. Corresponding glial postsynaptic currents can be distinguished by their current kinetics and their pharmacological profiles. Modified from Jabs et al. (2005).

fluctuate from trial to trial, as expected from the stochastic nature of vesicle release (Fig. 1) (Paukert and Bergles, 2006). Moreover, AMPA receptor currents can be induced repeatedly for more than one hour with repetitive stimulation (Ziskin et al., 2007), indicating that such signalling is likely to take place at stable junctions where terminals possess all the machinery necessary to release, recycle and refill vesicles with glutamate.

Evoked and spontaneous AMPA receptor-mediated currents with characteristics similar to those observed in the hippocampus also have been recorded from NG2 cells in other grey matter regions, such as the cerebellum (Lin et al., 2005), cortex (Chittajallu et al., 2004) and brainstem (Muller et al., 2009), as well in white matter regions such as the corpus callosum (Kukley et al., 2007; Ziskin et al., 2007) and cerebellar white matter (Karadottir et al., 2005) that are largely devoid of neuronal dendrites. These studies suggest that synaptic signalling is a conserved property of this class of glia, and that this form of rapid neuron-glia communication is widespread in the brain. Inputs to these cells arise from axons that innervate neighbouring neurons; that is, they do not appear to be the target of a unique set of axons. For example, NG2 cells form synapses with Schaffer collateral axons of CA3 neurons in the CA1 region of the hippocampus (Bergles et al., 2000), with granule cells in the dentate gyrus (Mangin et al., 2008), with climbing fibres and parallel fibres in the molecular layer of the cerebellum (Lin et al., 2005), with axons of globular bushy cells of the cochlear nucleus in the medial nucleus of the trapezoid body (Muller et al., 2009), and with axons of cortical neurons in the corpus callosum (Ziskin et al., 2007). Although it is possible that these cells are influenced by long-range neuromodulatory projections (Papay et al., 2004; Papay et al., 2006), the predominant input, in terms of their ability to induce transmembrane ion flow, arises from projections that are not exclusive to NG2 cells. Indeed, paired recordings from Purkinje cells and NG2 cells in the cerebellum demonstrated that the same climbing fibres that innervate Purkinje cells also make synapses with NG2 cells (Lin et al., 2005), and ultrastructural analysis of NG2 cell processes in the hippocampus revealed single boutons that formed both axo-dendritic synapses and axon-NG2 cell synaptic junctions (Bergles et al., 2000).

What governs the formation of synapses with NG2 cells? Developmental studies indicate that synaptic inputs can be first recorded from these glial cells during the first week of life in rats and mice, and NG2 cells in mature tissue exhibit even more robust synaptic activity (Bergles et al., 2000), indicating that innervation of these cells closely parallels the maturation of synaptic connections between surrounding neurons. Given the small size and symmetrical shape of NG2 cells (Fig. 1), it is possible that these cells simply form synaptic junctions with a random selection of axons in their vicinity. Indeed, paired recordings from interneurons in the dentate gyrus and nearby "satellite" NG2 cells revealed that cells located within 200 μm typically received glutamatergic input from the same presynaptic neurons (Mangin et al., 2008). The density of neuron-NG2 cell synapses seems to vary considerably among brain regions. Hippocampal NG2 cells bear only a few synapses (7–19 per cell; Bergles et al., 2000). However, in the cerebellum (Lin et al., 2005) and in white matter (Kukley et al., 2007; Ziskin et al., 2007) single NG2 cells may form hundreds of synaptic contacts. It is unclear whether neurons select particular NG2 cells with which to form synapses, or vice versa. At present, the molecular mechanisms that guide the formation of glutamatergic and GABAergic synapses with NG2 cells have not been determined.

One of the most unexpected findings regarding glutamate receptor signalling in NG2 cells is that axon-glial synapses are also found in white matter. NG2 cells are slightly more abundant in white matter (Dawson et al., 2003) such as the corpus callosum, where their processes extend parallel to axons. Measurements of the conduction velocity of axons that form synapses with NG2 cells in the corpus callosum indicate that they preferentially receive input from unmyelinated axons (Kukley et al., 2007; Ziskin et al., 2007). As unmyelinated axons make up less than 30% of axons in the corpus callosum, it suggests that this pattern is unlikely to arise from random association. The specificity of these connections is surprising, as several anatomical studies have suggested that NG2 cells contact nodes of Ranvier along myelinated axons (Butt et al., 1999; Huang et al., 2005), where presumably glutamate could also be released. If NG2 cells initiate the formation of these junctions, the larger exposed area afforded by unmyelinated axons may increase the likelihood of synapse formation.

3.2. *Diversity among NG2 cell synapses*

Glutamatergic synaptic currents are remarkably similar among NG2 cells in different brain regions, exhibiting small amplitudes and rapid kinetics (Bergles et al., 2000; Chittajallu et al., 2004; Karram et al., 2008), and both evoked and spontaneous glutamatergic events are blocked by selective AMPA receptor antagonists (Fig. 1). Despite physiological evidence for expression of NMDA receptors by NG2 cells in white matter (Karadottir et al., 2005; Ziskin et al., 2007), there is little evidence for the contribution of NMDA receptors to synaptic responses in either white matter or gray matter. The inability of vesicular release of glutamate to reliably engage NMDA receptors at these neuron-glia junctions may indicate that these receptors are located primarily extrasynaptically; however, the summed NMDA receptor response induced by activation of receptors over the entire cell in response to exogenous agonists is very small in NG2 cells when compared to NMDA receptor mediated currents in neurons, suggesting that the density of these receptors is very low. Thus, it is possible that the summed response from a restricted group of synapses may simply fail to exceed detection threshold. This aspect of NG2 cell synaptic function may vary significantly between brain regions, as NMDA receptor-mediated currents have not been described in NG2 cells in gray matter and complex cells in the hippocampus lack NMDA receptor transcripts (see Section 2.1). Future studies will be necessary to determine the extent of NMDA receptor expression among NG2 cells in different brain regions, the mechanisms that regulate their expression, and the physiological or pathological conditions under which they become activated.

Axo-glial synapses apparently share an exclusive reliance on AMPA receptors for transduction, but the properties of AMPA receptors that underlie these currents also vary among brain regions (see Section 2.1). Although the functional implications of these differences have not been determined, Ca^{2+} influx through AMPA receptors following repetitive stimulation can trigger enhancement of synaptic currents in hippocampal NG2 cells (Ge et al., 2006), indicating that Ca^{2+} dependent mechanisms of potentiation also exist at axo-glial synapses.

3.3. *Morphological evidence for direct neuron-NG2 cell synaptic junctions*

Although there have been sporadic reports of synaptic-like or synaptoid contacts between axons and glial cells, anatomical studies have not described the consistent innervation of glial cells in the mature CNS. This discrepancy between physiological studies and the anatomical record may reflect difficulty in identifying the processes of NG2 cells in electron micrographs (EMs); indeed, it is only recently that NG2 cells were recognized as a distinct class of glial cells with unique ultrastructural characteristics (Peters, 2004; Reyners et al., 1982). It is also likely that these junctions evaded detection because they are relatively rare and less distinct than neuronal synapses. Nevertheless, EM-level analysis of physiologically-identified NG2 cells revealed the presence of anatomically distinct junctions between axons and NG2 cell membranes that exhibit many features of traditional synapses, including rigid alignment and consistent spacing of axonal and NG2 cell membranes over a restricted area, the presence of electron dense material in the space where these junctions are formed, and localization of small clear vesicles and mitochondria in region of the axon near the junction (Bergles et al., 2000; Lin et al., 2005; Ziskin et al., 2007). Moreover, confocal microscopic analysis has shown the accumulation of vesicular glutamate transporter 1 (VGLUT1)- and synaptophysin-immunoreactive puncta adjacent to the processes of NG2 cells in the corpus callosum, in regions where MAP2-immunoreactive dendrites were absent (Ziskin et al., 2007). These findings suggest that NG2 cells are a direct target of innervation, and support the hypothesis that AMPA receptor signalling occurs at discrete locations, rather than diffusely over the surface of these glial cells.

In accordance with these data, preliminary evidence suggests that AMPA receptors are not expressed uniformly over the surface of NG2 cells, but rather are clustered into discrete patches along their processes. Freeze-fracture immunolabeling of brain tissue from transgenic mice in which NG2 cells can be unambiguously identified through labeling with anti-GFP antibodies, show clusters of AMPA receptor-immunoreactive intramembrane particles along NG2 cell processes (Huck et al. 2008), and functional mapping of AMPA receptors using two-photon uncaging of MNI-L-glutamate has shown the presence of "hot spots" along NG2 cell processes where AMPA receptors are enriched (M. Paukert and Dwight Bergles, unpublished observations). Together, these anatomical studies indicate that rapid glutamatergic signalling between axons and NG2 cells occurs at *bona fide* synaptic junctions.

4. Functions and future studies

The transient activation of ionotropic receptors in NG2 cells at synapses provides one mechanism by which this population of cells can monitor ongoing activity in surrounding axons. The rapid kinetics of these events provide distinct advantages for following the timing of action potentials, rather than summed activity over extended periods, as would be more likely if metabotropic receptors were used for transduction. Moreover, this mechanism for communication has an inherently low threshold, as individual action potentials can induce quantal glutamate release, and spontaneous vesicular release occurs in the absence of activity. A few studies have shown that some NG2 cells in both white and gray matter can generate Na^+-dependent spikes reminiscent of action potentials in response to current injection (Chittajallu et al., 2004; Karadottir et al., 2008), although the question of excitability within the NG2 cell population has not been thoroughly examined. If synaptic currents trigger spikes, they could promote Na^+ influx, inhibit K^+ channels and affect cellular development (Gallo et al., 1996). Such activity could also facilitate the activation of other voltage-gated channels to induce secretion of growth factors, neurotransmitters, or factors to influence the growth of axons. However, it is has not yet been shown that neuronal input under physiological conditions produces Ca^{2+} transients in these cells. NG2 cells possess several alternative pathways which might serve to induce intracellular Ca^{2+} transients, including Ca^{2+} permeable AMPA receptors (cf. 2.1) and voltage-gated Ca^{2+} channels (Akopian et al., 1996), which at least in cultured cells can be activated through depolarizing $GABA_A$ receptor-mediated responses (Kirchhoff and Kettenmann, 1992). Combined electrophysiological and Ca^{2+} imaging analyses *in situ* will help address the contribution of ionotropic receptors to Ca^{2+} dynamics in NG2 cells.

As NG2 cell processes do not extend for more than 100 μm (Fig. 1), and these cells are not coupled via gap junctions (Bergles et al., 2000; Wallraff et al., 2004), the consequences of NG2 cell activation would be expected to manifest locally. At present, the incidence of Na^+ spikes among NG2 cells in various brain regions at different times of development, the ability of synaptic inputs to induce these events, and the consequences of this excitability have not been determined.

Genetic fate mapping studies indicate that in white matter, a majority of NG2 cells differentiate into oligodendrocytes. In contrast, most NG2 cells in adult grey matter maintain their phenotype and only a minority differentiate into oligodendrocytes or astrocytes (Dimou et al., 2008; Rivers et al., 2008; Zhu et al., 2008). It is possible that synaptic signalling serves to alert white matter NG2 cells to the state of myelination of nearby axons, inducing proliferation or differentiation if certain patterns of activity are observed. The involvement of glutamatergic signalling in NG2 cell development is supported by *in vitro* studies (Gallo et al., 1996; Yuan et al., 1998), where glutamate receptor signalling also has been shown to influence the growth and migration of progenitors (Gudz et al., 2006). In addition, $GABA_A$ receptor activation has also been shown to influence NG2 cell migration in cultured tissue explants (Tong et al., 2009). Thus, it is possible that both glutamatergic and GABAergic synaptic input, by promoting an increase in $[Ca^{2+}]_i$, regulate the migration of NG2 cells during development and after brain injury.

It has been reported that NG2 cells have the capacity to differentiate into neurons and astrocytes *in vitro* when exposed to certain growth factors (Kondo and Raff, 2000). These findings raise the possibility that subpopulations of NG2 cells may act as multipotent progenitors with capacity for cell replacement. Further analysis using genetic techniques under both physiological and pathological conditions will help us to understand the potential of these cells to undergo further

differentiation and to assess whether neuronal activity plays a role in this process.

Increasing evidence suggests that NG2 cells are physiologically and morphologically diverse (Chittajallu et al., 2004; Karadottir et al., 2008; Karram et al., 2008), and that only some of these cells may serve as progenitors. The development of new transgenic mouse lines in which fluorescent proteins (Karram et al., 2008; Ziskin et al., 2007) and Cre recombinase (Rivers et al., 2008; Zhu et al., 2008) are selectively expressed under control of the NG2 promoter will allow direct manipulation of NG2 cell properties and analysis of the outcomes *in vivo*. Such studies will undoubtedly help define the functions of these enigmatic glial cells under both physiological and pathological conditions.

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BRAIN RESEARCH REVIEWS

Review

The contribution of astrocyte signalling to neurovascular coupling

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ABSTRACT

The tight spatial and temporal coupling between neuronal activity and blood flow ensures that active brain regions receive an adequate supply of oxygen and energetic metabolites. There clearly is still an enormous amount of experimental and theoretical work to be done to unravel the precise mechanism of neurovascular coupling, but over the last decade significant advances have been made. The most recent studies confirm the original finding that the activation of Ca^{2+} elevations in astrocyte endfeet is an essential step but also reveal new levels of complexity in the astrocyte control of neurovascular coupling. The recent evidence for a link between Ca^{2+} signalling in astrocytes and local metabolic states of the brain tissue has broad implications for the interpretation of data from functional brain imaging studies. Unraveling the full molecular mechanism of the astrocyte control of cerebral blood flow represents a formidable challenge in neurobiological research in the years to come that might also create opportunities for the development of new therapeutic strategies for cerebrovascular diseases such as ischemic stroke, hypertension and migraine as well as neurodegenerative diseases as Alzheimer's disease.

Contents

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Abbreviations: AA, arachidonic acid; BOLD, blood oxygenation level dependent; CBF, cerebral blood flow; COX, cyclooxygenase; DHK, dihydrokainate; TBOA, dl-threo-b-benzyloxyaspartate; EET, epoxyeicotrienoic acid; fMRI, functional magnetic resonance imaging; GABA, gamma-aminobutyric acid; GABAT, GABA transporter; Glu, glutamate; GLAST, glutamate aspartate transporter; GLT-1, glutamate transporter-1; IP3, inositol 1,4,5-trisphosphate; iGluR, ionotropic glutamate receptor; mGluR, metabotropic glutamate receptor; NPY, neuropeptide Y; NO, nitric oxide; PLA2, phospholipase A2; PGE2, prostaglandin E2; SOM, somatostatin; t-ACPD, 1-aminocyclopentane-trans-1,3-dicarboxylic; 20-HETE, 20-hydroxyeicosatetraenoic acid; VIP, vasoactive intestinal peptide

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1. Introduction

Functional hyperemia is a fundamental phenomenon in normal brain function. First discovered by A. Mosso in the late 1800s (Mosso, 1880), and later confirmed by Roy and Sherrington (1890), functional hyperemia reflects the dilation of arterioles and capillaries of a restricted brain region and perhaps also the constriction of other blood vessels from adjacent and distant regions, in response to a local episode of high neuronal activity. This event is spatially restricted, occurs within a few seconds of the onset of an episode of intense neuronal activity, and it ensures that active neurons can be sustained by adequate amounts of oxygen and metabolic substrates. Initially, the local accumulation of metabolic products was proposed to directly control blood flow, but it soon became clear that the time course of this process was not consistent with the rapid response observed in blood vessels upon increased neuronal activity (Lou et al., 1987). Indeed, results obtained over the last few years provide significant support for the view that CBF is directly coupled to neuronal activity rather than to local energy needs (Attwell and Iadecola, 2002). Although important questions remain unanswered, during the same period our knowledge of the mechanisms at the basis of functional hyperemia has increased significantly revealing not only the contribution of multiple cellular and molecular signalling pathways, but also the central role of neuron-to-astrocyte signalling (Zonta et al., 2003b; Zonta et al., 2003a; Filosa et al., 2004; Mulligan and MacVicar, 2004; Lovick et al., 2005; Metea and Newman, 2006b, 2007).

A crucial premise for the identification of the role of astrocytes in functional hyperemia was the discovery that astrocytes can respond to transmitters released by neuronal activity with intracellular Ca^{2+} elevations and signal back to neurons by releasing chemical transmitters (Dani et al., 1992; Nedergaard, 1994; Parpura et al., 1994; Newman, 1995; Porter and McCarthy, 1996; Pasti et al., 1997; Mothet et al., 2000, 2005), now termed gliotransmitters. These observations promoted an emerging new understanding of the functional roles played by these glial cells in the brain. It is now recognized that astrocytes listen and talk to synapses exerting both excitatory and inhibitory actions on neurons (Araque et al., 1999b; Brockhaus and Deitmer, 2002; Zhang et al., 2003; Pascual et al., 2005; Panatier et al., 2006; Serrano et al., 2006; Jourdain et al., 2007; Perea and Araque, 2007). Astrocytes are now considered intrinsic elements of the neuronal circuit that compose a tripartite synapse with the pre- and post-synaptic neuronal membrane (Araque et al., 1999a; Carmignoto, 2000; Haydon and Carmignoto, 2006; Halassa et al., 2009; Perea et al., 2009).

Beside their role as local modulators of neuronal excitability and synaptic transmission, astrocytes may also serve a hub-like function by integrating the signal received from thousands of synapses and then transferring it to other cells in the neuron-astrocyte network, including the cerebral vasculature that is intimately enwrapped by the astrocytic processes, the so-called endfeet (Peters et al., 1991; Ventura and Harris, 1999; Simard et al., 2003). It is because of this polarized anatomical structure and the vicinity of their endfeet to contractile elements of blood vessels, such as smooth muscle cells in arterioles and pericytes in capillaries, that astrocytes have been long proposed to contribute to the regulation of cerebral blood flow (CBF) during neuronal activity. Additional clues for such a role were the ability of astrocytes to produce and release a number of vasoactive substances, including nitric oxide (NO) (Murphy et al., 1993; Wiencken and Casagrande, 1999; Li et al., 2003), cycloxygenase (COX) and epoxygenase activity-derived products (Pearce et al., 1989; Oomagari et al., 1991; Amruthesh et al., 1992, 1993; Alkayed et al., 1997; Shi et al., 2008) and ATP (Queiroz et al., 1999; Arcuino et al., 2002; Coco et al., 2003). Only recently, however, did a number of studies provide convincing evidence that the neuron-to-astrocyte signalling pathway indeed serves a major role in functional hyperemia, the coupling between local episodes of intense neuronal activities and CBF (Fig. 1).

A number of comprehensive reviews have recently discussed our current understanding of how astrocytes are activated by neuronal signals and release vasoactive agents to regulate vascular tone (Haydon and Carmignoto, 2006; Filosa and Blanco, 2007; Gordon et al., 2007; Iadecola and Nedergaard, 2007; Koehler et al., 2009). We will thus restrict our review to the most recent observations that represent remarkable advances in our understanding of the amazing complexity of this important phenomenon.

2. Astrocyte activation by neuronal activity in neurovascular coupling: initial findings

While one of the first hypotheses on the putative role of astrocytes in directly regulating CBF was proposed in 1998 (Harder et al., 1998), direct experimental evidence for a distinct role of these cells in neurovascular coupling was provided only a few years later in brain slice preparations (Zonta et al., 2003b). In these experiments, Ca^{2+} elevations evoked in astrocyte processes by synaptically released glutamate were observed to propagate to perivascular endfeet with a timing correlated with an increase in the diameter of the cerebral blood vessels in contact with the activated endfeet. The activation of astrocytes depends on metabotropic glutamate receptors (mGluRs) since antagonists of these receptors were found to inhibit both the Ca^{2+} elevation in endfeet and the vasodilating response without affecting the response of neurons to neuronal afferent stimulation. Laser Doppler blood flow measurements also revealed that mGluR antagonists reduced the CBF increase evoked in the somatosensory cortex by forepaw stimulation *in vivo*, without affecting evoked field potentials, and thus confirmed, although indirectly, the role of astrocytes in functional hyperemia (Zonta et al., 2003b). Astrocyte-mediated vasodilation was found to depend mainly on arachidonic acid metabolites, such as prostaglandin E_2 (PGE_2) (Zonta et al., 2003a).

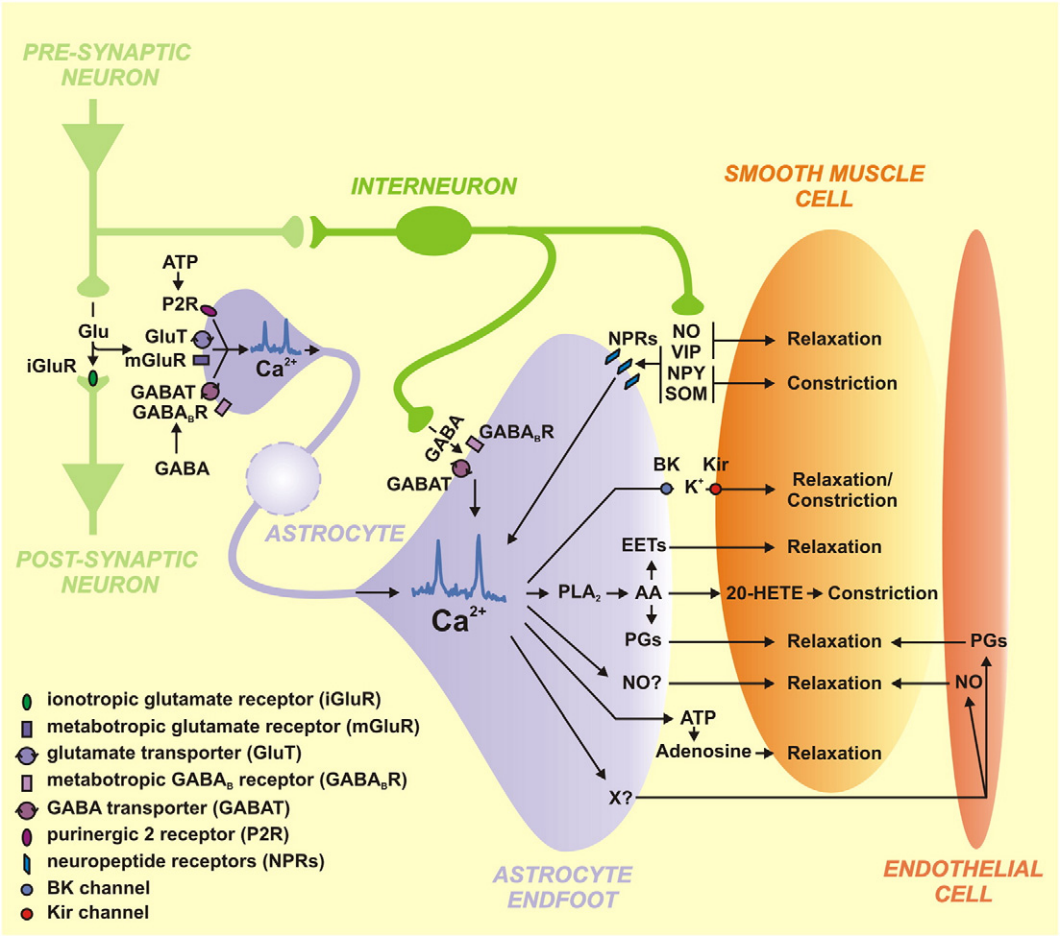

Fig. 1 – Astrocyte-mediated control of neurovascular coupling. Schematic view of the different signalling pathways that control the cerebral blood vessels response to neuronal activity. The Ca^{2+} elevation in endfeet that represents a crucial step in neurovascular coupling mechanism is here proposed to be mediated not only by glutamate but also by GABA and neuropeptides released form activated interneurons.

Subsequent studies in brain slice and retina preparations essentially confirmed that evoking Ca^{2+} elevations in astrocyte endfeet either directly (using photolysis of Ca^{2+}- and inositol 1,4,5-trisphosphate (IP3)-caged compounds) or indirectly (through neural stimulation) triggers a blood vessel response (Metea and Newman, 2006a). Astrocytes were found to favour vasodilations by releasing not only PGE_2 but also other dilating agents, such as epoxygenase derivatives (EETs) (Amruthesh et al., 1993; Alkayed et al., 1997; Metea and Newman, 2007; Shi et al., 2008). The complexity of the astrocyte action on CBF regulation was enriched by the observations that the release of K^+ through Ca^{2+}-sensitive K^+ channels activated by Ca^{2+} elevations in astrocyte endfeet modulates cerebral arteriole tone by Kir channel activation in smooth muscle cells (Filosa et al., 2004, 2006) (Fig. 1).

3. Astrocyte-mediated constrictions: role of NO and myogenic tone

Cortical slice preparation experiments that used the direct stimulation of astrocytes by Ca^{2+} uncaging through two-photon photolysis revealed that astrocytes can trigger also cerebral arteriole constrictions (Mulligan and MacVicar, 2004). At the basis of this constrictive effect, it was proposed that arachidonic acid (AA) produced by astrocytes diffused to smooth muscle cells where it is converted to the constrictive agent 20-HETE (Mulligan and MacVicar, 2004). This finding raised the intriguing possibility that upon activation astrocytes can release both vasodilating and vasoconstrictive agents. How can an astrocyte-mediated constriction be reconciled with a role of astrocytes in functional hyperemia? Results obtained in whole-mounted retina experiments then suggested that nitric oxide (NO) may regulate the conversion of AA to either EETs or 20-HETE, thus providing a plausible mechanism for the different response of blood vessels to astrocyte activation (Metea and Newman, 2006a). Support for such a modulatory role of NO was the observation that an activation of astrocytes that evoked a vasoconstriction changed to a vasodilation after inhibition of NO synthase by L-NAME (Mulligan and MacVicar, 2004). Notably, L-NAME was also used in the original experiments that revealed astrocyte-mediated vasodilations (Zonta et al., 2003b).

An additional factor that contributes to dictate the type of vasomotor response to astrocyte signalling is the resting state of cerebral arterioles. In the intact brain, the arteriole resting

state is controlled by the contraction of smooth muscle cells. The myogenic tone is, indeed, regulated by an interplay of pressure-mediated stretching of the smooth muscle cell membrane, intraluminal blood flow and modulatory factors released by neurons, astrocytes and endothelial cells (Davis and Hill, 1999; Hill et al., 2001; Dora, 2005). Cerebral arterioles are thus characterized by a variable resting state that depends on tonic and dynamic factors (Faraci and Heistad, 1998). Results obtained from brain slices should thus be interpreted with caution since the myogenic tone is reduced or lost in this experimental preparation due to the lack of blood flow and cerebral vessel inner resistance. The importance of the myogenic tone in determining the type of the arteriole response to astrocyte activation has been recently specifically addressed by Blanco et al. (2008) in cortical slice preparations. These authors used different concentrations of the thromboxane A_2 receptor agonist U-46619 to induce different degrees of preconstriction to arterioles – and thus to mimic the natural occurring myogenic tone of blood vessels – before stimulating Ca^{2+} elevations in astrocytes. Results obtained clearly showed that the polarity and magnitude of the vasomotor response to astrocyte activation were dictated by the particular tone of the arteriole. Thus, when the myogenic tone is low, arterioles tend to be in a dilated state and thus to respond with a constriction to dilating and constrictive agents released upon astrocyte activation. Similarly, when the myogenic tone is high, arterioles are constricted and can more easily respond to astrocyte activation with a dilation (Fig. 2a).

The ultimate effect of astrocyte activation on blood vessels may depend on the one hand, on a competitive action of dilating and constrictive agents, and on the other, on arteriole resting state. By opposing the powerful action of the dilating agents, the constrictive agents may ultimately serve to modulate the amplitude of the neuronal activity-dependent increase in CBF.

4. Relationship between astrocyte signalling and metabolic state

It has long been hypothesized that the sudden increase in energy demand by neurons at the site of activation might lead to a significant reduction in oxygen and glucose that initiates the rapid CBF response. While the hypothesis of a direct link between cellular energy state and CBF regulation remains controversial, a CBF increase in the retina and visual cortex associated with sensory stimulation was observed to correlate with an increase in the plasma lactate level (Ido et al., 2004; Mintun et al., 2004). These studies, however, failed to clarify the nature of the link between CBF and the energy state of the cell.

In a series of elegant experiments in brain slice preparations, Gordon et al. (2008) recently provided evidence for a direct link between lactate levels in the brain and the astrocyte signalling that control blood vessel tone. These authors showed that at low oxygen levels – a condition that the authors imposed experimentally by perfusing cortical slices with 20 % O_2 / 5 % CO_2 – Ca^{2+} elevations in astrocytes triggered by the mGluR agonist t-ACPD caused PGE_2-mediated vasodilation. Notably, upon slice perfusion with 95 % O_2 / 5 % CO_2, the same astrocyte stimulation evoked a vasoconstriction. The authors then showed that low oxygen *per se* as well as astrocyte activation by t-ACPD applied at low oxygen stimulated a large production of lactate by astrocyte glycolytic metabolism, that after its extracellular accumulation inhibited prostaglandin transporters. A crucial step in the dilating action of astrocytes at low oxygen is thus this latter action of lactate that allows PGE_2 released from activated astrocytes to exert its dilating effect on blood vessels.

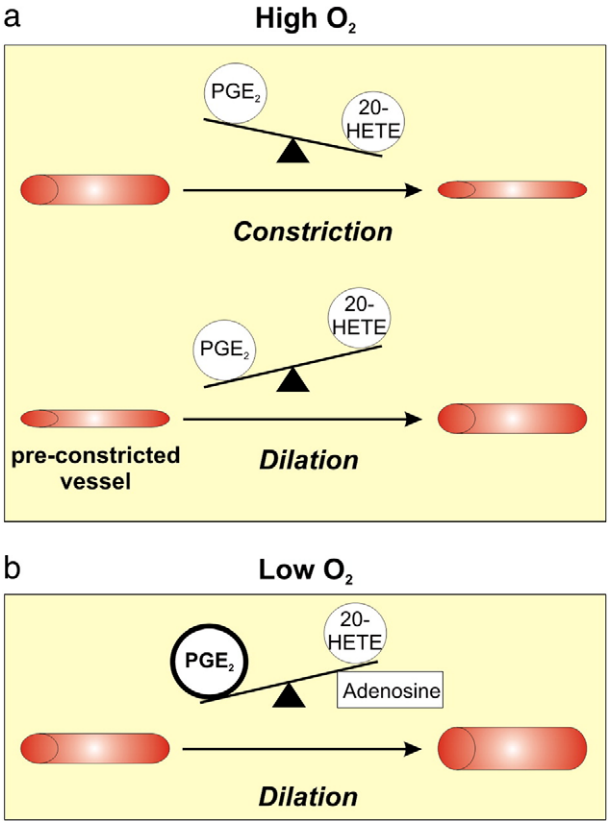

Fig. 2 – The vasomotor response to astrocyte-derived vasoactive agents depends on the resting state of cerebral arterioles and brain tissue metabolic state. Schematic view of the influence of non-astrocytic factors on the action of vasodilating (PGE_2) and vasoconstrictive (20-HETE) agents released from astrocytes. (a) A reduced myogenic tone in arterioles tips the balance in favour of the constrictive action of 20-HETE (upper panel). Conversely, arterioles with a higher tone respond to astrocyte vasoactive agents more easily with a dilation (lower panel). (b) Upon a decrease in ambient O_2 both lactate and adenosine accumulate in the extracellular space, reducing the uptake of PGE_2 and counteracting the effects of 20-HETE, respectively. These conditions favour the vasodilating response to astrocyte activation.

Gordon et al. (2008) also showed that at low oxygen, adenosine also accumulates in the extracellular space and it down-regulates L-type Ca^{2+} channel activity by acting on A2 receptors in smooth muscle cells (Murphy et al., 2003). Adenosine thus limits the constrictive effect of astrocyte-derived arachidonic acid and favours the action of the dilating agents. Astrocyte Ca^{2+} elevations can ultimately lead to a CBF increase when ambient oxygen is low and lactate inhibits

prostaglandin transporters allowing PGE_2 to act on blood vessels, while adenosine favours PGE_2 dilating action by counteracting astrocyte-mediated vasoconstriction (Fig. 2b).

It is noteworthy to recall here the astrocyte–neuron lactate shuttle hypothesis proposed by Magistretti and collaborators (Pellerin and Magistretti, 1994; Magistretti et al., 1999). This hypothesis generated a considerable interest (and also controversies) since it fits well with the experimental data on the neurometabolic coupling in the brain. Briefly, the hypothesis postulates that (i) neuronal activity increases extracellular glutamate which is taken up via a Na^+-dependent mechanism by the specific glial glutamate aspartate transporter (GLAST) and glutamate transporter-1 (GLT-1); (ii) the following intracellular increase of Na^+ activates Na^+-/K^+-ATPase and by stimulating aerobic glycolysis into astrocytes lead to a large increase in the production of lactate; and (iii) lactate is released as an energy substrate for neurons.

The direct link between the metabolic state of the cells and astrocyte signalling described in Gordon et al. (2008) adds to the "lactate shuttle" hypothesis a novel level of complexity and represents a clear breakthrough in our understanding of the basic mechanisms underlying neurometabolic and neurovascular coupling. By sensing synaptically released glutamate – through mGluR-mediated Ca^{2+} signalling and glutamate uptake – astrocytes can coordinate both the CBF response and the change in metabolism that are necessary to meet the increased energy requirements of active neurons and astrocytes.

A number of issues remains, however, unsolved and further supportive evidence is needed. Some key questions are related to the decrease in oxygen concentration during functional hyperemia. Do available data from *in vivo* experiments provide convincing evidence for a significant decrease of oxygen at the site of activation? Is the initial decrease in oxygen at the site of activation sufficient to affect astrocyte signalling? As to the first question, although some debate is still existing in the field (Vanzetta and Grinvald, 1999, 2001; Yamamoto and Kato, 2002; Logothetis and Pfeuffer, 2004; Lauritzen, 2005; Vanzetta and Grinvald, 2008), over the past few years, results obtained from functional neuroimaging studies provided important insights into the spatial and temporal dynamics of the change in blood deoxyhemoglobin and oxyhemoglobin concentrations and also convincing evidence for an oxygen decrease in the living brain caused by functional stimulation. These studies described a bi-phasic response in local oxygen consumption. Immediately after the stimulus onset (300–500 ms delay), due to the activation of neurons (and astrocytes), the local oxygen concentration decreases. This oxygen decrease is indirectly revealed in functional magnetic resonance imaging (fMRI) by a transient increase in blood deoxyhemoglobin concentration, the so-called "initial dip" of the blood oxygenation level dependent (BOLD) signal (deoxyhemoglobin, but not oxyhemoglobin, is paramagnetic, and a deoxyhemoglobin increase is detected as a downward deflection of the fMRI signal). The initial dip precedes a large deoxyhemoglobin decrease in the fMRI signal that reflects a large oxygen supply to the active region due to blood vessel dilation.

Regarding the astrocyte component in the mechanism proposed in Gordon et al. (2008), the question remains whether the initial decrease in oxygen at the site of activation is sufficient to affect astrocyte signalling. A direct answer to this question would be provided by measurements of the change in oxygen concentrations at the site of functional activation. Unfortunately, although brain oxidative metabolism and oxygen tension have been studied with a variety of technical approaches and mathematical models, the actual level of human brain oxygen and its change upon stimulation are difficult to estimate and are subjects of an intense debate in the field (Mintun et al., 2001; Hayashi et al., 2003; Sharan et al., 2008; Masamoto and Tanishita, 2009). Noteworthy is that, concerning the point raised, this task appears even more complex and difficult since it is not the average oxygen tension in the brain tissue that is of importance but rather the drop in oxygen at the site of activation. Of note is also that oxygen in the cerebral tissue is distributed in a spatially and temporally heterogeneous manner and its concentration changes significantly over different distances from the oxygen source, i.e., the blood vessels, being relatively high around capillaries and arterioles, but likely low, perhaps very low, at more distant sites (Hudetz, 1999; Mintun et al., 2001; Lecoq et al., 2009). All in all, while a low oxygen environment might normally exist for neurons, it remains unclear whether oxygen in the living normal brain tissue is closer to the low rather than to the high level that Gordon et al. (2008) imposed to their brain slice preparations to reveal the vasodilating effects of astrocyte-derived agents.

Noteworthy is that fMRI studies in humans during appropriate task performances often reported that the CBF increased at the site of functional activation, but it decreased in other regions, as measured by negative BOLD signals. Experimentally, data confirm that during physiological neuronal activation functional hyperemia in the active region is accompanied by hypoperfusion in surrounding areas, a sort of center-surround effect that may be functional to increase the redistribution of the blood flow to the active region (Harel et al., 2002; Shmuel et al., 2002; Devor et al., 2007a,b). While the positive BOLD signal is commonly recognized to result from an elevated oxygen level in brain tissue due to increased CBF, cerebral blood volume, and metabolic rate of oxygen utilization, the source of the negative BOLD signal change is much less defined. The local increase in deoxyhemoglobin content, that necessarily reflects the negative BOLD signal, may be due to a relative CBF reduction in regions adjacent to the site of activation by a steal phenomenon secondary to the local increase in CBF (Shmuel et al., 2002) or by an increase in neuronal inhibition that within a local cortical network leads to a decrease in neuronal activity (Schwartz and Bonhoeffer, 2001; Zhao et al., 2009). Negative BOLD signals could also be due to a larger fractional increase in cerebral metabolic rate of oxygen compared with CBF or to a larger fractional decrease in CBF compared with oxygen metabolic rate (Harel et al., 2002; Gotman et al., 2006).

The recognized ability of astrocytes to release both vasodilating and vasoconstrictive agents adds a new level of complexity in an already rich mechanism and suggests that these cells may contribute also to the control of CBF changes in the surrounding regions. It would be, thus, of interest to investigate whether or not astrocytes (and their endfeet) are activated in the area surrounding the activated region, where the decrease in oxygen concentration is likely less than that at

the site of activation. The different effect of astrocyte signalling on blood vessels that Gordon et al. (2008) observed to be linked to the brain tissue metabolic state is consistent with both the CBF increase at the site of activation and its decrease in the surrounding regions. Vasodilating and constrictive agents may be both released from activated astrocytes, but their ultimate effect on blood vessels may depend on external factors such as the oxygen and lactate level (Fig. 3).

An additional key question is related to the timing of the blood vessel response. Does the initial dip in oxygen concentration affect astrocyte signalling rapidly enough to mediate the blood vessel dilation that occurs within 1–2 s after functional activation? More specifically, is the time course of the extracellular lactate accumulation and the consequent inhibition of PGE_2 uptake consistent with the initiation of stimulus-evoked CBF response? Can it be more consistent with the maintenance of the CBF increase during prolonged activation?

5. Is the timing for astrocyte activation by neuronal activity in the living brain compatible with functional hyperemia?

To be consistent with a central role in functional hyperemia, Ca^{2+} elevations in astrocyte endfeet should be activated by neuronal signals before or in coincident with the increase in CBF that occurs, in general, 1–2 s after the stimulus onset. Over the last years, the improved time resolution of the Ca^{2+} fluorescence images acquired through two-photon laser scanning microscope allowed to gain important insights into the temporal features of the astrocyte response to sensory activation in the living brain. Most of the results obtained are consistent with a role of astrocyte Ca^{2+} signal in the initiation of the blood vessel response, but conflicting data also exist. An initial study that used two-photon laser scanning microscope technique and imaging of Ca^{2+} signals in astrocytes confirmed the astrocyte control of neuronal activity-dependent CBF changes (Takano et al., 2006). However, in this study the temporal correlation between the activation of astrocytes and the onset of the CBF change could not be appropriately evaluated since an extracellular electrode, and not a physiological stimulus, was used to evoke the blood vessel response. A delay of several seconds observed in the response of astrocytes and endfeet to sensory stimulation was reported by (Wang et al., 2006) and this led to the suggestion that astrocyte activation might not be required for the initiation of the blood vessel dilating response and it rather contribute to its maintenance during prolonged activation. Subsequent studies described a shorter delay (Winship et al., 2007) and a tight correlation between activation of Ca^{2+} signals in endfeet and the initiation of the blood vessel response in the neocortex upon sensory stimulation (Schummers et al., 2008). The role and timing of astrocyte Ca^{2+} signal in neurovascular coupling mechanisms has been also investigated in the olfactory bulb. This model has several advantages with respect to other *in vivo* models (Shepherd and Charpak, 2008), and among these are the anatomical location and the layered organization that allow the glomeruli, i.e., the functional units of the olfactory bulb, to be easily imaged *in vivo* by two-photon laser scanning microscopy. Furthermore, in this well-defined system neurovascular coupling mechanism can be studied by using the specific physiological stimulus, i.e., an odor, that activates a selective blood flow response at the level of individual glomeruli. In this ideal model, glomerular astrocytes were observed to respond with a Ca^{2+} increase to a physiological stimulation in an odor-specific fashion, and the activation of their endfeet to occur shortly, approximately 1 s, after stimulus presentation, before or in coincidence to the dilation of the arterioles that penetrate the glomerular layer (Petzold et al., 2008). As a further support, the authors reported that the absence of dilation in some arterioles was accompanied by a failure of Ca^{2+} elevations in the surrounding endfeet. As to the molecular mechanism, this study essentially confirmed previous findings on the role of mGluR and COX activation, but it also revealed that in the presence of DL-threo-b-benzyloxyaspartate (TBOA), a general inhibitor of neuronal and astrocytic glutamate transporters, or dihydrokainate (DHK), a specific inhibitor of the astrocytic glutamate transporter GLT-1, the odor-evoked increase in CBF was surprisingly reduced. The uptake of glutamate into astrocytes ultimately accounted for about one third of the CBF response. It thus appears that additional astrocytic signalling pathways, apart from that mediated by glutamate activation of mGluR, may be involved in astrocyte control of neurovascular coupling (Fig. 1). The intracellular cascade of events linked to glutamate uptake remains to be elucidated. According to results reported by Petzold et al. (2008), it might be independent of Ca^{2+} signals, although these authors recognized that glutamate uptake may have induced small,

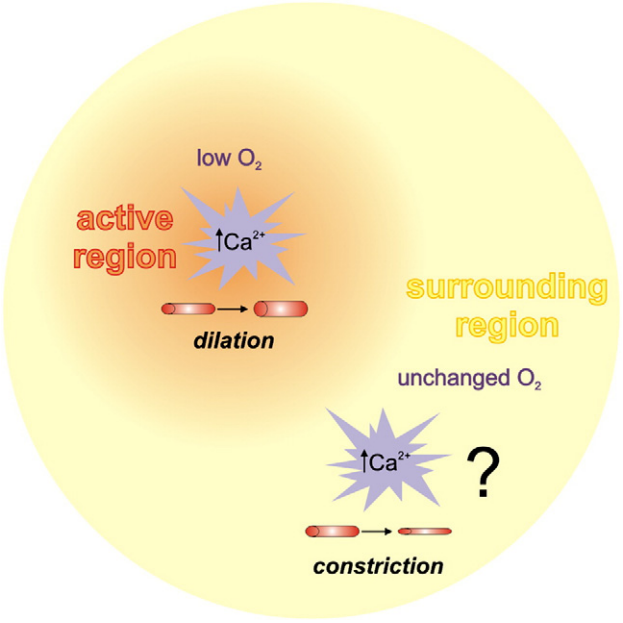

Fig. 3 – Hypothesis for a different role of astrocyte signalling at the site of functional activation and in the surrounding regions. The local O_2 decrease caused by neuronal activity may favour the action of vasodilating agents released from activated astrocytes, while in the surrounding regions an unchanged O_2 level may favour astrocyte-derived vasoconstrictive agents.

local Ca^{2+} elevations that were hardly detectable by the *in vivo* imaging that they performed. The possible contribution of glutamate uptake by astrocytes in neurovascular coupling was also suggested by an additional study that focused on the activity of astrocytes in the ferret visual cortex (Schummers et al., 2008). This study revealed that astrocytes, as neurons, respond with robust Ca^{2+} elevations to distinct features of the visual stimuli, such as orientation and spatial frequency, and also showed that the Ca^{2+} response of astrocytes, but not that of neurons, was drastically reduced by inhibiting glutamate transporters with TBOA. The authors then reported that in the presence of TBOA the blood flow response to visual stimuli, as measured by intrinsic optical signals, was also drastically reduced. This result confirms the observation reported by Petzold et al. (2008) concerning the role of glutamate uptake on CBF regulation and strengthens the view that different pathways contribute to the neuronal activity-dependent Ca^{2+} elevations in astrocytes. In contrast to results reported by Petzold et al. (2008), the activation by visual stimulation of glutamate uptake in astrocytes resulted in large Ca^{2+} elevations that, in principle, account for the contribution of this signalling pathway in the control of neurovascular coupling. Further experiments that address specifically the role of these different pathways in astrocyte Ca^{2+} signalling are, however, needed to reconcile these conflicting observations and clarify the mechanism at the basis of astrocyte-mediated CBF regulation.

The above mentioned *in vivo* studies substantially confirm the central role of astrocytes in functional hyperemia and also revealed that Ca^{2+} elevations at the level of the endfeet can occur within approximately 1–2 s after stimulus onset, i.e., a delay consistent with an astrocyte role in the fast vasomotor response that characterizes functional hyperemia. However, the mechanism at the basis of such a rapid Ca^{2+} response in endfeet remains unclear. If we assume that the same astrocytes that sense neuronal activity are in contact with blood vessels, the Ca^{2+} increase activated at the processes in contact with active synapses should propagate intracellularly to the endfeet with an estimated travel of 50–100 μm. Given that the upper limit of Ca^{2+} signal propagation speed in living cells is about 50 μm/s, it appears that this mechanism may, in principle, be consistent with the 1- to 2-s delay of the Ca^{2+} activation in endfeet by neuronal activity.

Can alternative pathways be more suitable to activate a rapid Ca^{2+} elevation in astrocyte endfeet? Clues for the existence of such a pathway derives from the finding that the activation of specific subsets of GABAergic interneurons can affect the tone of nearby cerebral arterioles (Cauli et al., 2004; Kocharyan et al., 2008). The original study by Cauli et al. (2004) elegantly showed that the selective stimulation of vasoactive intestinal peptide (VIP)- and nitric oxide synthase (NO)-positive interneurons leads to vasodilation, while that of somatostatin (SOM)-positive interneurons to vasoconstrictions. Given that the direct application of these peptide evoked similar responses in the arterioles, the authors suggested that the specific vasomotor effect observed after interneuron stimulation is consistent with the release of the peptides from the stimulated interneurons. Given that processes of the interneurons are probably in direct contact with cerebral blood vessels, released peptides may act directly on smooth muscle cells (Fig. 1). However, receptors for these SOM and VIP are also expressed by astrocytes (Rougon et al., 1983), and a direct application of vasoactive peptides triggers Ca^{2+} elevations in endfeet (Straub et al., 2006). The release of gamma-aminobutyric acid (GABA) by interneurons can also contribute to trigger Ca^{2+} elevations through either a $GABA_B$-dependent or -independent signalling pathway (Fig. 1). This latter pathway has been recently described to depend on GABA transporter activity and the accompanying intracellular Na^+ rise that reduces the Na^+/Ca^{2+} exchange activity leading to a Ca^{2+} rise sufficient to trigger Ca^{2+}-induced Ca^{2+} release from intracellular stores. Most interestingly, this signalling pathway mediates vasoconstriction in the olfactory bulb (Doengi et al., 2009). It seems thus possible that an interneuron-to-astrocyte endfeet signalling might contribute to neurovascular coupling mechanism. It is noteworthy that interneurons are activated along with principal neurons by sensory stimulation and through neuropeptide release they can, in principle, convey this activation to endfeet. Accordingly, the timing for a Ca^{2+} elevation evoked in endfeet by interneurons can be much more rapid than that linked to the slow intracellular propagation of a Ca^{2+} signal arising at the processes in contact with the glutamatergic synapse.

6. Astrocyte control of CBF in awake animals

With the advances in imaging techniques and with the discovery that mice can be trained to familiarize to restraining conditions, the activity of neurons and astrocytes has recently started to be investigated also in awake, behaving animals (Margrie et al., 2002; Crochet and Petersen, 2006; Dombeck et al., 2007). A unique advantage of this approach is the absence of anesthetics that are known to affect to different degrees both neuronal dynamics (Rinberg et al., 2006; Greenberg et al., 2008) and the magnitude of the CBF response (Nakao et al., 2001). Given the reciprocal signalling systems existing between neurons and astrocytes, it is not surprising that the dynamics of astrocyte Ca^{2+} signalling are also affected by anesthetic regimen. In the ferret visual cortex, the response of astrocytes to visual stimuli have been, indeed, reported to be even more sensitive than that of neurons to isoflurane, a widely used anesthetic for *in vivo* studies (Schummers et al., 2008). To study the possible correlation between neuron-astrocyte network activities and animal behavior as well as the astrocyte control of blood flow, astrocyte signalling should thus be ideally monitored in awake animals.

A recent study in awake, behaving mice addressed this issue by monitoring Ca^{2+} signals in Bergmann glia, a type of astrocytes of the cerebellar cortex (Nimmerjahn et al., 2009). Different forms of activities have been observed in these cells: isolated Ca^{2+} elevations, termed *sparkles*, and radially expanding Ca^{2+} waves, termed *bursts* and *flares*, that involved tens and hundreds of cells, respectively. While sparkles and bursts occurred spontaneously and were relatively independent on neuronal activity, flares required neuronal activity. Indeed, this type of Ca^{2+} change arose at the onset of locomotion, when mice ran voluntarily on the exercise ball, and involved practically all the Bergmann glia present in the field of view. The Ca^{2+} flares evoked in these cells by locomotion coincided

with the onset of the hemodynamic change, as measured by laser Doppler. However, locomotion-evoked Ca^{2+} flares in Bergmann glial cells have a refractory period and a limited duration, while the cerebellar blood flow increase persisted throughout motor behavior. Further investigations are needed to clarify whether Bergmann glia contribute to regulate the initial phase or rather the maintenance of the CBF response in a brain area, such as the cerebellum, in which functional hyperemia seems to depend almost exclusively on nitric oxide released from interneurons (Yang et al., 1999, 2000; Rancillac et al., 2006).

The findings of Nimmerjahn et al. (2009) have also additional implications: (i) they suggest that the Ca^{2+} signal in astrocytes can have a richness of forms that may reflect a richness of functional roles. Bergmann glia Ca^{2+} flares likely have a role in blood flow regulation, and perhaps in locomotor behavior, but the role of sparkles and bursts, that are typically observed in awake animals at rest, remains undefined; (ii) in mice under isoflurane anesthesia, Ca^{2+} flares were abolished and sparkle rates reduced, indicating that different forms of Ca^{2+} excitability in astrocytes can be selectively affected by anesthetics. These observations imply that results from *in vivo* studies in anaesthetized animals should be interpreted with some cautious; (iii) in another study previously performed by the same authors in awake animals (Dombeck et al., 2007), the massive Ca^{2+} signal that characterizes flares in Bergmann glia has not been observed in cortical astrocytes. This latter observation strengthens the emerging view that astrocytes represent a heterogeneous population of cells that have different properties and functions within the same as well as across different brain regions (Matthias et al., 2003).

7. Concluding remarks

The experimental research on functional hyperemia blossomed over the last few years and has lead to significant advances in our understanding of the mechanisms at the basis of this phenomenon. Due to progress made in neuroimaging techniques, we began to visualize neuron and astrocyte signalling in the living brain of anaesthetized as well as freely moving animals, monitoring at the same time CBF changes associated with functional stimulation. This review focuses on the most recent studies that, on one hand, confirmed the important role of astrocytes in neuronal activity-dependent CBF changes and, on the other, revealed that at the basis of this complex phenomenon is a sequence of events that involves also inhibitory interneurons, vascular architecture, and metabolic factors. Key issues remain unclear, however, and a full understanding of the mechanisms underlying functional hyperemia represents a formidable challenge in neurobiological research in the years to come. The recent discovery of a direct link between the metabolic state in brain tissue and astrocyte signalling opens new perspectives for a better understanding of the cerebrovascular dysregulations that characterize brain pathologies, such as ischemic stroke, hypertension, migraine and also Alzheimer's disease. It is thus time to move a step forward and start gaining insights into the distinct roles of astrocyte signalling in these brain disorders.

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BRAIN RESEARCH REVIEWS

Review

Glucose and lactate supply to the synapse

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ABSTRACT

The main source of energy for the mammalian brain is glucose, and the main sink of energy in the mammalian brain is the neuron, so the conventional view of brain energy metabolism is that glucose is consumed preferentially in neurons. But between glucose and the production of energy are several steps that do not necessarily take place in the same cell. An alternative model has been proposed that states that glucose preferentially taken by astrocytes, is degraded to lactate and then exported into neurons to be oxidized. Short of definitive data, opinions about the relative merits of these competing models are divided, making it a very exciting field of research. Furthermore, growing evidence suggests that lactate acts as a signaling molecule, involved in Na^+ sensing, glucosensing, and in coupling neuronal and glial activity to the modulation of vascular tone. In the present review, we discuss possible dynamics of glucose and lactate in excitatory synaptic regions, focusing on the transporters that catalyze the movement of these molecules.

Contents

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1. The stage

The synaptic region is divided into three major compartments: interstitium, and the cytosolic spaces of neuronal and astrocytic processes (see also Deitmer and Rose, this volume). The combination of short distances, high prevailing concentrations of glucose and lactate and slow turnover rate of the transporters and the enzymes involved predicts that the three compartments are internally homogeneous, i.e. there are no significant glucose or lactate gradients inside each membrane-bound space (Barros and Martinez, 2007). Concentration gradients may of course appear for longer distances, e.g. between different areas of the neuropil and between neuronal cell bodies and their far away processes. The interstitial compartment comprises the space between endothelial cells and astrocytic end feet (the basal lamina) and also the space between neuronal and astrocytic processes. Electron microscopy and tracer diffusion studies have shown that these two regions are well connected, so that no significant concentration gradients are expected between these compartments for abundant molecules like glucose and lactate (Brightman and Reese, 1969; Barros et al., 2007; Simpson et al., 2007). In other words, glucose and lactate are homogenously distributed around astrocytes and neurons. In contrast, the concentration of other molecules around these cells can be highly heterogeneous. Glutamate for instance varies from transient 1 mM near an active synaptic cleft to under 3 μM only a few micrometers away in extrasynaptic domains (Rusakov and Kullmann, 1998).

A consensual model for glucose and lactate fluxes in the mammalian neuropil is provided in Fig. 1A. Blood-derived glucose flows into the parenchyma at an average rate of about 6.5 μM/s, i.e. 0.3 mg ml^{-1} min^{-1} (Pappenheimer and Setchell, 1973). Autoradiography and PET have shown that the rate of glucose consumption differs between brain regions, with higher values in grey matter, and also varies with time, with active areas capturing glucose more avidly (Raichle and Mintun, 2006). In the case of lactate, its net production by the brain is normally negligible (Dienel and Cruz, 2004). Overall concentrations are also known, with congruent estimates from several techniques at 0.5–1.5 mM for both glucose and lactate (Barros et al., 2007; Dienel and Cruz, 2004). Given these consumption rates and steady-state concentrations, it can be calculated that if the glucose supply to the brain were to stop, the glucose pool may keep the average tissue functioning for about 150 s and that the lactate pool would contribute about 75 s of fuel. These add to the largest energy pool in the brain, astrocytic glycogen, worth about 500 s of normal fuel consumption in the human brain (Oz et al., 2007). These times may be considerable shorter if energy demand is higher, for instance during ischemic excitotoxicity. Another reason that makes the dynamics of glucose and lactate in the brain of topical interest is recent evidence that lactate acts as a signaling molecule involved in Na^+ sensing (Shimizu et al., 2007) and in glucosensing (Lam et al., 2005), and that lactate is responsible for coupling metabolic activity to the modulation of vascular tone in the brain (Gordon et al., 2008).

After entering neurons and astrocytes, glucose is phosphorylated by hexokinase, a reaction that in the brain is irreversible due to lack of significant glucose-6-phosphatase activity (Dienel et al., 1988; Dringen et al., 1993). The latter also means that astrocytic glycogen can not be used to produce glucose and may only be transferred to neurons in the form of lactate, which is the case during hypoglycemia (Oz et al., 2007; Walls et al., 2009). Another consequence of the lack of glucose synthesis in the brain is that on average glucose moves from interstice to cells. However, this does not necessarily has to happen all the time. Low resolution biochemical and NMR spectroscopy measurements indicate that in the brain intracellular glucose is relatively high and not much lower than that in the interstitium (Pfeuffer et al., 2000), so it seems possible that some cells may export glucose transiently, perhaps during acute changes in demand, an intriguing possibility that underscores the need for high-resolution measurements. For lactate the situation is even more uncertain, for it can be metabolized and produced in both cell types. Net efflux of lactate from the brain is negligible compared with the rate of lactate turnover, so its balance depends largely on local production and usage. As with glucose, there are no available measurements of lactate with cellular resolution, and it is not possible to tell which cell type is the lactate source and which is the lactate sink, and whether these roles may be region-specific or affected by activity. Glucose and lactate are also exchanged between neighbouring regions of the neuropil via the interstitial space (Cruz et al., 2007), an exchange that is

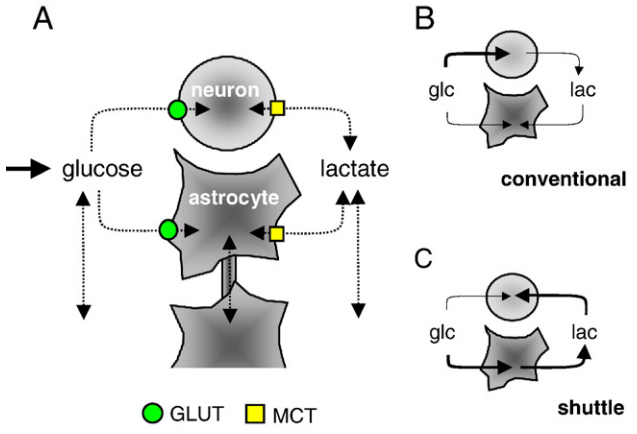

Fig. 1 – Modes of neurometabolic coupling in the neuropil. (A) Neurons and astrocytes are surrounded by interstitial fluid, which contains glucose and lactate, both at about 1 mM. The glucose pool feeds the cells and is replenished by blood-derived glucose. Lactate flows from and towards the cells, and is cleared into the blood at a very low rate. The astrocytic glucose and lactate pools are communicated with neighbouring regions by gap junctions. The extracellular pools are well connected with neighbouring regions through the interstitium. (B) The conventional model of coupling proposes major uptake of glucose by neurons and net production of lactate by neurons and consumption by astrocytes. (C) The lactate shuttle model proposes major uptake of glucose by astrocytes and net production of lactate by astrocytes and consumption by neurons. In B and C the transporters have been omitted for clarity and the thickness of the arrows represent the respective fluxes.

however limited by the random nature of diffusion, for it takes over 30 min to equilibrate these substrates between two regions separated by only 1 mm. In addition to the extracellular pathway, glucose and lactate can reach neighbouring regions through inter-astrocytic gap junctions, a connection that is modulated by activity (Rouach et al., 2008; Gandhi et al., 2009).

2. Fuelling synaptic activity: glucose versus lactate

Excitatory synaptic transmission is a very energy-consuming process. Most ATP expenditure occurs in neurons, for recovery of the ion homeostasis dissipated by excitatory postsynaptic potentials (Attwell and Laughlin, 2001; Alle et al., 2009), so it is widely accepted that the main site of ATP synthesis is the neuron. Several studies *in vitro* have shown that neurons can obtain ATP from metabolizing glucose as well as from lactate, in order to maintain their electrical and synaptic activity (Schurr et al., 1988, 1999; Izumi et al., 1997). During energy deprivation, the addition of monocarboxylates has been shown to restore synaptic function and to be neuroprotective in acute rodent brain slices, isolated optic nerve and neuronal cultures (Izumi et al., 1997; Schurr et al., 1997; 2001; Maus et al., 1999; Cater et al., 2001; Rouach et al., 2008). Glucose and lactate could provide sufficient support for energy-dependent processes in isolated nerve terminals, allowing effective functioning of neurotransmitter release and reuptake systems (Tarasenko et al., 2006). In another study, lactate appeared to behave in similar manner to glucose for sustaining synaptic vesicle turnover in cultured cortical neurons during activity (Morgenthaler et al., 2006). Repetitive stimulation sequences of FM1-43 uptake and release recorded by fluorescence microscopy in primary cultures of mouse cortical neurons in the absence of any metabolic substrate showed that the number of active sites dramatically decreased after the first cycle of vesicle loading/unloading, while adding 1 mM glucose or lactate was sufficient to sustain synaptic vesicle recycling. Under some conditions, lactate may even be superior to glucose as an energy substrate (Bouzier-Sore et al., 2003), preferentially metabolized in a pyruvate-carboxylase deprived, i.e. neuronal, compartment (Bouzier et al., 2000), but a recent study on cultured cerebellar granule cells using NMDA stimulation concluded that lactate on its own is not sufficient to sustain synaptic function and that glucose is required (Bak et al., 2006). Because of its relatively small impact on ion gradients, inhibitory neurotransmission is much less energetically demanding than excitatory neurotransmission (Attwell and Laughlin, 2001). Consistently, it was reported that GABA uptake into astrocytes does not cause an activation of glycolysis such as observed with glutamate (Chatton et al., 2003).

Microsensor and microdialysis studies *in vivo* have shown that excitatory neuronal activity causes an increase in extracellular lactate that accompanies increases in glucose and oxygen consumption (Hu and Wilson, 1997a,b; Caesar et al., 2008). In one of these studies the lactate rise was preceded by a transient fall in lactate concentration (Hu and Wilson, 1997b), which was also observed by NMR spectroscopy in human subjects (Mangia et al., 2003). Given the simultaneous increase in oxygen consumption, these changes suggest an increase in local lactate demand followed by a larger increase in local lactate production. The identities of both the lactate sink and the lactate source are controversial. There are at present two extreme views of metabolic coupling in the brain, which accommodate the higher rate of oxygen consumption and ATP synthesis in neurons, but differ sharply in the identity of the lactate source during activity, and therefore in the direction of lactate flux. The default or conventional model states that neurons capture most of the glucose flux and release some lactate, which is taken up by astrocytes (Fig. 1B). According to this model, astrocytes consume less oxygen but are more aerobic than neurons, i.e. their oxygen to glucose index (OGI) is higher. The alternative model is termed the "astrocyte-to-neuron-lactate shuttle hypothesis" (ANLSH; Pellerin and Magistretti, 1994), which states that astrocytes take most of the glucose and export it in the form of lactate, which is then taken up and oxidized by neurons (Fig. 1C). In this model, the more aerobic cell is the neuron. Several scholarly reviews that ponder the evidence for and against the two models of neurometabolic coupling have been published in recent years (Gjedde et al., 2002; Hertz and Dienel, 2002; Gladden, 2004; Chih et al., 2001; Bonvento et al., 2005; Leybaert, 2005; Dienel and Cruz, 2004; Pellerin et al., 2007; Nehlig and Coles, 2007; Simpson et al., 2007; Mangia et al., 2009a). A fresh addition to the debate is the finding that neurons–but not astrocytes–are constitutively deficient in the activator of glycolysis Pfkfb3. As a result glucose in neurons is diverted towards the pentose phosphate pathway, resulting in high antioxidant status at the expense of energy production (Herrero-Mendez et al., 2009). There are also mathematical models, whose conclusions obtained under different assumptions have supported one model or the other (Aubert et al., 2005; Hyder et al., 2006; Aubert and Costalat, 2007; Mangia et al., 2009b). Our aim here is to focus on the functional properties of the transporters that mediate the flux of glucose and lactate between the perisynaptic compartments.

3. GLUTs and MCTs

The transport of glucose and lactate between the compartments of the mammalian neuropil is respectively mediated by glucose transporters (GLUTs) and monocarboxylate transporters (MCTs), integral membrane proteins of about 500 amino acid residues that belong to the Solute Carrier (SLC) superfamily of membrane transporters (SLC2 and SLC16). The 2D structure of these proteins is conserved, with 12 membrane-spanning helices and three cytosolic domains, the carboxy and amino terminal ends and a large loop between helices 6 and 7 (Uldry and Thorens, 2004; Halestrap and Price, 1999). These cytosolic domains are the main regions of variation between isoforms. Highly hydrophobic, these transporters have been notoriously difficult to crystallize, and more than 20 years after the cloning of the glucose transporter GLUT1 (Mueckler et al., 1985), we are still hoping to peruse their 3D projection. Structure models have been proposed by analogy to other transporters for which 3D structures are available (Salas-Burgos et al., 2004; Simpson et al., 2008; Manoharan et

al., 2006). Functionally, the glucose transporters (GLUTs) are uniporters, which need only one substrate for a full catalytic cycle. Their main physiological substrate is glucose, although they can also transport other minor endogenous molecules like mannose, galactose and dehydroascorbate (Carruthers, 1990; Vera et al., 1993; Dringen et al., 1994; Nualart et al., 2003). The permeation of lactate across the membranes is catalyzed by MCTs, symporters that mediate the obligatory cotransport of a lactate anion and a proton. Lactate transport therefore affects and is affected by pH, an important aspect that will be dealt with below. MCTs also transport pyruvate and ketone bodies, which under some conditions, like lactation and fasting, may be at high enough concentrations to interfere with the movement of lactate (Broer et al., 1998, 1999). All these transporters are bidirectional, moving their substrates down chemical potential gradients. Because they do not move net charges, their driving forces are insensitive to membrane potential. Another common property of GLUTs and MCTs is that they translocate much more efficiently when loaded with substrate than when empty, a phenomenon termed accelerated exchange or transacceleration, which makes them sensitive to the actual concentration of substrate at both sides of the membrane.

In the adult brain, astrocytes express the glucose transporter GLUT1, and the lactate transporters MCT1 and MCT4. Neurons express the glucose transporter GLUT3 and the lactate transporter MCT2 (Simpson et al., 2008; Dienel and Cruz, 2004; Hertz and Dienel, 2002; Pierre and Pellerin, 2005). Specialized glucosensing cells in the hypothalamus express the low affinity GLUT2 (Garcia et al., 2003), whereas the insulin-sensitive GLUT4 is found in some neurons of the hippocampus, the cerebellum and scattered throughout the brain (Choeiri et al., 2002; Piroli et al., 2007; Bakirtzi et al., 2009). GLUT8 has been detected in intracellular organelles in neurons (Schmidt et al., 2009). These distributions are not rigid and can vary during development, between different brain regions and in response to perturbations, for instance after ischemia (Simpson et al., 2008), likely shifting the balance along the spectrum described in Fig. 1.

4. What are the kinetic properties of the transporters and how informative are they?

The kinetic parameters of a transporter may only be used to predict flux if substrate concentrations at both sides of the membrane are known, which unfortunately is not the case in the brain. However, some conclusions may still be drawn. Transport is a saturable process that can be described by a parameter of affinity, K_m, and a parameter of capacity, V_{max}. The parameter K_m is not a direct indicator of the affinity of binding between transporter and substrate for it is also affected by the rates of binding-site translocation. For this reason it is said that the K_m of a transporter is an apparent affinity constant (explained in detail in Barros et al., 2007). The neuronal transporters, GLUT3 and MCT2, show higher apparent affinity for their substrates than their astrocytic counterparts GLUT1, MCT1 and MCT4 (Simpson et al., 2007; Broer et al., 1998, 1999; Halestrap and Price, 1999; Leybaert, 2005; Barros et al., 2007; Simpson et al., 2008). GLUT3 has a K_m of 1–2 mM whereas GLUT1 has a K_m of 5–8 mM; for the lactate carriers, the difference is more marked, since MCT2 has a K_m of 0.5 mM, whereas MCT1 has a K_m of 3–5 mM and MCT4 of 15–30 mM. So what is the significance of these differences in apparent affinity between neuronal and astrocytic transporters?

An obvious consequence of having higher affinity transporters is that neurons are less vulnerable to external fuel shortages. For example, by using the Michaelis–Menten equation, it can be shown that a fall in extracellular lactate from 2 mM to 1 mM would cause a 42% influx decrease in MCT1-endowed astrocytes (K_m of 5 mM), but only a 17% influx decrease in MCT2-endowed neurons (K_m of 0.5 mM). A similar argument is valid for glucose, whose interstitial concentration is not controlled by the brain and can fall significantly during activity and during hypoglycemia. Most cells in the body express low-affinity glucose and lactate transporters, so it seems likely that the presence of high-affinity fuel transporters may have provided an evolutionary advantage, e.g. protecting neuronal demands in times of fuel shortage. The affinity of the transporters may also affect the preferential direction of flux. The glucose transporters and the lactate transporters are functionally symmetrical, and therefore not particularly suited for influx or efflux, regardless of affinity (Carruthers, 1990; Halestrap and Meredith, 2004). However, an apparent asymmetry emerges in the presence of substrate gradients, which is more marked for high-affinity transporters, behaviour that was demonstrated for the lactate transporter by plotting flux versus intracellular concentration at a fixed extracellular concentration (Aubert et al., 2005). The explanation for this apparent asymmetry is that at a fixed extracellular concentration, influx occurs at lower concentration than efflux, and because the process is saturable, influx appears more efficient. Using numerical simulation, it can be shown how high-affinity carriers such as GLUT3 or MCT2 are more efficient at driving influx than efflux; e.g. with an inward gradient of 2 mM driving an influx 300% larger than the efflux driven by a 2 mM outward gradient (Fig. 2A). For a low-affinity carrier like GLUT1 or MCT1, influx is also favoured, but now the difference is only a 70% (Fig. 2B). A third consequence of high-affinity transport can be illustrated using the same plot (Fig. 2C). If, for instance, in the presence of constant extracellular substrate, intracellular substrate concentration decreases because of higher metabolic demand, a cell with a high-affinity carrier will be able to take up more substrate at a given gradient, thus avoiding intracellular fuel shortage. In summary, by virtue of their high-affinity carriers, neurons are better equipped than other cell types, including astrocytes, to endure fuel shortages and react to their own increases in demand. The direction of flux will ultimately be determined by the chemical gradient, but neurons seem better equipped to take up fuels than to release them.

The other parameter of transport is V_{max}, which depends on the turnover (K_{cat}) and on the number of transporters. Knowing V_{max} and K_m for the same cell type *in vivo* has not been possible because of the limited spatial resolution of standard techniques for measuring glucose transport. Short of a complete set of kinetic parameters for astrocytic GLUT1, recent mathematical modelling considered kinetic parameters obtained in erythrocytes, cells that have been extensively characterized and also express the isoform

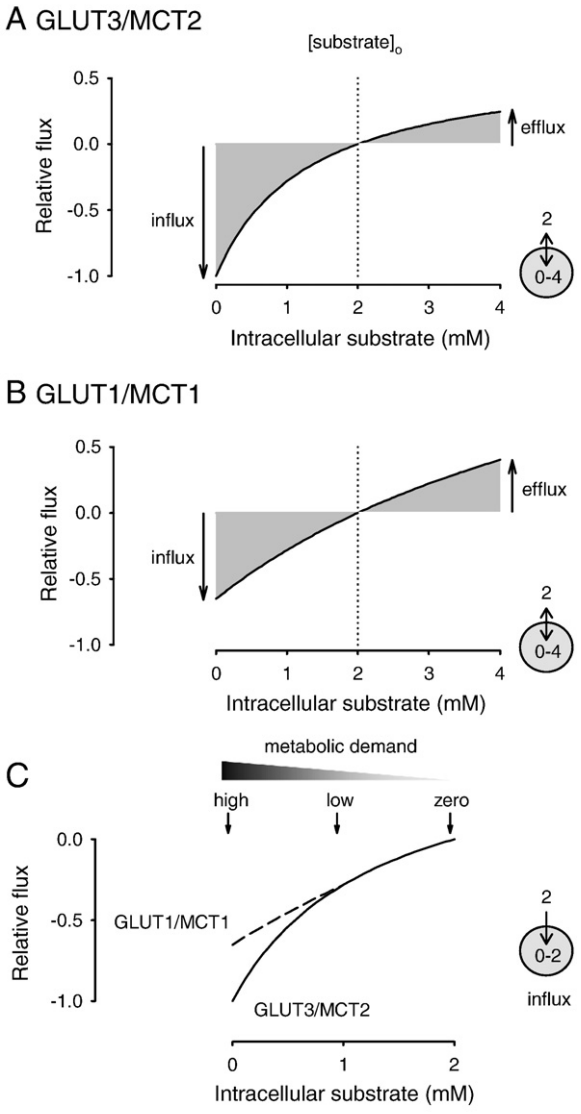

Fig. 2 – Differential behaviour of high-affinity and low-affinity transporters. Fluxes through membrane transporters were simulated at a fixed extracellular substrate (2 mM) and increasing intracellular substrate (0 to 4 mM) using the simple carrier model as described previously (Barros et al., 2007). This model is also valid for the lactate transporter if pH is assumed to be the same at both sides of the membrane. (A) Behavior of a high-affinity transporter like GLUT3 or MCT2 (K_m = 0.5 mM). (B) Behavior of a low-affinity transporter like GLUT1 or MCT1 (K_m = 5 mM). (C) Comparison of high- and low-affinity transporters in terms of their response to a reduction in intracellular substrate concentration by enhanced usage.

GLUT1, and compared them with data obtained in cerebellar granule cells in culture, neurons that express GLUT3 (Simpson et al., 2007). These calculations suggested that GLUT3 K_{cat} may be 7-fold higher than that of GLUT1. By combining with the relative expression of GLUT1 and GLUT3 as assessed by immunoblotting and cytochalasin B binding, it was concluded that neurons transport glucose with a higher capacity, about 12 times more than astrocytes (Simpson et al., 2007). If these extrapolations were representative of the situation in brain tissue, two extreme scenarios may be realized depending on assumptions made about intracellular glucose concentration. If neurons and astrocytes are assumed to present similar intracellular glucose concentrations, i.e. similar transmembrane glucose gradients, it would mean that the glucose flux is 12 times higher in neurons than in astrocytes, supporting the conventional model pictured in Fig. 1B (Simpson et al., 2007; Mangia et al., 2009b). At the opposite end of the spectrum, neurons may contain more glucose than astrocytes and sustain smaller fluxes despite higher transport capacity, thus compatible with the shuttle model in Fig. 1C. Of note, high transport capacity, as described for cultured neurons, does not necessarily correlate with high metabolic rate; for instance the human erythrocyte possesses a high glucose transport capacity but its rate of glucose metabolism is small, resulting in high steady-state glucose concentration. High resolution measurements of glucose transport parameters and glucose concentration in neurons and glial cells in the same region of the brain tissue would seem to be required in order to make progress with respect to these questions. In line with this, using multiphoton microscopy we recently observed that in acute cerebellar slices, Bergmann glial cells transport fluorescent glucose analogs at higher rates than neighbouring Purkinje cells and granule cells (Barros et al., 2009b).

5. Control of flux versus control of concentration

In the steady-state, a change in the kinetic properties of a transporter will affect flux, concentration or both. This will depend on how the transporter is poised in relation to the other components of the pathway. For instance, the glucose transporter GLUT1 in the human erythrocyte is so abundant relative to the activity of hexokinase, that the intracellular concentration of glucose in the steady-state is close to that in the plasma, and the first downstream enzyme, hexokinase, is highly saturated. Given this disposition, a moderate change in the efficacy of the erythrocytic glucose transporter will cause a small change in intracellular glucose and virtually no change in glycolytic flux. Adipocytes are at the opposite end of the spectrum, expressing few GLUT4 glucose transporters at the cell surface under resting conditions. This determines a resting glucose concentration close to the K_m of hexokinase and allows the transporter to exert a high degree of control over glycolytic flux in response to insulin, a regulation that becomes defective in diabetes mellitus. So how do transporters in the brain behave in relation to metabolic flux? Glucose concentration falls from a maximum in the blood to a minimum in the cytosol of astrocytes and neurons. At normoglycemia of 5.5 mM, the average resting concentration of glucose in the human brain tissue is close to 1 mM, well established by the concourse of several techniques including NMR spectroscopy (reviewed in Barros et al., 2007). Thus, the glucose gradient between blood and tissue is 4.5 mM, about 80% of the maximum possible gradient. This means that any increase in metabolic flux

larger than 20% necessarily requires an increase in permeability, i.e. stimulation of the glucose transporters (Barros et al., 2005). Measurements of resting interstitial glucose concentration with microprobes and microdialysis are typically in the range of 1–2 mM, suggesting that the endothelium plays an important role. However, since we do not know the exact intracellular concentrations, it is neither possible to tell how much control is exerted by parenchymal cells, nor how different neurons and astrocytes are in this respect. Measurements in cultured cells using a FRET-based glucose nanosensor show that non-stimulated astrocytes bathed in 1 mM glucose keep an intracellular glucose level of about 0.3 mM, which falls to near zero in response to stimulation of glycolysis by neuronal signals (Bittner CX and Barros LF, unpublished data). So at least in culture, the glucose transporter of astrocytes is poised to regulate glycolytic flux, much in the same way as the adipocyte transporter. There is no available information yet about neuronal glucose concentrations, *in vivo* or in culture.

The question of flux control versus concentration control also applies for lactate/pyruvate and the MCTs in neurons and astrocytes. The acute increase in interstitial lactate observed during neuronal activity (Hu and Wilson, 1997b; Caesar et al., 2008) suggests that an even larger increase occurs inside the cells that produce the lactate. Astrocytes and neurons differ in their lactate dehydrogenase (LDH), the enzyme that metabolizes lactate to pyruvate. In contrast to hexokinase, which is unidirectional, LDH is bidirectional and may produce or consume lactate depending on the concentrations of lactate, pyruvate and the NADH/NAD^+ ratio. Neurons and astrocytes differ in the kinetic properties of LDH, with the neuronal isoform LDH1 presenting a higher affinity for lactate and pyruvate than the astrocytic isoform LDH5 (Bittar et al., 1996). It can be shown that the consequences of this differential affinity are analogous to those discussed for the transporters: i.e. high affinity LDH1 makes neurons better than LDH5-endowed astrocytes at enduring lactate shortages and also more efficient at coping with their own rises in pyruvate demand. The high affinity LDH1 isoform also makes them better equipped to metabolize lactate into pyruvate rather than the reverse reaction.

6. Regulation of GLUTs by synaptic activity

In the first seconds of activation, the concentration of glucose in the brain interstitium goes down (Silver and Erecinska, 1994; Hu and Wilson, 1997a), consistent with increased glucose demand by parenchymal cells. Experiments in cultured cells with fluorescent glucose analogs that permeate through the glucose transporters (Barros et al., 2009a) have shown that within seconds of exposure to glutamate, the activity of the astrocytic glucose transporter GLUT1 increases by a factor of 2–3 (Loaiza et al., 2003). This effect is mediated by synergistic increases in intracellular Na^+ and Ca^{2+}, secondary to the activation of the Na^+/glutamate cotransporter, an effect that requires an active Na^+/K^+ATPase (Porras et al., 2008). Several studies *in vitro* and *in vivo* have shown the involvement of both the Na^+/glutamate cotransporter and the Na^+/K^+ ATPase in the regulation of glycolytic flux in astrocytes (Voutsinos-Porche et al., 2003; Pellerin et al., 2007). Activation of glutamate uptake into astrocytes resulted in the generation of propagated Na^+ waves, which gave rise to a spatially correlated increase in glucose uptake (Bernardinelli et al., 2004). In marked contrast to astrocytes, neurons in the same culture responded to glutamate with an inhibition of their glucose transporter GLUT3, an effect that also required Na^+ and Ca^{2+} signals, mediated by ionotropic glutamate receptors (Porras et al., 2004). The possible impact of these regulations for the tissue depends on how glycolysis responds to neuronal activity in the short term. If astrocytic glycolysis is activated swiftly by synaptic activity, the simultaneous activation of GLUT1 will limit the decrease in intracellular glucose and prevent hexokinase desaturation, allowing an increase in flux. A higher level of intracellular glucose will also moderate the rate of glucose influx from neighbouring astrocytes via gap junctions (Rouach et al., 2008). In neurons, inhibition of GLUT3 by glutamate may hamper glycolytic flux, sparing NAD^+ for the use of LDH and thus promote the uptake of lactate (Cerdan et al., 2006). Taken together, these effects would redirect glucose from neurons to astrocytes, thus supporting the lactate shuttle model (Fig. 1C).

In addition to the effects of glutamate on glucose transport in astrocytes and neurons, which develop over seconds, there have been reports of slower modulation of glucose uptake in these cells. In cultured neurons, deoxyglucose uptake was inhibited by ascorbic acid loading (Patel et al., 2001; Castro et al., 2007). An intriguing aspect of these data is that ascorbic acid inhibited the uptake of deoxyglucose, a substrate for hexokinase, but not the uptake of methylglucose, which is transported but not phosphorylated (Carruthers, 1990). This divergence may be explained by the presence of cross-talk between hexokinase and GLUT3 and/or to a direct effect of ascorbic acid on metabolism as reported by Patel et al., (2001). A recent study of cerebellar granule cells concluded that GLUT3 translocates to the cell surface in response to glutamate, an effect that was detected 30 minutes after transient exposure to the neurotransmitter (Weisova et al., 2009). However, these data are controversial, because surface GLUT3 was detected with an antibody against the COOH terminus domain of the protein, a region that is thought to reside intracellularly and which is unavailable to antibodies (Baldwin, 1993; Simpson et al., 2008).

7. Regulation of MCTs by synaptic activity

As H^+ is a co-substrate, transport activity through MCT is dependent on the pH gradient across the cell membrane (Broer et al., 1998, 1999). Thus, extracellular acidification and intracellular alkalinization would promote lactate uptake into cells, and extracellular alkalinization and intracellular acidification would promote lactate release. Particularly at low lactate concentrations, lactate flux across the cell membrane is largely dominated by the H^+ gradient (Becker and Deitmer, 2008). If larger amounts of lactate are produced, as occurs during activity or under hypoxic conditions, lactate could be carried out of the cells even against a H^+ gradient. Neuronal activity leads to glial alkalinization, a phenomenon observed the rat cortex *in vivo* (Chesler and Kraig, 1987) and in several

preparations *in vitro*, where it was found to be due to HCO_3^- entry through the Na^+/HCO_3^-cotransporter NBC mediated by K^+-induced depolarization (Deitmer and Szatkowski, 1990; Pappas and Ransom, 1994; Deitmer and Rose, 1996). A second mechanism of glial alkalinization was also HCO_3^- dependent and mediated by metabotropic glutamate receptors (Amos and Chesler, 1998). Simultaneously neurons acidify, through a number of mechanisms including production of metabolic acid, H^+ entry through the Ca^{2+} pump, and others (reviewed in Chesler, 2003). The neuron-to-astrocyte H^+ gradient that builds up during activity may reach up to 0.4 pH units or 2.5-fold (Chesler, 2003), and would have to be counterweighed by an opposite lactate gradient of at least 2.5-fold, for lactate to be shuttled from astrocytes to neurons.

In glial cells, where MCT1 and NBC operate together, the shuttling of bicarbonate by NBC can support lactate transport by increasing the effective H^+ buffering capacity (Becker and Deitmer, 2004). Heterologously expressed in *Xenopus* oocytes, it was shown that the presence of NBC doubles the lactate transport capacity of MCT1 (Becker et al., 2004). This suggests that the base-transporting NBC also cooperates with the MCT1 in astrocytes, which show high expression of both MCT1 and NBC (Schmitt et al., 2000; Anneke, 2003; Pierre and Pellerin, 2005). Furthermore, carbonic anhydrase isoform II (CAII), but not carbonic anhydrase isoform I (CAI), enhances lactate transport via MCT1, a process that does not depend on the catalytic activity of the enzyme, but presumably on binding of the two proteins (Becker et al., 2005; Becker and Deitmer, 2008). It was suggested that CAII helps to clear H^+ from the mouth of MCT1 during lactate uptake, thus slowing the dissipation of the H^+ gradient across the oocyte membrane. Hence, by means of protein–protein interaction, MCT activity appears to be subject to regulation or modulation by associated and/or functionally related proteins, which may directly bind to the carriers, or help to maintain the cellular H^+ gradient. There is also evidence for extracellular (interstitial) location of CA activity (Chen and Chesler, 1992; Deitmer, 1992; Tong et al., 2000), which could enhance lactate transport across cell membranes in general (Becker et al., 2005), and in membranes of glial cells and neurons in particular (Svichar and Chesler, 2003).

In a hypothetical scheme, lactate flux between perisynaptic astrocytes and pre-and postsynaptic neuronal elements may be promoted during synaptic activity (Fig. 3). During synaptic activity the exocytosis of acid vesicles, estimated as having a pH of 5.2 to 5.7 (Miesenbock et al., 1998), a considerable, dynamic fall of pH in the synaptic cleft is expected, which may approach the pH of the vesicles during high frequency exocytosis, i.e. below 6. The stronger acidication in the cleft and its immediate vicinity would favour lactate uptake into neurons via MCT2, which in addition would acidify the neuronal compartment. Since the lactate concentration would rapidly decline in this small synaptic domain, perisynaptic astrocytes, which may experience a less aggravated acidity than the neuronal synaptic compartments may then re-supply lactate to the synaptic domain, driven mainly by the lactate gradient. Thus, the transient, strong, local acidification in the synaptic cleft would promote a net uptake of lactate in the pre- and/or postsynaptic compartments, even at lactate concentrations below 1 mM, attributable to the high affinity of neuronal MCT2 and the transiently large H^+ gradient across the neuronal cell membranes. Secretion of bicarbonate via the glial sodium/bicarbonate cotransporter NBCe1 from astrocytes, may counteract the acidosis in the immediate glial environment, thus suppressing lactate uptake by perisynaptic astrocytes, and hence allow more lactate release via MCT1 and MCT4.

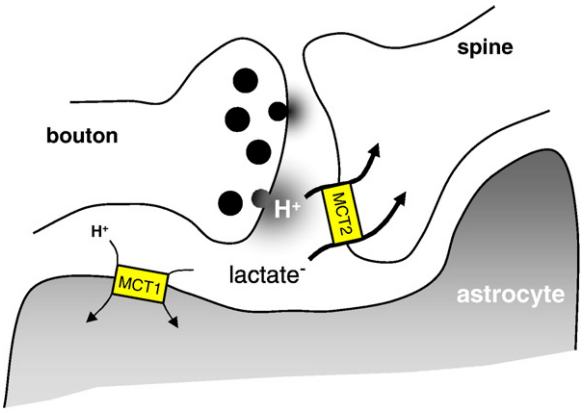

Fig. 3 – Preferential uptake of lactate by neurons in response to synaptic activity. Synaptic activity causes local acidification in the synaptic cleft, predicted to drive the uptake of lactate preferentially into neurons. This is due to the postsynaptic location and the higher lactate affinity of MCT2, whereas the astrocytic isoform MCT1 is located further away and shows a lower lactate occupancy than MCT2.

There are other proteins, which have been found to be essential for functional expression of MCTs in the cell membrane. Both MCT1 and MCT4 appear to require co-expression of the accessory protein CD147, also known as basigin, an extracellular matrix metalloproteinase inducer (Kirk et al., 2000). The elimination of the basigin gene greatly reduced the strength and distribution of MCT1 in tissues where both proteins are normally expressed. On the other hand, localization of MCT1 in tissues, where basigin was absent in wild-type, was not affected in the basigin knockout mouse model. Since MCT1 mRNA and protein levels were normal in basigin knockout animals, it was concluded that basigin is required for incorporation into the membrane (Nakai et al., 2006). In the retina, however, a dramatic decrease of MCT1 expression was observed after loss of the basigin gene (Philp et al., 2003). A different accessory protein, gp70 or embigin, is needed for the functional expression of MCT2 in neurons (Wilson et al., 2005). Both accessory proteins are widely expressed membrane proteins with immunoglobulin-like extracellular domains and a short intracellular C-terminus. The association with these different proteins apparently also confers the different sensitivity of MCT1 and MCT2 to the thiol reagent p-chloromercuribenzene sulfonate (*p*CMBS; (Wilson et al., 2005).

Bergersen et al. (2005) demonstrated, using quantitative double-labeling immunogold cytochemistry, that MCT2 and AMPA receptor GluR2/3 subunits have a similar postsynaptic distribution at asymmetric synapses with high levels expressed within the postsynaptic density. MCT2 was found

at the postsynaptic density of asymmetric synapses, in the stratum radiatum of both rat hippocampal CA1 and CA3 regions, as well as at parallel fibre-Purkinje cell synapses in mouse cerebellum. MCT2 levels were significantly lower at mossy fibre synapses on CA3 neurons, and MCT2 was almost absent from symmetric synapses on CA1 pyramidal cells. More recently, it was shown that MCT2 is associated in a common trafficking process with AMPA receptor GluR2/3 subunits and that MCT2 translocates upon neuronal activation, increasing the rate of lactate transport (Pierre et al., 2009). These results indicate that MCT2 is not merely a passive link subject to its substrate gradients but is also subject to a complex modulation imposed by neuronal activity.

8. Conclusions

Synaptic domains are not only the pivotal structures for information processing, but also the sites with the highest energy demands in the brain, attributable to the maintenance of ion gradients across the postsynaptic membranes, which are constantly challenged during synaptic activity. The supply of energy to synaptic regions, the distribution of fuels in the tissue and the utilization of these compounds by neurons and glial cells involve complex and highly regulated processes. The proteins that mediate the transport of glucose and lactate between neuronal and astrocytic compartments are increasingly regarded as prime sites for the regulation of local energy flux, but high spatiotemporal resolution recordings of glucose and lactate concentrations, both intra- and extracellularly, are urgently needed to ascertain the roles of the transporters in the control of flux and concentrations. Based on low resolution isotope measurements, current models of energy distribution in the brain picture a time-invariant tissue where all neurons behave similarly, but electrophysiology and neurobiology make it increasingly clear that there may not be such thing as the “typical” neuron or even the “typical” astrocyte, so that a great diversity of strategies whereby cells solve their individual energy problem ought to be expected. The combination of powerful imaging techniques like multiphoton microscopy and increasingly available fluorescent probes for ions and metabolites should open the way to a new level of understanding of glucose and lactate dynamics in the brain tissue, at the single cell level and with resolution of seconds.

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BRAIN RESEARCH REVIEWS

Review

Pharmacological and genetic approaches to study connexin-mediated channels in glial cells of the central nervous system

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ABSTRACT

This review gives an overview of connexin expression in glial cells of the central nervous system, the different modes of connexin action, including gap junctional channels and hemichannels, as well as the available methodologies to measure their activity. We summarize the strengths and limitations of current pharmacological and genetic approaches to interfere with connexin channel functions. We outline new avenues not only to study specific mechanisms by which connexins exert these functions but also to selectively investigate well-defined coupling compartments among glial networks.

Contents

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Abbreviations: CNS, central nervous system; Cx, connexin; GJC, gap junction channel; HC, hemichannel; Panx, pannexin; PNS, peripheral nervous system; RG, radial glia; SGZ, subgranular zone

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1. Introduction

Neurons and glial cells (astrocytes, oligodendrocytes, NG2, and microglia) represent the two main cell populations in the central nervous system (CNS). Throughout phylogeny, their respective proportion reverts, and in higher vertebrates, the number of glial cells surpasses that of neurons. Indeed, while nematodes have only 25% of glia compared to neurons, this ratio grows to 65% in mice and reaches 91% in humans (Allen and Barres, 2009). Although the exclusive function of the brain is the processing of information pertaining to the relationship of the body with its environment, it is not yet understood what are the meaning and consequences of such a change in the relative ratio of glia to neurons. Indeed, while it is now well known how neurons work, an active contribution of glia to information processing only starts to be considered. Interestingly, there is increasing evidence that glial cells participate in this task by receiving and integrating neuronal signals and, in turn, by sending signals targeted to neurons (Haydon and Carmignoto, 2006; Verkhratsky and Toescu, 2006; Volterra and Meldolesi, 2005). Such achievement requires a number of glial properties that have started to be identified (Kettenmann and Ransom, 2005) including the need for cells to communicate together efficiently and specifically as neurons do, thanks to synaptic transmission. To achieve this goal, several alternative pathways for intercellular communication exist in glia. Among them, one that is particularly expressed in glia is mediated by gap junctions. Such membrane specializations are also found between neurons at "electrical" synapses, but in much lower amounts since chemical synapses prevail. This mode of intercellular communication is based on membrane channels made by a family of multigenic proteins named connexins (Cxs). Indeed, at the plasma membrane, Cxs are organized as hexamers (connexons) forming a ring around a central pore, and when two connexons are face-to-face, they constitute an intercellular channel. These channels aggregate at areas of close apposition between cells and form junctional plaques called gap junctions. In fact, Cx channels provide the structural basis for two types of intercellular communication: (i) gap junction channels (GJCs) that allow direct cell-to-cell exchanges with a poor selectivity for ions and for small molecules with a molecular weight up to 1–1.2 kDa (Harris, 2007); (ii) alternatively, connexons can also function as hemichannels (HCs) that enable, under well-defined conditions, the exchange of molecules and ions between the cytoplasm and the external medium and thus support autocrine and paracrine actions (Spray et al., 2006). Furthermore, HCs can also be formed by pannexins (Panxs), another family of membrane proteins homologous to innexins, the GJC-forming proteins in invertebrates (Scemes et al., 2007). Sequence analysis indicates a transmembrane topology of Panxs similar to that of Cxs but a great divergence in primary sequence (Baranova et al., 2004; Panchin et al., 2000). Moreover, Panxs do not seem to form GJCs (Scemes et al., 2007; but see Bruzzone et al., 2003), while to a certain extent, Panx and some Cx HCs exhibit similar unitary conductance and pharmacology (see below) in spite of strong sequence diversity. The present review will be mostly focused on how to study Cx channel properties, while Panx channels will be solely evoked when tools and approaches can help to discriminate between the molecular constituents of these two types of HC components.

Furthermore, besides forming membrane channels, glial Cxs are also involved in other functions that can affect neuroglial interactions. Indeed, they act as adhesive molecules contributing to neuronal migration without the need for functional GJCs (Cotrina et al., 2008; Elias et al., 2007). The cytoplasmic C-terminal domain of Cx43 was reported to modulate activity of the P2Y1 receptor in astrocytes (Scemes, 2008). Also, pharmacological or genetic disruption of astrocytic

gap junctions decreased the expression of glial glutamate transporters (Figiel et al., 2007). Finally, in astrocytes from Cx43 knockout mice, the lack of this Cx was shown to affect gene expression suggesting the occurrence of Cx-dependent transcellular transcriptomic networks in the brain (Iacobas et al., 2007). Although these aspects are important, here we will restrict our focus to the contribution of Cxs to neuroglial interactions based on their channel functions. Also, as most information related to this topic comes from studies on astroglial Cxs, the present review will discuss on how to study Cxs in these glial cells. Within such frame, we will list and consider which tools are available to study the role of Cx channels in the context of neuroglial interactions and information processing and we will discuss the pros and cons of the two main attempts generally used to achieve this goal, i.e., pharmacological and genetic approaches.

2. Molecular nature of glial connexins and functional consequences

2.1. *Molecular constituents of Cx channels in glia*

So far, 21 different genes encoding for Cxs have been identified in the human genome and 20 genes in the mouse. Eleven Cxs have been detected in the rodent brain. Recently, several reviews have summarized and discussed what is known about the pattern of expression of these Cxs in the different brain cell types (Nagy et al., 2004; Ransom and Ye, 2005; Rouach et al., 2002; Theis et al., 2005). The main message is that all brain cell populations express more that one Cx in a combination which is unique for each cell type. This statement implies that two adjacent cells can be connected by more than one Cx type. Such richness of expression offers multiple combinations and opens the way to encounter homotypic and homomeric as well as heterotypic and heteromeric channels between brain cells (Bruzzone and Giaume, 1999). However, although data are available for GJCs made by various combinations of several brain Cxs in transfected cells (Bukauskas and Verselis, 2004; Gonzalez et al., 2007), so far there is no evidence for such diversity in glia based on *in situ* analysis. Indeed, so far, no functional data (single channel recording) have permitted to distinguish between these various molecular organizations in glia, and the only evidence for heterotypic GJCs comes either from ultrastructural and freeze-fracture immunogold labeling observations (Nagy et al., 2004) or from examples of dye coupling between astrocytes and neurons in cocultures (Froes et al., 1999) or acute slices from developing brain (Alvarez-Maubecin et al., 2000; Bittman et al., 2002; Pakhotin and Verkhratsky, 2005) as well as between astrocytes and oligodendrocytes in cocultures (Venance et al., 1995) or in the intact retina (Robinson et al., 1993). Table 1 summarizes the pattern of expression of Cxs in different CNS glial cell types based on previous works on this topic. Although astrocytes and oligodendrocytes have clearly different sets of Cxs (Fig. 1), the oligodendrocytic Cx29 is also present in Bergmann glia, an astroglial cell subtype in the cerebellum (Altevogt and Paul, 2004; Eiberger et al., 2006; Theis et al., 2005) and the oligodendrocytic Cx47 shows some overlap with the marker S100β (Odermatt et al., 2003). In addition, the expression patterns of Cxs in the CNS are rather plastic: in mice lacking Cx43 in astrocytes, the protein level of Cx30 is increased (Nakase et al., 2004; Theis et al., 2003, 2004) and Cx30 transcripts are upregulated in astrocytes and neurons upon induction of seizures (Condorelli et al., 2002). The repertoire of Cx-expressing glial cells in the adult mouse brain has received a recent addition since Cx expression was reported in radial glia (RG) of a neurogenic niche in the hippocampus (Kunze et al., 2009). In the subgranular zone (SGZ) of the adult dentate gyrus, RG-like cells, a specialized subtype of astrocytes, represent stem cells giving rise to new neurons and glial cells (Kempermann et al., 2004). Recent work shows that a majority of RG-like cells is coupled and that both Cx43 and Cx30 mediate intercellular coupling between RG-like cells in the adult dentate gyrus. Interestingly, Cx26 was also found in RG-like cells at the transcript level, but did not seem to have an impact on interastroglial communication in the stem cell population (Kunze et al., 2009). Finally, it should be noted that the expression of Cx26 in astrocytes of the brain is still a matter of debate (Rouach et al., 2002; Theis et al., 2005) and remains to be clarified, using mice with deletion of Cx26 in the CNS, to verify the specificity of available Cx26 antibodies.

Table 1 – Cellular distribution of connexins and pannexins in brain glial cells.

Cell types	Connexins GJCs	Connexins HCs	Panxs HCs
Astrocytes	Cx43, Cx30, Cx26	Cx43	Panx1
Oligodendrocytes	Cx32, Cx47, Cx29	ND	ND
Microglia	Cx43, Cx36, Cx32	ND	ND
Astrocytes/ oligodendrocytes	Cx30/Cx32, Cx43/ Cx47	—	—
Astrocytes/neurons	Cx43, Cx26/Cx32	—	—

The two communicating functions of these membrane proteins are considered, i.e., GJCs and HCs. This table summarizes data that have been previously reviewed and discussed (Nagy et al., 2004; Orellana et al., 2009; Rouach et al., 2002; Theis et al., 2005). Note that the expression Cx43 in microglia was only reported when these cells are activated by peptidoglycans or interferon γ. In contrast, there is no functional or immunological evidence for the expression of Cx43 in microglia that is either resting or is activated by compounds other than peptidoglycans or interferon γ (Meme et al., 2006). Also, Cx32 and Cx36 have been detected in microglia (Takeuchi et al., 2006; Dobrenis et al., 2005). Gap junctions between astrocytes and neurons are rather infrequent but positive immunolabeling has been reported in the locus coeruleus (Alvarez-Maubecin et al., 2000) and in the cortex (Bittman et al., 2002; but see Rash et al., 2001). Finally, so far, neither Cx nor Panx expression has been reported in NG2 cells, and several studies indicate that these cells are not coupled by GJCs (Houades et al., 2008; Wallraff et al., 2004).

2.2. *Glial coupling compartments and networks*

Interastroglial gap junctions are prominent and contribute to decrease elevated extracellular potassium concentrations near synapses of active neurons by a process called spatial buffering (Wallraff et al., 2006) as well as to provide glucose from the bloodstream for neurons to sustain their activity. However, GJC function can be subjected to modulation (Rouach et al., 2008) and interastroglial coupling is not

uniform, but is instead favored within anatomical or functional neuronal compartments (Fig. 1), forming coupling compartments in the barrel cortex (Houades et al., 2008) and in the olfactory glomeruli (L. Roux and C.G., unpublished data; but see Houades et al., 2006 and Konietzko and Müller, 1994 for hippocampus). In addition, the RG-like cells in the subgranular zone of the hippocampus expressing Cx43 and Cx30 show only very weak intercellular coupling (Kunze et al., 2009) while at the same time the astrocytes in the hippocampus expressing the same Cxs are extensively coupled to each other (Rouach et al., 2008; Wallraff et al., 2004). Since the term "syncytium" implies unrestricted intercellular coupling, we suggest using the term "networks" to refer to the cellular pathways designed by Cx-mediated communication between astrocytes, between oligodendrocytes and between astrocytes and oligodendrocytes, a term that better reflects the spatial limitation, the regulation and heterogeneity in gap junctional coupling.

The close apposition of astrocytic and oligodendrocytic Cxs (Lutz et al., 2009; Nagy and Rash, 2000) as well as the demonstration of a heterotypic coupling in cocultures between both cell types (Venance et al., 1995) and in the intact retina (Robinson et al., 1993) suggest functional heterocellular coupling (Fig. 1; see Theis et al., 2005, and references therein), and the term of a panglial syncytium was coined following the demonstration of such frequent apposition. Now, we further suggest applying the term "panglial networks" to the oligodendroglial–astroglial (panglial) coupling. In such a panglial compartment, the combined activity of astroglial and oligodendroglial Cxs might mediate spatial buffering by clearing potassium ions from the adaxonal space and subsequently utilizing astrocyte-mediated spatial buffering (Theis et al., 2005).

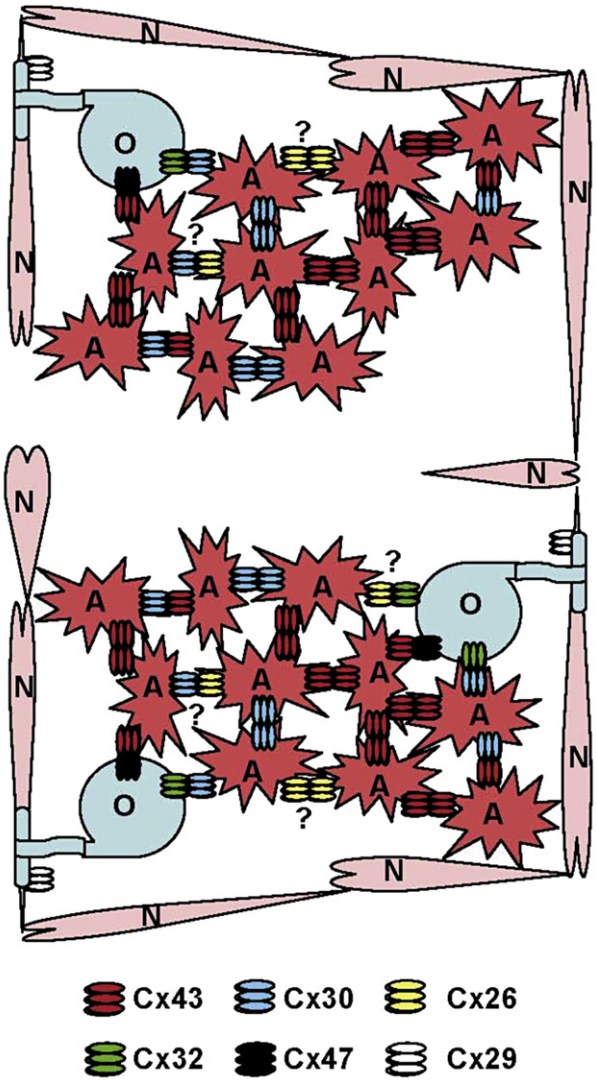

Fig. 1 – Schematic view of glial connexin distribution and arrangement of coupling compartments. For simplicity, only homomeric gap junctions are shown in this drawing. Interastroglial gap junctions are prominent, but interastroglial coupling may be restricted by anatomical or functional borders and astroglial coupling is not uniform and subject to modulation. Close apposition of astroglial and oligodendroglial Cxs suggests functional heterocellular coupling which might contribute to mediate spatial buffering by clearing potassium ions from the adaxonal space. However, data on panglial coupling *in situ* are not conclusive and convincing functional evidence for such panglial coupling compartments in the brain does not exist yet. Note that Cx29 is regarded as forming hemichannels only. The separation indicates functional or anatomical borders of intercellular coupling leading to the formation of coupling compartments. Note that, although represented here, the expression of Cx26 in astrocytes is still under debate. N, neuron; A, astrocyte; O, oligodendrocyte. Modified from Theis et al. (2005).

3. Methods to study Cx channel function in glia: advantages and limits

Typically, functional study of Cx channels is based on their permeability properties for ions (ionic coupling) or for small molecules (biochemical or metabolic coupling). While the electrophysiological recording of HC activity can be performed by a classical patch-clamp approach, monitoring of GJC currents needs to be achieved with the double patch-clamp technique (Giaume et al., 1991). Alternatively, intercellular passage and uptake of fluorescent dyes or tracers provide an index of GJC or HC activities, respectively. In these cases, the use of fluorescent dyes provides a useful tool to evaluate coupling and uptake processes, although they are not themselves biologically relevant. In fact, several laboratories start to use probes with a biological relevance to follow the activity of GJCs and HCs, and this is certainly a direction to pursue since they could help to understand the role of these channels in the glial contribution to brain physiology and pathology. Finally, when these approaches are applied to glial cells, several technical limitations have to be kept in mind to avoid overinterpretation.

3.1. *Electrophysiological study of GJCs*

As GJCs connect the cytoplasm of adjacent cells, electrophysiological recording of their activity requires the simultaneous control of voltage in the two connected cells (Spray et al., 1979). In cultured cells, this can be achieved by recording junctional currents in pairs of astrocytes (Dermietzel et al.,

1991; Giaume et al., 1991) or oligodendrocytes (Venance et al., 1995) using the double patch-clamp technique. Such an approach allowed monitoring of single-channel events in astrocytes. In contrast, when double whole-cell recordings are performed in acute brain slices, the morphology and cable properties of glial cells do not favor accurate recording of junctional currents (Meme et al., 2009; Xu et al., 2009). Moreover, this drawback is enhanced by the fact that the pair of recorded cells is also coupled to other cells in which voltage cannot be controlled. Consequently, junctional currents monitored in this configuration are underestimated. Nevertheless, as illustrated in Fig. 2, this technique permits to follow the kinetics of changes in ionic coupling during pharmacological treatments (Meme et al., 2009).

3.2. *Electrophysiological study of HCs*

In glial cells, the main membrane conductances are sustained by potassium channels (Olsen et al., 2006). As astrocytes express numerous ionic channels, HC activity has to be studied under conditions which block other channels. Once these channels are inactivated by external and/or internal channel blockers (TEA, 4-AP, etc.), HC currents can be recorded, at macroscopic or unitary levels, in the whole-cell

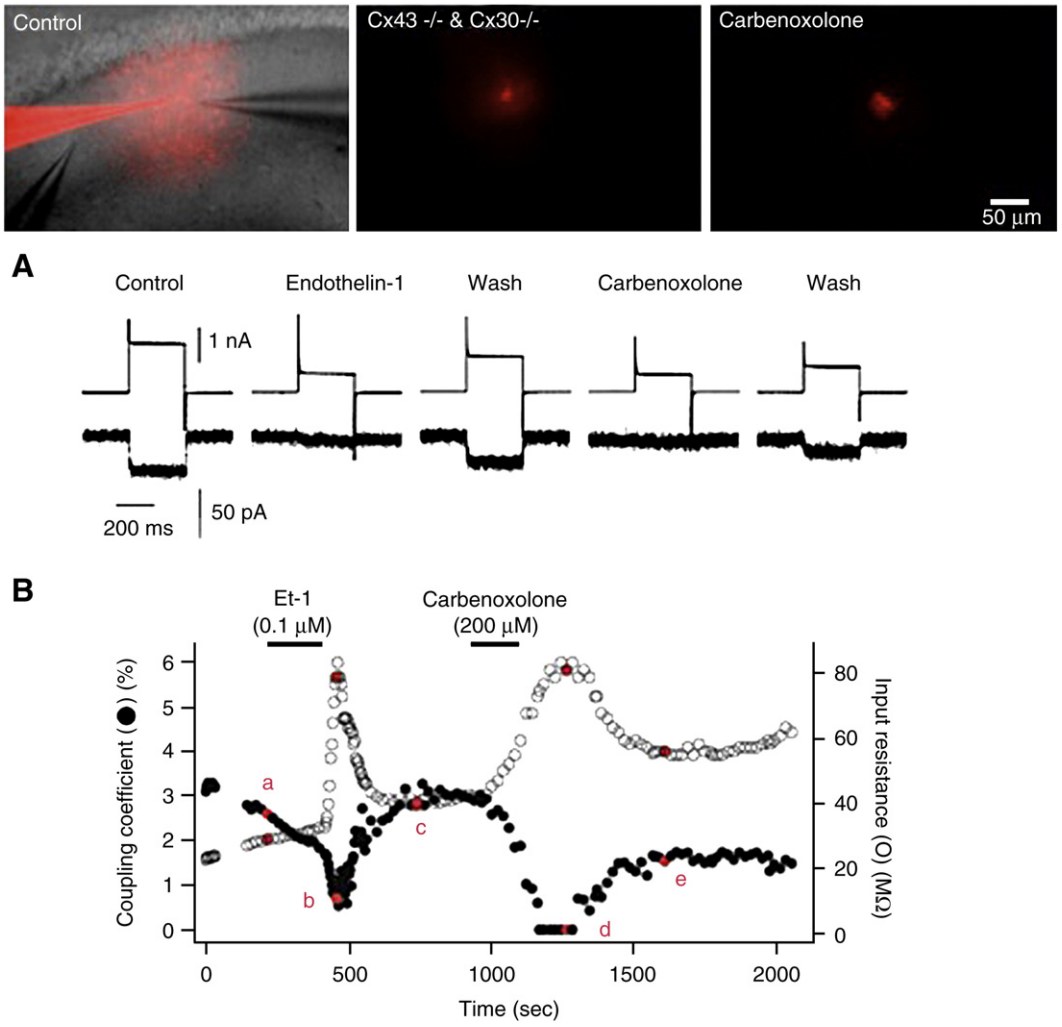

Fig. 2 – Dye coupling experiments and recording of junctional currents in astrocytes studied from acute hippocampal slices. Upper part: dye injection of an hippocampal astrocyte with a patch pipette filled with sulforhodamine B in slices from a wild type mouse (left), from a double knockout for Cx43 and Cx30 mouse (center) and from a wild type slice treated with carbenoxolone 150 µM (right) (data provided by Dr. N. Rouach). Lower part (A) Example of membrane currents (I_1 and I_2; upper and lower traces, respectively) in electrically coupled pairs of astrocytes recorded from a hippocampal slice. Membrane potentials (V1 and V2; not illustrated in the figure) were both held at –80 mV while +40 mV depolarizing pulses were applied for 300 ms to cell 1 (V1) that generated currents I_1 (upper traces) in the stepped cell. Junctional currents I_2 (lower current traces) recorded in cell 2 to maintain membrane potential at –80 mV were abolished after 90 seconds of bath application of endothelin-1 (0.1 µM) or after 5 minutes of bath application of carbenoxolone (200 µM). (B) Time course analysis of the coupling coefficient k (black circles) and input resistance R_i (white circles) of the injected astrocyte in which the depolarizing voltage pulse was applied during transient exposure to gap junction inhibitors. Note the reverse relationship between k and R_i values during the decoupling and recoupling phases of the gap junctional currents. Modified from Meme et al. (2009).

configuration at positive potentials (Contreras et al., 2003; Iglesias et al., 2009), but also for negative potentials in inflammatory astrocytes (Retamal et al., 2007). However, as discussed below, the lack of pharmacological specific tools for Cx channels makes it difficult to formally attribute the recording currents to HC activity. Hence, an alternative way is to compare recordings obtained from wild type astrocytes with those monitored either from Cx/Panx knockout astrocytes or from siRNA silenced astrocytes. Finally, in brain slices, unitary events have also been observed in cell-attached and inside-out recordings and were defined as potentially being due to HC activity (Kang et al., 2008; Lin et al., 2008). Their analysis indicates a very low open probability under resting conditions.

3.3. *Dye coupling experiments*

The most extensively used approach to study GJCs in astrocytes is based on the intercellular diffusion of dyes or tracers with a low molecular weight (< 1 kDa): this includes Lucifer yellow, sulforhodamine B, Alexa dyes, biocytin, and neurobiotin. While the first demonstration of the regulation of astroglial GJCs has been performed in acute cortical slices with sharp electrodes (Connors et al., 1984), a number of subsequent studies were carried out in primary cultures (Giaume and McCarthy, 1996). Now several attempts have been done in hippocampal slices, with patch electrodes, and showed dye spread between more than one hundred astrocytes, indicating that under resting conditions the coupling level is high (Fig. 2). Furthermore, regulation of dye coupling in astroglial networks has been investigated in hippocampal slices using agonists of membrane receptors. Indeed, inhibition of GJCs has been demonstrated by applying endogenous peptides such as endothelins (Blomstrand et al., 2004), while an increase in dye coupling was induced by NMDA application (Serrano et al., 2008). Nevertheless, so far, this approach used to assess the functional status of GJCs remains qualitative and does not inform about the kinetics of these regulatory processes. Another way to look at GJC permeability is to follow the intercellular exchanges of biologically relevant molecules. This was carried out in primary cultures of astrocytes using radiolabeled glucose and lactate (Tabernero et al., 1996) and more recently in culture and in brain slices using fluorescent glucose derivatives (Blomstrand and Giaume, 2006; Rouach et al., 2008).

3.4. *Dye uptake through HCs*

As the opening of HCs permeabilizes cell membranes with a low selectivity, many low molecular weight dyes (Lucifer yellow, ethidium bromide, YoPro, etc.) can be taken up by open HCs. In cultured cells, such property permits kinetic studies. Indeed, time-lapse recording provides access to the kinetics of cell permeabilization when the dyes used become fluorescent only upon binding to DNA, such as ethidium bromide or YoPro. These uptake experiments are easy to perform in cell cultures and can also be achieved in acute brain slices if the identity of the cells that take up the dye is established either by using hGFAP–EGFP mice (see additional information in Rouach et al., 2008) or by performing immunostaining after fixation of the slice. Here also, HC activity has started to be studied by monitoring efflux or influx of molecules having a biological significance. This is the case for the release of "gliotransmitters" by astrocytes, such as demonstrated for ATP using a bioluminescent imaging detection assay (Kang et al., 2008) and for glutamate release analyzed by HPLC (Ye et al., 2003).

A critical limitation of these experimental approaches, in particular dye coupling and dye uptake experiments, is that in most cases the probes used have no biological significance for the studied cells which limits the interpretation. However, recently, the use of fluorescent glucose derivatives (2- and 6-NBDG) has allowed to visualize glucose uptake through Cx HCs (Retamal et al., 2007) and to observe GJC-mediated coupling in confluent cultured astrocytes (Blomstrand and Giaume, 2006). When used in brain slices, NBDGs allow studying metabolic networks in hippocampal astrocytes and their activity-dependent regulation. Moreover, adding glucose or lactate to the patch pipette solution has demonstrated the role astroglial networks play in maintaining neuronal activity when external glucose is absent (Rouach et al., 2008). Finally, intracellular injection and photo-activation of caged-IP3 have permitted to demonstrate permeability of GJCs to this compound involved in calcium signaling between astrocytes studied in acute brain slices (Kang et al., 2008; Mulligan and Macvicar, 2004).

4. Why a need to block connexin channels or to prevent their expression in glia

As Cx-mediated channels are a typical feature of glial cells (see above) and are the basis of glial networks, neuroglial interaction should be dependent of their activity (for example, Kunze et al., 2009; Rouach et al., 2008; Wallraff et al., 2006). To determine the contribution of glial GJCs and HCs in neuronal activity and survival, the way to address this question is either to block their activity by a pharmacological approach or to develop molecular tools to interfere with their expression. This strategy should suppress Cx-mediated glial communication, allowing to determine its consequence on neuronal activity and fate. However, several conditions have to be fulfilled to address these questions and to make correct interpretations. Indeed: (i) the selected approach must have neither side effects nor affect *per se* neuronal properties, development, and/or survival; (ii) as numerous Cx types are expressed in glia (Table 1) and in neurons, the approach should be selective for a defined Cx; (iii) as more than one Cx is expressed in a defined glial cell type, several Cxs have to be targeted to fully prevent intercellular communication; (iv) as Cxs support two channel functions, it is important to be able to discriminate between GJC and HC activity; and finally, (v) since Cxs but also Panxs can form HCs, their respective contribution should be distinguished.

5. Pharmacology of connexin channels in glial cells

For several decades it is known that the activity of Cx channels is regulated by intracellular factors such as calcium concentration and pH (Peracchia, 2004). In addition, transjunctional

and transmembrane voltages also control their activity with specific features depending on the identity of the Cxs involved in channel formation (Bukauskas and Verselis, 2004; Gonzalez et al., 2007). However, as these three parameters are involved in many regulatory processes, their use is excluded when the objective is to suppress specifically GJC or HC activity. Accordingly, along the years several attempts have been carried out to select molecules and design pharmacological strategies that abolish intercellular communication mediated by Cx (or Panxs) channels (Juszczak and Swiergiel, 2009; Rozental et al., 2001).

5.1. *Pharmacology of gap junction channels*

The quest for specific blockers of GJCs has a long history that started more than 30 years and encountered several "fashions" which all failed to reach the Holy Grail. Initially, long-chain alcohols such as octanol and heptanol (Johnston and Ramon, 1981) and well as volatile anesthetics, such as halothane (Johnston et al., 1980), have been extensively used to block GJCs in various cell types, including astrocytes (Giaume et al., 1991; Mantz et al., 1993). However, very rapidly, it became clear that at the efficient concentrations (i.e., mM to μM range, respectively) used to block glial gap junctions none of these compounds were specific. Alternatively, glycyrrhetinic acid derivatives offered another means to inhibit gap junctional communication (Davidson et al., 1986), but with time, it appeared that nonspecific side effects were also present. Through the years, all these molecules have been also used in glial cells and were shown to be efficient in blocking GJCs using *in vitro* as well as *in vivo* preparations. However, side effects in glial cell properties as well as in neurons were also reported including, in some cases, neurotoxicity at the concentrations used to inhibit Cxs channels (Juszczak and Swiergiel, 2009; Rozental et al., 2001). Actually, the most extensively used GJC inhibitor is carbenoxolone, a water-soluble glycyrrhetinic acid derivative, which, when used at 20 μM in cultures (Meme et al., 2006, 2009) and at 100–150 μM in acute brain slices (Blomstrand et al., 2004), has a rapid (<1 min) and reversible inhibitory action on GJCs, as shown by double patch-clamp recording in hippocampal slices (Meme et al., 2009). However, this compound, like other uncouplers, was shown to affect neuronal properties through side effects on voltage-dependent potassium (Rouach et al., 2003) and calcium channels (Vessey et al., 2004), P2X7 purinergic receptors (Suadicani et al., 2006) and NMDA-evoked currents (Chepkova et al., 2008). Also, carbenoxolone has been reported to induce acute mitochondrial damage *in vitro* (Salvi et al., 2005) and *in vivo* (Pivato et al., 2006). This may not be a problem for electrophysiological studies in which the intercellular medium is controlled but makes its use unsuitable for studies in living cells. Finally, several compounds have been shown to preferentially block the activity of GJCs or HCs formed by certain Cxs in transfected cell systems. This could be particularly helpful in the future to selectively withdraw the contribution of a defined Cxs in a defined brain cell population. This is the case of quinine and its derivatives. Indeed, quinine (300 μM) blocks neuronal GJCs made by Cx36 while Cx45 GJCS are moderately affected and Cx26, Cx32, and Cx43 GJCs are not blocked (Srinivas et al., 2001). In addition, quinidine used at the same concentration blocks Cx36 GJCs but so far has not been tested on other Cx GJCs of the CNS (Cruikshank et al., 2004). Also mefloquine at a low concentration (3 μM) completely blocks Cx36 GJCs and has no effect on Cx26, Cx32, and Cx43 GJCs, while at a higher concentration (30 μM), it inhibits almost completely Cx43 GJCs and significantly affects those composed by Cx26 and Cx32 (Cruikshank et al., 2004). Interestingly, Panx1 HCs are 1,000- to 10,000-fold more sensitive to mefloquine than Cx43 GJCs (Cruikshank et al., 2004; Iglesias et al., 2008). Alternatively, compounds of the fenamates family have also been reported to inhibit GJCs composed by Cx43 with the following sequence of efficiency: meclofenamic acid > niflumic acid > flufenamic acid (Harks et al., 2001). In addition, flufenamic acid was shown to inhibit with a low selectivity GJCs composed of Cx26, Cx32, and Cx43 (Srinivas and Spray, 2003). Finally, Panx1 HCs have the following sequence of sensitivity for several of the above cited family of blockers mefloquine > carbenoxolone > flufenamic acid (Iglesias et al., 2008). However, like other Cx channel blockers mentioned above, quinine derivatives and fenamates have also side effects and bind to other targets such as ionic channels, membrane receptors, and glycoproteins (Juszczak and Swiergiel, 2009).

5.2. *Pharmacology of Cx HCs*

Due to their similar molecular constitution, HCs are sensitive to most of the regulatory factors and compounds listed above for GJCs. But the options for targeting HCs also differ from that of GJCs because one extremity of the channel is directly accessible from the extracellular space. For instance, divalent cations act as HC blockers with Zn^{2+} being the most efficient (Sun et al., 2009) and La^{3+} also being a good inhibitor of Cx HC activity (Kondo et al., 1998). Interestingly, when applied extracellularly La^{3+} does not affect the activity of GJCs in astrocytes (Contreras et al., 2002) and thus can be used to discriminate between GJC and HC Cx functions. Finally, while under resting conditions the activity of HCs is low, the withdrawal of external calcium leads to their activation in astrocytes studied either in culture (Stout and Charles, 2003) or in organotypic (Stridh et al., 2008) and acute hippocampal slices (see additional information in Rouach et al., 2008).

5.3. *Distinct pharmacology of Cx versus Panx hemichannels*

The pharmacological profile of Panx HCs differs from that of Cx HCs in several points. The sensitivity of Panxs to carbenoxolone occurs at significantly lower concentrations (5- to 20-fold less). In addition, Panx HCs currents are independent of external calcium, are not sensitive to La^{3+} and are inhibited by probenecid (Suadicani et al., 2006), a compound that also acts on membrane ionic transporters (Iglesias et al., 2008; Ma et al., 2009). Moreover, niflumic acid that inhibits Cx HCs but not Panx HCs (Bruzzone et al., 2005) blocks Cx43 HCs in cultured astrocytes (Retamal et al., 2007). Finally, Panx1 has been shown to form protein–protein association with the purinergic P2X7 receptor, whose activation by external ATP opens a typical cationic channel within milliseconds followed seconds later by an opening or activation of a large pore permeable to molecules up to 900 Da

(Surprenant et al., 1996; Pelegrin and Surprenant, 2006). Accordingly, antagonists of P2X7 receptors also affect Panx HCs as for instance brilliant blue G (Qiu and Dahl, 2009).

5.4. *Engineered agents targeting Cxs and Panxs channels*

An alternative approach pursued by several laboratories has been to apply polypeptides corresponding to extracellular domains of Cx or Panx sequences, i.e., mimetic peptides (Evans and Boitano, 2001). Initially, these peptides were developed to interfere with the docking between two connexons thus preventing the formation of GJCs. While this strategy was initially described to be efficient in endothelial cells (Dora et al., 1999), so far there is no report indicating inhibition of GJCs using these polypeptides in glial cells (Rozental et al., 2001; N. Rouach and C.G., unpublished data). However, Cx mimetic peptides have been reported to be efficient in blocking dye uptake in cultured astrocytes suggesting that they block HCs (Retamal et al., 2007; Orellana et al., 2009). Similarly, mimetic peptides have also been designed for Panx1. In this case, they were reported to be efficient in macrophages expressing Panx1 while Cx mimetic peptides had no effect (Pelegrin and Surprenant, 2006). Reversibly, in cultured astrocytes expressing Cx43, the Cx43 mimetic peptides were able to block dye uptake generated by hypoxia in high glucose, while Panx mimetic peptides did not (Orellana et al., 2009). A controversy on the specificity of these polypeptides exists since in oocytes injected with mRNA encoding for either Cxs or Panxs, the two categories of peptides seem to have nonspecific effects (Dahl, 2007; Wang et al., 2007). HC single-channel recordings of transfected cells in the presence of the different mimetic peptides could address this issue in the near future.

6. siRNA approaches

RNA interference is a commonly used technique to down-regulate gene expression using small RNAs with designed target mRNA complementary sequences (He and Hannon, 2004). RNA interference has been quite successfully used to study the role of Cxs CNS in neurogenesis (Cina et al., 2009; Elias et al., 2007), in the control of astroglial gene expression (Figiel et al., 2007; Iacobas et al., 2008; Olk et al., 2009), as well as in astrocyte proliferation and glucose transport (Herrero-Gonzalez et al., 2009). The siRNA approaches may suffer from specificity problems generally referred to as "off-target effects" since complementarity between siRNA and target mRNA does not need to be complete and thus many mRNAs with sufficient sequence similarity might be affected besides the desired target (Svoboda, 2007). Such off-target effects can be controlled by the use of independent siRNAs targeting different sequences on the same mRNA (Elias et al., 2007; Olk et al., 2009). In addition, many studies have investigated Cx knockout animals and cultures for comparison (Figiel et al., 2007; Herrero-Gonzalez et al., 2009; Iacobas et al., 2008), which is probably the best control since it is based on an entirely different methodology. A limitation of siRNA approaches compared to gene ablation is their lower efficacy compared to global gene deletion (knockdown versus knockout). A clear advantage is the flexibility and short preparation time of siRNA approaches compared to gene-deficient mice and the exquisite temporal control by timing the application of siRNA.

7. Transgenic approaches

7.1. *Cx transgenic animals*

The major advantage of using transgenic animals compared to pharmacological agents is the specificity for selected Cxs and for defined cell types (see above). Now, mice deficient for all the main glial connexins known are available (Table 2). Since global deficiency for Cx26 and Cx43 is lethal (Gabriel et al., 1998; Reaume et al., 1995), conditional knockout mice were also engineered. Cell type-restricted deletion can be achieved by the Cre/loxP system (Nagy, 2000). Mice with astrocyte-directed deletion of Cx43 (Theis et al., 2003; Wiencken-Barger et al., 2007) are viable and mice with astrocyte-directed deletion of a conditional Cx26 allele (Cohen-Salmon et al., 2002) are expected to be viable, as well. It is important to note that the GFAP-cre transgenes used in most studies are not restricted to astrocytes but mediate deletion of conditional alleles in neurons and oligodendrocytes as well (Zhuo et al., 2001; but see Requardt et al., 2009). Astrocyte-specific gene deletion can rather be achieved by tamoxifen-inducible cre recombinase transgenes such as Cx30-CreERT2 (Slezak et al., 2007), GLAST-CreERT2 (Slezak et al., 2007), GLASTki-CreERT2 (Mori et al., 2006), GFAP-CreERT2 (Hirrlinger et al., 2006), or Cx43ki-CreERT (Eckardt et al., 2004). Such mouse lines are also required for timed gene inactivation of Cxs to avoid pleiotropic effects resulting from developmental disturbances. In most cases, however, such cell type restriction of gene deletion is not necessary, since oligodendrocytic and astrocytic Cxs do not overlap significantly in their expression pattern (Fig. 1; Table 1).

7.2. *Functional compensation among glial Cxs: use of double deficient mice*

In mice lacking astrocytic Cx43, residual gap junction coupling was observed that was most likely due to the expression of Cx30 (Rouach et al., 2008; Theis et al., 2003; Wallraff et al., 2006). Consistently, in mice lacking both Cx43 and Cx30, astrocytes were devoid of tracer coupling (Rouach et al., 2008; Wallraff et al., 2006). Similarly, mice lacking Cx29, Cx32 or Cx47 show no or only relatively mild phenotypical changes (Altevogt and Paul, 2004; Anzini et al., 1997; Menichella et al., 2003; Odermatt et al., 2003) while mice lacking both Cx32 and Cx47 exhibit a severely decreased life expectancy (Menichella et al., 2003; Odermatt et al., 2003). In Bergmann glia, Cx43, Cx29 (detected at transcript level only) and Cx30 are present (Theis et al., 2005) and the absence of phenotypical changes in mice lacking Cx43 in Bergmann glial cells indicates the occurrence of compensation. Thus, a combination of multiple Cx-deficient mice is required for an analysis of Cx function in Bergmann glia (Tanaka et al., 2008).

7.3. *Pannexin-deficient mice*

Three pannexin genes have been described so far (Baranova et al., 2004; Bruzzone et al., 2003; Scemes et al., 2007). A gene trap

Table 2 – Mouse mutants for the main glial connexins.

Name	Alteration	Affected cell type	References
Cx26 cKO	Gene disruption	Conditional	Cohen-Salmon et al. (2002)[a,b]
Cx29 KO	Gene disruption	Global	Altevogt and Paul (2004)[a,b]
Cx30 KO	Gene disruption	Global	Teubner et al. (2003)[a,b]
Cx32 KO	Gene disruption	Global	Nelles et al. (1996)[a], Anzini et al. (1997)[b]
Cx43 KO	Gene disruption	Global	Reaume et al. (1995)[a], Dermietzel et al. (2000)[b]
Cx43cKO (hGFAP-cre)	Gene disruption	Astrocytes, RG, RG-like cells	Theis et al. (2003)[a,b]
Cx43cKO (GFAP-cre)	Gene disruption	Astrocytes	Wiencken-Barger et al. (2007)[a,b]
Cx43cKO (nes-cre)	Gene disruption	Neural progenitors	Cina et al. (2009)[a,b]
Cx43K258 Stop	C-terminal truncation	Global	Maass et al. (2004)[a], Cina et al. (2009)[b]
Cx43G60S	Point mutation channel dead	Global	Flenniken et al. (2005)[a]
Cx43I30T	Point mutation channel dead	Global	Kalcheva et al. (2007)[a]
Cx43G318R	Point mutation channel dead	Conditional	Dobrowolski et al. (2008)[a]
tetO-Cx43	Forced expression	Conditional	Roell et al. (2007)[a]
Cx30/Cx43 DKO (hGFAP-cre)	Gene disruption	Global (Cx30), RG, RG-like cells, astrocytes (embryonic onset)	Wallraff et al. (2006)[a,b]
Cx30/Cx43 DKO (mGFAP-cre)	Gene disruption	Global (Cx30), astrocytes (postnatal onset)	Lutz et al. (2009)[a,b]
Cx47 KO	Gene disruption	Global	Menichella et al. (2003)[a,b], Odermatt et al. (2003)[a,b]
Cx32/Cx47 DKO	Gene disruption	Global	Menichella et al. (2003)[a,b], Odermatt et al. (2003)[a,b]

A number of global and conditional knockout mice have been generated, as well as knockin mice with modifications of the Cx43 coding region.
[a]The first description of a mouse mutant.
[b]Where applicable, the first description of CNS effects.
KO, knockout; cKO, conditional knockout; KI, knockin; DKO, double knockout.

ES cell line is available from the International Gene Trap Consortium (www.genetrap.org; sequence tag: DD0964) in which the Panx1 gene has been disrupted; in addition, a Panx1 KO mouse has been described recently (Anselmi et al., 2008). Unlike most Cxs that show a more stringent cell type specificity of expression, Panx1 is expressed in neurons and astroglia (Huang et al., 2007; Iglesias et al., 2009; Ray et al., 2005). This will pose an interpretation problem when it comes to the cell type specificity of Panx1 action studied in such global knockout mice, but a possible solution is the generation of a conditional Panx1 knockout mouse.

8. Functional impairments of glial connexins and their consequences

8.1. *Neuron–glia interactions*

Transgenic animal models with selective modulation of gap junctional coupling in astrocytes were instrumental to understand more about the specific role of astrocytic GJCs in the CNS. Much research on the function of astrocytic Cx43 has been done in fetal or neonatal Cx43-deficient astrocyte cultures (Naus et al., 1997; Scemes et al., 1998; Dermietzel et al., 2000) due to early postnatal death of Cx43 knockout mice (Reaume et al., 1995). Cx43-deficient cultures showed strongly decreased tracer coupling and partially impaired propagation of intercellular calcium waves, impaired growth, and decreased saturation density (Naus et al., 1997; Dermietzel et al., 2000). Subsequent work further specified that impaired growth in Cx43-deficient astrocyte cultures was not due to different growth rates but rather to a delayed onset of growth following replating of cultured astrocytes (Theis et al., 2004). A decreased dye uptake upon metabolic inhibition was also observed which is probably mediated by HC function either directly exerted by Cx43 or at least dependent on the presence of Cx43 (Contreras et al., 2002). Although the strong decrease in tracer coupling indicated that Cx43 was the major gap junction protein in cultured astrocytes, other connexins such as Cx26, Cx30, Cx40, Cx45, and Cx46 have been detected in astrocytes *in vitro* (Kunzelmann et al., 1999; Spray et al., 1998; Dermietzel et al., 2000) and in acute slices by single cell RT–PCR (Blomstrand et al., 2004). The expression of Cx30 was shown to be promoted by the presence of neurons cocultured with astrocytes (Koulakoff et al., 2008). It is likely that compensation by other Cxs in Cx43-deficient astrocyte cultures accounts for residual electrical and dye coupling (Naus et al., 1997; Scemes et al., 1998; Spray et al., 1998; Dermietzel et al., 2000).

The gross morphology of the brain was normal in Cx43-deficient neonatal mice (Dermietzel et al., 2000), but later stages could not be evaluated due to the early postnatal lethality of the global Cx43-deficiency (Reaume et al., 1995). Subsequently, the implementation of the Cre/loxP system for cell type-restricted gene inactivation allowed investigation of Cx43 function in the CNS of adult mice (Kunze et al., 2009; Lin et al., 2008; Lutz et al., 2009; Rouach et al., 2008; Theis et al., 2003; Wallraff et al., 2006; Wiencken-Barger et al., 2007). The investigation of adult mice

lacking Cx43, one of the major astroglial Cxs, showed an increased velocity of spreading depression (Theis et al., 2003) which provided the first direct indication that astroglial Cxs in grey matter may be involved in spatial buffering of K^+ released into the extracellular space by neuronal activity (Theis et al., 2005) and that impaired spatial buffering could increase the susceptibility for epileptiform activity. Recent work substantiated the role of astroglial GJCs in spatial buffering using mice lacking both Cx43 and Cx30 and showed that genetic ablation of both Cxs in astrocytes lowers the threshold for generation of spontaneous and induced epileptiform discharges in acute hippocampal slice (Wallraff et al., 2006). At first sight, this finding seems to be in contrast to recent observations showing that Cx43 and Cx30 in astrocytes help to provide glucose to neurons for their sustained activity (Rouach et al., 2008). However, in the study of hyperexcitability in acute slice preparations, glucose was not a limiting parameter. Both roles of astrocytic Cxs, i.e., spatial buffering of potassium ions and glucose supply need to be taken into account and may be operative in epilepsy. Mice lacking Cx30 and Cx43 in astrocytes further show a deficiency in ischemic preconditioning (Lin et al., 2008) and provided that epileptic preconditioning (Ferriero, 2005) utilizes similar mechanisms, lack of Cx30 and Cx43 may likewise affect the propensity for epileptiform neuronal activity at yet another level.

8.2. *Myelin status*

Knockout mice for the known oligodendrocytic Cxs have been generated. Cx29-deficient mice did not show any signs of myelin abnormalities, gross anatomical changes or motor deficits (Altevogt and Paul, 2004; Eiberger et al., 2006). Aged mice deficient for Cx32 experienced a progressively demyelinating peripheral neuropathy starting around 3 months of age (Anzini et al., 1997; Scherer et al., 1998), but only mild phenotypical changes in the CNS, i.e., a smaller diameter of axonal myelin sheaths in the neocortex and a decreased volume of myelin within the neuropil of Cx32-deficient mice (Sutor et al., 2000). Functional loss of Cx47 leads to pronounced myelin vacuolation (Menichella et al., 2003; Odermatt et al., 2003). Mice lacking both Cx47 and Cx32 developed an action tremor before death around postnatal week 6 and vacuolation of the central white matter was much more severe when compared to that evoked by Cx47 deficiency alone (Menichella et al., 2003; Odermatt et al., 2003). Also, double deficiency of Cx43 and Cx30 in astrocytes was recently shown to lead to a white matter pathology, i.e., myelin-associated vacuoles and dysmyelination (Lutz et al., 2009) that was similar to that of Cx32/Cx47 double KO mice although less pronounced (Menichella et al., 2003; Odermatt et al., 2003) yet the Cx43/Cx30 double KO mice exhibited a normal life expectancy. Thus, while the vacuolization and dysmyelination might reflect a common cause, i.e., impairment of panglial coupling compartments, additional deficiencies restricted to oligodendrocytic functions are likely responsible for the more severe phenotypical changes in Cx32/Cx47 mice.

8.3. *Neurogenesis*

Cxs expressed in radial glia have a strong impact on neurogenesis, and disruption of Cxs by RNA interference or transgenic mouse technology led to deficits in neuronal migration that were mainly attributed to adhesive functions of Cxs, although the role of the C-terminal cytoplasmic tail of Cx43 in this process is under debate (Cina et al., 2009; Elias et al., 2007). Notably, deficits in neuronal migration observed in Cx43-deficient mice seem to be compensated during development (Fushiki et al., 2003), and consistently, the overall brain architecture of newborn mice with global deletion of Cx43 or of adult mice with astrocyte-specific deletion of Cx43 is normal (Dermietzel et al., 2000; Theis et al., 2003). Another study described severe morphological changes in the brain of mice lacking Cx43 in astrocytes, which indicated a role for Cx43 during neurodevelopment, but the phenotype occurred only in a defined genetic background, i.e., 129SVEV (Wiencken-Barger et al., 2007).

Recently, Cx43 and Cx30 were found to mediate intercellular coupling between RG-like cells in the adult dentate gyrus (Fig. 3). Moreover, both Cxs are required for proliferation of these cells and subsequently for neurogenesis.

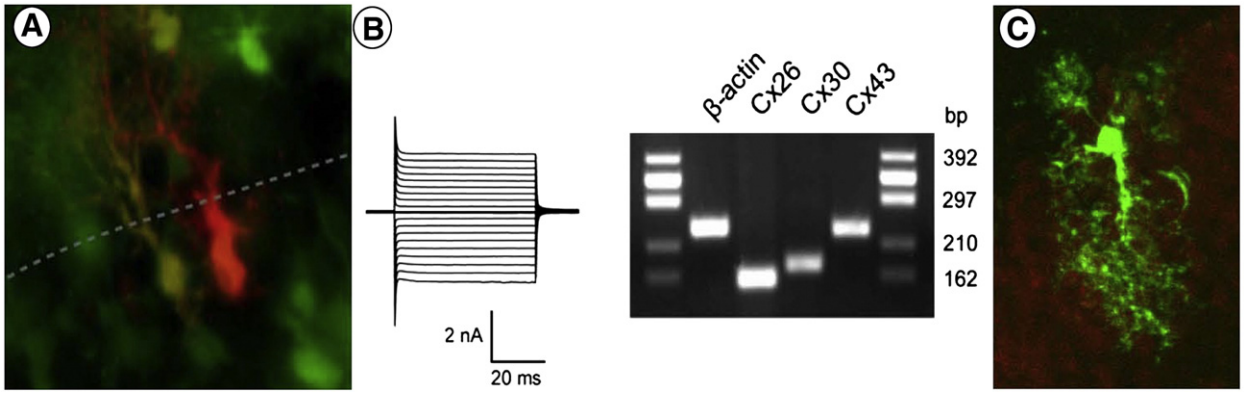

Fig. 3 – Connexins in radial glia-like cells. (A) Transgenic hGFAP-EGFP mice with fluorescently labeled RG-like cells (green) show that the cells are coupled to each other by gap junctions when filled with the tracer biocytin (red). Dotted line indicates the location of the subgranular zone. (B) Fluorescently labeled RG-like cells were electrophysiologically characterized and subjected to single-cell RT–PCR, showing expression of Cx26, Cx30, and Cx43 in RG-like cells. (C) Biocytin filled RG-like cells from a Cx43/Cx30 double knockout mouse show that the tracer (green) is confined to the injected cell. nA, nanoampere; ms, milliseconds; bp, base pairs. Modified from Kunze et al. (2009).

Indeed, mice lacking Cx43 and Cx30 in astrocytes show an almost complete inhibition of proliferation in the subgranular zone as revealed by staining against Ki67 protein, and a 20% decrease in the number of granule cells in the dentate gyrus. These findings suggest the requirement for gap junction coupling of RG-like cells for intact neurogenesis in the adult brain (Kunze et al., 2009).

8.4. *Higher-order brain function*

On the behavioral level, mice lacking Cx43 in astrocytes and Cx30-deficient mice have shown no or only mild behavioral impairments based on short- and long-term memory tests (Dere et al., 2003; Frisch et al., 2003). However, mice lacking Cx43 in astrocytes showed increased locomotory activity in the open field and impaired motor capacities on the rotarod (Frisch et al., 2003; Theis et al., 2003). A steeper learning curve in the water maze indicated deficits in novel task situations that were, however, fully compensated during consecutive trials. This compensation was paralleled by an increase in acetylcholine levels in the frontal cortex exclusively after training (Frisch et al., 2003). Mice with postnatal onset of Cx43 deletion in the cerebellum did not exhibit behavioral impairments (Tanaka et al., 2008). Cx30-deficient mice showed changes in the open field test that are opposed to those observed in mice lacking astroglial Cx43 and were linked to increased emotionality and decreased exploratory activity, while motor capacities were not impaired. Neurochemical changes were also observed (Dere et al., 2003). Cx43/Cx30 double KO mice showed a deficit in novel object recognition indicating impairment of spatial memory exceeding that of single deficiencies, while the motor impairment observed was similar to that found in Cx43-deficient mice (Lutz et al., 2009).

8.5. *Mutations of glial connexins*

Several hundreds of mutations in the Cx32 gene were discovered in human patients presenting with the X-linked form of Charcot–Marie–Tooth disease, a demyelinating peripheral neuropathy (Abrams et al., 2000; Orthmann-Murphy et al., 2008). Mutations led to a wide range of changes from complete loss of expression to more selective impairments, such as incorrect Cx trafficking, disrupted current–voltage relationships or a reduction in the pore diameter which may selectively restrict the passage of important signaling molecules (cf., Abrams et al., 2000). Mice expressing selected Cx32 mutant forms in oligodendrocytes and in Schwann cells of the PNS have been generated and show progressive demyelinating peripheral neuropathy along with mild CNS myelination deficits (Sargiannidou et al., 2009). Several mutations in Cx47 cause Pelizaeus–Merzbacher-like disease, a dysmyelinating CNS disorder (Uhlenberg et al., 2004; Bugiani et al., 2006; Orthmann-Murphy et al., 2007a), while another Cx47 mutation was identified as being causative for hereditary spastic paraplegia (Orthmann-Murphy et al., 2009). Several mutant Cx47 forms identified have been tested in cultured cells and a deficiency in heterologous coupling of mutant Cx47-expressing cells with Cx43-expressing cells was observed (Orthmann-Murphy et al., 2007b, 2009), but so far, no data are available on transgenic mice expressing mutant Cx47. Oculodentodigital dysplasia (ODDD) is a predominantly autosomal dominant disorder caused by mutations in the Cx43 gene; currently, 62 different nonconservative nucleotide exchanges in the coding region of Cx43 are known (Paznekas et al., 2009). This syndrome is characterized by craniofacial, ocular, dental, and digital abnormalities, which are most common to the disease. Neurological manifestations are less common and mostly arise in the second decade of life (Paznekas et al., 2009). About a quarter of the known mutations has been characterized in cultured cells, where reduced gap junction plaque formation and loss of junctional conductance were generally observed (Dobrowolski et al., 2007; see Paznekas et al., 2009). A random mutagenesis screen resulted in the identification of a mouse model for oculodentodigital dysplasia with a Cx43 missense mutation for which no human counterpart has been reported so far (Flenniken et al., 2005). In addition, two different lines of transgenic mice have been generated with point mutations in Cx43 mimicking mutations observed in human patients (Kalcheva et al., 2007; Dobrowolski et al., 2008), but functional impairments or phenotypical abnormalities in the CNS have not yet been reported in such mice.

8.6. *Limits of the genetic approach*

While Cx gene ablation is specific to individual Cxs and mostly to specific glial cell types, it often lacks sufficient temporal control. In global knockout mice, the gene is lacking throughout development, giving rise to pleiotropic effects (Reaume et al., 1995). Mice with conditional deletion are often limited by the onset of promoter activity of the respective cre transgene, although careful comparison of cre transgenes with early (Kunze et al., 2009; Wallraff et al., 2006) and late (Lutz et al., 2009) onset of deletion may give a clue about developmental effects. Mice with inducible gene deletion may allow precise temporal control, but as a rule, inducible cre transgenes are less efficient than their constitutive counterparts (see, for example, Hirrlinger et al., 2006; Zhuo et al., 2001). Retroviral delivery of the cre recombinase is especially suited to study the impact of gene function in dividing cell populations (Kunze et al., 2009), but gene ablation may be locally restricted. There are two additional major limitations of gene ablation studies: (i) numerous changes in gene expression were observed in Cx43 KO mice (Iacobas et al., 2007), questioning a direct role of the ablated Cxs in the phenotypical changes observed; (ii) in gene ablation studies, the Cx subunits are missing, but it is far from clear what is their major mechanism of action under normal conditions, i.e., intercellular coupling, HC activity, adhesion, or control of gene expression. A solution to this problem may come from the generation of Cx43 mutant mice in which only some functions of Cx43 are impaired while other functions are preserved, as discussed in more detail in the next section.

9. Conclusions and perspectives

In the CNS, Cxs are expressed in almost all cell populations and they have been extensively studied in neurons and glia. In the mammalian brain, Cx channels are important in neurons

as they provide the morphological basis for electrical synapses (Connors and Long, 2004). In glia, Cxs are expected to play a more critical role because they are less challenged by an alternative mode of intercellular communication and they are much more widely expressed from developmental to adult stages. In astrocytes, their role is even more important since up to several hundred astrocytes can be coupled together (D'Ambrosio et al., 1998; Blomstrand et al., 2004). They are involved in the propagation of intercellular calcium waves (Scemes and Giaume, 2006) that constitute a specific glial mode of intercellular signaling in the brain. This is particularly relevant because astrocytes are not excitable cells and do not generate action potentials, but the richness of their calcium signaling has led to propose that it could represent their mode of cell excitability. In fact, besides the implication of astroglial Cxs in potassium buffering and water regulation which are based on ionic coupling properties, it seems that biochemical and metabolic coupling properties are likely more important than ionic coupling for astrocyte physiology. To understand their role in the interactions that they establish either with neurons at the tripartite synapse or with the blood circulation at the glio-vascular unit, there is a critical need to specifically act on their expression and their function without affecting the other cell partners. To reach this goal, cell type-specific and Cx-specific tools are required. As discussed above, so far the pharmacology available for brain Cxs does not allow satisfying this requirement. This is particularly true for GJCs that are not easy targets for pharmacological tools since the two channel faces are cytoplasmic. However, the demonstration that Cx HCs operate in astrocytes, particularly in pathological situations (Orellana et al., 2009), gives more hope for the successful development of new molecules that could act specifically at the HC level. Indeed, in the case of HCs, the external face of the channel can be more easily reached. Nevertheless, although GJC and HC blockers cannot be used to study the role of Cxs or Panxs in various brain functions or dysfunctions, it remains true that the blockers listed above can be used to determine whether a defined cell type is coupled or not to its neighbors, but nothing more. Thus, based on these limitations the pharmacological strategy seems to be inappropriate, giving more credit to the genetic approach. However, also in this domain, improvements remain to be done.

The future challenges which lie ahead will be (i) to elucidate the actual mechanisms by which Cxs exert their function, i.e., GJC function, HC function, adhesion, or control of gene expression (Theis et al., 2005) using a combination of genetic approaches; (ii) to determine which respective contribution has to be attributed to Cx versus Panx HCs; (iii) to investigate cause–effect relationships of Cx function in normal and pathological conditions, which requires to overexpress or misexpress Cxs in addition to ablating their functions and to increase temporal control of gene manipulation in disease models; (iv) to test experimentally the hypothesized panglial coupling compartment governing spatial buffering of potassium ions by electrophysiological and tracer coupling studies as well as phenotypical assessment of glial defects using deficient mice in which the panglial coupling is selectively disrupted while interastroglial and interoligodendroglial coupling are mostly preserved.

9.1. *Investigating the mechanisms of astroglial Cx action using Cx43 mutant mice*

By comparing a panel of Cx43 mutant mice (Table 2), it might be possible to elucidate the actual mechanisms of Cx function in astrocytes: astrocyte-directed Cx43-deficient mice lack a major astrocytic Cx and thus none of the known Cx functions, i.e., intercellular coupling HC activity, adhesion, or control of gene expression can be exerted by Cx43. In combination with deficiency for Cx30, astrocytic Cx expression is completely or largely abolished (see (Rouach et al., 2008; Wallraff et al., 2006). A recent work describes a mouse carrying a conditional point mutation of Cx43 (Dobrowolski et al., 2008, 2009) as found in some patients with oculodentodigital dysplasia (Paznekas et al., 2009). This particular mutant (Cx43G138R) exhibited lack of intercellular coupling but preserved HC activity. This mutant still contains the C-terminal cytoplasmic tail and thus one can assume that adhesion and control of gene expression exerted by Cx43 are still preserved; however, this still needs experimental verification. Mice with an astrocyte-directed conditional replacement of Cx43 by a point mutation of Cx43 (Cx43G138R mice; (Dobrowolski et al., 2008, 2009) should lack Cx43-mediated intercellular coupling and in combination with Cx30 deficiency, astrocytes should be uncoupled while other mechanisms of Cx43 action should be preserved. Finally, the Cx43K258Stop mouse lacks the C-terminal tail (implicated in adhesion and control of gene expression) but the mutant protein allows, at least in transfected Hela cells, robust intercellular coupling (Maass et al., 2004). First studies in the CNS using these mice have already been performed (Cina et al., 2009). Again, with the correct allelic setting, including Cx30 deficiency, one can investigate astrocytes expressing Cx43 without C-terminus, which should still exhibit intercellular coupling but should lack adhesive properties and Cx43-mediated control of gene expression. Thus, by comparing the various phenotypical changes observed in Cx43/Cx30 double KO mice with those observed in Cx43G138R/Cx30 KO mice and Cx43K258STOP/Cx30 KO mice, we should learn more about the most relevant mode of Cx43 action in astrocytes, under conditions where the second major astrocytic connexin, Cx30 has been removed.

9.2. *Cx overexpression and misexpression*

Another direction of manipulating Cx function would be to force Cx43 expression in astrocytes or neurons. This is now possible using mice carrying a GFAP-tTA transgene (Fiacco et al., 2007) or a CamKIIα-tTA transgene (Mayford et al., 1996) and a tetO-Cx43 transgene (Roell et al., 2007) with doxycycline-regulated expression of Cx43 in astrocytes or neurons (Table 2). Such approaches could allow rescue experiments or the establishment of new coupling compartments.

9.3. *Investigating panglial coupling*

The proposed concept of panglial coupling compartments that, besides astroglial networks, are in continuity with oligodendroglial compartments still needs experimental verification (Theis et al., 2005). In this context, it should be noted that mice lacking both Cx32 and Cx47 as well as mice lacking both Cx43 and Cx30 should not only be completely uncoupled in their respective coupling compartments (i.e., oligodendroglial for

Cx32/Cx47 and astroglial for Cx30/Cx43) but should have also lost their panglial coupling. The phenotypical changes in Cx32/Cx47 double KO mice (Menichella et al., 2003; Odermatt et al., 2003) are very strong, and thus, it is difficult to pinpoint any effect on the impairment of panglial coupling compartments. Phenotypical aberrations observed in Cx30/Cx43 double knockout mice (Kunze et al., 2009; Lin et al., 2008; Lutz et al., 2009; Wallraff et al., 2006; Rouach et al., 2008) probably mainly reflect disturbance of astrocytic Cx function but should also include changes resulting from abrogation of panglial coupling compartments. In fact, white matter damage observed in Cx30/Cx43 double knockout mice (Lutz et al., 2009) may be a first hint at a specific role of the panglial coupling compartments. The use of Cx43/Cx32 or Cx30/Cx47 double deficient mice in which the remaining astroglial and oligodendroglial Cxs are not compatible with each other is well suited to provide the most specific insight into the biological role of presumed panglial coupling compartments. In such mice, astroglial coupling and oligodendroglial coupling are only partially impaired, but communication between astrocytes and oligodendrocytes should be abolished (Theis et al., 2005).

9.4. *Lentiviral approaches*

Finally, an alternative approach to the generation of transgenic animals could be the development of a lentiviral strategy to specifically enhance or silence the expression of Cxs in astrocytes. Indeed, recently engineered lentiviral constructs that specifically target astrocytes have been generated (Colin et al., 2009). In addition, such approach has already been applied for several Cxs, including Cx43, in insulin-secreting pancreatic cells (Caton et al., 2003). Once validated *in vitro* in cultured astrocytes, lentiviral constructs could be used *in vivo* by stereotaxic injection. As the expression of the transgene will be restricted to the injected brain region, it will be possible to compare the effect of such manipulation to the contralateral side, which can be taken as a control. A similar approach has been used to study the impact of astroglial Cxs in adult neurogenesis, whereby a Cre expressing retrovirus preferentially targeting proliferative cells was used to downregulate Cx43 expression postnatally (Kunze et al., 2009).

Finally, from this overview, one can conclude that pharmacology, siRNA techniques, and transgenic/viral approaches have to be used in combination, rather than alone, being considered as partners to team up in the study of how Cxs exert their function in glial cells, i.e., by intercellular coupling, HC activity, control of gene expression, or adhesive action.

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BRAIN RESEARCH REVIEWS

Review

The cytosolic redox state of astrocytes: Maintenance, regulation and functional implications for metabolite trafficking

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ABSTRACT

Astrocytes have important functions in the metabolism of the brain. These cells provide neurons with metabolic substrates for energy production as well as with precursors for neurotransmitter and glutathione synthesis. Both the metabolism of astrocytes and the subsequent supply of metabolites from astrocytes to neurons are strongly affected by alterations in the cellular redox state. The cytosolic redox state of astrocytes depends predominantly on the ratios of the oxidised and reduced partners of the redox pairs NADH/NAD^+, NADPH/$NADP^+$ and GSH/GSSG. The NADH/NAD^+ pair is predominately in the oxidised state to accept electrons that are produced during glycolysis. In contrast, the redox pairs NADPH/$NADP^+$ and GSH/GSSG are biased towards the reduced state under unstressed conditions to provide electrons for reductive biosyntheses and antioxidative processes, respectively. In this review article we describe the metabolic processes that maintain the redox pairs in their desired redox states in the cytosol of astrocytes and discuss the consequences of alterations of the normal redox state for the regulation of cellular processes and for metabolite trafficking from astrocytes to neurons.

Contents

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Abbreviations: BDNF, brain derived neurotrophic factor; CtBP, C-terminal binding protein; EAAC1, excitatory amino acid carrier 1; γGT, γ-glutamyl transpeptidase; G6PDH, glucose-6-phosphate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPx, glutathione peroxidase; GR, glutathione reductase; GS, glutamine synthetase; GSH, glutathione; GSSG, glutathione disulfide; H2B, histone 2B; ICAM-1, intercellular adhesion molecule 1; ICDH, isocitrate dehydrogenase; IP3, inositol-1,4,5-trisphosphate; LDH, lactate dehydrogenase; ME, malic enzymes; Mrp, multidrug resistance protein; NF-κB, nuclear factor-κB; NRSF, neural restrictive silencing factor; NRE, NADPH-regenerating enzyme; 6PGDH, 6-phosphogluconate dehydrogenase; PPP, pentose phosphate pathway; ROS, reactive oxygen species; RyR, ryanodine receptor

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1. Introduction

The endfeet of astrocytes occupy a strategically special location in brain between the endothelial cells of the capillaries and neurons. In addition, astrocytes as a component of the tripartite synapse remove efficiently neurotransmitters such as glutamate from the synaptic cleft and also have important functions in regulating the extracellular ion homeostasis (Allen and Barres, 2009). Due to these extensive contacts with both blood vessels and neurons, astrocytes are key players in the control of cerebral metabolism and in the supply of metabolites to neurons. Metabolite trafficking from astrocytes to neurons is important in the metabolism of energy substrates (Gandhi et al., 2009a; Magistretti, 2009; Nehlig and Coles, 2007; Pellerin et al., 2007; Pellerin, 2008; Barros and Deitmer, 2010), of neurotransmitters such as glutamate (Albrecht et al., 2007; McKenna, 2007; Yang et al., 2008), of thiol containing compounds (Banerjee et al., 2008; Dringen, 2009) and of cholesterol (Mulder, 2009). Astrocyte functions and astrocyte–neuron interactions are also very important for synaptic plasticity and neural networks (Fellin, 2009; Vijayaraghavan, 2009). For these reasons, alterations in astrocyte metabolism are expected not only to affect the astrocytes themselves but also to compromise neuronal function and viability. There is therefore a potential involvement of astrocytes in almost every type of pathological condition of the brain (Chvatal et al., 2008; De Keyser et al., 2008).

The redox state of cells is predominately established by the ratios of the redox-active cofactors NADH/NAD+, NADPH/NADP+ and glutathione (GSH)/glutathione disulfide (GSSG). These substances are essential for cellular catabolism, anabolism and antioxidative defense. Depending on the availability of energy substrates, on the ATP demand of astrocytes and on the prevailing physiological conditions, the ratio of the partners in the redox pairs NADH/NAD+, NADPH/NADP+ and GSH/GSSG is altered. This in turn will affect the supply of metabolites from astrocytes to neurons. In this review article we describe the maintenance and regulation of the redox state in the cytosol of astrocytes, discuss how the redox state can regulate cellular functions and speculate on how alterations in the astrocytic redox potential could affect the trafficking of metabolites from astrocytes to neurons.

2. The cytosolic redox state in astrocytes

The redox state of astrocytes depends predominantly on the cellular concentrations and the ratios of NADH/NAD+, NADPH/NADP+ and GSH/GSSG (Figs. 1 and 2). Under unstressed conditions the NADH/NAD+ pair in cultured astrocytes is predominately in the oxidised state (Chen, 2007; Wilhelm and Hirrlinger, 2009; Zhu et al., 2005) to accept electrons that are produced during glycolysis in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) reaction. In contrast, cultured astrocytes maintain the redox pair NADPH/NADP+ in a more reduced state (Chen, 2007) to provide electrons for reductive biosyntheses, while the concentration of GSH strongly exceeds that of GSSG (Dringen and Hamprecht, 1997, 1998) to support efficient antioxidative defense. These ratios of the redox pairs in cultured astrocytes are similar to those reported for brain (Adams et al., 1993).

2.1. NADH/NAD+

The cytosolic redox state of the NADH/NAD+ pair is intimately linked to the pathways that contribute to the synthesis of ATP, i.e., glycolysis and oxidative phosphorylation. This is of special interest within the brain since the human brain accounts for 20% of the total consumption of both oxygen and glucose by the body (Clarke and Sokoloff, 1999). Synaptic functions require particularly large amounts of energy (Attwell and Laughlin, 2001) which are mainly generated by the oxidation of NADH in mitochondrial respiration. During catabolic oxidation, glucose is metabolized via glycolysis and the citric acid cycle to CO_2 and the electron acceptor NAD+ becomes reduced to NADH (Fig. 1). However, since NAD+ is continuously required as acceptor for electrons, it has to be efficiently regenerated by oxidation of NADH. While NADH produced in mitochondria can be directly used as substrate for complex I of the respiratory chain, glycolytically generated reduction equivalents have to be transported into mitochondria or are used to reduce the glycolysis endproduct pyruvate to lactate. In the latter case, lactate is efficiently exported from astrocytes and can be used by neurons as an energy substrate, thereby linking astrocytic and neuronal NADH/NAD+ redox states (Cerdan et al., 2006; Pellerin et al., 2007; Barros and Deitmer, 2010). In astrocytes, the ratio of cytosolic NADH/

NAD^+ is determined by the availability of glucose (or other substrates) and oxygen, as well as by the cellular ratio of pyruvate to lactate, and therefore reflects the rate of catabolism in these cells. In addition to their function as cosubstrates of oxidoreductases, NAD^+ and NADH have additional functions in the brain and other tissues (Berger et al., 2004; Ying, 2007).

2.2. *NADPH/NADP$^+$*

The cellular ratio of NADPH/$NADP^+$ in astrocytes depends on the consumption of NADPH in antioxidative and biosynthetic enzyme reactions and on the regeneration of NADPH by NADPH-regenerating enzymes (NRE) (Dringen et al., 2005, 2007). A large number of astrocytic metabolic reactions are known to rely on NADPH as an electron donor. Among these are the NADPH-dependent oxidoreductases of the pathways that catalyze the synthesis of fatty acids and cholesterol, the degradation of heme, the active production of the radicals superoxide and NO as well as the regeneration of the antioxidant GSH from its oxidation product GSSG (Brown, 2007; Dringen et al., 2007; Sorce and Krause, 2009). In order to provide NADPH-dependent enzymes with sufficient amounts of cosubstrate, the cellular ratio of NADPH/$NADP^+$ is maintained in astrocytes in the reduced state by NRE (Fig. 1). Within the cytosol of astrocytes, these include the two enzymes of the oxidative part of the pentose phosphate pathway (PPP) glucose-6-phosphate dehydrogenase (G6PDH) and 6-phosphogluconate dehydrogenase (6PGDH), the cytosolic $NADP^+$-dependent isocitrate dehydrogenase (ICDH) and the cytosolic malic enzyme (ME) (Dringen et al., 2007; Garcia-Nogales et al., 1999; Kurz et al., 1993; Minich et al., 2003; Rust et al., 1991; Schmidt and Dringen, 2009).

The balance of cytosolic NRE activities contributing to NADPH regeneration in astrocytes depends on the metabolic activity of the cells. The cytosolic isoforms of ICDH and ME use isocitrate and malate as substrates, respectively, which are intermediates of the citric acid cycle and have to be released from mitochondria to serve as substrates for cytosolic NADPH regeneration (Fig. 1). Thus, these enzymes are likely to supply NADPH for the synthesis of fatty acids and cholesterol under metabolic conditions that favour the release of mitochondrial

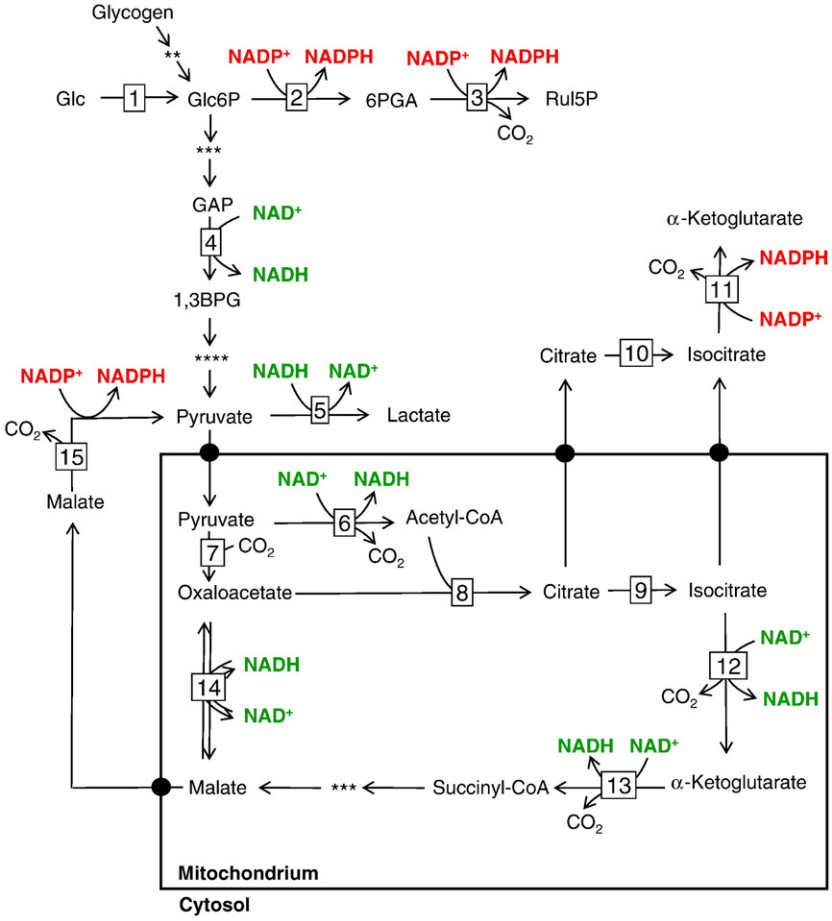

Fig. 1 – Metabolic pathways that determine the cytosolic redox states of NADH/NAD^+ and NADPH/$NADP^+$. 1: hexokinase, 2: glucose-6-phosphate dehydrogenase, 3: 6-phosphogluconate dehydrogenase, 4: glyceraldehyde-3-phosphate dehydrogenase, 5: lactate dehydrogenase, 6: pyruvate dehydrogenase, 7: pyruvate carboxylase. 8: citrate synthase, 9: mitochondrial aconitase, 10: cytosolic aconitase, 11: cytosolic $NADP^+$-dependent isocitrate dehydrogenase, 12: NAD^+-dependent isocitrate dehydrogenase, 13: α-ketoglutarate dehydrogenase, 14: malate dehydrogenase, 15: malic enzyme; Glc6P: glucose-6-phosphate, 6PGA: 6-phosphogluconate, Rul5P: ribulose-5-phosphate, GAP: glyceraldehyde-3-phosphate, 1,3BPG: 1,3-bisphosphoglycerate, Each * indicates an enzymatic reaction that is not separately mentioned.

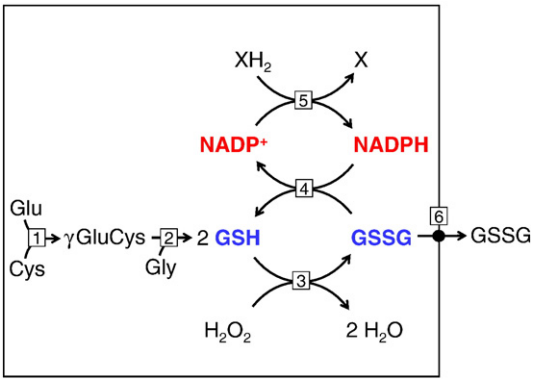

Fig. 2 – GSH and NADPH regeneration. GSH is synthesized from glutamate, cysteine and glycine in the consecutive reactions of glutamate cysteine ligase (1) and GSH synthetase (2). Glutathione peroxidases (3) use GSH as electron donor for the reduction of hydrogen peroxide to water. The GSSG formed in the GPx reaction is reduced by glutathione reductase (4) in a reaction that uses NADPH as electron donor. NADPH is regenerated by NADPH-regenerating enzymes (5) during the oxidation of their respective substrates. Alternatively to the GR reaction, GSSG can be exported from the cells via multidrug resistance protein 1 (6).

intermediates. In contrast, for a quick supply of large amounts of NADPH in astrocytes, e.g., during acute oxidative stress, the PPP enzymes appear to play a major role, since the substrate of G6PDH, glucose-6-phosphate, is continuously generated in astrocytes by phosphorylation of glucose and is therefore continuously available. If the rate of glucose uptake and phosphorylation is insufficient to supply G6PDH with its substrate, glycogen can be recruited in astrocytes as an additional source of glucose-6-phosphate (Rahman et al., 2000).

The activity of the oxidative part of the PPP depends strongly on the availability of both glucose-6-phosphate and $NADP^+$ and is regulated by the cytosolic concentration of NADPH, which is an effective competitive inhibitor of brain G6PDH (Askar et al., 1996). Therefore, the cytosolic ratio of $NADP^+$ to NADPH determines the flux of metabolites through G6PDH. Unstressed cells have a high NADPH/$NADP^+$ ratio (Adams et al., 2001) and the activity of G6PDH is rather low under such conditions due to the low concentration of the substrate $NADP^+$ and the presence of the inhibitor NADPH. Therefore, the PPP is only a minor contributor of cellular glucose-6-phosphate consumption under unstressed conditions (Dringen et al., 2007). In contrast, if NADPH is rapidly consumed for example for the regeneration of GSH during peroxide disposal, the flux of glucose-6-phosphate into the PPP becomes strongly activated in astrocytes (Ben Yoseph et al., 1994, 1996) as a consequence of the simultaneous removal of the G6PDH inhibitor NADPH and the generation of the G6PDH substrate $NADP^+$ (Askar et al., 1996; Dringen et al., 2007).

2.3. *GSH/GSSG*

The antioxidant GSH is essential for the detoxification of reactive oxygen species (ROS) that are continuously generated as products and by-products of oxidative metabolism. The redox state of the redox pair GSH/GSSG in astrocytes depends on the rates of (i) the synthesis of GSH, (ii) the oxidation of GSH to GSSG, (iii) the reduction of GSSG to GSH, (iv) the consumption of GSH for other cellular processes and (v) the export of GSH or GSSG from the cells (Fig. 2). Cultured astrocytes contain a cytosolic total glutathione concentration of 8 mM (Dringen and Hamprecht, 1998) which represents almost exclusively GSH (Dringen et al., 1997; Dringen and Hamprecht, 1996; Liddell et al., 2006a,b). The high ratio of GSH to GSSG in unstressed astrocytes is maintained by cellular glutathione reductase (GR). This enzyme is present in cultured astrocytes at a specific activity of about 33 nmol/(min mg protein) (Gutterer et al., 1999). Due to the low micromolar K_M values for both NADPH and GSSG (Acan and Tezcan, 1989, 1991; Dringen and Gutterer, 2002), the GR activity of cultured astrocytes is sufficient to rapidly reduce the low amounts of GSSG that are produced during normal metabolism.

During oxidative stress, GSH becomes rapidly oxidized to GSSG either by glutathione peroxidase activity (GPx) or by enzyme-independent chemical reactions. Exposure of cultured astrocytes to peroxides leads to a short and transient increase in the cellular concentration of GSSG (Dringen et al., 1997; Kussmaul et al., 1999; Liddell et al., 2006a,b). Under such conditions, GR becomes limiting for the GSH/GSSG redox cycling in astrocytes and the GSSG content can accumulate to 50% of total glutathione within a few minutes. The GPx isoform 1 (GPx1) is exclusively responsible for the transient cellular accumulation of GSSG after peroxide application, since GSSG was not detectable during peroxide clearance in GPx1-deficient astrocytes (Liddell et al., 2006a,b). In contrast to the transient accumulation of cellular GSSG after a bolus application of peroxides, the cellular GSSG to GSH ratio is maintained at a high level during exposure of astrocytes to peroxides for a prolonged time period (Dringen et al., 2006; Hirrlinger et al., 1999). Under these conditions, the cellular GSSG content remains at around 50–70% of total glutathione due to the permanent reduction of peroxide via GPx (Hirrlinger et al., 2001; Minich et al., 2006). In addition to the redox cycling driven by GPx and GR, the GSH/GSSG ratio in astrocytes can also be modulated by the export of GSH (Dringen et al., 1997; Hirrlinger et al., 2002b; Minich et al., 2006), the export of GSSG after oxidation of GSH (Hirrlinger et al., 2001, 2002a; Minich et al., 2006) and the conjugation of GSH to xenobiotics and subsequent export of the conjugates (Schmidt and Dringen, 2009; Waak and Dringen, 2006). The multidrug resistance protein (Mrp) 1 is involved in these export processes in cultured astrocytes (Hirrlinger et al., 2001, 2002b; Minich et al., 2006; Waak and Dringen, 2006).

2.4. *Interdependence of the redox pairs that define the cytosolic redox state*

The three cytosolic redox pairs NADH/NAD^+, NADPH/$NADP^+$ and GSH/GSSG cannot be considered independently from each other. NAD^+ is the substrate for the synthesis of $NADP^+$ by NAD kinase and $NADP^+$ can be hydrolysed to NAD^+ (Magni et al., 2008). In addition, the GSH/GSSG ratio is determined by the NADPH consuming enzyme GR (Fig. 2), while glycolysis

and PPP, which are predominately responsible for the cytosolic reduction of NAD^+ and $NADP^+$, respectively, share the common substrate glucose-6-phosphate (Fig. 1). Thus, alterations of the redox state of one of these redox pairs in the cytosol will also have consequences for the others. This connection has been demonstrated e.g. by overexpression of G6PDH, which increases the levels of NADPH and NADH as well as the GSH/GSSG ratio (Legan et al., 2008). Similarly, inhibition of GR not only lowers the GSH/GSSG ratio but simultaneously increases the ratios of NADH/NAD^+ and NADPH/$NADP^+$ (Zhao et al., 2009). The direct coupling of GSSG reduction and NADPH regeneration by PPP has been clearly demonstrated in astrocytes by the strong activation of the PPP after application of H_2O_2 (Ben Yoseph et al., 1994, 1996) by the lowered clearance rate of peroxides in the absence of glucose (Dringen et al., 1998; Dringen and Hamprecht, 1997; Kussmaul et al., 1999) and by the rapid mobilisation of glucose from glycogen after peroxide exposure (Rahman et al., 2000).

3. Analysis of alterations in NAD(P)H metabolism by fluorescence microscopy

The analysis of the NADH/NAD^+ redox state has been greatly facilitated by the fact that NADH is a fluorescent molecule while NAD^+ is not. Therefore, the fluorescence signal emitted by cells or tissue preparations after appropriate excitation in the UV range or by two-photon lasers allows a semiquantitative analysis of the NADH/NAD^+ redox state. Although NADPH fluorescence is indistinguishable from that of NADH, the main proportion of the observed cellular signal has been attributed to NADH due to the different magnitudes in intracellular concentrations of NADH and NADPH (Kasischke et al., 2004). We will refer to this fluorescence signal as NAD(P)H fluorescence to indicate that a discrimination of NADH and NADPH solely from fluorescence intensity is not possible. Using this technology it has been shown that the NADH/NAD^+ redox state is regulated by neuronal activity in various preparations (Duchen, 1992; Kann et al., 2003; Kasischke et al., 2004; Lipton, 1973; Mironov and Richter, 2001; Shuttleworth et al., 2003). Interestingly, the changes in NAD(P)H fluorescence under these conditions show a biphasic response consisting of a fast decrease ("dip") and a delayed increase ("overshoot") (Duchen, 1992; Kann et al., 2003; Kasischke et al., 2004; Lipton, 1973; Mironov and Richter, 2001; Shuttleworth et al., 2003). For example, in slice preparations from the brain stem that contain the main rhythm generators of the respiratory centres, each burst of neuronal activity is accompanied by these biphasic changes in NAD(P)H fluorescence (Mironov and Richter, 2001). Similarly, exogenous electrical stimulation of cultured or acutely isolated slices from the hippocampus also elicits a biphasic response of NAD(P)H fluorescence (Kann et al., 2003; Kasischke et al., 2004; Shuttleworth et al., 2003). The increase in NAD(P)H fluorescence has been reported to correlate strongly with the stimulation intensity as well as with the induced change in mitochondrial Ca^{2+} (Kann et al., 2003) and is mirrored by changes in FAD fluorescence (Brennan et al., 2006; Duchen, 1992; Mironov and Richter, 2001; Shuttleworth et al., 2003), consistent with the view that NAD(P)H fluorescence transients are mainly attributable to mitochondria (Brennan et al., 2006; Schuchmann et al., 2001). In contrast, using high-resolution two-photon laser scanning microscopy the dip has been attributed to neuronal NADH oxidation through the respiratory chain, while the latter increase originates from the astrocytic compartment, most likely from cytosolic glycolysis (Kasischke et al., 2004). The latter is supported by the early findings in cortical slices that addition of pyruvate in high concentrations decreases the overshoot component of the NAD(P)H fluorescence response indicating an involvement of cytosolic (i.e., most likely glycolysis-derived) NADH (Lipton, 1973), a finding that was not confirmed for hippocampal slices (Brennan et al., 2006). It should be noted that NADH and NADPH can diffuse through gap junctions between coupled astrocytes (Gandhi et al., 2009b), thereby complicating the interpretation of results obtained by NAD(P)H fluorescence imaging.

Using NAD(P)H fluorescence, functional *in vivo* evidence for the relevance of the NADH/NAD^+ redox state to brain physiology has been obtained. During cortical spreading depression, a pathological state of the brain characterized by a wave of interruption of synaptic activity, tissue depolarization and increased metabolic demands, NAD(P)H fluorescence has been found to change in animals (Mayevsky et al., 1980, 1982; Mayevsky and Chance, 1975; Takano et al., 2007) and even in humans (Mayevsky et al., 1996; Mayevsky and Chance, 2007). However, the detailed kinetics seems to be more complex *in vivo* than in tissue preparations (Takano et al., 2007). Indeed, it has been shown that during spreading depression in the cortex, a decrease in NAD(P)H fluorescence (indicating oxidation of NADH) occurs close to blood capillaries while an increase in NAD(P)H fluorescence (the overshoot component) occurs in areas further away from the blood supply (Takano et al., 2007). This contradicts findings for acutely isolated hippocampal slices that localize the dip in NAD(P)H fluorescence to neurons and the overshoot to astrocytes (Kasischke et al., 2004) but is consistent with the idea that differences between *in vitro* and *in vivo* results might be due to differences in oxygen availability (Turner et al., 2007). Nevertheless, these data show that NAD(P)H fluorescence is a sensitive indicator for activation of metabolism in a given brain area and may help to reveal the connection between brain signaling and metabolism.

4. Regulation by the cellular redox state

4.1. *Regulation by the NADH/NAD^+ redox state*

Besides their well known functions as co-factors in dehydrogenase reactions, NAD^+ and NADH have been shown to have numerous other functions. These include redox-responsive regulation of cellular signaling processes, regulation of Ca^{2+} signaling following conversion to cyclic ADP-ribose and serving as redox-independent substrates for a range of enzymes including poly-ADP-ribose-polymerase and members of the sirtuin family of histone-deacetylases. We will focus here on the redox related regulatory functions of NADH/

NAD^+ and the reader is referred to recent reviews for discussion of the other topics (Berger et al., 2004; Lin and Guarente, 2003; Ying, 2007, 2008). In contrast to the NADH/NAD^+ redox pair, relatively little information is available on the regulation of cellular signaling processes by the redox state of NADPH/$NADP^+$ (Berger et al., 2004; Ying, 2008).

4.1.1. *Control of cellular signaling*

Ca^{2+} signals are considered as the major signaling pathway used by astrocytes (Araque, 2008; Fellin, 2009; Fiacco and McCarthy, 2006; Scemes and Giaume, 2006). Inositol-1,4,5-trisphosphate (IP3) receptors, which play an important role in mediating cytosolic Ca^{2+} increases, are modulated by the intracellular NADH/NAD^+ redox state (Kaplin et al., 1996). IP3-induced Ca^{2+} fluxes through these receptors are potentiated by the presence of NADH, but not by NAD^+. In addition, hypoxia-increased intracellular NADH elevates Ca^{2+} signaling in PC12 cells, an effect blocked by inhibition of glycolysis (Kaplin et al., 1996). A similar metabolic modulation of Ca^{2+} signals in astrocytes has the potential to affect numerous astrocytic functions as well as intercellular signaling both to other astrocytes and to neurons. Furthermore, GAPDH has been found to interact directly with IP3-receptors, suggesting that the NADH involved in IP3 receptor regulation might be locally produced via glycolysis (Patterson et al., 2005). A similar direct interaction of GAPDH has also been reported for the $GABA_A$ receptor (Laschet et al., 2004). Here GAPDH does not function as oxidoreductase, but rather acts as a kinase phosphorylating the $GABA_A$ receptor, thereby maintaining $GABA_A$ receptor mediated currents. NADH stimulates this kinase activity of GAPDH and improves the inhibition of current run-down, indicating that the local redox state can contribute to the regulation of $GABA_A$ receptor mediated signals (Laschet et al., 2004).

4.1.2. *Control of transcription*

Several transcription factors have been reported to sense the NADH/NAD^+ redox state. The transcription factors Clock and NPAS2, which are essential for the circadian clock in the mammalian superchiasmatic nucleus and the forebrain, respectively, increase their binding to their partner BMAL1 and to their DNA target sequence with increasing concentrations of both NADH or NADPH (Rutter et al., 2001). The activation by the reduced dinucleotide is strongly inhibited by the oxidized form resulting in a very steep activation curve with an apparent Hill coefficient of 15 (Rutter et al., 2001). One target gene of the NPAS2:BMAL1 transcription factor complex is lactate dehydrogenase (LDH) A (Rutter et al., 2001), which uses NAD^+ or NADH for the conversion of lactate to pyruvate or vice versa and is mainly expressed in astrocytes (Pellerin et al., 1998). It has been proposed that glutamate released from active neurons stimulates astrocytic glycolysis and lactate production (Magistretti, 2006). While not shown directly, this might also increase intracellular NADH levels, thereby inducing LDH expression and entraining the circadian clock via the NPAS2:BMAL1 complex (Rutter et al., 2001).

The transcription repressor C-terminal binding protein (CtBP) is also regulated by the NADH/NAD^+ redox state (Fjeld et al., 2003; Garriga-Canut et al., 2006; Zhang et al., 2002). The binding to its interaction partners such as the transcriptional repressors zinc finger E-box-binding homeobox protein (ZEB) and neural restrictive silencing factor (NRSF) is modulated by NADH, and results in regulation of target genes including E-cadherin, brain derived neurotrophic factor (BDNF) and the BDNF receptor TrkB (Garriga-Canut et al., 2006; Zhang et al., 2002). In addition, recent *in vivo* evidence connects the effect of 2-deoxyglucose on the rat kindling model of epilepsy to NADH and CtBP dependent regulation of TrkB and BDNF expression (Garriga-Canut et al., 2006). These findings provide strong *in vivo* evidence that the regulation of gene expression can be modulated by the NADH/NAD^+ redox state and that this regulation has a major impact on brain physiology. Since this regulation is mediated by NRSF and since numerous other genes expressed within the brain contain the respective regulatory sequences (Roopra et al., 2001), it is tempting to speculate that a similar regulation by the NADH/NAD^+ redox state will also be discovered for other genes.

A connection between the NADH/NAD^+ redox state and the cell cycle has been described via the control of histone 2B (H2B) expression during the S-phase (Dai et al., 2008; Zheng et al., 2003). This regulation is accomplished by the Oct-1 transcription factor in combination with the "Oct-1 coactivator in S-phase"-complex (OCA-S). This protein complex includes GAPDH and LDH, both of which are essential for proper expression of H2B (Dai et al., 2008; Zheng et al., 2003). The assembly of this complex is inhibited by NADH and stimulated by increasing concentrations of NAD^+. However, increasing concentrations of NAD^+ finally lead to the repression of H2B expression (Dai et al., 2008). These properties link H2B expression to the metabolic state of the cell and might therefore contribute to a cell cycle checkpoint allowing G1–S cell cycle transition only in the case of a proper redox state of the cell (Dai et al., 2008). These findings fit into the increasing amount of evidence that enzymes such as GAPDH can serve numerous functions distinct from their primary role, e.g., in glycolysis (Sirover, 2005).

4.2. *Regulation by glutathionylation*

Glutathionylation of protein sulfhydryl groups has emerged as a rather new aspect of cellular regulation. Numerous proteins have been identified as potential targets of this regulatory mechanism (Mieyal et al., 2008). Although the mechanisms of formation of mixed protein–glutathione disulfides have not yet been resolved, they most likely involve reactive intermediates such as S-nitroso-glutathione (Dalle-Donne et al., 2008; Gallogly and Mieyal, 2007; Mieyal et al., 2008). A direct disulfide exchange of GSSG reacting with a free cysteine sulfhydryl group in proteins is unlikely since, in unstressed cells, the ratio of GSH to GSSG is very high. During oxidative stress, however, the GSH to GSSG ratio decreases drastically in astrocytes (Dringen et al., 1998, 1999a, 2005; Hirrlinger et al., 2001). Under these conditions, it is feasible that disulfide exchange reactions may occur at least with reactive protein sulfhydryl groups as has been documented, e.g., for c-Jun (Klatt et al., 1999).

Little information is available regarding regulation by glutathionylation in astrocytes or neurons. Therefore, most

information has to be extrapolated from data obtained from other cells. Only a few aspects will be discussed here as recent comprehensive reviews cover glutathionylation of proteins in depth (Dalle-Donne et al., 2009; Gallogly and Mieyal, 2007; Mieyal et al., 2008). Glutathionylation of GAPDH lowers its enzymatic activity (Mohr et al., 1999; Ravichandran et al., 1994). As GAPDH catalyses a step of glycolysis, its glutathionylation is likely to impact energy production as well as the NADH/NAD^+ redox state. In addition, glutathionylation of GAPDH affects the GAPDH-dependent regulation of expression of endothelin-1 (Rodriguez-Pascual et al., 2008), a signaling molecule involved in the proliferation and glucose uptake of astrocytes (Herrero-Gonzalez et al., 2009). Both the cytosolic and mitochondrial isoforms of ICDH are also subject to glutathionylation (Kil and Park, 2005; Shin et al., 2009). At least for mitochondrial ICDH, an increase in glutathionylation has been reported in the MPTP model of Parkinson's disease (Kil and Park, 2005), indicating a potential impact of regulation of this NRE by glutathionylation.

Ryanodine receptors (RyR) are Ca^{2+} channels localized in the membrane of the endoplasmatic reticulum, which contribute to Ca^{2+} signaling in numerous types of cells. The RyR subtype 1 has been shown to be glutathionylated and to change its properties as a result (Aracena et al., 2003). Since RyR1 is most likely not present in astrocytes (Beck et al., 2004; Matyash et al., 2002) and a similar regulation of the astrocyte-expressed RyR3 (Hertle and Yeckel, 2007; Matyash et al., 2002) has not yet been analysed, it remains unclear whether or not glutathionylation of RyRs contribute to the regulation of Ca^{2+} signaling in astrocytes. However, RyR1 is expressed in neurons (Beck et al., 2004; Hertle and Yeckel, 2007) raising the possibility of glutathionylation-based regulation of Ca^{2+} signals in neurons and especially at synapses. Indeed, in cultured hippocampal neurons RyR-glutathionylation is induced by exposure to H_2O_2 and coincides with RyR-mediated Ca^{2+} signals (Kemmerling et al., 2007).

Furthermore, actin has been shown to be subject to glutathionylation, which regulates its polymerization (Wang et al., 2001). Processes of astrocytes close to synapses are highly motile (Hirrlinger et al., 2004) and neuronal dendritic spines also show a high degree of actin-dependent remodeling (Matus, 2000; Schubert and Dotti, 2007). It is therefore possible that glutathionylation influences morphological plasticity within the brain. Finally, glutaredoxin, the enzyme responsible for removing glutathione from glutathionylated proteins, has been implicated in nuclear factor-κB (NF-κB) dependent regulation of intercellular adhesion molecule 1 (ICAM-1) expression in retinal Müller cells (Shelton et al., 2007) as well as in proinflammatory responses of those cells to diabetic conditions (Shelton et al., 2009).

Taken together, substantial evidence has been presented that glutathionylation is likely to be an important regulatory mechanism for various cellular processes. If alterations in the ratio of cellular GSH to GSSG or in the cellular concentration of S-nitroso-glutathione are able to induce or modulate the glutathionylation of enzymes and regulatory proteins, the occurrence of oxidative or nitrosative stress in cells could be translated by glutathionylation directly into effects on metabolism and cell functions. However, the currently available information regarding the regulation and consequences of glutathionylation of proteins in brain cells is sparse and needs further investigation.

5. Consequences of alterations in astrocytic redox state for metabolite trafficking

Astrocytes provide their neighboring neurons with a variety of metabolites that include energy substrates (Nehlig and Coles, 2007; Pellerin et al., 2007; Pellerin, 2008; Schousboe et al., 2007), precursors of neurotransmitters such as glutamine (Albrecht et al., 2007; McKenna, 2007; Yang et al., 2008) and precursors for the neuronal synthesis of the antioxidant GSH (Banerjee et al., 2008; Dringen, 2009; Dringen and Hirrlinger, 2003). Thus, any disturbance of the metabolic pathways of astrocytes required for the formation of such metabolites – for example by oxidative stress – is likely to also markedly affect neuronal functions.

Lactate is a valuable metabolic intermediate that is produced in large amounts in astrocytes. This astrocyte-derived lactate is provided as an energy substrate to neighboring neurons, although the extent and the importance of this lactate supply for the *in vivo* situation is still a matter of debate (Dienel and Cruz, 2008; Gandhi et al., 2009a; Magistretti, 2009; Nehlig and Coles, 2007; Pellerin et al., 2007; Pellerin, 2008; Schousboe et al., 2007; Barros and Deitmer, 2010). The rate of lactate production by astrocytes depends on the cytosolic redox state. Inhibition of mitochondrial ATP production for example by azide will increase lactate production by the LDH reaction, thereby regenerating the NAD^+ that is required as substrate of the GAPDH reaction (Dringen et al., 1993). In addition, the rates of glucose consumption and glycolysis in cultured astrocytes are also strongly affected by oxidative stress. Sustained peroxide stress lowers the rate of lactate production and the lactate release from astrocytes by up to 40% (Liddell et al., 2009). Partially responsible for this reduced lactate production is the loss of carbon due to the rapid activation of the PPP that is a direct consequence of the demand of NADPH for GR-dependent GSSG reduction (Liddell et al., 2009). In addition, the strong inactivation of GAPDH that has been observed for astrocytes exposed to sustained peroxide stress (Liddell et al., 2009) is likely to contribute to the reduction in lactate formation.

Astrocytes are essential partners of neurons at the tripartite synapse (Araque et al., 1999) and are responsible for the highly efficient removal of synaptic glutamate that is released from glutamatergic neurons into the synaptic cleft. The excitatory neurotransmitter glutamate is quickly removed from glutamatergic synapses by uptake into the perisynaptic processes of astrocytes. This glutamate is then amidated by glutamine synthetase (GS) to glutamine, released from astrocytes, taken up by neurons and hydrolysed to glutamate via glutaminase (Fig. 3) (Albrecht et al., 2007; McKenna, 2007; Yang et al., 2008; Zwingmann and Leibfritz, 2007). This glutamate–glutamine cycle between neurons and astrocytes provides neurons with glutamine which serves as precursor for glutamate that is required as neurotransmitter but also for neuronal protein and GSH synthesis (Kranich et al., 1996). In addition, quick removal of glutamate by

astrocytic uptake is highly important to protect neurons against glutamate-dependent excitotoxicity (Dong et al., 2009). Astrocytic GS is an essential component of the glutamate-glutamine cycle and thereby plays a central role in the astrocytic supply of precursors for neuronal glutamate (Fig. 3). Inactivation of GS slows the clearance of excitotoxic glutamate and depletes the supply of glutamine to neurons (Hertz et al., 1999; Pow and Robinson, 1994). Astrocytic GS is strongly affected by oxidative stress. Sustained peroxide stress diminished the specific activity of GS in cultured astrocytes in a process that is accelerated in GPx1-deficient cells (Dringen et al., 2006; Knorpp et al., 2006). Thus, the availability of an intact GSH/GSSG redox cycling that includes GPx1 appears to contribute to the protection of functional astrocytic GS against oxidative damage.

The amino acid cysteine limits neuronal GSH synthesis (Dringen et al., 1999b) and is supplied to neurons by neighboring astrocytes. The cysteine supply involves the export of GSH from astrocytes by Mrp1, the extracellular cleavage of GSH by the ectoenzymes γ-glutamyl transpeptidase (γGT) and aminopeptidase N, and the uptake of cysteine into neurons by the sodium-dependent excitatory amino acid carrier 1 (EAAC1) (Aoyama et al., 2006, 2008; Dringen, 2009; Dringen and Hirrlinger, 2003). With the release of the glutamate precursor glutamine by astrocytes and the extracellular generation of CysGly (Fig. 3), all three constituent amino acids of GSH are provided to neurons by astrocytes.

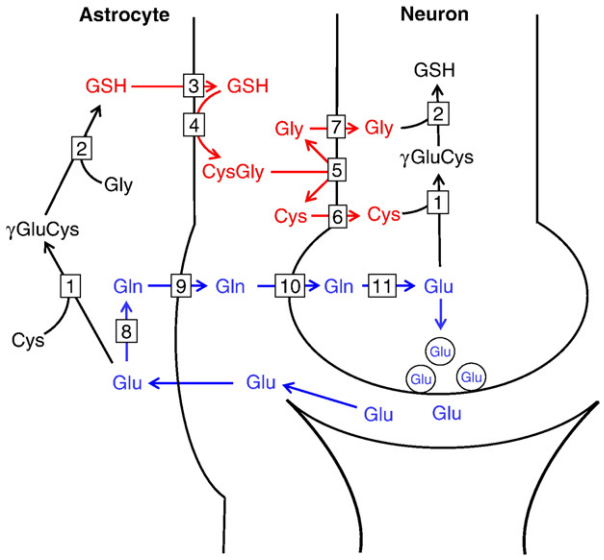

Fig. 3 – Metabolic interaction between astrocytes and neurons in GSH (red) and glutamate (blue) metabolism. Astrocytes and neurons synthesize GSH in the consecutive reactions of glutamate cysteine ligase (1) and GSH synthetase (2). GSH is exported from astrocytes by Mrp1 (3) or other transporters and serves as substrate of the astrocytic ectoenzyme γGT (4). This enzyme generates the dipeptide CysGly by transfering the γ-glutamyl moiety from GSH onto an acceptor. CysGly is hydrolysed by neuronal aminopeptidase N (5) and the generated amino acids cysteine and glycine are taken up into neurons by active transport (6, 7). The glutamate that is required as neurotransmitter or for GSH synthesis in neurons is generated from the glutamine provided by astrocytes (9–11). Glutamate that leaves the synaptic cleft is taken up by astrocytes and is amidated by glutamine synthetase (8) to glutamine that is subsequently released by glutamine transporters (9). Extracellular glutamine is taken up into neurons (10) and hydrolysed by phosphate-activated glutaminase (11) to glutamate.

The rate of GSH export from astrocytes depends on the cellular concentration of GSH (Sagara et al., 1996). Thus, every process that lowers cellular GSH concentration in astrocytes will reduce the rate of GSH export and subsequently the supply of GSH precursors to neurons. Astrocytic GSH concentrations are markedly lowered by the presence of xenobiotics that react with GSH (Schmidt and Dringen, 2009; Waak and Dringen, 2006). As a consequence of such conditions, the export of GSH from astrocytes is drastically slowed and extracellular GSH that is required as substrate of γGT is barely detectable. GSH export is also markedly compromised by alterations in the cytosolic GSH/GSSG ratio. The rapid oxidation of GSH to GSSG following peroxide exposure reduces the concentration of cytosolic GSH, resulting in reduced export of GSH. In addition, astrocytes exposed to sustained peroxide stress efficiently export GSSG via Mrp1 (Hirrlinger et al., 2001, 2002a; Minich et al., 2006). This situation deprives the cells of cytosolic GSSG that could serve as substrate for cytosolic GSH regeneration by GR (Fig. 2). ATP-dependent synthesis of GSH is then required for the replenishment of cellular GSH. Therefore, a decrease in the cellular GSH/GSSG ratio during oxidative stress almost completely prevents the export of GSH from astrocytes and compromises the supply of GSH precursors to neurons.

6. Conclusions

The cellular redox state is highly important for metabolism and signaling of astrocytes and of the brain in general. Numerous processes are influenced either directly or indirectly by the redox balance in the NADH/NAD^+, the NADPH/$NADP^+$ and the GSH/GSSG pools. Therefore, disturbances or dysregulation of these redox states are likely to markedly affect energy homeostasis, signal processing and plasticity in the brain. An improved knowledge of the events that determine the cytosolic redox state of astrocytes, the interrelated signaling pathways and the metabolite trafficking between astrocytes and neurons may help to develop strategies to promote neuronal survival and recovery in brain pathologies (Escartin and Bonvento, 2008). However, most data reported so far in this field have been obtained on cell culture models or on brain slices without (sub-)cellular resolution. While these approaches are well suited for elucidating experimental strategies and for screening approaches providing important basic and mechanistic information, they fail to show the functional relevance of the observed phenomena at the local microenvironment of synapses. To elucidate the relevance of the crossroads of redox regulation, metabolism and signaling at synapses in the brain *in vivo*, it will be essential to develop new animal model systems which allow the modulation of metabolic pathways and redox states cell type-specifically *in vivo*.

Acknowledgments

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Review

Neuroglia in neurodegeneration

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ABSTRACT

Neuroglial cells are fundamental for control of brain homeostasis and they represent the intrinsic brain defence system. All forms in neuropathology therefore inevitably involve glia. The neurodegenerative diseases disrupt connectivity within brain circuits affecting neuronal–neuronal, neuronal–glial and glial–glial contacts. In addition neurodegenerative processes trigger universal and conserved glial reactions represented by astrogliosis and microglial activation. The complex of recently acquired knowledge allows us to regard the neurodegenerative diseases as primarily gliodegenerative processes, in which glial cells determine the progression and outcome of neuropathological process.

Contents

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1. Neuroglia—the concept

The complexity of the cellular circuitry of human brain is unparalleled by any other living system known so far. The neural cells are exceedingly densely packed within a strictly limited volume of the skull, thus requiring a specific attention to the control of brain homeostasis throughout early development and postnatal functioning. This specific requirement is manifested in the highly developed brain–blood barrier, which essentially limits the impact of bodily homeostatic systems on the central nervous system (CNS). The brain homeostasis therefore is entrusted to specific population of neural cells known as neuroglia.

The concept of neuroglia as a connective tissue into which all elements of the central nervous system (CNS) are embedded was introduced by Rudolf Virchow (Kettenmann and Verkhratsky, 2008; Virchow, 1856, 1858). In the course of late 19th–early 20th century the cellular nature and morpho-functional heterogeneity of neuroglial cells were firmly established (Andriezen, 1893; Golgi, 1903; Kölliker, 1889; Retzius, 1894–1916; Schleich, 1894; Weigert, 1895). In the recent decades the functional relevance and versatility of neuroglia that is involved in all activities of the CNS, from structural and metabolic support to information processing, has started to be fully appreciated (Haydon and Carmignoto, 2006; Kettenmann and Ransom, 2005; Perea et al., 2009; Ransom et al., 2003; Verkhratsky, 2006b, 2009; Verkhratsky and Toescu, 2006; Volterra and Meldolesi, 2005). Furthermore, the evolutionary uniqueness of human glial cells (Oberheim et al., 2009, 2006) indicates their role in the formation of human intellect.

The neuroglia appeared early in evolution, when primitive nervous systems began to emerge, and became the predominant cellular type in the brain of *Homo sapiens*. The main types of neuroglia are represented by astrocytes (named so by Michael von Lenhossek (Lenhossek, 1893)), oligodendrocytes (christened by Pio del Rio-Hortega (Rio-Hortega, 1921)) NG2 positive glia (initially revealed by William Stallcup (Levine et al., 1986; Stallcup, 2002) and identified as a separate class of glia in the recent decade (Berry et al., 2002; Butt et al., 2005; Nishiyama et al., 2009) and microglia (discovered by Rio-Hortega (Rio-Hortega, 1919a,b, 1920)). The cellular elements comprising each of these groups, although having common features, demonstrate a profound functional heterogeneity in different brain regions and at different developmental stages. Nonetheless, each and every neuroglial cell has a conceptual countenance to keep the brain homeostasis, starting from the control of local molecular environment to providing the intrinsic brain defence system. These glial homeostatic functions are many, and their failure inevitably signals brain pathology. This essay is specifically dedicated to the role of neuroglia in neurodegenerative processes, yet prior to embark into the realms of disease we shall provide a brief overview of physiological functions of main types of glial cells.

2. Physiological functions of glial cells: Neuroglia as the brain homeostatic machinery

2.1. *Astrocytes*

2.1.1. *Astrocytes organise the brain matter*

The astrocytes provide for the micro-architecture of the grey matter by dividing it (through the process known as "tiling" (Bushong et al., 2004)) into relatively independent structural units. Each protoplasmic astrocyte dwelling in the grey matter establishes its own territory within the limits of its elaborated arbour of processes; this domain organisation exists in both rodents and humans (Bushong et al., 2002; Nedergaard et al., 2003; Oberheim et al., 2009; Wilhelmsson et al., 2006). Within these anatomical domains astroglial membranes cover synaptic contacts and establish contacts with neuronal membranes as well as with blood vessels. Evolution of the CNS and appearance of the intellect coincided with a remarkable increase in the size and complexity of astroglial cells (Oberheim et al., 2009). That is the average diameter of the domain belonging to a human protoplasmic astrocyte is ~2.5 times larger that the domain formed by a rat astrocyte (142 vs. 56 μm). The volume of the human protoplasmic astrocyte domain was ~16.5 times larger than that of the corresponding domain in a rat brain. Furthermore, human protoplasmic astrocytes have ~10 times more primary processes emanating from their somatas, and correspondingly much more complex processes arborisation. Likewise, the fibrous astrocytes, populating the white matter are ~2.2 times larger in humans when compared to rodents. The astroglial domains that

parcellate the grey matter can be the unifying structures, which, by covering many synapses (about 20.000–120.000 in rodents and 270.000 to 2.000.000 in humans), can integrate and regulate the activity of large synaptic sets (Halassa et al., 2007b).

The single astrocytic domains are further integrated by virtue of gap junctions (Bruzzone and Giaume, 1999; Dermietzel et al., 2000) into astroglial syncytia. The gap junctional contacts are localised on the peripheral processes where two astroglial domains overlap; the actual areas of contacts between neighbouring astrocytes are quite small. It is interesting that the overlapping areas of human astrocytes were ~20 times larger than in rodents (Oberheim et al., 2009), which possibly indicates a higher degree of coupling. Gap junctions provide for a glial information-transfer system, as they form pathways for intercellular diffusion of many molecules, which convey the long-range signalling. One of these signalling pathways, represented by diffusion of second messenger $InsP_3$ with subsequent Ca^{2+} release is well characterised as propagating Ca^{2+} wave (Cornell Bell et al., 1990; Dani et al., 1992; Scemes and Giaume, 2006); both Ca^{2+} release and Ca^{2+} waves are considered to be a substrate for astroglial excitability (Agulhon et al., 2008; Deitmer et al., 1998; Verkhratsky, 2006a; Verkhratsky and Kettenmann, 1996; Verkhratsky et al., 1998). However, other molecules, such as, for example, metabolic substrates (Rouach et al., 2008) can form alternative inter-glial signalling systems. The astroglial syncytia are formed within larger anatomical structures: for example, in the somatosensory cortex, astroglial networks are confined to individual barrels with very weak (if any) inter-barrel coupling (Giaume et al., 2009; Houades et al., 2008).

2.1.2. *Astrocytes form neuronal–glial–vascular units and provide neurones with metabolic support*

The concept of astrocytes forming a metabolic connection between neurones and blood vessels was introduced by Camillo Golgi after his discovery of astroglial endfeet embracing brain capillaries (Golgi, 1903). The astroglial domains are instrumental in establishing such a connection through moulding the neuronal–glial–vascular units, which integrate neural circuitry with local blood flow. Indeed, most protoplasmic astrocytes contact neighbouring capillaries through the perivascular processes forming an endfoot (Oberheim et al., 2009). Astrocytes also forge a functional link between neurones and blood vessels. An increase in focal neural activity triggers rapid vasodilatation, the phenomenon known as functional hyperaemia (Roy and Sherrington, 1890). An increase in neuronal activity within the astroglial domain triggers Ca^{2+} signals, which enter astrocyte endfeet and result in the release of vasoactive substances. The latter can trigger either vasoconstriction or vasodilatation (Iadecola and Nedergaard, 2007; Metea and Newman, 2006; Mulligan and MacVicar, 2004; Takano et al., 2006; Zonta et al., 2003).

The metabolic support of neurones is achieved through a glucose-lactate shuttle operative within the astroglial domains. Astrocytes accumulate about 50% of glucose entering the brain tissue, and store it in the form of glycogen. An increase in neuronal activity, accompanied with an increased glutamate release, results in Na^+-dependent glutamate transport into the astrocytes. An increase of Na^+ concentration in the cytosol of astrocytes stimulates glycolysis, which results in synthesis of lactate; the latter is then transported to neurones by monocarboxylase transporters 1 and 2, thus providing active cells with much needed energy substrate (Magistretti, 2006, 2009; Pellerin et al., 2007).

2.1.3. *Astrocytes control extracellular homeostasis of ions and neurotransmitters*

Control of the extracellular concentrations of ions, metabolites and neuroactive molecules is of a paramount importance for brain function. Neuronal excitability is maintained by relatively large transmembrane fluxes of ions, which are moved by electro-chemical gradients. These fluxes affect the extracellular concentrations of ions, which in turn change these gradients. The accumulation of extracellular K^+ is particularly important as it accompanies the repolarisation phase of action potentials; under physiological conditions the extracellular potassium concentration ($[K^+]_o$) can rise up to 10–12 mM (Kofuji and Newman, 2004). Control of extracellular K^+ concentration is accomplished by astrocytes through local K^+ uptake involving inward rectifier K^+ channels and K^+ spatial buffering (Coles and Orkand, 1983; Kofuji and Newman, 2004; Newman, 1995). The spatial K^+ buffering provides for the redistribution of K^+ from the areas with elevated $[K^+]_o$ to the regions with low $[K^+]_o$. This spatial buffering occurs either in glial syncytia or within the confines of single radial Muller glial cells, through the process known as K^+ siphoning (Newman et al., 1984).

The astroglial transport of ions, which accompanies neuronal activity (e.g. K^+ uptake through K_{IR} channels, or Na^+ accumulation alongside with glutamate transportation) requires concomitant movement of water. Control of water homeostasis is also accomplished by astrocytes. High synaptic activity is associated with local shrinkage of the extracellular space, which is regulated by water transport across astroglial membranes and water redistribution through the glial syncytium. Water enters and leaves the astroglial syncytia through aquaporins channels (mostly of AQP4 type) which are concentrated in perisynaptic processes and in the perivascular and subpial endfeet (Simard and Nedergaard, 2004). Furthermore, K^+ buffering and water redistribution are tightly coordinated and alteration of water flux impairs upon K^+ buffering (Eid et al., 2005).

Astrocytes play the central role in the extracellular homeostasis of neurotransmitters and most importantly of glutamate. Glutamate, despite being the main excitatory transmitter in the CNS, is the most powerful neurotoxin, and every excess of glutamate in the extracellular spaces triggers excitotoxic neuronal death. Astrocytes are the main sink of glutamate in the brain; from the bulk of glutamate released during synaptic transmission, about 20% is accumulated into postsynaptic neurones and the remaining 80% is taken up by perisynaptic astrocytes (Swanson, 2005; Verkhratsky and Butt, 2007; Verkhratsky and Kirchhoff, 2007a).

Glutamate transport is accomplished by specific glutamate transporters (Gadea and Lopez-Colome, 2001), represented by several types (EAAT1 to EAAT5 in human brain). The EAAT1 and EAAT2 (known in rodent brain as glutamate/aspartate transporter, GLAST, and glutamate transporter-1, GLT-1) are

expressed exclusively in astrocytes (Danbolt, 2001) and are responsible for the bulk of glutamate uptake. The translocation of glutamate is powered by transmembrane ion gradients, and the transport of a single glutamate molecule requires an influx of three Na^+ ions and one H^+ ion coupled with the efflux of one K^+ ion (Owe et al., 2006; Zerangue and Kavanaugh, 1996). As a result Na^+/glutamate transporter is electrogenic and its activation produces a net inward sodium current (Kirischuk et al., 2007), which may substantially affect intracellular Na^+ concentration. The excessive sodium accumulation accompanying glutamate accumulation can be counterbalanced by Na^+ efflux through Na^+/Ca^{2+} exchanger working in the reverse mode (Kirischuk et al., 1997).

Astrocytes are also crucial for the recovery of glutamate to the presynaptic terminal. After entering the astroglial cells glutamate is converted into glutamine by the astrocytic-specific glutamine synthetase (Martinez-Hernandez et al., 1977). Glutamine, being non-toxic, can subsequently be safely transported back to the presynaptic terminal through the extracellular space; after entering the neuronal compartment glutamine is converted into glutamate, thus accomplishing the glutamate–glutamine shuttle.

2.1.4. *Gliotransmission*

The ability of astrocytes to release chemical transmitters (named gliotransmitters in order to distinguish them from neurotransmitters) is fundamental for their involvement in information processing in neuronal–glial networks. The gliotransmitters include glutamate, ATP, D-serine, GABA, taurine and possibly other molecules (see Angulo et al., 2008; Bezzi et al., 1998; Fellin et al., 2004; Jourdain et al., 2007; Kozlov et al., 2006; Oliet and Mothet, 2009; Panatier et al., 2006; Volterra and Meldolesi, 2005 for review). The gliotransmitters can be released from astrocytes through Ca^{2+}-dependent exocytosis (Bezzi et al., 2004), by diffusion though large pore channels (e.g. $P2X_7$ receptors, hemichannels or volume-activated Cl^- channels (Abbracchio et al., 2009; Duan et al., 2003; Kang et al., 2008; Pankratov et al., 2006)) or through transporters (reversed glutamate transporter (Rossi et al., 2000) or a cystine-glutamate antiporter (Warr et al., 1999)).

2.1.5. *Astrocytes mould the CNS synapses and participate in synaptic transmission*

Most of the CNS synapses are formed by three elements—the astroglial perisynaptic process, the presynaptic neuronal terminal and the postsynaptic neuronal membrane—the structure generally known as a tripartite synapse (Araque et al., 1999; Halassa et al., 2007a; Perea et al., 2009). The astrocyte has a dual role in this tripartite synapse. First, by the virtue of neurotransmitter receptors expressed in the astroglial membrane, the astrocyte can sense the transmitter release from the neuronal terminal, and secondly by releasing gliotransmitters the astrocyte can modulate the efficacy of the synapse. Astroglial cells can potentially express virtually every neurotransmitter receptor (Verkhratsky and Kettenmann, 1996; Verkhratsky et al., 2009, 1998; Verkhratsky and Steinhauser, 2000). This expression however, is strictly controlled *in vivo* and astrocytes from different brain regions are endowed with very distinct complement of receptors. The cortical astrocytes, for example, express functional NMDA and $P2X_{1/5}$ receptors, which are absent in hippocampus (Jabs et al., 2007; Lalo et al., 2006, 2008; Verkhratsky and Kirchhoff, 2007b). Gliotransmitter release actively modulates synaptic transmission by activating various neuronal receptors such as, for example, NMDA or adenosine receptors (Lee and Haydon, 2007; Parpura and Haydon, 2000; Pascual et al., 2005).

Astrocytes do not only participate in synaptic transmission—they act as key elements in synaptogenesis, in synaptic maturation and maintenance. In the *in vitro* condition, the addition of astrocytes triggers very substantial increase (up to seven times) in synapse formation (Pfrieger and Barres, 1996). Astrocytes produce and secrete cholesterol (Nieweg et al., 2009), which is critically important for synapse formation and secrete variety of factors needed for both synaptic maturation and maintenance (Pfrieger, 2009; Pfrieger and Barres, 1996). Furthermore astrocytes synthesise and release thrombospondins 1 and 2 that promote synaptogenesis both in vivo and in vitro and are critically important for post-lesion synaptic plasticity, remodelling and regeneration (Christopherson et al., 2005; Liauw et al., 2008).

2.2. *Oligodendrocytes*

The function of oligodendrocytes is to produce the myelin sheaths that insulate axons in the CNS. The myelin sheath is composed from several specific proteins including myelin proteolipid protein (PLP), myelin basic protein (MBP) and myelin associated glycoprotein (MAG) (Butt, 2005; Ndubaku and de Bellard, 2008). The myelin sheath being a fatty insulating layer facilitates the saltatory conduction of action potentials (Butt, 2005). So far four different phenotypes (I–IV) subdivide oligodendrocytes. Developmentally, all four types of oligodendrocytes are likely to originate from common oligodendrocyte progenitor cells (OPCs) nestled in the SVZ. After the migration to their target regions OPCs differentiate and mature up-regulating the expression of myelin proteins, and begin to form the myelin sheath (McTigue and Tripathi, 2008).

2.3. *NG2 glial cells*

The NG2 glial cells (identifiable by the expression of NG2 chondroitin sulphate proteoglycan (Levine et al., 1986)), which are also known as synantocytes (from the Greek *synanto* for contact (Butt et al., 2005)), are present throughout the developing and adult brain (Karram et al., 2008; Nishiyama et al., 2009). Although NG2 cells express various markers, which are characteristic for oligodendrocyte progenitor cells, they have some distinct features, which permit classifying them as a separate type of glia. The NG2 cells have a stellate morphology with many primary processes, which bifurcate to form a process arborisation with a diameter of about 100 μm (Butt et al., 2005).

Physiologically many NG2 cells express voltage-gated Na^+ channels (Bergles et al., 2000; Chittajallu et al., 2004), which, at least in cortical NG2 cells are dense enough to generate action potentials (Chittajallu et al., 2004). In addition, the NG2 glia express Ca^{2+} permeable AMPA receptors, GABA receptors (Lin and Bergles, 2002) and, most likely, purinoceptors (Butt et al., 2005). In the hippocampus the NG2 glia receive functional

synaptic inputs from CA3 pyramidal neurones and GABAergic interneurones (Lin and Bergles, 2002). The NG2 cells may be important for integration in the brain because their processes pass through several neuronal layers and traverse grey and white matter. Finally, the NG2 glial cells are highly plastic progenitor cells that can give rise to astrocytes (Leoni et al., 2009) and may be even to neurones.

2.4. *Microglia—the brain surveillance system*

Microglial cells are the resident macrophages of the CNS. Microglia constitute around 10% of all cells in the nervous system. These cells are of myeloid origin (Ransohoff and Perry, 2009) and they enter CNS during the early postnatal period through the so-called "fountains of microglia" (Kershman, 1939). After entering the CNS, these cells disseminate through the parenchyma and transform into the resting microglia. The resting microglial cells have small somatas and multiple fine processes. Every microglial cell occupies the defined territorial domain, which does not overlap with neighbouring microglia. In the physiological conditions, microglial processes are constantly moving scanning the microenvironment in their anatomical domains (Davalos et al., 2005; Nimmerjahn et al., 2005). Microglia represent the innate immune system in the brain and thus are the first line of defence against invading pathogens and serve as specialised sensors for brain tissue injury (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009). Insults to the nervous system trigger a complex and multi-stage activation of microglia, which results in both phenotypic and functional changes (Hanisch and Kettenmann, 2007; Kreutzberg, 1996). This process manifested by microglia transition from a surveillance state to an activated state is controlled by multiple extracellular signals acting through a multitude of receptors. These "danger" signals are represented either by the disappearance of certain molecules, indicative of normal brain functioning or by the appearance of new molecules associated with infectious agents, debris from damaged or dying cells or misfolded and aggregated proteins appearing in response to a primary degenerative process (the concept of "on" and "off" signalling—(Biber et al., 2007; Hanisch and Kettenmann, 2007)).

Under pathological situations, such as neurodegenerative diseases, strokes, traumatic injuries and tumour invasions, these cells become activated, migrate to and surround damaged or dead cells, and subsequently clear cellular debris from the area, similarly to the phagocytic macrophages of the peripheral immune system (Fetler and Amigorena, 2005). Activated microglia up-regulate a variety of surface receptors, including major histocompatibility complex and complement receptors (Hanisch and Kettenmann, 2007; Liu and Hong, 2003). They also undergo fundamental morphological changes from a ramified phenotype to motile activated amoeboid cells (Kreutzberg, 1996). Once they are immunostimulated in response to neurodegenerative events, these microglia cells release a variety of proinflammatory mediators including cytokines, reactive oxygen species, complement factors, neurotoxic secretory products, free radical species and NO, all of which can contribute to neuronal dysfunction and cell death, ultimately creating a vicious cycle (Heneka and O'Banion, 2007).

3. Neuroglia determines the outcome of neurological pathology

Glial cells are fundamental for the control of brain homeostasis, and they represent the intrinsic brain defence system. First, the homeostatic systems expressed in astrocytes prevent homeostatic imbalances triggered by various types of stressors applied to CNS. Second, two types of glia—the astrocytes and microglia—possess evolutionary conserved programs of activation in response to brain damage. A variety of brain insults trigger a condition generally referred to as reactive gliosis, which includes astrogliosis and activation of microglia. The astrogliosis (Li et al., 2008; Pekny and Nilsson, 2005; Rolls et al., 2009) is essential for both limiting the areas of damage (by scar formation through anisomorphic astrogliosis) and for the post-insult remodelling and recovery of neural function (by isomorphic astrogliosis). The activation of microglia is fundamental for the brain immune response as well as for the removal of both invading infectious agents and posthumous cell debris (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009).

All these glial defence mechanisms are genuinely survivalistic, and yet, the glial cells being homeostatic tools possess (as many other biological homeostatic systems do) an inherent dichotomy—they can be protective as well as deleterious (Fig. 1). In fact, stronger brain insults may push glial homeostatic systems towards a damage exacerbating mode. The severe stress on astroglial energetics with a subsequent loss of ion homeostasis may trigger a massive release of glutamate (through reversed transporters or large pore channels), a substantial leak of K^+ ions, release of NO and reactive oxygen species—i.e. agents promoting neurotoxicity (Nedergaard and Dirnagl, 2005). The activation and overactivation of microglia may have similar deleterious effects through both phagocytic activity and release of pro-inflammatory and neurotoxic factors. All these glial reactions are intimately involved into acute brain damage such as trauma or stroke. All in all "glia appears as a brain warden, and as such it is intrinsically endowed with two opposite features: it protects the nervous tissue as long as it can, but it also can act as a natural killer, trying to eliminate and seal the damaged area, to save the whole at the expense of the part (Giaume et al., 2007)".

Neuroglia is also thoroughly involved in pathogenesis of many chronic neurological disorders (Halassa et al., 2007a; Rossi and Volterra, 2009). Astrocytes in epileptic foci in the temporal lobe epilepsy undergo both morphological and functional changes (see Jabs et al., 2008; Seifert et al., 2006 for review). In humans, epilepsy triggers reactive astrogliosis and an increase in GFAP expression (Cohen-Gadol et al., 2004; Gurnett et al., 2003). The role of glutamate release from astrocytes in synchronous discharges triggering epileptiform seizures has been proposed (Tian et al., 2005), although the exact degree of astroglial involvement in this process remains controversial (Fellin et al., 2006). At any rate astrocytes from a human epileptic brain display spontaneous Ca^{2+} oscillations (Manning and Sontheimer, 1997) and have an increased gap junctional coupling (Lee et al., 1995), which indicates remodelling of signalling cascades. In addition, astroglial glutamate

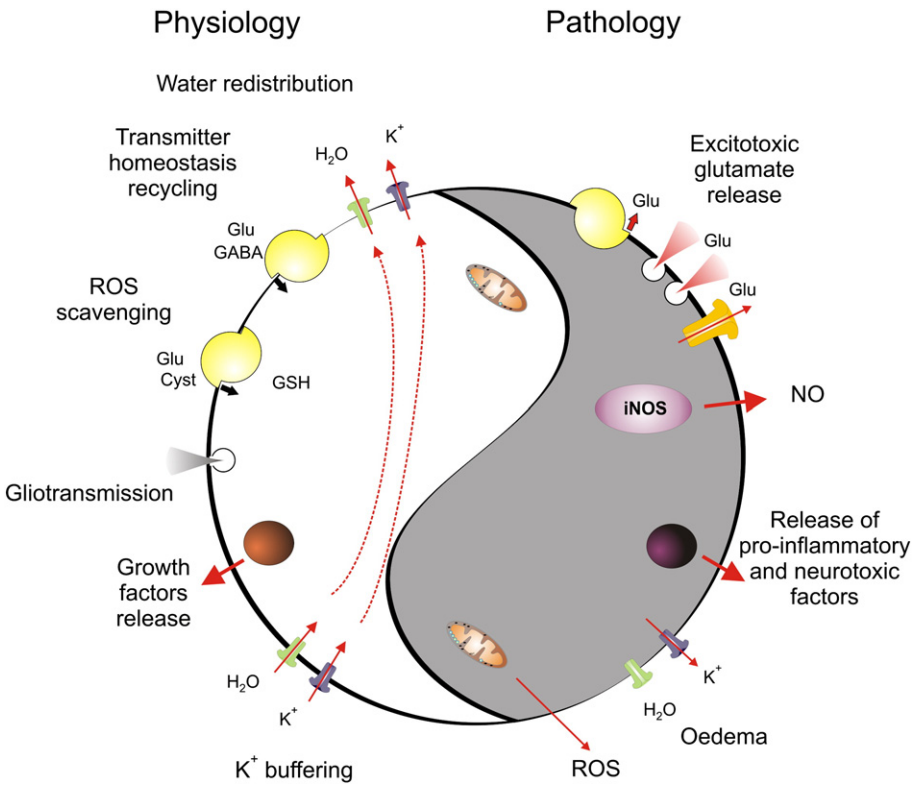

Fig. 1 – Dual role of astroglial homeostatic cascades. The homeostatic cascades expressed in astrocytes control extracellular ion homeostasis through K^+ buffering, regulate movements and distribution of water, control extracellular concentration of neurotransmitters and provide main reactive-oxygen species scavenging system. In pathological conditions, when astrocytes experience metabolic stress, the same systems may contribute to brain damage. Failure in water transport triggers brain oedema, reversal of neurotransmitter transporters together with Ca^{2+}-dependent exocytosis and opening of high-permeability plasmalemmal channels contributes to glutamate excitotoxicity; inadequate K^+ buffering promotes further overexcitation of neural cells, and glial cells begin to release ROS and pro-inflammatory factors, further exacerbating brain damage.

transport can also be important for controlling seizures development, as was directly demonstrated in genetically modified mice, which lack astroglial transporter GLT-1. These animals develop spontaneous and lethal seizures which killed half of homozygous mice before they reach 6 weeks of age (Tanaka et al., 1997).

Astroglial cells are also involved in a variety of psychiatric disorders. The loss of astrocytes was observed in patients suffering from depression (Rajkowska et al., 1999). The astrocytes may also play a role in the pathogenesis of schizophrenia, through their control over glutamate homeostasis and gliotransmission. The recently developed glutamate theory of schizophrenia (Tsai and Coyle, 2002) stresses the role of the hypofunction of NMDA receptors, which are under positive control of the gliotransmitter D-serine. The latter was used in clinical trials with certain beneficial effects (Tsai et al., 1998), once more indicating that deficient gliotransmission may be involved in schizophrenia pathogenesis.

Pathological changes in oligodendroglia are central to the broad class of diseases of white matter. Oligodendrocytes and oligodendrocyte precursors are highly vulnerable to excitotoxic insults. This excitotoxic death is mediated by Ca^{2+} influx following overactivation of ionotropic glutamate receptors and possibly $P2X_7$ purinoceptors (Matute, 2008; Matute et al., 2007). The oligodendroglial death can directly affect axons, causing their degeneration and profound damage of the white matter that may result in severe dementia. A particular type of post-stroke dementia is represented by Binswanger's disease (or subcortical dementia), which is a form of vascular dementia characterised by diffuse white matter lesions; it leads to progressive loss of memory, cognition and behavioural adaptation (van Swieten and Caplan, 1993). The infarct occurring in white matter triggers progressive death of oligodendrocytes, activation of astrocytes and microglia and degeneration of axons (Akiguchi et al., 1997). The primary pathological steps most likely are associated with ischaemic death of oligodendrocytes.

Another ischaemia-related disease arising from death of oligodendrocytes is periventricular leucomalacia; a condition that causes diffused cerebral white matter injury (Blumenthal, 2004). This occurs mostly in prematurely born infants. The roots of this pathology can be found in (1) poor vascularisation of white matter in premature infants and (2) the prevalence of oligodendrocyte progenitors, which are particularly sensitive to ischaemia, reactive oxygen species and glutamate excitotoxicity. Thus, periods of even comparatively mild ischaemia result in profound damage to white matter and the demise of many oligodendrocyte progenitors. This, in turn, leads to defective myelination, which further alters cerebral cortex development and leads to the impairment of pyramidal tracts, with subsequent neurological disorders, including cerebral

palsy and cognitive deficits. Last but not least, the oligodendroglia plays a central role in various demyelinating disorders, including multiple sclerosis. The pathogenesis and molecular mechanism of these diseases was a subject of many reviews (e.g. Antel and Arnold, 2005; Ercolini and Miller, 2006; Jessen and Mirsky, 2008; Nave et al., 2007; Smith and Hall, 2001; van der Valk and Amor, 2009) to which we address the curious reader.

Finally, the microglia controls the immune response and phagocytotic after-damage clearance system of the brain. Through multiple stages of activation, microglia grades the reaction to brain lesion, being one of the most important determinants of the course of CNS pathology (Hanisch and Kettenmann, 2007; Ransohoff and Perry, 2009).

Our knowledge about the pathological potential of neuroglia is still rudimentary, as major attention has always been diverted to the pathology of neurones. Nonetheless, it becomes increasingly obvious that it is the glia which determine the initiation, course and outcome of majority (if not all) diseases of the nervous system. Indeed, loss of glial support inevitably signals neuronal demise, and glial performance decides upon the balance of neuroprotection, neuroregeneration and neural death, thus controlling the pathology of the brain. Specifically, glial reactions are instrumental in shaping various neurodegenerative processes, which we shall discuss in subsequent chapters that are specifically dedicated to the role of astroglia and microglia in neurodegenerative processes.

4. Astrocytes in neurodegeneration and AD

The causes of neurodegenerative diseases are many, from traumatic or infectious attacks to intrinsic processes associated with genetic predispositions or the accumulation of sporadic errors of yet unknown origins. The neurodegenerative disorders, which affect the main human asset, the intellect, are in essence the failures of connectivity within brain circuitry. The astrocytes, being involved in synaptic birth, maturation and maintenance, as well as in controlling the brain homeostasis and neurotransmitters balance, are strategically important for preserving connection in brain networks, and their malfunction can be critical for the development of neurodegeneration. Indeed, recently the pathological potential of astroglia in neurodegenerative diseases started to be experimentally revealed.

4.1. *Amyotrophic lateral sclerosis*

Amyotrophic lateral sclerosis (ALS, known in the United States as “Lou Gehrig’s disease”; named so after a baseball player who suffered and died from this pathology) was described by Jean-Martin Charcot in 1869 (Charcot, 1881; Charcot and Joffroy, 1869). The ALS is manifested by degeneration of motor neurones located in the cortex, in the brain stem and in the spinal cord. Clinically the ALS appears in the form of progressive paralysis and muscle atrophy. The ALS appears in both familial (~10% cases) and sporadic forms. About 20% of ALS cases are associated with dominant mutations in the gene coding for Cu–Zn superoxide dismutase (SOD1) (Rosen et al., 1993); this mutated gene become instrumental in generating animal models of ALS (Turner and Talbot, 2008). The initial hypothesis of direct toxicity of the mutant gene on neurones mediated through reduced free-radical buffering was not confirmed (Turner and Talbot, 2008), while recent studies have demonstrated the key role of glial impairments in the ALS pathogenesis.

The ALS is associated with astrogliosis and microglial activation, which was described in both humans and transgenic animals (McGeer and McGeer, 2002a; Rossi and Volterra, 2009). The astrogliosis, however, is preceded by astroglial degeneration and atrophy, which occurs before neuronal death and the appearance of clinical symptoms in the $hSOD1^{G93A}$ transgenic mouse (Rossi et al., 2008). At later stages of the disease the reactive astroglia appears, although atrophic astrocytes are also present close to lesion sites. The astroglial degeneration was a property of SOD1 bearing astrocytes, which demonstrated an increased vulnerability to glutamate, mediated through mGluR5 receptors (Rossi et al., 2008). The hSOD1 bearing astrocytes also release neurotoxic factors and assist microglial activation (Di Giorgio et al., 2007; Nagai et al., 2007; Rossi and Volterra, 2009). Finally, selective silencing of the SOD1 mutant gene in astrocytes significantly slowed the progression of ALS in transgenic mice (Yamanaka et al., 2008). Notably, a recent PET study by Johansson et al. (2007) targeting MAO-B, which in the CNS is almost confined to astrocytes, using C^{11} (L)-deprenyl, described a profound astrocytic proliferation in ALS patients.

Therefore, astrocytes can be considered as central players in APS pathology. At the initial stages, glutamate induces gliotoxicity. The atrophic astrocytes in turn reduce synaptic coverage and fail to perform their homeostatic and neurone-supportive functions. This initiates neurodegeneration, which triggers reactive gliosis; reactive astrocytes release neurotoxic factors and stimulate microglial activation thus supporting the vicious circle of neurodegeneration.

4.2. *Wernicke encephalopathy*

The combination of ataxia, ophthalmoplegia, and mental changes, reflecting encephalopathy with deep thalamo-cortical lesions was initially described by Carl Wernicke (Wernicke, 1881–1883). This encephalopathy is generally caused by a deficiency of thiamine. Although the nature of neuronal death remains unclear, the possible failure of glutamate homeostasis can assume the leading role. Indeed, specific analysis revealed a substantial reduction (60–70%) of the expression of astroglial transporters EAAT1 and EAAT2 in cortical samples from human tissues obtained from confirmed cases of Wernicke encephalopathy. A similar profound decrease in astroglial glutamate transporters was found in the rat thiamine deficiency model of the disease (Hazell et al., 2009). The failure of astroglial glutamate uptake can be the reason for neuronal excitotoxicity and subsequent lesions. In addition, a significant decrease in expression of GFAP, astrocytic glutamine synthetase and astrocytic GAT-3 GABA transporter, all indicative of astroglial dystrophy or death, was observed in the thalamus of thiamine deficient rats (Hazell, 2009).

4.3. *Parkinson’s disease*

The disturbed locomotive and motor functions (which include akinesia, rigidity, tremor at rest, and postural abnormalities) are

prevailing clinical symptoms of the Parkinson's disease (PD—(Lees, 2009; Parkinson, 1817, 2002)). These symptoms arise from the specific extermination of dopaminergic neurones in substantia nigra with a subsequent severe impairment of nigrostriatal dopaminergic transmission. The systematic investigation of the astroglial involvement into the pathogenesis of PD has not yet been performed, although existing data allow suspecting the pathological potential for astrocytes. At the late stages of the disease, profound astrogliotic changes were identified in substantia nigra, reflecting the inflammatory state accompanying neurodegeneration (McGeer and McGeer, 2008; Mena and Garcia de Yebenes, 2008). The early changes in astroglia are unknown and yet they may play an important role in the progression of PD.

The substantia nigra has less astrocytes compared to other brain region; therefore it is tempting to speculate that when stressed these astrocytes fail and cease to support the dopaminergic neurones, which in turn contributes to the degeneration of the latter. The support and protection of dopaminergic neurones by astroglia is well documented *in vitro* (Mena et al., 2002; Mena and Garcia de Yebenes, 2008). The addition of astrocytes to midbrain cultures increases the percentage of tyrosine hydroxylase-positive (i.e. dopaminergic neurones) from 2–5% to ~40%; the effect, which is mimicked by glial-conditioned medium collected from cultures of mesencephalic astrocytes (Mena et al., 1996). The same medium also protects dopaminergic neurones against cell death triggered by 1-methyl-4-phenylpyridinium (MPP^+; the latter is a selectively toxic for dopaminergic neurones and is the active agent in 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, MPTP induced akinetic rigid syndrome, considered to be the relevant animal model of PD) or by NO (Mena et al., 1999), promotes neurite growth and affects signalling cascades in these neurones (Mena and Garcia de Yebenes, 2008).

Furthermore, astrocytes play a central role for L-DOPA-dependent PD therapy. The L-DOPA (the main agent used in clinical treatment of PD) is toxic for dopaminergic neurones in the culture (Mena and de Yebenes, 2006; Mena and Garcia de Yebenes, 2008), albeit it is beneficial for these neurones *in vivo*. The difference is all in astroglia, and indeed addition of glial-conditioned medium to cultures of dopaminergic neurones prevented L-DOPA neurotoxicity and turned it into trophic agent (Mena and de Yebenes, 2006; Mena and Garcia de Yebenes, 2008). Finally, astrocytes may promote differentiation of stem cells into dopaminergic neurones and facilitate their incorporation into the neuronal circuitry (Mena and de Yebenes, 2006; Mena and Garcia de Yebenes, 2008).

Therefore, the early astroglial atrophy and failure to support dopaminergic neurones may be an important pathological step in the development of PD.

4.4. *Non-AD dementia*

Astrocytes are affected in many types of dementia. Depending on the type and progression of the disease, both astroglial atrophy and astrogliosis are observed; these two processes can develop in parallel depending on the pathological stage. In the frontotemporal dementia (the clinical term covering several types of sporadic non-Alzheimer cognitive disruptions, which include e.g. Pick's disease and frontotemporal lobar degeneration) early and dramatic apoptotic death and dystrophy of astrocytes is observed (Broe et al., 2004); the degree of glial atrophy displayed direct correlation with the severity of dementia. In another study however, prominent astrogliosis and profound increase in astrocyte density (up to four to five times) was found in post-mortem tissues (Kersaitis et al., 2004).

Early and prominent astrogliosis also accompanies thalamic dementia. In this form of pathology a specific proliferation of perivascular and perineuronal astroglial processes changes are observed. These changes in astroglia are considered to be the primary pathological change, which can produce dementia even in the absence of severe neuronal loss (Potts and Leech, 2005).

Astrocytes (and microglia) also play a primary neurotoxic role in immunodeficiency virus-1 (HIV-1) associated dementia, or HAD (Kaul et al., 2001). In HAD significant astrogliosis and an increase in GFAP expression is observed in the entorhinal cortex and the hippocampus (Vanzani et al., 2006). The progression of HAD, however, leads to a significant astroglial cell loss through apoptosis, which is specifically prominent in the subjects with rapidly progressing cognitive deficits (Thompson et al., 2001). In this type of pathology, the virus infects exclusively microglia, although the dementia progresses due to NMDA-receptor mediated neuronal death through necrosis or apoptosis (Toggas et al., 1996). The glutamate excitotoxicty can result from TNF-α release from infected and activated microglia. This triggers a massive release of glutamate from astrocytes following a TNF-α-mediated activation of chemokine receptors of the CXCR4 type expressed in astroglial membranes (Bezzi et al., 2001). Incidentally, the same CXCR4 receptors can also be activated by the isoform of HIV-1 coat protein $gp120_{IIIB}$, implicated in HAD pathology. The neurotoxicity can also be exacerbated by the release of additional inflammatory and death factors from both astrocytes and activated microglia (Deshpande et al., 2005; Nardacci et al., 2005).

Various types of non-AD dementia (e.g. progressive supranuclear palsy, corticobasal degeneration and Pick's disease) are associated with the appearance of tau protein inclusions in astroglial cells, which normally express very little (if at all) of tau protein (Komori, 1999). A targeted expression of FTDP-17 tau protein (the FTDP-17 gene is associated with parkinsonism and frontotemporal dementia linked to chromosome 17) into astrocytes in a transgenic mouse model triggered age-dependent neurodegeneration, thus directly indicating that astroglia can indeed be a primary cause of a chronic neurodegenerative disease (Dabir et al., 2004; Forman et al., 2005).

4.5. *Alzheimer's disease*

The glial involvement in the pathogenesis of Alzheimer's disease (AD) was initially suggested by Alois Alzheimer himself (Alzheimer, 1910). He had demonstrated that the neuritic plaques (the extracellular deposits of fibrillar β-amyloid, which together with the tau neurofibrillary tangles represent the major histopathological markers of AD) include glial cells (Fig. 2). The AD brains are characterised by prominent astrogliosis, mostly observed in the cells surrounding amyloid plaques with processes of activated astrocytes participating in formation of neuritic plaques (Nagele et al., 2004; Rodriguez et al., 2009).

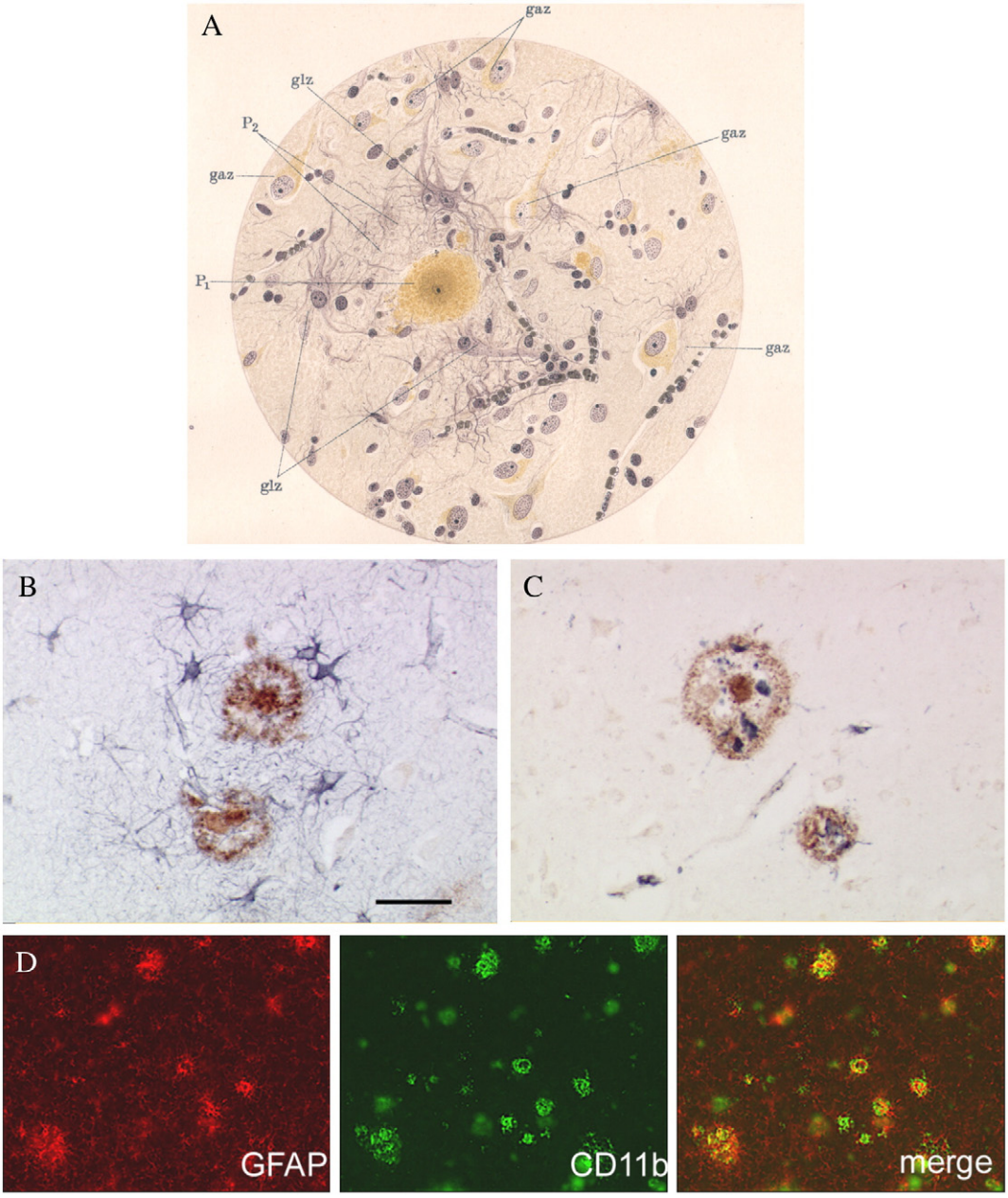

Fig. 2 – Activation of glial cells at sites of β-amyloid deposition in human brain and in APP transgenic mice. (A) Neuritic plaques as seen and drawn by Alois Alzheimer (Alzheimer, 1910). The plaque is surrounded by activated astrocytes; and activated microglia is present at the peripheral region. Abbreviations: P1—the central part of the plaque (amyloid deposition); P2—periphery of the plaque; gaz—neurone; glz—glial cell. (B) Double immunostaining of a human brain section derived from a 70 years old Alzheimer disease (AD) patient reveals GFAP positive astrocytes (blue) surrounding 6E10 positive β-amyloid deposits (brown). (C) Double-staining for β-amyloid (brown) and CD68-positive microglia cells. Scale bar = 50 μm. (D) Double immunostaining of GFAP and CD11b in a cortical section of a 12 month old APP23 transgenic mouse shows a focal and close association of both markers for astro- and microglial reactivity.

The β-amyloid peptide presents activating signals for astrocytes; the exposure of cultured glial cells to aggregated β-amyloid or to amyloid plaques isolated from human AD brains trigger reactive astrogliosis (DeWitt et al., 1998). The Aβ also induces functional changes in astrocytes *in vitro*: the β-amyloid peptide ($A\beta_{1-42}$) and its toxic fragment ($A\beta_{25-35}$) induced spontaneous $[Ca^{2+}]_i$ elevations and $[Ca^{2+}]_i$ oscillations in astrocytes growing in mixed astroglial–neuronal cultures. The Aβ-induced $[Ca^{2+}]_i$ oscillations lasted for many hours and were linked to neuronal death, which occurred 24 h after the administration of Aβ to the cultures. The inhibition of $[Ca^{2+}]_i$ oscillations prevented neuronal death (Abramov et al., 2003). In the same mixed culture model Aβ was also shown to induce mitochondrial depolarisation and oxidative stress in astrocytes; the release of reactive oxygen species from stressed astrocytes caused neuronal death (Abramov et al., 2004).

The abnormalities in astroglial Ca^{2+} signalling were observed in the brains of transgenic AD mice. In these experiments, employing *in vivo* multiphoton confocal microscopy, the general elevation of resting $[Ca^{2+}]_i$ was observed throughout the astroglial syncytia. In addition, astrocytes located in the vicinity of plaques triggered spontaneous long-distance propagating Ca^{2+} waves, which were absent in control animals (Kuchibhotla et al., 2009).

The participation of astrocytes in plaque formation initiated the hypothesis of the Aβ-clearing role of astroglia (Nagele et al., 2003, 2004); with subsequent astroglial degeneration triggered by the accumulated β-amyloid peptide. Indeed, the plating of isolated healthy astrocytes on the slices prepared from transgenic (APP) AD mice resulted in astrocytes migration towards the plaques with subsequent accumulation and degradation of Aβ. To support this finding, some evidence suggests that astroglial cells are able to phagocyte Aβ peptides, a process which may depend on their apolipoprotein E (ApoE) status, suggesting that ApoE polymorphisms may influence the risk to develop AD by affecting astroglial Aβ phagocytosis (Jiang et al., 1998; Niino et al., 2001). In contrast, endogenous astrocytes surrounding the Aβ plaques were unable to accumulate and remove Aβ (Wyss-Coray et al., 2003). In the triple transgenic mouse model of AD (3xTg-AD; harbouring the mutant genes for amyloid precursor protein (APP_{Swe}), presenilin $1PS1_{M146V}$ and tau_{P301L} (Oddo et al., 2003)) very little (if any) Aβ accumulation by reactive astrocytes was observed (Rodriguez et al., 2009). These data clearly indicate the phenotypic difference between normal astroglia and astrocytes affected by the AD pathology. Another kind of phenotypic difference was observed in astrocytes from an AD model expressing double mutated K670N-M671L APP; these astrocytes began to express β-secretase, thus becoming possible producers of Aβ (Hartlage-Rubsamen et al., 2003; Heneka et al., 2005b; Rossner et al., 2005). While it remains unclear to which degree astrocyte activation contributes to Aβ generation or its clearance, it seems apparent that astrocytes contribute to the inflammatory component of AD. For example, astrocytes have been shown to express iNOS and the L-arginine-supplying enzyme argininosuccinate synthetase and consequently contribute to NO- and peroxynitrite mediated neurotoxicity (Heneka and Feinstein, 2001; Heneka et al., 2001). Although astrocytes serve as a constant and important source of neurotrophic factors under physiological conditions, *in vitro* and *in vivo* experiments suggest that chronically activated inflammatory astrocytes may not generate significant amounts of these molecules (Nagatsu and Sawada, 2005).

The AD may also impair other astroglial homeostatic functions. For example, Aβ affects astroglial ability to accumulate glutamate: treatment of rat cultured astrocytes with $Aβ_{1–40}$ reduced both expression and capacity of GLAST- and GLT-1 mediated glutamate uptake (Matos et al., 2008). Reactive and pathologically changed astrocytes are also responsible for failures in the functional activity of neuronal–glial–vascular units. Indeed, the vascular dysfunctions, perivascular amiloidosis and compromised blood–brain barrier are inseparable parts of AD pathology (Bell and Zlokovic, 2009). How astroglial cells are participating in these changes remains, however, an open question.

Nevertheless, the astrogliosis is not the only astroglial reaction in the AD brains. In our recent studies, performed on different regions of the brains of triple-transgenic (3x-Tg-AD—(Oddo et al., 2003)) mice, both astrogliosis and astroglial atrophy were found (Rodriguez et al., 2009; Rodriguez and Verkhratsky, papers in preparation; Fig. 3). The decrease in complexity of astrocytes, which indicated their atrophy, began to be observed before the formation and consolidation of neuritic plaques. In a plaque infested brain the reactive astrocytes were concentrated around the Aβ plaques, whereas astroglial cells distant to the plaques had an atrophic features.

5. Microglia in neurodegeneration and AD

5.1. *Amyotrophic lateral sclerosis*

The primary pathological feature of ALS is the loss of motor neurones (Talbot, 2002), which is accompanied by a robust glial response including the activation of microglia and astrocytes as well as the expression of cyclooxygenase 2 (COX-2) and nitric oxide synthase (iNOS) in the spinal cord (Almer et al., 1999; Barbeito et al., 2004; Phul et al., 2000; Yasojima et al., 2001). The histological studies of post-mortem brains and spinal cord tissue were recently supported by a study using the PET ligand PK1195, which labels the peripheral benzodiazepine receptor being expressed by activated microglia in the brain. In this study, Turner et al. (2004) found evidence for increased microglial activation in the prefrontal cortex, the motor cortex, the thalamus and the pons of ALS patients. Both pathologies—the loss of motor neurones and neuroinflammation, can be found in transgenic mice overexpressing mutant variants of the human gene encoding for copper/zinc superoxide dismutase (SOD1), which have been linked to inherited ALS (Gurney et al., 1994; Hensley et al., 2003, 2002; Kunst, 2004; Yoshihara et al., 2002). The massive appearance of activated microglia and astrocytes already at an early, presymptomatic stage of the disease in SOD1 transgenic mice suggests that an inflammation may contribute to motor neurones degeneration and the suppression of the inflammatory component could be neuroprotective. Indeed, increasing experimental evidence suggests an active and contributory role of microglia in ALS. Thus, the overexpression of mutant human SOD1 in motor neurones alone did not result in significant neuronal degeneration in transgenic mice (Lino et al., 2002; Pramatarova et al., 2001). A similar approach expressing mutant SOD1 under control of the astrocytic promoter GFAP, thus causing the astroglial expression of protein, also failed to induce motor neurones death (Gong et al., 2000). In contrast, the selective deletion of mutant SOD1 in microglia increased the survival rate in the SOD1 transgenic mouse model, indicating that the presence of mutant SOD1 in microglial cells is fundamental for their detrimental effect on motor neurone integrity (Boillee et al., 2006). The latter hypothesis was further corroborated by bone marrow transplantation experiments in mice deficient for myeloid cells but harbouring the human SOD1 mutant. In these mice, the transplantation of wild type bone marrow cells but not SOD1 mutant bone marrow cells significantly delayed the progression of the disease (Beers et al., 2006).

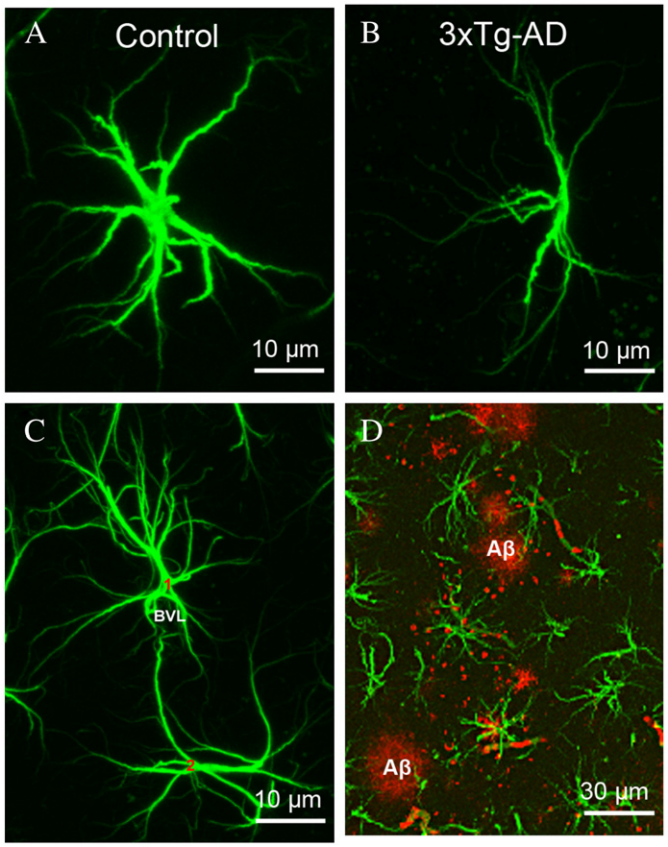

Fig. 3 – Astroglial atrophy and astrogliosis accompany the development of AD pathology in the brains of triple transgenic mouse. (A, B) Representative confocal micrographs illustrating normal control astrocytes (A) compared to the astrocytic atrophy observed in the dentate gyrus of 3xTg-AD mice (B). The atrophy is manifested by a reduction of the size of somatas as well as in the reduction of number of primary processes and their branching. (C) Confocal micrograph showing perivascular astrocytes in normal animals. (D) Confocal dual labelling images (GFAP in green and Aβ in red) in 3xTg-AD mice showing the close apposition of astrocytes with the Aβ accumulations. Astrocytes surround Aβ plaques and undergo astrogliosis.

Although the exact molecular mechanism by which microglial cells become activated in ALS remains to be determined, various approaches targeting the inflammatory component of the disease demonstrated beneficial effects. Thus, the treatment of mutant SOD1 transgenic mice with minocycline improved motor performance and survival (Kriz et al., 2002; Zhu et al., 2002). Likewise, the inhibition of COX2 by celecoxib or nimesulide delayed the onset of the disease (Drachman et al., 2002; Pompl et al., 2003). Using a further anti-inflammatory treatment strategy, two studies with SOD1-G93A transgenic mice, an established model of ALS, independently found that oral treatment with the PPARγ agonist pioglitazone extended the survival of these mice (Kiaei et al., 2005; Schutz et al., 2005). Pioglitazone treatment delayed the onset of disease and prevented a decrease in body weight in these mice in comparison with untreated SOD1-G93A mice. The quantification of spinal cord motor neurones revealed neuroprotection in mice treated with pioglitazone, whereas non-treated SOD1-G93A mice had lost 30–40% of these neurones at comparable time points. This neuroprotective effect was paralleled by the preservation of the median fibre diameter of the quadriceps muscle in treated mice, indicating not only a functional but also a morphological protection of motor neurones by pioglitazone. This finding was further substantiated by the superior motor performance in the RotaRod35 and grip latency tests of mice treated with pioglitazone. The numbers of activated microglia were markedly reduced at sites of neurodegeneration in pioglitazone-treated SOD1-G93A mice compared with non-treated mice, as were levels of COX2 and iNOS proteins. Kiaei et al. (2005) also provided evidence in a mouse model of ALS that nitric-oxide-dependent peroxynitrite generation was reduced by pioglitazone.

5.2. *Parkinson's disease*

PD is characterised by a progressive degeneration of dopaminergic midbrain neurones in the substantia nigra (SN) and becomes clinically apparent when more than 50% of SN neurones have been lost. Despite the decades of intensive research, the cause of the neurodegeneration in PD is still poorly understood. Microglial activation has been found in the substantia nigra (SN) at sites of dopaminergic cell loss in post-mortem human brains derived from PD patients. Likewise, activated microglial cells are found in all animal models of PD, together suggesting that neuroinflammatory mechanisms are involved in the disease process. Structurally modified α-

synucleins (α-SYN), particularly nitrated species, which are released as a consequence of dopaminergic neurodegeneration, have been found to act as potent microglial immunostimulant (Kim et al., 2009; Standaert et al., 2009; Theodore et al., 2008). Additional experimental evidence leads to the hypothesis that neuroinflammation plays an active and promoting role in the disease process based on the finding that suppression of inflammatory signalling cascades substantially improved the phenotypic outcome as well as protected from dopaminergic cell loss and subsequent neurochemical changes in SN projection areas. Thus, iNOS inhibition by either genetic deficiency or pharmacological treatment has been found to exert neuroprotection (Dehmer et al., 2000; Liberatore et al., 1999). Similarly, more general anti-inflammatory approaches including the activation of the peroxisome proliferator activated receptor gamma pioglitazone (Breidert et al., 2002; Dehmer et al., 2004) or treatment with the semisynthetic tetracycline minocyline significantly protected from dopaminergic neurodegeneration. All of these experimental approaches aim to block or at least interact with mechanisms that finally execute neuronal cell death such as oxidative stress and cytokine-receptor-mediated apoptosis.

Microglia may, however, have also protective functions in this disease, for example by secretion of anti-inflammatory and neuroprotective cytokines such as TGF-β (Polazzi et al., 2009). An important question is, as to what extent the local inflammatory process within the substantia nigra can be influenced by peripheral inflammatory events. In a recent study Godoy et al. tested whether a sub-toxic dose of bacterial lipopolysaccharide (LPS) is actively modulating microglia from an anti- to a pro-inflammatory state and thereby exacerbate disease progression. In this experiment, LPS injection in the degenerating SN exacerbated neurodegeneration, worsened the behavioural phenotype and caused an increase in microglial IL-1 secretion. Of note, IL-1 inhibition reversed these effects. Importantly, chronic systemic IL-1 also exacerbated neurodegeneration and microglial activation in the SN (Godoy et al., 2008). Interestingly, in a very recent paper, Smeyne et al. showed that abdominal infection with the H5N1 influenza virus resulted in a very rapid viral migration into the CNS and viral presence predominantly within brainstem and midbrain nuclei (Jang et al., 2009). Viral neurotrophism caused a robust inflammatory reaction within these nuclei including the locus coeruleus and SN. Importantly, the H5N1 virus induced neuroinflammation, which persisted beyond the presence of the virus itself and caused α-SYN aggregation and progressive neurodegeneration. Together, these data suggest that the brainstem nuclei such as the SN are vulnerable to peripheral infection and immunological processes. In keeping with this, anti-inflammatory therapeutic strategies that target proinflammatory microglial activation may represent a future therapeutic avenue.

5.3. *Alzheimer's disease*

Next to the classical neuropathological features of AD, namely Aβ deposition and neurofibrillary tangle formation, neuroinflammatory changes have been identified as the third important component of the disease. The inflammatory reactions of microglia and astroglia are intimately associated with the pathogenesis and progress of AD. The activated microglial cells are associated with neuritic plaques (McGeer and McGeer, 1999) and they secrete a wide variety of pro-inflammatory molecules (Heneka and O'Banion, 2007). Furthermore the microglia is implicated in active phagocytosis of Aβ, thus counterbalancing the Aβ load (Bolmont et al., 2008; Frautschy et al., 1998). The activation of microglia occurs in response to the formation of neuritic plaques. Several amyloid peptides and APP itself can act as potent glial activators (Barger and Harmon, 1997; Dickson et al., 1993; Schubert et al., 2000), whereas the disruption of the APP gene and its proteolytic products delay and decrease microglial activation (DeGiorgio et al., 2002). Microglial cells have been suggested to be preferentially associated with certain amyloid plaque types indicating that plaque development and the degree of microglial reaction are interrelated (D'Andrea et al., 2004). However, it remains unclear whether Aβ plaque deposition is an absolute requirement for microglial activation, or whether this can already be evoked by soluble and toxic Aβ species. This hypothesis is supported by a recent study where the focal activation of microglial cells becomes apparent at 3 months of age in APP V717I transgenic mice, which usually start to deposit Aβ in plaque like structures much later—at around 10–12 months (Heneka et al., 2005a). In contrast, studies using *in vivo* multiphoton microscopy using 5–6 month old B6C3-YFP transgenic mice (bearing APP_{swe} and PS1d9x-YFP genes) suggested that microglial are recruited to Aβ plaques only after they have been formed (Meyer-Luehmann et al., 2008).

The mechanisms of microglial activation by Aβ depositions are not yet clear, although several receptor systems are directly implicated in this process. In particular, the activation of microglia requires $P2X_7$ purinoceptors and Ca^{2+} signalling. The exposure of cultured microglial cells to $Aβ_{25–35}$ triggers Ca^{2+} influx, the IL-1β release and $P2X_7$-dependent membrane permeabilisation, all being absent in cells prepared from $P2X_7$ KO mice (Sanz et al., 2009). Furthermore, the intra-hippocampal injection of $Aβ_{1–42}$ failed to induce microglial activation (as judged by IL-1β accumulation) in animals deficient in $P2X_7$ receptors (Sanz et al., 2009).

The activation of microglial cells by aggregated Aβ involves Toll-like receptors (Okun et al., 2009) of TLR4 type; the TLR4 receptors are up-regulated in both AD brain preparations and in APP transgenic mice. A spontaneous loss-of-function mutation in the TLR4 gene significantly reduced Aβ-induced microglial activation (Walter et al., 2007). Exposure of microglial cultures to Aβ also stimulated TLR2 receptors, while inhibiting TLR9 receptors (Lotz et al., 2005). The stimulation of the TLR-associated signalling system may have dual effect in AD progression. On one hand, the activation of TLRs increases microglial phagocytosis of Aβ (this involves p38 MAPK signalling and expression of G-protein-coupled formyl peptide receptor-like 2, mFPR2; the latter likely being the sensor for Aβ (Chen et al., 2006; Iribarren et al., 2005; Tahara et al., 2006)). At the same time, however, the over-stimulation of TLRs may trigger excessive release of cytokines, proteases and other cytotoxins thus promoting neural cell death (Okun et al., 2009).

The Aβ stimulates a nuclear factor kappa B (NFκB)-dependent pathway that is required for cytokine gene transcription (Combs et al., 2001a) within activated microglia and reactive astrocytes. Not only Aβ, but also the carboxy-terminal 100 amino acids of APP (CT100) (which are present in senile plaques) can induce astrogliosis and neuronal death. Exposure to CT100

results in activation of the mitogen-activated protein kinase (MAPK) pathways as well as NFκB (Bach et al., 2001). Additionally, other proteins involved in APP processing have been implicated in the inflammatory response. Loss of presenilin function in presenilin conditional knockout mice leads to differential up-regulation of inflammatory markers in the cerebral cortex, such as strong microglial activation, complement component C1q, and cathepsin S (Beglopoulos et al., 2004).

Once stimulated, microglia participate in the generation and release of a wide range of inflammatory mediators including complement factors, chemokines and cytokines. The complement system represents a complex and tightly regulated attack cascade designed to destroy invaders and assist in the phagocytosis of waste, one of the key microglial tasks under physiological and pathophysiological conditions. The components of this system carry out four major functions: recognition, opsonisation, inflammatory stimulation and direct killing through the membrane attack complex (MAC) (McGeer and McGeer, 2002b). In addition to triggering the generation of a membranolytic complex, complement proteins interact with cell surface receptors to promote a local inflammatory response that contributes to the protection and healing of the host. Microglial complement activation causes inflammation and cell damage, yet it is essential for eliminating cell debris and potentially toxic protein aggregates. The complement system consists of some 30 fluid-phase and cell-membrane associated proteins that can be activated by three different routes. The classical pathway (involving C1q, C1r, C1s, C4, C2, and C3 components) is activated primarily by the interaction of C1q with immune complexes (antibody–antigen), but activation can also be achieved after the interaction of C1q with non-immune molecules such as DNA, RNA, C-reactive protein, serum amyloid P, bacterial lipopolysaccharides, some fungal and virus membranes, and most importantly, by fibrillar Aβ. The initiation of the alternative pathway (involving C3, factor B, factor D, and properdin) does not require the presence of immune complexes and leads to the deposition of C3 fragments on target cells. Mannose-binding lectin (MBL), a lectin homologous to C1q, can recognise carbohydrates such as mannose and *N*-acetylglucosamine on pathogens and initiate the complement pathway independently of both the classical and the alternative activation pathways. Like the C1 complex in the classical pathway, MBL is associated with two serine proteases that cleave C4 and C2 components, leading to the formation of the classical C3 convertase (van Beek et al., 2003).

Microglial cells can produce complement proteins to recognise and kill pathogens locally. Studies using RT-PCR have shown locally up-regulated complement mRNA in AD brain, especially in the areas of primary pathology: the entorhinal cortex, the hippocampus, and the midtemporal gyrus (Yasojima et al., 1999). Numerous groups have reported the association of complement proteins of the classical pathway, particularly the MAC, with amyloid plaques and neurofibrillary tangles in AD brains (Webster et al., 1997). Information about the functional role comes from studies of mutant mice lacking complement proteins, which suggest that impaired phagocytosis can result in immunomediated tissue damage and inflammation (Botto, 1998; Taylor et al., 2000). However, the complement system may be Janus-faced and also provide beneficial action to the brain during AD. Thus, Wyss-Coray and Mucke (2002) demonstrated that complement activation can protect against Aβ-induced toxicity and may reduce the accumulation or promote the clearance of senile plaques. The AD mice expressing a soluble form of the complement inhibitor Crry, which blocks C3 activation, under the control of the glial fibrillary acidic protein promoter displayed higher Aβ deposition and more prominent neurodegeneration than age-matched control mice. However, more recently it was reported that transgenic mouse models of AD lacking C1q showed reduced pathology, consisting of decreased numbers of activated microglia and improved neuronal integrity, without changes in plaque area. These data suggest that at stages when fibrillar plaque pathology is present, C1q exerts a detrimental effect on neuronal integrity, most likely through the activation of the classical complement cascade.

In AD, unlike in the aforementioned neurological disorders characterised by leukocyte infiltration, abnormal or excessive migration of inflammatory cells into the CNS has not been definitively shown to occur. Nonetheless, there is growing evidence that chemokines and chemokine receptors are up-regulated in resident CNS cells in an AD brain (Ransohoff and Perry, 2009), and chemokines may contribute to plaque-associated inflammation and neurodegeneration. Up-regulation of CXCR2 expression has been observed on some dystrophic neurites in senile plaques (Horuk et al., 1997; Xia and Hyman, 1999). In addition, the expression of CCR3 and CCR5 is increased on some reactive microglia in AD, and MIP-1α is found in a subpopulation of reactive astrocytes (Xia et al., 1998). MCP-1 has also been localised to mature senile plaques and reactive microglia, but is not found in immature senile plaques. Furthermore, *in vitro* studies have demonstrated the ability of Aβ to stimulate the production of IL-8, MCP-1, MIP-1α and MIP-1β from human monocytes (Meda et al., 1999). For example, microglia cultured from rapid autopsies of AD and non-demented patients exhibit significant, dose-dependent increases in IL-8, MCP-1 and MIP-1α after an exposure to Aβ (Lue et al., 2001). Although more studies are certainly needed, it is likely that plaque-associated chemokine production plays a role in the recruitment and accumulation of microglia to the neuritic plaques. Future studies using targeted disruption of chemokines and chemokine receptors in mouse models of AD should help to clarify the role of chemokines in plaque-associated inflammation and neurodegeneration.

Microglia derived cytokines associated with AD include several interleukins (ILs), TNF-α and TGFβ amongst others. In general, cytokine production is increased in inflammatory states and they function by regulating the intensity and duration of the immune response (Heneka and O'Banion, 2007; Tuppo and Arias, 2005). Thus, IL-1 induces IL-6 production, stimulates iNOS activity (Rossi and Bianchini, 1996), and induces the production of M-CSF (Aloisi et al., 1992; Frei et al., 1992; Thery et al., 1992). In addition, IL-1 enhances neuronal acetylcholinesterase activity, microglial activation and additional glial IL-1 production, with consequent activation, and expression of the cytokine S100β by astrocytes, thereby establishing a self-propagating cycle (Griffin, 2000; Mrak and Griffin, 2001). The IL-6 promotes astrogliosis (Selmaj et al., 1990), activates microglia (Heyser et al., 1997), and stimulates the production of acute phase proteins (Castell et al., 1989). The

knockout mice deficient in IL-6 exhibit a facilitation of radial maze learning over 30 days and show a faster acquisition, suggesting a possible negative regulation of memory formation and consolidation processes by IL-6 (Braida et al., 2004). TNF-α has both pro-apoptotic and anti-apoptotic effects. This proinflammatory cytokine accounts for most of the neurotoxic activity secreted by monocytes and microglia (Combs et al., 2001b). On the other hand, TNF-α has been reported to have neuroprotective properties (Akiyama et al., 2000) in the AD brain.

In addition to the general role of cytokines, AD-specific interactions of certain cytokines with the APP processing pathway and Aβ may be pathophysiologically relevant. For example, IL-1 can regulate APP processing and Aβ production *in vitro* (Blasko et al., 1999). In turn, fibrillar Aβ has been reported to increase neurotoxic secretory products, proinflammatory cytokines and reactive oxygen species (Eikelenboom and van Gool, 2004; Eikelenboom et al., 1994; McGeer and McGeer, 1995). Cultured rat cortical glia exhibit elevated IL-6 mRNA after exposure to the carboxy-terminal 105 amino acids of APP (Chong, 1997). Incubation of cultured microglia with Aβ increased IL-1, IL-6, TNF-α MIP-1α and MCP-1 in a dose-dependent manner (Benveniste et al., 2001; Butovsky et al., 2005; Floden and Combs, 2006; Hanisch, 2002; Lindberg et al., 2005; Veerhuis et al., 2005). Altogether, Aβ stimulated production of interleukins and other cytokines and chemokines and their feedback activation of APP production or BACE1 (Sastre et al., 2003, 2006) may establish a self-perpetuating, vicious cycle. A second general category of cytokine action is manifested by inhibitory, anti-inflammatory cytokines such as IL-1 receptor antagonist (IL-1Ra), IL-4, IL-10 and TGF-β. Some of these are reportedly elevated in AD, consistent with induction of homeostatic mechanisms in neuroinflammation (Grammas and Ovase, 2001; Rota et al., 2006; Szczepanik et al., 2001). The use of anti-inflammatory cytokines such as IL-4 and TGF-β could be beneficial, because they are able to inhibit CD40 and class II MHC by restricting their expression and activity (Benveniste et al., 2001). However, an overexpression of TGF-β in transgenic mice leads to changes in the microvasculature, including age related amyloid deposition (Wyss-Coray et al., 2000), reflecting the multi-functional nature of many cytokines. In addition to the above described evidence from the analysis of human brain tissue, cell culture and transgenic animal studies, an association of AD with several polymorphisms of proinflammatory genes has been described, including IL-1 (Nicoll et al., 2000), IL-6 (Papassotiropoulos et al., 1999), TNF-α (McCusker et al., 2001; Perry et al., 2001), and α1-antichymotrypsin, an acute phase protein (Kamboh et al., 1995). However, none of the various members of the interleukin cytokine family that are associated with AD actually map to chromosomal regions with evidence of genetic linkage (Tanzi and Bertram, 2005). Thus, although inflammation and the up-regulation of inflammatory mediators like the interleukins are regularly observed in AD brain, it appears less likely that variation at the genomic level of these proteins makes a large contribution to AD risk in general.

Next to the complement factors, chemokines and cytokines, activated microglia can also serve as a chief source of prostanoids. Two isoforms of cyclooxygenases, the mainly constitutively expressed COX-1 and the inducible COX-2, catalyze key steps of prostanoid synthesis in mammalian cells. Downstream of both COX-1 and COX-2 several other enzymes regulate the generation of a whole spectrum of prostanoids, some of which may be neuroprotective and others neurodestructive. Thus, the composition and proportion of all prostanoids together may actually determine whether the activity of COX enzymes is beneficial or detrimental.

In vitro, LPS activated microglial cells and IL-1β-stimulated astroglial cells are capable of synthesising COX-2 (Almer et al., 2001; Bauer et al., 1997; O'Banion et al., 1996). In contrast to peripheral monocytes, cultured rat microglia cells do not synthesise COX-2 in response to IL-1 or IL-6 (Bauer et al., 1997), suggesting that COX-2 regulation differs between CNS and peripheral cells. In rat microglial cell cultures, the major enzymatic product of COX-2 appears to be prostaglandin E_2 (PGE_2). Because PGE_2 itself is able to induce COX-2 in microglia (Minghetti et al., 1997), some sort of autocrine or paracrine amplification of the COX-2 induction in microglial cells or a spreading of the COX-2 expression between neurones and microglial cells is possible. The PGE_2 acts on four different receptors designated as EP1 to EP4 (Narumiya et al., 1999). EP1 and EP2 receptors have been detected in cultured microglia, while EP3 receptors are also present in activated microglia *in vivo* (Slawik et al., 2004). Microglial EP2 receptors inhibit phagocytosis and enhance neurotoxic activities of microglia (Shie et al., 2005a,b). PGE_2 may also act on the neuronal EP2 receptor, which is involved in apoptosis, although investigations of the role of EP2 activation on neuronal cell death have yielded conflicting results and somewhat suggested a neuroprotective role of neuronal EP2 stimulation under several pathophysiological circumstances (Bilak et al., 2004; Hensley et al., 2003, 2002; Lee et al., 2004; McCullough et al., 2004; Takadera et al., 2004; Yasojima et al., 2001). This is further exemplified in a recent report where the knockout of EP2 in a double transgenic (APP/PS1) mouse led to decreased evidence of oxidative stress and decreased Aβ production, associated with lower levels of BACE (Liang et al., 2005). In conclusion, the neuronal and glial secretion of PGE_2 may impair the phagocytotic clearance of Aβ by binding to the microglia EP2 receptor and enhancing microglial toxicity. However the role of PGE_2 in neurodegeneration may be far more complex due to the presence of other EP receptor subtypes on microglial cells and the effects of PGE_2 on other cell types. Neuronal death elicited by excitotoxins is elevated in transgenic animals with high expression of COX-2, suggesting that the COX-2 expression may further interact with other pathogenetic mechanisms (Kelley et al., 1999).

It should be noted that some aspects of microglial function may be beneficial, since activated microglia are able to reduce Aβ accumulation by increasing its phagocytosis, clearance and degradation (Frautschy et al., 1998; Qiu et al., 1998). Thus, secreted $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides are constitutively degraded by the insulin degrading enzyme (IDE), a metalloprotease released by microglia and other neural cells. Finally, microglia can also secrete several trophic factors, such as the glia-derived neurotrophic factor (GDNF), which exert a well documented neuroprotective function (Liu and Hong, 2003).

6. Concluding remarks

The neurodegenerative diseases result from the failure in brain connectivity, which is formed by neuronal–neuronal,

neuronal–glial and glial–glial contacts. Contrary to past beliefs, which regarded neuropathology to be solely associated with neuronal malfunction and neuronal cell death, the new doctrine, which regards glia as a central element in neurological diseases is emerging. The neurodegeneration is driven by complex astroglial reactions which include astroglial atrophy with a subsequent impairment of synaptic transmission and astrogliosis, which control both neuroprotective and neurotoxic reactions. The activation of microglia, associated with the secretion of pro-inflammatory factors further contributes to regulation of neuroprotection/neurotoxic balance. This recently acquired knowledge allows us to regard the neurodegenerative diseases as primarily gliodegenerative processes, in which glial cells determine the progression and outcome of neuropathological process.

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BRAIN RESEARCH REVIEWS

Review

Astrocyte dysfunction in epilepsy

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ABSTRACT

Epilepsy comprises a group of disorders characterized by the periodic occurrence of seizures. Currently available anticonvulsant drugs and therapies are insufficient to controlling seizure activity in about one third of epilepsy patients. Thus, there is an urgent need for new therapies that prevent the genesis of the disorder and improve seizure control in individuals already afflicted. The vast majority of epileptic cases are of idiopathic origin, and a deeper understanding of the cellular basis of hyperactivity and synchronization is essential. Neurosurgical specimens from patients with temporal lobe epilepsy typically demonstrate marked reactive gliosis. Since recent studies have implicated astrocytes in important physiological roles in the CNS, such as synchronization of neuronal firing, it is plausible that they may also have a role in seizure generation or seizure spread. In support of this view, several membrane channels, receptors and transporters in the astrocytic membrane have been found to be deeply altered in the epileptic brain, and they are now gradually emerging as new potential targets for antiepileptic therapeutic strategies. This review summarizes current evidence regarding astroglial dysfunction in epilepsy and discusses presumed underlying mechanisms.

Contents

Abbreviations: AED, antiepileptic drug; ALS, amyotrophic lateral sclerosis; AQP4, aquaporin4; BBB, blood–brain barrier; EAAT, excitatory amino acid transporter; GS, glutamine synthetase; HS, hippocampal sclerosis; mGluR, metabotropic glutamate receptor; mTOR, mammalian target of rapamycin; SE, status epilepticus; TLE, temporal lobe epilepsy; TLR, toll-like receptor; TS, tuberous sclerosis

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1. Introduction

Epilepsy is a condition of the brain characterized by the periodic and unpredictable occurrence of seizures. Even with optimal current antiepileptic drug (AED) therapy, about one third of patients have poor seizure control and become medically refractory. Many AEDs act as CNS depressants and must be taken chronically for seizure suppression, which often leads to marked side effects on cognition. Thus, there is a need for the development of more specific AEDs that may target cellular and molecular abnormalities responsible for epilepsy but not globally affect cerebral function. In this regard, recent developments in the understanding of glial (especially astrocytic) changes in epilepsy can potentially provide novel therapeutic targets.

Recent work has identified glial cells and astrocytes in particular, as active partners in neural information processing. Application of advanced electrophysiological and Ca^{2+} imaging techniques unraveled that astrocytes in acute brain slices or after fresh isolation from the tissue express a similar broad spectrum of functional ion channels and transmitter receptors as neurons (Verkhratsky and Steinhäuser, 2000). The presence of ionotropic and metabotropic neurotransmitter receptors led to the conclusion that astrocytes are endowed with the machinery to sense and respond to neuronal activity. In 1994 two groups discovered that elevation of the intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ in cultured astrocytes upon membrane receptor activation can induce glial release of glutamate (Nedergaard, 1994; Parpura et al., 1994). This astonishing finding for the first time demonstrated that astrocytes are sensing neuronal activity and feed back to neurons to modulate CNS signaling (Pasti et al., 1997). Later studies corroborated the view that astrocytes are direct communication partners of neurons and dynamically interact with synapses through the uptake of neurotransmitters, receptor-mediated Ca^{2+} signaling and subsequent gliotransmitter release. The intimate morphological and physiological interconnection between both cell types gave rise to the term tripartite synapse which comprises not only pre- and postsynaptic elements but also the astrocytic process (Araque et al., 1999; Halassa et al., 2007).

According to a long-standing concept, astrocytes supply neurons with nutrition metabolites and oxygen. Fundamental new insight into this aspect of astrocyte function was gained through the discovery that astrocytes control in an activity-dependent manner cerebral blood flow, by releasing vasoactive substances such as polyunsaturated fatty acids, adenosine and prostaglandins (Koehler et al., 2009; Mulligan and MacVicar, 2004; Zonta et al., 2003). In addition to the only recently discovered modulatory actions on brain signaling and circulation, astrocytes are known for decades to serve homeostatic functions, including the clearance of neuronally released K^+ and glutamate from the extracellular space.

Despite the fact that the pathways enabling activation of these cells under physiological conditions are still ill-determined, evidence is emerging suggesting a critical role of astrocyte dysfunction in the pathogenesis of neurological disorders (Seifert et al., 2006). In this article, we focus on alterations of astrocytes that are currently thought to be associated with human or experimental epilepsy. Most of these data were obtained from focal epilepsies. In addition a chapter on tuberous sclerosis is included because astrocytes are thought to play a crucial role in the pathogenesis of this disorder. While different types of cells with astroglial properties have been described to co-exist in the brain (Matthias et al., 2003) in this article only alterations of *bona fide* astrocytes are discussed.

2. Impaired K^+ buffering in temporal lobe epilepsy

During seizure activity *in vivo*, the extracellular K^+ concentration, $[K^+]_o$, increases from 3 mM to a ceiling level of 10–12 mM (Heinemann and Lux, 1977). Such high $[K^+]_o$ levels can generate epileptiform activity in acute brain slices. Because of its presumed role in K^+ homeostasis, properties of astroglial Kir channels have been investigated in experimental and human epilepsy. Measurements of $[K^+]_o$ with ion sensitive microelectrodes and patch-clamp studies suggested that the impaired K^+ buffering in sclerotic human hippocampus resulted from altered Kir channel expression. Differences were observed in the effect of Ba^{2+} on stimulus-induced changes in $[K^+]_o$ in the CA1 region of hippocampal brain slices obtained from temporal lobe epilepsy (TLE) patients with hippocampal sclerosis (HS) or without sclerosis (non-HS). In non-HS tissue, Ba^{2+} application significantly enhanced $[K^+]_o$ while this effect was not observed in HS specimens. Since Ba^{2+} is a blocker of Kir channels in astrocytes of the hippocampus (Seifert et al., 2009), these findings suggested impaired function of these channels in the sclerotic tissue (Kivi et al., 2000). The hypothesis could be confirmed with patch-clamp analyses demonstrating downregulation of Kir currents in the sclerotic human CA1 region of TLE patients (Bordey and Sontheimer, 1998; Hinterkeuser et al., 2000). Accordingly in HS, impaired K^+ buffering and enhanced seizure susceptibility result from reduced expression of Kir channels.

Dysfunction of the blood–brain barrier (BBB) seems to be involved in seizure generation. Indeed, a recent study suggested that transient opening of the BBB is sufficient for induction of focal epileptogenesis in rat (Seiffert et al., 2004). BBB lesion is also a primary event in human TLE leading to the extravasation of serum albumin taken up by neurons, astrocytes and microglia (Seiffert et al., 2004; Van Vliet et al., 2007). In astrocytes, albumin is taken up through a TGF-β receptor-mediated mechanism, resulting in downregulation of Kir4.1 and Kir2.3 channels, impaired buffering of extracellular K^+ and induction of NMDA receptor-mediated hyperexcitability (Perillan et al., 2002; Tomkins et al., 2007; review by Friedman et al., 2009).

A prerequisite for the operation of the so-called spatial buffering of K^+ is the expression of Kir channels and connexins forming gap junctions (Kofuji and Newman, 2004; Walz, 2000). According to this concept, K^+ entry into the astrocytic network occurs at sites of maximal extracellular K^+ accumulation, driven by the difference between glial syncytium membrane potential and the local K^+ equilibrium potential. Since K^+ propagates through the glial network, at sites distant to elevated $[K^+]_o$ a driving force for K^+ efflux results because here local depolarization exceeds the K^+ equilibrium potential. Surprisingly, clearance and redistribution of K^+ were still preserved in the hippocampal stratum radiatum (but not in the lacunosum-moleculare) of mice with coupling deficient astrocytes, indicating that gap junction independent mechanisms add to K^+ homeostasis in the brain (e.g. indirect coupling; Wallraff et al., 2006). Nevertheless, genetic deletion of astrocyte gap junctions leads to impaired K^+ buffering, spontaneous epileptiform activity and a decreased threshold for eliciting seizure activity (Wallraff et al., 2006).

Genetic downregulation of Kir4.1, the main Kir channel subunit in astrocytes (Kofuji et al., 2000; Neusch et al., 2006; Olsen et al., 2006; Seifert et al., 2009), profoundly reduced the ability of astrocytes to remove glutamate and K^+ from the extracellular space, both in cell culture (Kucheryavykh et al., 2007) and *in vivo* (Djukic et al., 2007). General knockout of Kir4.1 leads to early postnatal lethality (Kofuji et al., 2000) while mice with astrocytic deletion of the channel developed a pronounced behavioral phenotype, including seizures (Djukic et al., 2007).

Ultrastructural analyses in rat demonstrated a spatial overlap of Kir4.1 and the water channel aquaporin 4 (AQP4) in astroglial endfeet contacting the capillaries (Higashi et al., 2001; Nielsen et al., 1997). This finding gave rise to the hypothesis that K^+ clearance through Kir channels might be critically dependent on concomitant transmembrane flux of water in a given cell, to dissipate osmotic imbalances due to K^+ redistribution. Subsequent functional work corroborated this idea, by showing that in mice the clearance of extracellular K^+ is compromised if the number of perivascular AQP4 channels is decreased (Amiry-Moghaddam et al., 2003b). Similarly, impaired K^+ buffering and prolonged seizure duration were observed in AQP4 knockout mice (Binder et al., 2006). In TLE patients presenting with HS, immunostaining indicated a loss AQP4 in vasculature-associated astrocyte endfeet as compared with specimens from non-HS patients (Eid et al., 2005). The decrease of perivascular AQP4 channels might be secondary, following disruption of the dystrophin complex that is essential for anchoring of AQP4 in the plasma membrane (Amiry-Moghaddam et al., 2003a). Together, these findings suggest that in HS, dislocation of water channels in concert with decreased expression of Kir channels in astrocytes might underlie impaired K^+ buffering and increased seizure propensity.

In addition to spatial buffering, transient K^+ accumulations can be counterbalanced by net K^+ uptake through Na,K–ATPase and the Na–K–Cl co-transporter NKCC1, at the cost of cell swelling due to concomitant water influx (reviewed by Kofuji and Newman, 2004). In rodent hippocampus, the Na–K–ATPase was reported to have a potential role in maintaining the low $[K^+]_o$ level and to clear elevations in $[K^+]_o$ after epileptiform activity (D'Ambrosio et al., 2002; Xiong and Stringer, 2000). However, whether alterations in net K^+ uptake contribute to the enhanced $[K^+]_o$ levels seen in epileptic tissue has still to be elucidated.

3. Ambiguous role of gap junctions in epileptogenesis

Cx43 and Cx30 comprise the main connexins forming gap junctions in astrocytes of the CNS (Nagy et al., 2004) and as discussed above, its cell type-specific deletion in mice led to the generation of spontaneous epileptiform activity and a decreased threshold for evoking seizure activity (Wallraff et al., 2006). Disruption of the BBB and albumin-dependent generation of epilepsy in rat are accompanied by a transient decrease of both connexion transcripts (Cacheaux et al., 2009). These findings are in line with the long-standing concept that astrocyte gap junctions are essential for proper K^+ regulation (Orkand et al., 1966) and help counteract the generation of epileptiform activity. However, an opposite effect was observed in organotypic hippocampal slice cultures where long-term block of gap junctions through Cx43 mimetic peptides attenuated spontaneous seizure like events (but not evoked epileptiform responses; Samoilova et al., 2008). The authors also observed that serum deprivation strongly reduced spontaneous recurrent network activity and assigned this effect to a neuroprotective role of gap junctional communication. Recent work revealed that inhibition of gap junction coupling not only enhances glucose uptake, synthesis of nucleic acids and proliferation of astrocytes (Tabernero et al., 2006). Rather, gap junctions formed by Cx43 and Cx30 allow intercellular trafficking of glucose through the astrocytic network and deliver energetic metabolites from blood vessels to neurons, to maintain synaptic transmission in the murine brain (Rouach et al., 2008). This study also showed that glutamate released during epileptiform activity increases astrocyte glucose trafficking and that, in turn, glucose delivery through the astrocytic network is needed to sustain epileptiform activity. Hence, a decrease of gap junction permeability seems to exert opposite effects on excitability: a fast onset, pro-convulsive effect due to impaired K^+ redistribution, but a delayed antiepileptic effect because of disruption of neuronal energy supply.

Despite these intriguing new insights into astrocyte function, the role of gap junctions in human epilepsy is still unresolved. Published data are not always consistent, reflecting that i) human epilepsy cannot be considered a uniform condition, ii) that most of the currently available gap junction blockers do not distinguish between neuronal and glial gap junctions and iii) that these blockers usually have dramatic side effects (Steinhäuser and Seifert, 2002). Moreover, analysis of tissue samples is restricted to the chronic phase of the disorder and is likely to be affected by patients' long-lasting treatment with different AEDs, a problem inherent to experiments with neurosurgically resected specimens. Increased expression of Cx43 protein was observed in low-grade tumors and reactive astrocytes of human epileptic cortical tissue surrounding tumors although high-grade gliomas exhibited great variations in Cx43 (Aronica et al., 2001). Specimens from

pharmacoresistant TLE patients with HS showed strongly enhanced Cx43 immunoreactivity and transcript levels (Collignon et al., 2006; Fonseca et al., 2002; Naus et al., 1991). The authors speculate that upregulation of connexins might represent a compensatory response of astrocytes to cope with the enhanced K^+ release during seizure activity. However, in the light of the aforementioned findings enhanced coupling could also serve to fuel hyperactivity and thereby exacerbate generalized seizures. Importantly, it has to be emphasized that any functional evidence of enhanced gap junction coupling in human epilepsy is yet missing, which considerably limits conclusions that can be drawn from the above studies.

4. Glutamate uptake in epilepsy

The uptake of glutamate that helps to terminate the action of this neurotransmitter at CNS synapses is mainly mediated by transporters localized at the astrocytic membrane. Alteration in the activity of the astrocytic glutamate transporters, EAAT1 and EAAT2, has been revealed to be a common feature of TLE and other brain disorders (Seifert et al., 2006). Excess of extracellular glutamate is found in human epileptogenic tissue and can induce recurrent seizures and neuronal cell death (Glass and Dragunow, 1995). Different reports exist about the regulation of glial glutamate transporters in patients presenting with pharmacoresistant TLE. Employing *in situ* hybridization and Western blot in specimens from patients with HS, Tessler et al. (1999) did not find changes of EAAT1 or EAAT2. In contrast, other groups reported downregulation of EAAT2 immunoreactivity in the CA1 region displaying profound neuronal loss in human HS (Mathern et al., 1999; Proper et al., 2002). EAAT1 was found to be increased in the sclerotic CA2/3 region (Mathern et al., 1999). Later work showed downregulation of EAAT1 and EAAT2 in the CA1 region in HS and emphasized that currently it is still unclear whether this reduction is causative of the condition or rather represents a compensatory effect (Sarac et al., 2009). The latter hypothesis would be in line with recent work reporting that expression of EAAT2 is critically dependent on synaptic activity. In this study, EAAT2-mediated uptake was decreased after nerve fiber transection or neurodegeneration in a mouse model of amyotrophic lateral sclerosis (ALS) (Yang et al., 2009). Beta-lactam antibiotics increased glutamate uptake in primary human astrocytes through NFκB mediated EAAT2 promoter activation (Lee et al., 2008). Hence, the antibiotics might represent a therapeutical tool to counteract glutamate transporter dysfunction in neurological disorders such as ALS, epilepsy, stroke, or hepatic encephalopathy (Rothstein et al., 2005).

In a rat model of cortical dysplasia, pharmacological inhibition of glial glutamate transporters in the lesion area led to the opening of neuronal NMDA receptors, prolonged synaptic currents and decreased threshold for the induction of epileptiform activity (Campbell and Hablitz, 2008). This enhanced activity of NMDA receptors also triggered dephosphorylation of Kv2.1 K^+ channels, produced a negative shift of its voltage-dependent activation, and hence modulated excitability and neuronal plasticity in mice (Mulholland et al., 2008).

For effective removal of excess extracellular glutamate, the transmitter must be converted by glutamine synthetase (GS) into the receptor-inactive substrate glutamine, under consumption of ATP and ammonia. Increasing evidence indicates a loss of this astrocyte-specific enzyme in epilepsy. In patients presenting with HS, loss of GS in the hippocampus was accompanied by elevated extracellular glutamate levels (Eid et al., 2004; van der Hel et al., 2005; reviewed by Eid et al., 2008b). In experimental epilepsy, upregulation of GS and GFAP was observed in the latent phase, prior to recurrent seizure onset while in the chronic phase GS was downregulated with elevated GFAP immunoreactivity persisting (Hammer et al., 2008). By contrast, glutamate dehydrogenase, another glutamate degrading enzyme, remained unaltered in this rat model. Compatible with a potential causative role of GS loss in initiating epilepsy was the finding that pharmacological inhibition of GS produced recurrent seizure activity and a rat brain pathology resembling human HS (Eid et al., 2008a).

Inhibition of GS in astrocytes and/or glutamine transporters in neurons reduced amplitudes of evoked IPSCs and GABA release from interneurons in the hippocampus. Hence, in rat the glial glutamate–glutamine cycle is a major contributor to synaptic GABA release and regulates inhibitory synaptic strength (Liang et al., 2006), while inhibition of GS did not significantly affect glutamatergic transmission in the same species (Kam and Nicoll, 2007). Together, these results indicate that protecting GS function might represent a promising therapeutical strategy to prevent seizures.

5. Astrocyte Ca^{2+} signaling in epilepsy: Release of gliotransmitters and synchronization of neuronal activity

5.1. *Release of glutamate*

Astrocytes signal to neurons by releasing a number of gliotransmitters that can have both excitatory and inhibitory actions. In rodents this gliotransmitter release is stimulated by neuronal activity-dependent elevation of intracellular Ca^{2+} (Halassa et al., 2009; Petzold et al., 2008; Schummers et al., 2008; Wang et al., 2006) and may occur through Ca^{2+} dependent exocytosis (Bezzi et al., 2004; Chen et al., 2005). In the normal brain Ca^{2+} elevations in astrocytes are mediated mainly by metabotropic receptors. Indeed, a large body of evidence has shown that the activation of astrocytes by neuronal activity-derived glutamate is due to activation of metabotropic glutamate receptors (mGluRs) mGluR3 and mGluR5. Activation of these receptors affects cAMP accumulation and leads to increases in intracellular Ca^{2+}, respectively. The Ca^{2+} rise may oscillate and initiate Ca^{2+} wave propagation within the astrocyte network, activate Ca^{2+}-dependent ion channels and induce glutamate release from astrocytes (cf. above). In epilepsy models, elevated protein levels for mGluR3, mGluR5 and mGluR8 have been found (Steinhäuser and Seifert, 2002). High-resolution analysis of hippocampal specimens from TLE patients detected mGluR2/3, mGluR4 and mGluR8 in reactive astrocytes, suggesting an involvement of these mGluRs in gliosis (Tang and Lee, 2001). Enhanced levels of astroglial mGluR2/3 and mGluR5 were also observed

in epileptic specimens from patients with focal cortical dysplasia (Aronica et al., 2003b). Since their activation affects expression of EAAT1 and EAAT2 (Aronica et al., 2003a) and elevates $[Ca^{2+}]_i$, astrocytic mGluRs might contribute to the generation of seizure foci.

The aforementioned observation that astrocytes exhibit Ca^{2+}-induced release of glutamate providing direct excitation to neighboring neurons is of particular interest in the context of epileptogenesis. Accordingly, alterations in this glia-derived excitatory pathway in concert with impaired glutamate uptake, might increase excitability of the neuron–astrocyte network, favor neuronal synchronization and ultimately predispose neurons to seizures. Recent work suggested that in chemically induced, acute epilepsy models astrocytic Ca^{2+} oscillations and glutamate release generate paroxysmal depolarizing shifts, i.e. interictal spikes (Kang et al., 2005; Tian et al., 2005), although this conclusion is disputed by others (Fellin and Haydon, 2005). Nevertheless, glutamate released from rodent astrocytes during status epilepticus (SE) contributes to neuronal death. This process could be averted by inhibiting glia–neuron signaling and by applying mGluR5 and NR2B NMDA receptor antagonists which suppress astrocytic Ca^{2+} increase and block extrasynaptic NMDA receptors activated by glia-derived glutamate (Ding et al., 2007).

To better understand the role of gliotransmission in epilepsy a number of important issues remain to be clarified. Glutamate from astrocytes can modulate synaptic transmission by affecting presynaptic receptors and regulate exocytosis, or by acting postsynaptically on extrasynaptic NMDA receptors, which can counteract or promote seizure activity (reviewed by Halassa et al., 2007; Wetherington et al., 2008). Moreover, recent evidence suggests that the increase in intracellular Ca^{2+} *per se* might not be sufficient for the release of astroglial glutamate. Rather, other factors such as spatial relations between Ca^{2+} increase and release sites or different modes of exocytosis might be of critical importance (Agulhon et al., 2008; Shigetomi et al., 2008).

5.2. *Release of ATP*

ATP can be released from rodent astrocytes upon the same stimuli that trigger the release of glutamate from astrocytes (Montana et al., 2006; Zhang et al., 2007). The potential impact of astroglial ATP release on seizure activity is now gradually emerging. Under physiological conditions, released ATP is rapidly degraded to adenosine through ectonucleotidases. By acting on presynaptic A1 receptors adenosine leads to inhibition of transmitter release and heterosynaptic depression (Pascual et al., 2005; Serrano et al., 2006; Zhang et al., 2003). The ambient adenosine level is controlled by the activity of the astrocytic enzyme adenosine kinase which phosphorylates adenosine to 5′-AMP (Boison, 2008; Martin et al., 2007). In experimental epilepsy, seizure induction results in the upregulation of adenosine kinase and decreased ambient adenosine concentration, while genetic reduction of adenosine kinase prevents seizures (Li et al., 2008). These findings gave rise to the adenosine kinase hypothesis of epileptogenesis, which considers this enzyme both a diagnostic marker as well as a potential therapeutic target to prevent epileptogenesis (Boison, 2008).

Hence by releasing glutamate and ATP, astrocytes may potentially exert pro- or anticonvulsive actions, respectively. Full clarification of the functional significance of these different astrocyte-to-neuron signaling pathways represents an intriguing challenge. Notably, use of molecular genetics is providing important tools that allow us to selectively affect the different signaling pathways. Gliotransmission may soon be recognized as target for developing new antiepileptic therapies.

6. Tuberous sclerosis

Tuberous sclerosis (TS) is an autosomal dominant genetic disorder caused by mutations of the tumor suppressor genes TSC1 and TSC2, encoding hamartin and tuberin, respectively. The pathology is characterized by non-malignant tumor growth in the brain and other organs such as lung, skin, kidney and muscle. A large majority (80–90%) of TS patients suffer from epileptic seizures which are often medically refractory. In addition to neurons, astrocytes are affected by the disease which develop a reactive or gliotic phenotype (Sosunov et al., 2008). Similar to human TLE, astrocytes in specimens from TS patients displayed increased levels of GFAP, S100β and CD44 while Kir4.1 and GS proteins were dramatically decreased.

Using a genetic approach to delete TSC1 in astrocytes, downregulation of Cx43 protein in astrocytes of the cortex and hippocampus was observed, preceding the onset of seizures (Erbayat-Altay et al., 2007; Xu et al., 2009). Cx43 downregulation led to reduced gap junction coupling of astrocytes in the stratum radiatum of the murine CA1 region and impaired K^+ buffering in the study of Xu et al. (2009).

A typical feature of TSC1/TSC2 mutations is the activation of the mammalian target of rapamycin (mTOR) cascade in astrocytes, indicated by the upregulation of phospho-p70 S6 kinase, phospho-S6 and phospho-stat 3. mTOR is encoded by the FK506 binding protein 12-rapamycin associated protein 1 gene (FRAP1) and acts as a serine/threonine protein kinase. This kinase regulates a plethora of enzymes affecting gene transcription, protein translation, cell growth and motility (reviewed by Inoki et al., 2005b). mTOR acts as a molecular integrator receiving input from nutrients (glucose, amino acids, O_2), growth factors and the TSC1/TSC2 complex. Dysregulation of effectors impinging on the mTOR pathway causes hamartoma syndromes in various organs including the brain (Inoki et al., 2005a). In addition, in acute human reactive astrocytosis, mTOR activation results from responses to local cellular stress (Sosunov et al., 2008). Rapamycin reverses abnormal activation of the mTOR pathway. Treatment of targeted $TSC1^{GFAP}$ CKO mice with rapamycin prevented Cx43 downregulation and reversed impaired K^+ buffering (Xu et al., 2009).

The mTOR pathway is activated not only in TS but also in experimental TLE. After i.p. injection of kainate in rats, biphasic activation of mTOR has been observed in the hippocampus. It started 1 h after seizure onset and persisted several weeks into the chronic epileptic state (Zeng et al., 2009). Treatment with the mTOR inhibitor rapamycin before kainate injection attenuated neuronal death, neurogenesis,

mossy fiber sprouting and development of spontaneous epileptic seizures. Thus, the mTOR pathway is critically involved in epileptogenesis, provoking molecular and cellular changes in astrocytes and neurons (Buckmaser et al., 2009). Hence, mTOR inhibitors might play a beneficial role in antiepileptic treatment.

7. Astrocyte immune responses and epilepsy

Astrocytes contribute to the inflammatory environment of the CNS by producing and responding to immunologically relevant molecules, including a variety of chemokines and cytokines (Dong and Benveniste, 2001). Fever provokes seizures in 1 out of 20–50 children, termed febrile seizures, and is the most common form of pathological brain activity during early development. Prolonged or focal febrile seizures predispose to the development of intractable TLE (Dube et al., 2007). Both in rodents and patients, such febrile seizures have been shown to be associated with increased IL-1β levels in CNS and blood plasma (Dube et al., 2005; Virta et al., 2002) which enhances neuronal excitability, in part by augmenting glutamate receptor function and inhibiting GABAergic transmission (Vezzani et al., 2008). However, IL-1β and other cytokines

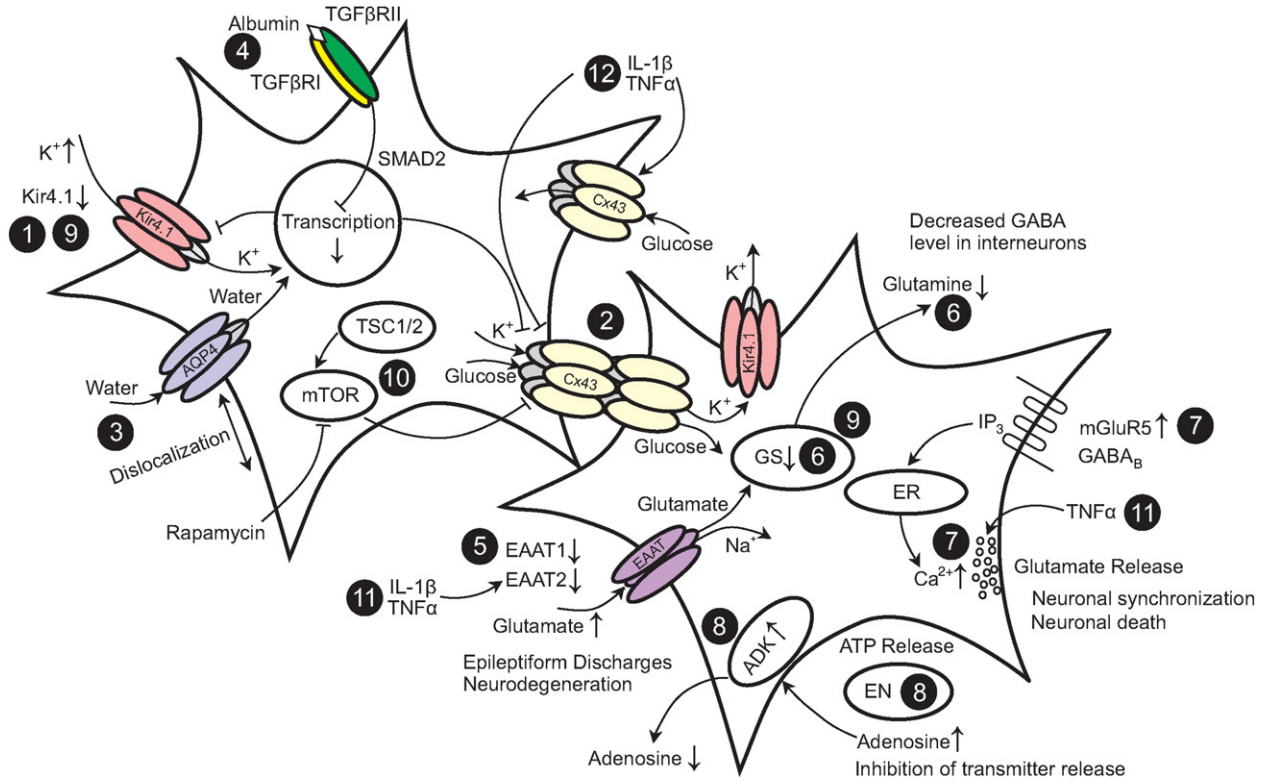

Fig. 1 – Epilepsy-associated alterations of functional properties in astrocytes. (1) Seizure activity leads to an increase in extracellular K^+ concentration. Downregulation of Kir channels was observed in astrocytes in human and experimental epilepsy. (2) Gap junctions mediate spatial redistribution of K^+. Genetic ablation of gap junction entails impaired K^+ buffering and hyperactivity. (3) Dislocation of water channels contributes to impaired K^+ buffering. (4) Blood–Brain barrier disruption, albumin intake and activation of TGFβ receptors lead to downregulation of Cx43, Cx30 and Kir4.1 transcripts and impaired K^+ buffering. (5) Astrocytes are primarily responsible for glutamate uptake. Reduction of EAAT1 and EAAT2 protein is observed in human epileptic hippocampus. Elevated extracellular glutamate decreases the threshold for seizure induction. (6) Glutamate is converted into glutamine through glutamine synthetase (GS). In human epileptic hippocampus, loss of GS resulted in elevated extracellular glutamate levels. In experimental epilepsy, downregulation of GS was observed in the chronic phase. (7) Ca^{2+} elevations in astrocytes are mediated by metabotropic receptors. Activation mGluR5 leads to increases in intracellular Ca^{2+}. Enhanced mGluR5 levels are observed in experimental and human epileptic tissue. (8) Ambient adenosine levels are controlled by ATP release, endonucleotidases (EN) and the activity of the astrocytic enzyme adenosine kinase (ADK). In experimental epilepsy, seizure induction results in upregulation of ADK and decreased ambient adenosine concentration, while genetic reduction of ADK prevents seizures. (9) Astrocytes from epilepsy patients presenting with tuberous sclerosis (TS) display decreased levels of Kir4.1 and GS protein. (10) TSC1 deletion in astrocytes entails downregulation of Cx43 and impaired K^+ buffering. This is prevented by rapamycin which inhibits the mammalian target of rapamycin (mTOR) cascade. (11) The inflammatory cytokines IL-1β and TNFα inhibit glutamate uptake and increase glial glutamate release, which produces hyperactivity. TNFα release as observed during inflammation may entail overexcitation and neurodegeneration. (12) IL-1β and TNFα inhibit Cx43-mediated gap junction communication, but increase activity of Cx43 hemichannels. This might represent an alternative pathway for glucose entry under pathological condition.

can also act on astrocytic receptors. Thus in culture or acute rat brain slices, IL-1β and TNFα were reported to inhibit glutamate reuptake and increase glial glutamate release (Bezzi et al., 2001; Hu et al., 2000; Ye and Sontheimer, 1996), which can be expected to produce hyperactivity. TNFα released from rodent astrocytes controls synaptic efficiency by increasing surface expression of AMPA receptors (Beattie et al., 2002) and causing endocytosis of $GABA_A$ receptors which decreases inhibitory synaptic strength (Stellwagen et al., 2005). Hence, excess TNFα release as observed during inflammation may entail overexcitation and neurodegeneration. In addition, Ca^{2+} increase and glutamate release from astrocytes might be mediated through other stimuli of the NFκB pathway in HS (Steinhäuser et al., 2007).

IL-1β and TNFα also interfere with astrocyte gap junction communication. In cell culture, release of these proinflammatory cytokines from activated microglia leads to a closure of Cx43-mediated gap junctions in astrocytes (Meme et al., 2006). Since in mice SE induces massive microglial activation *in vivo* (Avignone et al., 2008), inhibition of gap junction communication in astrocytes appears to represent a very early alteration in the process of epileptogenesis. On the other hand, proinflammatory cytokines have been reported to open Cx43 hemichannels in cultured astrocytes, which might represent an alternative pathway of glucose entry under pathological condition when intercellular astrocytic junctional communication is impaired (Retamal et al., 2007). It has still to be clarified whether the opposite effects of the cytokines on gap junction coupling and disruption of neuronal energy supply (see point 3) or hemichannel activity have a pro- or anticonvulsive outcome.

Inflammation causes loss of polarized localization of AQP4, as recently shown in a mouse model of experimental autoimmune encephalomyelitis (Wolburg-Buchholz et al., 2009), which might contribute to increased seizure propensity as discussed in chapter 2. It is tempting to speculate that inflammatory changes after seizures (such as release of IL-1β and TNFα) may account for changes in the distribution and function of several other astrocyte membrane channels or receptors.

Infections of the brain, e.g. through meningitis, human herpes virus 6 or herpes simplex virus, have also been associated with TLE, and again astrocytes might be important in this context. Human astrocytes express toll-like receptors (TLRs) (Bsibsi et al., 2002) which recognize various microbial infections (Medzhitov, 2001). Several TLRs are expressed by astrocytes, including those that recognize and respond to viruses and bacteria. Activation of TLRs, similar to interleukin 1 receptors, induce signal transduction pathways leading to the stimulation of the transcription factor NFκB which accordingly might add to enhanced release of astroglial glutamate in the sclerotic hippocampus (Steinhäuser et al., 2007).

8. Concluding remarks

The novel view that considers astrocytes as communication partners of neurons rather than 'brain glue' has rekindled the question regarding the role of these cells in neurological disorders such as epilepsy. Indeed, an increasing body of evidence has documented astroglial dysfunction, and even dysregulation of astroglia-specific functions in human and experimental epilepsy (Fig. 1). A number of key questions need, however, to be addressed before a unifying picture can be proposed. For example it is still unclear whether the reported glial alterations are causative of the condition or rather represent a compensatory phenomenon. In addition, difficulties arise from the fact that the term "astrocyte" covers a heterogeneous group of cells, and this complicates comparison of individual studies. It is worth, however, underlining that the molecular, functional and structural characterization of astroglial heterogeneity is a rapidly evolving field that may soon lead to a better definition of astroglial subtypes. In a comprehensive approach that uses modern molecular genetics and *in vivo* models we may have now the opportunity to clarify the specific roles of astroglia in epilepsy and to develop novel therapeutic approaches to fight this disorder.

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BRAIN RESEARCH REVIEWS

Review

Pain and purinergic signaling

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ABSTRACT

A growing body of evidence indicates that extracellular nucleotides play important roles in the regulation of neuronal and glial functions in the nervous system through P2 purinoceptors. P2 purinoceptors are divided into two families, ionotropic receptors (P2X) and metabotropic receptors (P2Y). P2X receptors (seven types; P2X1–P2X7) contain intrinsic pores that open by binding with ATP, and P2Y receptors (eight types; P2Y1, 2, 4, 6, 11, 12, 13 and 14) are activated by nucleotides and couple to intracellular second-messenger systems through heterotrimeric G-proteins. Nucleotides are released or leaked from non-excitable cells as well as neurons in physiological and pathophysiological conditions. Studies have shown that microglia, a type of glial cells known as resident macrophages in the CNS, express several subtypes of P2X and P2Y receptors, and these receptors play a key role in pain signaling in the spinal cord under pathological conditions such as by peripheral nerve injury (called neuropathic pain). Within the spinal dorsal horn, peripheral nerve injury leads to a progressive series of changes in microglia including morphological hypertrophy of the cell body and proliferation, which are considered indicative of activation. These activated microglia upregulate expression of P2X/Y receptors (e.g., P2X4 and P2Y12). Importantly, pharmacological, molecular and genetic manipulations of the function or expression of these microglial molecules strongly suppress neuropathic pain. We expect that further investigation to determine how ATP signaling via P2X receptors participates in the pathogenesis of chronic pain will lead to a better understanding of the molecular mechanisms of pathological pain and provide clues for the development of new therapeutic drugs.

Contents

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1. Introduction

In 1972, Burnstock proposed new roles of nucleotides as neurotransmitters (Burnstock, 1972) even though it was primarily recognized that intracellular ATP is the source of free energy to maintain life and nucleotides are key molecules within cells. In 1993, the first receptors for nucleotides, called P2 purinoceptors, were cloned (Lustig et al., 1993; Webb et al., 1993). Afterwards, numerous subtypes of these receptors were also cloned, and subsequently scientists began to gradually accept the "purinergic nervous system". Now P2 purinoceptors are divided into two families, ionotropic receptors (P2X) and metabotropic receptors (P2Y) (Abbracchio et al., 2006; Khakh et al., 2001; Ralevic and Burnstock, 1998). Accumulating evidence indicates that nucleotides are released and leaked from non-excitable cells as well as neurons and are involved in cell-to-cell communication in physiological and pathophysiological conditions (Burnstock, 2008). We finally have learned that the playground of nucleotides may expand throughout the entire body. One of the most exciting findings is that glia cells make up over 70% of the total cell population in the central nervous system (CNS). Glial cells are classified into astrocytes, oligodendrocytes and microglia. Astrocytes express many types of P2X/Y receptors and release ATP in response to various stimuli or even spontaneously, and communicate with neurons at synapses, microglial cells and vascular walls at capillaries (Abbracchio et al., 2006; Inoue et al., 2007). Microglia, known as resident macrophages of the CNS, also express many types of P2 receptors (Farber and Kettenmann, 2006; Inoue et al., 2007; Inoue, 2008). Although it was long believed that glial cells in the nervous system were only physical and nutrient supports for neurons, a growing body of evidence has dramatically changed this classical view and indicates that neuron–glia interactions are a key component to understand CNS functions. In addition to crucial roles in normal physiological conditions, glial cells also play important roles in pathophysiological conditions of the CNS including psychiatric disorders, physical trauma, and chronic pain (Miller, 2005).

Pain in response to noxious stimuli can act as an early warning device that alerts us to the presence of damaging stimuli. This normal 'good' pain usually goes away soon after the nociceptive stimulus is removed. In contrast, sometimes pain persists for a long time. In general, chronic pain may be categorized into inflammatory pain and neuropathic pain. Inflammatory pain is the result from inflammatory responses to trauma in peripheral tissues. This type of pain, however, still has a physiological significance in that it may assist wound repair since contact with the damaged area is minimized. Also, inflammatory pain usually goes away after the tissue damage is repaired and can be generally managed by treatment with known analgesics. However, neuropathic pain does not go away even though the initial damage has healed. It typically occurs after nerve damage that can be induced by bone compression in cancer, diabetes, infection, autoimmune disease or physical injury (Baron, 2006). In addition to spontaneous pain and hyperalgesia (the increased pain perception of noxious stimuli), a troublesome symptom of neuropathic pain is pain hypersensitivity to normally innocuous stimuli, a phenomenon known as tactile allodynia. This type of pain is refractory to currently available treatments, such as non-steroidal anti-inflammatory drugs and opioids (Scholz and Woolf, 2002; Woolf and Mannion, 1999). We are now beginning to understand that neuropathic pain is not just a symptom of disease but is a consequence of disordered functioning of the nervous system (Scholz and Woolf, 2002; Woolf and Mannion, 1999; Woolf and Salter, 2000). Unraveling the mechanisms of pain hypersensitivity caused by nerve damage is therefore essential for the development of new therapeutic drugs for neuropathic pain.

Accumulating evidence from diverse animal models of neuropathic pain suggests that neuropathic pain might involve aberrant excitability of the nervous system. Notably, in primary sensory ganglia and in the dorsal horn of the spinal cord multiple functional and anatomical alterations follow peripheral nerve injury (Costigan et al., 2009; Scholz and Woolf, 2002; Woolf and Salter, 2000). While it has long been considered that there are relevant changes in neurons, emerging lines of evidence have revealed that they also occur in glial cells, especially microglia (Marchand et al., 2005; Scholz and Woolf, 2007; Suter et al., 2007; Tsuda et al., 2005; Watkins et al., 2001). After peripheral nerve injury, microglia in the normal state (traditionally called 'resting' microglia) in the spinal dorsal horn are converted to an activated state through a series of cellular and molecular changes (Echeverry et al., 2008; Suter et al., 2007; Tsuda et al., 2005). Microglia change their phenotype to the activated form following altered expression of multiple molecules, including cell surface receptors, intracellular signaling molecules, and diffusible factors. By responding to extracellular ligands, activated microglia can evoke various cellular responses, such as migration toward afflicted sites and secretion of proinflammatory factors. Importantly, pharmacological, molecular and genetic manipulations of the function or expression of these microglial molecules strongly modulate neuropathic pain (Clark et al., 2007; Griffin et al., 2007; Jin et al., 2003; Obata et al., 2007; Tanga et al., 2005; Tsuda et al., 2003, 2004; Zhuang et al., 2005) and hyperexcitability of dorsal horn neurons (Coull et al., 2005; Keller et al., 2007). A growing body of evidence indicates that purinergic receptors are expressed in activated microglia under such pain conditions and that P2X and P2Y receptors-mediated signaling critically contributes to the development and maintenance of neuropathic pain. In this article, we focus on recent developments that

further the understanding of mechanisms by which P2X and P2Y receptors on microglia in the spinal cord participate in the pathogenesis of neuropathic pain. In addition, we summarize the accumulated evidence from immunohistochemical, biochemical, molecular and behavioral studies, and we also provide a framework to address the major questions that currently remain unanswered.

2. Molecular structure and pharmacological profiles of purinergic receptors

2.1. *P2X receptors*

P2X receptors belong to a family of ligand-gated ion-channels and are cation-selective channels with almost equal permeability to Na^+ and K^+, and significant permeability to Ca^{2+} (Khakh et al., 2001; North, 2002; Ralevic and Burnstock, 1998). Molecular cloning has so far identified seven genes encoding P2X receptor subunits (P2X1–P2X7). All P2X subunits possess two transmembrane regions and have intracellular N- and C-terminals and a long extracellular loop between transmembrane domains (Khakh et al., 2001; North, 2002; Vial et al., 2004). One third of the amino acids in the extracellular loop are conserved in at least six P2X subunits, suggesting they are involved in ATP binding (Vial et al., 2004). While the intracellular N-terminal regions have relatively similar lengths of amino acids, the length of the C-terminals diverges considerably from 30 residues in P2X6 to 240 in P2X7 (North, 2002; Vial et al., 2004). In addition, there is a putative motif for phosphorylation by protein kinase C and A in the N- and C-terminals, respectively (North, 2002), and by ecto-protein kinase C at the ectodomain (Wirkner et al., 2005).

One functional P2X receptor channel is presumably formed by a number of P2X subunits, as is considered to be the case in other ligand-gated ion channels. The number of subunits of a P2X receptor has been proposed to be three (Jiang et al., 2003; Nicke et al., 1998, 2003; Stoop et al., 1999) or four (Ding and Sachs, 2000; Kim et al., 1997). Recently, we analyzed the architecture and ATP-induced structural changes in P2X4 receptors using fast-scanning atomic force microscopy (AFM) and revealed a trimer structure (Shinozaki et al., 2009). The trimer structure of P2X receptors is also demonstrated in P2X2 receptors (Barrera et al., 2005). The trimer structure of P2X channels is strongly supported by a recent study revealing the three-dimensional crystal structure of P2X4 receptor at a high resolution (Kawate et al., 2009). It appears that all three subunits are either identical, making P2X4 receptors homomeric, rather than identical, or heteromeric receptors. Biochemical studies have shown the co-assembly of P2X1/5 (Torres et al., 1998), P2X2/3 (Lewis et al., 1995), P2X2/6 (King et al., 2000), P2X4/6 heteromeric receptors (Le et al., 1998). In addition, there is a report that P2X2/3 receptors are likely to contain one P2X2 and two P2X3 subunits (Jiang et al., 2003; Wilkinson et al., 2006).

Each of the seven homomeric P2X receptors and at least four heteromeric receptors show different (but partly overlapping) electrophysiological and pharmacological properties in terms of current kinetics, desensitization rates and sensitivities to agonists and antagonists (Jarvis and Khakh, 2009; Khakh et al., 2001; North, 2002; Ralevic and Burnstock, 1998). When each one or two of them are expressed heterologously in cells, ATP evokes a rapid- or slow-inactivating inward current. The former is seen in cells expressing either P2X1 or P2X3 receptors, and the latter is seen in those expressing all other receptors including the four heteromeric receptors (Khakh et al., 2001; North, 2002). Furthermore, repetitive activation of P2X1 and P2X3 receptors by ATP causes a marked desensitization of ATP-induced responses. α,β-Methylene ATP (αβmeATP), an analogue of ATP, is a useful agonist for basic identification of P2X receptors containing P2X1 or P2X3 (i.e., P2X1, P2X3, P2X1/5 and P2X2/3), although αβmeATP also activates P2X4/6 (Khakh et al., 2001; North, 2002; Ralevic and Burnstock, 1998). As for antagonists, suramin and pyridoxalphosphate-6-azophenyl-2′,4′-disulfonic acid (PPADS) are antagonistic to almost all P2X receptors except rat P2X4 receptors (Buell et al., 1996). However, it should be noted that suramin also blocks several neurotransmitter receptors (e.g., NMDA and $GABA_A$ receptors) (Nakazawa et al., 1995; Peoples and Li, 1998). 2′,3′-O-(2,4,6-trinitrophenyl)-ATP (TNP-ATP) at a nanomolar range selectively blocks P2X1, P2X3 and P2X2/3 receptors (Virginio et al., 1998) and at high concentration also blocks P2X4 receptors (Virginio et al., 1998). Furthermore, diinosine pentaphosphate (Ip_5I) is a very potent antagonist to P2X1 (King et al., 1999) and P2X3 receptors but, interestingly, not to P2X2 and P2X2/3 receptors (Dunn et al., 2000). In 2002, the first selective antagonist for P2X3 (and P2X2/3), A-317491, was developed (Jarvis et al., 2002). A-317491 blocks the responses mediated by P2X3 or P2X2/3 receptors in a competitive fashion without any effect on other receptors, enzymes or ion-channels (Jarvis et al., 2002). Furthermore, several selective antagonists for P2X7 receptors, A-740003 (Honore et al., 2006), A-438079 (McGaraughty et al., 2007), A-804598 (Donnelly-Roberts et al., 2009) and AZ11645373 (Stokes et al., 2006), have been developed recently. A-317491 and A-438079 are commercially available.

2.2. *P2Y receptors*

The first G protein-coupled P2Y receptors were cloned in 1993 (Lustig et al., 1993; Webb et al., 1993), and there are currently eight accepted P2Y receptors: P2Y1, P2Y2, P2Y4, P2Y6, P2Y11, P2Y12, P2Y13, and P2Y14. The missing numbers represent either nonmammalian orthologs or receptors that have some sequence homology to P2Y receptors but for which there is no functional evidence of responsiveness to nucleotides (Abbracchio et al., 2006). P2Y receptors are 308–377 amino acid proteins with a mass of 41–53 kDa after glycosylation. The seven transmembrane (TM) domain tertiary structure of P2Y receptors is common to other G protein-coupled receptors (Fig. 1). P2Y are activated by purine or pyrimidine nucleotides or sugar-nucleotides with subtype-dependent heterotrimeric G-proteins (Abbracchio et al., 2006). Site-directed mutagenesis studies have shown that some positively charged residues in TM3, 6 and 7 are crucial for receptor activation by nucleotides (Jiang et al., 1997). Each P2Y receptor binds to a single heterotrimeric G protein ($G\alpha_{q/11}$ for P2Y1, 2, 4, 6), although P2Y11 can couple to both $G\alpha_{q/11}$, and $G\alpha_s$, whereas P2Y12 and P2Y13 couple to $G\alpha_i$ and P2Y14 couples to $G\alpha_{i/o}$ (Abbracchio et al., 2006). In response to nucleotide activation, recombinant P2Y receptors either activate the phospholipase C (PLC)-IP_3 pathway through $G\alpha_{q/11}$, which in turn releases intracellular calcium and activates protein kinase C, or affect adenylyl cyclase and alter cAMP

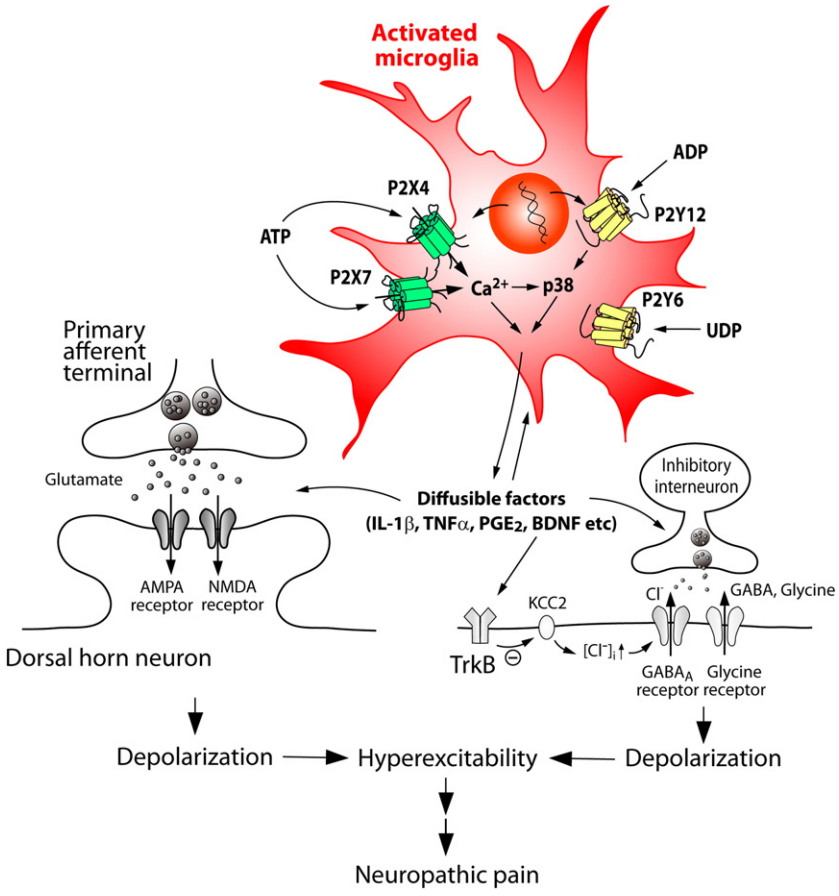

Fig. 1 – Schematic illustration of potential mechanisms by which P2X and P2Y receptors in activated microglia modulate pain signaling in the dorsal horn. Activated microglia after nerve injury increase the expression of P2X4, P2Y6 and P2Y12. The P2X4 and P2X7 receptors are activated by ATP which is presumably released from primary sensory neurons (or astrocytes), and in turn cause a rise in the intracellular calcium and activation of p38MAPK. P2X receptors activation leads to the release of bioactive diffusible factors, such as BDNF and other proinflammatory factors (cytokines and chemokines). BDNF causes a collapse of the transmembrane anion gradient in dorsal horn lamina I neurons presumably through the downregulation of KCC2, which in turn renders GABA and glycine affects depolarizing, rather than hyperpolarizing, in these neurons. Microglial factors may also interact with excitatory synapses of neighboring dorsal horn neurons and enhance the excitability in dorsal horn neurons. The net hyperexcitability in the dorsal horn pain network by these factors from activated microglia may be responsible for neuropathic pain.

levels through $G\alpha_{i/o}$ (Abbracchio et al., 2006). Furthermore, recent studies have shown that several P2Y receptors (P2Y2, P2Y6 and P2Y12) also coupled to $G\alpha_{12/13}$, and nucleotide stimulation also activates Rho (Liao et al., 2007; Nishida et al., 2008).

In contrast to P2X receptors, P2Y receptors are activated not only by ATP but also by its metabolite ADP and other nucleotides such as UTP and UDP (Abbracchio et al., 2006). For P2Y1 receptors, ADP is a more potent agonist than ATP (their 2-methylthio derivatives are even more potent). The most effective antagonists to display selectivity for the P2Y1 receptor are MRS2179 (Boyer et al., 1998), MRS2279 (Boyer et al., 2002) and MRS2500 (Kim et al., 2003). P2Y2 receptors are fully activated by equivalent concentrations of ATP and UTP, whereas ADP and UDP are much less effective agonists. P2Y6 receptors are selective for UDP (Abbracchio et al., 2006). A 1,4-di-(phenylthioureido) butane derivative (MRS2578) (Mamedova et al., 2004) has been shown to selectively inhibit UDP-induced phospholipase C activity through P2Y6 receptors. P2Y12 receptors are also activated selectively by ADP and blocked by the 5′-triphosphate derivative AR-C69931MX (Ingall et al., 1999) (although this antagonist also blocks P2Y11 and P2Y13 receptors (Abbracchio et al., 2006; Communi et al., 1999)). Recent studies have demonstrated that the active metabolite of the antiplatelet drug clopidogrel is a potent and selective antagonist of P2Y12 receptors (Savi and Herbert, 2005).

3. Purinergic signaling and neuropathic pain

3.1. *P2X4 receptor*

A clue to identifying $P2X_4Rs$ in the spinal cord as required for neuropathic pain first came from pharmacological investigation of pain behavior after nerve injury using the P2X receptor antagonists TNP-ATP and PPADS (Tsuda et al., 2003) that are

known to be sensitive and insensitive to P2X4, respectively (Jarvis and Khakh, 2009; Khakh et al., 2001). Because nerve injury-induced allodynia was effectively suppressed by TNP-ATP but not by PPADS, it was inferred that tactile allodynia depends upon P2X4Rs in the spinal cord (Tsuda et al., 2003). This hypothesis is substantially supported by recent findings showing that both mice treated spinally with a P2X4 antisense oligonucleotide and mice lacking P2X4 show attenuated tactile allodynia after nerve injury (Tsuda et al., 2003, 2009; Ulmann et al., 2008). Expression of P2X4 receptors in the spinal cord, after nerve injury, markedly increased specifically in microglia, indicating that tonic activation of P2X4 receptors in microglia is necessary for sustaining allodynia (Tsuda et al., 2003). Moreover, it was found that spinal administration of P2X4-stimulated microglia caused otherwise normal rats to develop allodynia. Therefore, P2X4 receptor activation in microglia is not only necessary but is also sufficient to cause tactile allodynia (Tsuda et al., 2003).

The mechanisms by which microglia are crucial for producing neuropathic pain must involve signaling from activated microglia to dorsal horn neurons, but how? Coull et al. (2005) demonstrated that P2X4-stimulated microglia induced the hyperexcitability of dorsal horn neurons. In spinal cord slices of rats that had displayed allodynia after intrathecal administration of ATP-stimulated microglia, a positive shift of the anion reversal potential (E_{anion}) in spinal lamina I neurons was found, which caused GABA-evoked depolarization, rather than hyperpolarization, in these neurons (Coull et al., 2005). Moreover, TNP-ATP acutely reversed the depolarizing shift in E_{anion} in lamina I neurons after nerve injury (Coull et al., 2005). These results imply that spinal microglia stimulated by P2X4 receptors cause neuropathic pain through a rise in intracellular Cl^- in spinal lamina I neurons. Furthermore, Keller et al. (2007) have shown that local spinal administration of ATP-stimulated microglia changed the phenotype of *in vivo* spinal lamina I output neurons, such that they relay innocuous mechanical input (Keller et al., 2007), which may account for allodynia. Recently, Coull et al. (2005) determined the role of brain-derived neurotrophic factor (BDNF) as a signaling factor between microglia and dorsal horn lamina I neurons. It was found that intrathecal application of BDNF mimicked tactile allodynia and the depolarizing shift in E_{anion} in lamina I neurons by peripheral nerve injury or by intrathecal administration of P2X4-stimulated microglia. Interference of signaling between BDNF and its receptor (TrkB) prevented tactile allodynia caused by peripheral nerve injury or by intrathecal administration of P2X4-stimulated microglia. Activating P2X4 on microglial cells caused the release of BDNF(Coull et al., 2005; Trang et al., 2009), an effect that is dependent on activation of p38 (Trang et al., 2009), a member of mitogen-activated protein kinase that has been reported to be implicated in neuropathic pain (Jin et al., 2003; Tsuda et al., 2004). Interestingly, P2X4-mediated BDNF release was abolished by inhibiting SNARE (soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor)-mediated exocytosis. Thus, these results indicate that P2X4-stimulated microglia release BDNF as a crucial factor to signal to lamina I neurons, causing a collapse of their transmembrane anion gradient and subsequent neuronal hyperexcitability (Fig. 1). The γ-aminobutyric $acid_A$ ($GABA_A$) receptor-mediated depolarization might also produce excitation through voltage-sensitive Ca^{2+} channels and *N*-methyl-D-aspartate (NMDA) receptors. There is evidence that several proinflammatory cytokines that are known to be released from microglia (Inoue, 2002; Inoue, 2006) modulate excitatory synaptic transmission. Spinal injection of interleukin-1β (IL-1β) enhanced C-fiber-evoked responses and windup in wide-dynamic-range dorsal horn neurons (Reeve et al., 2000). Exogenous application of IL-1β enhanced NMDA receptor-mediated Ca^{2+} responses via activation of the tyrosine protein kinase Src (Viviani et al., 2003). IL-1β was also reported to decrease $GABA_A$ receptor-mediated currents (Wang et al., 2000). A recent study has clearly demonstrated a powerful role for proinflammatory cytokines in excitatory or inhibitory synaptic transmission and on neuronal activity in the superficial dorsal horn neurons (Ikeda et al., 2007; Kawasaki et al., 2008b). Also, inhibition of microglial ERK decreased hyper-responsiveness of dorsal horn neurons in spinal cord injured rats through a reduction of prostaglandin E_2 production (Zhao et al., 2007). Thus, the net enhanced transmission in the dorsal horn pain network by these factors might be responsible for nerve injury-induced neuropathic pain.

From the above evidence, upregulation of P2X4 receptor expression in microglia is therefore a key process in neuropathic pain. How peripheral nerve injury increases expression of P2X4 receptors in microglia is not clearly understood. Activating both toll-like receptors (TLRs) and nucleotide-binding oligomerization domain 2 (NOD2, another pattern-recognition receptor) in cultured microglia increased the expression of P2X4 at the mRNA level (Guo et al., 2006), but the functional relevance of these receptors *in vivo* is still unknown. Studies in our laboratory have recently shown that the extracellular matrix protein, fibronectin, increased expression of mRNA and protein of P2X4 receptors in primary cultured microglial cells (Nasu-Tada et al., 2006; Tsuda et al., 2010). Cultured microglia stimulated by fibronectin also showed an enhanced ATP-induced Ca^{2+} influx (Nasu-Tada et al., 2006). The level of fibronectin protein was elevated in the dorsal horn 3–7 days after nerve injury (Nasu-Tada et al., 2006), the time when P2X4 protein levels start to increase (Tsuda et al., 2003). Blockade of fibronectin receptors attenuated nerve injury-induced P2X4 upregulation and allodynia (Tsuda et al., 2008a). It was also found that the upregulation of P2X4 receptors in the spinal cord and allodynia after spinal nerve injury was significantly suppressed by intrathecal administration of echistatin (Tsuda et al., 2008a). Furthermore, intrathecal delivery of fibronectin increased P2X4 expression and produced allodynia, a behavior that was not evoked in P2X4-deficient mice. These results suggest that the fibronectin-integrin signaling system participates in the upregulation of P2X4 expression after nerve injury and subsequent neuropathic pain (Tsuda et al., 2008a).

We recently identified Lyn as a critical signaling molecule for P2X4 upregulation in microglia (Tsuda et al., 2008b). Lyn-deficient microglial cells showed a deficit in increased P2X4 expression caused by fibronectin. The level of Lyn expression was increased exclusively in microglia after nerve injury, and Lyn-knockout mice exhibited a striking reduction in upregulation of P2X4 and tactile allodynia after nerve injury. Lyn tyrosine kinase distinctly activated the phosphatidylinositol 3-kinase

(PI3K)-Akt and mitogen-activated protein kinase kinase (MAPK kinase, MEK)-extracellular signal-regulated kinase (ERK) signaling cascades (Tsuda et al., 2010). Signaling through the PI3K-Akt pathway induced degradation of p53 via mouse double minute 2 (MDM2) in a proteasome-dependent manner. The consequence of an attenuated repressive effect of p53 may be associated with enhanced P2X4 gene expression. On the other hand, activated MEK-ERK signaling in microglia exposed to fibronectin enhanced eukaryotic translation initiation factor 4E (eIF4E) phosphorylation status via activated MAPK-interacting protein kinase-1 (MNK1), which may play a role in regulating P2X4 expression at translational levels. The molecular machinery for the upregulation of P2X4 expression in microglia is now considered to be as shown in Fig. 2.

There is evidence that P2X4 receptors in microglial cells are located predominantly within lysosomal compartments and are targeted there by their N- and C-terminal motifs (Qureshi et al., 2007). Notably, P2X4 receptors remain stable within lysosomes and resist degradation by virtue of their N-linked glycans. It is of particular interest that stimulating lysosome exocytosis, for example, using a Ca^{2+} ionophore, resulted in the trafficking of P2X4 to the plasma membrane (Qureshi et al., 2007). Given that P2X4 activity on the plasma membrane of microglia is critical for microglial function, it is conceivable that mechanisms underlying P2X4 recycling between the lysosome and the plasma membrane are important for neuropathic pain.

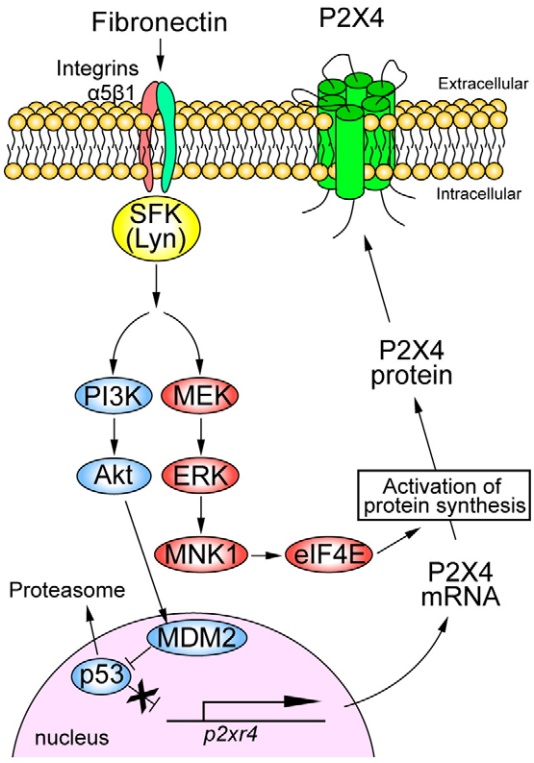

Fig. 2 – Schematic representation of the hypothetical mechanisms involved in fibronectin-induced P2X4 upregulation in microglia. Extracellular fibronectin binds to α5β1 integrins on microglial cells, which leads to activation of the PI3K-Akt and MEK-ERK signaling cascades through Lyn tyrosine kinase. Signaling through the PI3K-Akt pathway induces degradation of p53 via MDM2 in a proteasome-dependent manner. The reduction of the repressive effect of p53 may enhance P2X4 gene expression. Activated MEK-ERK signaling in microglia exposed to fibronectin enhances eIF4E phosphorylation status via activated MNK1, which may play a role in regulating P2X4 expression at translational levels.

The above evidence indicates that P2X4 might be a potential therapeutic target to treat neuropathic pain. However, there is no antagonist to strongly inhibit P2X4 receptors. We have recently demonstrated that some antidepressants and anticonvulsants clinically used in patients with neuropathic pain have inhibitory effects on ATP-evoked Ca^{2+} response in cells expressing recombinant P2X4 receptors and primary cultured microglial cells (Nagata et al., 2009). Among the drugs used, paroxetine has the most potent inhibitory effect. Intrathecal administration of paroxetine and fluvoxamine, but not citalopram, produced an anti-allodynic effect in an animal model of neuropathic pain, which correlated with the potency of inhibition of rat P2X4 receptors. Interestingly, it was found that the anti-allodynic effect of paroxetine is insensitive to 5-HT receptor antagonists and was also observed in neuropathic pain animals with depleted 5-HT in the spinal cord. These results suggest that the anti-allodynic effect of antidepressants may be independent of the spinal 5-HT system and may be mediated by inhibiting P2X4 receptors (Nagata et al., 2009).

3.2. *P2X7 receptor*

The first evidence indicating the involvement of P2X7 receptors in pain was provided by a study using P2X7-deficient mice. These mice exhibited reduced thermal and mechanical hypersensitivities after partial sciatic nerve ligation (Chessell et al., 2005). Recently, several groups have developed novel, selective inhibitors of P2X7 receptors and have examined their effects on neuropathic pain. Systemic (i.p.) administration of A-740003 and A-438079, structurally unrelated P2X7 antagonists, reduced tactile allodynia in three different models of neuropathic pain in rats (Honore et al., 2006). Furthermore, A-438079 (i.v.) reduced innocuous stimuli-evoked activity of *in vivo* dorsal horn neurons in neuropathic rats, but not in sham-operated rats (McGaraughty et al., 2007). The role of microglial P2X7 receptors in neuropathic pain remains unclear because of a lack of data showing the effect of intrathecal administration of these antagonists. Interestingly, recent evidence has indicated a structural and functional interaction between P2X7 and P2X4 receptors (Casas-Pruneda et al., 2009; Guo et al., 2007). Studies have shown co-immunoprecipitation of both receptor subtypes (Boumechache et al., 2009; Guo et al., 2007), but it might not be due to the formation of heterotrimeric complex but to the close association of neighboring P2X4 and P2X7 homotrimers (Boumechache et al., 2009; Nicke, 2008). Furthermore, in contrast to the predominant localization of P2X4 in intracellular lysosome (as mentioned above), P2X7 is predominantly distributed in the cell surface. If the trafficking of P2X4 to the plasma membrane is enhanced in activated microglia, a functional interaction between these two receptors might occur. Therefore, it is of particular interest to investigate the interaction between P2X4 and P2X7 in neuropathic pain.

3.3. *P2Y6 receptor*

Microglial cells also express several G-protein-coupled receptors. In our recent study, it was found that microglial cells in culture express P2Y6 receptors (Koizumi et al., 2007) and that after peripheral nerve injury P2Y6 mRNA expression is markedly increased in the spinal cord, the time course of which is parallel with that of tactile allodynia (unpublished observation). The function of P2Y6 in microglia remains unknown, but we recently revealed that this receptor mediated microglial phagocytosis. It was found that applying UDP, an agonist for P2Y6, to primary cultured microglial cells facilitated phagocytosis (Koizumi et al., 2007). Neuronal injury induced by administration of kainic acid (KA) caused upregulation of P2Y6 receptors in microglia in the hippocampus. KA-evoked neuronal injury resulted in an increase in extracellular UTP, which was immediately metabolized into UDP *in vivo* and *in vitro*. UDP, leaked from injured neurons, caused P2Y6 receptor-dependent phagocytosis. The P2Y6 receptor is upregulated when neurons are damaged, and could function as a sensor for phagocytosis by sensing diffusible UDP signals. However, the role of upregulated P2Y6 in the spinal cord under neuropathic pain conditions remains unknown, and further studies are required to determine its role.

3.4. *P2Y12 receptor*

Two recent studies have revealed that P2Y12 receptors are one of the key regulators for neuropathic pain (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008). It was demonstrated that intrathecally administered P2Y12 receptor antagonists, such as MRS2395 and AR-C69931MX, or antisense oligonucleotide for P2Y12 receptors significantly suppressed development of neuropathic pain after spinal nerve injury (Tozaki-Saitoh et al., 2008) and partial sciatic nerve injury (Kobayashi et al., 2008). A crucial finding was established using P2Y12-knockout mice. Genetic ablation of P2Y12 receptors failed to produce tactile allodynia (Tozaki-Saitoh et al., 2008). Strategic advances in targeting P2Y12 receptors were considered because of its restricted expression in the CNS. Several lines of evidence revealed that P2Y12 receptor expression is specifically observed in brain and spinal cord resident microglia but is not observed in fms- or CD11b-positive peripheral macrophages in spleen (Haynes et al., 2006; Kobayashi et al., 2006; Pausch et al., 2004; Sasaki et al., 2003). In line with these facts, we could consider that P2Y12 receptor inhibition results from P2Y12 receptor-gated signal modulation in the CNS. p38MAPK is a signaling molecule whose phosphorylation after partial sciatic nerve injury is almost completely suppressed by an antagonist or antisense oligonucleotide for P2Y12 receptors (Kobayashi et al., 2008). We have also shown that a single administration of the P2Y12 receptor antagonists AR-C69931MX (intrathecally) or clopidogrel (orally) to nerve-injured rats produced a striking alleviation of established tactile allodynia (Tozaki-Saitoh et al., 2008). These effects by P2Y12 receptor antagonists are in contrast to the effects of another P2Y12 receptor antagonist, MRS2395, and a P2Y12 receptor antisense oligonucleotide, which did not reverse pain behaviors (Kobayashi et al., 2008). The exact reason for this difference remains open to question. In addition, it must be noted that P2Y12 receptors are predominantly expressed in platelets in the periphery and inhibition of this receptor is one of the adopted mechanisms for anti-platelet agents (Gachet, 2005).

Expression levels of P2Y12 receptors are dramatically increased in microglial cells ipsilateral to peripheral nerve injury in the spinal cord after nerve injury (Kobayashi et al., 2008; Tozaki-Saitoh et al., 2008) but down-modulated in brain microglia activated by slice preparation (Haynes et al., 2006). A major difference in each experiment was the proximal or distal neuronal damage, which might induce different signal cascades to invoke microglial responses. Indeed, upregulation of P2Y12 receptor mRNA is also observed in a facial nerve injury model (Sasaki et al., 2003). Detailed mechanisms of expressional regulation of P2Y12 receptors remain to be elucidated.

Many studies that have investigated in depth the mechanism of ATP-mediated chemotaxis in microglia have identified P2Y12 and P2X4 receptors as primary sensors for extracellular ATP (Honda et al., 2001; Irino et al., 2008; Kanazawa et al., 2002; Ohsawa et al., 2007). Further studies have added evidence indicating that P2Y12 receptors are implicated in the motility of microglial cell bodies and processes *in vivo* or *ex vivo* (Davalos et al., 2005; Haynes et al., 2006; Kurpius et al., 2007). Thus, low ATP concentrations in the extracellular space activate chemotaxis of microglia at the injured or inflamed site. We already know that P2Y12 receptors are unlikely to be necessary to convert spinal microglia to the activated state and to increase their cell numbers because morphological and numerical alterations of microglia were not influenced by the lack of P2Y12 receptors or by the administration of the P2Y12 receptor antagonist AR-C69931MX in the spinal dorsal horn after spinal nerve injury (Tozaki-Saitoh et al., 2008). One of remaining possibilities regarding ATP-induced microglial chemotactic activity is that P2Y12 receptor activity in microglia may influence the abilities of microglia to extend the tips of their branched processes toward neighboring pain transmission neurons, which may in turn affect microglia–neuron communications.

4. Concluding remarks

We have primarily focused on the role of purinoceptors expressed in spinal microglia in neuropathic pain caused by peripheral nerve injury. Pharmacological, molecular and genetic manipulations of the function or expression of these purinoceptors have influenced nerve injury-induced pain behaviors and hyperexcitability of the dorsal horn pain pathway. Therefore, purinoceptor-mediated spinal microglia activity make a critical contribution to pathologically enhanced pain processing in the dorsal horn, and microglial purinoceptors might be promising targets for treating neuropathic pain. Recently, novel and established drugs that target P2X and P2Y receptors have been reported to be effective in models of pain. Of note, a more recent study has revealed the three-dimensional crystal structure of P2X4 receptor. Although the structure was solved in the absence of ATP, cavities between subunits thought to be the ATP binding site were also found. Thus, the P2X4 structure will be highly informative for designing new drugs targeting P2X4 receptor.

A predicted therapeutic benefit of interfering with microglial P2X and P2Y receptors may be that normal pain sensitivity would be unaffected because expression or activity of most of these molecules are upregulated or enhanced predominantly in activated microglia in the spinal cord where damaged sensory fibers project. In addition to microglia, recent studies have also identified astrocyte-specific molecules and demonstrated a critical role of spinal astrocytes in neuropathic pain, particularly in the maintenance phase (Ji et al., 2006; Katsura et al., 2008; Kawasaki et al., 2008a; Zhuang et al., 2006). Extracellular nucleotides are also a strong modulator of astrocytes in the brain including the dorsal horn of the spinal cord (Fam et al., 2000; Salter and Hicks, 1994). How astrocytic purinoceptors participate in neuropathic pain and interact with microglial purinoceptors is now open for investigation. It is expected that an increased understanding of the functions of purinoceptors will provide us with exciting insights into pain mechanisms and clues to develop new therapeutic agents for the management of neuropathic pain.

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