Sodium dynamics in neurons and astrocytes under epileptiform conditions

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Science, my lad, is made up of mistakes, but they are mistakes which it is useful to make, because they lead little by little to the truth.

Jules Verne, A Journey to the Center of the Earth

Abstract

In the mammalian central nervous system, astrocytes monitor and regulate their local microenvironment. Their multiple fine processes, which make contact to neurons, blood vessels and adjacent astrocytes, are appropriately equipped to control homeostasis as well as to detect and actively modulate information processing. Many of these functions such as regulation of ion and neurotransmitter levels as well as energy supply are closely linked to the Na^+ gradient as a key transmembrane driving force. Therefore, to maintain this driving force, the intracellular Na^+ concentration is stabilized by the ATP-consuming activity of the Na^+/K^+ATPase.

Hence, we propose that, due to the variety and functional relevance of Na^+-dependent processes, transient as well as prolonged changes of Na^+ levels in both neurons and astrocytes could be involved in information processing and thereby constitute yet another component of neuron-glial crosstalk.

Although previous studies showed the appearance of Na^+ concentration changes in neurons and astrocytes upon external, artificial stimulation, it remained unclear to which extent intracellular Na^+ fluctuations occur during activity of the endogenous brain circuitry. To tackle that question, we performed quantitative fluorescence imaging of the sodium sensitive fluorescent dye SBFI and local field potential recordings during recurrent epileptiform discharges in acute hippocampal slices of adolescent mice. We observed synchronous, large and long-lasting Na^+ transients in neurons and astrocytes with average peak changes of 7 mM and 3 mM as well as decay time constants of 18 s and 6 s, respectively. Thus, despite the steady Na^+/K^+ATPase activity, considerable Na^+ changes were generated. Still, although challenged by strong, recurrent activation, cells maintained their overall homeostasis for prolonged periods. In contrast, pharmacological inhibition of glutamate transporters induced neuronal Na^+ accumulation, followed by swelling, and excitotoxic damage. Moreover, impairment of astroglial energy production during recurrent discharges increased the Na^+ concentration in astrocytes and neurons and prolonged both discharge duration and Na^+ transients.

In conclusion, our results show that large and long-lasting Na^+ transients occur during synchronized activity of the endogenous circuitry. The experiments suggest that metabolic support of neurons during enhanced activity, provided by astrocytes, is necessary to maintain neuronal Na^+ homeostasis.

Zusammenfassung

Das lokale Milieu im zentralen Nervensystem von Säugetieren wird durch Astrozyten reguliert, welche über eine Vielzahl feiner Ausläufer mit Neuronen, Blutgefäßen sowie benachbarten Astrozyten in Kontakt stehen. Mittels dieser Fortsätze sind Astrozyten in der Lage, die Homöostase von Ionen, Neurotransmittern und des Energiehaushalts zu kontrollieren. Zusätzlich nehmen sie neuronale Aktivität wahr und modulieren diese aktiv. Ein Großteil dieser Funktionen ist eng mit dem Na^+-Gradienten verknüpft, welcher als wichtige Triebkraft für Transportprozesse über die Membran dient und daher unter Energieverbrauch von der Na^+/K^+-ATPase aufrecht erhalten wird.

Aufgrund der Vielfalt und funktionellen Relevanz Na^+-abhängiger Prozesse liegt die Annahme nahe, dass vorübergehende sowie langanhaltende Konzentrationsänderungen sowohl in Neuronen als auch in Astrozyten zur Informationsverarbeitung beitragen können. Na^+-Signale könnten somit als zusätzlicher Faktor in der bidirektionalen Kommunikation zwischen Neuronen und Astrozyten angesehen werden.

Bisherige Untersuchungen konnten zeigen, dass Änderungen der Na^+-Konzentration in Neuronen und Astrozyten durch artifizielle Stimulation hervorgerufen werden können. Ob und in welchem Maße die Aktivität des intrinsischen Netzwerkes Na^+-Fluktuationen hervorbringen kann, ist dagegen bislang unbekannt. Zur Klärung dieser Fragestellung untersuchten wir wiederkehrende, epileptiforme Entladungen in akuten, hippocampalen Hirnschnitten adoleszenter Mäuse. Mittels quantitativer Fluoreszenzmikroskopie des Na^+-sensitiven Farbstoffs SBFI und simultaner Aufzeichnung der lokalen Feldpotentiale konnten wir synchrone, große und langanhaltende Na^+-Transienten in Neuronen und Astrozyten beobachten. Trotz der stetigen Pumpaktivität der Na^+/K^+-ATPase erreichten die Transienten mit einer mittleren Amplitude von rund 7 mM in Neuronen und 3 mM in Astrozyten sowie einer Abfallszeitkonstante von jeweils 18 s bzw. 6 s nennenswerte Dimensionen. Darüber hinaus waren die Zellen trotz der Beanspruchung durch die starke, wiederkehrende Aktivität in der Lage, ihre grundlegende Homöostase zu bewahren. Pharmakologische Blockade der Glutamattransporter dagegen induzierte zunächst die Akkumulation von Na^+ in Neuronen, gefolgt von Zellschwellung und exzitotoxischer Schädigung. Die Hemmung der astroglialen Energiegewinnung führte während wiederkehrender Entladungen zum Anstieg der Na^+-Konzentration in Neuronen und Astrozyten. Zusätzlich war die Dauer der Entladungen sowie der Na^+-Transienten verlängert.

Zusammenfassend zeigen diese Experimente, dass große, langanhaltende Na^+-Transienten während synchroner Aktivierung des intrinsischen Netzwerkes auftreten. Des Weiteren scheinen Neurone zur Aufrechterhaltung ihrer Na^+-Homöostase bei verstärkter Aktivität auf die metabolische Unterstützung durch Astrozyten angewiesen zu sein.

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Introduction and Résumé

1. Glia

Thinking of the central nervous system (CNS) one primarily imagines the intricate network of neurons, which generate the classical electrical signals and communicate among one another mainly via synaptic contacts. Nevertheless, the brain contains a multitude of other cell types of paramount importance for the functionality of the organ.

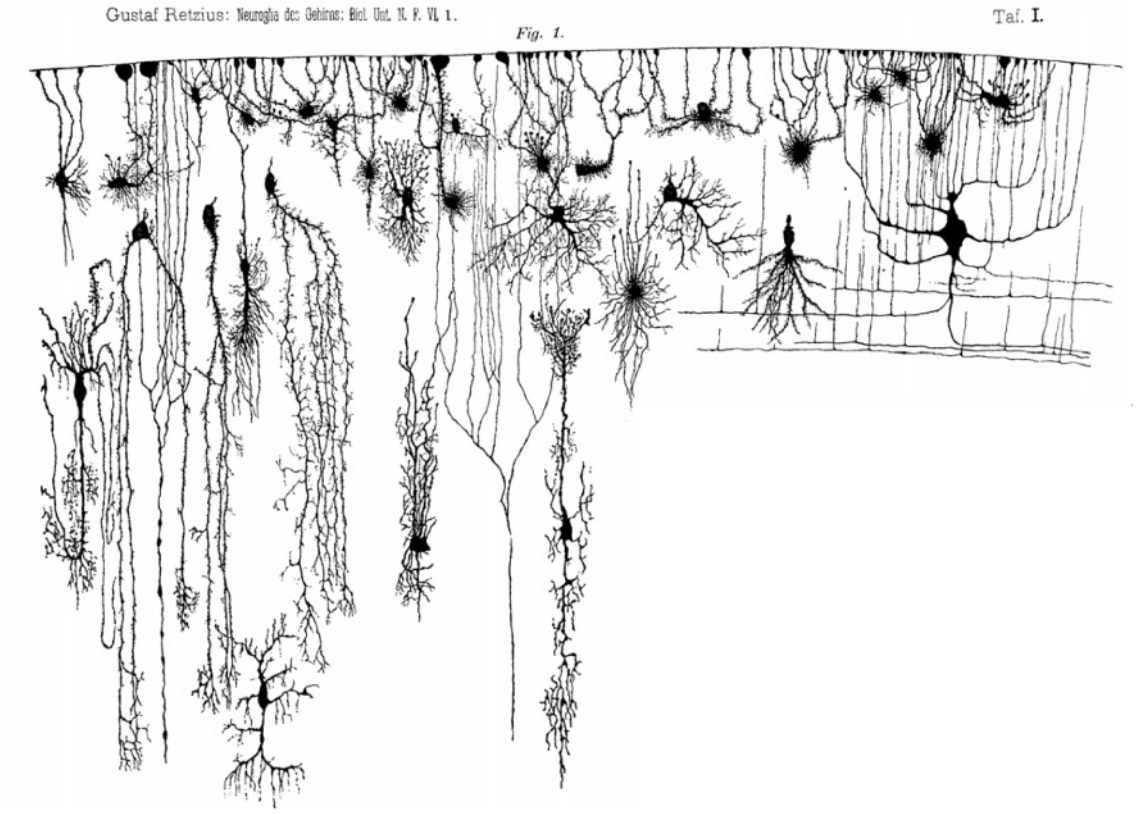

Figure 1 Neuroglial cells of the human cortex. Drawing of a cortical brain slice derived from a 6.5 month old human fetus depicting the morphological diversity of neuroglial cells as seen by Gustav Retzius. (Retzius, 1893)

The diversity of brain cells was already described in the mid- to end-19th century, when intricate analyses of cell morphology resulted in the realisation that, next to neurons, the brain contains various other cells. At that time, theories on cell function were purely speculative and derived from morphological and anatomical observation. Based on his observations, Rudolph Virchow introduced the term glia for non-neuronal brain cells in 1858. He derived the name glia from the Greek word “γλια” for glue and was convinced that glia were basically a cell free connective tissue providing a scaffolding to maintain brain structure (Virchow, 1858). Anatomists,

among them Camillo Golgi, Santiago Ramón y Cajal, Gustav Retzius and many others took advantage of the progress in microscopy and staining techniques, studied brain cell morphology extensively and depicted glial cells in explicit detail (see Fig.1 and 2A) (Oberheim et al., 2012). Today, the cells described back in the 19th century are still collectively referred to as glia. Nevertheless, this group comprises morphologically and functionally distinct cell types of even different developmental origins. They are generally subdivided into macroglia derived from the neuroectodermal lineage and microglial cells which originate from the mesoderm and invade the CNS during embryonic development.

The term **macroglia** includes astrocytes, oligodendrocytes and ependymal cells, and each of these groups again comprises several distinct subtypes. **Astrocytes** are present virtually everywhere in the brain and spinal cord. They are complex, ramified cells which are thought to exert many regulatory functions such as the maintenance of ion and neurotransmitter homeostasis. Additionally, they connect neuronal elements and blood vessels. **Oligodendrocytes**, on the other hand, are also found throughout the whole CNS but their most prominent function is the formation of myelin sheath in the white matter. The insulation of axons by myelin facilitates fast and efficient electrical transmission. **Ependymal cells** are involved in the production of the cerebrospinal fluid and line the ventricular walls as well as the central canal of the spinal cord.

Microglia are the resident phagocytic cells of the CNS. They are primarily concerned with the disposal of necrotic and apoptotic cells as well as clearance of extracellular debris. Yet, these cells are thought to also participate in the pruning of dispensable synapses as well as in the maturation of glutamatergic connections. Thereby, microglia contribute to plastic adaptations of the (developing) brain circuitry (Salter and Beggs, 2014).

Astrocytes are the most diverse type of macroglial cells. Still, they have long been regarded as passive bystanders of neuronal activity that merely provide a scaffold to stabilize tissue architecture. Nowadays, they are being recognized more and more for their active involvement in neurotransmission and information processing (Araque et al., 1999; Haydon, 2001; Volterra and Meldolesi, 2005). Therefore, this class of cells has received a lot of attention and research effort throughout the last decades.

1.1 Astrocytes – morphology and subtypes

Astrocytes have been originally named by Michael von Lenhossek based on their “star-like” morphology formed by several primary processes, which protrude from the cell soma (von Lenhossek, 1893). These processes branch further to form a complex tree of higher order ramifications.

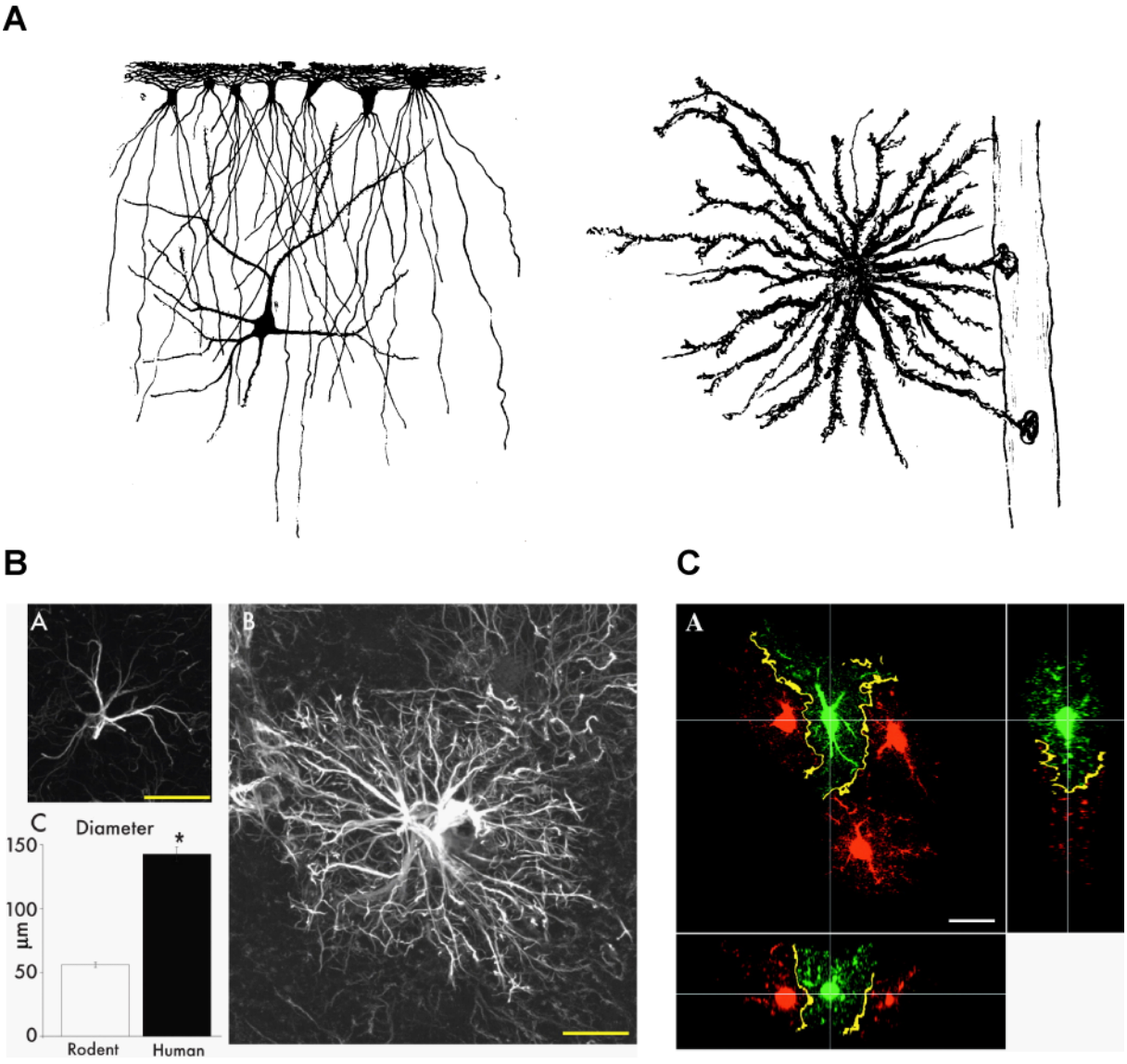

Figure 2 A Drawings of fibrous astrocytes and a pyramidal neuron (left) and of a protoplasmic astrocyte contacting a blood vessel (right) as seen in the human cortex. (Andriezen, 1893) **B** Human protoplasmic astrocytes (B) are larger and more complex than those of rodents (A) as quantified in C. GFAP stained astrocytes, scale bar 20 µm. Reprinted from (Oberheim et al., 2009) with permission from SfN © 2009. **C** Astrocytes occupy separate domains as shown by cells filled with different dyes (shown red and green). Yellow areas label overlapping processes. scale bar 20 µm. Reprinted from (Bushong et al., 2002) with permission from SfN © 2002.

Morphologically, astrocytes have been subdivided into protoplasmic and fibrous astrocytes (see Fig. 2A) (Andriezen, 1893) as well as radial glia. **Radial glial**

cells are mainly found during development in which they act as neural progenitor cells and migration scaffold. They are bipolar cells contacting both the ventricular walls as well as the pial surface. While most radial glia disappear during CNS maturation, the retinal Müller glia as well as Bergmann glia cells of the cerebellum represent two distinct types of radial glial cells that persist in adulthood. In the white matter, astrocytes show several long processes, which are specialized as perinodal, perivascular or subpial endfeet. These cells are termed **fibrous astrocytes**. **Protoplasmic astrocytes**, on the other hand, are the most abundant type of astroglial cells and located in the gray matter. They have a spongiform appearance made up by an elaborate tree of numerous fine processes. Many of these are contacting either blood vessels with perivascular endfeet or synapses as perisynaptic processes. Thereby, they establish a neurovascular unit and the tripartite synapse, respectively. Beyond these main types, several other, regionally specialized astrocyte subtypes have been introduced (Emsley and Macklis, 2006; Oberheim et al., 2012).

The hippocampal CA1 region, as a part of the gray matter, mainly contains protoplasmic astrocytes. Within the last 20 years, these protoplasmic astrocytes have been extensively studied. Each cell occupies a separate domain with only minor overlap of the most distal processes (cf. Fig. 2C) (Bushong et al., 2002). During early postnatal development, immature astrocytes show extensive overlap which regresses from beginning of the third postnatal week onwards, when protoplasmic astrocytes attain their mature, spongiform morphology with clear-cut borders (Bushong et al., 2004). While neuronal morphology and synapse density per volume are thought to be relatively stable throughout phylogeny (DeFelipe et al., 2002), the abundance of astrocytes as well as size and ramification of the cells increases in higher organisms and with brain complexity (Nedergaard et al., 2003; Oberheim et al., 2012). The diameter of human protoplasmic astrocytes has been found to be about threefold larger than in rodents and within this increased territory they extend up to ten times more primary processes (see Fig. 2B) (Oberheim et al., 2006). Within its ovoid territory, a single astrocyte can cover about ~140,000 synapses in the rodent brain (Bushong et al., 2002) and even up to 2 million synaptic sites in humans (Oberheim et al., 2009). Thus, if astrocytes act as local integration units, they receive even more complex inputs and modulate a larger group of target cells and synapses in humans compared to rodents. In addition, astrocyte subtypes have been described which are exclusively found in human brain (Oberheim et al., 2006; Oberheim et al., 2009).

Although each astrocyte occupies a separate domain, the cells are extensively coupled among each other via gap junctions building a functional syncytium. Astrocytes mainly express the connexins Cx43, Cx30 and Cx26, which can assemble to form homo- or heteromeric hemichannels (connexons). Connexons of adjacent astrocytes then combine to form a large diameter (~1.5 nm) gap junction channel, which can be permeated by molecules up to 1 kDa. Several tens to hundreds of these channels cluster to form a gap junction. Moreover, astrocytes also form heterocellular gap junctions contacting neurons or oligodendrocytes. Dye coupling studies revealed the vast extent of astroglial gap junction coupling. Functionally, gap junction coupling allows for the spread of signals including Ca^{2+} ions, the targeted distribution of metabolic substrates (flowing along their concentration gradients) as well as for the redistribution and removal of ions and water (Giaume et al., 2010).

With the advent of immunological techniques, the search for a characteristic astroglial label started, but even today no universal astrocyte marker protein has been identified (Kimelberg, 2010; Oberheim et al., 2012). The intermediate filament glial fibrillary acidic protein (GFAP) represents the classical astrocyte marker. It is still widely used although its expression is downregulated in many mature astrocytes, and GFAP re-expression is only initiated upon induction of gliosis. Many other proteins, predominantly expressed in astrocytes, have been proposed to specifically label these cells. Among these are the enzymes glutamine synthetase (GS) and aldehyde dehydrogenase 1 family member L1 (Aldh1L1), transporters such as the glutamate transporters Glt-1 and GLAST or the Ca^{2+}-binding protein S100β. Currently, Aldh1L1 is thought to label the broadest spectrum of astroglial cells (Cahoy et al., 2008; Yang et al., 2011; Tien et al., 2012), but still, none of the markers is accepted to unequivocally identify all and only astrocytes. Thus, in addition to marker expression, to assign a cell to the group of astrocytes, morphological and physiological criteria have to be applied. These include electrical nonexcitability, a very negative membrane potential as well as the presence of two types of contact sites, one with neuronal membranes and one with the borders of the CNS, e.g. the blood vessels or the ventricular walls (Verkhratsky et al., 1998; Kimelberg, 2010). The problem to find a unifying astrocyte marker highlights the heterogeneity of this group of cells.

2. Astrocytes – functions and interaction

Protoplasmic astrocytes have been described to regulate brain homeostasis in general as well as the local neuronal and synaptic microenvironment. This involves ion and water homeostasis, metabolic status and substrate supply as well as neurotransmitter clearance. Moreover, many studies provide evidence that astrocytes actively participate in neurotransmission and modulate synaptic function and plasticity (Haydon, 2001; Volterra and Meldolesi, 2005; Allaman et al., 2011; Rose and Karus, 2013).

But how can astrocytes perceive ongoing neuronal activity and what mechanisms for regulation, feedback and influence do they have?

2.1 Tripartite synapse

Today, synapses are no longer viewed as contacts exclusively formed between neurons. Instead, synapses are thought to consist of three components, namely the presynaptic terminal, the postsynaptic site and the perisynaptic glial process (see Fig. 3). This so called concept of the **tripartite synapse** was first introduced by Alfonso Araque, Vladimir Parpura and Philip G. Haydon in 1999 to combine the evidence of neuron-glial crosstalk which accumulated throughout the preceding decade (Araque et al., 1999). Since then, the concept has been expanded and refined to describe astroglial involvement in almost all brain functions (Haydon, 2001; Volterra and Meldolesi, 2005; Fiacco and McCarthy, 2006; Theodosis et al., 2008; Deitmer and Rose, 2010; Kimelberg, 2010; Allaman et al., 2011; Oberheim et al., 2012; Rose and Karus, 2013).

The percentage of synapses covered by astroglial perisynaptic processes is highly variable between different brain areas indicating local specialization of neuron-glial interaction (Theodosis et al., 2008). In the cerebellum, Bergmann glia processes cover virtually all synapses (Grosche et al., 2002), while cortical synapses were found to be only partially enwrapped by astroglial membrane sheath. In the adult rat hippocampus (perfusion-fixed) a glial coverage of the axon-spine interface was observed for 60-99% of the larger synapses, while coverage was lower at small, thin spines (Witcher et al., 2007). Thus, glial processes are ideally positioned to perceive and influence synaptic function. For example, synaptic activity, LTP induction and brief exposure to oxygen glucose deprivation (OGD) were shown to induce

alterations of glial morphology and increase synaptic coverage (Lushnikova et al., 2009). Another well described case of the plastic glial coverage derives from experiments in the rat supraoptic nucleus (SON). Here, under normal conditions, 90% of the somata of oxytocin secreting neurons are covered by astroglial processes. During lactation, however, the ensheathment is reduced to about 70% (Theodosis et al., 1986; Theodosis et al., 2008). Moreover, it was shown that astrocytes modulated synaptic transmission of SON neurons via glutamate removal from the extracellular space (ECS) depending on the degree of coverage (Oliet et al., 2001; Theodosis et al., 2008).

Hence, astrocytes are in the position to detect and integrate neuronal activity and to respond by various mechanisms from homeostatic control over morphological rearrangement to active feedback.

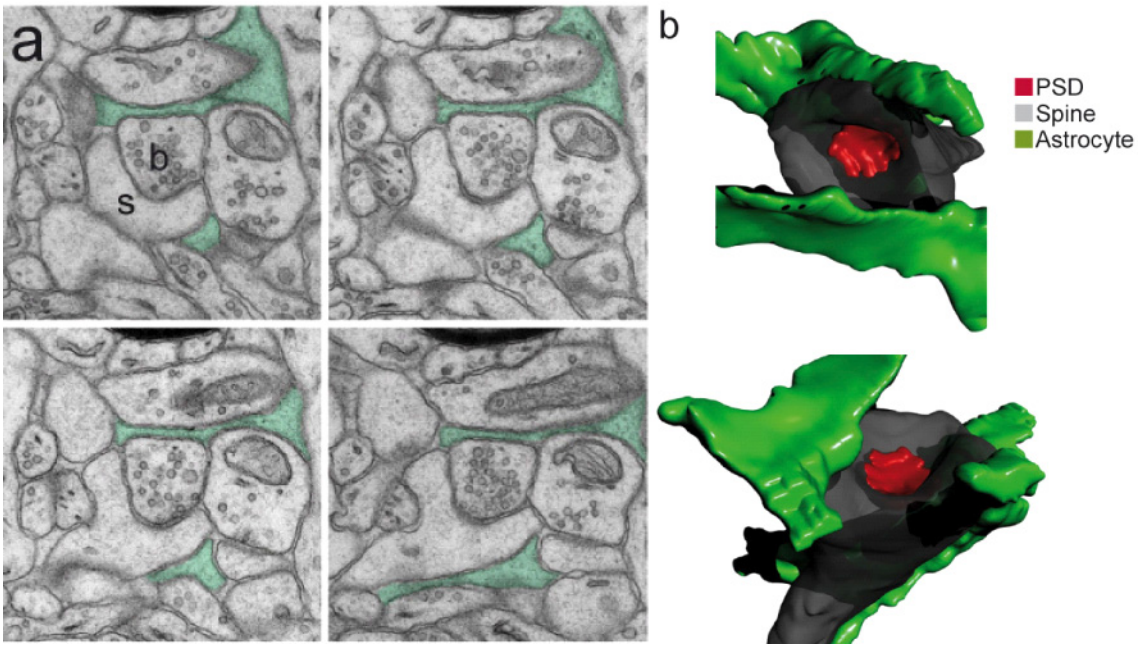

Figure 3 A Serial electron microscopic (EM) images showing an astroglial process (green) contacting and ensheathing a presynaptic terminal (b) and the target dendritic spine (s). Slices prepared from mouse hippocampus. **B** 3D reconstruction of EM images presented in A. The astroglial process (green) surrounds the dendritic spine (gray). The postsynaptic density is highlighted in red. Reprinted with permisison from Macmillan Publishers Ltd: (Pannasch et al., 2014) © 2014.

2.2 Neurotransmitter transporters and receptors

One of the main functions of perisynaptic astroglial processes is the regulation of the synaptic microenvironment. This includes removal of neurotransmitters such as glutamate as well as the uptake and redistribution of K^+ released during neuronal activity (see below) (Kimelberg, 2010). These are critical mechanisms to prevent

hyperexcitable conditions and excitotoxic damage and shape the course of synaptic transmission.

Glutamate is the most important excitatory transmitter in the CNS. Astrocytes remove glutamate from the ECS via high affinity glutamate transporters driven by the transmembrane Na^+ gradient. One molecule of glutamate is cotransported with 3 Na^+ and one H^+ while one K^+ is exported (compare Fig. 4) (Zerangue and Kavanaugh, 1996; Danbolt, 2001). Astrocytes express the glutamate transporters GLAST (human: EAAT1) and Glt-1 (human: EAAT2) with the former one being the predominant type in Bergmann glia cells, while Glt-1 was described as the most abundant isoform in

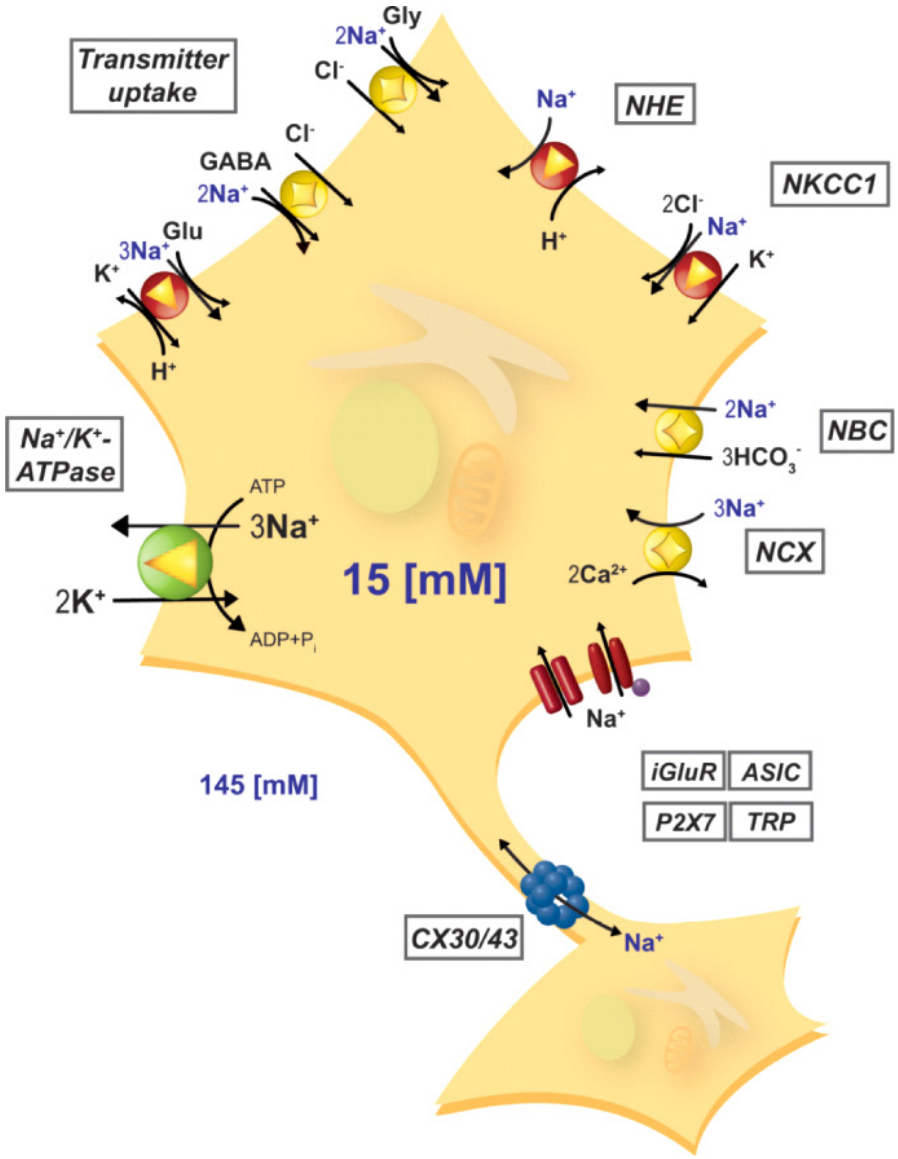

Figure 4 Mechanisms of Na^+ homeostasis and dynamics in astrocytes. Various neurotransmitter and ion transporters depend on the transmembrane Na^+ gradient which is maintained by the Na^+/K^+ ATPase. Astrocytes also express Na^+ permeable ionotropic transmitter receptors. Moreover, astrocytes form a functional syncytium due to gap junction coupling. (see text for abbreviations). Reprinted with permission from John Wiley and Sons: (Rose and Karus, 2013) © 2013 .

mature hippocampal astrocytes (Chaudhry et al., 1995; Lehre et al., 1995; Lehre and Danbolt, 1998). During development, GLAST expression occurs first in the hippocampus, whereas Glt-1 is upregulated only at later developmental stages coincident with synaptic maturation (Schreiner et al., 2014). Neurons also express glutamate transporters (EAAC1; human: EAAT3), but their contribution to overall glutamate removal was estimated as minor (Danbolt, 2001; Schousboe et al., 2004). Glutamate transporter activity was shown to shape synaptic transmission by limitation of receptor activation as well as by assuring synapse independence via restriction of glutamate spillover to adjacent synaptic sites (Tzingounis and Wadiche, 2007).

γ-aminobutyric acid (GABA), the most important inhibitory transmitter in the brain, is also removed from the synaptic cleft via uptake into neurons and astrocytes (see Fig. 4). In contrast to glutamate, direct reuptake into neurons is thought to represent an appreciable percentage GABA removal (Schousboe et al., 2004). Neurons predominately express GABA transporter 1 (GAT1; human: GAT-1), which is also found in cerebellar Bergmann glia cells, while cortical and hippocampal astrocytes mainly express GAT3 (Bak et al., 2006). GABA transporters are electrogenic with a stoichiometry of one GABA molecule being cotransported with two Na^+ and one Cl^- (Scimemi, 2014). In contrast to glutamate transporters, the reversal potential of GABA transporters is close to the resting membrane potential, and cell depolarization as well as increased intracellular Na^+ may result in reverse operation. GABA release from astrocytes by reverse transport was shown to modulate synaptic efficacy and tonic inhibition (Richerson and Wu, 2003; Wu et al., 2007; Unichenko et al., 2013).

Glutamate transporters are not the only way for astrocytes to detect ongoing neuronal communication. Expression of virtually all types of neurotransmitter receptors has been described for astrocytes. Specific cells usually produce those types of receptors which are also present in adjacent neurons. Hence, astrocytes are specialized to match the local type of neurotransmission. Although astrocytes do not show electrical excitability, alterations of the glial membrane potential still change functional components like electrogenic transport processes and the activation of neurotransmitter receptors and result in changes of the intracellular ion concentrations (mainly Na^+ and Ca^{2+}). These changes induce responses within the astroglial syncytium as well as bi-directional communication between astrocytes and

neurons (Lalo et al., 2011). Moreover, astroglial cells can detect most neuromodulators and –hormones (Verkhratsky et al., 1998).

The expression of several types of ionotropic receptors in astrocytes has been described (cf. Fig. 4) (Lalo et al., 2011). Cells, previously categorized as glutamate receptor expressing (GluR) type astrocytes (complex astrocytes), are now distinguished from protoplasmic astrocytes and separately classified as NG2 glia. NG2 cells express fast desensitizing AMPA type glutamate receptors in the hippocampus (Seifert and Steinhäuser, 1995). Moreover, Bergmann glia cells have been found to express Ca^{2+}-permeable AMPA receptors (lacking the GluR2 subunit) (Müller et al., 1992). The presence of NMDA type glutamate receptors (lacking the Mg^{2+} block typical for neuronal NMDA receptors) was proposed in cortical astrocytes, but only weakly indicated in the hippocampus and cerebellum. Additionally, the expression of ATP-responsive purinergic P2X receptors (Lalo et al., 2011) and Cl^--permeable $GABA_A$ receptors was observed (Fraser et al., 1995).

Nevertheless, the majority of neurotransmitter receptor proteins expressed in protoplasmic astrocytes are metabotropic, mostly G protein-coupled receptors (GPCR) relaying to intracellular signaling cascades, which often involve Ca^{2+} signaling. Basically all metabotropic receptors present in neurons have also been observed in astrocytes (Porter and McCarthy, 1997). In hippocampal astrocytes, Ca^{2+} responses to activation of metabotropic glutamatergic (mGluR) (Fellin et al., 2004), GABAergic ($GABA_B$) (Kang et al., 1998), purinergic (P2Y, A_1-A_3), adrenergic and various other receptors have been demonstrated (Agulhon et al., 2008).

Astrocytes were found to not only “listen” but also “talk” back to the synapse via the release of transmitter molecules (Haydon, 2001). Most important among these “gliotransmitters” are glutamate, the NMDA receptor modulator D-serine, ATP and its metabolite adenosine. Different release mechanisms for these transmitters have been proposed including volume-activated anion channels, pore-forming P2X receptors, gap junction hemichannels, reversal of glutamate and GABA transporters as well as Ca^{2+}-dependent exocytosis (Halassa et al., 2007; Agulhon et al., 2008). Still, whether exocytotic release, comparable to that in neurons, really occurs *in vivo* is a matter of intense discussion (Hamilton and Attwell, 2010; Kimelberg, 2010).

Various *in situ* studies indicate a functional impact of gliotransmitter release on synaptic function and network activity (Todd et al., 2006; Perea and Araque, 2010). Glutamate released from astrocytes has been proposed to synchronize neuronal

activity (Angulo et al., 2004; Fellin et al., 2004), increase spontaneous AMPA currents in CA1 neurons (Fiacco and McCarthy, 2004) and enhance presynaptic glutamate release (Perea and Araque, 2007). Moreover, ATP/adenosine release from astrocytes upon $GABA_B$ receptor activation has been involved in heterosynaptic depression (Serrano et al., 2006; Andersson et al., 2007). These and many other modulations imposed by gliotransmission have been described and introduce astrocyte-neuronal communication as an additional level of synaptic plasticity (Halassa et al., 2007).

2.3 Metabolic and neurovascular coupling

Astrocyte endfeet are tightly coupled to brain capillaries. During development, the interaction of astrocytes and vessels is necessary to induce the formation of interendothelial tight junctions to establish the blood brain barrier (BBB). Apart from this developmental effect, perivascular endfeet remain in contact with the vasculature during the whole life.

Astrocytes control cerebral blood flow as a function of local neuronal activity by release of vasoconstrictive or vasodilating agents (see Fig. 5) (Gordon et al., 2007). Moreover, astrocytes are regarded as a kind of distributive system, which takes up metabolic substrates from the blood and provides them upon demand. Additionally, the opposite route, i.e. disposal of waste material and detoxification products, is thought to work via release from astrocytes to the blood stream. Beyond that, ion and water content of the brain is coupled to the controlled exchange with the peripheral environment, although it is tightly regulated and relatively stable towards transient fluctuations in the blood (see below) (Somjen, 2002; Abbott et al., 2006).

Astrocytes, together with their adjacent neurons and blood vessels are viewed as neurovascular units, interacting to coordinate blood flow and BBB functions in accordance with network activity (Leybaert et al., 2007). Astrocytes sense neuronal activity via the activation of neurotransmitter receptors as well as by transmitter uptake. Intracellular signaling cascades are initiated which finally translate neuronal activation into a haemodynamic (see Fig. 5) and metabolic response (cf. Fig. 6). On the one hand, neuronal transmission is thought to increase the blood flow through arterioles in active brain regions to provide sufficient oxygen. Upon induction of Ca^{2+} signals by glutamatergic neuronal activity, astrocytes have been shown to release vasoactive agents produced from arachidonic acid (AA) (e.g. vasodilating

prostaglandin E2 (PGE_2) or release AA itself, transformed to vasoconstricting 20-hydroxyeico-sateraenoic acid (20-HETE)) in smooth muscle cells (Zonta et al., 2003; Metea and Newman, 2006; Koehler et al., 2009). Which type of response is initiated, critically depends on the vascular tone set by the level of NO (Gordon et al., 2007).

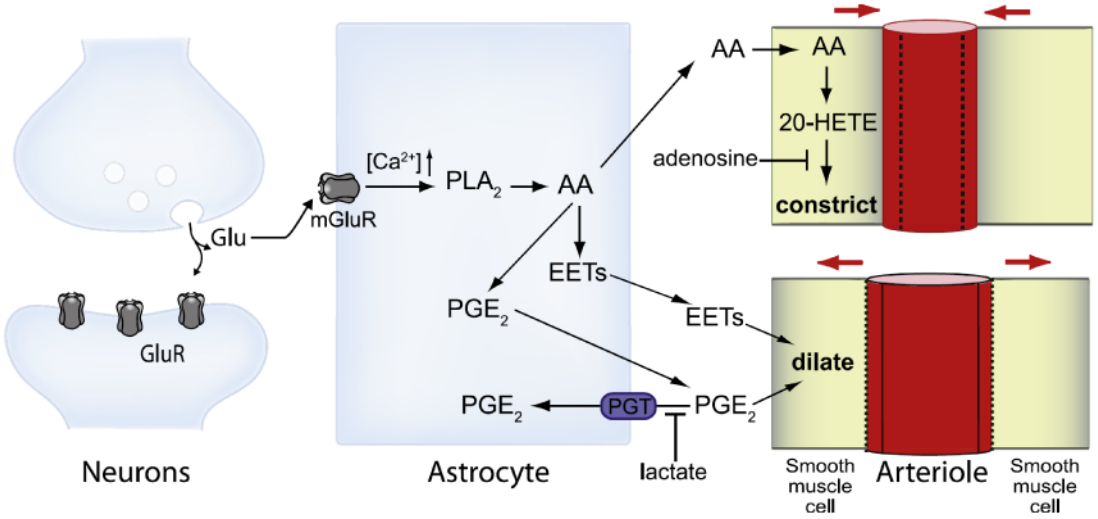

Figure 5 Astrocytes couple neuronal activity to the blood flow by the release of vasoconstricting or vasodilating agents in response to Ca^{2+} signals. (see text for abbreviations). Reprinted from (Belanger et al., 2011) with permission from Elsevier © 2011.

On the other hand, supply with glucose, the major metabolic substrate in the brain, has been linked to regulation by astrocytes. Under resting conditions, the glucose transporter 1 (GLUT-1) in vascular endothelial cells is not fully activated but still imports sufficient amounts of glucose to meet the metabolic needs of the tissue. During activity, the energy demand increases massively and additional glucose uptake into the brain is initiated. This is achieved by membrane incorporation of additional GLUT-1 molecules from intracellular pools as well as by stimulation of transport activity via astrocyte derived signals, for instance angiotensin II or glutamate (Leybaert et al., 2007). Although neurons themselves express glucose transporters (GLUT-3), astrocytes are thought to take up most of the glucose transported through the BBB. They express GLUT-1, which is clustered in the perivascular membrane and therefore closely associated with blood vessels.

Glucose utilization in the brain is thought to be compartmentalized. Glycogen is stored almost exclusively in astrocytes which are also thought to be the primary site of glycolysis, while oxidative metabolism is predominantly localized in neurons. Hence, neurons seem to rely on the cooperation with astrocytes to access metabolic substrates. This has been first put forward by Luc Pellerin and Pierre Magistretti as

the “astrocyte neuron lactate shuttle” hypothesis (Pellerin and Magistretti, 1994). In astrocytes, glucose is either stored as glycogen or broken down to pyruvate by glycolysis. Pyruvate can enter the tricarboxylic acid (TCA) cycle for oxidation or is converted to lactate by the enzyme lactate dehydrogenase (LDH). While astrocytes express LDH5, which preferentially produces lactate, neurons contain LDH1, which rather converts lactate to pyruvate (Bittar et al., 1996). Lactate is transported across the cell membrane via the monocarboxylate transporter system (MCT4 in astrocytes and MCT2 in neurons). Via this route, astrocytes were proposed to support neuronal energy production. For example, an experiment in living rats showed that a rescue of memory formation by lactate supplementation was only possible upon knockdown of the astroglial MCT4 but not upon knockdown of the neuronal MCT2. This result emphasizes the importance of lactate supply to neurons for physiological brain function *in vivo* (Suzuki et al., 2011).

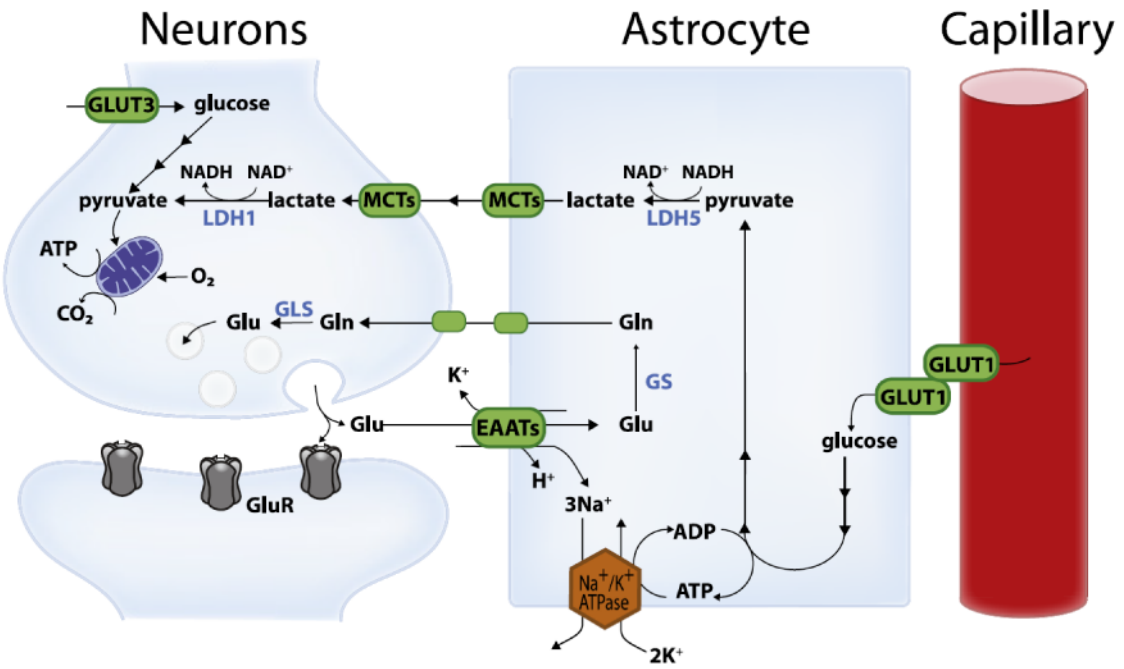

Figure 6 Astrocyte can provide lactate to neurons as a metabolic substrate. Astroglial glucose uptake and glycolysis are stimulate by neuronal activity. Reprinted from (Belanger et al., 2011) with permission from Elsevier © 2011.

Astroglial glucose uptake and glycolysis are thought to be stimulated by glutamatergic neurotransmission. Glutamate uptake into astrocytes increases the intracellular Na^+ concentration ($[Na^+]_i$) and thereby activates ATP consuming activity of the Na^+/K^+ATPase (NKA). Low ATP in turn augments the level of glucose uptake and glycolysis resulting in enhanced release of lactate to the ECS (Chatton et al., 2000). Glucose uptake was shown to be stimulated by glutamate in astrocytes, while

neuronal glucose import was inhibited upon glutamate exposure (Loaiza et al., 2003; Porras et al., 2004; Porras et al., 2008). Furthermore, it was shown, that glucose withdrawal suppressed stimulated fEPSPs as well as epileptiform activity in acute hippocampal slices. Intracellular delivery of glucose or lactate into an astrocyte rescued both types of activity as long as gap junction coupling functionally connected single cells to form a syncytium. The extent of astroglial coupling is regulated by network activity, and increased activity results in an enlarged network of functionally connected astrocytes. This shows the relevance of intercellular coupling for substrate supply from astrocytes to neurons (Rouach et al., 2008).

Next to the lactate shuttle, many other substrate cycles have been introduced as connectors of neuronal and glial metabolism. Among them is the “glutamate-glutamine cycle”. Astrocytes rapidly remove glutamate from the ECS, thereby shaping neurotransmission and preventing glutamate induced excitotoxicity (Schousboe and Waagepetersen, 2005). After the uptake into astrocytes, glutamate is converted to the inactive glutamine by the enzyme glutamine synthetase (GS), which is exclusively expressed in astrocytes in the CNS (Norenberg, 1979). Glutamine is then returned to neurons to be transformed back into glutamate or as a substrate to produce GABA. Both transmitters cannot be synthesized *de novo* in neurons (Bak et al., 2006). Hence, the “glutamate-glutamine shuttle” is of paramount importance to replenish the neuronal transmitter pool.

These findings emphasize the direct, metabolic connection of neuronal activity and glial function and highlight the important role of their interaction for maintenance and modulation of brain function.

3. Ion homeostasis and dynamics in astrocytes and neurons

In the brain, as in virtually all tissues, cells maintain steep ion gradients across their plasma membrane. Extracellularly, concentrations of Na^+, Ca^{2+} and Cl^- are high as compared to the cytoplasm, while the opposite is true for K^+ and organic anions. These gradients, in combination with the ion permeability of the membrane, set the membrane potential of cells. Neurons typically show a resting membrane potential of -60 to -70 mV, while classical passive astrocytes present with a more negative membrane potential of -80 to -90 mV. The astroglial membrane is almost exclusively but highly permeable for K^+ and thus the membrane potential is close to the K^+ equilibrium potential. Drastic changes of the membrane potential caused by transient

alterations in ion permeability and subsequent ion fluxes are a characteristic feature of electrically excitable neurons. Via the opening of ligand-gated ion channels, neurons generate synaptic potentials, which spread electrotonically. Additionally, activation of voltage-gated ion channels produces regenerative, self-sustaining action potentials.

As introduced above, ion gradients and membrane potential do not only serve electrical signaling. They are also the prerequisite for other cell functions such as transmembrane substrate transport, energy production as well as the regulation of cell volume and pH. Moreover, the steep inward gradient for Ca^{2+} enables this cation to act as a potent second messenger. All ions are subject to diverse transport processes and, moreover, there are multiple types of channels through which ions may pass the membrane. Hence, there is a steady leakage of ions which cells need to counteract to maintain the gradients. As a consequence, a lot of energy is consumed by ion pumps. Most important are the NKA, the plasma membrane Ca^{2+} ATPase (PMCA) and the sarcoendoplasmic Ca^{2+} ATPase (SERCA) (Alberts, 2009).

Thus, to maintain cell function and viability, ion gradients are strictly controlled by homeostatic mechanisms. Nevertheless, these homeostatic processes are interdependent and modulated by the cell and tissue status. Hence, the regulatory machinery also serves adaptive functions and, therefore, homeostatic responses may also be interpreted as signals.

3.1 K^+ regulation and clearance

The K^+ gradient is one of the main determinants of the neuronal resting membrane potential and the extracellular baseline concentration of K^+ ($[K^+]_o$) in the brain is maintained at a level of about 3 mM. During neuronal activity, K^+ enters the ECS via ionotropic transmitter receptors as well as through voltage-gated ion channels. Even moderate activity causes measurable $[K^+]_o$ transients (Sykova et al., 1974; Connors et al., 1979) and stronger stimulation can induce changes by several mM up to a “ceiling level” of about 8-12 mM (Heinemann and Lux, 1977; Somjen, 2002). Under pathological circumstances such as spreading depression or ischemia, $[K^+]_o$ may reach even higher values when regulatory mechanisms are overwhelmed (Somjen, 2002). The K^+ gradient mainly defines the neuronal membrane potential and elevated extracellular concentrations result in a depolarization of neurons. This depolarization can influence the efficacy of synaptic and action potential generation

(Kofuji and Newman, 2009). Increased $[K^+]_o$ alone can be sufficient to induce neuronal hyperactivity and cause spontaneous, epileptiform discharges (Traynelis and Dingledine, 1988) or even render neurons inexcitable via depolarization block. Hence, rapid restoring of $[K^+]_o$ to its baseline level is of utmost importance for proper neuronal activity. Recovery of $[K^+]_o$ is achieved via passive, spatial buffering as well as net uptake and active clearance by astrocytes (cf. Fig. 7) (Walz, 2000).

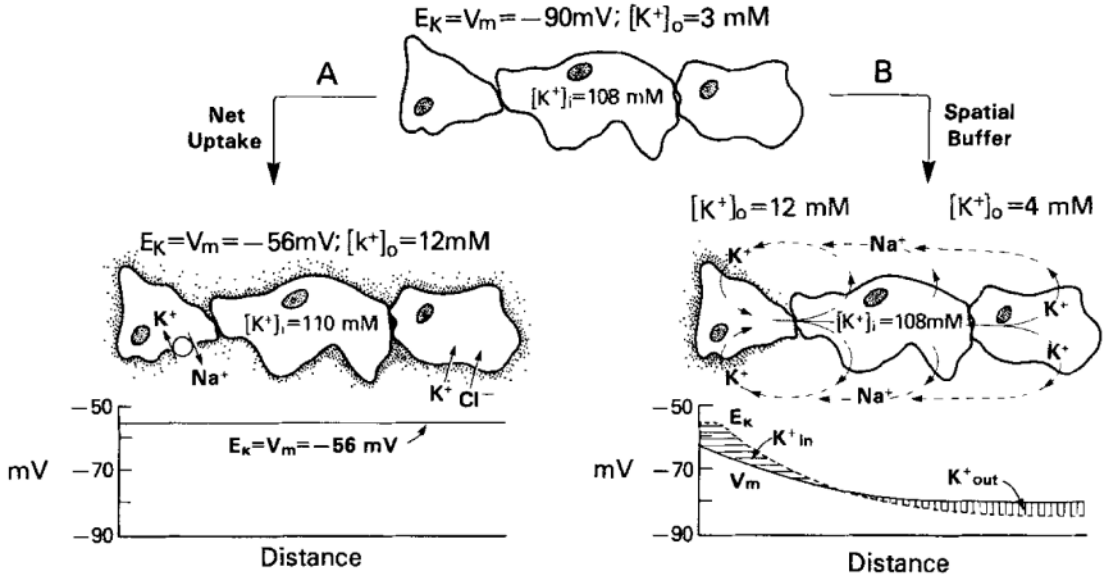

Figure 7 K^+ clearance from the ECS by astrocytes can occur via net uptake into the cells (A) and spatial buffering (B). While spatial buffering requires a localized $[K^+]_o$ increase and membrane potential gradient for spatial redistribution of ions without increase of $[K^+]_i$, net uptake operates at more global $[K^+]_o$ increases via NKA activity and KCl uptake causing $[K^+]_i$ rise. Reprinted with permission from John Wiley and Sons: (Orkand, 1986) © 2006.

Astrocyte membranes are rich in K^+ channels. The term spatial buffering describes the influx of K^+ via these channels at the site of K^+ increase, its redistribution within the glial syncytium and release at distant sites, where a lower $[K^+]_o$ is present. Among others, astroglial cells express the inwardly rectifying K_{ir} channels (mainly $K_{ir}4.1$), which favor inward movement of K^+ and show a higher permeability at increased $[K^+]_o$ levels. Additionally, due to gap junction coupling, the cells form a large syncytium in which K^+ can spread. Hence, astrocytes are well equipped for spatial buffering, which is uptake of K^+ at the site of activity and release at a distant, inactive location. Nevertheless, this mechanisms is only functional for locally restricted $[K^+]_o$ transients, because it is driven by K^+ gradients and differences in astrocyte membrane potential and cannot operate during widespread, uniform $[K^+]_o$ elevations (Traynelis and Dingledine, 1988; Walz, 2000; Kimelberg, 2010). In addition

to the gradient-dependent redistribution, net uptake of excess K^+ via K^+ channels and active pump activity of the NKA can recover $[K^+]_o$ to baseline levels. This net uptake is always accompanied by a compensatory influx of water resulting in glial cell swelling (Dietzel et al., 1980). Then, after $[K^+]_o$ and neuronal activity have reached resting levels, K^+ is released and returned to the neurons again. Furthermore, it was suggested that the $Na^+/K^+/Cl^-$ cotransporter 1 (NKCC1) may be activated by K^+ increases above 10 mM and add to the removal of ions from the ECS (Hertz et al., 2013).

Thus, astrocytes define the neuronal environment and therefore also neuronal properties by the regulation of K^+ homeostasis. These homeostatic processes do not only set the baseline $[K^+]_o$ but also impact neurotransmission, cell volume regulation and transporter function.

3.2 Water homeostasis and cell volume regulation

As pointed out for the example of K^+ above, considerable net ion fluxes are accompanied by water movement to maintain isoosmolarity. Likewise, fluctuations of the intra- or extracellular osmolarity alter the transmembrane driving forces for ions and result in the redistribution of ions and water. Movement of water into or out of the cytoplasm changes the volume of cells and ECS. Shrinkage of the ECS reduces the distance between neuronal membranes and facilitates nonsynaptic, ephaptic transmission via local electrical fields. Ephaptic transmission was proposed as a mechanism to synchronize neuronal firing (Anastassiou et al., 2011). Moreover, the efficacy of synaptic transmission is thought to be enhanced by ECS shrinkage due to increased transmitter concentration and reduced spillover from the synapse.

Cells are endowed with an elaborate machinery to regulate their volume (see Fig. 8). Astrocytes express Aquaporin 4 (Aqp4), a water permeable transmembrane pore which allows for rapid transfer of water (Solenov et al., 2004). Aqp4, together with $K_{ir}4.1$, is distributed in a polarized fashion within the cell. Some channels face the neuropil but the majority is clustered in perivascular endfeet. Thereby, Aqp4 enables water exchange with the blood (Nagelhus et al., 2004). Net accumulation of ions in astrocytes during neuronal activity due to K^+ removal and Na^+- dependent glutamate uptake draws water into the cells and results in an increased volume. This swelling subsequently dissipates via regulatory volume decrease (RVD). RVD basically depends on the activation of solute export systems including K^+ and Cl^-

channels and volume regulated anion channels (VRAC). The release of ions is then followed by water. RVD is further supported by the spread and dilution of locally accumulated water and ions in the glial syncytium. The opposite effect, the regulatory volume increase (RVI), is facilitated by solute uptake e.g. via NKCC1, NHE and NBC with the respective flow of water (Kahle et al., 2009). Thus, cell volume regulation and water homeostasis depend on the maintenance of ion gradients and, accordingly, on the ATP-consuming activity of ion pumps. Accordingly, pathological situations that comprise energy production, e.g. ischemia, result in cell swelling and brain edema (Chen and Sun, 2005; Kahle et al., 2009).

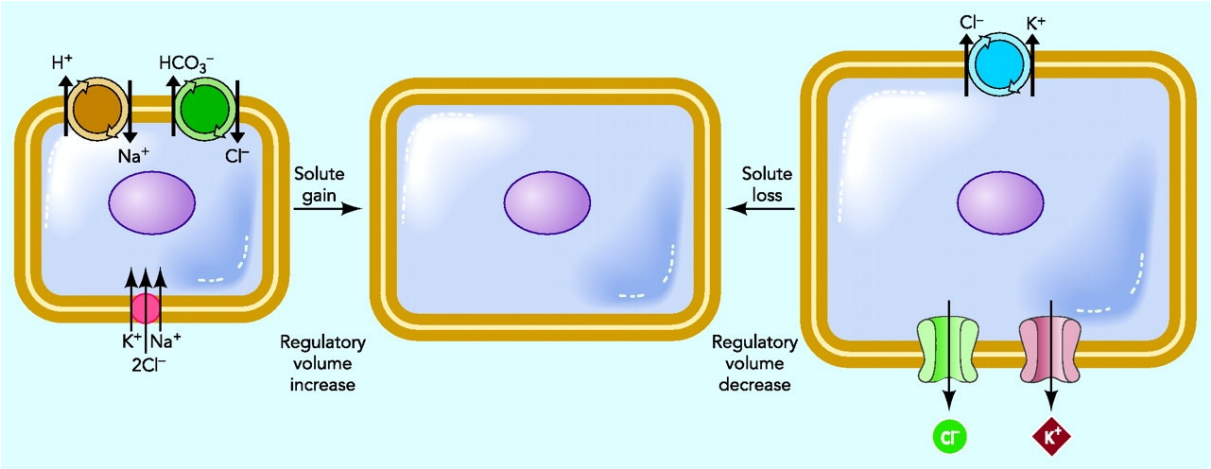

Figure 8 Mechanisms of cell volume regulation. Regulatory volume increase is obtained by cytoplasmic gain of solutes and subsequent passive influx of water to maintain isoosmolarity. The regulatory volume decrease employs the opposite mechanism which is the export or loss of solutes and concomittant release of water molecules. Reprinted from (Kahle et al., 2009) © 2009.

The water homeostasis of the brain as a whole is defined by the action of several neuropeptides that also act on and via astroglial cells. Vasopressin increases glial water permeability and whole brain water content while atriopeptin, produced by astrocytes, counteracts this effect. Additionally, the renin-angiotensin system is involved in water and blood pressure regulation. Astrocytes are the main source of angiotensinogen in the brain, which is further processed to angiotensin II (Simard and Nedergaard, 2004).

3.3 Regulation of intra- and extracellular pH

Maintenance of a physiological pH is a prerequisite for the normal function of proteins including enzymes and ion channels. The pH of the ECS (pH_o: 7.1 - 7.3) and that of the cytoplasm (pH_i: 7.0 - 7.4) are strictly controlled and regulated interdependently. Free intra- and extracellular H^+ concentrations are similar (40-

100nM) but, due to the negative membrane potential of neural cells, this results in an inwardly directed driving force for H^+. This force is used to drive transport processes whose operation may result in intracellular acidification (Deitmer and Rose, 2010).

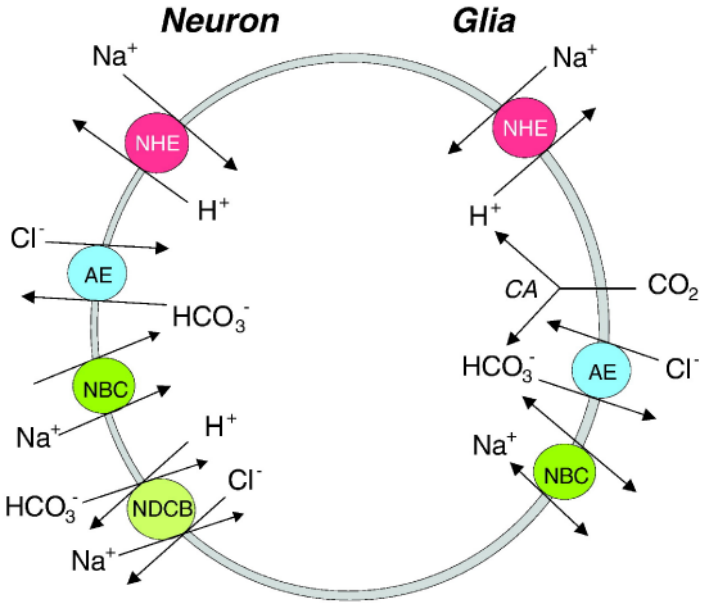

Figure 9 Neuronal and glial pH are regulated by transmembrane transport of H^+ and HCO_3^- as well as by the acitivity of the carboanhydrase in astrocytes. See text for abbreviations (AE – Cl^-/HCO_3^- anion exchanger; NDBC – Na+-dependent Cl^-/HCO_3^- exchanger) Reprinted from (Deitmer and Rose, 2010) with permission from Elsevier © 2010.

In vesicles, ATP-consuming pumps accumulate H^+ and establish a gradient that drives the import of molecules e.g. glutamate via the H^+-dependent vesicular glutamate transporter (VGLUT). Thus, the intravesicular pH is low and transmitter exocytosis causes acidification of the ECS due to released protons. Moreover, acid is constantly produced in metabolically active cells by production of CO_2, which is equilibrate with HCO_3^- and H^+ as part of the physiological tissue pH buffering system. This reversible reaction is catalysed by the enzyme carboanhydrase (CA). CA is primarily expressed in glial cells in the CNS and especially important during phases of high neuronal activity and energy demand (Deitmer, 2002). The control of pH depends on transmembrane transport processes mainly driven by the sodium gradient (see Fig. 4 and 9). Acid is continuously produced by metabolic reactions and extruded from the cells by the Na^+/H^+ exchanger (NHE) as well as the Na^+ bicarbonate cotransporter (NBC). The NBC has a transport stoichiometry of 1 Na^+: 2 HCO_3^-.

Therefore, NBC transport is electrogenic. Its activity is modulated by the membrane potential and can even reverse transport direction (Chesler, 2003;

Deitmer and Rose, 2010). Accordingly, when astrocytes are depolarized by extracellular K^+ increases and Na^+-dependent glutamate uptake during neuronal activity, NBC activity is enhanced and astrocytes are alkalinized (Chesler and Kraig, 1989; Deitmer and Rose, 2010). The uptake of HCO_3^- into the cytoplasm adds to the cellular buffer capacity and supports the activity of transport processes depending on the H^+ gradient such as glutamate uptake (Danbolt, 2001) and operation of Ca^{2+} pumps (Clapham, 2007). pH responses to neuronal activity are quite complex, involve various processes and are often multiphasic (Deitmer and Rose, 1996, 2010).

Beyond homeostatic regulation, pH fluctuations accompanying neuronal activity were also proposed to have signaling function (Deitmer and Rose, 1996). NMDA receptors are blocked by H^+ and gating or inactivation properties of ion channels were found to be modulated by pH (Deitmer and Rose, 2010). The neuronal acid sensing ion channel 1a (ASIC1a), which is predominantly expressed in inhibitory interneurons, was shown to be activated by extracellular acidification and implicated in the termination of epileptic seizures (Ziemann et al., 2008). Moreover, transfer of metabolic substrates such as lactate or pyruvate from astrocytes to neurons via MCTs is linked to the electroneutral cotransport with protons. Hence, among other effects, pH alterations were proposed to serve as signal to coordinate energetic support and to provide feedback on neuronal activity and excitability (Deitmer and Rose, 1996; Deitmer, 2002; Deitmer and Rose, 2010).

3.4 Calcium regulation and signalling

Ca^{2+} is ubiquitously used as a signaling molecule in all cells. It operates as one of the most important second messengers and is involved in many signaling cascades. Ca^{2+} binds to and modulates the conformation and activity of various proteins including enzymes, ion channels and transcription factors. Some of these interactions result in immediate effects such as the induction of Ca^{2+}-dependent exocytosis or muscle contraction while others operate on a long term scale e.g. by induction or suppression of gene expression (Alberts, 2009).

While the extracellular brain milieu contains about 1-2 mM Ca^{2+}, the free cytoplasmic Ca^{2+} concentration is maintained at a very low level of less than 100 nM. Low $[Ca^{2+}]_i$ is stabilized by the ATP-consuming activity of PMCA as well as by NCX and its K^+-dependent version (NCKX), which rely on the transmembrane Na^+ gradient as a driving force (see Fig. 4 and 10). In addition to export to the ECS, Ca^{2+} is

accumulated intracellularly in organelles, most importantly in the endoplasmic reticulum. Here, SERCA concentrates Ca^{2+} ions to up to a concentration of 0.1-1 mM. Via a Ca^{2+} uniporter, Ca^{2+} can also enter mitochondria during intracellular Ca^{2+} elevations and serve as a signal to stimulate ATP synthesis (Clapham, 2007). Due to the high abundance of Ca^{2+} binding proteins in the cytoplasm, the Ca^{2+} concentration is highly buffered and Ca^{2+} signals can be restricted to subcellular microdomains (cf. Fig. 10) (Rizzuto and Pozzan, 2006; Shigetomi et al., 2010).

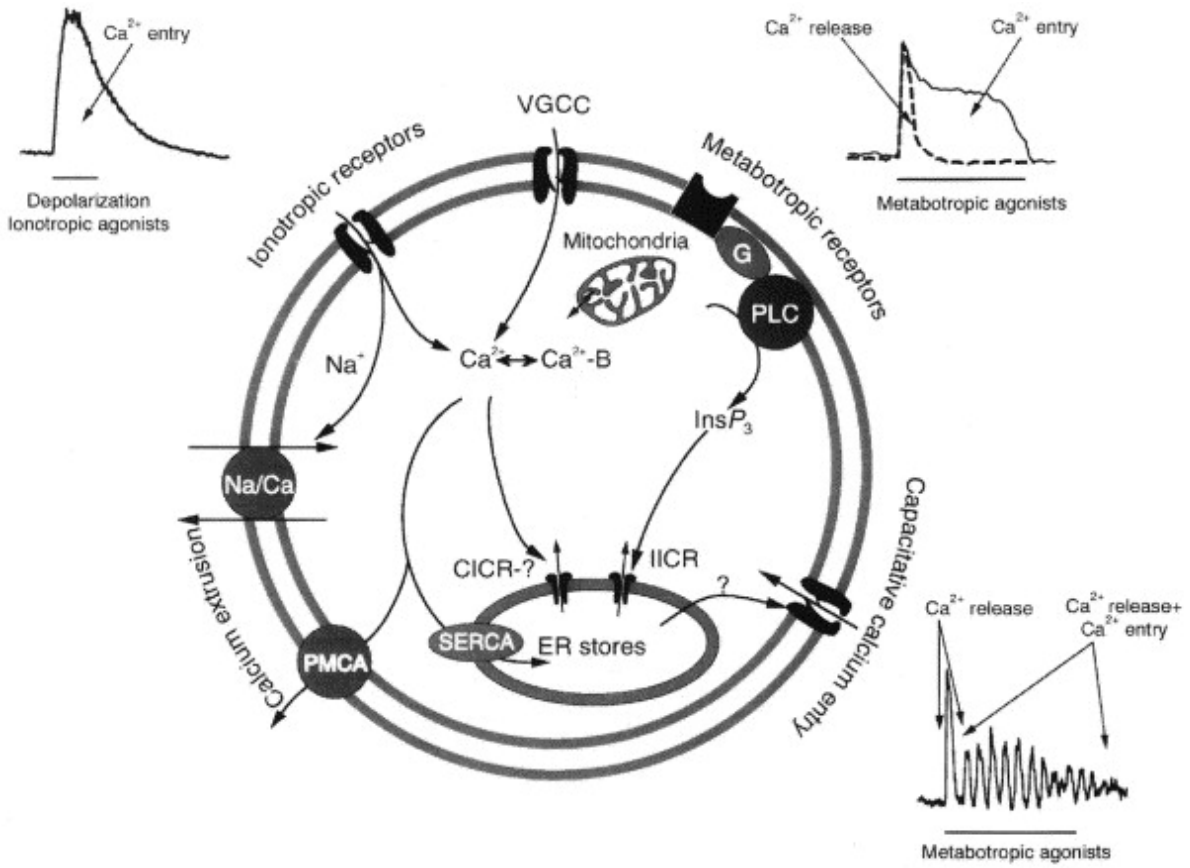

Figure 10 Ca^{2+} signals and regulation. The course of an intracellular Ca^{2+} signal depends on the trigger and the activated mechanisms of Ca^{2+} increase and subsequent removal. Ca^{2+} signals can be single sharp peaks, prolonged plateaus as well as oscillating Ca^{2+} transients. See text for abbreviations (Ca^{2+}-B – Ca2+ buffer; IICR – IP_3-induced Ca^{2+} release; CICR – Ca^{2+}-induced Ca^{2+} release; G – G-protein). Reprinted from (Verkhratsky and Kettenmann, 1996) with permission from Elsevier © 1996.

Although neurons are associated with electrical signaling in the first place, Ca^{2+} signals are known to serve very important and diverse functions in neuronal information processing. Among them are, on the one hand, short term effects such as the induction of neurotransmitter release from the presynaptic site. Voltage-gated Ca^{2+} channels open due to membrane depolarization and Ca^{2+} binding to synaptotagmin which subsequently activates vesicular exocytosis. On the other hand,

Ca^{2+} influx via NMDA receptors and somatic Ca^{2+} channels is responsible for the induction of synaptic plasticity in terms of long term potentiation and depression (LTP and LTD) (Nicholls et al., 2012).

For astrocytes, in contrast to neurons, Ca^{2+} transients have been postulated as the primary route of excitability, information processing and signal transmission (Verkhratsky et al., 1998). The spectrum of ligands, which induce Ca^{2+} signals in astrocytes, is very broad including the classical neurotransmitters, chemokines, ATP, hormones and many others (Verkhratsky et al., 1998; Halassa et al., 2007; Agulhon et al., 2008; Perea and Araque, 2010). Most of the astroglial transmitter receptors trigger Ca^{2+} signals in the perisynaptic astrocyte process upon detection of neurotransmission. Glutamatergic transmission and concomitant glial Ca^{2+} signaling have been extensively studied in the rodent hippocampus. Here, protoplasmic astrocytes express group I mGluRs, which, upon glutamate binding, activate the phospholipase C (PLC). PLC liberates inositol-1,4,5-trisphosphate (IP_3) from phosphatidylinositol bisphosphate (PIP_2). IP_3 then binds to and activates the IP_3 receptor in the ER membrane and induces Ca^{2+} release. In addition to Ca^{2+} release from internal stores, which is the main source of $[Ca^{2+}]_i$ elevations in astrocytes, Ca^{2+} entry into the cytoplasm may also arise from other sources. Voltage-gated Ca^{2+} channels (VGCC) as well as unspecific cation channels or the reversal of electrogenic transporters (e.g. NCX) can cause Ca^{2+} entry from the ECS. Above this, Ca^{2+} signals may even potentiate themselves via mechanisms such as store operated Ca^{2+} release and Ca^{2+}-activated Ca^{2+} channels. Thus, astrocytes respond to neuronal activity with $[Ca^{2+}]_i$ changes, and the type of the triggered Ca^{2+} signal can be used to discriminate different input pathways (Perea and Araque, 2005). Ca^{2+} signals can be restricted to subcellular microdomains or spread to the soma depending on the intensity of the stimulus (Pasti et al., 1997; Verkhratsky et al., 1998; Honsek et al., 2012). Moreover, signals can also extend further within the glial syncytium in the form of regenerative Ca^{2+} waves (Cornell-Bell et al., 1990), which depend on both Ca^{2+} diffusion via gap junctions as well as activation of metabotropic P2Y receptors by paracrine ATP signaling. In addition to evoked Ca^{2+} transients, spontaneous oscillations, which persist during a block of metabotropic signaling, have been observed in hippocampal astrocytes (Aguado et al., 2002; Nett et al., 2002).

As introduced before, astrocytes do not only detect neuronal activity but also release signaling molecules themselves and provide feedback to neurons. Ca^{2+} elevations in astrocytes have been described to elicit slow inward currents (SICs) in neurons mediated by extrasynaptic NMDA receptor activation (Fellin et al., 2004; Tian et al., 2005). Ca^{2+} signal-dependent release of the NMDA receptor coagonist D-serine from astrocytes was even described as a prerequisite for LTP induction in the mouse hippocampus (Henneberger et al., 2010). Furthermore, exocytotic glutamate release from astrocytes was proposed to be involved in network excitability as indicated by a lower seizure probability in mice expressing a dominant negative SNARE domain in glia (Clasadonte et al., 2013). Astroglial detection of neuronal activity and the subsequent glial Ca^{2+} signaling directly couple activity to the regulation of cerebral blood flow. Ca^{2+} signals propagate to the perivascular endfeet and induce vasoconstriction or –dilation through the release of vasoactive compounds (Fiacco and McCarthy, 2006). Furthermore, it was shown, that rapid arteriole dilation during ictal epileptiform discharges critically depends on the propagation of astroglial Ca^{2+} signals to perivascular endfeet (Gomez-Gonzalo et al., 2011).

Ca^{2+} changes are not only acting as adaptive signals but they can also initiate programmed cell death, and Ca^{2+} dysregulation may permanently damage cells. This is a well explored phenomenon in neurons, which undergo excitotoxic cell death upon overstimulation by glutamate and intracellular Ca^{2+} overload (Wang and Qin, 2010). Astrocytes, in contrast, are thought to be more resistant to damage induced by prolonged presence of high glutamate concentrations (Verkhratsky et al., 1998). Nonetheless, astrocytes do show alterations such as process swelling in response to intracellular Ca^{2+} accumulation (Finkbeiner, 1993).

Ca^{2+} signaling is involved in almost all functions of cells. It provides a mechanism for local as well as widespread signal processing in astrocytes and, moreover, operates as trigger for homeostatic as well as active feedback reactions. The regulation of Ca^{2+} homeostasis is important for the physiological integrity of the cells. It is tightly connected to the cellular metabolism and $[Ca^{2+}]_i$ is regulated by the ATP-consuming activity of pumps as well as transporters driven by the transmembrane sodium gradient.

3.5 Sodium regulation and signaling

The extracellular Na^+ concentration of about 140 mM in the brain is much higher than the $[Na^+]_i$ in both neurons and astrocytes, being about 12-15 mM and 10-13 mM, respectively (Langer and Rose, 2009; Kelly and Rose, 2010). The cells maintain this steep gradient via the NKA, which moves 2 K^+ into and 3 Na^+ out of the cell driven by the hydrolysis of 1 molecule ATP. This action is electrogenic, moving more positive charges to the ECS. Thereby, the NKA adds to the negative membrane potential. The NKA is constantly active in astrocytes (Rose and Ransom, 1996a) and considered to be one of the major energy consumers in cells. In unstimulated, cultured astrocytes, NKA was described to consume about 20% of the ATP content and a block of its activity with ouabain resulted in the dissipation of both Na^+ and K^+ gradient and loss of membrane potential (Silver and Erecinska, 1997). Moreover, during energy deprivation in simulated ischemia, Na^+ concentration in astrocytes also massively increased (Rose et al., 1998). To maintain a low internal Na^+ level, the ion is not only steadily extruded to the ECS, but also equilibrated via gap junctions within the glial syncytium (Rose and Ransom, 1997; Langer et al., 2012). Nevertheless, local transient fluctuations still occur and even spread within and between astrocytes (Bennay et al., 2008; Langer and Rose, 2009; Langer et al., 2012).

During network activity, considerable additional sodium fluxes occur, which are counteracted by enhanced NKA pump activity. Accordingly, energy consumption and demand are increased and thereby couple neuronal activity to energy metabolism (Pellerin and Magistretti, 1997; Pellerin et al., 2007). Intracellular sodium transients and cell acidification in astrocytes were proposed to be transferred into mitochondria and serve as a signal to lower mitochondrial activity, which would favor lactate transfer to neurons (Bernardinelli et al., 2006; Azarias et al., 2011). In addition, Na^+-dependent glutamate uptake was shown to increase the uptake of glucose into astrocytes (Voutsinos-Porche et al., 2003; Bernardinelli et al., 2004). Another observation, supporting the involvement of Na^+ signals in metabolic coupling, is the facilitation of glutamine release following Na^+ increases in astrocytes. This glutamine may replenish neuronal transmitter pools (Broer et al., 2002).

Energy stored in the transmembrane Na^+ gradient drives many secondary active transport processes, which control substrate uptake, neurotransmitter removal as well as the homeostasis of the other ions. Thus, Na^+ homeostasis and fluctuations

are tightly connected to various processes such as Ca^{2+} signaling, pH transients and the course of synaptic transmission. The electrogenic NCX exchanges 3 Na^+ for 2 Ca^{2+} and uses the Na^+ gradient as driving force. Alterations of the gradient or membrane depolarisation can shift driving forces and induce a switch of the transporter to the reverse mode. Reverse operation of NCX imports Ca^{2+} into the cells generating a Ca^{2+} increase and potentially a signal (Goldman et al., 1994). In Bergmann glia cells *in situ*, an increase of $[Na^+]_i$ to about 30 mM by kainate application and the concomitant depolarization were shown to cause reversal of the NCX and to enhance the observed Ca^{2+} signal (Kirischuk et al., 1997). Concerning many aspects, sodium and Ca^{2+} signals in astrocytes appear to be interconnected, but they also show differences. For example, glial Ca^{2+} waves can propagate in a regenerative fashion and, while sodium signals also spread within the syncytium, the amplitude of Na^+ signals dissipates with growing distance to the site of initiation (Bernardinelli et al., 2004; Langer et al., 2012). Thereby, Na^+ signals could indicate the localization of the triggering signal.

While $[Ca^{2+}]_i$ is not only controlled by Na^+-dependent transporters but also by the pump activity of PMCA and SERCA, pH regulation is primarily dependent on Na^+ driven mechanisms. Transport capacity and direction of the main pH regulators NHE and NBC directly depend on the sodium gradient (Chesler, 2003; Deitmer and Rose, 2010). Both transporters constantly extrude acid from the cytoplasm, and a weaker sodium gradient would lower their efficacy and result in an intracellular acidification. This could, on the one hand, lower mitochondrial activity (Azarias et al., 2011) and, on the other hand, enhance lactate export via the cotransport with H^+ using MCT4 (Deitmer, 2002). Thus, pH changes, caused by an altered Na^+ gradient, constitute one of the mechanisms of metabolic coupling between neurons and astrocytes.

The precise function of neurotransmission requires a tight control of pH_o and the supply with energy. But, efficient neurotransmission also relies on the appropriate timing of neurotransmitter release and subsequent inactivation, which is often achieved by uptake into astrocytes. The removal of the neurotransmitters GABA (via GAT3) and glutamate (via GLT-1 and GLAST) is operated by Na^+-dependent transport systems and, hence, their uptake generates considerable intracellular Na^+ elevations (see Fig. 11) (Langer and Rose, 2009; Unichenko et al., 2013). The efficacy of glutamate transporters strongly depends on the maintenance of the inward driving force by Na^+ and H^+ gradients (Zerangue and Kavanaugh, 1996) and an

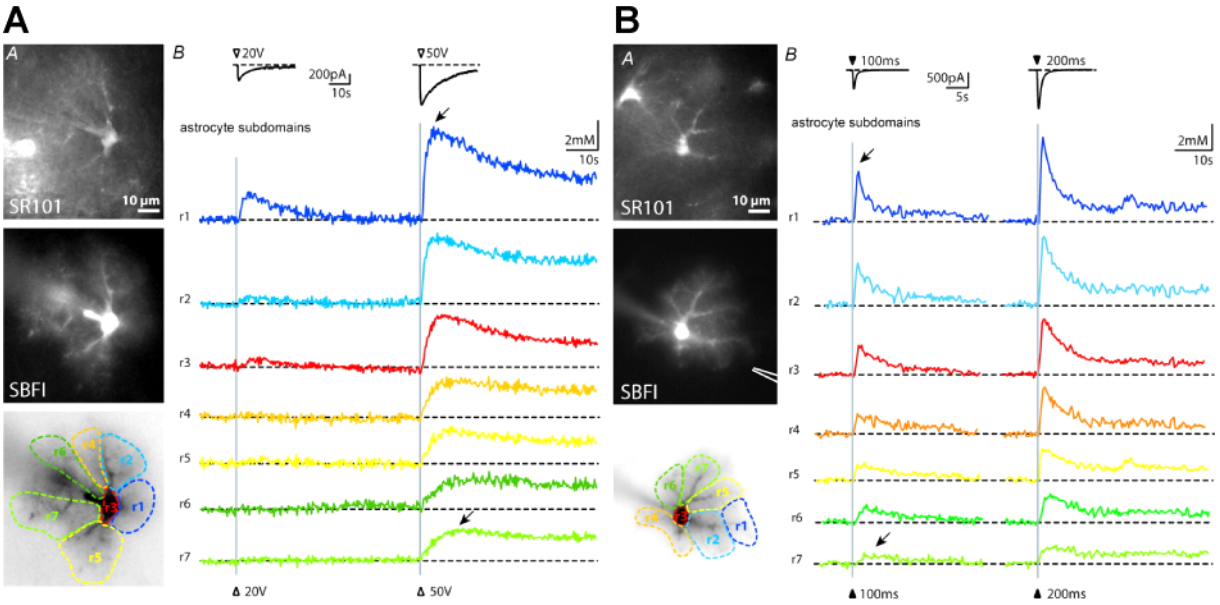

Figure 11 Na^+ increases in astrocytes can be induced by synaptic stimulation (A) or local D-asp application (B). The extent of the signal spread depends on the intensity of stimulation. Reprinted with permission from John Wiley and Sons: (Langer and Rose, 2009) © 2009.

increased Na^+ content in astrocytes as e.g. during exposure to NH_4^+, impairs glutamate removal (Kelly et al., 2009). Moreover, glutamate transporters in astrocytes may even switch to reverse transport releasing glutamate, especially during pathological conditions such as ischemia (Szatkowski et al., 1990; Rossi et al., 2000). While glutamate transporters robustly work in the forward mode of operation in healthy tissue, the reversal potential of GABA transporters is close to the resting membrane potential and transport direction can reverse easily upon minor changes of $[Na^+]_i$ and depolarization, thereby releasing GABA (Kirischuk et al., 2012; Unichenko et al., 2013). This GABA release was proposed as a mechanism to negatively regulate network activity by translation of glutamatergic excitation into tonic GABAergic inhibition (Heja et al., 2009; Heja et al., 2012).

In addition to import by transmembrane transporters, other routes for sodium entry into astrocytes could be ion channels such as ionotropic glutamate receptors, P2X purinergic receptors, acid sensing ion channels (ASIC) and transient receptor potential channels (TRP) (cf. Fig. 4) (Lalo et al., 2011; Kirischuk et al., 2012; Rose and Karus, 2013). According to the broad spectrum of processes, which hold the potential to generate $[Na^+]_i$ changes, an equally diverse range of reactions may be modulated or even induced and controlled by Na^+. In the past, sodium ions were thought to be immediately and locally expelled from the cells. Thus, a signaling function for Na^+ fluctuations was not assumed. The data presented above show, that,

in contrast to previous assumptions, considerable sodium transients are present in astrocytes upon appropriate, physiological stimulation. Moreover, these transients potentially affect all different aspects of cell physiology and therefore require further in depth analysis in healthy as well as pathological settings.

4. Astrocytes and epileptiform activity

Epilepsy is a very common disease of the CNS affecting about 0.5-1% of the population (Thurman et al., 2011). The umbrella term epilepsy comprises a broad diversity of disorders (Christensen and Sidenius, 2012), which are collectively characterized by recurring seizures (ictus) and additional alterations of the interictal electroencephalogram (EEG) (Fisher et al., 2005). On the level of neural circuits, epilepsy is thought to arise from an imbalance of excitation and inhibition, which shifts the network towards a hyperexcitable state (McCormick and Contreras, 2001). This state facilitates the occurrence of hypersynchroneous network discharges, which constitute the neurophysiological correlate of interictal and ictal EEG signals.

The causes for epileptogenesis, which is the development of a hyperexcitable network, are diverse and often unknown. Only a minority of epileptic syndromes are linked to known genetic causes. Among them are Dravet`s syndrome, associated with mutations of the SCN1A gene coding for $Na_v1.1$ (Marini et al., 2011), or tuberous sclerosis (Wong and Crino, 2012). Most epilepsies are cryptogenic with unknown cause or symptomatic, which means that they arise secondary to previous insults such as febrile seizures, ischemic insults or brain injury (Scharfman, 2007).

The excitability of a neuron can be defined as its probability to generate action potentials. This probability depends on both intrinsic properties of the single cell (e.g. expression of transmitter receptors and ion channels) as well as on the status of the cellular environment in terms of connectivity (synaptic plasticity) and composition of the ECS fluid. Neurons receive excitatory and inhibitory synaptic input, which is integrated and weighted for controlled action potential generation. Moreover, expression of specific proteins such as ligand- and voltage-gated ion channels, electrogenic transporters and ion pumps define the membrane excitability. As introduced above, astrocytes are critically engaged in the control of the neuronal environment and actively support und modulate neuronal activity. Hence, alterations of these cells likely affect network excitability and may be involved in discharge

generation and epileptogenesis (see Fig. 12) (Wetherington et al., 2008; Seifert et al., 2010; Heuser et al., 2014). Some recent studies even assigned a causal role to astrocytes claiming that released glutamate may generate paroxysmal depolarization shifts, and thereby induce and support hyperexcitability (Rogawski, 2005; Tian et al., 2005). Still, this view has been critically discussed and other studies found that astroglial Ca^{2+} signals were not necessary for their epileptiform discharge generation. Nevertheless, these studies observed that astroglial Ca^{2+} oscillations and glutamate release support and enhance epileptiform discharge induction and spread (Fellin and Haydon, 2005; Fellin et al., 2006; Gomez-Gonzalo et al., 2010).

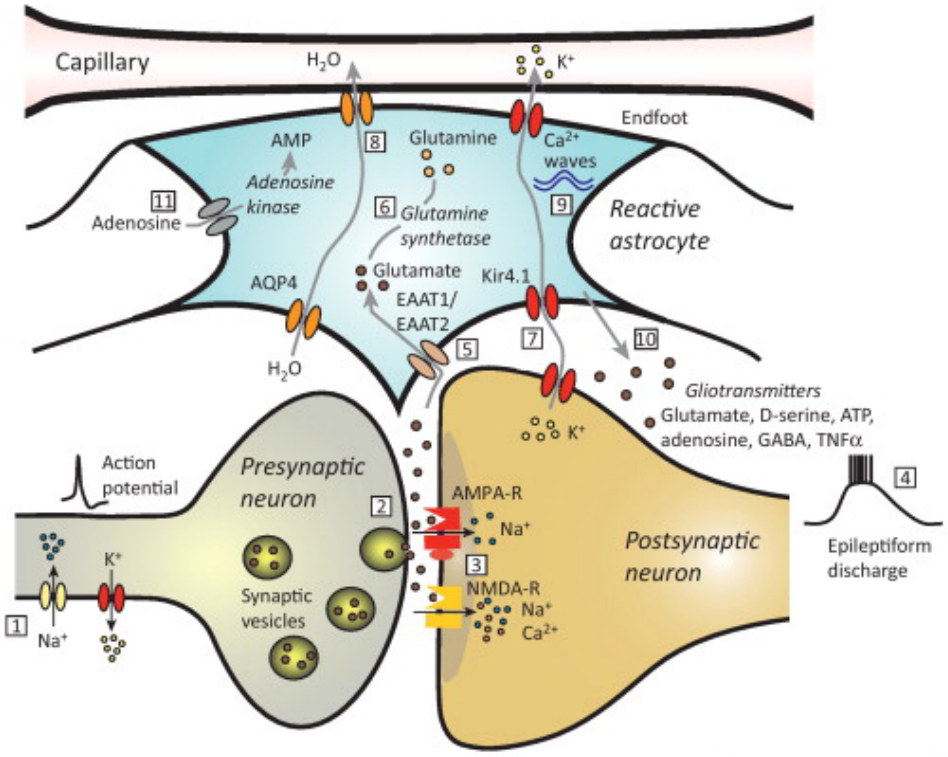

Figure 12 Selected interactions of neurons and astrocytes that have been proposed to be altered in epilepsy and involved in discharge generation. Among these are K^+ and water homeostasis, glutamate uptake, glutamate-glutamine shuttle, Ca^{2+} signaling and gliotransmission. Reprinted from (Devinsky et al., 2013) with permission from Elsevier © 2013.

Further indications for a role of astrocytes in epilepsy and epileptogenesis were derived from temporal lobe epilepsy (TLE), which is one of the most common types of epilepsy with seizure foci in the hippocampus. Hippocampal sclerosis with significant neuronal loss and prominent astrogliosis was found in about 65% of human hippocampal tissue specimen from epilepsy surgery (de Lanerolle et al., 2010) and various alterations of astroglial characteristics have been reported in TLE.

These changes include the upregulation of mGluRs, changes in glutamate transporter levels, upregulation of GAT3 and lower levels of GS. Furthermore, disruption of K^+ clearance by impaired K_{ir} channel function, altered gap junction coupling and a loss of the focal distribution of Aqp4 have been proposed (see Fig. 12) (Wetherington et al., 2008; de Lanerolle et al., 2010; Seifert et al., 2010). Whether these changes of glial properties are cause or consequence of the epileptic syndrome, is not known. Still, it is most likely that the alterations do modify astroglial responses to neuronal activity and change subsequent feedback mechanisms. Moreover, the basic homeostatic parameters controlled by astrocytes could also be subject to functional changes in gliotic tissue and affect epileptogenesis and seizure generation.

$[K^+]_o$ increases have been consistently observed in different models of epileptiform activity *in situ* and *in vivo* (Lux et al., 1986; Wallraff et al., 2006). Moreover, intracellular Ca^{2+} transients are known to occur in neurons during discharges and astroglial Ca^{2+} signals as well as Ca^{2+} oscillations have been shown to be enhanced under hyperexcitable conditions (Carmignoto and Haydon, 2012). In addition, a reduction of the extracellular sodium concentration was observed in the cat cortex *in vivo* during epileptiform afterdischarges (Dietzel et al., 1982). A recent computational study predicted considerable intracellular sodium changes in neurons during induced discharges and proposed an involvement of Na^+ transients in the unknown mechanisms of spontaneous seizure termination (Krishnan and Bazhenov, 2011). Still, up to now, no data on intracellular Na^+ fluctuation in neurons and astrocytes during epileptiform, hyperexcitable network activity are available.

Aim of the study

Astrocytes have been shown to be actively involved in information processing in the brain. Ca^{2+} signaling and the release of gliotransmitters are the main processing mechanisms that have been described up to now. Nevertheless, other tasks performed by astroglial cells, often closely linked to Na^+-dependent processes, such as glutamate and K^+ removal from the ECS as well as metabolic support of neurons may not only serve mere homeostatic functions. Illustrating this aspect, the present work is complemented by a comprehensive review addressing sodium homeostasis and its putative signaling function in astrocytes in physiological and pathophysiological states (Rose and Karus, 2013).

The transmembrane Na^+ gradient provides cells with a driving force for a multitude of transport processes. Hence, its maintenance is thought to be of critical importance for cell function. Nevertheless, appreciable sodium transients have been observed in primary cultures of neurons and astrocytes as well as *in situ* upon exogenous stimuli such as electrical, mechanical or pharmacological activation. Still, it is unknown, whether endogenous activity of the brain is associated with intracellular Na^+ transients.

The goal of the present work was to determine whether activity of the intrinsic cellular network is capable of inducing sodium shifts in neurons and astrocytes. To that end, the endogenous activity of the hippocampal circuitry in acute tissue slices was enhanced pharmacologically resulting in a status of hyperexcitability and epileptiform discharges. To obtain information about Na^+ dynamics within the cells, the Na^+-sensitive fluorescent dye sodium-binding benzofuran isophthalate (SBFI) was used in wide field imaging experiments. These were complemented with extracellular field potential recordings to monitor the ongoing network activity. Furthermore, extracellular K^+ concentration was recorded with ionsensitive microelectrodes and the membrane potential dynamics were probed in whole-cell patch-clamp experiments.

The presence and function of neuronal and astroglial sodium dynamics and regulatory mechanisms were analysed in epileptiform conditions as a model for strong activity generated by the intrinsic network (Karus et al., unpublished).

Summary and discussion

Decades ago, extracellular microelectrode recordings already showed $[Na^+]_o$ decreases during Schaffer collateral stimulation (Zanotto and Heinemann, 1983) and $[Na^+]_o$ fluctuations in stimulation-induced epileptiform hyperactivity (Dietzel et al., 1982; Lux et al., 1986). However, detailed analyses of Na^+ dynamics at single cell level are still missing. To address this matter, we applied Mg^{2+}-free artificial cerebrospinal fluid (ACSF) containing bicuculline ($0Mg^{2+}$/BIC) in acute hippocampal slices of adolescent mice. These treatments, individually and in combination, are established protocols to induce epileptiform activity *in situ*. Removal of Mg^{2+} from the ACSF relieves the magnesium block from NMDA receptors, which are then available for activation at resting membrane potential. In addition, application of the $GABA_A$ receptor blocker bicuculline depletes GABAergic, inhibitory transmission. Thus, both manipulations shift the balance of excitation and inhibition towards hyperexcitability. To study Na^+ dynamics, we applied the Na^+-sensitive fluorescent dye SBFI and measured its fluorescence changes during recurrent network activity.

The experiments presented in this study show, for the first time, synchronized sodium transients within the local network of neurons and astrocytes during endogenously generated network activity. This activity was represented by epileptiform discharges induced by $0Mg^{2+}$/BIC observed in field potential recordings from the CA1 pyramidal cell layer. The discharge activity commenced within few minutes of treatment with $0Mg^{2+}$/BIC and remained stable for at least up to 95 minutes. Intracellular Na^+ transients in neurons and astrocytes during spontaneous, recurrent epileptiform discharges were present in all analyzed cells in a field of view. Neuronal sodium transients reached an average peak $[Na^+]_i$ increase by 7 mM and signals lasted for several tens of seconds. The signals in astrocytes were smaller (peak change about 3 mM) and shorter (<10 s) and were often followed by an undershoot below baseline. In between discharges, $[Na^+]_i$ recovered to a concentration similar to baseline level.

In contrast to synchronized Na^+ transients, widespread Ca^{2+} fluctuations are well described under epileptiform conditions. Such fluctuations may involve several neurons or even extend throughout the whole network and have been previously observed both in culture (Bacci et al., 1999; Verderio et al., 1999) and *in situ* (Gomez-Gonzalo et al., 2010; Losi et al., 2010). In brain slices, astrocytes also

showed enhanced and often synchronized Ca^{2+} oscillations under hyperexcitable conditions (Fellin et al., 2006; Gomez-Gonzalo et al., 2010). These Ca^{2+} oscillations, induced by proconvulsive agents such as $0Mg^{2+}$ or bicuculline, were described to precede epileptiform field potential shifts and neuronal activation (Tian et al., 2005). Moreover, the aforementioned studies found that astroglial Ca^{2+} increases were insensitive towards tetrodotoxin (TTX), an inhibitor of voltage-gated sodium channels and, accordingly, not dependent on neuronal action potential generation and subsequent neurotransmission. Based on these observations, the authors concluded that astrocyte Ca^{2+} oscillations might play a causal role in discharge generation (Tian et al., 2005; Fellin et al., 2006). The results of the present study indicate that astroglial Na^{+} transients, as opposed to astroglial Ca^{2+} oscillations, do not arise spontaneously but in response to neurotransmission. Supporting this view, Na^{+} transients occurred without detectable delay in both neurons and astrocytes indicating a common origin. Moreover, epileptiform discharges as well as Na^{+} transients were completely suppressed by TTX. Additionally, we observed, that interfering with glutamatergic transmission blocked neuronal and glial Na^{+} transients as well as field potential discharges partially, using the AMPA receptor antagonist CNQX, or even completely upon application of the NMDA receptor blocker APV. This further strengthens the hypothesis, that Na^{+} transients are tightly connected to glutamatergic neurotransmission and additionally indicates a considerable release of glutamate to the ECS during discharges. These results show that Na^{+} transients do not constitute a primary cause of discharge generation but rather occur as a response to ongoing network activity.

Ca^{2+} signaling is thought to operate via direct modification of Ca^{2+} sensitive proteins, whereas no Na^{+}-binding enzymes are known. Instead, Na^{+} transients may affect cell physiology via altered driving forces for Na^{+}-dependent transport processes. In our study, neuronal Na^{+} transients exhibited much longer durations than Ca^{2+} signals described in CA3 neurons in a similar model of epileptiform activity (Fellin et al., 2006). Na^{+} transients of similar amplitudes as those described here were observed by (Langer and Rose, 2009) in hippocampal slices upon Schaffer collateral stimulation. Their experiments also showed a relatively slow time course of neuronal Na^{+} signals as compared to Ca^{2+} transients elicited by similar stimulation. Moreover, a slow time course was described for kainate induced sodium transients in Bergmann glia cells (Kirischuk et al., 1997) as well as in electrically stimulated

cerebellar granule cells (Regehr, 1997). Such prolonged shifts of the Na^+ gradient may alter the driving forces for Na^+-dependent transport processes to a physiologically relevant extent. Variations of glutamate transporter efficacy in terms of Na^+-dependent short term plasticity have been described for cortical astrocytes (Unichenko et al., 2012) and reversal of GABA transporters due to Na^+ transients induced by glutamate uptake have been observed (Unichenko et al., 2013). Hence, Na^+ transients can actually function as an adaptive signal and these Na^+ signals are fundamentally distinct from Ca^{2+} transients.

In current clamp recordings of CA1 pyramidal neurons, we measured burst discharges similar to typical paroxysmal depolarisation shifts accompanied by superimposed action potentials (Johnston and Brown, 1984). Simultaneous sodium imaging confirmed the concomitant occurrence of somatic sodium transients. Although the results from this study provide only limited information on the precise routes of sodium entry into neurons because TTX, APV and CNQX depressed the generation of epileptiform discharges, previous studies indicated that the majority of sodium enters neurons via ionotropic glutamate receptors and especially via NMDA receptors (Rose and Konnerth, 2001). Hippocampal, protoplasmic astrocytes do not express ionotropic glutamate receptors. Thus, astroglial Na^+ transients induced by recurrent epileptiform discharges likely depend on the activation of glutamate transporters. It has been established that synaptic glutamate release and high-affinity transport into astrocytes result in an increase in $[Na^+]_i$ (Rose and Ransom, 1996b; Chatton et al., 2000; Langer and Rose, 2009). Along these lines, we were able to induce similar $[Na^+]_i$ increases by exogenous application of glutamate.

In about 30% of the Na^+ signals in astrocytes, we observed an undershoot below baseline following the initial Na^+ increase. This decrease in astrocyte Na^+ during periods of glutamatergic activity might be caused by activation of NKA in response to increases in $[K^+]_o$ (Grisar, 1984; Blanco and Mercer, 1998; Rose and Karus, 2013). Extracellular K^+ recordings, performed in this study, showed transient increases of $[K^+]_o$ by about 1.7 mM during epileptiform. Due to the high K^+ permeability, the astroglial membrane potential is considered to reflect extracellular K^+ changes and indeed, patch clamp recordings from astrocytes revealed transient membrane depolarizations by about 10 mV. According to the measured change of $[K^+]_o$, the expected potential shift, as calculated via the Nernst equation, is closely matched by the observed depolarisations. Thus, both patch clamp and K^+-sensitive

microelectrode recordings show transient, moderate increases of $[K^+]_o$ reflecting the ongoing network activity. In line with this, bath application of 10 mM K^+ resulted in a decrease of $[Na^+]_i$. Furthermore, in some astrocytes, combined application of glutamate and K^+ mimicked the biphasic $[Na^+]_i$ response with initial increase and subsequent undershoot seen during discharge activity. In opposition to astrocytes, neuronal $[Na^+]_i$ did not respond to the application of 10 mM K^+, which might be explained by the cell type specific expression of NKA isoforms. The NKA consist of the catalytic α and accessory β subunits (Crambert and Geering, 2003). The different α subunits are thought to define the affinities to Na^+, K^+ and ATP resulting in different kinetic properties. While astrocytes express α1 and α2 subunits, neurons primarily express α1 and α3 subunits (Blanco and Mercer, 1998). The α1 isoenzyme is uniformly distributed in the glial membrane and thought to mainly fulfil "housekeeping" tasks (Juhaszova and Blaustein, 1997). Under resting conditions, the α1 isoenzyme is saturated with K^+ (as is the α3 in neurons). In contrast, the α2 isoform was found to have a lower K^+ affinity (α2β2: $K_{0.5}(K^+)$ ~ 4.8mM; (Blanco and Mercer, 1998)), and thus, can be further activated by $[K^+]_o$ increases. This feature of astroglial NKA might explain the faster decay kinetics of Na^+ signals in astrocytes as compared to neuronal. In addition to the low K^+ affinity, the α2 isoform of the NKA is found in close spatial association with GLAST and GLT-1 (Cholet et al., 2002; Rose et al., 2009) as well as mGluR5 and Aqp4 (Illarionova et al., 2010). Hence, astroglial NKA seems to be of paramount importance for activity related ion regulation, and the α2 isoform likely plays an important role in the context of synaptic transmission (Rose and Karus, 2013). Our observation of astrocyte specific stimulation of NKA activity highlights the role of active K^+ uptake into glial cells for K^+ clearance and suggests involvement of NKA activation in the undershoots of astroglial Na^+ signals.

Transient sodium increases in astrocytes, due to glutamate uptake, provide an additional activator for the NKA (Blanco and Mercer, 1998) and promote the K^+ clearance. But, equally important, glutamate uptake regulates neuronal excitation by limitation of synaptic transmitter exposure. Accordingly, we observed, that blocking glutamate transporters with the antagonist TFB-TBOA during epileptiform discharges abolished astroglial Na^+ increases while the discharge frequency strongly increased. In parallel, neuronal Na^+ transients no longer recovered to baseline levels but $[Na^+]_i$ accumulated massively. Under these conditions, neurons are likely to swell (Kahle et al., 2009) and our observations support this notion. Discharge activity ceased within

several minutes and did not recover after TFB-TBOA washout indicating permanent, presumably excitotoxic, damage to the neurons (Sonnewald et al., 2002).

Taken together, these experiments suggest that astroglial Na^+ transients during epileptiform bursts are caused by the combined action of high-affinity glutamate uptake (mediating Na^+ influx) and NKA (mediating Na^+ efflux). Furthermore, they show that glutamate uptake is a necessary requirement for the maintenance of neuronal viability and network activity. During otherwise undisturbed conditions, epileptiform discharges in $0Mg^{2+}$/BIC persisted for prolonged periods without striking signs of altered cellular properties. Hence, although the cells were challenged by recurrent phases of appreciably enhanced activity they were still well capable of maintaining their intra- as well as extracellular conditions close to normal. Nonetheless, the observation that a block of glial glutamate transporters resulted first in an enhancement of discharges and subsequently in a deterioration of neurons indicates a vital role of astrocytes in maintaining tissue function.

To further address the role of glial function during recurrent network activity and especially the interaction of neurons and astrocytes in terms of energy supply, we made use of the toxic compound sodium fluoroacetate (NaFAc). Astrocytes specifically take up NaFAc (Hassel et al., 1992; Sonnewald et al., 1993), which interferes with the TCA cycle by inhibition of the enzyme aconitase (Fonnum et al., 1997). Increased tissue Na^+ concentrations with a concomitant drop of ATP levels have been observed by (Banay-Schwartz et al., 1974), though whether glial ATP concentration is actually altered by NaFAc treatment is a matter of debate (Keyser and Pellmar, 1994; Fonnum et al., 1997). Here, we treated acute slices with 1 mM NaFAc and observed increases of $[Na^+]_i$ baseline after 30 to 60 minutes treatment which predominantly occurred in astrocytes indicating NaFAc specificity. Most likely, basic NKA activity was impaired by reduced ATP supply which revealed constant Na^+ influx via pathways such as NKCC1 (Kelly et al., 2009), NBC (Rose and Ransom, 1996a) and NHE (Chesler, 2003). Moreover, we found a significant reduction of NKA stimulation by application of 10 mM K^+ in NaFAc. This observation provides further evidence for the interference of NaFAc with fundamental astroglial function.

Additionally, the capacity of glutamate transporters in astrocytes was tested by application of the transported agonist D-aspartate (D-asp). D-asp, like glutamate, induced Na^+ transients in astrocytes, which were significantly decreased by NaFAc treatment. While Na^+-coupled neurotransmitter uptake is not thought to substantially

add to $[Na^+]_i$ under resting conditions (Marcaggi and Attwell, 2004; Langer and Rose, 2009; Rose and Karus, 2013), the efficiency of glutamate transporters directly depends on the Na^+ gradient. Therefore, a higher $[Na^+]_i$ directly interferes with the glutamate uptake capacity of astrocytes (Kelly et al., 2009; Unichenko et al., 2012). The driving force for transporters can be calculated as the difference between membrane potential and reversal potential of the transport (Zerangue and Kavanaugh, 1996). Accordingly, the observed increase of $[Na^+]_i$ baseline by 12 mM would cause a decrease of transport driving force by about 20%. This is well in line with the 26% reduction of the D-asp induced Na^+ signal that was determined in NaFAc treated cells. In addition, D-asp activates neuronal NMDA receptors and generates Na^+ transients, which are not altered by NaFAc. Hence, under resting conditions, neurons were not strongly affected by NaFAc treatment and thus by the interference with glial metabolism indicating their metabolic independence at rest. Consequently, NaFAc can be used as a specific tool to increase $[Na^+]_i$ and attenuate both NKA as well as glutamate transporter activity in astrocytes.

In primary cultures, NaFAc was observed to reduce spontaneously occurring epileptiform discharges. This effect was attributed to a suppression of the glutamate-glutamine cycle (Bacci et al., 1999). In contrast to these findings, in acute slices, we observed enhanced epileptiform activity in NaFAc during $0Mg^{2+}$/BIC treatment. While the discharge frequency was unaltered, discharges were prolonged and contained a higher number of population spikes. We noticed increased Na^+ transient amplitudes and signal durations in neurons as well as prolonged $[K^+]_o$ transients. All of these effects point towards a longer presence of glutamate in the ECS and enhanced stimulation of neuronal postsynaptic elements due to reduced glutamate removal by astrocytes. The observed augmentation of sodium transients does not argue for a depletion of neuronal glutamate stores. The decay time constants of sodium signals in both neurons and astrocytes were strongly increased indicating impaired NKA function probably due to lowered ATP availability in both cell types. Hence, under conditions with increased energy demand, neurons seem to require an intact astroglial metabolism to meet their metabolic needs. This supports the theory of metabolic coupling between neurons and astrocytes as a means to satisfy activity-induced energy requirements. The apparent enhancement of Na^+ increases in astrocytes in NaFAc can be explained by the impaired NKA function. Usually, due to the close spatial association of NKA and glutamate transporters (Cholet et al., 2002;

Rose et al., 2009), most Na^+ is thought to be expelled immediately at the site of entry, whereas upon NKA impairment, diffusion allows for its detection in the soma. NKA dysfunction is further supported by the fact that, in NaFAc, we observed hardly any Na^+ undershoots and decay of transients in astrocytes was strongly attenuated. While K^+ clearance was also slowed, the relatively minor changes of the amplitude of $[K^+]_o$ transients may be attributed to the clamping effect of the perfusion solution.

In sum, this study establishes the occurrence of simultaneous, long lasting sodium transients of considerable amplitude in neurons and astrocytes during strong, recurrent network activity. Although these transients are thought to impose a considerable energetic burden on the cells, cellular vitality and function were not significantly altered after extended periods of discharge activity as long as astrocytes were healthy. On the contrary, if astrocyte function was impaired, either by pharmacological interference with glutamate removal or energy metabolism, neuronal functions were likewise affected. These findings reveal the intense interactions and interdependence of both cell types and furthermore show that astrocytes are necessary to limit neuronal excitation and prevent damage caused by glutamatergic overstimulation. Moreover, the presence of long lasting Na^+ transients during endogenously generated network activity highlights the possible role of Na^+ dynamics as signals to adapt the multiple Na^+-dependent processes to the activity level of the local circuitry as well as the detrimental effects of Na^+ dysregulation (Rose and Karus, 2013). While the functional relevance Ca^{2+} signals in neurons and astrocytes is widely accepted and intensively investigated (Volterra and Meldolesi, 2005; Fiacco and McCarthy, 2006; Grienberger and Konnerth, 2012), dynamic fluctuations of $[Na^+]_i$ have only recently started to gain attention (Rose, 2002; Deitmer and Rose, 2010; Kirischuk et al., 2012) and the nature of possible Na^+ signaling is thought to differ fundamentally from the mechanisms described for Ca^{2+} signals (Rose and Karus, 2013).

Publications and Manuscripts

For reasons of copyright protection, the published version of this thesis does not contain reprints of the articles. Instead, full bibliographic references are reported.

“Two sides of the same coin: sodium homeostasis and signaling in astrocytes under physiological and pathophysiological conditions”

Rose C.R., Karus C. (2013)

Glia 61(8): 1191-1205

I drafted the text passages discussing pathophysiological conditions (sections: Astrocyte Sodium Dysbalance Under Conditions of Metabolic Stress; Astrocyte Sodium Dysbalance: Consequences for Transmitter Uptake and Volume regulation).

I contributed to the draft and revision of the whole manuscript as well as the choice and preparation of figures.

“Astrocytes restrict discharge duration and neuronal sodium loads during recurrent network activity”

Karus C., Mondragao M.A., Ziemens D., Rose C.R.

(Glia; submitted 19.09.2014, under revision)

I performed all experiments and analyses except for K^+-sensitive microelectrode recordings and patch clamp experiments (including simultaneous imaging). The data I obtained represent about 95% of results presented in the manuscript and are illustrated in 7 out of 8 figures.

I drafted the first version of the manuscript, prepared the figures and revised manuscript and figures together with C.R. Rose.

I contributed to the experimental design and data interpretation.

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Erklärung

Ich versichere an Eides Statt, dass die Dissertation von mir selbständig und ohne unzulässige fremde Hilfe unter Beachtung der „Grundsätze zur Sicherung guter wissenschaftlicher Praxis an der Heinrich-Heine-Universität Düsseldorf“ erstellt worden ist.
Diese Arbeit wurde weder vollständig noch in Teilen einem anderen Prüfungsamt zur Erlangung eines akademischen Grades vorgelegt.

Claudia Karus