Na^+ signaling in white matter glial cells

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Abstract

The vertebrate brain consists of so-called grey and white matter regions. While the latter mainly hosts axonal fiber tracts and different macroglial cells, grey matter harbors neuronal somata and dendritic areas on which chemical synapses are located. The most prevalent excitatory transmitter is glutamate, which upon its synaptic release, is taken up by the high-affinity, sodium-dependent transporters GLT-1 and GLAST mainly expressed by astrocytes. As a result, astrocytes in grey matter experience sodium signaling in response to glutamatergic synaptic activity. These intracellular sodium signals have been suggested to serve important functional roles under both physiological as well as pathophysiological conditions. In contrast to grey matter, sodium signaling and possible sodium influx pathways in macroglial cells of white matter were not studied and are thus unknown so far.

To address these questions, I analyzed white matter sodium signaling in acute *corpus callosum* sections taken from juvenile mouse brains. To this end, I adopted the dynamic ion imaging technique, using the sodium indicator SBFI-AM, and stimulated the cells both via focal pressure application of glutamate or by electrical stimulation of *corpus callosum* fibers to evoke axonal action potentials. Both approaches evoked robust sodium transients in astrocytes, but also in oligodendrocytes and NG2 glia. Blockers of ionotropic glutamate receptors revealed a differential involvement of AMPA and NMDA receptors between the groups, but inhibition of glutamate transporters with TFB-TBOA showed a strong effect across all glial cells. Using a GLT-1 specific antagonist and GLAST-KO animals in combination with studies of specific protein expression indicated that, compared to grey matter regions of the same age group, white matter astrocytes and cells of the oligodendrocyte lineage both display a clear involvement and expression of GLAST as well as GLT-1.To test if sodium signals spread intracellularly via a panglial syncytium, I directly stimulated an astrocyte, resulting in a steep sodium transient not only in the stimulated but also in neighboring glial cells, most likely by passage of sodium through gap-junctions. In summary, the results of this study thus demonstrate for the first time that white matter macroglial cells display activity-related sodium signaling and reveal the different pathways for sodium influx. Moreover, I could show that sodium efficiently spreads in the panglial syncytium.

Sodium signals could thus represent a key factor in panglial communication and coordination of physiological processes in white matter. These could include for instance glial metabolism, which is of utmost importance for axonal support or production of myelin, which enables rapid signal conduction along fiber tracts.

Zusammenfassung

Das Wirbeltiergehirn besteht aus Regionen grauer und weißer Substanz. Während die weiße Substanz vor allem axonale Faserbahnen und verschieden Makrogliazellen beherbergt, finden sich in der grauen Substanz neuronale Somata und dendritische Bereiche, an denen sich chemische Synapsen befinden. Der häufigste erregende Neurotransmitter Glutamat wird nach synaptischer Freisetzung vor allem von Astrozyten über die hoch-affinen und Natrium-abhängigen Transporter GLT-1 und GLAST aufgenommen. Dadurch entstehen in Astrozyten der grauen Substanz nach glutamaterger synaptischer Aktivität Natriumsignale. Es wird vermutet, dass diese intrazellulären Natriumsignale sowohl unter physiologischen als auch pathophysiologischen Bedingungen wichtige Funktionen erfüllen. Im Gegensatz zur grauen Substanz wurden Natriumsignale und deren mögliche Entstehungswege in Makrogliazellen der weißen Substanz noch nicht erforscht und sind somit unbekannt.

Um diesen Fragen nachzugehen, wurden Natriumsignale im *Corpus callosum* in akuten Hirnschnitten der juvenilen Maus untersucht. Hierfür wurde die Technik des dynamischen Ionenimaging mithilfe des Natriumindikators SBFI-AM angewandt. Zellen wurden sowohl durch lokale Druckapplikation von Glutamat als auch durch elektrische Stimulation von *Corpus callosum*-Fasern zur Hervorrufung von Aktionspotenzialen angeregt. Durch beide Ansätze wurden robuste Natriumsignale in Astrozyten, sowie in Oligodendrozyten und NG2-Zellen, ausgelöst. Antagonisten ionotropischer Glutamatrezeptoren zeigten, dass AMPA- und NMDA-Rezeptoren in beiden Gruppen unterschiedlich stark involviert sind, während die Inhibition von Glutamattransportern durch TFB-TBOA einen starken Effekt in allen Gliazellen hervorrief. Mithilfe eines GLT-1-spezifischen Antagonisten und unter Verwendung transgener GLAST-knock-out-Tiere, zeigte sich, dass anders als in der grauen Substanz gleichen Alters, sowohl Astrozyten als auch Oligodendrozyten und NG2-Zellen Aktivität beider Transportertypen aufweisen. Diese Resultate wurden durch die Ergebnisse von Studien zur Proteinexpression unterstrichen. Um zu prüfen ob Natriumsignale sich über ein pangliales Netzwerk ausbreiten, wurde ein einzelner Astrozyt direkt stimuliert. Ein so ausgelöstes Natriumsignal war nicht auf diese Zelle beschränkt, sondern konnte auch in benachbarten Gliazellen beobachtet werden, was wahrscheinlich auf die Ausbreitung von Natrium über *gap-junctions* zurück zu führen ist. Die Ergebnisse der Studie zeigen zum ersten Mal, dass sich in der weißen Substanz aktivitätsabhängige, komplexe Natriumsignale abspielen. Zudem wird gezeigt, dass sich Natrium effektiv über das pangliale Netzwerk ausbreitet.

Natriumsignale könnten somit ein Schlüsselfaktor in panglialer Kommunikation und Koordination physiologischer Prozesse in der weißen Substanz sein, wie etwa der gliale Metabolismus, welcher für Axone von größter Bedeutung ist, sowie Myelinproduktion für beschleunigte Signalweiterleitung an Nervenfasern.

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1. Introduction

1.1 Preface

One way to classify the areas of the vertebrate central nervous system (CNS) is to subdivide it into grey and white matter regions. While grey matter contains the majority of neuronal cell bodies and synapses, white matter primarily consists of axonal tracts (figure 1) conducting electrical signals from the somata to the synaptic terminals (Luo, 2015). White matter constitutes about half of the human brain volume (Groeschel et al., 2010) and it is estimated that the total length of axons in the human brain is 160.000 km (Marner et al., 2003). The term 'white matter' is based on the myelin, a dense lipid-rich structure, formed by resident oligodendrocytes (Luo, 2015). Myelination of axonal segments serves as an effective insulation and thus immensely facilitates and accelerates action potential propagation along fiber tracts, a process also called saltatory propagation (Luo, 2015). As opposed to other strategies that facilitate action potential propagation, like an increase of axon diameter, myelination results in a much more compact structure and thus allowed for the evolution of a highly complex CNS (Salzer and Zalc, 2016).

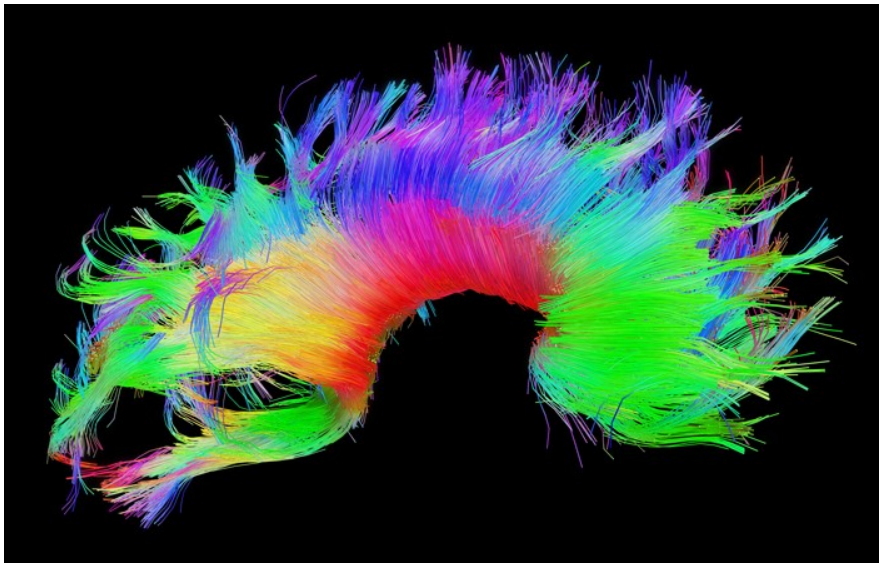

Figure 1: representation of the human white matter fiber architecture revealed by a connectome scanner performing diffusion spectrum imaging. Colors indicate the direction of the fibers: red = left-right; green = anterior-posterior, blue = ascending-descending (RGB = XYZ). Courtesy of the Laboratory of Neuro Imaging and Martinos Center for Biomedical Imaging, Consortium of the Human Connectome Project – www.humanconnectomeproject.org (http://www.humanconnectomeproject.org/gallery, accessed on 23/05/2018. Courtesy of the Laboratory of Neuro Imaging and Martinos Center for Biomedical Imaging, Consortium of the Human Connectome Project – www.humanconnectomeproject.org).

While past research on brain physiology, particularly metabolism and plasticity, has put emphasis mainly on grey matter, interest in white matter has grown in recent years (Filley and Fields, 2016). Especially for astrocytes, past research has overwhelmingly focused on grey matter, where astrocytic processes contribute to the so-called 'tripartite synapse' and help shape neuronal signal transmission on the one hand (Haydon, 2001), while supporting neuronal metabolism by acting as a mediator between vasculature and neuropil on the other (Magistretti, 2006). Here, Na^+ signals play a pivotal role, as they are involved in glutamate clearance at the synapse and potentially many more processes (Kirischuk et al., 2016; Rose and Chatton, 2016). The present work provides insight into white matter physiology, more specifically the role of Na^+ signals in macroglia – a group comprising mainly astrocytes, oligodendrocytes and NG2 (neural/glial antigen 2) cells.

In order to address the role of astrocytes in white matter, it is important to revise well-established **astrocyte functions in grey matter** with particular regard to **Na^+** homeostasis and signaling. While I initially laid focus on astrocytes as they have the highest relevance among glial cells in grey matter physiology, I soon incorporated **oligodendrocytes and NG2** cells into my studies, as they equally displayed complex Na^+ activity upon stimulation. Oligodendrocytes and NG2 cells will be introduced later and lead into a summary of **white matter physiology**.

1.2 Na^+ in brain physiology

First and foremost, Na^+ fluxes are essential in providing the basis for neuronal excitability and signal conduction (Bear et al., 2015). At resting conditions, ions are distributed unequally across the plasma membrane, resulting in distinct electrochemical gradients for each specific type of ion (Bear et al., 2015). Besides Na^+, which has an inwardly directed gradient (figure 2), the ions that are considered to be most important in this context are K^+, Cl^- and Ca^{2+}, as well as organic anions (Bear et al., 2015). While K^+ and the organic anions are more highly concentrated in the cytosol than in the extracellular space, the remaining ions follow an inwardly directed gradient as their concentration within the cell is lower than outside (Luo, 2015, Bear et al., 2015). These gradients and the specific membrane conductance define the neuronal membrane potential at rest, which is at -60 to -70 mV (Luo, 2015). In astrocytes, the resting potential is typically lower, ranging from -80 to -90 mV, and thus being close to the K^+ equilibrium potential (-90 mV) (Verkhratsky and Butt, 2013).

In terms of neuronal excitability and signal conduction, glutamate is the most common excitatory neurotransmitter in the brain (Niciu et al., 2012) and will therefore be in the focus of the following passages. In glutamatergic systems, during neuronal activity, glutamate is released by the presynaptic neuron into the synaptic cleft and binds to ionotropic receptors in

the postsynaptic density that, upon opening, allow for an influx of positively charged ions, specifically Na^+ and Ca^{2+} (Squire et al., 2013). The receptors which are involved here are AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid), NMDA ((2R)-2-(methylamino)butanedioic acid), and kainate receptors (figure 2) (Squire et al., 2013). AMPA receptors are activated first and allow for Na^+ influx, moderately depolarizing the membrane (Luo, 2015; Squire et al., 2013). NMDA receptors are blocked by Mg^{2+}, which is removed upon this slight depolarization, leading to further influx of Na^+ and additionally Ca^{2+} (Luo, 2015; Squire et al., 2013). Concurrently, K^+ flows out of the cells through the same channels (Luo, 2015). The thereby generated postsynaptic potentials propagate in an electrotonic manner along the postsynaptic dendrites and the somatic membrane and are summated at the initiation zone - at the axon hillock (Luo, 2015). Here the activation of densely distributed voltage gated Na^+ (Na^+_v) channels can lead to the generation of an action potential (AP), if the depolarization reaches the threshold of ~-50 mV (Bostock et al., 1998). After the influx of Na^+ into the neuron at the axon, the Na^+_v channels inactivate for a certain period of time - causing a so-called refractory time - and voltage gated K^+ channels open, leading to an efflux of K^+ and repolarization (Luo, 2015). The depolarization caused by the AP activates neighboring Na^+_v channels resulting in the directed - due to the refractory time - propagation of the AP along an axon (Luo, 2015).

At the axonal terminal, voltage gated Ca^{2+} channels open and the inwardly directed flux of Ca^{2+} triggers the fusion of neurotransmitter-filled vesicles with the presynaptic membrane and the release of transmitters e.g. glutamate into the synaptic cleft (Luo, 2015). Due to the strong ion fluxes across the neuronal plasma membrane, ion distribution is altered after activity. The ion gradients are restored by the NKA (Na^+/K^+-ATPase), which consumes ATP (adenosine triphosphate) to constantly pump K^+ into the cell and Na^+ out of the cell, thus maintaining physiological ion homeostasis (figure 2) (Morth et al., 2011).

While Na^+ is evidently crucial for neuronal excitability and signal transduction, it is equally important for a variety of processes involving glial cells, specifically grey matter astrocytes (Kirischuk et al., 2016; Rose and Chatton, 2016).

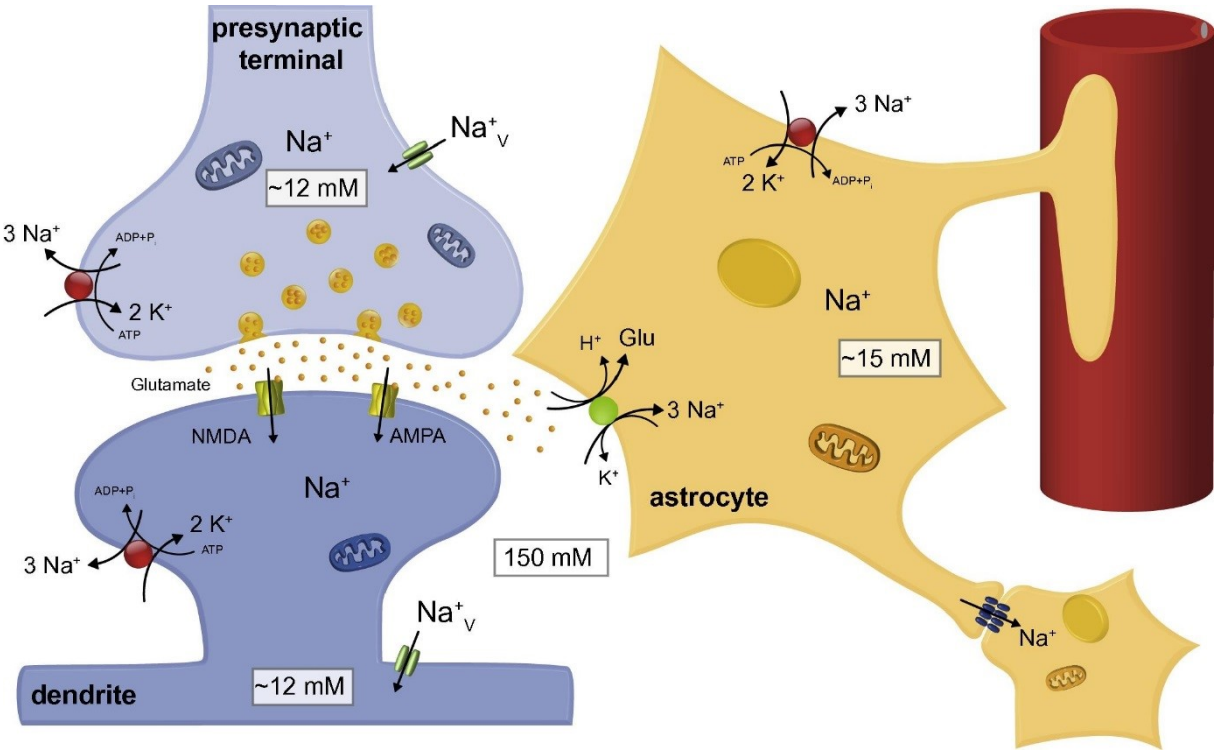

Figure 2: Na^+-dependent perisynaptic glutamate uptake at the tripartite synapse. Synaptically released glutamate acts on ionotropic receptors allowing for Na^+ influx into the postsynapse. Excess glutamate in the extracellular space is taken up by Na^+-dependent glutamate transporters expressed in astrocytic processes surrounding the synapse to prevent excitotoxicity. Na^+ elevations in both neurons and astrocytes are counteracted by ATP-dependent NKA. Abbreviations: NMDA, (2R)-2-(methylamino)butanedioic acid; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; Glu, glutamate; ADP, adenosine diphosphate; ATP, adenosine triphosphate (Rose and Chatton, 2016).

1.3 Astrocytes

Astrocytes are a heterogeneous group of glial cells found in the vertebrate brain (Schitine et al., 2015). Named after their star-shaped appearance, they were initially thought to merely provide structural support for neurons, but by now a multitude of functions has been revealed (Verkhratsky and Nedergaard, 2018; Volterra and Meldolesi, 2005). Astrocytes in grey matter, also named protoplasmic astrocytes, have the typical star-like morphology (figure 3) with numerous finely branched processes protruding from the soma, resulting in a spongiform structure (Squire et al., 2013). As perivascular endfeet, these processes contact and largely cover blood vessels, contributing to the blood-brain barrier which is furthermore formed by pericytes, endothelial cells and smooth muscle cells (Ballabh et al., 2004). On the other end, the astrocytic perisynaptic processes closely surround synapses, constituting a part of the ‘tripartite synapse’ (Araque et al., 1999).

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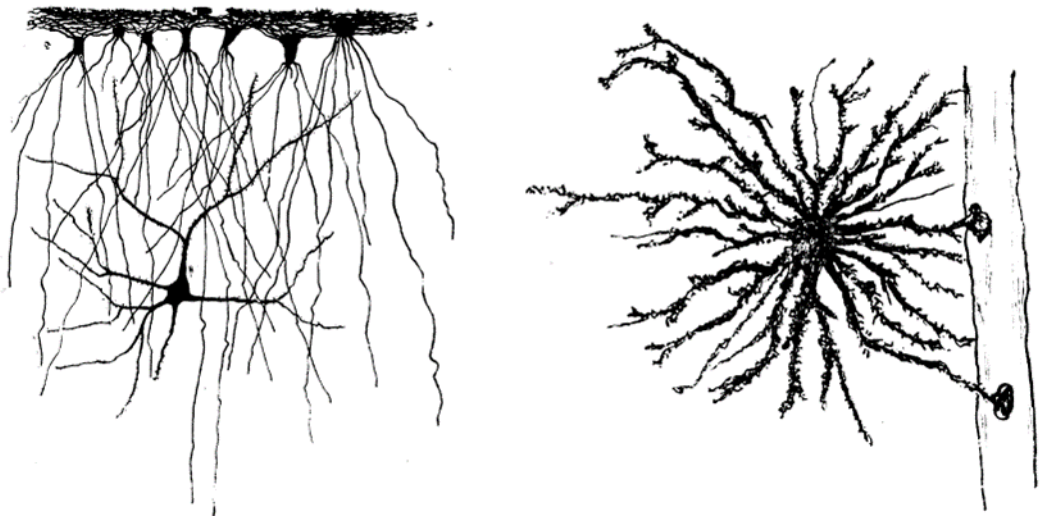

Figure 3: drawings depicting the heterogeneous morphology of human cortical astrocytes. Left: fibrous astrocytes and a pyramidal neuron. Right: protoplasmic astrocyte with endfeet contacting a blood vessel (Andriezen, 1893).

Another subset of this glial type are fibrous astrocytes predominantly found in white matter. These cells are elongated with less branched processes that closely run along white matter fibers (Verkhratsky and Nedergaard, 2018). It has been shown that these processes contact neurons at nodes of Ranvier along myelinated axons (Serwanski et al., 2017). The properties and functions of this potential neuron-glia interaction will be in the focus of the present study. Besides protoplasmic and fibrous astrocytes, there are additional subsets of specialized cells ascribed to this group of macroglia that will not be introduced in detail, including radial glia, retinal Müller cells and cerebellar Bergmann glia (Emsley and Macklis, 2006).

Generally, astrocytes fulfil a wide range of functions in the brain, including, K^+ buffering (Kofuji and Newman, 2004), water homeostasis (Solenov et al., 2004) and regulation of pH (Deitmer and Rose, 1996) - only to name a few. In the following, I will focus on tasks that are particularly related to Na^+ homeostasis and Na^+ signaling, as this aspect of glial physiology was the subject of my research.

1.4 Astrocyte Na^+ signaling

Glutamate transport

In grey matter, synaptically released glutamate binds to ionotropic receptors expressed primarily by the postsynaptic compartment to allow for influx of Na^+ and Ca^{2+} and the generation of postsynaptic potentials (Luo, 2015). However, the glutamate has to be removed

to prevent an extended activation of those channels and excess influx of ions into the cells, which may result in processes detrimental for the postsynaptic neuron summarized in the term 'excitotoxicity'. Here, the excessive influx of Ca^{2+} surpasses the cell's ability to buffer the cation, leading to an activation of enzymes such as proteases or phospholipases, resulting in neuronal damage and potentially cell death (Manev et al., 1989; Wang and Qin, 2010).

This is where astrocytes intervene. They are equipped with specialized glutamate transporters that import one glutamate molecule together with 3 Na^+ and 1 H^+, while exporting 1 K^+ (figure 2), thus being electrogenic (Grewer and Rauen, 2005). Glutamate transporters, with their human homologs named EAATs (excitatory amino acid transporters), can be divided into 5 isoforms (EAATs 1-5), whereas GLAST and GLT-1 (the mouse equivalents of EAAT1 and 2, respectively) represent the main subtypes expressed by astrocytes (Danbolt, 2001). All isoforms show similar structural properties and their transport processes follow the same mechanism, however, they display slight differences in glutamate affinity and transport rates (Fahlke et al., 2016; Rose et al., 2018; Vandenberg and Ryan, 2013). Neurons express a functional glutamate transporter subtype (EAAT3) as well, but it is thought to contribute to glutamate clearance only to a minor degree (Schousboe et al., 2004).

Looking at the stoichiometry of glutamate transporters, it becomes evident why Na^+ homeostasis, meaning the inwardly directed electrochemical gradient for Na^+ under resting conditions, is a fundamental prerequisite to maintain a physiological environment. At rest, astrocytes display a slightly higher intracellular Na^+ concentration than neurons (15 mM vs. 12 mM) (Rose and Karus, 2013; Rose and Ransom, 1996). Nevertheless, the difference to the extracellular Na^+ concentration is much higher. In combination with the low astrocytic membrane potential, the driving force of Na^+ to enter the cell is still present and provides the mechanism for glutamate uptake and prevention of excitotoxicity (Danbolt, 2001). The Na^+ dependence of glial glutamate transport has been verified in Bergmann glia by removing extracellular Na^+ and replacing it with NMDG (N-methyl-D-glucamine) or Li^+, which diminished glutamate-evoked signals (Kirischuk et al., 2007).

Similarly, block of the glutamate transporter with TFB-TBOA ((3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid) has been shown to completely abolish astrocytic Na^+ influx in the hippocampus (figure 4) (Langer et al., 2017). In the cerebral cortex however, Na^+ influx is not mediated by glutamate transporters exclusively, as cortical astrocytes additionally express functional Na^+-permeable NMDA receptors (Lalo et al., 2011).

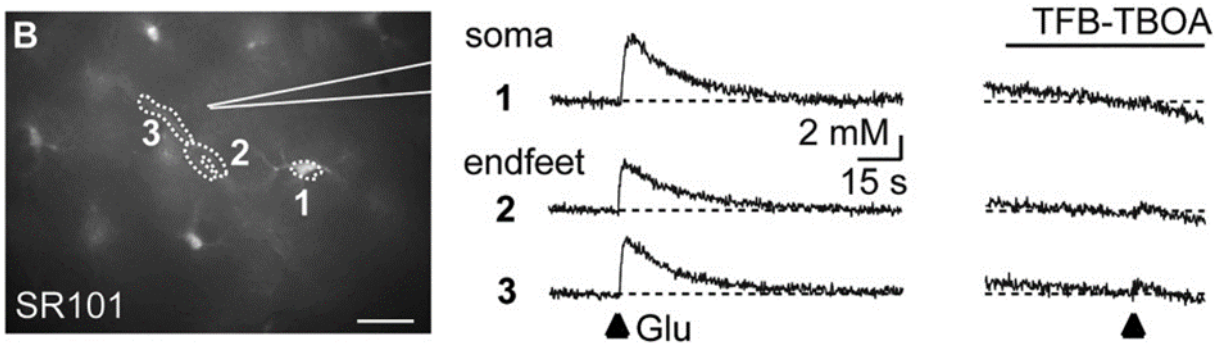

Figure 4: glutamate-evoked and TFB-TBOA-sensitive Na^+ transients in a grey matter astrocyte. Na^+ transients evoked by focal pressure application of 1 mM glutamate (500 ms) can be measured in astrocytic soma and endfeet. In the presence of glutamate transport antagonist TFB-TBOA (1 µM), those transients are diminished in the soma and strongly reduced in the endfeet. Scale bar is 20 µm. Abbreviation: SR101, sulforhodamine 101; Glu, glutamate; TFB-TBOA, (3S)-3-[[3-[[4-(Trifluoromethyl)benzoyl]amino]phenyl]methoxy]-L-aspartic acid (Langer et al., 2017).

Still, particularly during stimulation of recurrent network activity by pharmacological disinhibition, block of glutamate transport has drastic effects on neurons, which experience immense baseline increases in Na^+ and potentially cell death (Karus et al., 2015). This finding underlines the significance of glial glutamate uptake in maintaining physiological ion concentrations and, ultimately, ensuring neuronal viability. Once the glutamate enters the cell it is converted to glutamine by glutamine synthetase (GS) (Hertz et al., 1978; Suarez et al., 2002). Glutamine is chemically inert and can be transferred back to neurons to serve as a substrate for glutamate production by glutaminase and replenish the neuronal glutamate pool (Bak et al., 2006). Here too, astrocytic Na^+ transients have been observed to stimulate the release of glutamine, adding to the significance of glial Na^+ signaling (Broer et al., 2002).

Just like neurons, astrocytes display an altered ion distribution across the plasma membrane after neuronal activity, meaning that the intracellular Na^+ concentration is elevated. This leads to the activation of the NKA (Na^+/K^+-ATPase), which pumps Na^+ out of the cell and K^+ into the cell via ATP hydrolysation (Kaplan, 2002). The NKA is in fact the main energy consumer in the vertebrate brain and is expressed ubiquitously in neurons and glia (Attwell and Laughlin, 2001, Chakraborti and Dhalla, 2015). Its functioning is vital for generating and maintaining physiological ion concentrations (Attwell and Laughlin, 2001). Pharmacological inhibition of the NKA or removal of extracellular K^+ results in fast Na^+ baseline increases in astrocytes, which, in turn, breaks down the inwardly directed Na^+ gradient and prevents astrocytic glutamate uptake (Rose and Karus, 2013). Furthermore, this also demonstrates that there is a constitutive Na^+ influx even under resting conditions, which has to be counteracted by constant NKA activity.

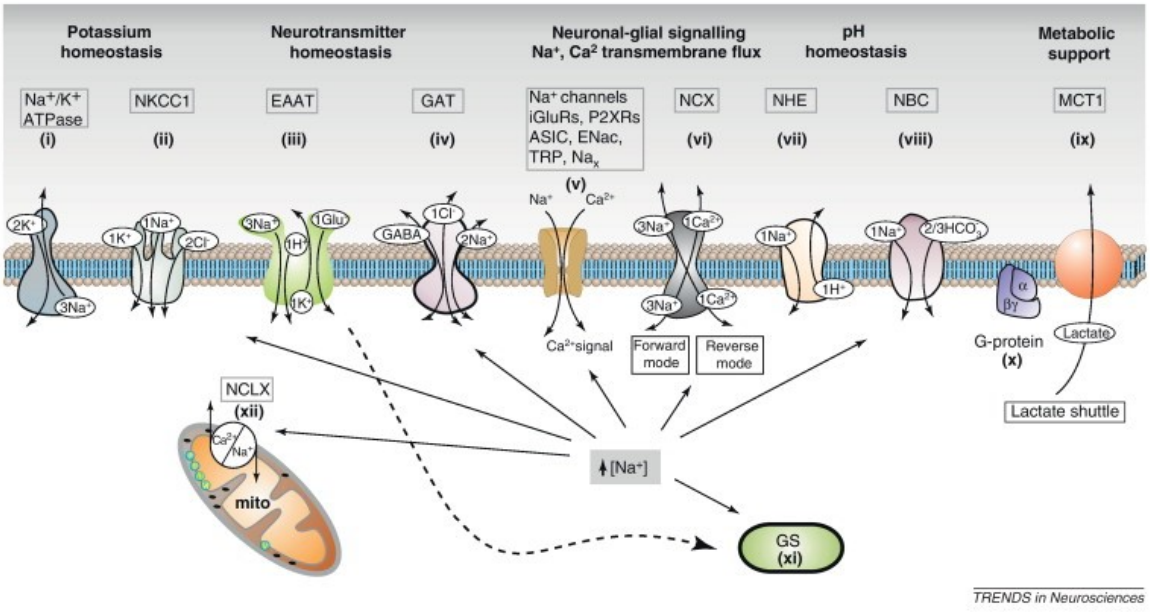

Figure 5: astrocytic transporters, exchangers and channels involving Na^+. Na^+ has an impact on a multitude of cellular transport and exchange processes affecting K^+ and neurotransmitter homeostasis, Ca^{2+} signaling, pH regulation and metabolic support. Abbreviations: NKCC1, $Na^+/K^+/Cl^-$ cotransporter, EAAT, excitatory amino acid transporters; GAT, GABA transporters; iGluRs, ionotropic glutamate receptors; P2XRs, ionotropic purinoceptors; ASIC, acid-sensing ion channels; ENac, epithelial sodium channels; TRP, transient receptor potential channels ;Na_x, Na^+ channels activated by extracellular Na^+; NCX, Na^+/Ca^{2+} exchanger; NBC, Na^+/HCO_3^- (sodium/bicarbonate) cotransporter; MCT1, monocarboxylase transporter 1; NCLX, mitochondrial Na^+/Ca^{2+} exchanger; GS, glutamine synthetase; mito, mitochondrion (Kirischuk et al., 2012).

Glutamate transport is not the only process that relies on the Na^+ gradient. In terms of neurotransmitters, the transport of e.g. GABA (γ-aminobutyric acid) depends on the inwardly directed electrochemical Na^+ gradient as well. Here, only 2 Na^+ are imported, while Cl^- is imported, making the transport process electrogenic, as well (Scimemi, 2014). GABA transport also easily reverses upon depolarization, contributing to tonic inhibition (Wu et al., 2006). Furthermore, Na^+ uptake is involved in intracellular pH changes via the NHE (Na^+/H^+-exchanger) and NBC ($Na^+/HCO3^-$-cotransporter) and other ions through transporters such as the NKCC1 ($Na^+/K^+/Cl^-$-cotransporter) and NCX (Na^+/Ca^{2+}-exchanger; figure 5).

Energy Metabolism

As mentioned above, astrocytes, as well as neurons and other glial cells, express the NKA, which is fundamental for maintaining physiological ion concentrations within the cell and in the extracellular space. Activation of the NKA, e.g. after neuronal activity and intracellular Na^+ increases, requires energy in the form of ATP, which is hydrolyzed during the process. One NKA pumping cycle consumes one molecule of ATP (Morth et al., 2011). Thus, activity at the tripartite synapses and concomitant Na^+ uptake stimulate astrocytic metabolism, as the consumption of ATP results in increasing energy demands of the cell (Chatton et al., 2000;

Pellerin and Magistretti, 1997). Additionally, neurons consume vast amounts of energy during activity, especially while recovering from postsynaptic potentials, and depend on external energy sources to function properly (Attwell and Laughlin, 2001).

As a result of higher energy demands, astrocytes show increased glucose uptake from the blood (Loaiza et al., 2003; Porras et al., 2008) and aerobic glycolysis resulting in the production of lactate. Lactate in turn, is then provided to neurons via specialized monocarboxylate transporters (MCT 1 and 4, figure 6) (Chatton et al., 2000). This 'lactate-shuttle' hypothesis is somewhat under debate, although there are several reports supporting the idea: In fact, there is evidence that neurons highly depend on astrocyte-derived lactate (Rose and Chatton, 2016; Suzuki et al., 2011). Moreover, they prefer lactate produced by astrocytes over their own ATP, produced through oxidative phosphorylation of glucose, which yields much more ATP compared to glycolysis (Bouzier-Sore et al., 2006; Itoh et al., 2003). Besides converting the blood derived glucose to lactate, astrocytes use it as a substrate for glycogenesis. The glycogen is then stored and metabolized during periods of energy shortage (Brown and Ransom, 2015).

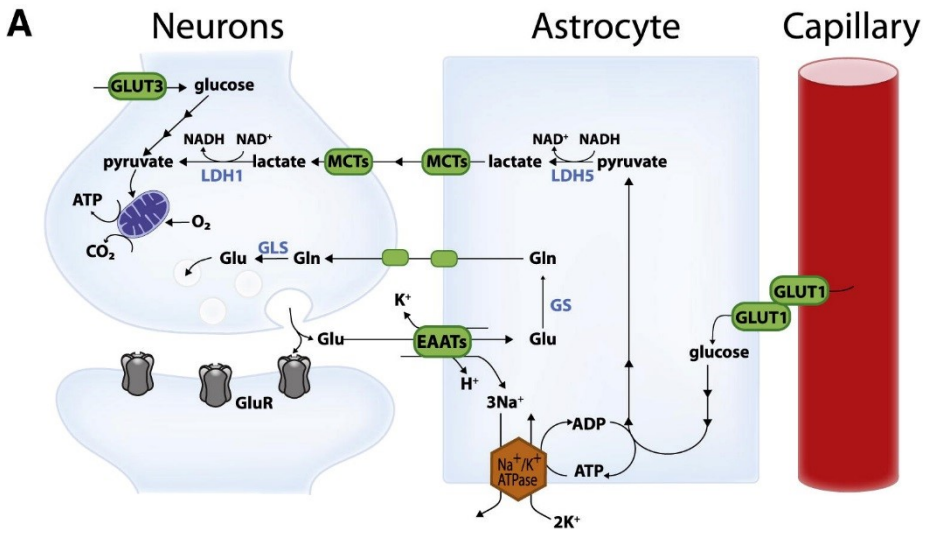

Figure 6: grey matter metabolic interactions between synaptic compartments and astrocyte during neuronal activity. Astrocytic Na^+ transients occurring due to neuronal activity and subsequent glutamate uptake stimulate glial metabolism through ATP-consuming NKA activity. This leads to increased astrocytic glucose uptake through GLUT1 and glycolysis, producing pyruvate and lactate, which are provided to neurons via MCTs for ATP production through mitochondrial respiration. Imported glutamate is recycled by astrocytes, generating glutamine that can be transferred back to the presynaptic compartment. Abbreviations: Glu, glutamate; GluR, glutamate receptor; ADP, adenosine diphosphate; ATP, adenosine triphosphate; GLS, glutaminase; LDH, lactate dehydrogenase; NAD^+, nicotinamide adenine dinucleotide ; NADH, reduced NAD^+,GLUT, glucose transporter; MCT, monocarboxylate transporter; GS, glutamine synthetase; Gln, glutamine (Belanger et al., 2011).

Astrocytes have efficient access to glucose from the blood as they possess perivascular endfeet that tightly ensheath capillaries and express specialized glucose transporter GLUT1 (Morgello et al., 1995). Microcirculation can be controlled by astrocytic release of substances stimulating vasoconstriction or vasodilation, with the latter resulting in increased local blood flow and consequently higher availability of glucose and oxygen (Gordon et al., 2007). These processes are affected by glial Ca^{2+} signaling, occurring especially after neuronal activity (Takano et al., 2006; Zonta et al., 2003). How astrocytic Ca^{2+} signaling is mediated and what other functional consequences it might entail will be described in the following section.

Na^+-mediated Ca^{2+} signaling

The physiological Na^+ gradient serves as a basis for numerous transport processes across the astrocytic plasma membrane. The ubiquitously expressed NCX imports 3 Na^+ while exporting one Ca^{2+} and thus maintains low intracellular Ca^{2+} concentrations at rest (Blaustein and Lederer, 1999). The NCX operates close to its reverse potential. Therefore, when intracellular Na^+ concentrations is increased during activity or due to pathophysiological conditions, the reverse mode is thought to be activated, leading to an export of Na^+ and an influx of Ca^{2+} (Gerkau et al., 2017; Goldman et al., 1994). Congruously, glutamate transporters and NCX appear to be co-localized in astrocytic processes (figure 7) (Minelli et al., 2007). Besides the NKA activity, the NCX reverse mode could thus be an additional mechanism to restore the Na^+ gradient necessary for glutamate uptake.

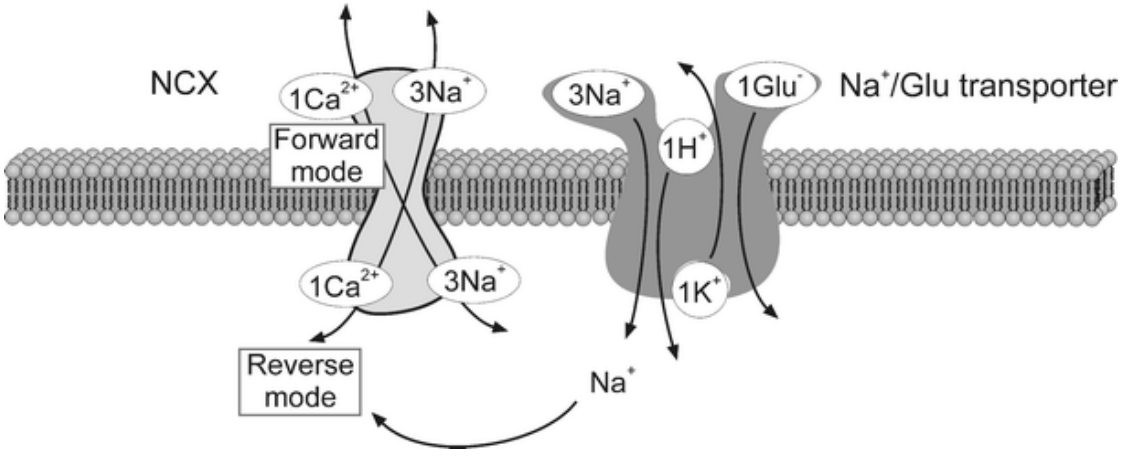

Figure 7: NCX modes of operation and interaction with glutamate transporter. At rest, NCX operates in the forward mode, exporting 1 Ca^{2+} and importing 3 Na^+. Stimulated by high intracellular Na^+, NCX is thought to enter the reverse mode, exporting 3 Na^+ and importing 1 Ca^{2+}, thereby contributing to Ca^{2+} signaling. Congruously, NCX is co-localized with Na^+-dependent glutamate transporters that allow for intracellular Na^+ transients during neuronal activity. Abbreviations: NCX, Na^+/Ca^{2+} exchanger; Glu, glutamate (Kirischuk et al., 2007).

Notwithstanding, NXC reverse activity might have more widespread functions, as it presents a mechanism for glial Ca^{2+} signaling. Consequently, Ca^{2+} elevations caused by NCX reverse activity are suggested to trigger Ca^{2+} mediated exocytotic release of gliotransmitters (Agulhon et al., 2008; Halassa et al., 2007). These transmitters bind to their respective receptors in other glial cells or at synaptic compartments, thereby shaping and fine tuning neuronal communication (Perea and Araque, 2010). Gliotransmitters comprise glutamate, D-serine, ATP and potentially more (Harada et al., 2015). NXC-evoked Ca^{2+} transients could furthermore lead to modifications of blood flow. Thus, similar to the activation of the NKA, Na^+ transients might have an impact on glial metabolism through modification of metabolite availability from the blood (Koehler et al., 2009). Interestingly, the reverse mode of the NCX has been a highly debated phenomenon considering its contribution to intracellular Ca^{2+} signaling in cardiomyocytes (Bouchard et al., 1993; Sipido et al., 1997).

Naturally, reversal of NCX is not the only process that can lead to the stimulation of Ca^{2+}-dependent processes. Other mechanisms that trigger astrocytic Ca^{2+} signaling include the activation of metabotropic glutamate receptors and second messenger-mediated Ca^{2+} fluxes, often involving Ca^{2+}-dependent Ca^{2+} release from intracellular stores (Verkhratsky and Parpura, 2014; Verkhratsky et al., 2012). Moreover, Ca^{2+} transients are rarely restricted to a single cell, but propagate to neighboring cells in a regenerative, wave-like manner. These Ca^{2+} waves are thought to be mediated by gliotransmission as well as gap-junction coupling (Scemes and Giaume, 2006).

Gap-junctions

Similar to Ca^{2+} waves, it has been shown that intracellular Na^+ elevations in astrocytes of various brain regions are not restricted to a single cell but rather spread along the astrocytic syncytium, which is realized by gap junction forming connexins (Cxs) (Giaume et al., 2010). These Cxs comprise different subtypes, with astrocyte-specific Cxs 30 and 43 coupling in a homotypic manner (Swenson et al., 1989; Werner et al., 1989). Gap junction-formed astrocytic syncytia are described to form a functional network involved in K^+ buffering (Kofuji and Newman, 2004) and the activity-dependent trafficking of glucose and metabolites which provides metabolic support to neurons (Rouach et al., 2008). Langer et al. (2012) showed in the CA1 region of the hippocampus that upon stimulation of a single astrocyte, Na^+ spreads to neighboring astrocytes over a limited distance, with the amplitudes of astrocytic Na^+ transients decreasing monoexponentially with higher distance from the stimulated cell (figure 8). Congruously, knock-out of both astrocyte-specific Cxs resulted in a complete abolishment of this Na^+ spread (Langer et al., 2012).

Gap junctions have been shown to be formed not exclusively between astrocytes but also between astrocytes and oligodendrocytes (Orthmann-Murphy et al., 2008). These

'panglial' syncycia can be found in several grey matter brain regions including the thalamus, neocortex and hippocampus (Griemsmann et al., 2015). Panglial gap junction coupling involves different Cxs and can thus only be formed in a heterotypic manner with oligodendrocytic Cxs 32 and 47 coupling to astrocytic Cxs 30 and 43, respectively (Dahl et al., 1996; Nagy et al., 2003; White et al., 1995). Whether these panglial connections allow for passage of Na^+ as well, has not yet been shown but will be addressed in the present study. However, panglial communication via gap junctions has been suggested to be involved in myelin maintenance in white matter tracts. The latter has been shown to be impaired in experiments that studied the effect of the deletion of specific Cxs (Tress et al., 2012). When talking about white matter physiology it is therefore necessary to refer to cells of the oligodendrocyte lineage, which make up the largest part of glial cells in white matter regions (Luo, 2015).

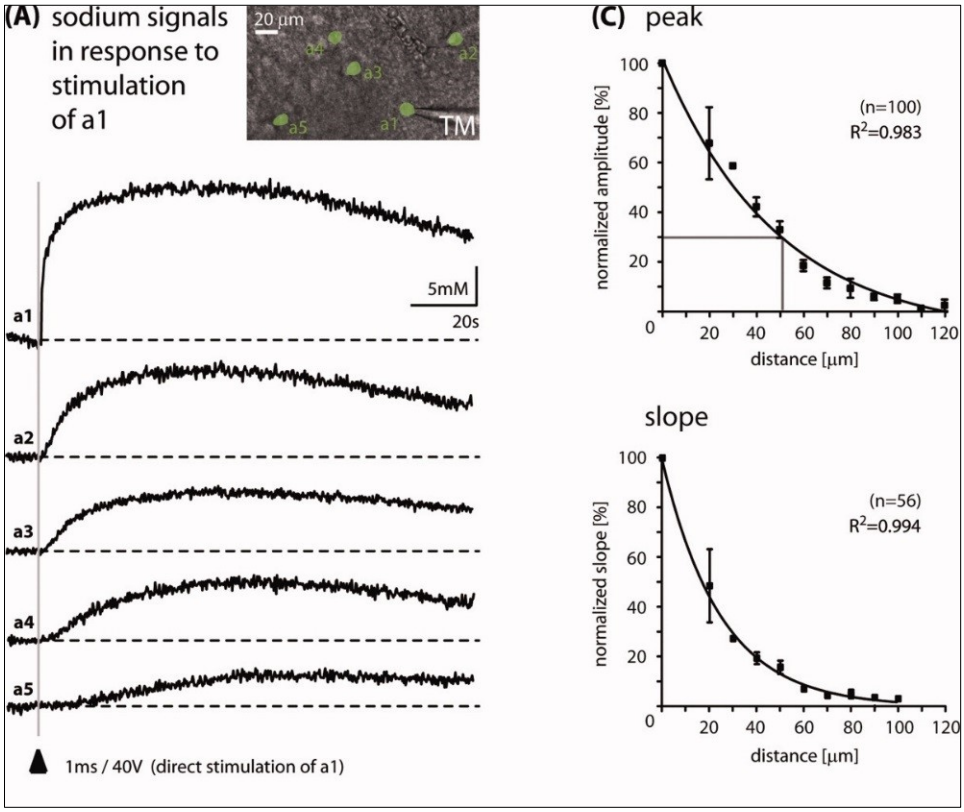

Figure 8: Na^+ spread between grey matter astrocytes after direct stimulation of a single cell. Direct electrical stimulation of a single astrocyte leads to a strong, long lasting Na^+ transient in that cell (A, a1), but also in neighboring astrocytes (a2-a5). Peak amplitudes and slopes depend on the distance to the stimulated cell, following a monoexponential decay function (C). Furthermore, the onset of the Na^+ signal is delayed with increasing distance. n is the number of cells analyzed, R^2 is the regression coefficient (Langer et al. 2012, modified).

1.5 Cells of the oligodendrocyte lineage

Oligodendrocytes

Oligodendrocytes are primarily known for their myelin-expressing processes (figure 9) which wrap around axonal segments to provide an insulation that has an immense impact on conduction speed and efficiency (Salzer and Zalc, 2016). The myelinated segments are known as internodes, while the gaps are called nodes of Ranvier (Luo, 2015). Internode length can vary greatly, whereas nodes of Ranvier usually have a length of about 1-2 µm (Arancibia-Carcamo and Attwell, 2014). APs are exclusively generated at the nodes, where a high density of Na^+_v channels allows for the influx of Na^+ necessary for depolarization (Luo, 2015). The neighboring myelinated internode membrane is then depolarized as well but due to a lack of channels, the voltage change spreads electrotonically along the axon to the next node, a process called salutatory conduction (Luo, 2015). The myelin membrane is composed of phospholipids and myelin-specific proteins. Dense layering of myelin sheaths by oligodendrocytic processes prevents ion leakage along the axon, thereby reducing membrane capacitance while increasing membrane resistance (Luo, 2015). Thus, electrotonic conduction speed at the internodes is enhanced enormously.

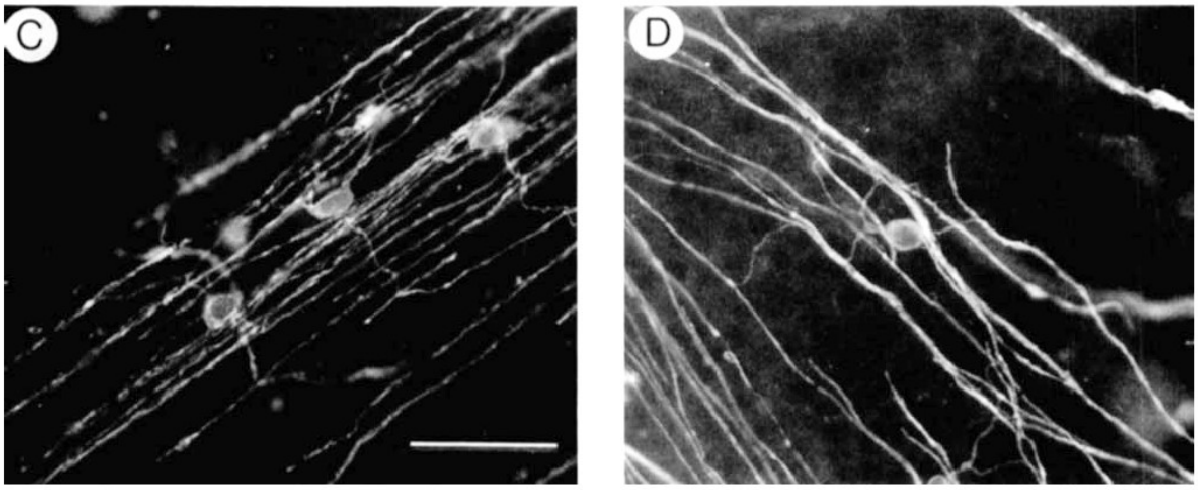

Figure 9: rat oligodendrocytes in the postnatal anterior medullary velum visualized by immunolabeling. The here used antibody Rip recognizes the oligodendrocyte specific protein CNP (2′,3′-cyclic nucleotide 3′-phosphodiesterase). Oligodendrocyte processes run parallel to each other and neuronal fibers to which they provide myelin sheaths. Scale bar is 50 µm (Butt et al., 1995).

Unlike their peripheral equivalent, Schwann cells, central oligodendrocytes do not myelinate axons in a 1:1 manner by completely wrapping around them, but their processes can myelinate up to 50 different axons (Baumann and Pham-Dinh, 2001). Another difference is the low potential for neuronal regeneration, due to inhibitory signaling molecules such as MAG (myelin-associated glycoprotein) or Nogo, that are expressed by myelinating cells of the

CNS, especially after injury (Xie and Zheng, 2008). Myelinating oligodendrocytes are prominent in white matter, but they are also present in grey matter, where they may insulate shorter fibers (Yeung et al., 2014). Furthermore, so-called satellite oligodendrocytes exist in grey matter that are not myelinating under physiological conditions, but are instead located close to neuronal somata, providing metabolic support for neurons and protecting them from apoptosis (Takasaki et al., 2010; Taniike et al., 2002). A more recent study suggests, however, that satellite oligodendrocytes may sense neuronal activity and form myelin at the perisomatic axon (Battefeld et al., 2016).

During development, oligodendrocytes undergo complex migration and differentiation processes. They are derived from their precursors, NG2 cells, which are now considered to be a separate major glial cell type with distinct characteristics (Peters, 2004), and will be further introduced below. Because of their complex physiology, oligodendrocytes are vulnerable to neurodegenerative diseases, one prominent example being multiple sclerosis (Cudrici et al., 2006; Dulamea, 2017; Prineas and Parratt, 2012). Also, white matter ischemic stroke severely affects oligodendrocytes (Dewar et al., 2003; Mifsud et al., 2014; Shindo et al., 2016). It is thus crucial to fully comprehend the physiology of white matter glia and glial interactions in addition to grey matter, which has been in the focus of brain research for decades.

NG2 glia

NG2 glia are found ubiquitously in white and grey matter regions of the developing and adult brain (Dawson et al., 2000; Levine and Reynolds, 1999). They have a distinct morphology with an often elongated soma and numerous slender processes protruding from it, although those processes are not as highly branched as those extending from protoplasmic astrocytes.

Interestingly, NG2 glia have been discovered and characterized on multiple occasions during the past two decades, leading to various names, such as GluR cells (glutamate receptor cells) (Wallraff et al., 2004), polydendrocytes (alluding to their morphology and cell fate) (Nishiyama, 2007) or synantocytes (Butt et al., 2005; Krawczyk and Jaworska-Adamu, 2010). Another common name which also describes a major function of this cell population is ‘OPCs’ (oligodendrocyte precursor cells), as they have been identified as part of the oligodendrocyte lineage. One evidence for this is the transcription factor Sox10, which is consistently expressed from NG2 cell to mature oligodendrocyte (Kuhlbrodt et al., 1998), suggesting a developmental relationship. However, this does not apply to all proteins that are found in the oligodendrocyte lineage. During development, NG2 cells can be easily distinguished from myelinating oligodendrocytes through the expression of various markers, such as the NG2 proteoglycan itself (Levine and Stallcup, 1987; Stallcup and Beasley, 1987) or PDGFRα (platelet derived growth factor receptor α) (Pringle et al., 1992), or by the lack of markers for more mature

oligodendrocytes like MBP (myelin basic protein) or PLP (proteolipid protein) (Frohlich et al., 2011; Nishiyama et al., 2009; Polito and Reynolds, 2005) (figure 10).

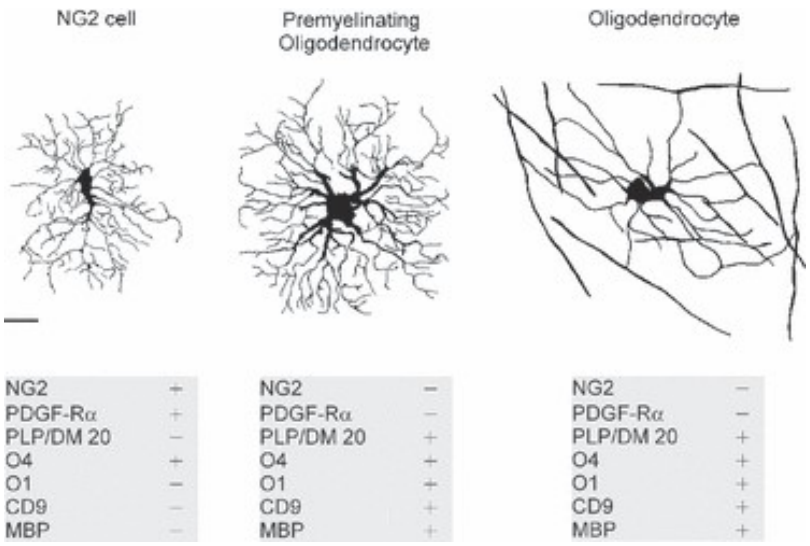

Figure 10: drawings of developmental stages and corresponding protein expression profiles (+ for present and - for absent) of cells of the oligodendrocyte lineage. From NG2 cell and premyelinating oligodendrocyte to mature oligodendrocyte, cells express specific proteins which are commonly used as markers to distinguish the different developmental stages. Abbreviations: NG2, neural/glial antigen 2; PDGF-R α, platelet-derived growth factor α; PLP, proteolipid protein; DM20, a PLP splice variant; CD9, tetraspanin-29, MBP, myelin basic protein. Scale bar is 10 µm (Frohlich et al. 2011).

Importantly, NG2 cells keep their proliferative potential throughout adulthood, thus being the most abundant self-renewing cell type in the adult brain outside of established neurogenic zones (Dawson et al., 2003; Woodruff et al., 2004). Furthermore, they seem to maintain the ability to differentiate during later developmental stages as well (Kang et al., 2010; Rivers et al., 2008), which has brought NG2 cells into the focus of studies revolving around regeneration and remyelination - both of which are vital after pathological processes or lesions leading to demyelination. In fact, it was shown in a mouse model for encephalomyelitis, among other pathologies, that NG2 proliferation is stimulated upon demyelination, resulting in an increased number of differentiated oligodendrocytes (Behrendt et al., 2013; Richardson et al., 2011; Tripathi et al., 2010; Zawadzka et al., 2010).

However, NG2 cells do not only represent progenitor cells for oligodendrocytes. As mentioned above, NG2 glia are considered to be a further class of macroglia next to astrocytes and oligodendrocytes (Peters, 2004). This is supported by a range of findings: first, it has been discovered that a subset of NG2 cells persists in the mature brain that does not obligatory follow the cell fate described above (Dimou et al., 2008). Second, NG2 cells have been shown to display (electro-) physiological characteristics that are more complex than what is expected from mere precursor cells. NG2 cells have been observed to closely interact with neurons, even receiving synaptic input involving AMPA receptors which mediate NG2 cell excitability

(Bergles et al., 2000). Furthermore, NG2 cells have been found to form GABAergic synapses with interneurons in the hippocampus (Lin and Bergles, 2004) and receive input from cerebellar climbing fibers (Lin et al., 2005). It is still unclear what the exact function of these synaptic contacts might be. One possibility is that the neuron-glia synapse allows NG2 cells to sense neuronal activity and that thus activity-dependent differentiation and subsequent myelination is stimulated (see next chapter and (Hill and Nishiyama, 2014)).

1.6 White matter physiology

The functions of grey matter astrocytes and especially glutamate uptake at the tripartite synapse have been the basis for the main hypothesis of the present study. As we will see in more detail below, white matter - just like grey matter - faces release of glutamate and it is conceivable that specialized mechanisms are put in place to handle extracellular glutamate loads and prevent the extended activation of glutamate-sensitive receptors present in surrounding cells. Thus, the question arises whether Na^+ homeostasis and intracellular Na^+ transients in glial cells fulfil a similar function in white matter as can be observed in grey matter.

During AP propagation, glutamate has been shown to be released by axons through Ca^{2+}-mediated vesicular exocytosis (Kukley et al., 2007). This relatively new finding is a first indication for white matter to display a ‘proper’ physiology, as opposed to the early view, that its only function is accommodating axonal tracts, where APs propagate without any further modification. It seems unlikely that an event as significant as neurotransmitter release is a mere side effect and does not imply any further consequences. Previous studies have already tackled the complex physiology of white matter, focusing on Ca^{2+} signaling, white matter plasticity and metabolic interactions, all of which might be as fundamental and sophisticated as related processes in grey matter.

White matter plasticity

Numerous reports have suggested that OPC differentiation and myelination might occur depending on AP firing rates (Nunez et al., 2000; Stevens et al., 2002). Activity-dependent myelination as a basic phenomenon was demonstrated in zebrafish, where the number of myelin sheaths was reduced after blocking axonal vesicle release (Mensch et al., 2015). During development, NG2 cells contact unmyelinated axons and form synapses, where AMPA receptors detect glutamate released from the axon (Micu et al., 2018) (figure 11). Thus, one hypothesis is that myelination is mediated by Ca^{2+}-dependent glutamate release and activation of glial glutamate receptors on processes of NG2 cells leading to a stop of proliferation in favor of differentiation into myelinating oligodendrocytes (Gallo et al., 1996; Yuan et al., 1998).

Similarly, it has been demonstrated that AMPA receptors mediate myelin production in cultured NG2 cells (Fannon et al., 2015).

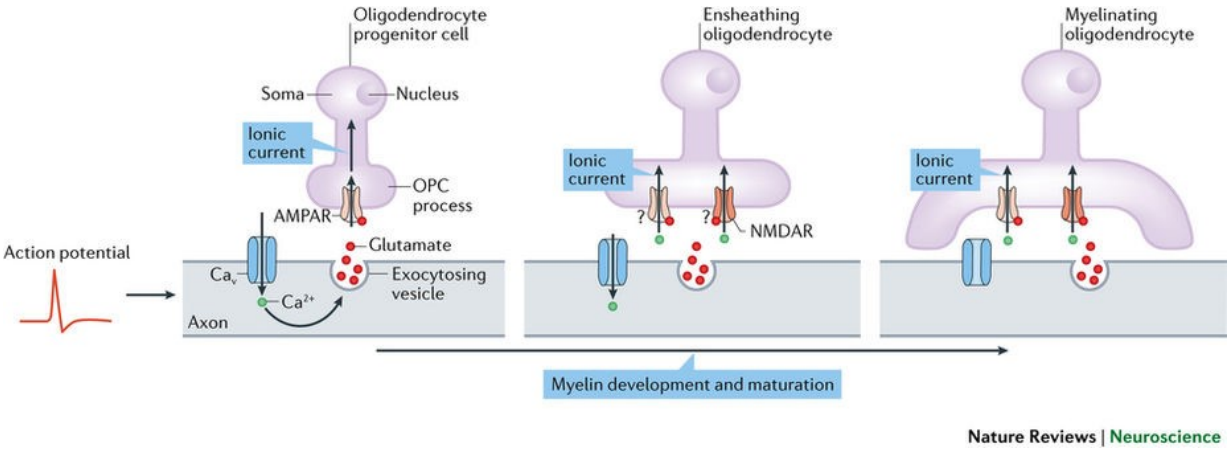

Figure 11: activity-dependent Ca^{2+} signaling and NG2 cell differentiation in white matter. Passing APs lead to a Ca^{2+}-mediated vesicular release of glutamate into the periaxonal space. The glutamate acts on AMPA-receptors in OPC (NG2 cell) processes resulting in detectable ionic (Ca^{2+}) currents, which are thought to stimulate OPC differentiation. Upon maturing, NMDA receptors expression is initiated. In myelinating oligodendrocytes, ionic currents are restricted to the periaxonal myelin. Abbreviations: AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; NMDAR, (2R)-2-(methylamino)butanedioic acid receptor; OPC, oligodendrocyte precursor cell (Micu et al. 2018).

Once differentiated, neuronal activity further facilitates initiation of myelin production by immature oligodendrocytes (Gibson et al., 2014). Here, NMDA rather than AMPA receptors might play a role, as it has been shown that neuronal activity induces NMDA receptor-mediated Ca^{2+} signaling in myelin (Micu et al., 2016) and glutamatergic signaling has been observed to stimulate mRNA translation of MBP in oligodendrocytes (Wake et al., 2011). However, myelination was not affected negatively in an NMDA receptor knock-out model (De Biase et al., 2011). Both receptors thus seem to fulfil different purposes depending on the developmental stage of oligodendrocyte lineage cells.

The aforementioned processes are not restricted to the establishment of myelination during brain development. On the contrary, the so-called white matter plasticity also occurs in the adult brain and includes not only new myelination of previously unmyelinated axons (Rivers et al., 2008) but also modification of already existing myelin sheaths (Young et al., 2013). This remodeling, meaning for example shortening or enlargement of internode segments, can have drastic effects on AP conduction speed and thus temporal fine-tuning and coordination of incoming electrical signals at synaptic terminals in the millisecond timescale. This has been studied extensively, e.g. in the auditory systems of birds (reviewed in (Seidl, 2014)).

Taken together, white matter plasticity appears to be equally complex as its synaptic counterpart. Furthermore, looking at the effect of myelination itself and modulation of internode length - not even taking into account variation of myelin thickness - or node length (Arancibia-

Carcamo et al., 2017), plasticity in white matter tracts might have similarly notable effects on physiology and consequently brain function or behavioral implications. While the exact mechanisms have not yet been revealed, it seems likely that Na^+ could pose a key factor in the communication between neuronal and glial compartments.

Metabolism

Neuronal activity does not only affect generation of oligodendroglia and production of myelin: another important aspect of white matter physiology is the metabolic support of axons provided by surrounding glial cells (Saab et al., 2013). Active axons are highly dependent on external energy sources; especially distal segments cannot rely on metabolic support from the soma only (Nave, 2010). Here, oligodendrocytes play a vital role, as they have been demonstrated in numerous studies to provide metabolites and maintain axonal integrity (Morrison et al., 2013). Oligodendrocytes sense neuronal activity via glutamate-mediated Ca^{2+} signaling in the periaxonal myelin, involving ionotropic and metabotropic receptors (Hamilton et al., 2008). By conditional deletion of NMDA receptors, Saab et al (2016) showed that these Ca^{2+} signals impact GLUT1 expression and insertion into the oligodendrocyte membrane, leading to an increased uptake of glucose, which serves as the substrate for production of lactate or pyruvate through glycolysis (Saab et al., 2016).

Furthermore, it has been shown that oligodendrocytes then release this lactate/pyruvate via MCT1, which is taken up by neurons via MCT2 and used for ATP production through mitochondrial respiration (Funfschilling et al., 2012; Lee et al., 2012) (figure 12). Underlining the importance of lactate for neuronal viability, Brown et al (2001) demonstrated that an isolated rat optic nerve can survive in the absence of glucose as long as lactate is present in the medium (Brown et al., 2001). Similarly, Trevisiol et al. (2017) showed in a novel ATP sensor mouse line that compound action potentials (CAPs) coincide with ATP consumption and that CAPs are attenuated during lactate deprivation (Trevisiol et al., 2017).

The functional principle of neuronal trophic support realized by oligodendrocytes is quite similar to the interaction between astrocytes and neurons in grey matter. Astrocytes are specialized in taking up glucose from the blood stream due to their perivascular endfeet and produce lactate to feed neurons (see according chapter). It seems intuitive that the same mechanism could work in white matter and that the lactate can then easily spread through the aforementioned panglial syncytium, passing oligodendroglia to finally reach the axon. Accordingly, Cx30/47 deficient mice are not viable due to a lack of complete myelination (Tress et al., 2012). However, as mentioned above white matter oligodendrocytes are able to take up glucose themselves and produce lactate during glycolysis (Funfschilling et al., 2012).

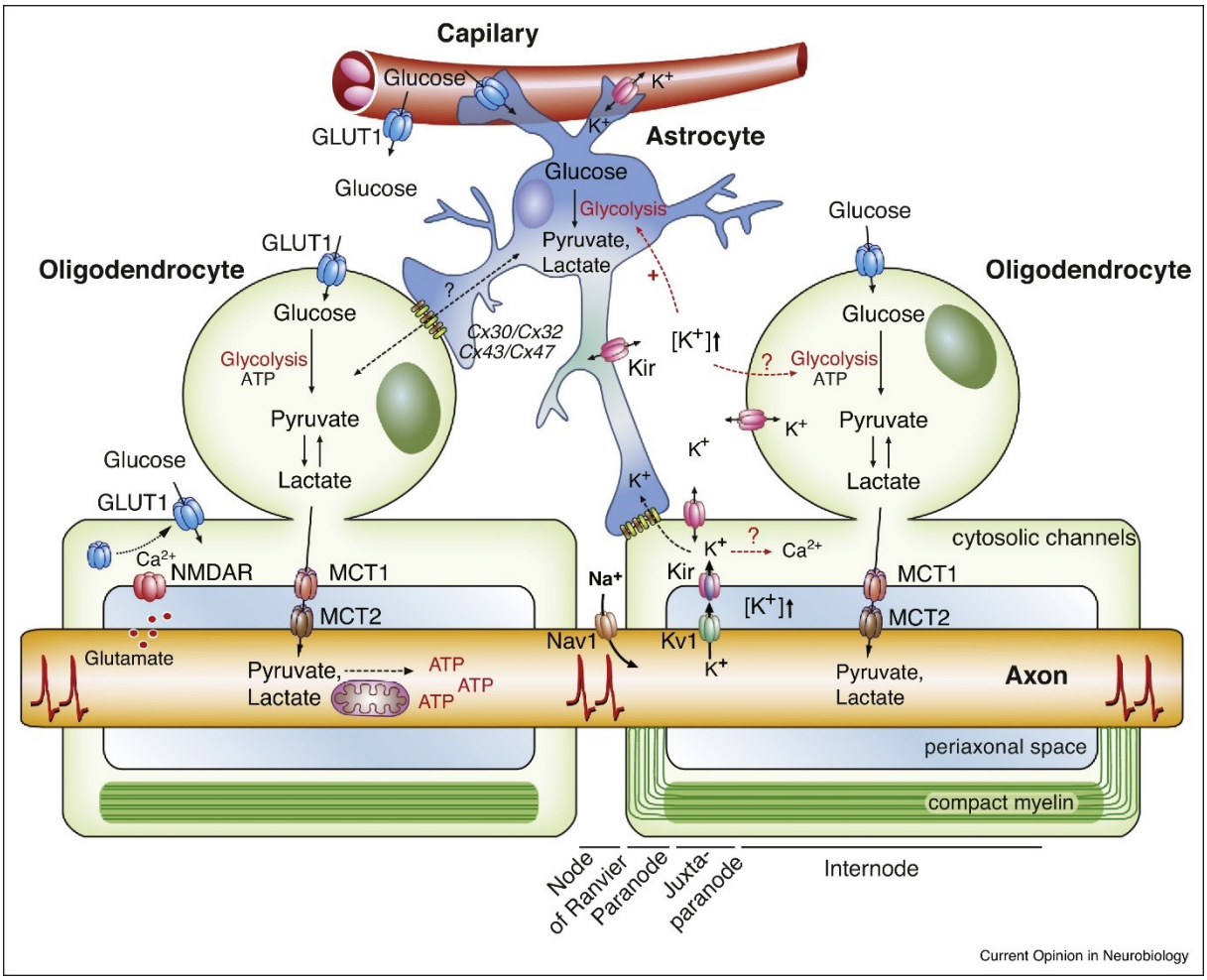

Figure 12: Metabolic interactions between axon and glial cells during neuronal activity within white matter. Passing APs mediate vesicular glutamate release. NMDA receptors in myelinating oligodendrocytes sense the glutamate and allow for Ca^{2+} influx. These Ca^{2+} transients stimulate glucose import via GLUT1. Glucose is then metabolized in oligodendrocytes, producing pyruvate and lactate, which are transferred to the axon via MCT1 (glial side) and MCT3 (neuronal side). Glucose can also be taken up and metabolized by white matter astrocytes. The generated metabolites can pass panglial gap junctions and contribute to trophic support of the axon. The panglial syncytium formed by gap junctions also provides an efficient path for K^+-buffering occurring after neuronal activity. Abbreviations: GLUT1, glucose transporter 1; NMDAR, (2R)-2-(methylamino)butanedioic acid receptor; ATP, adenosine triphosphate; Kv1, voltage gated K^+ channels; Kir, inwardly rectifying K^+ channel; Nav1, voltage-gated Na^+ channel ; Cx, connexin; MCT, monocarboxylase transporter (Saab and Nave, 2017).

Whether or not astrocyte glucose uptake and lactate production are vital in white matter thus seems disputable. It still seems likely that the coordination of these processes in both astrocytes and oligodendrocytes is necessary for proper metabolic support of neurons. Regardless of this, a crucial metabolic function of white matter astrocytes still is the production and storage of glycogen, which is metabolized during neuronal activity as shown in the murine optic nerve (Tekkok et al., 2005). Another similarity between grey matter astrocytes and white matter oligodendrocytes is the expression of glutamate transporters (Arranz et al., 2008; DeSilva et al., 2009; Desilva et al., 2007; Domercq and Matute, 1999). Na^+, which is taken up

by these transporters after axonal glutamate release could also play a role in stimulating oligodendrocyte metabolism through increased NKA activity. NKA expression and activity in oligodendrocytes has been demonstrated in numerous reports including cultured cells and pathophysiological conditions *in situ* (Annunziato et al., 2013; Dobretsov and Stimers, 1996; Fink et al., 1996; Martin-Vasallo et al., 2000).

Naturally, myelination and glial metabolism are tightly linked, as myelin production is highly energy consuming. In return, myelinated axons face lower energy consumption due to less AP generation (Nave and Werner, 2014; Wang et al., 2008). Again, Na^+ could pose as a mediator between white matter-specific processes impacting metabolism and signal conduction. To this end, Na^+ signaling was studied in the *corpus callosum*, a major white matter tract in the vertebrate brain.

1.7 *Corpus callosum*

The *corpus callosum* is the largest white matter tract of the brain, connecting various cortical regions of the two hemispheres (Aboitiz et al., 1992; Innocenti et al., 1986) and thus enabling interhemispheric communication (Anninos and Cook, 1988). Other major white matter regions include for example the optic nerve or the cerebellar white matter. Located in the forebrain between the hippocampus and the cerebral cortex (figure 13), the *corpus callosum* harbors approximately 200 million neuronal fibers (Innocenti et al., 1974), mostly running parallel to the coronal plane. Myelination of the *corpus callosum* is initiated at P11, while compact myelin is formed at P17. In contrast to the optic nerve, which is completely myelinated in the mature animal, only an average of 13.5% of the *corpus callosum* fibers are insulated at P45 (Sturrock, 1980). The *corpus callosum* is divided into 4 regions, which differ in structure, level of myelination, glial cell composition, and the target brain areas they connect. These areas are arranged as such from anterior to posterior: rostrum, genu, truncus and splenium (Witelson, 1989). Although the highest density of glial cells can be found in the genu region (Aboitiz et al. 1992), the present study was performed in the truncus, which contains the majority of myelinated axons. The latter is therefore easy to locate in a tissue section and has been proven suitable for the conducted imaging experiments. As it is typical for white matter regions, *corpus callosum* cell bodies are comprised almost exclusively of glial cells, with neurons being represented only by their axonal compartment (Sturrock, 1976).

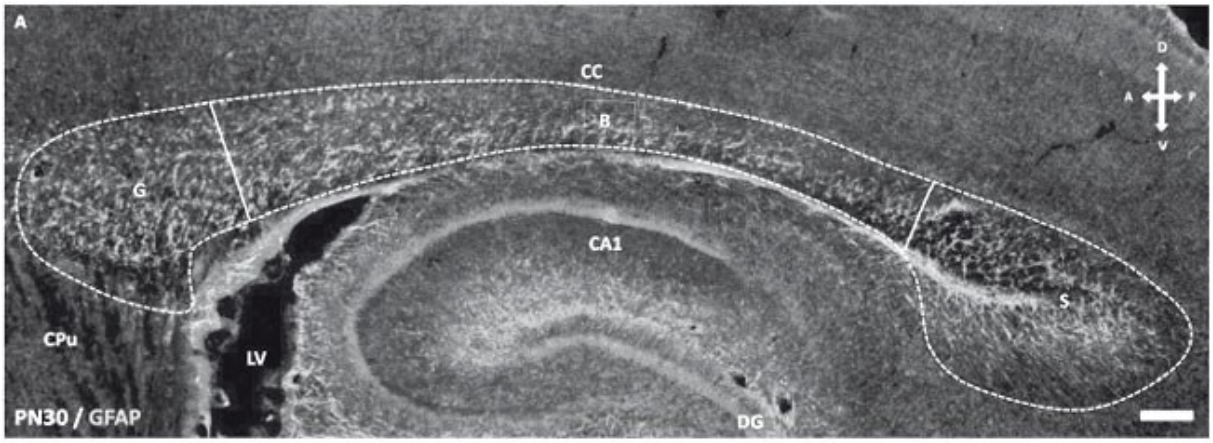

Figure 13: Sagittal view on *corpus callosum* structure and location in the mouse brain. The *corpus callosum* is located dorsal of the hippocampal CA1 region and ventral of the cerebral cortex (CC). It consists of 4 regions, named rostrum (not pictured), genu (G), truncus (body, B) and splenium (S, anterior to posterior). Abbreviations: DG, dentate gyrus; CPu (caudate-putamen); LV, lateral ventricle. Scale bar is 250 µm ((Reyes-Haro et al., 2013), modified).

2. Aim of the study

Intracellular Na^+ transients in grey matter glial cells, more specifically astrocytes, have been studied thoroughly in the recent years. Na^+ fluxes into astrocytes have been shown to present a vital mechanism for glial glutamate uptake and thus, through glutamate clearance, protection from overexcitation. Furthermore, there is increasing evidence for the reversal of the NCX and subsequent Ca^{2+} entry into cells after relevant Na^+ elevations. Additionally, it has been shown that Na^+ influx initiates glial metabolism because of energy consumption through the NKA and lactate production.

While it was believed for a long time that white matter is a mere passive fiber tract with myelinating oligodendrocytes for more efficient conduction of electrical signals, it has been shown in the recent past that there is a more complex interaction between resident glial cells and neuronal compartments. The increasing interest in white matter physiology combined with the immense relevance for astrocytic Na^+ signaling in grey matter raised the question whether there is similar complex Na^+ signaling in white matter astrocytes, as well.

The present study aimed at characterizing Na^+ signaling in white matter glial cells. To this end, *corpus callosum* slices were loaded with a Na^+ indicator dye SBFI-AM (Na^+ benzofuran isophthalate-acetoxymethyl ester) and widefield imaging experiments were performed. While it initially focused on astrocytic Na^+ transients, other glial cells were soon incorporated in the study as well, since they also showed Na^+ signaling with complex pharmacological profiles. Na^+ signaling was observed upon agonist application as well as electrical stimulation of *corpus callosum* axons. Electroporation experiments, where a single astrocyte was stimulated electrically to allow for a fast, reversible Na^+ entry shed some light on the panglial connectivity, possibly through gap junctions, as Na^+ spread not only between astrocytes but also from a stimulated astrocyte to other resident glial cells.

Thus, the present work is an important contribution to the recently increasing field of white matter studies, which not only focusses on axonal signal conduction but also on glial properties and interactions.

3. Summary of the work

As described in the introduction, Na^+ transients have multiple vital functions in the brain. The importance of Na^+ signaling has been established for numerous brain regions, most of which are within grey matter. The present study is the first to address the question, whether Na^+ transients can be evoked in glial cells of the *corpus callosum in situ* as well.

Na^+ imaging technique

To detect intracellular ion changes, ion-sensitive fluorescent dyes that change their fluorescent properties upon binding to a specific ion are applied. This means that the fluorescence intensities, which occur after appropriate excitation, depend on the ion concentration. In the present study, the Na^+ sensitive dye SBFI was applied and examined in an epifluorescent set-up where excitation was delivered by a monochromator, generating light of defined wavelengths. In slice preparations - as were used in the study - the AM (acetoxymethyl ester) version of a fluorescent indicator dye is commonly used. It acts as a membrane-permeant form that is injected into the tissue, where cells take it up, leading to a clear labelling of the cell bodies and thicker cellular processes. Within the cell, endogenous esterases cleave the -AM ester groups off, making the dye non-permeant. Remaining extracellular dye is washed out after a certain time, reducing background fluorescence in the slice (see (Stosiek et al., 2003)). SBFI is a ratiometric dye, meaning that it can be excited at two wavelengths, one of which is insensitive to ion concentration changes (the dye's isosbestic point) and one that is sensitive. By calculating the ratio of the captured fluorescence intensity at the two wavelengths, it is possible to obtain a value that is independent of changes in dye concentration that could occur due to efflux/leakage or other factors such as photobleaching. The resulting relative changes in intracellular Na^+ can be converted into millimolar changes by performing *in situ* calibrations. To this end, the cell membranes are permeabilized using ionophores and the NKA is inhibited, thereby equilibrating the intra and extracellular ion concentrations. By perfusing the slice with solutions containing defined concentrations of the respective ion, the dependence of concentration changes and changes in fluorescent intensities can be linearized, resulting in millimolar changes per relative change in fluorescent intensity.

Results and discussion

The present work was almost exclusively conducted in coronal tissue sections obtained from young juvenile (P15-P21) mice. In the coronal sections, *corpus callosum* fibers run parallel to the section plane, leaving most of the axons intact, which is advantageous for physiological studies.

As a starting point, the distribution and fraction of the different glial cells of the *corpus callosum* were analyzed using immunohistochemical stainings. To this end, PFA (paraformaldehyde)-fixed coronal tissue sections of transgenic reporter animals were used that feature cell-type specific expression of fluorescent proteins. In these established reporter mouse lines, the fluorescent proteins are expressed under the control of the respective promotors. These promotors and fluorescent proteins were hGFAP-GFP (human glial fibrillary acidic protein-green fluorescent protein) for astrocytes, PLP-GFP (proteolipid protein) for oligodendrocytes and NG2-EYFP (neural/glial antigen 2-enhanced yellow fluorescent protein) for NG2 cells. To enhance the intrinsic fluorescence of the proteins, they were again targeted with primary (goat anti-GFP) and secondary (fluorophore-tagged anti goat) antibodies, resulting in clear labelling of astrocytes, oligodendrocytes and NG2 cells. Additionally, the nuclei were labelled with DAPI, to determine the percentage of specific glial cells out of all cell bodies within the investigated region. Stainings were documented in the genu region of the *corpus callosum*, where physiological studies were performed as well.

The stainings revealed that the *corpus callosum* is comprised of 42% oligodendrocytes, 17% NG2 cells and 15% astrocytes. 18% of the cells could not be identified. Microglia were also labelled and constituted around 8% of all glial cells in *corpus callosum*. However, microglia have been proven to not take up ion indicator dyes (Eichhoff et al., 2011; Garaschuk, 2013) and were therefore neglected in the analysis of the experiments discussed below. In grey matter regions the glial cell composition is surprisingly similar. For example, Pelvig et al. (2003) report that the human neocortex contains 75% oligodendrocytes (most likely including NG2 cells), 19% astrocytes and 6% microglia (Pelvig et al., 2003).

Additionally to transgenic animals, wildtype animals were used as well. Here, conventional antibodies were applied to visualize the resident glial cells and furthermore characterize their morphology more adequately. The antibodies used were anti-GFAP- for targeting glial fibrillary acidic protein in astrocytes, anti-APC for recognizing oligodendrocyte specific protein adenomatous polyposis coli and anti-NG2. Astrocytes in particular showed a very distinct morphology when compared to typical grey matter astrocytes. These fibrous astrocytes feature an elongated soma with less branched processes which run parallel to the fibers.

Having established the glial cell composition within *corpus callosum* in fixed brain sections, dynamic ion imaging experiments were performed in acute tissue slices to study white matter Na^+ signaling. To distinguish astrocytes from other cells, vital dye SR101 is commonly used and has been established to reliably label astrocytes in various brain regions (Kafitz et al., 2008). To test whether SR101 is applicable in *corpus callosum* as well, acute slices from the aforementioned hGFAP-GFP mice were incubated in SR101 and co-labelling of the two markers was analyzed. While only 75% of al GFP-positive cells were labelled with

SR101, virtually all SR101-labelled cells were also GFP-positive, meaning that albeit not all astrocytes might be detected by SR101, it does not tag other (GFAP-GFP-negative) glial cells. Thus, astrocytes could clearly be identified while the other SBFI-loaded cells largely represent a heterogeneous group of cells of the oligodendrocyte lineage (oligodendrocytes and NG2 cells).

Along myelinated neurons, processes of astrocytes and NG2 cells have been observed contact axons at the nodes of Ranvier. Here, the activation of ionotropic and metabotropic glutamate receptors leads to intracellular Ca^{2+} signaling (Kriegler and Chiu, 1993; Serwanski et al., 2017). Glutamate receptor-mediated Ca^{2+} transients have been observed in oligodendrocytes as well, where they stimulate myelination and activation of glial metabolism (Butt et al., 2014; Gallo et al., 1996; Hamilton et al., 2008).

In the present study, 1 mM glutamate evoked strong Na^+ transients in astrocytes (5.4 ± 0.4 mM). Those transients had a typical monoexponential decay and lasted over ~120 s. White matter astrocytic signals were thus very similar to those detected in grey matter brain regions upon glutamate application (Bennay et al., 2008; Langer et al., 2017). While perfusion with antagonists of ionotropic glutamate receptors AMPA and NMDA had no effect on these Na^+ transients, glutamate transporter antagonist TFB-TBOA virtually omitted Na^+ signaling, indicating that glutamate transport presents the major influx pathway for Na^+. White matter astrocytes have indeed been shown to express glutamate transporters (Goursaud et al., 2009). Again, glutamate-evoked and TFB-TBOA-sensitive changes in the intracellular Na^+ concentration in astrocytes have also been observed before in grey matter areas such as the hippocampal CA1 region (Langer et al. 2017). Intriguingly, SR101-negative glia also displayed Na^+ signals. However, these were much smaller than those observed in astrocytes (1.8 ± 0.1 mM). Block of NMDA receptors had a minor effect, but again, the strongest reduction was caused by TFB-TBOA. Processes of mature myelinating oligodendrocytes have clearly been shown to express NMDA-receptors (Karadottir et al., 2005), which could explain the effect of NMDA receptor block. Oligodendrocytes, but not NG2 cells, have been shown to express glutamate transporters (Domercq and Matute, 1999). Following glutamate application, Na^+ transients of about 9 mM have been observed in cultured mouse oligodendrocytes (Ballanyi and Kettenmann, 1990). The present study is thus one of the first to provide evidence for Na^+ signaling in white matter oligodendrocytes *in situ*.

To attempt a more physiological approach and stimulate neuronal activity, *corpus callosum* fibers were directly depolarized electrically to generate APs and subsequent glutamate release. As discussed in the introduction, several studies have reported that neuronal activity triggers vesicular glutamate release and Ca^{2+} signaling in white matter. Generally, electrical stimulation is a well-established experimental approach - also referred to as ‘synaptic stimulation’ in grey matter regions. Bennay et al. (2008) and Langer and Rose

(2009) showed that synaptic stimulation results in Na^+ transients in astrocytes of the hippocampus and cerebellum (Bennay et al., 2008; Langer and Rose, 2009).

AP-evoked Na^+ transients in the *corpus callosum* differed greatly from those caused by glutamate application. Transients in astrocytes were much smaller (1.2 ± 0.1 mM) and lasted longer. It is easily conceivable that the glutamate that is released by axons during activity is considerably smaller than what is released into the synaptic cleft and sensed by postsynaptic receptors in grey matter (Kukley et al., 2007). However, exact data on the extracellular glutamate concentration in white matter during activity is still missing. Furthermore, astrocytes in myelinated white matter are most likely exposed to glutamate at the nodes of Ranvier only, which further limits the Na^+ influx under physiological conditions (Serwanski et al., 2017). Again, when applying antagonists of glutamate receptors and transporters, TFB-TBOA exhibited a strong effect, but peak amplitudes were also reduced following perfusion with AMPA-receptor blocker NBQX. Additionally to glutamate transporters, (Ca^{2+}-permeable) AMPA receptors have been shown to be expressed by astrocytes in hippocampal cultures and *in situ* (Fan et al., 1999; Seifert and Steinhauser, 1995). Compared to astrocytes, SR101-negative cells displayed similarly low peak amplitudes and decay times (1.4 ± 0.1 mM) after electrical fiber stimulation. The pharmacological profile was similar to that of astrocytes but showed an additional effect of NMDA-receptor blocker AP5. NG2 cells mainly contact unmyelinated axons but their processes also reach out to nodes of Ranvier when white matter is more developed (Butt et al., 1999; Sakry et al., 2011; Ziskin et al., 2007). On the other hand, mature myelinating oligodendrocytes enwrap large segments of axons and are thus more extensively exposed to glutamate that is released by neurons into the periaxonal space.

To test whether this structural difference between NG2 cells and mature oligodendrocytes is also reflected in distinct pharmacological profiles, NG2-EYFP mice were used. This allowed for the detection of NG2 cells and astrocytes with SR101, leaving the remaining (EYFP/SR101-negative) cells to be oligodendrocytes. Electrical stimulation evoked Na^+ signals with similar peak amplitudes in NG2 cells (1.7 ± 0.2 mM) and in oligodendrocytes (1.5 ± 0.1 mM). After application of the antagonists, both groups were affected to the same degree, again with the clearest inhibitory effect caused by TFB-TBOA. A visible albeit statistically insignificant difference was detected in the effect of NBQX on Na^+ signals, which might be due to AMPA-receptor expression in NG2 cells which has been demonstrated by several other groups (Patneau et al., 1994; Stegmuller et al., 2003; Yuan et al., 1998).

The results of the present study so far together with previous studies support the hypothesis that functional glutamate transporters are expressed by white matter astrocytes and cells of the oligodendrocyte lineage. Glial cells mainly express two types of glutamate transporters: GLT-1 and GLAST (Danbolt, 2001). To find out exactly which of these two subtypes are involved in the uptake of Na^+ in the *corpus callosum*, glutamate was applied again

in slices taken from wildtype animals in the presence of a subtype-specific antagonist and in slices obtained from transgenic knock-out mice. First of all, the effect of TFB-TBOA (without any blockers of glutamate receptors) was confirmed again to significantly reduce the peak amplitudes observed in astrocytes and SR101-negative cells. Then, in another set of experiments, wildtype slices were perfused with DHK (dihydrokainate), a GLT-1-specific antagonist. As a result, Na^+ amplitudes were only slightly - but still significantly - decreased in both groups. Lastly, glutamate was applied in the *corpus callosum* of GLAST knock-out animals, which thus only possess GLT-1 as a functional glutamate transporter. Interestingly, no effect on the Na^+ signals in SR101-negative cells could be detected, while astrocytic amplitudes were strongly dampened. These results indicate that different to grey matter of the same age (Rothstein et al., 1994), white matter astrocytes primarily rely on GLAST for glutamate uptake.

Several earlier studies suggested a general increase in glial glutamate transporter expression during development using immunohistochemical approaches (Kugler and Schleyer, 2004; Schreiner et al., 2014). However, there seems to be a developmental regulation concerning the expression of the specific subtypes. In grey matter regions, GLAST has been reported to be more relevant in immature astrocytes (Furuta et al., 1997; Rothstein et al., 1994), while GLT-1 is thought to be the predominant glutamate transporter in the here used age group (P15-P21) (Yang et al., 2009). While exact information on glutamate uptake by cells of the oligodendrocyte lineage is missing, the present work suggests that SR101-negative cells show a stronger involvement of GLT-1 in Na^+ signaling.

With regard to the mode of function, GLAST is described to be a higher affinity, lower frequency transporter (Danbolt, 2001; Wadiche and Kavanaugh, 1998), while GLT-1 has a slightly lower affinity to glutamate but operates at a higher frequency (Bergles and Jahr, 1998). It thus seems plausible when comparing SR101-positive and negative cells that astrocytic GLAST activity is sufficient to take up glutamate at the nodes of Ranvier, which is the only location where astrocytes contact myelinated axons. Oligodendrocytes are exposed to glutamate more extensively and high frequency GLT-1 activity may be needed to cope with higher concentrations of periaxonal glutamate, while affinity can be lower. Congruously, the present results suggest GLT-1 expression and significant activity in cells of the oligodendrocyte lineage. Arranz et al. (2008) reported contrasting results, as they observed high GLAST expression in oligodendroglia and GLT-1 in astrocytes in rodent optic nerves (Arranz et al., 2008). To address glutamate transporter expression with a molecular approach, indirect immunohistochemical stainings targeting GLT-1 and GLAST and SDS-PAGE/western blots from homogenized *corpus callosum* tissue were performed.

For immunohistochemistry, wild type sections were stained with aforementioned cell type-specific markers anti-GFAP, anti-APC and anti-NG2 and co-labelled with antibodies

targeting the glutamate transporters. The astrocyte marker anti-GFAP was clearly co-localized with GLT-1 and GLAST-labelling. On the other hand, oligodendrocyte marker anti-APC as well as NG2 were negative for GLAST-labelling. Instead, they showed co-localization with only GLT-1, although for NG2 cells this co-labelling was only partial. These data support the results from the physiological experiments, where astrocytes were affected by both GLAST and GLT-1 inactivation, while Na^+ transients in SR101-negative cells were not changed in the GLAST knock-out. The infrequent co-localization of NG2 and GLT-1 might be due to the comparatively fast differentiation rate of NG2 cells in white matter, where they predominantly generate oligodendrocytes (Dawson et al., 2003; Dimou et al., 2008). NG2 expressing cells detected here could therefore be in various developmental stages, where subsets already display GLT-1 expression, while others do not. Another factor could be the inherent heterogeneous character of NG2 cells (reviewed by (Vigano and Dimou, 2016)) that can also be observed in the variation of expression of different proteins. In an earlier part of the present work, the regional and developmental expression of Ca^{2+} binding protein S100B in NG2 cells has been addressed using immunohistochemistry, displaying immense heterogeneity. For example, it was found that labelling of S100B in *corpus callosum* NG2 cells was much lower than in hippocampal regions (Moshrefi-Ravasdjani et al., 2017). Even within the latter region, specifically the stratum radiatum, co-expression level of NG2 and S100B highly depended on the developmental stage and was only at 26% at postnatal days 25-35. It is therefore not surprising that expression of glutamate transporters in NG2 cells is not consistent and might depend on brain region and developmental stage.

Schreiner et al. (2014) studied the expression of GLT-1 and GLAST on a subcellular level in the developing mouse hippocampus (Schreiner et al., 2014). They found that GLT-1 is prominently present in clusters at perisynaptic processes of astrocytes. The same might apply to astrocytic processes contacting nodes of Ranvier in white matter. In the same study, a general presence and increase of both transporters was found throughout hippocampus development by SDS-PAGE and western blot using homogenized hippocampus tissue. The same is true for the present study working with *corpus callosum* tissue. However, similar to the aforementioned work, GLAST and GLT-1 had different relative expression levels in young postnatal mice with GLAST being already highly expressed when compared to P25 and GLT-1 showing a low initial expression level. At first glance, these results seem to contradict the data gained from the imaging and immunohistochemical experiments that indicate GLAST as a major contributor to astrocytic glutamate uptake. But bearing in mind that the number of oligodendrocytes - which rely more on GLT-1 - clearly outnumbers astrocytes, the high expression of GLT-1 makes sense again. It would be certainly worthwhile to compare GLT-1 and GLAST expression directly in each age group in addition to comparing them to their respective expression in an older age group.

This first part focused on the generation of Na^+ signals in single cells, assuming glial cells to present isolated domains. However, astrocytes have been demonstrated to form a gap-junction mediated network in several brain regions, allowing for a spread of ions or small molecules (Giaume et al., 2010; Langer et al., 2012; Volterra and Meldolesi, 2005). This raised the question whether Na^+ would spread within the glial network of the *corpus callosum* as well.

To this end, direct stimulation of single astrocytes was performed that allowed for a steep Na^+ increase in the stimulated cells (12.5 ± 1.8 mM). Moreover, following the stimulation, neighbouring cells displayed Na^+ transients as well. These cells comprised SR101-positive astrocytes as well as SR101-negative cells. The peak amplitudes occurring in the mentioned neighbouring cells were not equally high, but decreased with increasing distance from the stimulated cell, following a monoexponential decay function. When comparing the λ values - representing the distance at which the signals dropped to about 30% of the initial value (stimulated cell) - between cell groups, astrocytes displayed a much more effective spread of Na^+ signals than SR101-negative cells.

To find out whether the signals occuring in neighbouring cells are gap-junction-mediated or generated after gliotransmission, antagonists of metabotropic glutamate receptors combined with ATP receptor antagonists were washed in. In the presence of those compounds, Na^+ signals could still be observed in cells surrounding the stimulated cell. Moreover, Na^+ signals were unaltered when compared to the same experiment without antagonists, indicating a gap junction-mediated, passive spread of Na^+ between glial cells. Similarly, Langer et al. (2012) showed that a knock-out of Cx30 and 43 completely inhibited intercellular Na^+ spread between astrocytes in hippocampal CA1 region (Langer et al., 2012).

Other groups already observed gap-junction coupling between different glial cell types in several brain regions, including the hippocampus, neocortex, superior lateral olive and the thalamus (Augustin et al., 2016; Griemsmann et al., 2015). However, NG2 cells are not thought to be gap junction coupled, but instead to rely on paracrine communication, such as gliotransmission (Hamilton et al., 2010; Maglione et al., 2010; Wigley et al., 2007). The fact that block of gliotransmission in the aforementioned experiment did not exert any effect on the spread of Na^+ could be due to the high number of oligodendrocytes in comparison to NG2 glia.

To investigate panglial coupling more closely and to test if there is a detectable difference of Na^+ spread between oligodendrocytes and NG2 cells, transgenic reporter animals were used again and direct stimulation of astrocytes was performed. In PLP-GFP mice (which were employed to identify oligodendrocytes) Na^+ spread was shown to be less effective than between SR101-positive astrocytes. Interestingly, signals in NG2 cells, which were visualized using NG2-EYFP animals, dropped in a similar manner as in oligodendrocytes. In both mouse

lines, invasion of Na^+ signals between astrocytes was as effective as in slices taken from wildtype mice.

Having established the effective spread of Na^+ from a stimulated astrocyte to other glial cells, it would be interesting to test if the efficacy of the spread is any different in the opposite direction by stimulating an oligodendrocyte and observing the ensuing Na^+ signals in neighbouring glial cells. In dye coupling studies gap junction coupling among oligodendrocytes in the mouse *corpus callosum* was observed (Maglione et al., 2010), while ultrastructural studies claim that oligodendrocytes are not coupled with each other, but only with astrocytes (Orthmann-Murphy et al., 2008).

Because Na^+ is able to spread via gap-junctions between glial cells in the *corpus callosum*, it had to be tested if Na^+ transients evoked by e.g. glutamate are directly linked to the agonist activity or occur indirectly by stimulation of one cell type and subsequent spread via gap junctions. For this, Cx knock-out mice were used, which completely lack the astrocyte specific Cx30 and 43 and thus gap junction coupling among astrocytes, but also between astrocytes and other glial cells (Wallraff et al., 2004). In slices obtained from control animals as well as Cx knock-out mice glutamate induced intracellular Na^+ transients in both astrocytes and SR101-negative cells. Interestingly, both cell groups displayed signal amplitudes which were significantly larger in sections taken from Cx knock-out mice (2 ± 0.1 mM vs. 2.8 ± 0.4 mM in astrocytes and 1.2 ± 0.1 vs. 1.7 ± 0.2 mM in SR101-negative cells).

Thus, the results indicate that Na^+ transients occurring after glutamate application in SR101-positive, as well as SR101-negative cells are a direct effect of the agonist as opposed to diffusion-based signals. Furthermore, it appears that gap junctions limit glial Na^+ loads, as Na^+ peak amplitudes in slices taken from Cx knock-out mice were larger than in control sections. This finding supports the thesis, that gap junctions play a role in the maintenance of ion concentrations within the physiological range along the glial syncytium at rest and during activity, which results in high intracellular Na^+ loads. A similar observation was made in Rose and Ransom (1997), where they found that in cultured astrocyte gap junctions were required to maintain homeostatic Na^+ concentrations (Rose and Ransom, 1996).

The present study shows for the first time *in situ* that similar to grey matter, white matter astrocytes and cells of the oligodendrocyte lineage experience Na^+ signaling after agonist application or stimulation of neuronal activity through electrical stimulation. While glutamate receptor channels contribute to those signals to a certain degree depending on the cell types and the experimental approach, the major pathway for Na^+ influx appears to be glutamate transporter-mediated. The results presented above suggest that the impact of the different transporter subtypes and their cell type specific expression is different when compared to grey matter regions, adding to the field of glial heterogeneity that has gained much interest recently

and focusses on regional and developmental variety within glial subtypes. Furthermore, it has been shown that gap junction-mediated spread of Na^+ is not only restricted to one glial cell type but allows for panglial communication.

These results lead to the question - what are the functional consequences of white matter Na^+ signaling? First of all, as glutamate-evoked Na^+ signals in the *corpus callosum* seem to be mediated mainly via glutamate transport, the role of glial cells might indeed be very similar to grey matter, where glutamate has to be removed from the synaptic cleft. In white matter, the danger of neuronal excitotoxicity might be comparatively low as there are no postsynaptic densities with high concentrations of glutamatergic receptors. However, cells of the oligodendrocyte lineage have been shown to express glutamatergic receptors and those cells therefore face excitotoxic events when exposed to high extracellular glutamate loads (Deng et al., 2004; Domercq et al., 2005), even more so in combination with oxygen-glucose deprivation (Deng et al., 2006). Interestingly, strong Na^+ influx into oligodendrocytes might occur under pathophysiological conditions and mediate excitotoxic damage as well (Fern et al., 2014; Matute et al., 2013).

Whether or not Na^+ signals in white matter have a significance beyond reflecting glial glutamate uptake, has yet to be demonstrated. Regarding the glial functions presented in the introduction, the reversal of NCX and subsequent Ca^{2+} signaling may be one additional function. The NXC in Bergmann glia has been reported to enter the reverse mode after kainate-induced intracellular Na+ increases of 30 mM (Kirischuk et al., 1997). In a later study, Kirischuk et al. (2012) suggested that a depolarized membrane potential of -30 mV, intracellular Na^+ increases by ~ 9 mM and concomitant Ca^{2+} influx are sufficient to initiate the reverse operating mode (Kirischuk et al., 2012). Na^+ amplitudes as measured in the present study could therefore be sufficient to trigger NCX reversal, assuming that Na^+ peak amplitudes are higher in the processes than in the soma. Glial somata were in the focus of the present study, but high resolving microscopic approaches could be adopted to resolve Na^+ activity in astrocytic or oligodendrocytic processes. Eventually, reversal of the NCX and subsequent Ca^{2+} signaling might then trigger gliotransmission involving e.g. glutamate or ATP.

The NCX has been shown to be not only expressed by astrocytes but also by cells of the oligodendrocyte lineage. Moreover, modulation of certain NCX subtypes during maturation has been demonstrated to drive myelin formation and differentiation under ischemic conditions (Boscia et al., 2013). In NG2 cells, NCX reversal is implicated in cell migration induced by GABA-receptor activation, which has been shown to lead to membrane depolarization in hippocampal NG2 cells (Lin and Bergles, 2004; Tong et al., 2009). Na^+ signals do not only exert physiological functions via the NCX. In oligodendrocyte development, AMPA receptor-mediated Na^+ influx leads to a block of K^+ channels and inhibits proliferation (Borges and

Kettenmann, 1995; Knutson et al., 1997). This finding strongly supports the idea of activity-dependent OPC differentiation and myelination, contributing to white matter plasticity.

Finally, several groups have found a clear link between astrocytic Na^+ transients, stimulation of glial metabolism and neuro-metabolic coupling in grey matter. Activity-dependent Na^+ influx stimulates the NKA and thereby causes the consumption of ATP. This leads to glycogen breakdown, glycolysis and lactate production (Chatton et al., 2016). The same might occur in white matter, where axons are particularly dependent on metabolic support coming from their surroundings. Metabolic interactions in white matter appear to be more complicated however, as oligodendrocytes seem to take over some of the functions typically fulfilled by astrocytes, such as the transfer of lactate to neurons (Funfschilling et al., 2012; Lee et al., 2012). On the other hand, astrocytic glycogen stores are still important to maintain axonal function, as has been shown in the mouse optic nerve during high neuronal activity (Brown and Ransom, 2007; Brown et al., 2005). Another task that, in grey matter, is almost exclusively exercised by astrocytes is the recycling of glutamate via the glutamine synthetase. Here, synaptically released glutamate is taken up by the astrocytic transporters and converted to glutamine, which can be transported back to neurons as it is physiologically/chemically inert. Interestingly, GS has been shown to be expressed by white matter oligodendrocytes as well (Anlauf and Derouiche, 2013; Tansey et al., 1991). Therefore, one question that remains is how exactly the different glial cells in white matter interact in terms of metabolism and handling of intracellular glutamate. It is evident now, that white matter physiology is much more complicated than initially thought.

In sum, the present work demonstrates that Na^+ signaling can be evoked in astrocytes and cells of the oligodendrocyte lineage in the *corpus callosum*. Those transients are mediated mainly by glutamate transport, but in a heterogeneous manner when compared to grey matter regions. Physiological implications beyond the involvement in glial glutamate uptake are not yet fully understood, but considering the established functions of glial cells, it is easily conceivable that Na^+ could affect a variety of cellular processes in white matter. Looking at panglial coupling, Na^+ could be a key factor in connecting these processes, as it easily spreads via gap-junctions. It might thus present a mediator between the different glial cell types, possibly coupling metabolism between those cells and linking neuronal activity to white matter plasticity via its impact on differentiation and myelination.

4. Publications

4.1 Changes in the proliferative capacity of NG2 cell subpopulations during postnatal development of the mouse hippocampus.

Moshrefi-Ravasdjani B, Dublin P, Seifert G, Jennissen K, Steinhauser C, Kafitz KW, Rose CR
Brain Struct Funct 222(2):831-847 (2017)

Parts of the data included in this work have been previously implemented in my master's thesis. During my PhD phase, I complemented the project for publication, performing the following immunohistochemistry experiments, analyses and preparation of the updated figures: I increased the sample size for NG2 cell density studies (Fig. 2) and NG2/S100B co-localization studies including confocal images (Fig. 3-6). I increased sample size and added age groups for Ki-67 studies (Fig. 7 and 8). I contributed to the first draft of the manuscript and was involved in the interpretation of the data and in the revision of the manuscript.

4.2 Rapid sodium signaling couples glutamate uptake to breakdown of ATP in perivascular astrocyte endfeet.

Langer J, Gerkau NJ, Derouiche A, Kleinhans C, Moshrefi-Ravasdjani B, Fredrich M, Kafitz KW, Seifert G, Steinhäuser C, Rose CR

Glia 65(2):293-308 (2017)

I conducted direct electrical stimulation imaging experiments measuring sodium and magnesium fluorescence in astrocytes. I analysed the obtained data, prepared the according figure (Fig. 7B) and drafted the corresponding text passage. I was involved in the interpretation of the data and in the revision of the manuscript.

4.3 Astrocyte sodium signalling and panglial spread of sodium signals in brain white matter.

Moshrefi-Ravasdjani B, Hammel EL, Kafitz KW, Rose CR

Neurochem Res 42(9):2505-2518 (2017)

I was involved in the planning and conceptualization of the entire work. I prepared the figure addressing *corpus callosum* microanatomy (Fig. 1). For the following figures I conducted the imaging experiments, analyzed the data and prepared the figures for presentation: astrocyte identification with hGFAP-GFP/SR101 and sodium signals induced by focal pressure application of glutamate (Fig. 2); interglial spread of sodium after the direct stimulation of a single astrocyte (Fig. 3); spread of sodium from astrocytes to PLP-GFP-positive oligodendrocytes (Fig. 4) and to NG2-EYFP-positive NG2 cells (Fig. 5). I contributed to the interpretation of the data and wrote the first draft of the manuscript. I was involved in the revision of the manuscript.

4.4 Action potential firing induces sodium transients in macroglial cells of the mouse *corpus callosum*.

Moshrefi-Ravasdjani B, Ziemens D, Pape N, Färfers M, Rose CR

Neuroglia 1, 106–125 (2018)

I was involved in the planning and conceptualization of the entire work. I contributed to the figure addressing action-potential induced sodium signals, performing and illustrating the imaging experiments shown in figure 1 B and C. For following figures, I conducted the imaging experiments, analyzed the data and prepared the figures for presentation: pharmacology of action potential-induced sodium transients in astrocytes and SR101-negative cells (Fig. 2) and specifically in NG2 cells and oligodendrocytes (Fig. 3); involvement of gap-junctions (Fig. 4); contributions of glutamate transporter subtypes (Fig. 5). I prepared the figures illustrating immunohistochemistry and western blots of glutamate transporters (Fig. 6 and 7). I contributed to the interpretation of the data and wrote the first draft of the manuscript. I was involved in the revision of the manuscript.

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Eidesstattliche Erklärung

Ich versichere an Eides Statt, dass die vorliegende 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. 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.

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