Sodium Signals in Astrocytes in Mouse Brain

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Zusammenfassung

Astrozyten erfüllen wesentliche Funktionen im zentralen Nervensystem von Vertebraten. Strategisch positioniert zwischen tausenden von Synapsen sowie zwischen Neuronen und Blutgefäßen, sind sie involviert in Synapsenfunktion, Informationsverarbeitung und Energieversorgung des Gehirns. Wichtige Aufgaben von Astrozyten, wie Ionenhomöostase und Transmitter-Aufnahme an Synapsen, sind eng an den Natriumgradienten über der Plasmamembran gekoppelt. Veränderungen der intrazellulären Natriumkonzentration hätten daher erhebliche Konsequenzen für die synaptische Übertragung sowie für Calcium- und pH-Signale, die ihrerseits synaptische und metabolische Vorgänge modulieren. Für Astrozyten in Kultur wurden Erhöhungen der intrazellulären Natriumkonzentration außerdem als Verbindung zwischen Glutamat-Aufnahme und verstärktem Zellmetabolismus identifiziert.

In dieser Studie sollte festgestellt werden, ob in Astrozyten im intakten Gewebe Veränderungen der Natriumkonzentration auftreten. Quantitative Fluoreszenzmessungen mit dem Natrium-sensitiven Farbstoff SBFI wurden verwendet um Natriumtransienten, welche synaptische Aktivität in Schnittpräparaten aus Hippocampus oder Cerebellum begleiten, zu charakterisieren.

In der Tat zeigt diese Studie zum ersten Mal, dass synaptische Aktivität zu lang anhaltenden Natriumtransienten in Astrozyten führt. Diese Natriumsignale sind hauptsächlich ein Resultat Natrium-abhängiger Glutamataufnahme. Die Aktivität verschiedener Synapsen führt dabei zu unterschiedlichen Signalmustern, die eine Unterscheidungsfähigkeit der Astrozyten für die jeweiligen Signalquellen nahe legen. Natriumtransienten können lokal begrenzt auftreten, sich in einer Zelle ausbreiten oder zu Nachbarzellen wandern, wobei ihre Form, in Kombination mit ihrer räumlichen Ausbreitung, Ort und Stärke der synaptischen Aktivität reflektiert.

In Anbetracht der Vielzahl von Auswirkungen, die eine veränderte Natrium-Triebkraft auf verschiedene zelluläre Vorgänge hat, sollten Natriumtransienten daher als Element der Neuron-Glia-Kommunikation gewertet werden. Sie erhöhen die Komplexität der Signalvorgänge in den ineinander greifenden Netzwerken von Astrozyten und Neuronen und rekrutieren benachbarte Astrozyten, den erhöhten Energiebedarf der Neurone nach synaptischer Aktivität zu decken.

Abstract

In the vertebrate central nervous system astrocytes are intimately involved in almost all aspects of brain function. Strategically localized between thousands of synapses as well as between neurons and blood vessels, they add their share to tissue architecture, synapse function, information processing and energy supply. Vital astrocytic functions like ion homeostasis and transmitter uptake at synapses are tightly linked to the sodium driving force over the plasma membrane. Changes of the intracellular sodium concentration in astrocytes thus might have important consequences for synaptic transmission as well as for calcium and pH signaling, which modulate synapse fine-tuning and metabolic responses. Additionally, for astrocytes in cell culture sodium elevations were identified as a link between glutamate uptake and metabolic responses.

This study was designed to elucidate whether significant sodium transients occur in astrocytes in the intact tissue. Quantitative fluorescence imaging with the sodium sensitive dye SBFI was employed to characterize intracellular sodium concentration changes, which accompany synaptic activity, in astrocytes in acute tissue slices of mouse hippocampus and cerebellum.

Indeed, for the first time, this study establishes that glutamatergic synaptic transmission in both the hippocampus and the cerebellum results in long lasting sodium transients in astrocytes, mainly as a result of sodium dependent glutamate uptake. Activity of different synapses induces distinct sodium signal patterns in astrocytes, indicating a capacity for input discrimination. These sodium signals can be locally restricted, spread within one cell or travel within the astrocyte network through gap junctions, their amplitude, time course and spatial profile reflecting the site and strength of synaptic activity.

Taken into account the multitude of effects a decrease in the sodium driving force would exert on cellular functions, sodium transients should thus be considered as an element of neuron to glia signaling. Sodium signals add further complexity to signaling processes in the interdigitated networks of neurons and astrocytes. They could convey increased metabolic needs to neighbouring astrocytes and would thus recruit several cells in the active area to meet the energy demand of neurons after synaptic activity.

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Introduction and Résumé

> […] Einstein had a higher astrocyte-to-neuron ratio in the area of the left parietal cortex […]. Einstein had so many more cells that it was "statistically significant", a scientific term to mean that it was "pretty different enough to say that it means something". ["The root of thought" from Andrew Koob]

For a long time neurons were believed to be the main players in the central nervous system. Due to their electrical properties they seemed so well suited for the processing of incoming and outgoing signals that research mainly focused on them. At the same time, the large group of glia cells located dispersed in the space between them was believed to serve only supporting duties.

Over the years this view changed slowly, for it became obvious that the former passive "brain glue" accomplishes not only service and maintenance but is of much more significance to brain function.

1. Astrocytes

In 1895 a subpopulation of glia cells in the vertebrate central nervous system (CNS) was termed astrocytes (von Lenhossék, 1895). The name was based on their most frequent morphology, comprising a soma surrounded by multiple processes, which makes them appear like stars.

Another two types of glia cells, identified a few years later, are microglia and oligodendrocytes. Microglia cells are of mesodermal origin and in charge of immune system functions in the CNS. Oligodendrocytes, like neurons and astrocytes, originate from the neuroectoderm. Their main assignment is to build the myelin sheaths of neurons. While neurons, oligodendrocytes and microglia express markers, which allow an unequivocal determination, astrocytes have no common feature in terms of morphology, physiology or on the molecular level,

which would allow the discrimination from other cells as one distinct homogeneous group. Therefore, astrocytes are rather united by not belonging to one of the aforementioned groups of cells. Ongoing studies still aim to define subgroups of astrocytes, considering their morphology and physiology.

Figure 1: morphological diversity of glia cells (human frontal lobe; Retzius 1894)

Based on morphological appearance, in the 19th century, astrocytes were divided into two classes, termed protoplasmic and fibrous astrocytes. Protoplasmic astrocytes of grey matter are characterized by highly complex processes, while in white matter fibrous astrocytes comprise processes with little to moderate branching. In addition a small set of morphologically distinct types of astrocytes had been termed with special names, such as the Müller cells of the retina or the Bergmann glia cells of the cerebellum (Reichenbach, Wolburg 2005).

A subset of astrocytes could be visualized by staining either the astrocyte marker glial fibrillary acidic protein (GFAP), an intermediate filament of the cytoskeleton, or the calcium binding protein S100β. It was found that within a given brain region, several types of differently shaped astrocytes can coexist, their density and morphology to a certain extent determined by the cytoarchitecture of the tissue (Emsley, Macklis 2006). At the same time, similarly shaped astrocytes exhibit different physiological properties, suggesting that astrocyte heterogeneity goes far beyond their diverse morphological appearance.

Since high resolution microscopy accomplished the examination of astrocyte morphology, astrocytes are described more sponge-like than star-like, because the whole of processes of a single astrocyte is much more complex (Fig. 2), than it was estimated using other methods. Clearly distinguishable primary and secondary processes give rise to highly ramified higher-degree processes and their finest terminals, which can appear as lamellipodia, filopodia or leaf like structures (Grosche *et al.* 2002; Witcher *et al.* 2007). Many of the thin cellular protrusions of astrocytes reach into the vicinity of neuronal synapses and are thus called perisynaptic processes. They show heterogeneous and highly dynamic morphologies, from loosely associated to the pre- and/or postsynaptic compartment up to ensheathing the synapse and sealing the synaptic cleft (Witcher *et al.* 2007; Grosche *et al.* 2002; Wenzel *et al.* 1991).

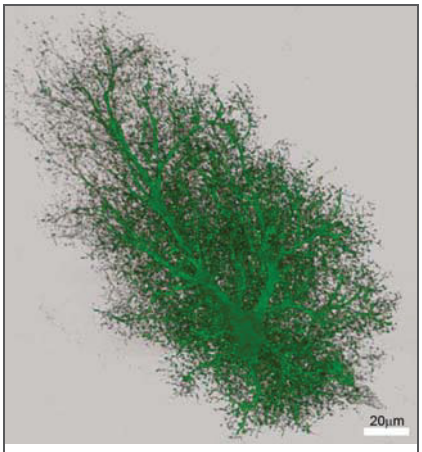

Figure 2: reconstruction of a single hippocampal astrocyte (Lucifer Yellow; Bushong *et al.* 2004)

In the human brain the astrocyte to neuron ratio is about 1.4 to 1, while in rodents it is about 0.4 to 1 (Nedergaard *et al.* 2003). In addition primate astrocytes are more diverse and complex, being larger and comprising more processes as compared to rodents. There are also distinct morphological subtypes of astrocytes described in human brain which are not to be found in rodents (Oberheim *et al.* 2009). Considering a synaptic density in cortex with 1400 million synapses/mm^3 in rat and approximately 1100 million synapses/mm^3 in human (DeFelipe *et al.* 2002), for rodents it was estimated that one protoplasmic astrocyte can contact 20000 to 120000 synapses, while for the larger human astrocyte 270000 to two million contacts were predicted (Oberheim *et al.* 2009).

Perisynaptic processes are commonly as narrow as 50 to 200 nm, containing only a small part of the cell cytoplasm but representing a major part of the cell surface (Grosche *et al.* 2002). Two of their main responsibilities are the uptake of neurotransmitters like glutamate or GABA and the normalization of the extracellular potassium concentration after neuronal activity (Kimelberg 2010).

These tasks are central to synapse function, since they prevent excitotoxicity and hyperexcitability of neurons. The dynamic maintenance of extracellular transmitter concentrations modulates the activation of extrasynaptic receptors, spillover of transmitter to adjacent synapses and the time course of synaptic transmission (Diamond, Jahr 1997; Huang, Bergles 2004; Tzingounis, Wadiche 2007).

Astrocytes express not only transporters (Danbolt 2001; Minelli *et al.* 1995) and potassium channels (Seifert *et al.* 2009; Bordey, Sontheimer 2000), which are involved in transmitter uptake and ion homeostasis, respectively, but also a range of different types of ionotropic and metabotropic receptors (Porter, McCarthy 1997; Lalo *et al.* 2010). Thus, transmitters released from neurons activate ion currents and second messenger cascades in perisynaptic processes of astrocytes.

Among the most important consequences of this neuron to glia signaling is the release of a wide variety of substances from astrocytes. Many of these molecules serve as signals between astrocytes and/or are involved in glia to neuron support and modulatory signaling (Fellin, Carmignoto 2004; Perea, Araque 2010). This includes for example the release of glutamate, D-serine, GABA and ATP (Fig.3), which, in this case, are called gliotransmitters (Hamilton, Attwell 2010; Parpura, Zorec 2010). They activate transporters and different receptors on pre- and post-synaptic neuronal compartments (Fig. 3) and thus can fine-tune neuronal activity and synaptic transmission (Newman 2003; Fellin *et al.* 2004; Kozlov *et al.* 2006; Jourdain *et al.* 2007). The proposal, that astrocytes play an active role as signaling elements, led to the concept of the “tripartite synapse”. In this model, in addition to pre- and postsynaptic terminals,

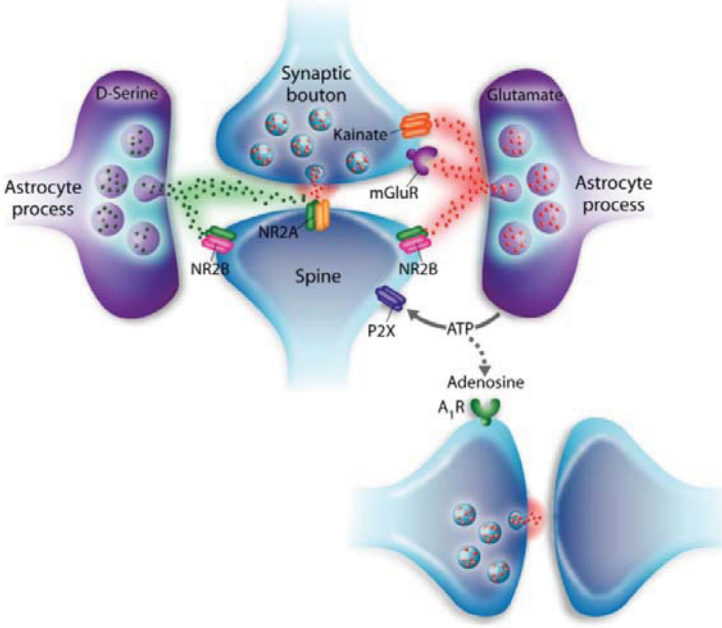

Figure 3: astrocytes modulate synaptic activity (Haydon, Carmignoto 2006) [for explanation see text]

perisynaptic processes of astrocytes are regarded as important structural components involved in the processing, transfer and storage of information (Perea *et al.* 2009; Haydon 2001).

Astrocytes usually express the connexins cx43, cx26 and/or cx30 (Rash *et al.* 2001), but show remarkable differences in the extent of gap junction coupling. Depending on age and precise location within a certain brain structure, they either form large syncytia, groups of various size or stay single and uncoupled. In addition, shape and orientation of the network vary, reflecting morphological and physiological characteristics of the brain region (Houades *et al.* 2006). Since astrocytes usually occupy separate domains, which overlap only very little (Fig. 4; Bushong *et al.* 2002; Nedergaard *et al.* 2003; Halassa *et al.* 2007), gap junction coupling is observed at the interface between such neighbouring domains, but exists also between processes of the same astrocyte (Rohlmann, Wolff 1998). There are as well reports suggesting that gap junction coupling is present not only among astrocytes but also between astrocytes and neurons or oligodendrocytes (Rozental *et al.* 2001; Nagy, Rash 2000; Orthmann-Murphy *et al.* 2008). Thus, a network-like communication contributes to signal integration in astrocytes.

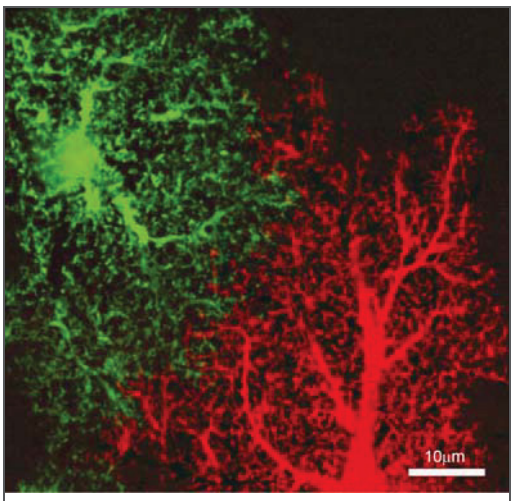

Figure 4: domains of neighbouring astrocytes overlap only very little (green: Lucifer Yellow, red: Alexa568; Bushong *et al.* 2004)

Among the consequences of the interaction between the neuronal and the astrocytic network is the introduction of additional levels to synaptic plasticity (Todd *et al.* 2006; Perea, Araque 2007; Wenzel *et al.* 1991). Since one astrocyte contacts usually several synapses and astrocytes communicate with each other via gap junctions or gliotransmitter release, their assignment in information processing can reach beyond the modulation of a single synapse. For example, upon activation of metabotropic receptors, ATP, which is released from astrocytes and degraded to adenosine, is responsible for activity-dependent heterosynaptic depression (Fig. 3; Zhang *et al.* 2003; Pascual *et al.* 2005; Serrano *et al.* 2006).

In addition to gliotransmitters, astrocytes can release a complex assortment of substances, which act in numerous ways on neuronal function and viability. These signal molecules include cytokines and neurotrophic factors (Farina *et al.* 2007; Liberto *et al.* 2004), glutathione (Dringen *et al.* 2000), hydrogen sulfide (Kimura *et al.* 2005; Lee *et al.* 2009), hormones, growth factors, thrombospondins, cholesterol and apolipoprotein E (Barres 2008; Pfrieger 2010). Astrocytes also exert important influence on synapse formation, stability, maintenance and plasticity by release of such factors (Ullian *et al.* 2004; Christopherson *et al.*, 2005; Pfrieger 2010) as well as by contributing to the extracellular matrix (Faissner *et al.* 2010).

The metabolic support astrocytes give to neurons reflects another aspect of the tight relationship between these two cell types. Two important theories have evolved over the years: The concept of the “glutamate-glutamine cycle” describes the link between the uptake of glutamate into astrocytes, its conversion to glutamine and the release of glutamine from astrocytes. Neurons subsequently take up glutamine as a resource for the production of glutamate and GABA (McKenna 2007; Albrecht *et al.* 2007; Bak *et al.* 2006). The concept of the functional connection between neuronal activity, astrocytic glucose metabolism and metabolite supply to neurons (Brown, Ransom 2007; Pellerin *et al.* 2007) was termed the “lactate shuttle” (Fig.6).

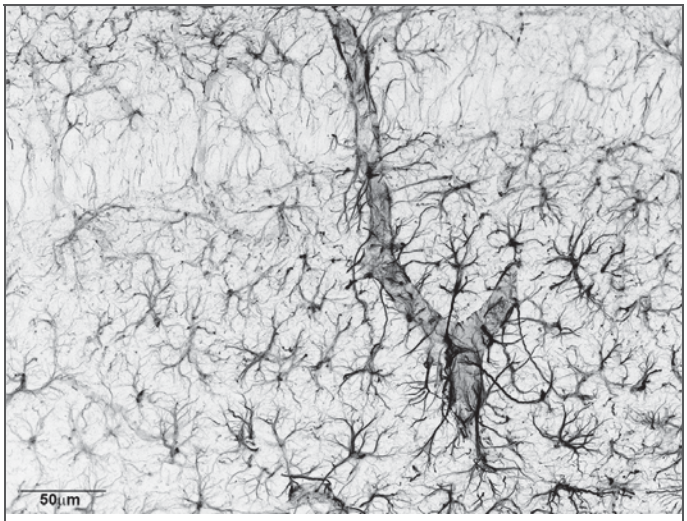

Figure 5: GFAP staining of hippocampal astrocytes, some of them contacting a blood vessel (courtesy of Alexandra Rduch)

Astrocytes are in the perfect anatomical position to sense neuronal activity on the one hand and support brain energy supply on the other hand, since in addition to contacting synapses, they get in touch to blood vessels (Fig. 5), forming structures called endfeet (Kacem *et al.* 1998). In fact, the cerebral vasculature is tightly enwrapped by these astrocyte processes, which contribute to the blood-brain-barrier (Abbot *et al.* 2006).

As already mentioned, transmitters, released at synapses, activate ionotropic and metabotropic receptors as well as transmitter uptake at perisynaptic astrocyte processes. Some of the subsequent ion currents and second messenger cascades could act as mediators in the intracellular chain of events, that connects sensing neuronal activity with a haemodynamic response, glucose uptake and increased astrocytic metabolism.

Intracellular calcium signals, as they are, for instance, elicited by activation of metabotropic glutamate receptors, were reported to be involved in the modulation of blood vessel diameter by astrocyte endfeet (Metea, Newman 2006; Zonta *et al.* 2003; Takano *et al.* 2006; Gordon *et al.* 2007; Koehler *et al.* 2009). The effect astrocytes exert on capillaries and arterioles was found to be precisely modulated not only by the energy need of the tissue but also by its oxygen demand (Gordon *et al.* 2008). Furthermore, ion channels and aquaporin-4, expressed in astrocyte endfeet are involved in water and ion homeostasis of brain tissue (Gunnarson *et al.* 2008; Simard, Nedergaard 2004; Zelenina 2010).

The glucose transporter GLUT-1 (Morgello *et al.* 1995; Vannucci *et al.* 1997) is expressed in astrocyte endfeet, facing the main source for glucose in the brain. Astrocytes take up glucose, exhibit a vivid glucose metabolism and contain high levels of glycogen (Magistretti 2006; Schousboe *et al.* 2007; 2010). For astrocytes in culture it was shown, that sodium elevations accompany glutamate uptake and subsequently stimulate the Na^+/K^+-ATPase. The increased ATP consumption enhances aerobic glycolysis, glycogen breakdown, glucose uptake and lactate release (Fig. 6; Pellerin, Magistretti 1994; Chatton *et al.* 2000; Loaiza *et al.* 2003; Porras *et al.* 2008), supporting the “lactate shuttle” concept.

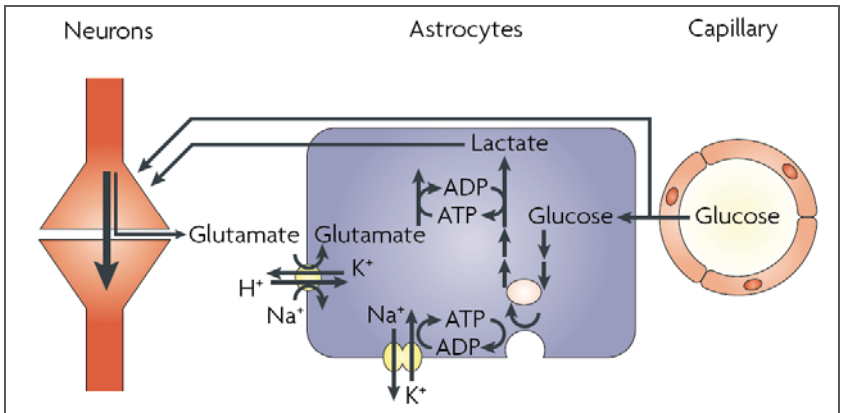

Figure 6: lactate shuttle theory (Giaume *et al.* 2010) [for explanation see text]

In the intact tissue metabolite diffusion in the astrocyte network is necessary to sustain neuronal function. There is evidence that AMPA receptor activation supports the diffusion of glucose and lactate through gap junctions (Rouach *et al.* 2008; Giaume *et al.* 2010). In addition AMPA receptor dependent lactate release via the monocarboxylate transporter MCT1 might contribute to targeted energy supply to neurons (Kleene *et al.* 2007). Furthermore, it was reported that glycogenolysis, lactate release from astrocytes and lactate uptake by neurons is critical for memory formation *in vivo* (Suzuki *et al.* 2011)

Taken all these findings together, in more than a hundred years of research it was revealed, that astrocytes are involved in almost all aspects of brain function. Strategically localized between synapses of the same and different neurons as well as between neurons and blood vessels, they add their share to tissue architecture, synapse function, information processing and energy supply. The intimate interplay between the neuronal and astrocytic network introduces complex levels of signal integration as well as structural and physiological plasticity.

2. Ion signaling in Astrocytes

In order to better understand the various duties astrocytes serve in brain function, it is necessary to take a closer look at the multitude of signaling events inter-coordinated and dynamically modulated in and around these cells.

A common feature of astrocytes is a negative membrane potential in the range of -60 to -90 mV, which is sustained by the Na^+/K^+-ATPase. As a result, astrocytes maintain a steep inwardly directed sodium gradient and an outwardly directed potassium gradient over the cell membrane. In addition there are strong inwardly directed gradients for calcium ions and protons. Many of the contributions astrocytes make to the complex signaling ambience of brain tissue are dependent on, or modulated by, processes, which rely on these ion gradients. Thus, it is important for the understanding of astrocyte physiology to thoroughly investigate the signal qualities of ion concentration changes as they appear for instance upon synaptic activity.

calcium

An ion well known as a signal transducer is calcium. Its concentration in the cytosol is kept close to 100 nM, in internal stores it is above 100 µM, and in the extracellular space it reaches 1-2 mM. The intracellular calcium concentration is actively kept low by the plasma membrane calcium ATPase (PMCA), the sodium/calcium exchanger (NCX) and the sodium/calcium/potassium exchanger (NCKX), which in forward-mode drive calcium out of the cell. In addition, the sarcoendoplasmic calcium ATPase (SERCA) transports calcium into internal stores (Fig. 7). Furthermore, calcium binding proteins of the cell cytoplasm provide a calcium buffer capacity (Verkhratsky *et al.* 1998; Clapham 2007).

A wide variety of signaling molecules, including hormones, growth factors, chemokines, prostaglandins, ATP and several neurotransmitters, induce calcium signals in astrocytes (Fiacco, McCarthy 2006; Bezzi *et al.* 1998; Porter, McCarthy 1997). Many of the corresponding receptors belong to the family of metabotropic receptors and are coupled via G-proteins to second messenger pathways which include IP3-mediated calcium release from internal stores (S/ER; Fig. 7; Verkhratsky *et al.* 1998). Apart from this, other sources for calcium signals in astrocytes can be voltage gated calcium channels, calcium permeable cation channels, or the reverse mode of calcium transporters. In addition, calcium signals themselves could modulate calcium activated ion channels, supporting calcium induced calcium signals or store-operated calcium entry (SOC; Fig. 7) from the extracellular space (ECF; Clapham 2007). Furthermore they can evoke calcium dependent potassium currents and adjust the affinity of membrane receptors for their substrates. Calcium signals are

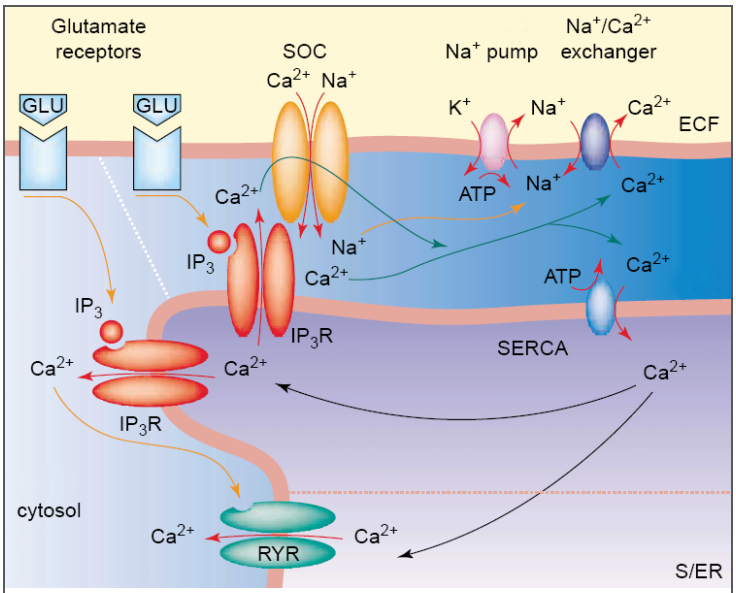

Figure 7: calcium homeostasis (Golovina, Blaustein 2001; modified) [for explanation see text]

also intimately linked to the function of numerous long-lasting intracellular processes via the regulation of enzymes, including protein kinases, protein phosphatases and adenylat cyclases, as well as via alteration of gene expression (Verkhratsky *et al.* 1998; Liu *et al.* 2010).

In several cell types the typical calcium signal is restricted to microdomains, its downstream effect largely determined by the local assortment of effectors. Interestingly, in astrocytes some spontaneous as well as stimulated calcium signals were found to travel from the site of their origin to other parts of the cell (Nett *el al.* 2002; Honsek *et al.* 2010). Beside this intracellular spreading signal, also intercellular calcium waves were recorded (Agulhon *et al.* 2008; Schipke, Kettenmann 2004; Parri *et al.* 2001). In astrocytes from different brain regions they occur either as spontaneous oscillations, or upon neuronal activity and their intercellular spread depends on gap junction coupling and/or ATP release (Wang *et al.* 2006; Nimmerjahn *et al.* 2009; Kuga *et al.* 2011; Scemes, Giaume 2006).

As already mentioned, astrocytes can release a variety of substances. Some of the proposed release mechanisms are calcium independent – like reverse of neurotransmitter transport (Szatkowski *et al.* 1990; Rossi *et al.* 2000) and efflux through connexin hemichannels (Ye *et al.* 2003), purinergic P2X7 receptors (Duan, Neary 2006; Haydon, Carmignoto 2006) or anion channels (Kimelberg *et al.* 2006). But there is also growing evidence for calcium dependent vesicular release of gliotransmitters. Many proteins belonging to the exocytose machinery have been found in astrocytes (Montana et al., 2004; Volterra, Meldolesi 2005; Hamilton, Attwell 2010). Consequently, calcium and SNARE complex dependent release of glutamate, d-serine and ATP from astrocytes was reported (Bezzi *et al.* 2004; Haydon, Carmignoto 2006; Mothet *et al.* 2005; Parpura, Zorec 2010).

Calcium dependent release of gliotransmitters supports the concept of the “tripartite synapse” and might be involved in several types of synaptic plasticity (Perea, Araque 2010). In addition calcium dependent intercellular signaling exists, which does not involve the classical exocytose machinery. For example, calcium signals reaching astrocyte endfeet add to the haemodynamic response by opening

calcium activated potassium channels, which would support "potassium syphoning" from areas of neuronal activity and dilate blood vessels (Gordon *et al.* 2007; Koehler *et al.* 2009). Furthermore, calcium rises activate phospholipase A_2 and the production of arachidonic acid (AA), which can be released from astrocyte endfeet and evoke vessel constriction (Fig. 8). Its metabolites, including prostaglandins (PGs) and epoxyeicosatrienoic acids (EETs), can evoke vessel dilatation (Fig. 8). Whether a calcium signal leads to vessel constriction or dilation, is determined by vessel tone, oxygen saturation and neuronal NO production (Gordon *et al.* 2007; Attwell *et al.* 2010; Koehler *et al.* 2009).

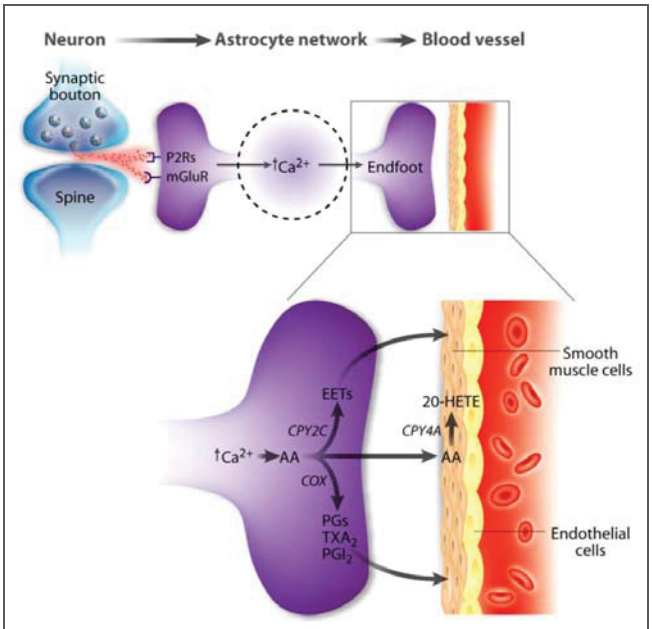

Figure 8: calcium signals connect synaptic activity to a haemodynamic response (Haydon, Carmignoto 2006) [for explanation see text]

In the light of neuron to glia and glia to neuron and/or blood vessel signaling, the release of gliotransmitters and signal molecules from astrocytes in response to synaptic activity is most important. The fact that calcium signals travel – inside one astrocyte or among several of them – reflects a way of information processing. Signals, derived from several synapses, might lead to modulation of several neurons and to the activation of astrocyte endfeet, thus regulating blood flow in order to meet oxygen and glucose demands. Accordingly, the spatial profile and time course of astrocytic calcium signals seem to be critical for the fine-tuning of brain functions.

pH

The intracellular pH of neurons and glia cells is actively maintained at a value between 7.0 and 7.4, similar to the extracellular pH in nervous tissue. As a result, because of the negative membrane potential of astrocytes, there is an inwardly directed electrical driving force for protons. This gradient delivers energy for

several transport processes, but also accounts for an intracellular acidification, whenever protons or bicarbonate ions are allowed to diffuse or are transported along their gradient (Deitmer, Rose 2010). The pH has a strong impact on functional properties of many proteins, such as enzymes, ion channels and connexins. Consequently, changes in pH modulate a variety of cellular functions, including metabolism, signaling processes and gap junction coupling.

The maintenance of the pH requires energy, usually provided by the sodium gradient over the plasma membrane, which drives the sodium/hydrogen exchanger (NHE) and the sodium/bicarbonate cotransporter (NBC; Fig. 9). The glial NBC is reversible and electrogenic, carrying one sodium ion together with two bicarbonate ions. Thus, it is modulated by the sodium, proton and bicarbonate concentrations as well as by the membrane potential (Deitmer, Rose 2010; Chesler 2003).

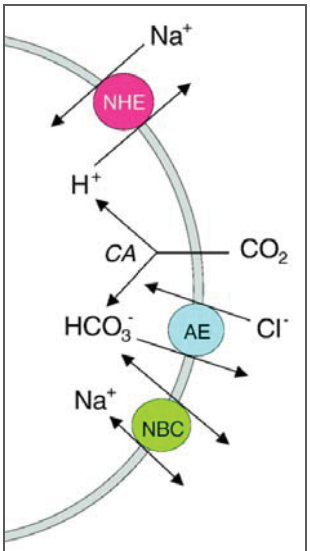

Figure 9: pH homeostasis (Deitmer, Rose 2010) [for explanation see text]

During neuronal activity an increased extracellular potassium concentration as well as the activation of astrocyte receptors and transmitter transport induces a depolarization of the astrocyte membrane potential. This depolarization is accompanied by an intracellular alkalinization due to the activation of the NBC (Pappas, Ransom 1994; Chesler 2003). This effect might counterbalance passive proton influx on the one hand and increase the driving force for proton dependent transport processes on the other hand, thus supporting calcium removal from the cytoplasm via the proton dependent PMCA and SERCA (Fig. 7; Clapham 2007) as well as glutamate uptake (Danbolt 2001) and vesicle loading (Schuldiner *et al.* 1995), processes that are accompanied by proton transport.

Also the uptake and release of glutamine via the glutamine transporter SNAT3 is pH dependent, since it cotransports glutamine with sodium ions in exchange for protons (Bröer *et al.* 2002; Chaudhry *et al.* 2001; Fei *et al.* 2000). Consequently, the steeper proton gradient after synaptic activity and the NBC dependent increase in pH buffer capacity might favor glutamine release over uptake (Wendel *et al.* 2008).

The $CO_2/H^+/HCO_3^-$ buffer system of living tissue is modulated by intra- and extracellular carbonic anhydrase (CA; Fig. 9), which contributes to the shaping of pH transients. In astrocytes CA activity is higher than in neurons, making astrocytes an important player in the maintenance of the tissue pH during phases of high neuronal activity and concomitant increased CO_2 production (Deitmer 2002). Some CA isoforms directly bind to and modulate the activity of acid/base transporters and the monocarboxylate transporter MCT1 (Deitmer, Rose 2010).

The MCT transporter family carries lactate and other monocarboxylate anions in cotransport with a proton across the cell membrane. Astrocytes can release these metabolites into the extracellular space via MCT1 and MCT4, while MCT2 enables neurons to take them up (Pierre, Pellerin 2005). These observations support the aforementioned “lactate shuttle hypothesis”, linking pH changes to the coordination of energy supply to neurons (Deitmer 2002; Brown, Ransom 2007; Pellerin *et al.* 2007). In addition, extracellular lactate stimulates accumulation of prostaglandin in the extracellular space, favoring vasodilatation (Gordon *et al.* 2008), which would support energy supply. Glutamate uptake and lactate production might induce a delayed acidification of astrocytes (Chesler 2003) after synaptic activity, which would favor lactate release.

Taken together, intra- and extracellular pH changes occur upon synaptic activity and have to be compensated, mainly by astrocytes, in order to maintain brain function. At the same time, changes of the intracellular pH and of the proton gradient over the plasma membrane serve signal functions, since they modulate a large group of cellular processes, including calcium signaling and metabolic responses.

sodium

The sodium gradient over the plasma membrane serves as an energy source for numerous transport processes. The NCX and NCKX, which transport three or four sodium ions, respectively, in exchange for every calcium ion (Fig. 7/10; Clapham 2007), tightly connect the time course of calcium signals and their downstream effects, such as vessel dilatation or fine tuning of synapses, to the sodium gradient. The maintenance of the pH, and thus the modulation of

enzymes, receptors, ion channels, gap junctions and proton dependent transport of lactate and calcium are linked to the sodium gradient via the NHE and NBC (Fig. 9/10; Deitmer, Rose 2010; Chesler 2003). Changes in the sodium gradient would modulate the capacity of these transporters and thus affect a wide variety of cellular functions, which were found to underlie central assignments of astrocytes.

While the mentioned transporters serve calcium and pH homeostasis, they are as well mediators of sodium influx (Fig. 10). Additional possible sources for sodium entry into astrocytes are, for example, cation channels, store operated calcium channels (SOC; Fig. 7; Golovina 2005), transient receptor potential channels (TRPC; Eder *et al.* 2005) and the sodium/potassium/chloride cotransporter (NKCC; Chen, Sun 2005). In order to sustain the persistently challenged gradient, sodium is constantly pumped out of the cell by the Na^+/K^+-ATPase (Fig. 10; Rose, Ransom 1996-a). In addition it was found, for astrocytes in culture, that baseline intracellular sodium concentration is equalized between neighbouring cells via gap junctions (Rose, Ransom 1997). This would support the maintenance of the sodium gradient on the one hand, but would also distribute the effect of a sodium concentration change in the astrocyte network on the other hand.

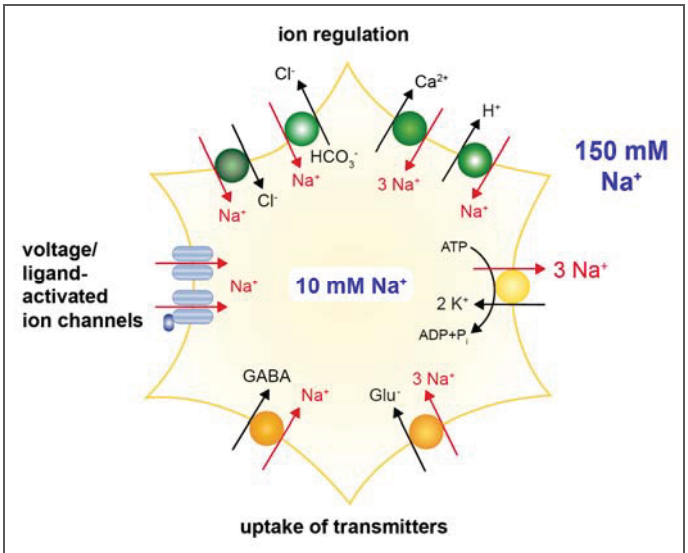

Figure 10: sodium homeostasis (courtesy of Christine Rose) [for explanation see text]

Apart from its association to calcium and pH signaling, the sodium gradient also serves as an energy source for transmitter uptake at synapses, another essential function of astrocytes. The glutamate transporters GLAST and Glt-1 cotransport three sodium ions and one proton and countertransport one potassium ion with every glutamate molecule (Fig. 10; Levy *et al.* 1998; Danbolt 2001; Owe *et al.* 2006). This stoichiometry suggests that high concentrations of extracellular glutamate would introduce a significant number of sodium ions into the cell.

The concept of the “lactate shuttle” involves transient sodium concentration changes as a link between glutamate uptake and activation of the Na^+/K^+-ATPase, which consumes ATP, while restoring the sodium gradient, and subsequently activates the cellular energy production (Voutsinos-Porche *et al.* 2003; Brown, Ransom 2007; Pellerin *et al.* 2007). This link was supported by results from astrocyte cell culture, where bath application of glutamate caused an elevation of the intracellular sodium concentration, primarily generated by activation of sodium-dependent glutamate uptake (Chatton *et al.* 2000; Rose, Ransom, 1996-b).

Additionally, a glutamate uptake induced sodium load in cultured astrocytes was found to reduce the cell surface expression of Glt-1 (Nakagawa *et al.* 2008) and to provide a negative feedback on glutamate uptake by reducing the driving force for glutamate transport (Kelly *et al.* 2009). This feedback might even result in glutamate release by reverse of transport (Szatkowski *et al.* 1990; Rossi *et al.* 2000), for example under pathophysiological conditions.

A comparable modulation exists for GABA transporters. The GABA transporter family GAT (Ribak *et al.* 1996; Minelli *et al.* 1995) cotransports two sodium ions and one chloride ion with every transmitter molecule (Wu *et al.* 2007). Glutamate uptake induced elevations in the sodium concentration can promote the release of GABA by transport reversal (Héja *et al.* 2009; Barakat, Bordey 2002).

Furthermore, in response to glutamate application the facilitative glucose transporter GLUT1 is activated by simultaneous intracellular sodium and calcium signals in cultured astrocytes (Porras *et al.* 2008). Consequently, an intercellular traveling sodium signal was followed by a spatially correlated increase in glucose uptake (Bernardinelli *et al.* 2004). Together with a possible promoting action on glutamine release via the sodium dependent SNAT3 (Bröer *et al.* 2002), these different observations support the idea of astrocytic sodium elevations as a link between synaptic activity, glial metabolism and metabolite supply to neurons (McKenna 2007; Pellerin *et al.* 2007).

While calcium and pH changes are well established as intra- and intercellular signals, changes in the sodium concentration were less intense studied in the past. It was assumed that the fast binding and transport of sodium by the Na^+/K^+-

ATPase would reduce amplitude and time course of possible physiological sodium transients to insignificant values. Still, it would add an important puzzle piece to the signaling mosaic to know, whether considerable sodium transients are induced in astrocytes in the intact tissue. The findings from cell culture suggest that glutamate uptake might be a source for sodium influx into astrocytes that would exert important consequences on calcium and pH signaling, maintenance of brain function by transmitter uptake and metabolic responses.

The present study should provide new insight into the complex signaling ambience that includes astrocytes as a crucial structural element for information processing and transport. It focuses on two types of astrocytes in two different well characterized brain regions: passive astrocytes in the CA1 *stratum radiatum* of the hippocampus and Bergmann glia cells in the cerebellum.

3. Classical Astrocytes of the Hippocampus

The hippocampus is a specialized structure of the limbic system in mammals and central to the formation of spatial memory. It is found bilateral symmetric in both hemispheres of the brain. One hippocampus has the shape of a curved tube and consists of the *cornu ammonis* and the *gyrus dentatus*, which pervade the structure along its longitudinal axis. In a transverse slice bowed layers of neuronal cell bodies become visible, the *stratum granulare* of the *gyrus dentatus* and the *stratum pyramidale* of the *cornu ammonis* (CA; Fig. 11). In the CA region, which is divided into CA1-4, the neighbouring layer towards the periphery of the “tube” was termed *stratum oriens*, and the layers towards the center are called *stratum radiatum* and *stratum lacunosum moleculare*.

Input reaches the hippocampus from the entorhinal cortex via the perforant path, a fiber track that projects mainly onto the granule cells of the dentate gyrus but also onto apical dendrites of CA1 and CA3 pyramidal neurons. Subsequently, granule cells project onto CA3 pyramidal neurons via the mossy fibers. The axons

of CA3 pyramidal neurons, the Schaffer collaterals, project into the CA1 *stratum radiatum*, where they make glutamatergic synapses onto the dendrites of CA1 pyramidal neurons. Finally the axons of CA1 neurons leave the structure into the subiculum and deeper layers of the entorhinal cortex. The cytoarchitecture of the hippocampus was intensively studied and the internal connections were found to transport and process information in a mainly unidirectional way (Fig. 11). The engaged synapses are well characterized, with a special focus on the phenomenon of long-term-plasticity, since it is believed to be the physiological manifestation of memory formation.

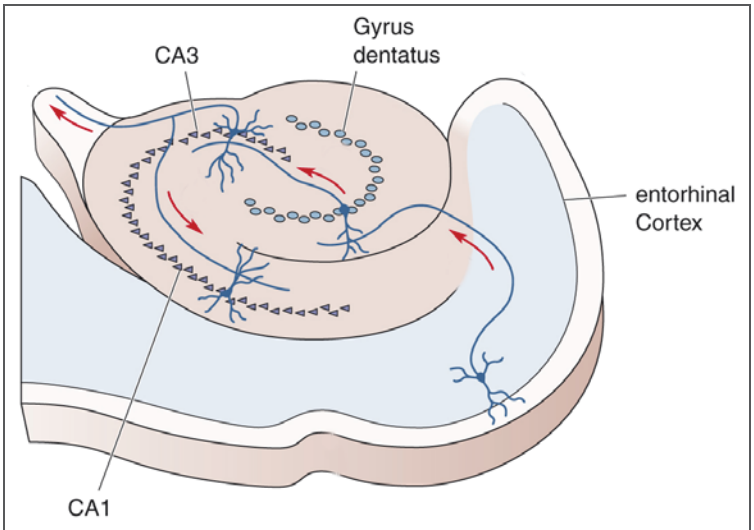

Figure 11: transverse slice of the hippocampus
(Baer *et al.* 2009; modified)

Astrocytes in the *stratum radiatum* of rodents exhibit a dense ramification of fine processes. Their complex shapes become more defined as well as restricted to distinct domains during maturation (Fig. 2/12; Nixdorf-Bergweiler *et al.* 1994; Bushong *et al.* 2004; Ogata, Kosaka 2002). For adult rodent distances of about 20 to 60 μm between neighbouring somata were reported (Xu *et al.* 2010).

Perisynaptic processes of astrocytes contact about 64% of hippocampal synapses to different extent (Witcher *et al.* 2007). At the same time, astrocytic cellular compartments sum up to only about 7% of the tissue volume (Ventura, Harris 1999). Thus, at many synapses the astrocytic coverage leaves enough space for glutamate to diffuse away from the synaptic cleft, to extrasynaptic regions and other synapses. The extent of astrocytic coverage of synapses determines the capacity for transmitter uptake, the activation of extrasynaptic receptors and the effect of gliotransmitters released by astrocytes (Lehre, Rusakov 2002). Therefore, a certain degree of morphological plasticity adds its share to the modulation of synaptic communication (Wenzel *et al.* 1991).

In the CA1 *stratum radiatum* of the hippocampus two types of astrocytes were found, which differ in their electrophysiological properties (Zhou, Kimelberg 2000). The membrane conductance of classical astrocytes is largely dominated by passive potassium currents in adult mice (Seifert *et al.* 2009), while another type, termed complex cell, expresses also voltage gated potassium and sodium channels (Steinhäuser *et al.* 1994-b).

Since passive astrocytes are characterized by a highly negative membrane potential, the expression of GABA (Ribak *et al.* 1996) and glutamate transporters (Steinhäuser *et al.* 1994-a; Zhou, Kimelberg 2001) as well as gap junction coupling (Wallraff *et al.* 2004) and a prominent potassium conductance (Seifert *et al.* 2009), they are well suited to fulfill the classical duties assigned to astrocytes. During maturation of the hippocampus, the percentage of passive astrocytes (Fig. 12), which can be reliably stained with SR101, increases to 90% (Kafitz *et al.* 2008; Zhou *et al.* 2006).

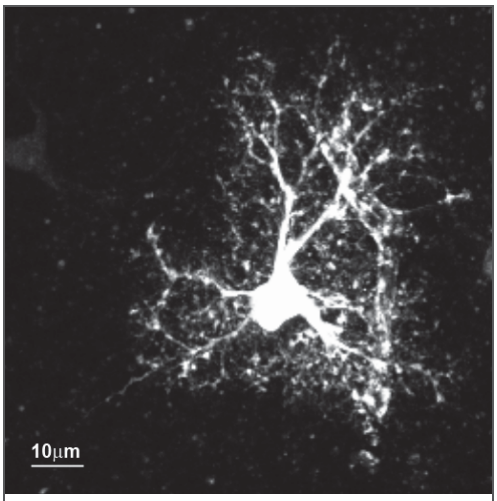

Figure 12: passive hippocampal astrocyte contacting a blood vessel (SBFI; two-photon)

GLAST and Glt-1 are the main glutamate transporters expressed by classical hippocampal astrocytes (Rothstein *et al.* 1994; Lehre *et al.* 1995). Correlating with the upregulation of glutamate transporter expression during the development of the hippocampus, glutamate dehydrogenase activity also increases (Kugler, Schleyer 2004), gearing astrocytes as a sink for glutamate. Adult hippocampal passive astrocytes show a low expression of AMPA type ionotropic glutamate receptors (Zhou, Kimelberg 2001) and a substantial expression of metabotropic glutamate receptors (Schools, Kimelberg 2001; Honsek *et al.* 2010). Purinergic receptors are a feature of only a subset of hippocampal astrocytes (Zhu, Kimelberg 2004).

At inhibitory synapses of interneurons, GABA is taken up into astrocytes via the transporters GAT-1 and GAT-3 (Ribak *et al.* 1996). Activation of astrocytic $GABA_A$ receptors, at the same time, induces a chloride conductance (Zhou,

Kimelberg 2001), which leads to a membrane depolarization and causes calcium influx through voltage-gated calcium channels (Fraser *et al.* 1995). Metabotropic $GABA_B$ receptors mediate calcium release from internal stores in passive astrocytes (Meier *et al.* 2008).

Astrocytes of the hippocampus exhibit spontaneous calcium oscillations as well as calcium signals upon neuronal activity (Nett *et al.* 2002; Honsek *et al.* 2010). Calcium signaling discriminates and integrates synaptic activity (Perea, Araque 2005) and thus reflects a level of information processing. It can be restricted to cell processes, travel throughout a single cell (zur Nieden, Deitmer 2006; Honsek *et al.* 2010) or propagate between astrocytes in a wave like manner that is dependent on ATP release (Sul *et al.* 2004; Kuga *et al.* 2011).

While the impact of astrocytes on short- and long-term-potentiation in the hippocampus is still discussed controversially (Andersson, Hanse 2010; Agulhon *et al.* 2010; Henneberger *et al.* 2010; Yang *et al.* 2003), the modulation of neuronal activity by astrocytes is proposed to at least include neuron synchronization (Angulo *et al.* 2004; Fellin *et al.* 2004), potentiation of transmitter release (Perea, Araque 2007; Fiacco, McCarthy 2004; Jourdain *et al.* 2007) and heterosynaptic depression (Serrano *et al.* 2006; Andersson *et al.* 2007).

Thus, sensing and modulating synaptic activity of the hippocampus, classical astrocytes are intimately involved in the information processing of a brain structure central to memory formation.

4. Bergmann Glia cells of the Cerebellum

The cerebellum is a structure of the metencephalon, which is involved in motor coordination and motor learning. It is largely separated from the rest of the brain, all its connections traveling through the pons. It is divided into two hemispheres with a connecting midline zone along the axis of the brain. The cerebellar cortex is strongly folded, giving rise to an enormously large surface.

The principal neurons of the cerebellum are the Purkinje cells. Their large cell bodies are organized in the Purkinje cell layer, while their huge dendritic trees reach towards the surface of the cerebellum into the molecular layer (Fig. 13). The dendritic trees of these neurons have a very characteristic shape – although highly branched, they extend in an almost two-dimensional manner in sagittal planes.

Underneath the Purkinje cell layer, the granule cell layer is located. Granule cells receive input from mossy fibers, which project into the cerebellum from several different brain regions. The granule cells themselves give rise to the parallel fibers, which project into the molecular layer, where they split into two branches (Fig. 13). These axon branches pass vertically through the dendritic trees of several Purkinje neurons, making each about 100 synaptic contacts.

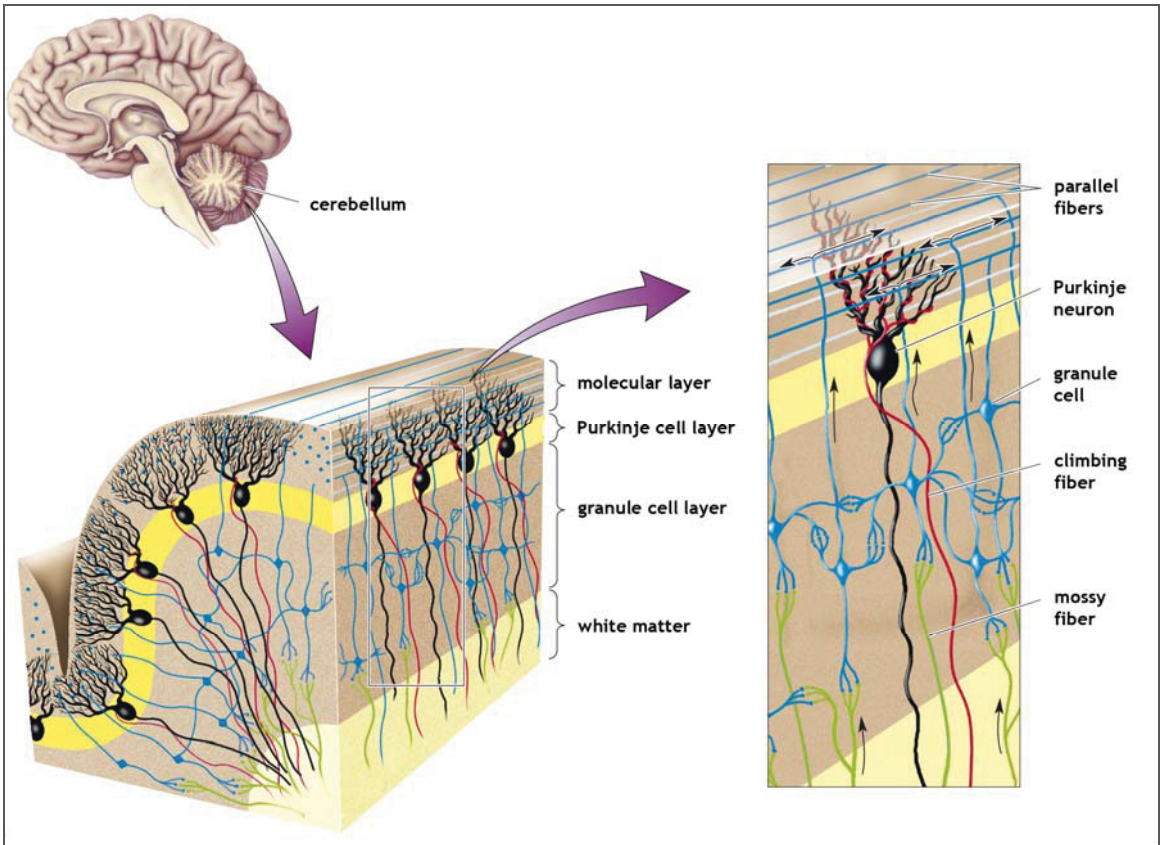

Figure 13: cerebellum (Bear *et al.* 2009; modified)

Each Purkinje neuron receives glutamatergic synaptic input from 60000 to 175000 granule cells (Ito 2000), which induce graded electrophysiological responses (Konnerth *et al.* 1990) and localized dendritic calcium transients (Eilers *et al.* 1995-a). In addition Purkinje neurons receive glutamatergic input from climbing fibers, which originate in the inferior olive of the *medulla oblongata*. One

climbing fiber makes several 10000s of synapses onto the dendritic tree of a single Purkinje neuron (Ito 2000), thus its activation results in an all-or-none electrical response (Konnerth *et al.* 1990) and global dendritic calcium increase in the targeted Purkinje neuron (Eilers *et al.* 1995-b).

Purkinje neurons (Fig. 14) are spontaneously active, their firing pattern modulated by the aforementioned excitatory inputs as well as inhibitory input from interneurons (Häusser, Clark 1997; Raman, Bean 1999). Together with direct projections of mossy fibers and climbing fibers, Purkinje neurons project into deep cerebellar nuclei, where they make inhibitory synapses releasing GABA. Therefore, long-term-depression (LTD) of the parallel fiber input (Barski *et al*. 2003; Ito 2000) and/or of the climbing fiber input (Hansel, Linden 2000) is believed to be the physiological event underlying cerebellar motor learning (Rose, Konnerth 2001-a).

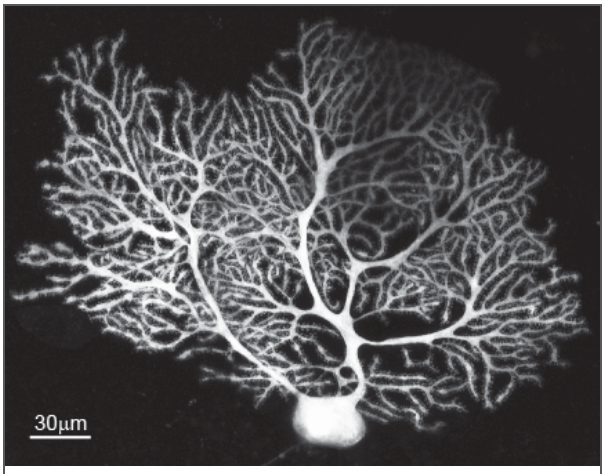

Figure 14: Purkinje neuron (Alexa594; two-photon)

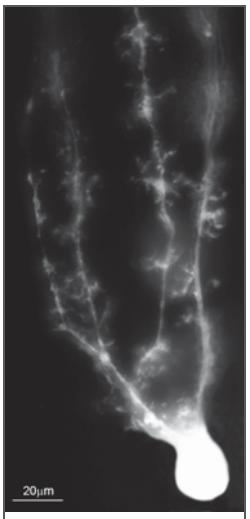

Figure 15: Bergmann glia cell (SBFI; wide-field; out-of-focus-light around cell body masked for clarity)

Bergmann glia cell somata are located in the Purkinje cell layer. Each of them sends four to five primary processes through the molecular layer (Fig. 15), terminating at the pial surface. Bergmann glia cells are coupled by gap junctions only in the direction orthogonal to the parallel fibers (Müller *et al.* 1996; Tanaka *et al.* 2008). The resulting planar networks form layers with the same orientation like the dendritic trees of the neighbouring Purkinje neurons. Thin protrusions originating from Bergmann glia primary processes enwrap tightly about 65% of parallel fiber synapses and about 87% of climbing fiber synapses on Purkinje neurons (Xu-Friedman *et al.* 2001). Bergmann glia cellular compartments make up about 33% of the tissue volume and leave only little space for spillover of transmitter (Lehre, Rusakov 2002).

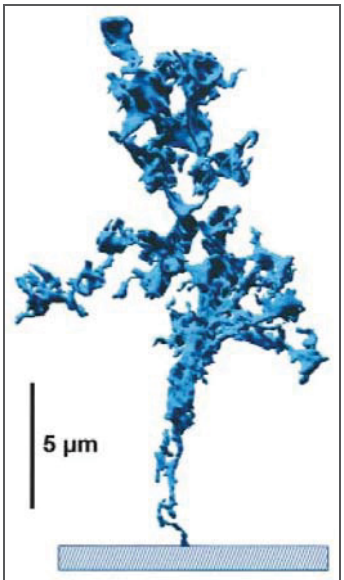

Figure 16: reconstruction of an appendage of a Bergmann glia cell (Lucifer Yellow; EM; Grosche *et al.* 1999)

The glial sheath of parallel fiber synapses increases during the first postnatal weeks, as the ramification of Bergmann glia fibers becomes more complex (Grosche *et al.* 2002). In the adult animal, side branches and lateral appendages (Fig. 16), which represent up to 90% of the cell surface, consist of repeats of so called microdomains (Grosche *et al.* 1999). On average, a glial microdomain makes contact to about five synapses, but every synapse is contacted by only one glia cell, its synaptic cleft always tightly sealed (Grosche *et al.* 2002).

The intimate relation between individual synapses and their glial microdomain might meet the functional diversity of Purkinje cell synapses. Calcium signals upon synaptic activity can be restricted to such microdomains or spread between them (Grosche *et al.* 1999; Beierlein, Regehr 2006). Furthermore, calcium signals can form spontaneous waves, which are based on ATP release and include parts of several cells (Hoogland *et al.* 2009). Calcium waves were also found in awake mice, their spread range depending on behavior (Nimmerjahn *et al.* 2009).

Thus, the downstream effects of those calcium signals, like gliotransmitter release (Oheim *et al.* 2006), could reach variable areas, modulating different numbers of synapses, depending on the input (Balakrishnan, Bellamy 2009).

Calcium signals in Bergmann glia cells can be mediated by activation of metabotropic glutamate, purinergic or adrenergic receptors (Meguro *et al.* 1999; Kirischuk *et al.* 1995; Piet, Jahr 2007; Kulik *et al.* 1999; Beierlein, Regehr 2006). In addition, the ionotropic AMPA type glutamate receptors of these cells are highly permeable for calcium, while there is no evidence for the expression of voltage gated calcium channels (Müller *et al.* 1992; Piet, Jahr 2007). Mature Bergmann glia cells are characterized by large, passive potassium currents (Müller *et al.* 1994). Upon calcium entry the resting potassium conductance is reduced, resulting in a membrane depolarization (Müller *et al.* 1994).

Bergmann glia cells express the GABA transporter GAT-1 (Barakat, Bordey 2002) and the glutamate transporters GLAST and Glt-1 (Takatsuru *et al.* 2007; Rothstein *et al.* 1994; Lehre *et al.* 1995). The expression level of glutamate transporters and the spatial relation of glial processes to synapses undergo plastic changes upon AMPA receptor activation (Bellamy, Ogden 2006; López-Bayghen *et al.* 2003). This plasticity phenomenon can lead to an input specific depression of glial activation (Balakrishnan, Bellamy 2009) with consequences for the glia to neuron signaling. At the same time, glutamate escaping the uptake into glia cells enhances postsynaptic currents in Purkinje neurons and alters the activation of pre- and extrasynaptic receptors (Clark, Cull-Candy 2002; Marcaggi *et al.* 2003). Activation of perisynaptic metabotropic glutamate receptors of Purkinje neurons was found to be involved in LTD (Rose, Konnerth 2001-a). Thus, the efficiency of glutamate uptake into Bergmann glia cells is centrally involved in different types of synaptic plasticity.

Bergmann glia cells have many properties in common with other astrocytes, which enables them to sense and modulate synaptic activity in addition to their assignment for maintenance of ion homeostasis and extracellular transmitter levels. Nevertheless, their principle shape and the complex morphology of their processes are quite special. Their extremely tight relation to distinct types of synapses and their different types of calcium signals – from extremely small microdomains up to waves, which reach several hundreds of cells – seem to add an advanced level to information processing in the cerebellum.

5. Aim of the study

As the introduction points out, the cerebellum and the hippocampus are well characterized in terms of their anatomy and central function as well as morphology of single cells, properties of neuronal circuits and some aspects of astrocyte physiology. The intimate interplay between the neuronal and the astrocytic network introduces complex levels of signal integration and plasticity in these brain structures, which might add as a physiological component to memory formation. Unfortunately, not all aspects of this interaction are yet unraveled.

Vital astrocytic functions like transmitter uptake at synapses and ion homeostasis are linked to the sodium driving force over the plasma membrane. Changes in this driving force thus might have important consequences for synaptic transmission as well as calcium and pH signaling, which modulate synapse fine-tuning and metabolic responses. For astrocytes in cell culture sodium elevations were identified as links between glutamate uptake and interstages of the “lactate shuttle”. Unfortunately, it was unknown whether under physiological conditions significant sodium transients occur in astrocytes in the intact tissue.

This question was addressed in the present study. Furthermore, the nature of such physiological sodium concentration changes was investigated in order to shed light on this formerly disregarded possible intra- and intercellular event.

Quantitative fluorescence imaging with the sodium sensitive dye SBFI was used to visualize intracellular sodium concentration changes, which accompany synaptic activity, in neurons and astrocytes. Synaptic stimulation or agonist application was performed *in situ* in acutely prepared tissue slices of hippocampus or cerebellum. The patch-clamp technique provided related electrophysiological data and allowed the selective dye-filling of single astrocytes in order to elucidate the spatial and temporal pattern of sodium transients in fine cellular branches with low background fluorescence. Pharmacological approaches revealed the source of sodium transients upon synaptic activity *in situ*. Additionally, pharmacological experiments and the employment of genetically modified animals granted information about intra- and intercellular sodium spread.

6. Summary of Results and Discussion

The experimental results presented in this study demonstrate for the first time that synaptic activity in the intact tissue induces significant sodium transients in Bergmann glia cells of the cerebellum (Bennay *et al.* 2008) and classical astrocytes of the hippocampus (Langer, Rose 2009). Furthermore, they reveal that sodium transients can propagate to neighbouring astrocytes via gap junctions in the hippocampus *in situ* (Langer *et al.* under revision).

In the cerebellum, well in line with earlier observations (Callaway, Ross 1997; Kuruma *et al.* 2003), parallel fiber stimulation induced inward currents and sodium transients in Purkinje neurons which were mainly caused by AMPA receptor activation. In agreement with a recent study (Kirischuk *et al.* 2007), somatic inward currents and long lasting sodium transients were also elicited in Bergmann glia cells. These sodium transients were found to result mainly from glutamate uptake, while a smaller component was due to AMPA receptor activation. Peak amplitude of sodium signals was dependent on stimulation strength and thus on the number of activated fibers. It reached up to several millimolar in fine cellular processes, which were probably closest to the activated synapses and thus represented the site of main glutamate uptake. Sodium signals had rise times of several seconds and lasted for tens of seconds. In neighbouring cellular domains sodium signals with smaller peaks and slower kinetics were recorded, indicating intracellular diffusion of sodium.

The stimulation of climbing fibers induced inward currents and sodium signals in Bergmann glia cells as well. These transients, however, exhibited similar kinetics, amplitudes and time courses throughout the entire tree of Bergmann glia cell processes.

These two different spatial patterns of sodium transients followed the profile expected from the synapse distribution of the two different inputs onto the dendritic tree of Purkinje neurons (Ito 2000). The different intracellular sodium signal patterns upon stimulation of parallel or climbing fibers could be interpreted as a capacity of Bergmann glia cells for discriminating the input source.

In the hippocampus Schaffer collateral stimulation evoked sodium transients in both pyramidal neurons and passive astrocytes of the CA1 stratum radiatum. Neuronal sodium signals were mainly attributable to sodium influx through ionotropic glutamate receptors, confirming earlier work (Rose, Konnerth 2001-b). Activation of ionotropic receptors also contributed to sodium transients of astrocytes, while glutamate transport was the major source. Similarly to Bergmann glia cells, synaptically induced sodium transients in hippocampal astrocytes reached amplitudes in the millimolar range and lasted for tens of seconds. At low stimulation intensities, sodium transients in astrocytes were confined to one or two primary branches and their adjacent fine processes, indicating that these regions were close to active synapses. Increasing the number of activated fibers by increasing the stimulation intensity elicited sodium transients which reached all processes and the somata of implicated astrocytes.

In order to analyze the characteristics of sodium propagation in the astrocyte network, single astrocytes in the hippocampus were selectively loaded with sodium by direct stimulation. Sodium signals were found to propagate in a radial manner to all neighbouring passive astrocytes within a radius of about 100 µm. Signal amplitude, slope and apparent velocity in downstream cells decayed monotonically with increasing distance from the centre cell. Expression of connexin Cx30/Cx43 containing gap junctions was essential for sodium propagation between astrocytes *in situ*. The calculated propagation speed ranged from more than 60 µm/s to less than 10 µm/s at distances greater than 60 µm from the centre cell. The true propagation speed in small cellular domains might be considerably larger, because of the elaborate morphology of astrocyte processes *in situ* (Bushong *et al.* 2004).

Intercellular calcium waves are well established as a basis for long-range signaling (Scemes, Giaume 2006; Agulhon *et al.* 2008), but this study shows, that in addition long lasting sodium signals rapidly propagate to neighbouring cells. Sodium propagation was independent of a concomitant calcium wave, but supported by release of glutamate. The study suggests that sodium signals generated in a single cell would reach about ten directly coupled astrocytes (Xu et al. 2010) appreciably faster than possible parallel calcium waves.

Considering the complex interdigitation of ion homeostasis, presented in the introduction, a significant sodium load in astrocyte processes would influence the time course of signaling events. It would reduce the driving force for NCX and NCKX and might thus prolong calcium signals and eventually support their downstream processes, such as haemodynamic responses (Gordon *et al.* 2007; Koehler *et al.* 2009) or fine tuning of synapses (Parpura, Zorec 2010; Perea, Araque 2010). Additionally, it would reduce the driving force for the NHE and NBC (Deitmer, Rose 2010; Chesler 2003), modulating pH changes and thus indirectly altering the activity of enzymes, receptors, ion channels, gap junctions and proton dependent transport of lactate and calcium. An intracellular sodium elevation might also promote glutamine release via SNAT3 (Bröer *et al.* 2002) after synaptic activity.

Similar to calcium and proton transporters, the sodium gradient over the plasma membrane provides energy for glutamate and GABA transporters (Danbolt 2001; Wu *et al.* 2007). Together with a membrane depolarization, induced by synaptic activity, sodium transients would thus significantly reduce the capacity of glutamate uptake (Kelly *et al.* 2009). After traveling intra- and intercellular, sodium transients might also reduce the glutamate or GABA uptake at other synapses. Elevations in the sodium concentration might even promote the release of GABA by transport reversal (Héja *et al.* 2009; Barakat, Bordey 2002).

Reduction of glutamate uptake would both change the time course of synaptic transmission and support the activation of pre- and extrasynaptic receptors (Marcaggi *et al.* 2003; Huang, Bergles 2004; Tzingounis, Wadiche 2007). In Purkinje neurons, for example, activation of perisynaptic metabotropic glutamate receptors was found to be involved in LTD (Rose, Konnerth 2001-a).

Furthermore, astrocyte sodium loads result in increased Na^+/K^+-ATPase activity and thus promote pump-mediated uptake of extracellular potassium (Kimelberg 2010). The propagation of sodium signals through gap junctions would recruit several cells in the active area for the restoration of the sodium and the potassium gradient, while at the same time lowering the burden imposed on a single cell.

Additionally, the increased ATP hydrolysis by the Na^+/K^+-ATPase has been suggested to promote aerobic glycolysis, glycogen breakdown, glucose uptake and production of lactate, which could then serve as metabolic fuel for activated neurons (Chatton *et al.* 2000; Loaiza *et al.* 2003; Brown, Ransom 2007; Pellerin *et al.* 2007). In cultured astrocytes the facilitative glucose transporter GLUT1 is activated by simultaneous intracellular sodium and calcium signals in response to glutamate application (Porras *et al.* 2008), and an intercellular traveling sodium signal was followed by a spatially correlated increase in glucose uptake (Bernardinelli *et al.* 2004).

In this study, propagation of sodium transients reached all cellular compartments, including astrocyte endfeet, where GLUT1 is expressed, facing the main glucose source (Morgello *et al.* 1995; Vannucci *et al.* 1997). This result suggests that sodium transients induced by activation of glutamate uptake in perisynaptic processes can propagate throughout the entire cell to astrocyte endfeet and thereby support glucose uptake upon synaptic activity.

Close to activated synapses, where sodium transients are largest, stimulation of glycolysis and lactate production will be strongest. Because sodium propagates through gap junctions, metabolic activation will, however, not be restricted to directly activated astrocytes, but include neighbouring cells (Gandhi *et al.* 2009), albeit to a lower degree. At the same time, gap junctions also provide the pathway for activity-dependent passage of glucose, which is directed to active sites, providing the fuel necessary for increased glycolysis (Rouach *et al.* 2008).

This study establishes that glutamatergic synaptic transmission in both the hippocampus and the cerebellum results in long lasting sodium transients in astrocytes. These transients can travel within the astrocyte network, and their amplitude and spatial profile reflect the site and strength of synaptic activity. Taken into account the multitude of effects, a decrease in the sodium driving force might exert on cellular functions, sodium transients should be considered as an element of neuron to glia signaling. Sodium transients could represent a signal, indicating increased metabolic needs in astrocytes, and thus support the proposed tight link between excitatory neuronal activity, glutamate transport, glucose utilization by astrocytes and metabolite supply to neurons.

Publications

For copyright reasons, this published version of the cumulative thesis document does not contain reprints of articles. Instead, only their abstracts are added along with the references on the following pages.

Pages 31-42:

Sodium Signals in Cerebellar Purkinje Neurons and Bergmann Glial Cells evoked by Glutamatergic Synaptic Transmission

Mustapha Bennay, Julia Langer, Silke D. Meier, Karl W. Kafitz, Christine R. Rose

GLIA 56:1138–1149 (2008)

ABSTRACT

Glial cells express specific high-affinity transporters for glutamate that play a central role in glutamate clearance at excitatory synapses in the brain. These transporters are electrogenic and are mainly energized by the electrochemical gradient for sodium. In the present study, we combined somatic whole-cell patch-clamp recordings with quantitative Na^+ imaging in fine cellular branches of cerebellar Bergmann glial cells and in dendrites of Purkinje neurons to analyze intracellular Na^+ signals close to activated synapses. We demonstrate that pressure application of glutamate and glutamate agonists causes local Na^+ signals in the mM range. Furthermore, we analyzed the pharmacological profile, as well as the time course and spatial distribution of Na^+ signals following short synaptic burst stimulation of parallel or climbing fibers. While parallel fibers stimulation resulted in local sodium transients that were largest in processes close to the stimulation pipette, climbing fibers stimulation elicited global sodium transients throughout the entire cell. Glial sodium signals amounted to several mM, were mainly caused by sodium influx following inward transport of glutamate and persisted for tens of seconds. Sodium transients in dendrites of Purkinje neurons, in contrast, were mainly caused by activation of AMPA receptors and had much faster kinetics. By reducing the driving force for sodium-dependent glutamate uptake, intracellular sodium accumulation in glial cells upon repetitive activity might provide a negative feedback mechanism, promoting the diffusion of glutamate and the activation of extrasynaptic glutamate receptors at active synapses in the cerebellum.

Impact factor 2008: 5.60

I performed

- about 30% of experiments and analysis

I contributed to

- the experimental design
- interpretation of data
- drafting and revision of figures and manuscript

Pages 44-62:

Synaptically induced Sodium Signals in Hippocampal Astrocytes *in situ*

Julia Langer, Christine R. Rose

THE JOURNAL OF PHYSIOLOGY 587(24):5859–5877 (2009)

ABSTRACT

Astrocytes are in close contact to excitatory synapses and express transporters which mediate the sodium-dependent uptake of glutamate. In cultured astrocytes, selective activation of glutamate transport results in sodium elevations which stimulate Na+/K+-ATPase and glucose uptake, indicating that synaptic release of glutamate might couple excitatory neuronal activity to glial sodium homeostasis and metabolism. Here, we analyzed intracellular sodium transients evoked by synaptic stimulation in acute mouse hippocampal slices using quantitative sodium imaging with the sodium-sensitive fluorescent indicator dye SBFI (sodium-binding benzofuran isophthalate). We found that short bursts of Schaffer collateral stimulation evoke sodium transients in the millimolar range in both CA1 pyramidal neurons and in SR101-positive astrocytes of the stratum radiatum. At low stimulation intensities, glial sodium transients were confined to one to two primary branches and adjacent fine processes and only weakly invaded the soma. Increasing the number of activated afferent fibers by increasing the stimulation intensity elicited global sodium transients detectable in the processes as well as the somata of astrocytes. Pharmacological analysis revealed that neuronal sodium signals were mainly attributable to sodium influx through ionotropic glutamate receptors. Activation of ionotropic receptors also contributed to glial sodium transients, while TBOA-sensitive glutamate transport was the major pathway responsible for sodium influx into astrocytes. Our results thus establish that glutamatergic synaptic transmission in the hippocampus results in sodium transients in astrocytes that are mainly mediated by activation of glutamate transport. They support the proposed link between excitatory synaptic activity, glutamate uptake and sodium signals in astrocytes of the hippocampus.

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I performed

- all experiments and data analysis

I contributed to

- the experimental design
- interpretation of data
- drafting and revision of figures and manuscript

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Intercellular Sodium Propagation between Hippocampal Astrocytes *in situ*

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GLIA (submitted 03.2011; under revision)

ABSTRACT

Activation of glutamatergic synapses results in long-lasting sodium transients in astrocytes that are mainly mediated by sodium-dependent glutamate uptake. Sodium elevations activate Na^+/K^+-ATPase and glucose uptake by astrocytes, representing a key signal for coupling glial metabolism to neuronal activity. Here, we analyzed the characteristics of sodium propagation in the astrocyte network *in situ*. Stimulation of a single astrocyte in a hippocampal slice preparation resulted in an immediate sodium elevation in the stimulated cell that propagated to neighbouring astrocytes within a distance of approximately 100 µm. Amplitude, slope and propagation speed of sodium elevations in downstream cells decayed monotonically with increasing distance. In contrast to sodium, calcium increases elicited by electrical stimulation were restricted to the stimulated cell and a few neighbouring astrocytes. Pharmacological inhibition of glutamate uptake, mGluR1 and 5, or gap junctions reduced sodium propagation, whereas inhibition of purinergic receptors had no effect. Propagation was also reduced in animals at P4 and virtually omitted in Cx30/Cx43 double-deficient mice. Our results indicate that glial calcium signaling and release of glutamate are supportive of, but are not prerequisites for, the propagation of sodium between hippocampal astrocytes *in situ*, whereas expression of Cx30 and Cx43 is essential. Cx30/Cx43-mediated sodium propagation could thus represent a signal indicating increased metabolic needs both in the presence and in the absence of concomitant calcium signaling. Sodium propagation in the astrocyte network might also serve a homeostatic function by supporting the re-establishment of a steep sodium gradient and by lowering the metabolic burden imposed on single cells.

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Addendum

For copyright reasons, this published version of the cumulative thesis document does not contain reprints of articles. Instead, only their abstracts are added along with the references on the following pages.

Pages 115-119:

Prion Protein regulates Glutamate-Dependent Lactate Transport of Astrocytes

Ralf Kleene, Gabriele Loers, Julia Langer, Yveline Frobert,
Friedrich Buck, Melitta Schachner

THE JOURNAL OF NEUROSCIENCE 27(45):12331–12340 (2007)

ABSTRACT

Prion-related protein (PrP) is a neural cell adhesion molecule involved in neurite outgrowth, neuronal survival, and synaptic function. In search of novel binding partners for PrP, we identified the $\alpha 2/\beta 2$-Na^+/K^+-ATPase and showed that this astroglial ATPase interacts directly with the immunoglobulin superfamily adhesion molecule basigin. In cultured astrocytes, PrP is involved in regulating lactate transport via the astroglial monocarboxylate transporter 1 (MCT1) and in conjunction with $\alpha 2/\beta 2$-ATPase and basigin. Lactate transport via MCT1 is glutamate dependent and regulated by glutamate receptor 2 (GluR2)-containing AMPA receptors with which PrP interacts. The functional interplay between PrP, GluR2, $\alpha 2/\beta 2$-ATPase, basigin, and MCT1 in regulating lactate transport of astrocytes may be functional in the metabolic cross talk between astrocytes and neurons, most likely under stress.

Impact factor 2007: 7.49

I performed

- the electrophysiological experiments
- design and analysis of electrophysiological experiments

I contributed to

- interpretation of data

Pages 121-140:

Contrasting Macrophage Activation by Fine and Ultra Fine Titanium Dioxide Particles is Associated with Different Uptake Mechanisms

Agnes M. Scherbart, Julia Langer, Alexey Bushmelev, Damiën van Berlo, Petra Haberzettl, Frederik-Jan van Schooten, Annette M. Schmidt, Christine R. Rose, Roel P.F. Schins, Catrin Albrecht

PARTICLE AND FIBRE TOXICOLOGY (submitted 12.2010; under revision)

ABSTRACT

Inhalation of (nano)particles may lead to pulmonary inflammation. However, the precise mechanisms of particle uptake and generation of inflammatory mediators by alveolar macrophages (AM) are still poorly understood. The aim of this study was to investigate the interactions between particles and AMΦ and their associated proinflammatory effects in relation to particle size and physico-chemical properties. NR8383 rat lung AM were treated with ultrafine (uf), fine (f) TiO2 or fine crystalline silica (DQ12 quartz). Physico-chemical particle properties were investigated by transmission electron microscopy, elemental analysis and thermogravimetry. Aggregation and agglomeration tendency of the particles were determined in assay-specific suspensions by means of dynamic light scattering. All three particle types were rapidly taken up by AM. DQ12 and ufTiO2, but not fTiO2, caused increased extracellular reactive oxygen species (ROS), heme oxygenase 1 (HO-1) mRNA expression and tumor necrosis factor (TNF)-α release. Only ufTiO2 enhanced inducible nitric oxide synthase (iNOS) mRNA expression, while DQ12 exclusively triggered interleukin (IL) 1β release. However, oscillations of intracellular calcium concentration and increased intracellular ROS were observed with all three samples. Uptake inhibition experiments with cytochalasin D, chlorpromazine and a Fcγ receptor II (FcγRII) antibody revealed that the endocytosis of fTiO2 by the macrophages involves actin-dependent phagocytosis and macropinocytosis as well as clathrin-coated pit formation, whereas the uptake of ufTiO2 was dominated by FcγIIR. The uptake of DQ12 was found to be significantly reduced by all three inhibitors. Our findings suggest that the contrasting AM responses to fTiO2, ufTiO2 and DQ12 relate to differences in the involvement of specific uptake mechanisms.

Impact factor: 5.54

I performed

- the analysis of calcium imaging experiments

I contributed to

- the design and interpretation of calcium imaging experiments
- drafting and revision of figure 4 and the corresponding part of the manuscript

Hiermit versichere ich, dass ich die vorliegende Arbeit selbständig angefertigt und keine anderen Hilfsmittel und Quellen verwendet habe, als die erlaubten. Textstellen oder Abbildungen, die wörtlich oder abgewandelt aus anderen Arbeiten stammen, habe ich mit einer Quellenangabe versehen. Diese Arbeit wurde weder vollständig noch in Teilen einem anderen Prüfungsamt zur Erlangung eines akademischen Grades vorgelegt.

Julia Langer Düsseldorf