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Heterogeneity of Astrocytes from the Cortical Gray Matter and White Matter: A Transcriptomic Approach

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Meiner Familie und Kristina

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1. Introduction

1.1 Overview

The notion of diversity among astrocytes dates back to over a century ago, when two morphologically distinct varieties were first described: protoplasmic astroglia, encountered in gray matter (GM) areas of the central nervous system (CNS), and fibrous astroglia, found in white matter (WM). Nevertheless, and owing in part to the dominance of neuronal research throughout much of the 20th century, astrocyte heterogeneity as a concept has only entered the field of neuroscience fairly recently.

Since then, a large number of morphological, molecular, and electrophysiological differences have been uncovered between astroglial cells from various brain regions and even between cells that share the same basic location. One question that arises from this surprising diversity is that of its origin: how much of astrocyte heterogeneity can be seen as preprogrammed in early development and to what degree is it subject to environmental cues?

In keeping with Rudolf Virchow's initial description of neuroglia as mere structural support for the neuronal elements of the CNS, astrocytes have long been regarded as essentially passive in nature. This notion, however, has been thoroughly turned on its head over the last couple of decades when astroglia has been identified as a crucial component of CNS physiology, directly involved in matters of homeostasis, metabolism, cerebral blood flow, and even synaptic transmission.

Apart from their multifaceted role in the healthy nervous system, astrocytes have also emerged as important elements in multiple pathologies of the brain and spinal cord. Reactive astrogliosis, a complex process of morphological and molecular changes in response to certain disease-associated triggers, can be found to varying degrees in virtually every CNS disorder. Both beneficial and harmful aspects of this reaction have been observed in different contexts, making astrocytes intriguing yet elusive targets for a number of potential therapeutic interventions.

The advent of next-generation sequencing, combined with the development and improvement of various cell-specific isolation methods in recent years, has allowed for

genome-wide approaches towards astrocyte heterogeneity. The enormous amounts of data such methods tend to produce can offer insights into cellular diversity at a previously unattainable level. However, the prudent application of interpretative and statistical tools is indispensable for any information so obtained to be meaningful.

Here we used two distinct methods to extract ribonucleic acid (RNA) samples from cortical GM and WM astrocytes, which were then sequenced and analyzed to gain a better understanding of the molecular and functional differences between these two glial subtypes. To our knowledge, this constitutes the first investigation of the molecular differences between protoplasmic and fibrous astrocytes on a transcriptomic level.

1.2 Historic Origins

It may be helpful to preface the actual discussion of astrocyte heterogeneity attempted in these pages with a short historical overview of how our present concept of the astrocyte has been shaped by a series of discoveries and technological advancements over the past 150 years. When talking about the inception of astrocyte – or more generally glia – research, the name of Rudolf Virchow is usually the first to be mentioned. And while there certainly is some justification to this attribution, the origins of our current understanding regarding astrocytic cells are quite a bit more tangled and complex.

1.2.1 Defining the Astrocyte

The first instance of the term ‘neuroglia’ can be found in Virchow’s collected papers from 1856 where he used it in a footnote to a previously published article. Here, it seems, his notion of this so-called ‘Nervenkitt’ (something along the lines of ‘nerve glue’) was one of a rather nondescript substance that fills the spaces between the cellular elements of the CNS (Virchow, 1856). This idea was picked up again and expanded upon only two years later in his landmark work on *Cellularpathologie* from 1858, a series of lectures delivered by Virchow to the medical students of the Charité Hospital in Berlin, where he worked as a pathologist. Although at this point Virchow acknowledged that this new form of ‘connective tissue’ contained a certain number of cellular components itself, he still saw it as essentially structural in nature, mere scaffolding for the functionally autonomous neurons (Virchow, 1858) – a view that was

elaborated by his colleague Carl Weigert and to some extent persists even today (Weigert, 1895).

It is worth noting that while the coinage of the term ‘neuroglia’ does indeed go back to Virchow’s writings, certain elements of this yet only vaguely defined class had already been described years earlier by people like Robert Remak in 1838 (sheaths around individual nerve fibers; Kettenmann and Verkhratsky, 2008) or Heinrich Müller in 1851 (radial fibers in the retina, still known today as ‘Müller glia’; Müller, 1851).

The second half of the 19th proved to be a very fruitful period for cellular research. As visualization methods both on a technical and biochemical level rapidly improved in quality, descriptions and pictures of a variety of glial elements were produced in short succession. In his posthumously published book *Untersuchungen über Gehirn und Rückenmark des Menschen und der Säugethiere* from 1865 Otto Deiters included histological images of stellate cells that are often made out to be the first depictions of astrocytes put on paper (Deiters, 1865). However, it is rather doubtful whether this is in fact the case. Judging from a modern perspective, they may well be oligodendrocytes or even neurons whose axons were not rendered adequately in the staining process (Somjen, 1988).

Perhaps the Italian biologist Camillo Golgi should be credited with the actual identification of astrocytes as a distinct type of cell. In 1872 he described a set of stellate cells the processes of which extend to the walls of nearby blood vessels, strongly suggestive of the glial-vascular interface formed by astrocytic endfeet as we know it today. His drawings from the period confirm that impression (Golgi, 1872).

When it became increasingly clear that Virchow’s concept of neuroglia was too general a term to account for the differences among the cellular elements it comprised, Michael von Lenhossék, an anatomist from Budapest, in 1893 proposed the name ‘astrocyte’ to refer to a subset of star-shaped cells that constituted one of the most common types of glia found in the CNS of vertebrates (Lenhossék, 1893). In the same year, the British physician William Lloyd Andriezen distinguished between fibrous astrocytes found in WM and protoplasmic astrocytes found in GM, a classification that – notwithstanding the ongoing emergence of ever subtler varieties – still holds today (Fig. 1; Andriezen, 1893).

Further diversifying the neuroglia palette, the Spanish neuroanatomist Santiago Ramón y Cajal, often referred to as the father of modern neuroscience, in 1913 described a “tercer elemento” or “third element” distinct from both neurons and astrocytes (Ramón y Cajal, 1913). His own improvements on Golgi’s revolutionary staining method had previously allowed him to visualize various cells of the CNS in hitherto unmatched detail. During the late 1910s and the 1920s, Ramón y Cajal’s disciple Pío del Río Hortega and the American-Canadian neurosurgeon Wilder Penfield managed to resolve this mysterious third element into two additional types of cells, microglia and oligodendrocytes, thereby completing our current picture of neuroglial elements (Binder and Hubbard, 2016) – excepting the only much later described class of Neuron-glial-antigen 2 (NG2) cells.

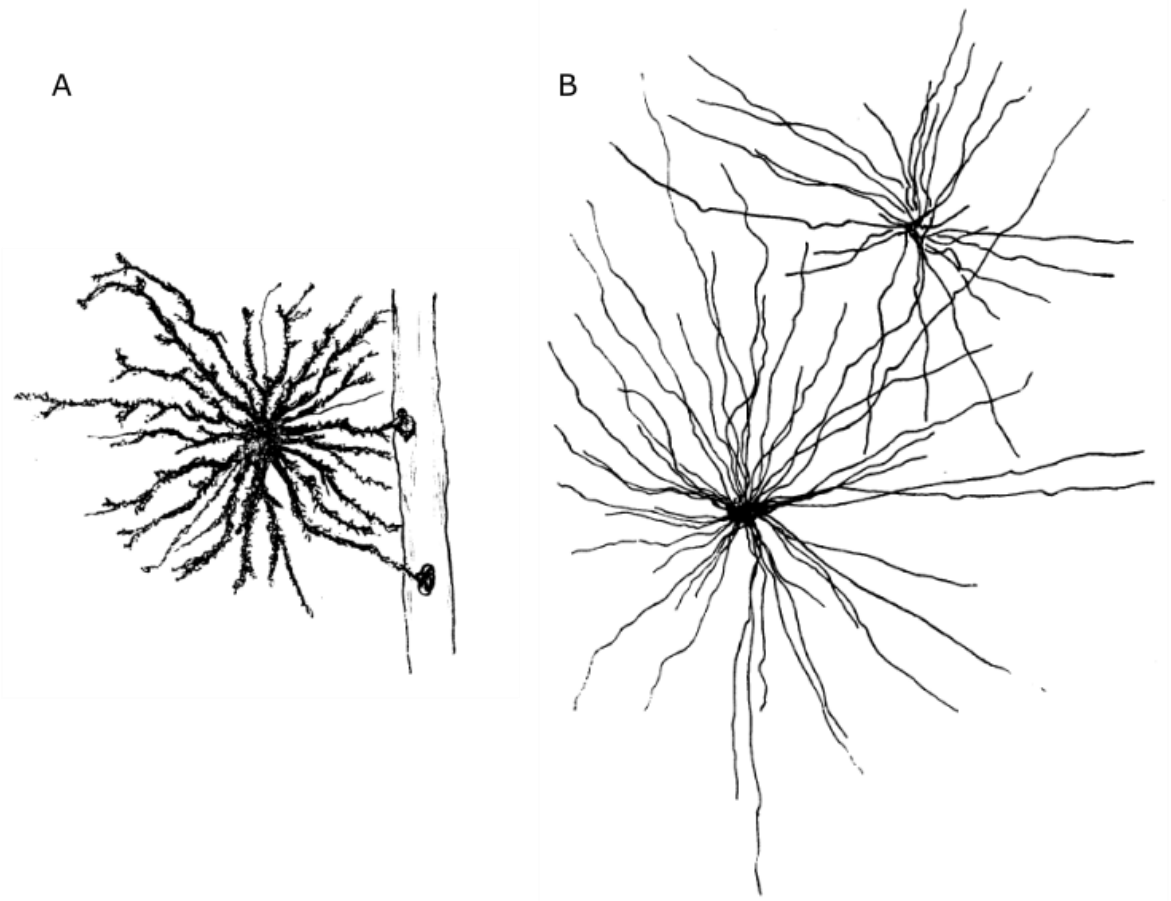


Fig. 1: Early depiction of protoplasmic and fibrous astrocytes by William L. Andriezen
Drawings of an astrocyte from human cortical GM contacting a neighboring blood vessel via its appendages (A) and of two WM astrocytes with their typical 'fibrous' morphology (B). Figure modified from Andriezen, 1893.

1.2.2 The Question of Function

While the widespread existence of astrocytes in virtually every part of the CNS was recognized early on, their actual purpose eluded most attempts at clarification (and in many ways continues to do so today). In his landmark *Histologie du Système Nerveux de l'Homme et des Vertébrés* from 1909, Ramón y Cajal put it thus:

> *What is the function of glial cells in neural centers? The answer is still not known, and the problem is even more serious because it may remain unsolved for many years to come until physiologists find direct methods to attack it.* (Ramón y Cajal, 1909; transl. in: Kettenmann and Verkhratsky, 2008)

However, a number of theories have been propounded during the initial years of glial research, some of which have since then seen a surprising amount of evidence confirming their basic tenets and building on them. One of those theories was put forward by Golgi who noticed the frequent association of astrocytic processes with the walls of blood vessels on the one hand, and the close spatial relationship of glial cells and neurons on the other. This observation led him to propose a nutritional role for astrocytes, rendering metabolic support to the energy-hungry neurons and supplying them with a means for the continual exchange of various substances (Golgi, 1885). Although refined and fleshed out in many respects, Golgi's notion of the astrocyte as an essential component of CNS metabolism has become a widely accepted cornerstone of modern neuroscience.

Another early speculation regarding the function of neuroglia came from the German surgeon Carl Ludwig Schleich. In his book *Schmerzlose Operationen* from 1894, a treatise dealing with the principles of local anesthesia, he hypothesized that by swelling in size to reach the spaces between two neighboring neurons and disrupt their intercellular communication, astrocytes could lower the excitability of the CNS (Schleich, 1894). Despite the fact that Schleich's suggested mechanism over-simplifies matters to a certain degree and in some ways actually runs counter to our current understanding of these issues, the general idea of glial cells influencing synaptic transmission has become a central tenet of modern neuroscience with the concept of the so-called tripartite synapse (Binder and Hubbard, 2016).

In a somewhat related manner, the Italian psychiatrist Ernesto Lugaro – originator of the term 'plasticity' in a neurophysiological context and the first to describe a class of cerebellar

interneurons bearing his name (Berlucchi, 2002) – in 1907 proposed the uptake and metabolism of chemical transmitters at 'neuronal articulations' (i.e. synapses) as another major task of astrocytes (Lugaro, 1907). This theory was confirmed decades later by the discovery that astrocytic glutamate transporters are responsible for the bulk of glutamate reuptake from the extracellular space.

Finally, a theory of astrocyte interconnectedness was put forward by the German physician Hans Held in his 1904 book *Über den Bau der Neuroglia und über die Wand der Lymphgefasse in Haut und Schleimhaut*. Held speculated that the fibers of glial cells in fact form a large syncytial network (Held, 1904), a notion that prefigures later findings of coupling via gap junctions in a variety of astrocytes from different brain regions.

1.3 Astrocyte Development

When investigating diversity among cells of the astrocytic lineage, their origin in CNS development is an obvious place to start. The generation of astrocytes in rodents begins around embryonic day 12.5 (E12.5) in the spinal cord and around E16-E18 in the cortex, when the bulk of neurogenesis has already taken place. While the concept of two different progenitor pools for neurons and glia held sway throughout much of the previous century, a substantial amount of evidence now suggests otherwise: cells considered part of the glial lineage – radial glia (RG) and their remnants in the adult CNS – have in fact been identified as precursor cells, giving rise to differentiated neurons and glial cells both in development and the postnatal brain (Malatesta et al., 2000; Götz et al., 2015).

The transition from neurogenesis to gliogenesis is a tightly regulated process involving a complex interplay of various molecular pathways. Similarly, the generation of astrocytes itself follows a distinctive time course and draws from various sources to build up the eventual population of the mature CNS. There is already an element of patterning present at this point, contributing to astrocyte heterogeneity right from the developmental stage.

1.3.1 Termination of Neurogenesis and Initiation of Astrogenesis

During the earlier part of embryogenesis, proneural transcription factors (TFs) like Neurogenin 1 (Ngn1) simultaneously drive the generation of neurons and block the initiation of

astrogenesis (Sun et al., 2001). The latter is mainly effected by repression of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway, the canonical signaling cascade promoting astrocyte differentiation (Bonni et al., 1997; He et al., 2005). By binding the p300/CBP co-activator complex, Ngn1 prevents it from interacting with STAT3, a crucial component of the JAK/STAT pathway (Sun et al., 2001).

In addition to this, STAT3 binding sites at the promoter of the astrocytic genes Glial fibrillary acidic protein (GFAP) and S100 calcium-binding protein β (S100β) are methylated during neurogenesis, further inhibiting gliogenesis (Takizawa et al., 2001). This was confirmed by conditional deletion of DNMT1, which lead to the initiation of astrogenesis (Fan et al., 2005).

A number of other factors and pathways contributing to the repression of astrogenesis have been identified, yet most of them are in some way or another associated with the JAK/STAT cascade. A notable exception is ErbB4-NCoR, a repressor complex whose signaling cascade is thought to be activated by Neuregulin (Sardi et al., 2006). The exact mechanisms and sequence of events are, however, still unclear.

For the switch from neurogenesis towards gliogenesis to occur, the chromatin conformation of signature astrocyte genes like GFAP has to be altered for transcription. This is accomplished in a number of different ways. The downregulation of Ngn1 – effected through the silencing of its promoter by PcG proteins (Hirabayashi et al., 2009) – releases p300/CBP, which in turn induces H3K9 and H3K14 acetylation at the STAT3 binding site of the GFAP promoter by its intrinsic acetyltransferase activity (Cheng et al., 2011).

Another important factor towards initiation of gliogenesis is the methylation status of STAT3 binding sites at astrocytic gene promoters (Takizawa et al., 2001). Here, Nuclear factor 1 A (NF1A) plays a crucial role, as its upregulation causes the dissociation of DNMT1 from the GFAP promoter (Deneen et al., 2006; Cebolla and Vallejo, 2006; Wilczynska et al., 2009). The lack of methylation structurally opens up the chromatin and allows for transcription to commence. Substantiating these relations is the fact that in NF1A knockout mice methylation is retained and GFAP expression is significantly reduced in various glial populations (Das Neves et al., 1999; Shu et al., 2003).

NF1A is itself a downstream target of Notch signaling (Namihira et al., 2009), a key regulator of neural stem cell (NSC) fate during development and a close functional relative to JAK/STAT

signaling in promoting astrogenesis (Kamakura et al., 2004). By repressing Hes1, another component of Notch signaling, NF1A creates a negative feedback loop that prevents excessive generation of astrocytes (Piper et al., 2010). Expression levels of NF1A are on their part tightly controlled by the inhibitors Lhx2 (Subramanian et al., 2011) and Emx2 (Gangemi et al., 2006; Brancaccio et al., 2010) as well as the positive regulator Sex determining region Y-box transcription factor 9 (Sox9) (Kang et al., 2012; Martini et al., 2013), another Notch downstream target.

Other pathways and extrinsic stimuli that seem to play a role in the relaxation of the GFAP promoter's chromatin state have been identified, among them FGF2 (Song and Ghosh, 2004; Irmady et al., 2011) and retinoic acid (Jepsen et al., 2007; Asano et al., 2009). The exact mechanisms involved, however, are as of yet only partially understood. Recently, Oasis-Gcm1 signaling, a pathway that bears on protein folding in the endoplasmic reticulum (ER), has been suggested as another element involved in the onset of astrogenesis, forging an interesting connection between ER function and astrocyte differentiation (Saito et al., 2012).

To initiate the differentiation of RG into astrocytes, gliogenic signals are necessary. Foremost among them is CT-1, a cytokine that is thought to be secreted by newly generated neurons, thus playing a crucial role in the temporal coordination of CNS development. Consequently, its loss results in severe defects concerning astrogenesis (Barnabe-Heider et al., 2005). A number of other related cytokines like LIF and CNTF have been identified in this context, although the phenotypical impact of their depletion was found to be significantly smaller (Bugga et al., 1998), mainly because their endogenous expression occurs at a later time point (Miller and Gauthier, 2007).

Although these gliogenic cytokines are the central players in promoting astrogenesis via activation of JAK/STAT signaling, they work in synergy with the class of Bone morphogenic proteins (BMPs) and their active agent SMAD1 (Nakashima et al., 1999). BMP2, by inducing expression of the Notch downstream target Hes5 (Nakashima et al., 2001), and BMP4, especially in later stages of development (Gross et al., 1996; Li et al., 1998), are assumed to be the principal family members in this regard. Recently, BMP7 has been identified as another important exponent by showing that its intraventricular injection can trigger premature astrogenesis (Ortega and Alcántara, 2010). Interestingly, GFAP expression due to BMP signaling was found to give rise to morphologically distinct astrocytes compared to LIF-induced

GFAP expression (Bonaguidi et al., 2005), providing a clue for astrocyte heterogeneity present at the level of development (cf. Fig. 2 for an overview of the various pathways described).

As will be apparent from the preceding paragraphs, the investigation into the mechanisms controlling astrogenesis has so far almost exclusively focused on the GFAP promoter – with a handful of studies looking at the S100β promoter as well. While GFAP is undoubtedly an important and well-established signature gene, a broadening of perspective will be indispensable if one wants to get a fuller picture of glial development and the origins of astrocyte diversity (Kanski et al., 2014).

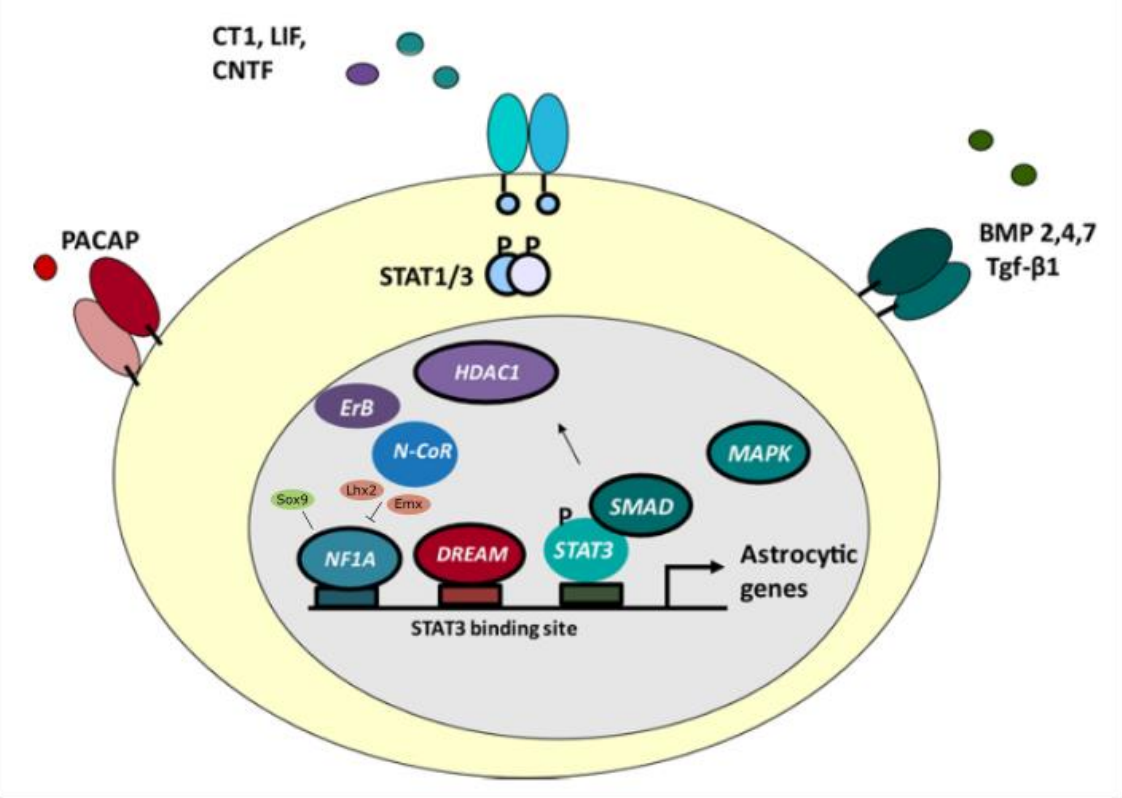


Fig. 2: Initiation of astrogenesis is regulated by a complex interplay of gliogenic cytokines and signaling factors
JAK/STAT signaling, the canonical pathway for astrocytic gene expression, is activated by cytokines such as CT-1, LIF, and CNTF. Additionally, these cytokines may release repressor complexes like ErbB4-NCoR and HDAC1 from astroglial gene promoters. Potentiating this effect are growth factors like various BMPs and TGF-β1, which signal via SMAD proteins on the STAT3 binding site of the GFAP promoter. Another important component in this context is the transcription factor NF1A, a downstream target of Notch signaling. Its upregulation opens up the chromatin, thereby allowing for the transcription of astrocytic genes to commence. Figure modified from Kanski et al., 2014.

1.3.2 Sources and Time Course of Astrogenesis

Once the transition from neurogenesis to gliogenesis has been accomplished, the actual generation of astrocytes begins. This process of astrogenesis – which precedes the formation

of oligodendrocytes yet also overlaps with it to some degree – sets in during late embryogenesis but reaches its apex around birth.

There are different mechanisms at work here: The first one of them is the direct transformation of RG, which after the termination of neurogenesis translocate from the ventricular zone (VZ) – a transient layer of tissue lining the ventricular system –, lose their apical processes, and become protoplasmic astroglia. This process was initially discovered in the brain of rhesus monkeys (Schmechel and Rakic, 1979), with similar observations later reported for the ferret brain (Voigt, 1989). More recently, RG-labeling experiments using dye in the brain of human fetuses (deAzevedo et al., 2003) and adenovirus-Cyclization recombination (Cre) infection in the mouse cortex (Tsai et al., 2012) also obtained results along those lines. RG-to-astrocyte transformation was even observed via live imaging, although cultured rat brain slices were used in this case (Noctor et al., 2004). Given that astrocyte morphology and protein expression tend to undergo significant changes under cultured conditions, a validation of these results *in vivo* would be desirable.

Direct transformation of RG may also be responsible for the generation of astrocyte-related NSCs or RG-like cells in the subventricular zone (SVZ) that persist and generate neurons throughout adult life (Merkle et al., 2004). Although these cells are known to express most astroglial marker proteins in varying degrees, Fluorescent-activated cell sorting (FACS) and subsequent transcriptomic comparison with parenchymal astrocytes revealed a distinct molecular profile that emphasizes their RG-like nature (Beckervordersandforth et al., 2010).

Cortical astrocyte generation in its second phase peaks between postnatal day 3 (P3) and P7, mainly because of local proliferation. Making use of retroviral injection and *in vivo* imaging, it has been shown recently that the symmetric division of differentiated parenchymal astroglia throughout the CNS contributes about half of the total adult astrocyte population (Ge et al., 2012). Due to the caudal to rostral maturation of the CNS, these timepoints occur around five to six days earlier in the spinal cord.

Interestingly, although local proliferation of astrocytes decreases drastically after the first two to three postnatal weeks, it persists at a low rate throughout adulthood (Messier et al., 1958). Early quantifications in P23, P100, P200, and P400 mice utilizing radioautography found the amount of proliferating astroglia to be highest in the corpus callosum with a mean percentage of 0.445% signal-positive cells (Dalton et al. 1968). Later experiments with P48-P52 transgenic

mice and Ki67 immunostaining – a marker for mitotic cells – observed 0.30% of dividing astrocytes averaged for the entire cortex (Ge et al., 2012).

Finally, NG2 glial cells have been suggested as another source of protoplasmic astroglia restricted to the ventral forebrain (Zhu et al., 2008a) and possibly the spinal cord (Zhu et al., 2008b). While several groups found the astrogenic potential of NG2 glia to be exhausted by the time of birth (Rivers et al., 2008; Kang et al., 2010; Zhu et al., 2011; Huang et al., 2014), one study came to different conclusions, detecting postmitotic, protoplasmic astrocytes in the GM of the spinal cord and ventral forebrain that originated from NG2 cells postnatally (Guo et al., 2009). An explanation for this discrepancy might be the difference in experimental paradigm: The groups that only encountered NG2-derived astrocytes during embryonic stages used transgenic mice carrying an inducible Cre gene under the control of either the Pdgfrα or the NG2 promoter, whereas the discovery of postnatal NG2-to-astroglia conversion was made in Cre-inducible Proteolipid protein (PLP) mice.

1.3.3 Developmental Patterning of Astrocyte Identity: Nature vs. Nurture

As our insights into the molecular and functional diversity of mature astroglia continue to advance, the question to what degree (and how far) this heterogeneity can be traced back to early development grows ever more relevant. In one extreme scenario, astrocyte fate would be entirely intrinsic, genetically preprogrammed from the very beginning and largely unresponsive to any environmental cues. On the other end of the spectrum, one could imagine all newly formed astrocytes to be essentially identical, their respective subtypes being shaped extrinsically by interaction with their surroundings. At this point, an intermediate scenario seems most likely, where astrocytes do indeed exhibit aspects that are prepatterned early on while at the same time remaining highly responsive to cues from their environment.

The patterning of the neural tube along its dorso-ventral axis by a variety of morphogens and their respective regulation of homeodomain TFs have been established for quite some time as regards the emergence of neuronal subtypes (reviewed for the spinal cord in Jessell, 2000). Interestingly, a number of more recent studies suggest that these very mechanisms are also instructive when it comes to the generation of different astrocyte populations.

A first confirmation of this principle was achieved for a restricted region of the spinal cord, where the bHLH TF Scl controls the generation of both V2b interneurons and ventral astrocytes from the p2 progenitor domain. Intriguingly, the same study found that Scl remains actively expressed in astroglia of the adult SVZ as well as the corpus callosum (Muroyama et al., 2005), possibly hinting at a transcriptional relation between the two subpopulations.

A few years later, another organizational homeodomain code was described operating in the chick ventral spinal cord, where graded expression of Reelin and Slit1 defines three positionally distinct subtypes of WM astrocytes. The location of these astrocytes along the dorso-ventral axis is governed by the combinatorial expression of the TFs Pax6 and Nkx6.1 and mirrors the arrangement of their progenitors in the p1-p3 domains (Hochstim et al., 2008).

Finally, the application of fate mapping techniques allowed for the finding that patterning during astrocyte development is a CNS-wide phenomenon, and that astrocytes are allocated to spatial domains in accordance with their embryonic sites of origin in the VZ. It was further shown that astrocytes stick to their respective domains throughout life, even after acute CNS injury: upon domain-specific depletion of astrocytes, their counterparts from adjoining regions were not able to invade and rescue the affected area, revealing fundamental limitations of the astroglial response to CNS injury (Tsai et al., 2012, Bardehle et al., 2013).

There is also a functional aspect to this developmental patterning of astrocytes, as was demonstrated for newly formed spinal cord astrocytes. Here, the protein Semaphorin3a (Sema3a) is expressed in a graded manner, with cells of the dorsal horn exhibiting low and cells of the ventral horn high levels of Sema3a messenger (m)RNA transcripts. Loss of astrocyte-encoded Sema3a led to a marked disruption of motor and sensory neuron circuit organization, comprising dysregulated axon initial segment orientation, abnormal synaptic inputs, eventual selective death of α-motor neurons, and ectopic ventral projections by a number of TrkA+ sensory afferents. *In vitro* studies suggested that these positional properties of spinal cord astrocytes are at least partly cell intrinsic because their properties were retained in co-cultures of varying composition (Molofsky et al., 2014).

In addition to being subjected to similar patterning mechanisms, newborn astrocytes and neurons originating from the same neural progenitor were found to be organized in discrete columnar structures throughout the neocortex (Noctor et al., 2001; Magavi et al., 2012). A striking visualization of this geometric fidelity was achieved by tracking astrocyte lineages

through the combinatorial expression of multiple fluorescent proteins (García-Marqués and López-Mascaraque, 2013). Conditional and spatiotemporally controlled ablation of the bHLH TF Oligodendrocyte transcription factor 2 (Olig2) revealed divergent regulatory mechanisms in the generation of cortical GM and WM astrocytes. Fibrous astrocytes, as opposed to their protoplasmic counterparts, proved to be crucially reliant on Olig2 expression, which possibly hints at a shared developmental origin between WM astroglia and oligodendroglia (Cai et al., 2007).

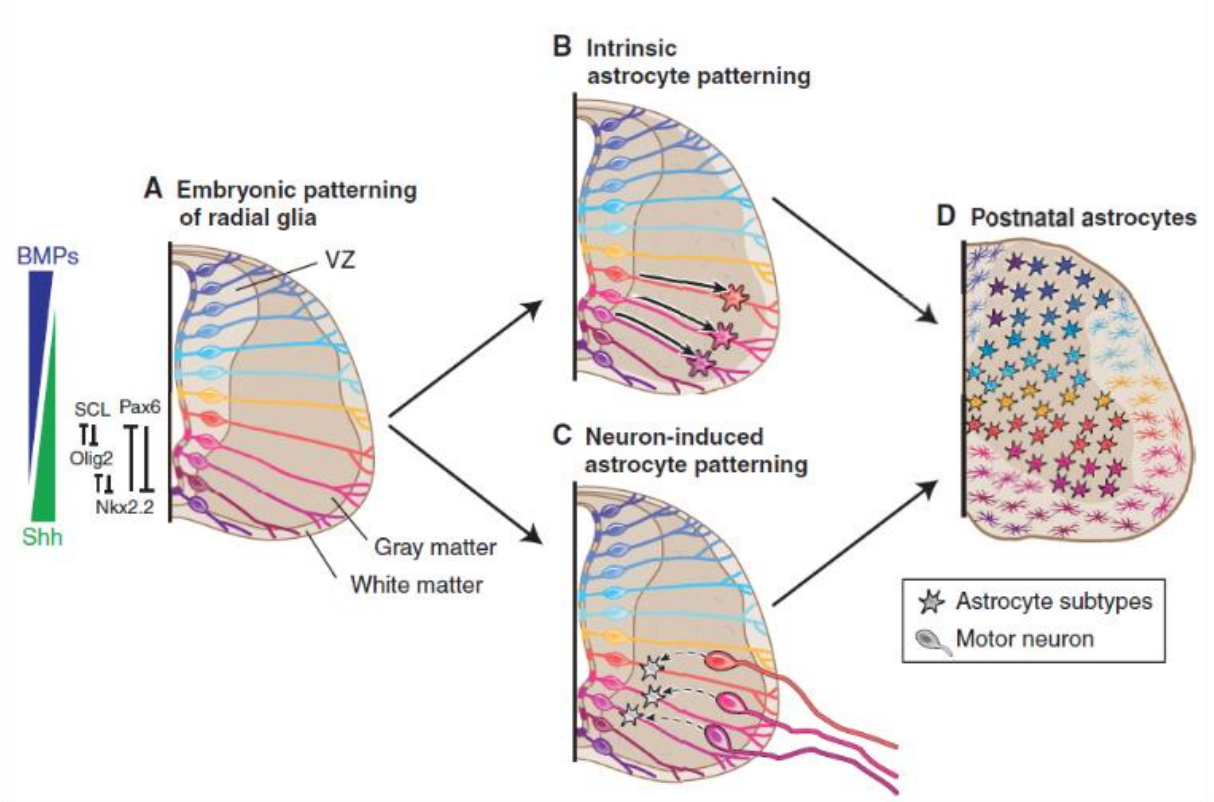


Fig. 3: Astrocyte heterogeneity is developmentally specified in an intrinsic manner as well as by environmental cues
During embryonic development, the graded expression of various transcription factors gives rise to regionally patterned RG (A), from which, in turn, spatially distinct astrocyte subpopulations emerge (B). Additionally, extrinsic cues of different origins (here exemplified by signaling from motor neurons) contribute to further refine this astroglial heterogeneity (C), leading to the establishment of molecularly and functionally distinct GM and WM astrocyte domains (D). The depiction here shows these processes for the spinal cord, yet similar mechanisms are most likely at play during cerebral development. Figure from Bayraktar et al., 2016.

However, there is evidence that astrocyte identity is not exclusively determined prenatally. A recent study looked at the role of Sonic hedgehog (Shh) signaling in regulating the molecular and functional profile of astrocytes, both shortly after birth (P2) and in the adult mouse CNS. In the cerebellum, neuron-derived Shh was found to be crucial for establishing (P2) and maintaining physiological properties of the local astrocyte population, i.e. Bergmann glial cells (BGs) and velate astrocytes. Interestingly, the molecular profile of velate astrocytes acquired

distinct features of the BG transcriptome when exposed to similarly high levels of Shh (Farmer et al., 2016).

What is more, Shh signaling was shown to act differently on astrocytes from various brain regions: While cerebellar BGs use Shh to promote a broader spectrum of functional properties, including glutamate detection/recovery and potassium (K^+) homeostasis, its role in hippocampal and cortical astrocytes seems to be restricted to the regulation of the K^+ channel K^+ inwardly rectifying 4.1 (Kir4.1), with glutamate receptors and transporters remaining largely unaffected by changes in expression (Farmer et al., 2016). This heterogeneous neuron-to-astrocyte communication via Shh in the adult mouse brain was also described by another group, who observed upregulation of GFAP and cellular hypertrophy – hallmarks of reactive gliosis – in some astrocytic subpopulations of the forebrain but not others upon attenuation of Shh signaling (Garcia et al., 2010; cf. Fig. 3 for an overview of developmentally based astrocyte heterogeneity).

1.4 Astrocyte Physiology: Diversity in Form and Function

While there had been some early attempts at clarifying the nature of astrocytes and their role in the CNS, as touched on above, this particular type of neuroglia spent most of the first half of the 20th century in the shadow of its more illustrious relative, the neuron. It was not until the late 1950s that astrocyte research can be said to enter its next phase, employing state-of-the-art technology and methodology to revisit previously made assumptions and gain new insights into its subject of investigation. As our understanding of glial physiology keeps expanding, a growing body of evidence suggests that astrocyte heterogeneity seems to be the rule rather than the exception.

1.4.1 General Characteristics

Besides their striking star-like appearance there are a number of characteristics both on an electrophysiological and a biochemical level that distinguish astroglia as a cell type. However, even in these very basic properties, astrocytes display a surprising amount of diversity both between different brain regions and within the same area, already hinting at a similarly heterogeneous situation in respect to functional aspects.

1.4.1.1 Morphology, Anatomical Organization and Interspecies Differences

Astrocytes take their name from their distinctive general shape (from Greek ἄστρον = star and κύτος = sheath, vessel; cell), although there are notable variations in appearance depending on anatomical location. The most basic subdivision between protoplasmic and fibrous astrocytes has already been suggested as early as the late 19th century (Andriezen, 1893) and retains much of its usefulness and validity even today. Protoplasmic astrocytes are encountered throughout the GM of the CNS, and their morphology is marked by comparatively large cell bodies with a number of stem branches radiating outward, which in turn give rise to further generations of ever more delicate processes. Fibrous astrocytes, on the other hand, are a variety found in WM areas, with rather smallish cell bodies that send out fewer and more elongated processes aligning along the myelinated fibers.

Contrary to the long-held assumption of astrocytes forming a tightly interdigitating lattice throughout the brain, it has been shown recently that they actually tend to command their own individual domains, with neighboring cells sharing only small common areas. Hippocampal astrocytes in mice were found to exhibit around 5% overlap with one another (Bushong et al., 2002; Ogata and Kosaka, 2002), and similarly low values have since been confirmed for cortical astrocytes (Livet et al., 2007; Halassa et al., 2007; Oberheim et al., 2008). As will be remarked upon in more detail later, there are a number of CNS pathologies that involve a disruption of this organization into domains, hinting at its crucial role in the healthy organism.

Interestingly, there are considerable differences both in size and morphological features of astrocytes between the rodent and the primate brain. Human astrocytes, for example, are multiple times larger and exhibit a vastly more complex morphology than their murine counterparts, allowing the processes extending from a single cell to encompass around 20 times more synapses (up to 120,000 in the rodent brain vs. up to 2,000,000 in the human brain). As a consequence of their increased size, human astrocytes – while still conforming to a basic domain organization – tend to overlap more fully with their neighboring glial cells (Fig. 4; Oberheim et al., 2006; Oberheim et al., 2009). Furthermore, studies of primate brains have led to the characterization of two additional morphologically distinct classes of cortical astrocytes that are absent from rodent nervous tissue: the so-called interlaminar astrocyte, found in the outermost layer I of the cortex (Colombo et al., 1995; Colombo and Reisin, 2004),

and the varicose projection astrocyte, whose domain are layers V-VI (Oberheim et al., 2009; Sosunov et al., 2014).

It is certainly tempting to assume that the increase in complexity from murine to human neuroglia might in some yet unknown way correlate with the tremendous cognitive abilities particular to our species. Seemingly bolstering this assumption are the often-read statements that (1) astrocytes are the most numerous class of cells in the whole human CNS and (2) the glia-to-neuron ratio grows progressively larger the further one goes up the evolutionary tree, with the human brain marking the apex in this regard – having a value of around 1.0 to 1.5 (reviewed in Bartheld et al., 2016). The germ of the latter idea can be dated back as far as 1898, when the German psychiatrist and pathologist Franz Nissl suggested as much in his treatise *Nervenzellen und graue Substanz*.

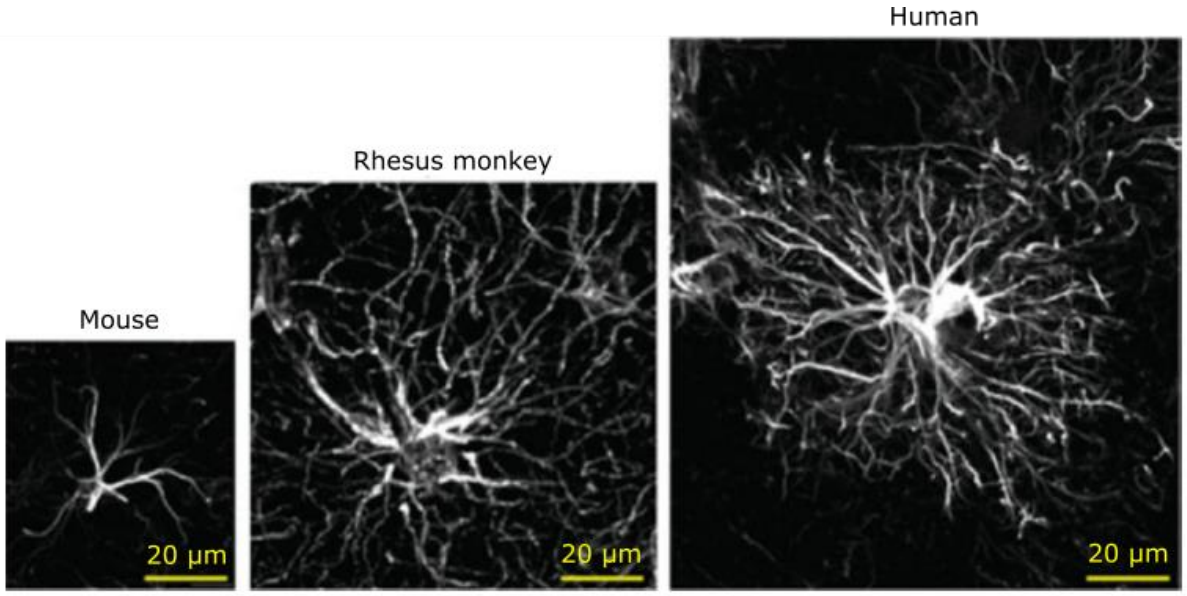


Fig. 4: Size and morphological complexity of astrocytes increase from mouse to rhesus monkey to human
GFAP staining of astroglia from mice, rhesus monkeys, and humans reveals considerable differences in both cell size and morphology among the three species. Human astrocytes are not only larger but significantly more complex than their animal counterparts, exceeding their number of cellular processes by a large margin. Figure modified from Oberheim et al., 2012.

It appears that in this case, however, conjecture has been uncritically taken for knowledge because the evidence for both of these claims is insufficient at best, and there is actual data that outright refutes them. For example, analyses of whale or elephant brains have yielded not only higher total numbers of glial cells than those counted in primates (which is to be expected) but have also shown that the glia-to-neuron ratios of both of these animals exceed that of humans by a large margin (Tower, 1954; Hawkins and Olszewski, 1957; Eriksen and

Pakkenberg, 2007; Goodman et al., 2009). The concept of a definite glia-to-neuron ratio is in itself questionable, considering that there is immense variation between different cerebral regions of the same species, ranging from 0.23 to 11.35 in the human brain (Herculano-Houzel, 2014). Moreover, the omnipresent claim that astrocytes constitute the most numerous fraction of cells in the CNS appears to be wrong, at least in the case of humans where oligodendrocytes likely outnumber them (Hess and Thalheimer, 1971; Pelvig et al., 2008). Thus, it is surely much too early to come to any definite conclusions in this matter, let alone jump to them.

1.4.1.2 Expression of Marker Proteins

The ability to reliably detect specific molecular markers in tissue samples using immunohistochemical (IHC) techniques is essential for any cell biological undertaking. In the case of astrocytes, GFAP was the first protein to be robustly associated with astroglia (Bignami et al., 1972) and has since come to be regarded as the prototypical astrocyte marker, being widely used in this capacity even today.

However, GFAP as a marker for astrocytes has a number of shortcomings and limitations that one needs to be aware of. It was initially isolated from brains of deceased multiple sclerosis patients, where demyelinated lesions showed very high concentrations of the protein (Eng et al., 1971; Uyeda et al., 1972). And while in a pathological context, GFAP does in fact prove to be a highly sensitive marker for astrocytes in the vicinity of lesion sites, its expression varies considerably across different regions of the healthy CNS, even exhibiting local differences. Fibrous WM astrocytes, for example, almost uniformly show robust expression of GFAP throughout the brain. Cortical GM astroglia, on the other hand, tends to display weaker levels of GFAP, and although many cells in superficial and deep layers express detectable amounts of GFAP, few do so in the middle layers (Ludwin et al., 1976).

In addition to its non-ubiquitous expression in astrocytes, detectable levels of GFAP are also found in cells that can at best be considered part of an extended astroglial family: the NSCs or RG-like cells inside the neurogenic niches, i.e. the SVZ (Doetsch et al., 1999) and the subgranular zone (SGZ) (Seri et al., 2001), that constitute the predominant source of adult neurogenesis (Garcia et al., 2004). They derive from RG and remain active throughout adult life, although their categorization as a subtype of astroglia (Kriegstein and Alvarez-Buylla,

2009) relies more on their expression of GFAP and a number of other glial proteins (Beckervordersandforth et al., 2010) than any functional association and is therefore open to debate (Sofroniew and Vinters, 2010).

Finally, a number of different isoforms and splice variants of GFAP exist – α, β (Feinstein et al., 1992; Galea et al., 1995), γ (Zelenika et al., 1995), δ (Condorelli et al., 1999) and κ (Blechingberg et al., 2007) –, and there is some evidence that they are heterogeneously expressed both in healthy and pathological CNS tissue (Roelofs et al., 2005; Andreiuolo et al., 2009), although their function remains unclear at this time.

Seeing itself confronted with these caveats, glial research has ever since been on the lookout for potential molecular markers that ideally would combine astrocyte exclusivity with pan-astrocytic expression. There have been some candidates over the years, each with its own strengths and weaknesses.

Glutamine synthetase (GS), a key enzyme in the metabolism of ammonia and various neurotransmitters, was found to be localized in rodent astrocytes some 40 years ago (Martinez-Hernandez et al., 1977; Norenberg and Martinez-Hernandez, 1979) and has since been used extensively as a marker protein. GS improves upon GFAP by being more generally expressed in astroglia throughout the rodent CNS, allowing for the IHC detection of protoplasmic and fibrous astrocytes in equal measure (Norenberg, 1979; Patel et al., 1985) – notwithstanding that one group claimed its absence in human WM astrocytes (Ong et al., 1993; Ong et al., 1995). However, there have been sporadic reports of GS expression in oligodendrocytes (Cammer, 1990; D'Amelio et al., 1990; Miyake and Kitamura, 1992), which adds some qualifications to its usage as a molecular marker for astrocytes.

Another well-established candidate is S100β, whose physiological and pathological functions in the CNS are manifold (reviewed in Donato et al., 2009). Although this particular protein was shown to be detectable in a significantly larger proportion of astroglia throughout the brain than GFAP (Savchenko et al., 2000, Ogata and Kosaka, 2002), it suffers from some of the same limitations as GS, namely the fact that it can possibly be found in other cell types as well. Reports exist for S100β expression in (predominantly WM) oligodendrocytes (Rickmann and Wolff, 1995b; Steiner et al., 2007) as well as different subpopulations of neurons (Rickmann and Wolff, 1995a). In the cerebellum, S100β appears to be a virtually exclusive astroglia marker, without any detectable expression in non-astrocytic cells (Ghandour et al., 1981).

Owing to the crucial role astrocytes play in the brain's amino acid metabolism, expression of glutamate transporter proteins was recognized early on as a potential avenue towards cell type-specific IHC labeling. There are two different forms of transporters, in particular, that have turned out to be relevant in this regard: the L-glutamate transporter 1 (GLT-1, a.k.a. SLC1A2 or EAAT2) (Pines et al., 1992) and the L-glutamate/L-aspartate transporter (GLAST, a.k.a. SLC1A3 or EAAT1) (Storck et al., 1992). While both of them are predominantly encountered in astroglia, there are notable differences in regional expression patterns: GLT-1 is detectable in astrocytes throughout the brain – with peak expression levels in the cerebral cortex (Lehre et al., 1995) – and to a lesser degree also in spinal cord (Regan et al., 2007), whereas GLAST is most abundant in BG of the cerebellar molecular layer, with weaker but still robust expression in astrocytes of other brain areas like the neocortex and the hippocampus (Rothstein et al., 1994; Schmitt et al., 1997).

GLAST is furthermore present in RG (Shibata et al., 1997) and was spotted in a subset of neurons, mainly located in the hippocampus and the deep cerebellar nuclei (Rothstein et al., 1994; Chaudhry et al., 1995). Other groups reported neuronal GLT-1 expression for various brain areas, including the neocortex, the hippocampus, and the olfactory bulb (Schmitt et al., 1996; Torp et al., 1997; Berger and Hediger, 1998). Weak but not negligible GLAST and GLT-1 expression was also observed in a number of WM oligodendrocytes (Regan et al., 2007).

Interestingly and in contrast to previously held assumptions, a comparative analysis using both GLAST and GLT-1 bacterial artificial chromosome (BAC) promoter reporter mice found that co-expression of both astrocytic glutamate transporters in the same cell seems to be the exception rather than the rule (Regan et al., 2007). Whether this surprising observation was due to better resolution or difference in approach, it certainly hints at a diversity in astroglial glutamate metabolism that goes beyond the interregional level.

Some 20 years ago, the water channel protein aquaporin-4 (AQP4) was isolated from rat brain (Jung et al., 1994) and found to be primarily expressed by astrocytes, barring its occurrence in ependymal and endothelial cells. A striking feature is its polarized distribution, as AQP4 is markedly enriched in endfeet membranes that come in close contact with intraparenchymal blood vessels or the subarachnoid space (Nielsen et al., 1997; Nagelhus et al., 1998; Rash et al., 1998). In this, it mimics the expression pattern of the inwardly rectifying K^+ channel Kir4.1 (Li et al., 2001). Kir4.1 expression was further found to be concentrated in astrocytes

surrounding synapses, while fibrous astroglia in the corpus callosum displayed only weak immunoreactivity (Higashi et al., 2001). Several studies have noted substantial Kir4.1 immunoreactivity in oligodendrocytes, which limits its use as a specific astrocyte marker (Poopalasundaram et al., 2000; Neusch et al., 2001; Kalsi et al., 2004).

In another intriguing instance of glial heterogeneity, astrocytic Kir channels were shown to occur either homomeric (Kir4.1/4.1) or heteromeric (Kir4.1/Kir5.1) throughout the brain. The proportional distribution of these subtypes varies from area to area, suggesting regional differences in mechanisms of K^+ buffering (Hibino et al., 2004). Finally, there is evidence that the colocalization of AQP4 and Kir4.1 actually betrays an association of the two proteins on a functional level (Guadagno and Moukhles, 2004; Nagelhus et al., 2004), the significance of which will be discussed below.

Among the large family of gap junction proteins, Connexin 43 (Cx43) and Cx30 – designated according to molecular weight in kDa – are the two interesting ones when it comes to astroglia. Originally isolated from cardiomyocytes (Beyer et al., 1987), Cx43 has since been identified as ubiquitously expressed by astrocytes throughout the CNS as well as by leptomeningeal and ependymal cells – both *in vivo* (Dermietzel et al., 1989; Yamamoto et al., 1990) and *in vitro* (Giaume et al., 1991; Dermietzel et al., 1991). In contrast, Cx30 is exclusively encountered in GM astrocytes where it is sometimes colocalized with Cx43 (Nagy et al., 1999). These differences in gap junction composition between fibrous and protoplasmic astrocytes entail certain physiological peculiarities expounded on below. Further examples of heterogeneous connexin expression have been described (Nagy et al. 1992), with one study observing both inter- and intraregional differences in Cx43 mRNA levels between rat hypothalamus and striatum (Batter et al., 1992).

There have been a number of potential astrocyte markers lately that were yielded by large-scale transcriptomic approaches to cell type diversity in the CNS, like Acyl-CoA synthetase bubblegum family member 1 (ACSBG1) or Aldehyde dehydrogenase 1 family member l1 (Aldh1l1) (Cahoy et al., 2008). The latter, a key enzyme of the folate metabolism, has been established as a new standard of sorts over the past couple of years, especially when used via a transgenic mouse line. The largely astrocyte-specific expression of Aldh1l1 was established some time ago (Neymeyer et al., 1997) and subsequently confirmed by a comparison to GLT-1 expression throughout the CNS, which revealed a remarkable amount of colocalization. It

was this same study, however, that yielded some important caveats: Aldh1l1 expression was found to change with age (esp. in the spinal cord) and pathology, and some cells that were Aldh1l1-positive exhibited traits typical of the oligodendroglial lineage (Yang et al., 2011).

Most recently, the TF Sox9 was proposed as yet another addition to the ever-growing toolbox of astrocyte marker proteins. Its properties look promising so far, and besides the fact that there are Sox9-positive cells in the neurogenic niches whose exact association with the astrocytic lineage remains to be defined, Sox9 seems to be expressed fairly ubiquitously, with the added advantage of not being diminished in aging or pathology (Sun et al., 2017).

1.4.1.3 Electrophysiological Properties and Calcium Signaling

The fact that astroglia has spent many years in the shadow of its more famous cousin, the neuron, might in parts be attributable to the fact that it is largely 'electrically silent': unlike neurons, astrocytes do not propagate action potentials along their numerous processes, making them seem rather ill-suited for electrophysiological purposes. Due to the dense expression of K^+ channels, their resting membrane potential as well as their membrane resistance are comparatively low – around – 80 mV and 10 MΩ, respectively (Mishima et al., 2007; Mishima and Hirase, 2010). Neonatal astrocytes of the hippocampal stratum radiatum proved to be even more hyperpolarized (Zhong et al., 2016).

Upon depolarization, astrocytes respond with a linear current-voltage relationship and are thus deemed not electrically excitable (Isokawa and McKhann, 2005; Olsen et al., 2006; Adermark and Lovinger, 2008; Du et al., 2015). Here, again, there are indications of differences between the rodent and the primate brain, with human astrocytes exhibiting slightly more positive resting membrane potentials and a membrane resistance that is about 15 times higher than that of their murine counterparts (Picker et al., 1981; Bordey and Sontheimer, 1998).

In spite of this relatively uniform conception of astrocyte electrophysiology, there are indications of diversity: Patch-clamp examinations in hippocampal slices of 10- to 12-day-old mice led to the characterization of four distinct groups of glial cells based on their membrane current patterns. These results must be taken with a grain of salt, however, as the study did not distinguish between astrocytes and oligodendrocytes (Steinhäuser et al., 1992).

Although astrocytes are not excitable in the traditional, neuron-derived sense, they do possess a mode of intercellular communication, namely the regulated increase of intracellular calcium (Ca^{2+}) concentrations, that might be seen as a form of astrocyte excitability. There is a vast body of evidence suggesting that these Ca^{2+} increases can be triggered in different ways – both extrinsically and intrinsically – and either occur highly localized or in coordinated intercellular waves (Fig. 5). They are believed to fulfill a variety of functions in the CNS, although much remains to be elucidated in this respect (Khakh and Sofroniew, 2015). For the purposes of this work, some instances of local and regional diversity in astrocyte calcium signaling behavior may be of particular interest.

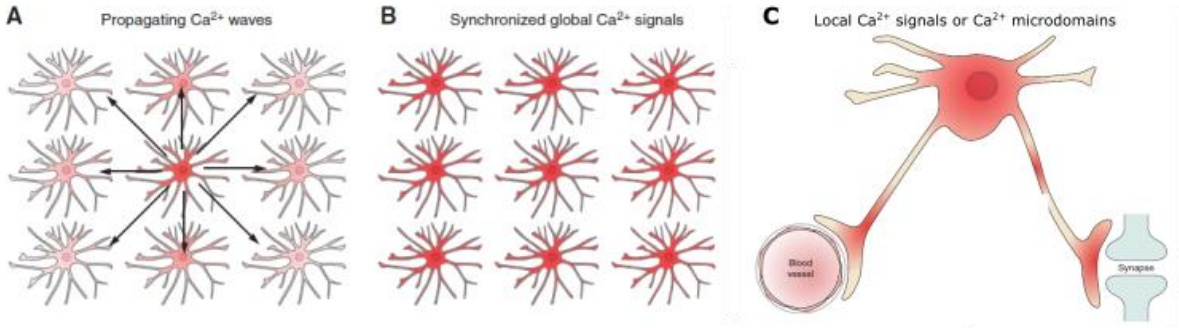


Fig. 5: Different modes of astroglial Ca^{2+} signaling
Ca^{2+} signals in astrocytes have been shown to occur in various forms, including propagating Ca^{2+} waves (A), globally synchronized Ca^{2+} signals over multiple individual cells (B), and strictly local microdomains (C). Figure modified from Verkhratsky and Nedergaard, 2018.

Heterogeneity in receptor expression among astrocytes from different CNS regions are reflected in their distinct Ca^{2+} responses to various neurotransmitters. Ca^{2+} increases in cortical astroglia, for instance, are triggered by glutamate (Wang et al., 2006; Schummers et al., 2008) or norepinephrine (Bekar et al., 2008). In the hippocampus, astrocytic Ca^{2+} responses are elicited by glutamate (Porter and McCarthy, 1996), acetylcholine (Araque et al., 2002), prostaglandins (Bezzi et al., 1998), adenosine triphosphate (ATP) (Bowser and Khakh, 2004), and endocannabinoids (Navarette and Araque, 2008). Still different combinations of trigger substances have been found for the cerebellum (Kulik et al., 1999; Matyash et al., 2001; Beierlein and Regehr, 2006; Piet and Jahr 2007), the retina (Newman, 2005) and the olfactory bulb (Rieger et al., 2007).

An *in vivo* study of astroglia in the somatosensory cortex revealed layer-specific differences in spontaneous oscillation patterns. Layer 2 and 3 astrocytes exhibited highly synchronized Ca^{2+} signals, whereas their layer 1 counterparts displayed rather asynchronous fluctuations at a significantly higher frequency (Takata and Hirase, 2008).

The manner in which Ca^{2+} waves are propagated between neighboring astrocytes is also subject to regional diversity. A comparison between protoplasmic astrocytes of the cortical GM and fibrous astrocytes of the corpus callosum showed that their respective coupling via gap junctions is remarkably distinct: Injection of biocytin into a single cell revealed a large syncytium of connected astrocytes in the GM, while no further WM astrocytes were labeled. This observation turned out to have direct consequences for the dynamics of Ca^{2+} waves in both brain regions: In the cortical GM of Cx43-deficient mice, no propagation of Ca^{2+} singaling could be observed, indicating a crucial reliance on gap junction coupling for the process (Haas et al., 2006). Generation of Ca^{2+} waves in WM astrocytes, on the other hand, were unaffected by the absence of Cx43 and shown to instead depend on purinergic signaling mechanisms (Schipke et al., 2002).

In keeping with the differences in numbers between rodent and human astroglia, the latter manages to propagate Ca^{2+} waves at around 40 µm/s (Oberheim et al., 2009), which is more than double the speed recorded in mouse astrocytes (Schipke at al., 2002).

1.4.2 Functions in the Healthy CNS

In stark contrast to their former reputation as essentially passive components of the nervous system, astrocytes are now known to fulfill a broad and diverse range of functions throughout the CNS. As our tools and methods of investigation continue to be refined, new functional aspects are uncovered, further illuminating the complex picture of astroglial physiology. Again, heterogeneity proves to be a central characteristic in many of these processes.

1.4.2.1 Homeostasis of the Nervous System

Control and regulation of various CNS parameters was among the earliest functions specifically attributed to astroglia. In a certain sense, this conception of the astrocyte as the homeostatic neural cell is still prevalent in most general accounts of the physiology of the nervous system. And while such a definition inevitably misses some of the more nuanced aspects, it does highlight one of the major tasks astrocytes perform in the CNS.

1.4.2.1.1 Ion and pH Homeostasis

The precise regulation of ionic gradients is of paramount importance for nervous functionality. Glial research has put a particular focus on the control of interstitial K^+ concentrations, and the crucial role of astrocytes in this context was recognized several decades ago by pioneers of the field like Leif Hertz, Steven Kuffler, and Richard Orkand (Hertz, 1965; Orkand et al., 1966).

Neuronal activity is closely associated with repolarizing efflux of K^+ and subsequent increases in local K^+ concentration. Under physiological conditions, these increases seem to be rather moderate, with observed numbers ranging from 0.4 mM (Heinemann et al., 1990) and 0.5 mM (Singer and Lux, 1975) to 1.0 mM (Ransom et al., 2000). However, noxious stimuli (Svoboda et al., 1988) and direct repetitive stimulation of certain brain areas (Heinemann and Lux, 1977) were found to cause much higher local K^+ accumulations.

Different mechanisms have been proposed for the astroglial regulation of extracellular K^+, one of them relying on K^+ clearance by diffusion: excess K^+ enters astrocytes through plasmalemmal channels and is then redistributed via neighboring gap junction-coupled cells to be released distantly, so the idea. A slight variation of this process was actually found to be at work in the Müller glia of the retina, where K^+ enters through Kir4.1 channels to be equilibrated inside the large cell body and eventually released from endfeet or from perivascular processes. This single-cell variant of K^+ buffering was termed K^+ siphoning (Newman et al., 1984).

The K^+ channel Kir4.1 was recognized early on to play a role in astrocytic K^+ buffering (Ballanyi et al., 1987). And while a handful of Kir4.1 knockout and knockdown studies – both *in vitro* and *in vivo* – have unanimously found alterations in the recovery kinetics following neuronal stimulation and a general depolarizing shift in the resting membrane potential when compared to wild-type (WT) animals, they disagree on the extent of these changes (Neusch et al., 2006; Kucheryavykh et al., 2007; Haj-Yasein et al., 2011a). Observed phenotypes range from relatively mild without any signs of general hyperexcitability (Chever et al., 2010) to significantly shortened lifespans with severe ataxia and stress-induced seizures (Djukic et al., 2007). Hence, the actual importance of Kir4.1 channels in K^+ clearance remains the subject of debate (Larsen et al., 2014; Nwaobi et al., 2016). Even more so, as Kir4.1 expression varies in

both a temporal (Kalsi et al., 2004; Seifert et al., 2009; Nwaobi et al., 2014) and a spatial (Olsen et al., 2007) manner.

A different mode of extracellular K^+ regulation mediated by the sodium (Na^+)/K^+-ATPase – thus being energy-dependent – was first described for cultured astrocytes (Hertz, 1979) and has since been confirmed multiple times using *in situ* preparations (Ransom et al., 2000; Xiong and Stringer, 2000; D'Ambrosio et al., 2002; Larsen et al., 2014). Here, K^+ that is taken up by astrocytes following synaptic activity is subsequently re-shuttled to the neuronal compartment by release through – among others – Kir4.1 channels, thereby restoring ionic gradients (Hertz and Chen, 2016; Larsen et al., 2016). Finally, the $Na^+/K^+/Cl^-$ transporter NKCC1 has been suggested as yet another participant in K^+ buffering, although this assumption is so far only based on cell culture data (Larsen et al., 2014).

Astrocytes further play a role in the maintenance of CNS chloride (Cl^-) concentrations. Strong activity by γ-aminobutyric acid (GABA)ergic neurons will deplete Cl^- levels in the synaptic cleft. Counteracting this, astroglia was found to respond with efflux of Cl^- to renormalize ionic concentrations and preserve the driving force for sustained inhibitory neurotransmission (Kettenmann et al., 1987). Moreover, gap junctional coupling of astrocytes was shown to be crucial in this regard, revealed by a collapse of the Cl^- gradient upon inhibition of connexons during stimulation of GABAergic transmission (Egawa et al., 2013).

Many biological processes in the CNS, like neuronal excitability, synaptic transmission, energy metabolism, or gap junction communication, are strongly affected by even small shifts in pH (Deitmer and Rose, 1996; Obara et al., 2008). Fittingly, astrocytes are equipped with a number of tools to keep the extracellular milieu from becoming either too acidic or too alkaline. They were found to express high levels of carbonic anhydrase, an enzyme that converts carbon dioxide (CO_2) into protons (H^+) and bicarbonate (HCO_3^-), effectively allowing them to function as a CO_2 reservoir (Kimelberg et al., 1982; Cammer and Tansey, 1988; Agnati et al., 1995). Extracellular pH regulation is further effected by a number of transporters expressed by astrocytes, such as Na^+/HCO_3^- cotransporters, Na^+/H^+ exchangers, and monocarboxylate transporters that cotransport one H^+ with one lactate molecule (Rose and Ransom, 1996; Deitmer and Schneider, 1998; Deitmer and Rose, 2010). Astroglial glutamate uptake plays a role in this context as well, as for every molecule of glutamate, one H^+ is shuttled along into the cell (Verkhratsky et al., 2015). Experiments with astrocytes from the rodent hippocampus

and optic nerve suggested that V-type H^+-ATPases might also be involved in pH regulation (Pappas and Ransom, 1993), and that the degree of their involvement differs markedly between *in situ* preparations and cultured cells (Hansen et al., 2015), paralleling an earlier study that noticed the significantly lower pH buffering capacity of cultured astrocytes when compared to their whole-mount counterparts (Bevensee et al., 1997).

1.4.2.1.2 Fluid Homeostasis and Oxidative Stress Management

The control and situational adaptation of fluid compartments and volume levels is a crucial aspect of brain physiology. Once again, astrocytes have different mechanisms at their disposal to influence these parameters. The close structural association of the astrocyte-specific water channel AQP4 and the K^+ channel Kir4.1 implies a functional synergy between the two, as mentioned above. And indeed, knockout of alpha-syntrophin – a protein that is closely associated with and critical to the function of AQP4 – significantly impaired the efficiency of K^+ removal in the rodent brain (Amiry-Moghaddam et al., 2003), strongly suggesting some sort of functional coupling. Very similar results were obtained by another study that assessed the effect of direct AQP4 knockout on extracellular K^+ levels after synaptic stimulation (Haj-Yasein et al., 2015). Nevertheless, there is also evidence to the contrary (Zhang and Verkman, 2008), so the purported interaction between AQP4 and Kir4.1 remains debatable for now.

Since water transport over AQP4 is bidirectional in nature, the consequences of its deletion are situational. In general, an increase in extracellular and overall brain water content was observed in AQP4-deficient mice (Yao et al., 2008; Haj-Yasein et al, 2011b). However, it has been known for quite some time now that changes in K^+ levels following neuronal activity also lead to a reduction in extracellular volume (Dietzel et al., 1980). This reduction, it was found, is noticeably accentuated in animals lacking AQP4 expression when compared to their WT littermates (Haj-Yasein et al., 2012).

In another intriguing instance of astrocyte heterogeneity, the subjecting of cortical slices to ischemic conditions revealed two populations of astroglia that responded distinctly to the challenge. One group exhibited substantial increases in intracellular volume and membrane potential depolarization, whereas the other showed only discrete volume changes with a slight hyperpolarization of membrane potential. Using IHC methods, the authors noticed marked difference of Kir4.1 and GFAP expression between the two populations (Benesova et

al., 2009). A similarly conceived study found differences in volume response to conditions simulating a hypoosmotic shock and pharmacological blockage of Kir4.1 channels in BG, retinal Müller cells, and hippocampal astrocytes (Hirrlinger et al., 2008).

The lack of a conventional lymphatic system in the CNS – a tissue known for its considerable metabolic activity – has long puzzled researchers. Recently, this conspicuous absence was partly addressed by the description of lymphatic vessels lining the dural sinuses (Aspelund et al., 2015; Louveau et al., 2015), although the originality of this finding is debatable (Bucchieri et al., 2015). More interesting, perhaps, is the discovery of what was termed the 'glymphatic system' of the brain, the name referring to the crucial involvement of astroglia in this new-found network. The perivascular space – a compartment unique to the CNS that is created by astrocytic endfeet and believed to cover the vast majority of its vasculature (Mathiisen et al., 2010) – was identified as the site of an organized pathway for interstitial fluid and solute clearance (Fig. 6). Once again, astrocytic AQP4 seems to play a crucial role in the directed flow of cerebrospinal fluid (CSF) from the periarterial towards the perivenous space, as shown by the disruption of the process in mice genetically engineered to lack AQP4 expression (Iliff et al., 2012).

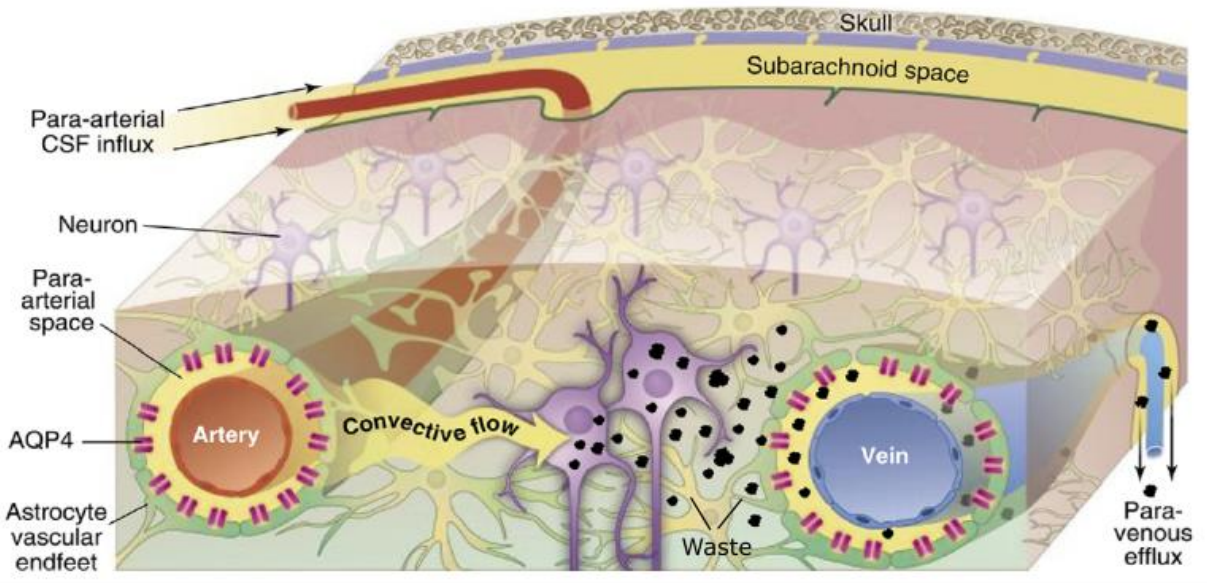


Fig. 6: Schematic outline of the glymphatic system
Waste products of cellular metabolism are swept into the paravenous space by convective fluxes of CSF and interstitial fluid. From there, they flow into lymphatic vessels and finally return to the general circulation. Figure modified from Nedergaard, 2013.

There is accumulating evidence that the glymphatic transport capacity and with it the clearance of toxic metabolites declines significantly with aging (Kress et al., 2014) and a

number of pathologies, including stroke (Gaberel et al., 2014), diabetes (Jiang et al., 2017), and Alzheimer's disease (Peng et al., 2016). Moreover, glymphatic activity was shown to peak during sleep (Xie et al., 2013) and be dependent to a certain degree on the position of the sleeper (Lee et al., 2015).

Finally, astrocytes are essential to another type of waste-removal process in the CNS, namely the antioxidative system. The energy metabolism of neurons is entirely reliant on oxidative phosphorylation and thus generates a huge amount of reactive oxygen species (ROS) (Bélanger et al., 2011). The two major components of the CNS antioxidative system are ascorbic acid and glutathione, with the latter occurring at significantly higher levels in astroglia when compared to neurons (Makar et al., 1994; Huang and Philbert, 1995). These findings are borne out by a number of experiments showing that neurons can better withstand stress from ROS when co-cultured with glial cells, as neuronal synthesis of glutathione is dependent on the external supply of cysteine furnished by astrocytes (Langeveld et al., 1995; Desagher et al., 1996; Lucius and Sievers, 1996; Dringen et al., 1999; Tanaka et al., 1999; Chen et al., 2001; Fujita et al., 2009).

Similarly, neurons rely on the support of astrocytes in their capacity to eliminate ROS using the ascorbic acid system. Mediated by the increased presence of glutamate, neuronal activity triggers the astroglial release of ascorbic acid (Wilson et al., 2000), which is then taken up by neurons and oxidized to dehydroascorbic acid in the process of scavenging ROS. The oxidized molecule is then again released into the extracellular space to be taken up and reduced by astrocytes, starting the cycle anew (Covarrubias-Pinto et al., 2015).

1.4.2.1.3 Transmitter Homeostasis

Consistent with their function as the homeostatic cell type of the CNS, astrocytes are crucially involved in the regulation of neurotransmitter metabolism. Since the processes of neuronal firing and synaptic transmission are heavily dependent on the tight control of extracellular signaling molecule concentrations, the removal, inactivation, or metabolic conversion of these substances is of paramount importance for neurophysiology.

Glutamate acts as the major excitatory transmitter in the CNS. As such, its precise metabolic regulation is indispensable for preventing excitotoxicity, a phenomenon that has been

recognized as a component of many brain disorders (reviewed in Takahashi et al., 2015). Astrocytes can influence CNS glutamate levels in a variety of ways, one of them being the uptake of excess glutamate from the synaptic cleft to keep concentrations from rising disproportionally, even during periods of intense synaptic activity. In this, they mainly rely on the above-mentioned transporters GLAST and GLT-1, the importance of which can be appreciated when looking at the consequences of their absence. Loss of either one resulted in elevated extracellular glutamate levels, progressive paralysis, and excitotoxic neurodegeneration (Rothstein et al., 1996). Another study found that GLT-1 knockout in mice led to a 95% reduction in overall glutamate uptake and early death from spontaneous seizures (Tanaka et al., 1997). Interestingly, GLAST-deficient mice were not prone to spontaneous seizures like their GLT-1-deficient counterparts, although upon pharmacological initiation of seizures, their duration and severity were increased when compared to WT animals (Watanabe et al., 1999). This might be interpreted as a functional correlate of the heterogeneity in astroglial transporter localization discussed above.

Once taken up into the astrocyte, glutamate is converted to glutamine by the mostly astrocyte-specific enzyme GS. Glutamine in turn is transferred back to neurons where it is converted to glutamate again via deamidation, thereby completing the well-established glutamate-glutamine cycle (Bak et al., 2006). At this point, the glutamate-glutamine cycle intersects with that of the major inhibitory transmitter of the CNS, GABA, as the latter is produced from glutamate via the enzyme glutamate decarboxylase in what has come to be known as the GABA-glutamine cycle (Walls et al., 2015). Like previously stated for glutamate, there is also a host of neuropsychiatric diseases that involve GABAergic dysfunction (reviewed in Wong et al., 2003).

Seeing how GS is central to the metabolism of both glutamate and GABA, it is hardly surprising that changes in its expression or activity are closely associated with various pathologies, e.g. epilepsy (Eid et al., 2004), schizophrenia (Steffek et al., 2008), or ischemic stroke (Lee et al., 2010). Prenatal excision of the GS gene in mice led to a progressive failure to feed, resulting in death due to hypoglycemia within three days after birth (He et al., 2010). In Müller cells, inhibition of GS led to a notable impairment of retinal response to light (Barnett et al., 2000).

Aside from ‘recycling’ glutamate, astrocytes are also its only *de novo* synthesizers in the CNS, as they alone express the necessary enzyme pyruvate carboxylase in sufficient quantities

(Shank et al., 1985). Furthermore, astroglia also constitutes an important element in the metabolism of other neurotransmitters, like adenosine or the group of monoamines (including norepinephrine, dopamine, and serotonin). Two of the essential enzymes in this regard, adenosine kinase (ADK) and monoamine oxidase B (MAO-B), respectively, are predominantly expressed in astrocytes (Studer et al., 2006; Levitt et al., 1982; Riederer et al., 1987; Saura et al., 1992; cf. Fig. 7 for an overview of astroglial involvement in neurotransmitter metabolism).

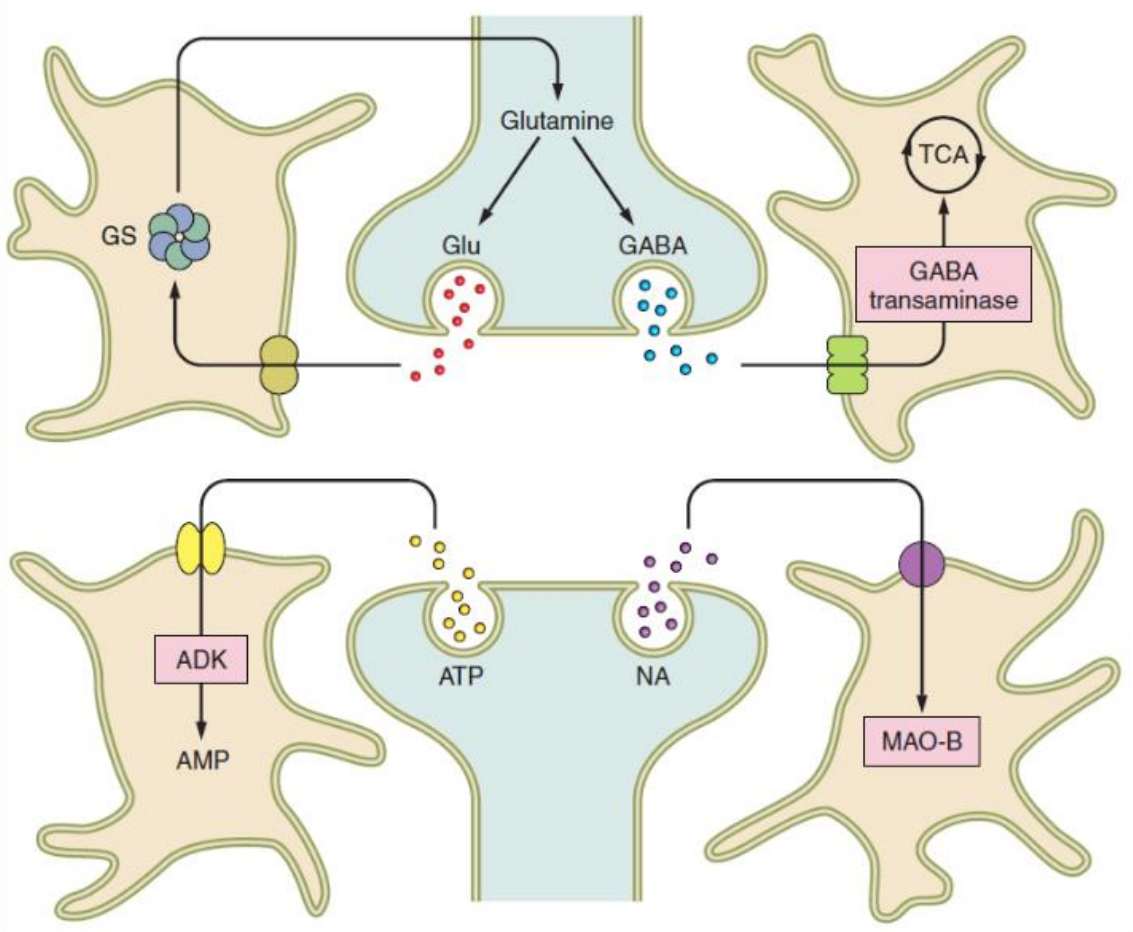


Fig. 7: Astrocytes are crucial to the metabolism of various neurotransmitters
Glutamate, GABA, adenosine, and monoamines are taken up by astrocytes for further processing. Glutamate is first converted to glutamine via GS and subsequenly shuttled back to neurons, where it is again converted to either glutamate or GABA. The latter is mainly processed by astrocytes in the tricarboxylic acid cycle. Adenosine is converted to adenosine monophosphate (AMP) via astroglial ADK, while monoamines, such as noradrenaline (NA), are deaminated by MAO-A and MAO-B (mainly expressed in adult astrocytes). Figure from Verkhratsky and Nedergaard, 2018.

1.4.2.2 Regulation of Blood Flow

The idea that increase in brain activity is associated with local vasodilatation – a principle known as 'functional hyperemia' or 'neurovascular coupling' and the basis for modern functional magnetic resonance imaging (fMRI) – goes back to the late 19th century, when pioneers of neurophysiology like Angelo Mosso or Charles Smart Roy and Charles Scott

Sherrington made initial observations to that effect (Mosso, 1880; Roy and Sherrington, 1890). Neurons are directly involved in the coupling of neuronal activity with local circulation by releasing a number of vasoactive agents and neurotransmitters, such as nitric oxide (NO), prostaglandins, acetylcholine, etc. (reviewed in Attwell et al., 2010 or Cauli and Hamel, 2010). Yet, it is once again the astrocyte that has been given special attention regarding the control of cerebral blood flow.

The most prominent hypothesis proposed in this context claims that glutamate released by neuronal activity acts on astroglial metabotropic glutamate receptors (mGluRs) to trigger an increase in astrocytic Ca^{2+}, which in turn initiates the production and release of different vasoactive substances. However, while several groups observed vasodilatation as the eventual outcome of this cascade (Zonta et al., 2003; Takano et al., 2006; Petzold et al., 2008; Schummers et al., 2008; Liu et al., 2011), rising Ca^{2+} concentrations led to vasoconstriction in another study (Mulligan and MacVicar, 2004).

And there are further problems to this theory: mGluR5, the glutamate receptor thought responsible for astrocytic Ca^{2+} increases, was actually found to be undetectable in rodents after postnatal week 3 (Sun et al., 2013), and its pharmacological blocking had no significant effect on the early hemodynamic response under physiological circumstances (Calcinaghi et al., 2011). Moreover, mice genetically manipulated to lack the inositol 1,4,5-triphosphate type-2 receptor (IP3R2), a crucial component of astrocytic Ca^{2+} release from intracellular stores, displayed intact stimulus-induced vasodilatation in three similarly conceived studies (Takata et al., 2013). Two of these studies also observed that astroglial Ca^{2+} increases did not seem to precede the relevant hemodynamic changes, calling into question the causality and timing of these events (Nizar et al., 2013; Bonder and McCarthy, 2014). Other groups, however, have found intracellular Ca^{2+} release in astrocytes to take place well before local vasodilatation (Lind et al., 2013; Otsu et al., 2015). Irrespective of the controversial involvement of astrocytic Ca^{2+} dynamics in neurovascular coupling, they do appear to play a role in the control of tonic local brain blood flow (Rosenegger et al., 2015) and pressure-evoked vasomotor tone (Kim et al., 2015).

An alternative explanation of functional hyperemia seeks to associate vasodilatation with the release of K^+ from astrocyte endfeet (Paulson and Newman, 1987; Filosa et al., 2006). The signal initiated by ionic influx through K^+ channels on capillary endothelial cells is then thought

to be communicated to upstream arterioles, propagating rapid hyperpolarization along the vasculature (Longden et al., 2017). However, this mechanism has also been called into question, when neurovascular coupling was found to be unaffected in Kir4.1 knockout mice (Metea et al., 2007).

A somewhat unifying perspective is offered by another group, reporting that astrocytes influence local blood flow mainly on the capillary level, whereas arteriole vasomotor tone depends mostly on NO generation by interneurons. And while increases in astrocytic Ca^{2+} levels were indeed observed to be essential to this process, they occur by entry through ATP-gated channels rather than by release from intracellular stores (Mishra et al., 2016).

1.4.2.3 Energy Metabolism

Over two decades ago, a hypothesis was formulated that sees astrocytes as instrumental in linking neuronal activity to CNS glucose uptake. The mechanism became known as the 'astrocyte-neuron lactate shuttle' (ANLS), and its core idea is that neuronal activity triggers glucose metabolism in astroglia, where glucose is glycolytically catabolized to pyruvate and converted to lactate, which is then released and taken up by neurons to fuel oxidative phosphorylation (Pellerin and Magistretti, 1994). The concept is made plausible by an apparent mismatch between cell-type specific energy expenditure, which is heavily dominated by neuronal signaling (Attwell and Laughlin, 2001), and glucose uptake, which is about equal for astrocytes and neurons at rest and even favors astroglia during periods of activity (Chuquet et al., 2010).

However, the actual *in vivo* reality of the ANLS is far from certain. On the one hand, a number of observations have been made over the years that support the notion of astrocytes providing neurons with lactate to metabolize during bouts of synaptic activity, like the identification of two mechanisms for astrocytic lactate release triggered by extracellular K^+ increases (Sotelo-Hitschfeld et al., 2015) and dips in Ca^{2+} levels (Karagiannis et al., 2016), respectively, or the description of a dedicated lactate oxidation complex in the mitochondria of neurons (Hashimoto et al., 2008). Moreover, there is evidence that electric stimulation of cerebellar Purkinje cells directly correlates with rising extracellular lactate levels (Caeser et al., 2008) and that synaptic function can be sustained in hippocampal slices by lactate as the sole energy substrate (Schurr et al., 1988; Izumi et al., 1997).

On the other hand, the claim that glycolysis is confined to astrocytes (Kasischke et al., 2004) was countered by studies observing neuronal enrichment of glycolytic enzymes and pyruvate, that stemmed from glucose originally taken up by neurons (Patel et al., 2014; Lundgaard et al., 2015). To complicate matters even further, there is evidence that up to 60% of activity associated lactate increase actually derive directly from the bloodstream (Boumezbeur et al., 2010), which might be taken up and subsequently released by perivascular astrocytes (Gandhi et al., 2009).

Another distinguishing feature of astroglia in CNS energy metabolism is its possession of glycogen granules, recognized almost a century ago (Holmes and Holmes, 1926). Except for small neuronal sub-populations, glycogen storage appears to be an exclusively astrocytic property (Phelps, 1972; Koizumi, 1974; Brown, 2004). Glial glycolysis was found to be regulated by noradrenaline, vasoactive intestinal polypeptide (Magistretti et al., 1981), and adenosine (Magistretti et al., 1986). Experimental disruption of the glycolytic cascade in astrocytes led to action potential degradation in the mouse optic nerve (Wender et al., 2000; Brown et al., 2005) and impairment of memory consolidation in young chicks (Gibbs et al., 2006) and mice (Suzuki et al., 2011). Interestingly, mild hypoxic preconditioning causes glycogen levels to rise, thus establishing a protective reserve against subsequent hypoxic-ischemic injury (Brucklacher et al., 2002; Herzog et al., 2008; Canada et al., 2011).

1.4.2.4 Regulation of Synaptic Connectivity and Transmission

Once more belying their reputation as the passive, merely supportive bystanders of the CNS, astrocytes are known to fulfill a variety of functions with regard to synaptic development and signaling. Evidence for the intricate interplay between neurons and astrocytes in signaling events has accumulated over time and eventually led to the concept of the tripartite synapse, comprising the presynaptic terminal, the postsynaptic compartment, and the surrounding astroglia. Interestingly, astrocytic coverage of synapses varies significantly between different brain areas (Verkhratsky and Nedergaard, 2018), hinting at regional diversity in synaptic transmission and regulation.

1.4.2.4.1 Synaptogenesis and Maintenance of Synaptic Coverage

Although the lifelong emergence, remodeling, and elimination of synapses throughout the CNS is the foundation of neuroplasticity and lies at the heart of the organism's capacity for learning and memory, it is the developing brain, in particular, that displays an inimitable capacity to generate new synapses. To find their way and make the right connections, growing axons rely on astrocyte-derived guidance molecules, such as proteoglycans or tenascin C (Powell and Geller, 1999). Cultured in the absence of glia, retinal ganglion cells exhibited drastically reduced synaptic activity, both in frequency and amplitude, and their rate of transmission failure was vastly increased (Pfrieger and Barres, 1997). Thrombospondins were identified as one class of molecules immature astrocytes secrete to facilitate presynaptic maturation in the developing CNS (Christopherson et al., 2005). And although mature astrocytes do not normally express thrombospondins at any significant level, a STAT3-mediated upregulation of thrombospondin 1 was observed following motor neuron injury (Tyzack et al., 2014).

An important role in astrocyte-related synaptic maturation is also played by cholesterol (Mauch et al., 2001). Mice deficient for certain regulatory elements in astrocytic cholesterol metabolism exhibited increased numbers of immature synapses as well as motor and behavioral defects (Ferris et al., 2017; Van Deijk et al., 2017). Another part of the astrocyte lipid metabolism, FABP7, was shown to be critical for excitatory transmission in the medial prefrontal cortex. Knockout mice displayed significantly impaired synaptic formation and maturation, both of which could be partially rescued by transplantation of WT astroglia (Ebrahimi et al., 2016).

The protein pair of Hevin and Secreted protein acidic and rich in cysteine (SPARC) was found by another study to be expressed by astrocytes of the superior colliculus, the synaptic target of retinal ganglion cells, with Hevin promoting and SPARC inhibiting synaptogenesis (Kucukdereli et al., 2011). Recently, astroglial glypicans 4 and 6 were identified as yet another group of molecules involved in synaptic maturation, more specifically the formation of the postsynaptic compartment, thus complementing the effect of thrombospondins (Allen et al., 2012). Intriguingly, the expression of these synaptogenic factors seems to be subject to tremendous regional heterogeneity, which has direct consequences for the synaptogenic potential of various astrocyte subpopulations (Buosi et al., 2018).

Furthermore, synaptic density in different brain regions is dynamically regulated by astrocytic ensheathment. For instance, formation of dendritic protrusions and subsequent maturation into functional spines was observed to be dependent on astrocytic motility and astroglia-to-neuron signaling in the hippocampus (Nishida and Okabe, 2007). Similarly, the degree to which BG covered the spines of Purkinje cells proved to be directly correlated with the number of functional synapses in the cerebellum (Lippman Bell et al., 2010).

To ensure proper development of the CNS, some incorrectly formed or otherwise unwanted synapses have to be eliminated, a process commonly referred to as 'synaptic pruning'. Synaptic terminals that are meant to be removed are labeled by astrocytes with the complement factor C1q, a tag that is subsequently recognized by microglia and induces selective phagocytosis (Fig. 8). Mice deficient in C1q or its downstream target C3 exhibit excessive synaptic connectivity and epileptiform activity (Stevens et al., 2007; Chu et al., 2010), although knockout of C3 was also associated with an attenuation of age-related hippocampal decline (Shi et al., 2015).

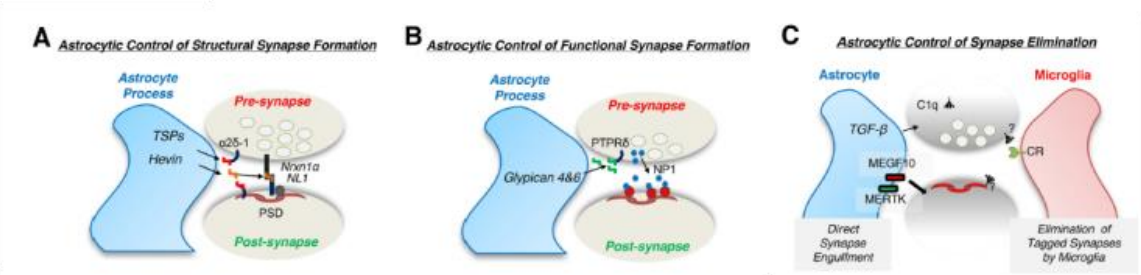


Fig. 8: Synapse formation, maturation, and elimination are controlled by astroglia
(A) Thrombospondins and Hevin are secreted by astrocytes to structurally induce synapse formation. Thrombospondins accomplish this via interaction with the neuronal calcium channel subunit α2δ-1. Hevin, on the other hand, contributes to synapse formation by bridging the neuronal receptors Neurexin-1a (Nrxn1a) and Neuroligin-1 (NL1).
(B) Functional synapse formation is induced by astroglial Glypican 4 (and 6), which binds to the presynaptic receptor protein tyrosine phosphatase delta (RPTPδ), leading to the release and postsynaptic binding of neuronal pentraxin 1 (NP1).
(C) Astrocytes contribute to the elimination of unwanted synaptic connections by two different ways: either through direct phagocytosis via the MEGF10 and MERTK pathways or by release of TGF-β, which induces neuronal complement protein C1q expression and subsequent recruitment of complement receptor (CR)-expressing microglia. Figure modified from Allen and Eroglu, 2017.

Furthermore, the above-mentioned SPARC has been found to trigger a cell-autonomous program of synapse elimination in cholinergic neurons at a certain time during normal development (Lopéz-Murcia, 2015). A recent study demonstrated that the IP3R2-dependent release of astrocytic ATP is necessary to induce the physiological elimination of the ventral posteromedial nucleus relay synapse, implicating purinergic signaling as another element of synaptic pruning (Yang et al., 2016). Finally, a mechanism of astrocytes actively engulfing

synapses independently of microglia has been identified, that plays a role in both the developing and the adult CNS (Chung et al., 2013).

1.4.2.4.2 The Tripartite Synapse

Aside from their contribution to the formation, maintenance, and elimination of synaptic connections, astrocytes are also directly involved in the processing of neuronal impulses. This close association of astroglial ensheathment and synaptic transmission underlies the notion of the tripartite synapse.

One hotly debated topic in this context is the secretion of neurotransmitters by astrocytes, commonly referred to as 'gliotransmission' (Hamilton and Attwell, 2010; Araque et al., 2014; Savtchouk and Volterra, 2018). While astrocytes have been shown to contain various neurotransmitters and to possess the necessary pathways for their exocytosis, the dynamics of their release are both slower and more diffuse when compared to neurons, as astroglia lacks any equivalent to presynaptic active zones where vesicular concentration might take place. Thus, the purpose of gliotransmission appears to consist in a more general modulation of neuronal activity rather than the specific interaction with individual synapses (Verkhratsky and Nedergaard, 2018).

Evidence supporting this view on glial release of neurotransmitters is abundant. Thus, astroglial glutamate secretion can result in inhibition (Araque et al., 1998; Liu et al., 2004) or potentiation (Kang et al., 1998; Jourdain et al., 2007) of inhibitory (IPSCs) and excitatory postsynaptic currents (EPSCs) in hippocampal neurons. Furthermore, glutamate-mediated increase in neuronal excitability (Bezzi et al., 1998), modulation of long-term potentiation (LTP) or depression (Han et al., 2012; Min and Nevian, 2012), and generation of slow inward currents (Angulo et al., 2004; Kang et al., 2005; Shigetomi et al., 2008; Chen et al., 2012, among many others) have been observed.

Similar data exist for astrocyte-derived ATP/adenosine and its manifold effects on synaptic transmission, which include suppression (Pascual et al., 2005; Martín et al., 2007) or potentiation (Gordon et al., 2005) of excitatory postsynaptic currents, increased neuronal excitability (Lee et al., 2013), and modulation of LTP (Pascual et al., 2005; Lee et al., 2013).

Sporadic reports also exist for astroglia-released GABA (Kozlov et al., 2006; Lee et al., 2010) and Tumor necrosis factor α (TNFα) (Stellwagen and Malenka, 2006; Kronschläger et al., 2016).

As mentioned before, the regulation of neurotransmitter concentrations in the synaptic cleft is one of the major functions fulfilled by astrocytes. Emphasizing the importance of this task are a number of studies that examined the effect of astroglial glutamate buffering on synaptic transmission. It was found to reduce both the amplitude and the duration of N-methyl-D-aspartate (NMDA) receptor-mediated EPSCs in hippocampal (Mennerick and Zorumski, 1994; Asztely et al., 1997; Diamond, 2001; Arnth-Jensen et al., 2002) and cerebellar (Overstreet et al., 1999) neurons. In a similar fashion, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor-mediated EPSCs in synapses located in the cerebellum (Takahashi et al., 1996; Overstreet et al., 1999) and in the auditory nerve (Otis et al., 1996) were observed to be shortened by astrocytic glutamate buffering. Interestingly, the kinetics of glial transmitter uptake seem to be in turn modulated by neuronal activity, as revealed by a short-term suppression of glutamate buffering following bursts of action potentials in the mouse cortex (Armbruster et al., 2016).

The role of astrocytic GABA transporters in the regulation of neurotransmitter concentrations has also been examined in some detail. In the hippocampus, tonic GABAergic transmission was found to be modulated by astroglial expression of the GABA transporter type 3 (GAT-3) (Kersanté et al., 2013). Thalamic astrocytes, known to express both GAT-1 and GAT-3 (De Biasi et al., 1998), affect the kinetics of $GABA_B$ receptor-mediated postsynaptic currents in two different ways: the influence of GAT-1 is most notable during the peak of IPSCs, while GAT-3 mainly prevents transmitter spillover (Beenhakker and Huguenard, 2010). The latter, i.e. the spatial isolation and maintenance of specificity in signaling events, is another general task fulfilled by astroglia in the context of the tripartite synapse. Thus, transgenic mice deficient for GLAST exhibited prolonged EPSCs and indications of significant glutamate spillover in cerebellar parallel fiber synapses compared to WT animals (Marcaggi et al., 2003). In another instance of astrocyte heterogeneity on a functional level, the degree to which neurotransmitter spillover is ‘allowed’ varies markedly between different regions of the CNS (Verkhratsky and Nedergaard, 2018).

Finally, perisynaptic coverage by astroglia and consequently the specifics of transmission regulation are far from static. Morphological plasticity of astrocytes in reaction to various

stimuli has been described *in vitro* (Vardjan et al., 2014), *in situ* (Oliet et al., 2001), and *in vivo* (Iino et al., 2001). Its induction seems to be activity-dependent, as suggested by an increase in perisynaptic coverage in the barrel cortex following prolonged whisker stimulation (Genoud et al., 2006) or by the enhanced motility of hippocampal astrocyte processes in response to LTP (Perez-Alvarez et al., 2014). Along the same lines, astroglial coverage of synapses was found to decrease during sleep (Bellesi et al., 2015).

1.4.3 Functional Differences between Fibrous and Protoplasmic Astrocytes

Now that an overview of the various roles fulfilled by astroglial cells in the CNS has been given, the question of differences in function underlying the morphological and biochemical diversity of GM and WM astrocytes arises. However, an answer remains largely elusive at this time, and despite some progress in this direction over the last couple of decades, the data available is still relatively scarce. The fact that two reviews explicitly dealing with the topic disagree on a major point, with one suggesting that "most findings implicate a substantially lower glutamate metabolism in WM" (Köhler et al., 2021) and the other presuming a "more effective glutamate clearance in white matter" (Lundgaard et al., 2014), may be seen as symptomatic of this situation.

As it is, the difference in micro-anatomical environment between protoplasmic and fibrous astroglia makes a corresponding difference on a metabolic level seem likely. GM astrocytes are directly involved in synaptic transmission through axonal ensheathment, and glutamatergic signaling is terminated by (re-)uptake into astrocytic processes. This circumstance forms the basis for the concept of the tripartite synapse, as detailed above. In contrast to this, fibrous astrocytes extend their processes along the axon tracts pervading the WM and can make direct contact only at the nodes of Ranvier or at unmyelinated axons (Köhler et al., 2021). Vesicular release of glutamate takes place at discrete sites along the axonal surface, and while the individual quantities of transmitter are much smaller than at GM synapses, the cumulative amount of glutamate released from the axons of longer projection neurons can represent a significant metabolic burden (Kukley et al., 2007; Ziskin et al., 2007).

The data concerning the respective glutamate handling properties of protoplasmic and fibrous astroglia is indeed inconsistent. One study comparing glutamate dynamics in GM and WM areas of the mouse brain using a proteoliposome assay and tissue homogenates found that

transporter expression as well as glutamate uptake and enzymatic activity was significantly lower in WM astroglia when compared to its GM counterpart (Hassel et al., 2003). These observations were confirmed in acute brain slices by another group (Regan et al., 2007). A comparison of cultured astroglial cells from the cortex and corpus callosum, however, showed the exact opposite, namely higher GLT-1 and GLAST expression levels in WM glia, as well as a significantly higher glutamate uptake capacity and GS activity (Goursaud et al., 2009). One explanation for this apparent discrepancy might be the fact that astrocytic density in WM tends to be lower than in GM, which is taken into account in cultured cells but not when working with brain slices or tissue homogenates (Lundgaard et al., 2014). Finally, the overall glutamate concentration in WM tissue was found to be half that in GM (Hassel et al., 2003), the implications of which, again, are ambivalent (more efficient glutamate clearing vs. less need for glutamate uptake).

The crucial involvement of astrocytes in the energy metabolism of the CNS has been delineated earlier. There is some evidence of functional heterogeneity between protoplasmic and fibrous astrocytes in this regard, although many of the particulars are still unknown. In general, the overall rate of glucose consumption was measured to be consistently lower in WM areas of the brain (Sokoloff et al., 1977). It seems, moreover, that the share of glycolytic activity without subsequent entry into the tricarboxylic acid cycle in WM metabolism is significantly greater than in the case of GM tissue, which is characterized by a rather tight glycolytic-oxidative coupling (Morland et al., 2007). These differences might at least partially account for the variously observed vulnerability of WM tissue to ischemic conditions (Pantoni et al., 1996; Shannon et al., 2007). The amount of intracellular glycogen deposits has been described as another point of distinction between protoplasmic and fibrous astroglia (Brown and Ransom, 2007), although once more, different studies came to opposite conclusions, finding higher concentrations of glycogen in GM (Oe et al., 2016) and WM astrocytes (Kong et al., 2002), respectively.

In accordance with the previously mentioned higher rate of glucose utilization in GM areas of the brain, the capillary density has been found to significantly exceed that of WM tissue (Borowsky and Collins, 1989; Murugesan et al., 2012). Interestingly, aging-related capillary loss seems to be more pronounced in the corpus callosum than in the cortex (Murugesan et al., 2012). Astroglia is an integral part of the neurovascular interface at the blood-brain barrier

(BBB), where one of its major tasks is the regulation of fluid homeostasis, as detailed earlier. When subjected to inflammatory or ischemic conditions, loss of perivascular AQP4 has been observed in GM astrocytes, which is usually interpreted as a protective mechanism to mitigate tissue damage through excessive swelling (Frydenlund et al., 2006; Steiner et al., 2012). However, a more recent comparison between GM and WM astroglia in postmortem brains of patients with focal ischemic stroke revealed an intriguing discrepancy: While protoplasmic astrocytes were again found to exhibit a reduction in perivascular AQP4 expression, fibrous astrocytes displayed a considerable increase in perivascular and plasmalemmal AQP4, resulting in parenchymal swelling of 40% in WM compared to a mere 9 % in GM tissue (Stokum et al., 2015). This observation may well constitute another reason for the sensitivity of WM areas to pathological challenges.

Mention has been made of the heterogeneity in connexin expression between protoplasmic and fibrous astrocytes, and how the former are reliant on coupling via Cx43 for the propagation of Ca^{2+} waves while a different mechanism is at play in the latter case. Another aspect of intercellular gap junction coupling especially relevant for WM astroglia is the pan-glial connection between astrocytes and oligodendrocytes. Here, astrocytic Cx30 and Cx43 are linked to oligodendroglial Cx32 and Cx47, respectively (Orthmann-Murphy et al., 2007; Maglione et al., 2010). The functional importance of this coupling for the maintenance of WM integrity becomes apparent on conditional deletion of the involved proteins. Both Cx30/Cx47 and Cx32/Cx43 double-deficient mice have been generated; they exhibit early onset myelin pathology with severe WM vacuolization, sensorimotor impairment, seizures and considerably shortened life-expectancy (Magnotti et al., 2011, Tress et al., 2012). These symptoms are reminiscent of Pelizaeus-Merzbacher-like disease 1, a leukodystrophy caused by a mutation in the Gja12 gene encoding for Cx47 (Orthmann-Murphy et al., 2007). It has been suggested that one of the main reasons for these alterations might be the lack of spatial K^+ buffering capacity normally provided by astro-oligodendroglial gap junctions within the context of neurotransmission (Nagy and Rash, 2000).

While it is problematic to pinpoint a definite function of the astroglial protein GFAP, there is some evidence for its importance concerning the integrity of WM architecture. GFAP knockout mice, perhaps surprisingly, are viable, but they display a whole slew of abnormalities, including hydrocephalus, abnormal myelination with actively myelinating oligodendrocytes in adult

animals, structurally and functionally impaired astrocytes, and pathological BBB changes (Liedtke et al., 1996). Moreover, glutamate metabolism was found to be significantly impaired in GFAP null mice (Hughes et al., 2004), which might be explained by the close cytoskeletal association between GFAP and the glutamate transporter GLAST (Sullivan et al., 2007). Taken together, these results suggest that astrocytes, and more particularly fibrous astrocytes, are indispensable for the normal development of oligodendrocytes and consequently the physiological process of axonal myelination.

1.5 The Role of Astroglia in CNS Pathologies: Friend or Foe?

As knowledge continues to accumulate, it becomes increasingly apparent that astrocytes are not only involved in a host of physiological processes but also figure prominently in various CNS pathologies. Their role in this context is usually referred to by the term 'reactive astrogliosis', which subsumes a multitude of molecular, cellular, and functional changes in astrocytes that take place in response to any internal or external insults to the integrity of the CNS. However, the details of this response can vary considerably depending on the location, time course, nature, and severity of the injury or disease – so much so that both detrimental and beneficial effects of astrocyte reactivity can often be observed side by side. This needs to be kept in mind when exploring potential therapeutic approaches involving astrocytes.

1.5.1 Hallmarks and Consequences of Astrocyte Reactivity

Despite its omnipresence in the literature of the field, reactive astrogliosis is actually not a very clearly defined concept. Rather than describing a single and distinct state, it should be thought of as a spectrum of potential alterations in astrocyte biology. The extent of these changes is determined by a variety of factors and ranges from slight cellular hypertrophy coupled with moderately upregulated expression of GFAP and other genes to severe astrogliosis with glial scar formation, heavily altered gene expression, loss of domain organization, and increased rate of proliferation (Fig. 9; Sofroniew and Vinters, 2010). The source of this proliferation was shown to be normally quiescent astrocytes that acquire stem cell properties under injury conditions (Buffo et al., 2008), with Shh signaling playing a central role in this context (Sirko et al., 2013). More specifically, selective proliferation of juxtavascular astrocytes could be identified as the mechanism behind the reactive increase in cell number

using two-photon microscopy (Bardehle et al., 2013). These juxtavascular astrocytes were recently found to exhibit drastic changes in their electrophysiological properties following traumatic brain injury when compared to their non-juxtavascular counterparts, alongside a pronounced downregulation of Kir4.1 (Götz et al., 2021). In focal pathologies, the degree of reactivity is inversely proportional to the distance from the lesion core (Sofroniew and Vinters, 2010).

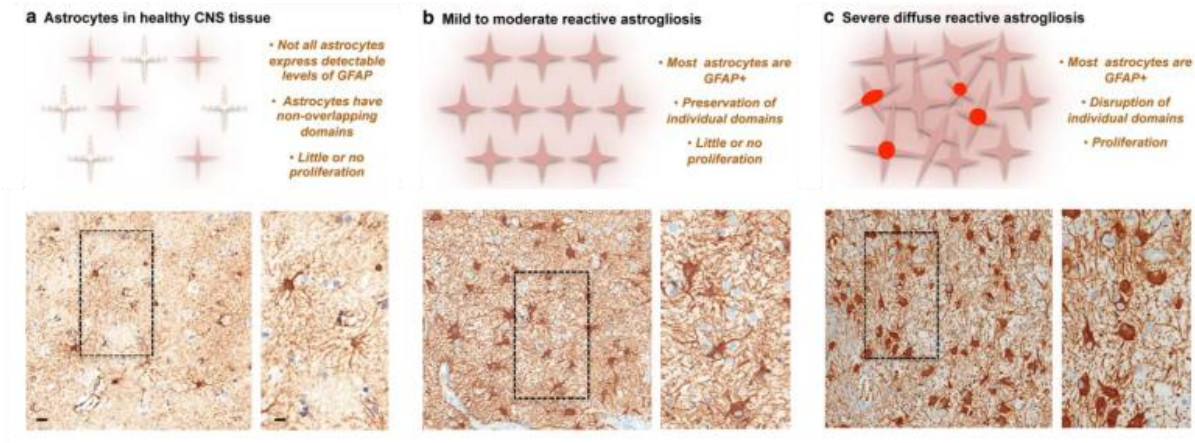


Fig. 9: Schematic and IHC representations of reactive gliosis in different gradations
The degree of reactive astrogliosis depends on the distance from the lesion site, as well as the severity and the type of lesion. Healthy astrocytes show almost no overlap in their individual domains and proliferate at a very low rate, if at all (a). Moderately reactive astroglia is defined by a more pronounced expression of GFAP and slightly more overlap between individual cellular domains combined with an increased but still very low rate of proliferation (c). Finally, sites of severe astrogliosis display very strong GFAP expression, a marked increase in astrocyte proliferation, and a disruption of individual domains (c). Scale bar in surveys = 25 µm, details = 10 µm. Figure modified from Sofroniew and Vinters, 2010.

Recent data shows that the composition and amount of proliferation among the various cell types involved in reactive gliosis is markedly different when comparing cortical GM with WM after stab wound lesion. Moreover, if the WM is included in the area of injury, astrocytes are significantly more prominent in the GM lesion site than in a GM only stab wound paradigm, suggesting a distinct assortment of cytokines and other signaling proteins depending on injury location (Mattugini et al., 2018).

Originally, reactive astrogliosis and glial scar formation in particular were considered to have mostly negative effects on disease outcome. The processes involved were found to inhibit axonal regeneration by astrocytic secretion of different molecules, most notably of the proteoglycan class (Rudge and Silver, 1990; McKeon et al., 1991).

Furthermore, electrophysiological approaches to astrocyte reactivity revealed a disruption of the glutamate-glutamine cycle in the mouse hippocampus, leading to deficits in neuronal

inhibition and a general hyperexcitability at the lesion site (Ortinski et al., 2010). In a series of papers, transgenic inactivation of the transcriptional regulator Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) in astrocytes, which is known to be highly active in the wake of trauma or disease, showed a number of similar results that pointed towards a deleterious component of astrocyte reactivity: reduction of inflammation and improved functional recovery after spinal cord injury (SCI) (Brambilla et al., 2005), increased axonal sparing and sprouting following SCI (Brambilla et al., 2009a), reduced inflammation and better functional outcome in experimental autoimmune encephalomyelitis (EAE) (Brambilla et al., 2009b), and improved survival of retinal neurons after ischemic insult (Dvoriantchikova et al., 2009).

At the same time, however, there is a wealth of evidence for the positive effects of reactive astroglia in CNS pathologies. Genetically targeted ablation of forebrain astrocytes adjacent to a stab wound injury led to a 25-fold increase in leukocyte infiltration, failure of BBB repair, and substantial neuronal degeneration. The latter could be attenuated by chronic glutamate receptor blockade, suggesting a role for reactive astrocytes in the prevention of glutamate excitotoxicity (Bush et al., 1999). Similarly conceived experiments by the same group, examining the consequences of astrocyte ablation in rodent SCI (Faulkner et al., 2004), EAE (Voskuhl et al., 2009), and traumatic brain injury (Myer et al., 2006) confirmed these observations.

Along the same lines, genetic disruption of STAT3 signaling – a key regulator of reactive gliosis – after SCI resulted in widespread infiltration of inflammatory cells and persisting motor deficits (Okada et al., 2006). Recently, and contrary to a number of earlier observations, glial scar formation was found to aid axon regeneration after SCI rather than hindering it (Anderson et al., 2016).

Mice deficient in both GFAP and vimentin, two major intermediate proteins associated with the reactive phenotype, showed significantly worse outcomes after brain ischemia, at least partly due to a marked decrease in glutamate transport capacity (Li et al., 2008). Paradoxically, the same experimental paradigm (i.e. GFAP and vimentin knockout) actually improved functional recovery after SCI (Menet et al., 2003). An explanation for this apparent contradiction – besides the different mechanisms of CNS insult – might be the time points at which the studies made their respective observations: one evaluated the outcome after seven

days (Li et al., 2008), whereas the other waited more than twice as long before looking at the results (Menet et al., 2003), so the effects of reactive astrogliosis might be more favorable on a subacute than on a chronic timescale.

A recent transcriptomic approach yielded further insights into the disparate effects reactive astrocytes tend to have on disease outcome: cells induced by ischemia exhibited very distinct expression profiles from those generated through neuroinflammation (Zamanian et al., 2012). The latter were shown to be induced by microglia-secreted cytokines, which lead to them acquiring a detrimental phenotype (Liddelow et al., 2017), whereas ischemic conditions seem to trigger a more beneficial, neurotrophic change in reactive astroglia (Liddelow and Barres, 2017). In another instance of glial diversity, the vulnerability of protoplasmic astrocytes to ischemic conditions seems to differ from that of their fibrous counterparts, although the two relevant studies in this context came to opposite conclusions (Lukaszevicz et al., 2002; Shannon et al., 2007).

1.5.2 Astrocytic Involvement in CNS Disorders

While some form of astrocyte reactivity is present in virtually every CNS pathology, the specifics of this association can vary significantly between different kinds of disorders. A short overview of some diseases where astroglia has been implicated as part of the pathogenesis might serve as an illustration.

Huntington's disease (HD) is a progressive brain disorder that is characterized by uncontrolled movements and cognitive decline. Its molecular cause is a polyglutamine expansion in the huntingtin protein, which leads to dysfunction and eventual death of striatal and cortical neurons (Khakh and Sofroniew, 2015). Several studies have proposed disruptions in glial glutamate buffering – caused by intracellular accumulation of the mutant protein and subsequent downregulation of GLT-1 expression – as an underlying disease mechanism (Liévens et al. 2001; Behrens et al., 2002; Shin et al., 2005; Bradford et al., 2009). In a recent paper, decreased expression of astrocytic Kir4.1 was suggested as another factor contributing to neuronal hyperexcitability in HD (Tong et al., 2014). Intriguingly, a marked increase in Cx43 expression was observed in the caudate nucleus of diseased HD patients, hinting at an expansion of astrocytic spatial buffering as a countermeasure against glutamate excitotoxicity (Vis et al., 1998). This ambivalent role of astroglia in HD is emphasized by another study, in

which the engraftment of glial progenitors with mutant huntingtin imparted a disease phenotype to WT mice, whereas striatal transplantation of healthy glia rescued some pathological aspects in transgenic HD animals (Benraiss et al., 2016).

Similar to HD, amyotrophic lateral sclerosis (ALS) is another CNS disorder with very limited and strictly symptomatic treatment options available. As a neurodegenerative disease, it is characterized by the loss of corticospinal and spinal motor neurons, which leads to progressive failure of the neuromuscular system (Xu et al., 2016). The role of astrocytes in the pathogenesis of ALS appears to be twofold since both loss of neuroprotective functions and gain of neurotoxic properties have been described. As in the case of HD, tissue of ALS patients displayed evidence of disrupted astroglial glutamate buffering associated with decreased GLT-1 expression (Rothstein et al., 1992; Fray et al., 1998; Howland et al., 2002). In rodent models of ALS, disease progression could be slowed by administration of the beta-lactam antibiotic ceftriaxone (Rothstein et al., 2005) and transplantation of healthy astrocyte progenitors (Lepore et al., 2008), both at least partly due to a modulation of glutamate transporter expression.

Approximately one in five cases of familial ALS exhibits missense mutations in the gene encoding Superoxide dismutase 1 (SOD1) (Rowland and Shneider, 2001). In animal models replicating SOD1 mutations in astrocytes, the genetically altered cells proved toxic for motor neurons (Di Giorgio et al., 2007, Marchetto et al., 2008), whereas spinal GABAergic neurons or interneurons were not affected (Nagai et al., 2007). Deletion of the mutant gene slowed disease progression significantly (Yamanaka et al., 2008; Wang et al., 2011). Different mechanisms underlying this astrocyte-induced neurotoxicity have been proposed: astroglial Transforming growth factor β (TGF-β) signaling was shown to interfere with neuroprotective properties of microglia and T cells, so that expression levels of TGF-β negatively correlated with the animals' lifespan (Endo et al., 2015). Another group observed astrocyte-mediated reduction of MHCI molecules on neurons, which made them more susceptible to cell death (Song et al., 2016). In both cases, disease progression could be slowed significantly by counteracting these processes, either by inhibition of TGF-β signaling (Endo et al., 2015) or by overexpression of a MHCI molecule (Song et al., 2016).

Alzheimer's disease (AD), the most prevalent cause of dementia and arguably the most societally impactful neurodegenerative disease, is characterized by marked deficiencies in

various cognitive abilities, combined with alterations in mood and behavior. Its pathological hallmarks are the accumulation of intracellular neurofibrillary tangles and extracellular plaques, the latter consisting mostly of misfolded Amyloid β (Aβ) protein (Scheltens et al., 2016). Centered around these plaques, reactive astrocytes with internalized deposits of Aβ are encountered (Nagele et al., 2003), where the misfolded protein is degraded (Wyss-Coray et al., 2003; Koistinaho et al., 2004; Pihlaja et al., 2008). Although this clearance mechanism would seem to be beneficial towards stalling the progression of AD, there is also evidence to the contrary: in neuronal-astrocytic cocultures, astrocyte interaction with Aβ actually resulted in increased neurotoxicity (Malchiodi-Albedi et al., 2001; Paradisi et al., 2004). Activation of astroglial NF-κB and subsequent release of C3 has been identified as an underlying mechanism (Lian et al., 2015), as has the Aβ-mediated decrease in astroglial TGF-β1 expression (Diniz et al., 2017).

Another aspect of astrocytic involvement in AD is the widespread alteration in Ca^{2+} signaling dynamics. Multiphoton fluorescence lifetime imaging microscopy revealed a general elevation of resting Ca^{2+} in astrocyte networks, independent of proximity to Aβ plaques (Kuchibhotla et al., 2009). Furthermore, increased frequencies of spontaneous Ca^{2+} increases and occasional intercellular Ca^{2+} waves were observed (Kuchibhotla et al., 2009), that were mediated by purinergic signaling (Delekate et al., 2014) and might contribute to vascular instability in AD (Takano et al., 2007). Finally, aberrantly high levels of GABA in AD-related reactive astroglia have been described independently by two different groups, leading to tonic inhibition in dentate granule cells and the impairment of memory and learning so characteristic for the disease. Interestingly, the deficits could be almost completely rescued by suppressing astrocytic GABA production or release, indicating a potential therapeutic avenue (Wu et al., 2014; Jo et al., 2014).

Besides neurodegenerative diseases, the role of astroglia in autoimmune inflammatory disorders has been investigated at some length. Most prominent among them is multiple sclerosis (MS), which is characterized by immune-mediated attacks leading to oligodendrocyte loss and demyelination. Reactive gliosis and glial scar formation are regularly encountered in (chronic) demyelinated lesions (Lundgaard et al., 2014), with MS-associated astrocytes exhibiting some distinctive features, such as multinucleation (Nishie et al., 2004),

emperipolesis of oligodendrocytes (Ghatak, 1992), and significantly altered protein expression (Holley et al., 2003).

Once again, our present picture of astroglial involvement in the pathogenesis of MS is ambivalent. Beneficial effects found in multiple studies include the support of oligodendrocytes in remyelination (Franklin et al., 1991; Talbott et al., 2005; Nash et al., 2011; Schulz et al., 2012), the clearance of obstructive myelin debris either directly (Lee et al., 1990) or through microglia recruitment (Skripuletz et al., 2013), and the control of inflammation-promoting immune cells (Voskuhl et al., 2009; Wang et al., 2013). On the other hand, there are reports of astroglial inhibition of remyelination (Hammond et al., 2014), astrocyte-secreted hyaluronan preventing the maturation of oligodendrocyte progenitors (Back et al., 2005), and transgenic suppression of reactive astrogliosis improving outcome in EAE (Brambilla et al., 2009b). As mentioned before, one explanation for this seeming paradox may be the essential time-dependency of the many processes involved (Iwama et al., 2011).

Recent observations have identified environmental factors (Wheeler et al., 2019) and aspects of the gut microbiome (Rothhammer et al., 2018) as modifiers of astrocyte activity in MS pathology. Intriguingly, the astrocyte-specific potassium channel Kir4.1 has been implicated as a direct immune target in a subgroup of MS patients (Srivastava et al., 2012). This purported mechanism somewhat resembles the pathogenetic basic of neuromyelitis optica, another autoinflammatory disease once considered a rare variant of MS. In that case, specific autoantibodies against the water channel AQP4 on astrocytic endfeet, which is part of the same dystroglycan protein complex as Kir4.1, have been identified as the underlying cause of inflammation (Lennon et al., 2004; Lennon et al., 2005).

1.5.3 Therapeutic Potential

With our understanding of both astrocyte physiology and pathology continuing to deepen, potential therapeutic applications of this new-found knowledge become increasingly conceivable. Tools like inducible knockout mice, that allow for precisely timed gene deletion in glial cells (Mori et al., 2006), or adeno-associated virus transduction (Foust et al., 2009) using astrocyte-specific promoters (Xie et al., 2010) can be employed to affect a large number of cells *in vivo* and examine the effects of direct genetic manipulation. However, due to the

multifaceted nature of reactive gliosis and its comprising both neuroprotective and neurotoxic aspects, any potential intervention must take into account the complexity of the situation.

Preventing astrocyte reactivity and glial scar formation has been attempted by various groups with a view to improving outcome in CNS pathologies. As already touched on above, the results have been mixed: on the one hand, inhibition of gliosis-associated pathways like NF-κB (Brambilla et al., 2005; Brambilla et al., 2009a; Brambilla et al., 2009b; Dvoriantchikova et al., 2009) or JAK/STAT (Ceyzériat et al., 2018) showed positive effects in different disease paradigms. Similarly, astrocyte-specific deletion of Sox2, a TF necessary for injury-induced activation of cortical astrocytes, was found to promote functional recovery after traumatic brain injury (Chen et al., 2019).

On the other hand, counteracting glial scar formation by deletion of STAT3 (Okada et al., 2006; Herrmann et al., 2008) or overexpression of purinergic signaling in astrocytes (Shinozaki et al., 2017) was associated with significantly worse outcomes. Interestingly, a recent study obtained promising results in a Parkinson's Disease model by specifically blocking the microglia-induced conversion of astrocytes to a neurotoxic phenotype (Yun et al., 2018).

Another potentially therapeutic approach is the transplantation of healthy glial progenitor cells. A number of studies have shown that engraftment of immature astrocytes and glial-restricted precursor cells can inhibit excessive scar formation and promote axonal regeneration and functional recovery after injury both in the rodent cortex (Smith et al., 1986; Wunderlich et al., 1994) and spinal cord (Cao et al., 2005). Olig2-positive progenitors derived from human embryonic stem cells were shown to differentiate into a neuroprotective type of astroglia upon transplantation into rat brains subjected to ischemic conditions (Jiang et al., 2013). As mentioned above, similar observations have been made in mice with lipid metabolism deficiencies (Ebrahimi et al., 2016) and in rodent models of ALS (Lepore et al., 2008) and HD (Benraiss et al., 2016).

At this point, there is *in vivo* evidence that transplanted astrocytes can survive for extended periods of time and functionally integrate into existing circuits of the host brain (Zhang et al., 2016). The experimental transplantation of human glial progenitor cells into the adult mouse forebrain has recently yielded some very intriguing results: the xenografts integrated into the recipient CNS, even forming gap junctions with host astrocytes, yet retained their unmistakable humanoid characteristics (size, morphology, etc.). Ca^{2+} signals were found to

propagate three times faster than in WT mice, with LTP and learning significantly enhanced (Han et al., 2013).

Finally, molecular reprogramming of astroglial cells into functional neurons has emerged as an elegant concept some 15 years ago. Pioneered by the lab of Magdalena Götz, its implications regarding neuronal repair are quite enticing. A multitude of TFs like Pax6 (Heins et al., 2002), Olig2 (Buffo et al., 2005), Neurogenin-2 and Mash1 (Berninger et al., 2007), Dlx2 (Heinrich et al., 2011), Nanog, Oct4, or Sox2 (Corti et al., 2012; Niu et al., 2013) have since been identified *in vitro* and *in vivo* that allow for the generation of subtype specific neurons when overexpressed or antagonized (Fig. 10). Interestingly, adult reactive astrocytes have proven equally susceptible to reprogramming as postnatal astroglia (Heinrich et al., 2010), in accordance with their display of stem cell properties after injury (Buffo et al., 2008; Sirko et al., 2013).

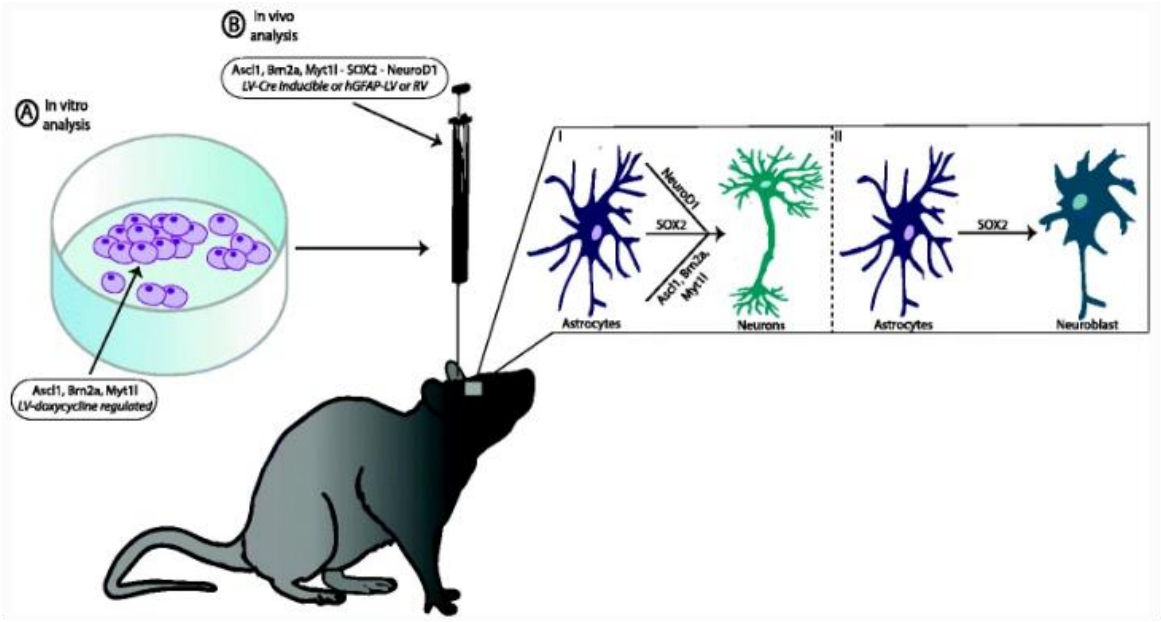


Fig. 10: Diagram of two different approaches to *in vivo* reprogramming
(A) After viral transfection *in vitro* (e.g. with a doxycycline-inducible lentivirus (LV) carrying the neural conversion factors Ascl1, Brn2a, and Myt1l), the cells are transplanted into the mouse brain where reprogramming is activated *in vivo*.
(B) Alternatively, the viral delivery system (e.g. lentiviruses or retroviruses) carrying one or multiple neural conversion factors is directly injected into the murine organism. Subsequently, astrocytes within the brain can be reprogrammed into different cell types, depending on the combination of factors involved. Figure from Dametti et al., 2015.

A number of studies have looked at the potential of astrocyte-to-neuron reprogramming in concrete disease paradigms. Retroviral expression of the TF NeuroD1 in cortical astroglia of stab-injured or AD model mice led to the generation of functional glutamatergic neurons (Guo et al., 2014). Similarly, overexpression of Sox2 after SCI resulted in the conversion of reactive

astrocytes to neuroblasts and eventually synapse-forming neurons (Su et al., 2014). Following stroke, striatal astrocytes were found to activate a latent neurogenic program by downregulating Notch1 signaling. This effect could be reproduced by inhibiting Notch signaling even in the absence of stroke (Magnusson et al., 2014). Once again emphasizing the impact of regional heterogeneity on CNS physiology and pathology, Neurogenin-2-induced reprogramming of non-neuronal cells differed markedly between neocortex and striatum in respect to reprogramming efficacy and molecular phenotype of newly generated neurons. Moreover, both regions responded in distinct ways to ischemic insult (Grande et al., 2013).

1.6 Decoding Astrocyte Heterogeneity: The Omics Approach

Traditionally, astrocyte heterogeneity was mainly appreciated through CNS-wide comparison of morphological features and IHC labeling with different protein markers. An often-cited study in this context uses a combination of protein expression (GFAP, S100β, and transgenic hGFAP-GFP), astroglial density, and proliferation rate across various CNS regions to define distinct subpopulations of astrocytic cells (Emsley and Macklis, 2006). As already mentioned earlier, a similar approach was taken by another group who compared astroglial expression of GLT-1 and GLAST using transgenic mice (Regan et al., 2007). While studies like these do provide interesting examples of astrocyte diversity in the CNS, their scope necessarily remains rather limited.

With the arrival of next-generation sequencing and the refinement of cell-specific isolation methods in recent years, a number of studies have emerged that attempt a more general approach towards astrocyte characterization (Fig. 11). Although some of them treat astroglia as a homogenous population of cells, their differently placed emphases lead to interesting observations in their own right. An early transcriptome study of astrocytes, neurons, and oligodendrocytes used FACS to isolate cells from the mouse forebrain at various ages between P1 and P30. Astrocytes were found to be enriched in a number of metabolic and, most notably, phagocytic pathways, suggesting a role as professional phagocytes (Cahoy et al., 2008). Data from another group that looked specifically at FACS-isolated protoplasmic astroglia support this special status regarding metabolism (Lovatt et al., 2007). Later, these results were complemented by analogous data for human astroglia, revealing significant differences between the human and rodent transcriptome (Zhang et al., 2016). A similarly conceived study

looked at alternative splicing events in different CNS cell types (Zhang et al., 2014), and recently, the task of generating a cell type-specific and region-resolved brain proteome was undertaken (Sharma et al., 2015).

More interesting, perhaps, for the purposes of this thesis are analyses that focus on astroglial subpopulations and the molecular differences between them. One early combinatorial examination of microarray data from both *in vitro* and *in vivo* astrocyte samples isolated from various brain regions at different time points serves as an extensive confirmation of basic astroglial diversity (Bachoo et al., 2004). Similar observations were made by another microarray study comparing postnatal astrocytes from four different CNS locations. Interestingly, expression of NF1, a known tumor suppressor gene, was found to vary significantly depending on brain region, suggesting a molecular basis for the propensity of certain human CNS tumors to develop in specific locations (Yeh et al., 2009).

By relying on a combination of transcriptomic, proteomic, electrophysiological, and IHC approaches, a recent study observed marked diversity of striatal and hippocampal astrocytes and their respective functions in neural circuits (Chai et al., 2017). Using translating ribosome affinity purification (TRAP), a method developed previously that allows for cell-type-specific and ribosome-associated mRNA isolation without the need for tissue fixation or single-cell suspensions (Heiman et al., 2008; Doyle et al., 2008), another group showed that the transcriptomic profile of astrocytes from different regions of the adult mouse brain closely follows the dorsoventral axis. Furthermore, cocultures of glial cells and neurons from multiple CNS locations revealed that neurite growth and synaptic activity of neurons are selectively promoted by astrocytes from the same brain region (Morel et al., 2017).

Somewhat in contrast to these observations, a different study of the same year identified five distinct subpopulations of astrocytes present across six CNS regions on the basis of cell surface marker expression. Intriguingly, correlative subpopulations were found in rodent and human gliomas, corresponding with certain pathophysiological aspects like onset of seizures and tumor progression (John Lin et al., 2017). In an attempt to further delineate this diversity, the same group recently compared subpopulations between different brain regions and investigated astroglial expression of long non-coding RNAs, again finding associations with elements of pathophysiology in glioma and neurodegenerative disease (Cuevas-Diaz Duran et al., 2019).

Intra-cortical astrocyte heterogeneity has recently been investigated by two studies, uncovering significant morphological and molecular differences (Lanjakornsiripan et al., 2018; Morel et al., 2019). Intriguingly, these differences were found to be reflected on an electrophysiological level (Morel et al., 2019) and were lost upon disturbance of neuronal layer formation via conditional gene knockout, suggesting an essential role of neuron-astrocyte interaction for the development of astroglial identity (Lanjakornsiripan et al., 2018).

Currently, the refinement of single-cell sequencing techniques is leading to a handful of pioneering brain transcriptome studies being published. So far, these investigations have mainly focused on identifying numerous molecularly distinct subclasses of neural cells at hitherto unseen resolution (Zeisel et al., 2015; Darmanis et al., 2015), although some functional correlates of this newly uncovered heterogeneity have already emerged (Tasic et al., 2016). A recent collation of these and additional gene expression studies aimed at uncovering cell type specific ‘signatures’ and potential marker genes conserved across the different data sets (McKenzie et al., 2018).

Even though there have been a number of studies that performed transcriptomic and proteomic analyses across different brain regions and glial subpopulations, none of them compared the molecular signatures of protoplasmic and fibrous astrocytes. As it stands today, the historically oldest distinction among astroglial cells, first described as far back as 1890, has not been systematically investigated using next-generation sequencing methods.

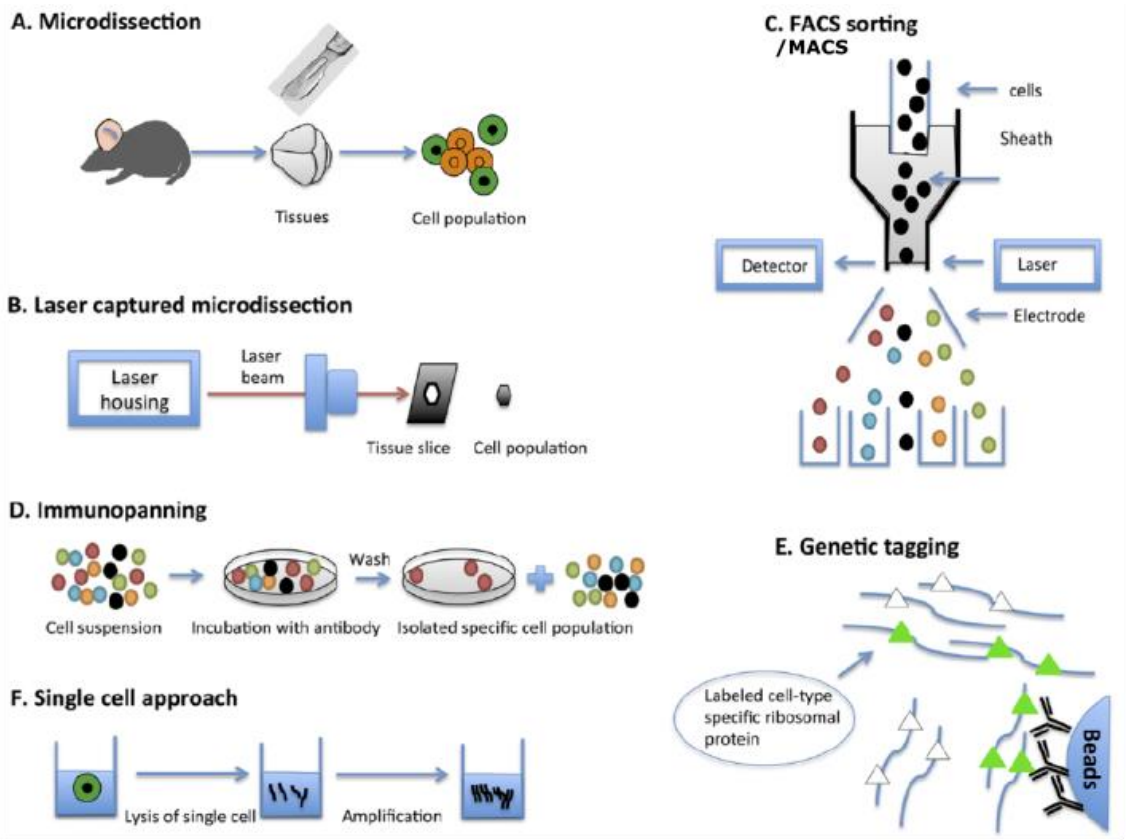


Fig. 11: Different ways of cell type-specific RNA isolation for transcriptomic analysis
Microdissection relies on morphological characteristics of a particular cell type to isolate it from its tissue of origin, either manually via scalpel (A) or by using a laser capture device (B). Both methods require a skilled and experienced researcher in order to obtain reliably pure yields. Other, more recent approaches, such as FACS and magnetic-activated cell sorting (MACS) or immunopanning, are based on the presence of specific cell surface markers. Cells of interest are isolated either by labeling with fluorescent antibodies/antibodies attached to magnetic beads (C) or by using antibodies immobilized on a solid surface (D). Methods like TRAP or RiboTag rely on genetic tagging for ribosome-associated mRNA isolation (E). Finally, single-cell sequencing has ermerged as an elegant and very powerful tool for high resolution RNA isolation in recent years (F). Figure modified from Dong et al., 2016.

1.7 Questions and Goal of Dissertation

Although the subject of astrocyte heterogeneity has seen increasing attention in recent years, it remains comparatively unexplored even today. Unlike in the case of neurons, where the existence of morphologically and functionally distinct subclasses is an established and well researched fact, the literature on astroglial subtypes is still rather sparse. One purpose of this introduction was to emphasize that examining the unique features of astrocytes from different brain regions is not only of academic interest but may have more practical applications as well, e.g. for the treatment of various CNS disorders or the promotion of cellular regeneration after injury or stroke.

This project takes as its object of investigation the two types of astroglia that were the first to be described as two separate entities over a century ago: protoplasmic and fibrous astrocytes (Andriezen, 1893). It aims to uncover their distinct transcriptomic signatures and, making use of the resulting data, gain a better understanding of functional differences between the two. To our knowledge, this study constitutes the first attempt to apply next-generation sequencing methods to specifically investigate astrocyte diversity in the cerebral GM and WM, as similar examinations have either focused on different cell types from whole brain homogenates or looked at astroglia from different brain regions without explicitly distinguishing between GM and WM areas.

In a first step, astroglial cells need to be isolated from cortical GM (for protoplasmic astrocytes) and WM tissue (for fibrous astrocytes). Two different methods will be employed for this purpose, the specific advantages of which will be discussed below. Immunocytochemistry (ICC) using various established astrocyte marker proteins will help us assess the overall purity of the sorted cells.

The cellular material yielded by the isolation procedure will then be processed for RNA Sequencing (RNA-Seq). To gain an initial overview of the resulting data, some general statistics including principal component analysis (PCA) and differential expression analysis will be performed. Here, we will look at the read counts of various astroglial and non-astroglial marker genes in our samples and also compare the expression values of numerous genes from literature associated with different levels of astrocyte maturity between our GM and WM data.

For a functional approach to our RNA-Seq data, a number of online resources will be used to screen for gene clusters and pathways enriched in the GM and WM samples. This way, we hope to shed some light on differences between protoplasmic and fibrous astrocytes that go beyond the merely morphological.

2. Materials and Methods

2.1. Materials

2.1.1 Equipment

Centrifuge 5424R	Eppendorf (Hamburg, D)
Centrifuge Mikro 22R	Hettich (Tuttlingen, D)
Centrifuge Heraeus Megafuge 8	Thermo Fisher Sc. (Waltham, MA, USA)
Cole-Parmer Stuart Rotator Disk	Thermo Fisher Sc. (Waltham, MA, USA)
Counting Chamber Neubauer improved	Brand (Wertheim, D)
Electrophoresis power supply LKB GPS 200/400	Pharmacia (not existent anymore)
Forceps	Fine Science Tools (Heidelberg, D)
Freezer (-20°C)	Liebherr (Bulle, CH)
Freezer (-20°C)	Bosch (Stuttgart, D)
Freezer (-80°C)	Thermo Fisher Sc. (Waltham, MA, USA)
Gel imaging system Chemi Doc XRS+	BIO-RAD (Hercules, CA, USA)
Gel system PerfectBlue	PEQLAB (Erlangen, D)
gentleMACS Octo Dissociater	Miltenyi Biotec (Bergisch Gladbach, D)
Ice machine	Scotsman (Vernon Hills, IL, USA)
Incubator Galaxy 170S	New Brunswick (Enfield, CT, USA)
Kryostat CM 3050	Leica (Wetzlar, D)
Kryostat CM 3050S	Leica (Wetzlar, D)
Laboratory balance ML 1602T	Mettler Toledo (Giessen, D)
Laboratory balance AT261 Delta Range	Mettler Toledo (Giessen, D)

Laboratory balance ABS 220-4	KERN (Balingen, D)
Laminar flow FlowSafe B-[MaxPro]²-130 und -160	Berner (Elmshorn, D)
LightCycler Instrument 480 II	Roche (Basel, CH)
Light source HXP-120	Visitron Systems (Puchheim, D)
MACS MultiStand	Miltenyi Biotec (Bergisch Gladbach, D)
Magnetic stirrer MSH 300	Lab4you (Berlin, D)
Magnetic stirrer IKA COMBIMAG RET	IKA Jahnke und Kunkel (Staufen, D)
Magnetic stand	VWR (Radnor, PA, USA)
Microscope Axiovert 40 C	Zeiss (Oberkochen, D)
Microscope AxioObserver Z1	Zeiss (Oberkochen, D)
Microscope DFC 3000 G	Leica (Wetzlar, D)
Microscope Leica MZ6	Leica (Wetzlar, D)
Microscope LSM 710	Zeiss (Oberkochen, D)
Microscope SZX10	Olympus (Tokio, J)
Microwave	SEVERIN (Sundern, D)
Mouse cages	Tecniplast (Buguggiate, I)
NanoDrop 1000 Spectrophotometer	PEQLAB (Erlangen, D)
OctoMACS Separator	Miltenyi Biotec (Bergisch Gladbach, D)
Oven BD 115	Binder (Tuttlingen, D)
pH-meter pH720	WTW inoLab (Weilheim, D)
Pipette controller accu-jet pro	Brand (Wertheim, D)
Pipettes (2.5 µl, 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Eppendorf (Hamburg, D)

Pipettes (2 µl, 20 µl, 100 µl, 200 µl, 1000 µl)	Gilson (Middleton, WI, USA)
Pipette (10 µl)	VWR (Radnor, PA, USA)
Refrigerator	Bosch (Stuttgart, D)
Refrigerator	Liebherr (Bulle, CH)
Shaker IKA-Vibrax VXR	IKA Jahnke und Kunkel (Staufen, D)
Shaker SU 1020	sunLab (Mannheim, D)
Syringe 5 µl 75N	Hamilton (Reno, NV, USA)
Thermocycler T3000	Biometra (Göttingen, D)
Thermomixer compact	Eppendorf (Hamburg, D)
Thermomixer F1.5	Eppendorf (Hamburg, D)
Vibratome VT1000 S	Leica (Wetzlar, D)
Vortex-Genie 2	Bender & Hobein (Bruchsal, D)
Vortex Reax top	Heidolph (Schwabach, D)
Vortex SU 1900	sunLab (Mannheim, D)
Water bath GFL 1002	GFL (Burgwedel, D)
Water bath GFL 1083	GFL (Burgwedel, D)
Water bath Haake D1/L	Haake (Karlsruhe, D)
Water purification system Milli-Q Reference	Merck Millipore (Billerica, MA, USA)

2.1.2 Consumables

Cell strainer 40 µm	Greiner Bio-One (Kremsmünster, A)
Coverslips	Carl Roth (Karlsruhe, D)
Eye and nose ointment Bepanthen	Bayer (Leverkusen, D)

Filaments (Vicryl)	Ethicon (Norderstedt, D)
Filter tips Biosphere	Sarstedt (Nümbrecht, D)
Filter papers	Macherey-Nagel (Düren, D)
Gloves	Meditrade (Kiefersfelden, D)
Injection needles Sterican 26G	B. Braun (Melsungen, D)
MACS C Tubes	Miltenyi Biotec (Bergisch Gladbach, D)
MACS MS Columns	Miltenyi Biotec (Bergisch Gladbach, D)
MACS Pre-Separation Filters 70 µm	Miltenyi Biotec (Bergisch Gladbach, D)
MACS SmartStrainers 70 µm	Miltenyi Biotec (Bergisch Gladbach, D)
Microscope slides	Carl Roth (Karlsruhe, D)
Microscope slides Superfrost Plus	Thermo Fisher Sc. (Waltham, MA, USA)
Pasteur pipettes	Hirschmann (Eberstadt, D)
Parafilm	Peckiney Plastic P. (Chicago, IL, USA)
PCR plates (96-well)	Brand (Wertheim, D)
Reaction tubes (0.5 ml, 1.5 ml, 2 ml)	Carl Roth (Karlsruhe, D)
Reaction tubes safelock (1.5 ml, 2 ml)	Eppendorf (Hamburg, D)
Reaction tubes (15 ml, 50 ml)	Greiner Bio-One (Kremsmünster, A)
Serolog. pipettes Falcon/Costar (5 ml, 10 ml, 25 ml)	Corning (Corning, NY, USA)
Syringes Omnifix-F (1 ml)	B. Braun (Melsungen, D)
Transfer pipettes	Sarstedt (Nümbrecht, D)
Well plates (24)	Sarstedt (Nümbrecht, D)

2.1.3 Chemicals and Reagents

Aceton	Carl Roth (Karlsruhe, D)
Acetic acid	Carl Roth (Karlsruhe, D)
Acrylamid (30%)	BIO-RAD (Hercules, CA, USA)
Agarose	Serva (Heidelberg, D)
Aprotinin	Biomol (Hamburg, D)
Bovine serum albumin (BSA)	Sigma-Aldrich (St. Louis, MO, USA)
Cycloheximide	Sigma-Aldrich (St. Louis, MO, USA)
4’,6-diamidino-2-phenylindole dilactate (DAPI)	Life Technologies (Carlsbad, CA, USA)
1,4-Dithiothreitol (DTT)	Sigma-Aldrich (St. Louis, MO, USA)
dNTPs (2 mM, 10 mM)	Thermo Fisher Sc. (Waltham, MA, USA)
Ethanol (> 99,8%)	Carl Roth (Karlsruhe, D)
Ethanol (70%)	Carl Roth (Karlsruhe, D)
Ethidium bromide	Carl Roth (Karlsruhe, D)
Ethylenediamine-tetraacetic acid (EDTA)	Sigma-Aldrich (St. Louis, MO, USA)
Ethylene glycol	Sigma-Aldrich (St. Louis, MO, USA)
First strand buffer 5x	Thermo Fisher Sc. (Waltham, MA, USA)
Glycerol	Sigma-Aldrich (St. Louis, MO, USA)
Glycine	Sigma-Aldrich (St. Louis, MO, USA)
Goat serum	Life Technologies (Carlsbad, CA, USA)
Heparin sodium salt	Sigma-Aldrich (St. Louis, MO, USA)
Hydrogen chloride (5 N)	Sigma-Aldrich (St. Louis, MO, USA)
Isopropanol	Carl Roth (Karlsruhe, D)

Ketaminhydrochlorid (Ketavet, 100 mg/ml)	Pfizer (New York City, NY, USA)
Leupeptin	Biomol (Hamburg, D)
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich (St. Louis, MO, USA)
Methanol	Merck (Darmstadt, D)
2-Mercaptoethanol	Sigma-Aldrich (St. Louis, MO, USA)
Mounting solution (AquaPolymount)	Polysciences (Warrington, PA, USA)
NaCl solution (Saline, 0,9%)	B. Braun (Melsungen, D)
Normal goat serum (NGS)	Rockland (Philadelphia, PA, USA)
NP-40	Sigma-Aldrich (St. Louis, MO, USA)
Paraformaldehyd (PFA)	Sigma-Aldrich (St. Louis, MO, USA)
Pepstatin	Biomol (Hamburg, D)
Pefabloc	Biomol (Hamburg, D)
Monopotassium dihydrogen phosphate	Merck (Darmstadt, D)
NEBuffer 10x	N. England Biolabs (Ipswich, MA, USA)
Potassium chloride (KCl)	Sigma-Aldrich (St. Louis, MO, USA)
Proteinase K	Carl Roth (Karlsruhe, D)
Random primers	Thermo Fisher Sc. (Waltham, MA, USA)
RNAse-free water	QIAGEN (Venlo, NL)
RNAse out	Thermo Fisher Sc. (Waltham, MA, USA)
RNasin Ribonuclease Inhibitor	Promega (Madison, WI, USA)
Sodium chloride	Sigma-Aldrich (St. Louis, MO, USA)
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich (St. Louis, MO, USA)

Sodium dihydrogen phosphate	Merck (Darmstadt, D)
di-Sodium hydrogen phosphate	Sigma-Aldrich (St. Louis, MO, USA)
di-Sodium hydrogen phosphate dihydrate	Merck (Darmstadt, D)
Sodium hydroxide	Sigma-Aldrich (St. Louis, MO, USA)
Sodium pyrophosphate	Sigma-Aldrich (St. Louis, MO, USA)
Sucrose	Merck (Darmstadt, D)
Superscript IV	Thermo Fisher Sc. (Waltham, MA, USA)
Triton X-100	Sigma-Aldrich (St. Louis, MO, USA)
TRISbase	Sigma-Aldrich (St. Louis, MO, USA)
TRISHCl	Sigma-Aldrich (St. Louis, MO, USA)
Tween 20	Sigma-Aldrich (St. Louis, MO, USA)
Xylacinhydrochlorid (Rompun, 2 Vol%)	Bayer (Leverkusen, D)
Yeast extract	BD (Franklin Lakes, NJ, USA)

2.1.4 Buffers and Solutions

2.1.4.1 RiboTag Immunoprecipitation

Homogenization buffer (HB)

15 ml	RNase-free water
2 ml	NP-40 (10%)
2 ml	KCl 1 M
670 µl	Tris 1.5 M, pH 7.4
240 µl	$MgCl_2$ 1 M

Supplemented homogenization buffer (HB-S)

19 ml	HB (see above)
400 µl	Cycloheximide (5 mg/ml)
10 µg	Leupeptin
5 µg	Aprotinin
20 µg	Pepstatin
2 mg	Pefabloc
200 µl	Heparin sodium salt (100 mg/ml)
100 µl	RNAsin Ribonuclease Inhibitor
20 µl	DTT 1 M

High salt buffer

10.5 ml	RNAse-free water
6 ml	KCl 1 M
2 ml	NP-40 (10%)
660 µl	Tris 1.5 M, pH 7.4
240 µl	$MgCl_2$ 1 M
400 µl	Cycloheximide (5 mg/ml)
10 µl	DTT 1 M

2.1.4.2 Tissue Preparation for Immunohistochemistry

Anesthesia solution

2.5 ml	Saline

1 ml	Ketavet
0.25 ml	Rompun

Paraformaldehyd stock (PFA 20%)

134 g	di-Sodium hydrogen phosphate dihydrate in 800 ml H_2Odd
400 g	Paraformaldehyd
ca. 10 ml	Sodium hydroxide

pass through paper filters; ad 2 l H_2Odd; pH 7,4

PFA (4%)

100 ml	PFA 20%
400 ml	H_2Odd

Sucrose solution (30% (w/v)) for cryoprotection:

15 g	Sucrose
ad 50 ml	PBS 1x

PO_4-buffer (10x; pH 7,2 - 7,4)

65 g	Sodium dihydrogen phosphate
15 g	Sodium hydroxide
2 ml	HCl (5 N)
ad 400 ml	H_2Odd

Storing solution for free floating brain tissue sections

150 ml	Glycerol
150 ml	Ethylene glycol
50 ml	PO_4-buffer (10x)
150 ml	H_2Odd

2.1.4.3 Immunohistochemistry/-cytochemistry

Phosphate buffer saline (PBS 10x)

58.75 g	di-Sodium hydrogen phosphate
10 g	Monopotassium dihydrogen phosphate
400 g	Sodium chloride
10 g	Potassium chloride
ad 5 l	H_2Odd; pH 7,4

PBS 1x

100 ml	PBS 10x
900 ml	H_2Odd

Blocking solution for brain tissue sections (10% (w/v) NGS)

1 ml	NGS
50 µl	Triton X-100
ad 10 ml	PBS 1x

Blocking solution for cells

0,2 g	BSA
50 µl	Triton X-100
ad 10 ml	PBS 1x

2.1.4.4 Quantitative Real-Time PCR (qPCR)

Master mix 1

1 µl	Random primers (0.5 mM)
1 µl	dNTPs (10 mM)
5 µl	RNA (or control equivalent)
6 µl	H_2Odd

Master mix 2

4 µl	First strand buffer 5x
1 µl	0.1 M DTT
0.2 µl	RNAse out
1 µl	Superscript IV
0.8 µl	H_2Odd

PCR mix

1 µl	cDNA (or control equivalent)
2 µl	NEBuffer 10x

2 µl	dNTPs (2 mM)
1 µl	FW primer
1 µl	RV primer
0.3 µl	Taq-Polymerase (self-made)
12.7 µl	H_2Odd

Probe mix

0.4 µl	Probe (for Roche LightCycler Instrument 480 II)
12 µl	PCR mix (replace generic primers with primers of interest)
2.6 µl	H_2Odd

2.1.5 Primers for qPCR

gene	left primer	right primer	amplicon length
Dmp1	agaaagtcaagctagcccagag	gtctgtactggcctctgtcgt	98
Igfbp2	gcgggtacctgtgaaaagag	cctcagagtggtcgtcatca	76
Fam216b	tcgaattcaagtcccagca	gcacctctgcccttgattta	70
Ttll6	tcttgttggaccggaaactc	tggagaagcttggagaatgg	63

2.2 Methods

2.2.1 Animals

Experiments not requiring a specific reporter gene were performed with adult C57BL/6J mice, an inbred mouse strain commonly used in lab work. Additionally, the following transgenic mouse lines were used:

BAC Aldh1l1-eGFP: This line was generated by the GENSAT project (Heintz, 2004) and uses enhanced green fluorescent protein (eGFP) expressed by the regulatory elements of the pan-astroglial marker Aldh1l1.

GLAST^{CreERT2} x RiboTag: This line uses the inducible form of the Cre recombinase (CreERT2) targeted to the locus of GLAST, a well-established astrocyte marker gene. Cre is fused to the ligand binding domain of the modified estrogen receptor (ERT2) and restricted to the cytoplasm. Nuclear translocation and recombination take place only upon administration of tamoxifen (Mori et al., 2006; Buffo et al., 2008). The GLAST^{CreERT2} line was crossbred with the commercially available RiboTag line to achieve astrocyte-specific labeling of ribosomal RNA (Sanz et al., 2009).

2.2.2 Magnetic-Activated Cell Sorting (MACS)

Kits used: Adult Brain Dissociation Kit and Anti-ACSA-2 MicroBead Kit

Tissue preparation and isolation of cells were performed according to the manufacturer's protocol (Miltenyi Biotec). Briefly, brains from adult Aldh1l1-eGFP mice were extracted, minced and mechanically dissociated with the gentleMACS Octo dissociator. The cells were then passed through a 70-µm strainer and incubated with the astrocyte-specific beads for 15 min. Afterwards, they were loaded on MS columns and separated by an OctoMACS magnet.

To consistently get satisfying cell yields after each sorting procedure, two details of the manufacturer's protocol were adjusted. First, more time was given to the phase formation during the crucial debris removal step (10 additional minutes after centrifuging), which notably improved overall cell number in the end. Secondly, the recommended red blood cell removal procedure was omitted entirely, since by carefully stripping away the brain's meninges during dissection, erythrocytic contamination posed no problem.

2.2.3 RiboTag Immunoprecipitation

Isolation of ribosomal RNA from the brains of RiboTag mice was performed over two days. After dissection of the tissue, samples were homogenized with a pestle in 200 µl of HB-S. Afterwards, 800 µl of HB-S were added to each homogenate and they were centrifuged for 10 min at 4°C. 800 µl were then collected from each tube, avoiding the tissue pellet at the bottom of the vial. Anti-HA (hemagglutinin) antibody was added, and the mixtures were placed in a cold room for 4 hours while resting on a gentle spinner.

30 minutes before the antibody-lysate solutions were taken out of cooling, 200 µl of Pierce Protein A/G magnetic beads (Thermo Fisher Sc., Waltham, MA, USA) were placed in a magnet for each sample. Storage buffer was removed and 800 µl of HB were added. After gentle resuspension on a rotator for 10 min, the tubes containing the beads were again placed in a magnet, the buffer was removed and the tubes were placed on ice. Afterwards, the antibody-tissue homogenates were added to the beads and the tubes were left to incubate overnight on a gentle rotator in a cold room.

The next day, the tubes were placed in a magnetic rack, the supernatant was removed and the beads were washed with 800 µl of high salt buffer. The tubes were then placed on a cooled rotator for 10 min, after which the washing step was repeated two more times. After removing the high salt buffer for the final time, 350 µl of lysis buffer (from the RNeasy Micro Kit by Qiagen, Venlo, NL) were added to the beads and the tubes were vortexed for 30 seconds to break up the antibody-bead-protein bonds. After placing the samples back in the magnetic rack, the volume was aspirated. All further steps were performed according to the manufacturer's instructions given by the RNeasy Micro Kit (Qiagen, Venlo, NL).

2.2.4 Histological and Cytological Procedures

2.2.4.1 Perfusion, Brain Sectioning and Storage of Slices

Animals were deeply anesthetized by injection of ketamine. Subsequently, they were perfused transcardially with PBS followed by 4% PFA (for a total of 100 ml per animal). Brains were postfixed in the same solution for 2 hours at 4°C and immersed in 30% sucrose for cryoprotection. After washing with PBS, the brains were cut using a Cryostat (Kryostat CM 3050S), and the slices were stored at -20°C in storing solution until further processing.

2.2.4.2 Immunohistochemistry

Slices were taken out of storage and washed in PBS for 10 min. They were pre-treated with 0.5% Triton X-100 and PBS along with 10 % NGS for 1 hour at room temperature. Primary antibodies were added to 500 µl of the blocking solution and the slices were incubated for 2 hours at room temperature and left in the solution overnight at 4°C.

The following primary antibodies were used:

- anti-GFP (chicken, 1:400, Rockland, Philadelphia, PA, USA)
- anti-GFAP (mouse IgG1, 1:500, Sigma-Aldrich, St. Louis, MO, USA)
- anti-GFAP (rabbit, 1:400, Abcam, Cambridge, UK)
- anti-ACSBG1 (rabbit, 1:250, Thermo Fisher Sc., Waltham, MA, USA)
- anti-S100β (mouse IgG1, 1:500, Sigma-Aldrich, St. Louis, MO, USA)
- anti-GS (mouse IgG2a, 1:200, Sigma-Aldrich, St. Louis, MO, USA)
- anti-HA (mouse IgG2b, 1:250, Thermo Fisher Sc., Waltham, MA, USA)
- anti-Sox9 (rabbit, 1:250, Sigma-Aldrich, St. Louis, MO, USA)
- anti-FGFR2 (rabbit, 1:100, Sigma-Aldrich, St. Louis, MO, USA)

The next day, the slices were washed in PBS and incubated with the appropriate secondary antibodies in blocking solution for 2 hours at room temperature. Secondary antibodies used were:

- rabbit 647 (1:500, Dianova, Hamburg, D)
- chicken 488 (1:500, Thermo Fisher Sc., Waltham, MA, USA)
- mouse IgG1 647 (1:500, Thermo Fisher Sc., Waltham, MA, USA)
- rabbit Cy3 (1:500, Dianova, Hamburg, D)
- mouse IgG2a 488 (1:500, Thermo Fisher Sc., Waltham, MA, USA)
- mouse IgG2b 555 (1:500, Thermo Fisher Sc., Waltham, MA, USA)

DAPI was added at a dilution of 1:1000 for 10 min, after which the slices were washed in PBS once more and mounted on glass slides with AquaPolymount.

In order to obtain a robust and reliable FGFR2-signal, we used the Pierce NHS-Rhodamine Antibody Labeling Kit (Thermo Fisher Sc., Waltham, MA, USA) according to the manufacturer's protocol.

2.2.4.3 Immunocytochemistry

Cells harvested though Magnetic Activated Cell Sorting (MACS) were first centrifuged at 1000 g for 5 min. The supernatant was removed, 100 µl of PBS were added per well, and the cells were left for 1 to 2 hours (until no movement was recognized on disturbance under the microscope). 150 µl of PBS + 250 µl of 8% PFA were added per well and removed after 7 min. The cells were washed three times with PBS and stored at 4°C until further processing.

100 µl of Blocking solution (0.5% Triton X-100 and 2% BSA in PBS) were added to the cells. After 30 min, primary antibodies were added and the whole was left to incubate for 1 hour at room temperature before being stored at 4°C overnight.

The following primary antibodies were used:

- anti-GFP (chicken, 1:1000, Rockland, Philadelphia, PA, USA)
- anti-GFAP (mouse IgG1, 1:1000, Sigma-Aldrich, St. Louis, MO, USA)
- anti-ACSBG1 (rabbit, 1:500, Thermo Fisher Sc., Waltham, MA, USA)
- anti-S100β (mouse IgG1, 1:1000, Sigma-Aldrich, St. Louis, MO, USA)
- anti-GS (mouse IgG2a, 1:500, Sigma-Aldrich, St. Louis, MO, USA)

After repeated washing in PBS, the cells were incubated with the appropriate secondary antibodies in blocking solution for 2 hours at room temperature. Secondary antibodies used were:

- chicken 488 (1:500, Thermo Fisher Sc., Waltham, MA, USA)
- mouse IgG1 647 (1:500, Thermo Fisher Sc., Waltham, MA, USA)
- rabbit Cy3 (1:500, Dianova, Hamburg, D)
- mouse IgG2a 488 (1:500, Thermo Fisher Sc., Waltham, MA, USA)

DAPI was added at a dilution of 1:1000 for 10 min, after which the slices were washed in PBS once more and mounted on coverslips with a single drop of AquaPolymount.

2.2.4.4 Microscopic Analysis

For tissue slices, analysis was performed with a laser-scanning confocal microscope (LSM 710). Images from equivalently stained slices were captured using the same settings in the

manufacturer's software (ZEN, Carl Zeiss, Oberkochen, D). Images for further analyses were either chosen individually from the Z-stack or acquired via merging the whole Z-stack through maximum intensity projection, depending on the question at hand.

Picture acquisition for ICC analyses was done on an epifluorescence microscope (AxioObserver Z1).

2.2.4.5 Cell Counts and Statistical Analysis

For purposes of cell counts, randomly selected sections of acquired images were used. Quantifications were done with the ZEN software and statistical analyses were performed using GraphPad Prism ver. 5 (GraphPad, San Diego, CA, USA). Values were given as mean ± SEM unless stated otherwise.

2.2.5 RNA Sequencing

mRNA was purified using RNeasy Micro Kit (Qiagen, Venlo, NL) and cDNA was synthesized using Ovation RNA-seq System v2 (NuGEN, Redwood City, CA, USA). Amplified cDNA was further fragmented, and libraries were prepared using the Ion Xpress Plus gDNA and Amplicon Library Preparation kit (Thermo Fisher Sc., Waltham, MA, USA). Samples were barcoded during the library preparation with sample specific indices. Equimolar ratios of each sample were then pooled and sequenced on an Ion Proton Sequencer.

The raw reads were demultiplexed based on the barcode sequences and the barcodes/adapters were trimmed. The pre-processed reads were checked for quality using FASTQC (Babraham Bioinformatics, Cambridge, UK) and then aligned using STAR software 2.4.2a (Dobin et al., 2013) to the Mus Musculus genome. The gene counts normalization and differential gene expression analysis was performed using DESeq2 (Love et al., 2014) and R (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org).

Library preparation, RNA-Seq and initial statistical analyses were performed by the lab of Prof. Moritz Rossner.

2.2.6 qPCR

Four genes identified by RNA-Seq to be enriched in GM or WM (2 each) were selected for qPCR validation. Primers were designed using the Roche Universal ProbeLibrary Assay Design Center (Roche, Basel, CH) and produced by Metabion (Metabion, Planegg, D). Alignment was ensured to be below 300 bp to rule out contamination with genomic DNA. Efficiency of the primers was assessed by creating standard curves through increasing dilutions in steps of x10.

cDNA synthesis was performed by first thermocycling Master mix 1 for 5 min at 65°C. After 1 min of cooling down at 4°C, the tube was centrifuged, and Master mix 2 was added for a total volume of 20 µl. This mixture was put into a thermocycler at 25°C for 5 min, 50°C for 60 min, and 70°C for 15 min before again cooling down at 4°C. The resulting cDNA was diluted 1:1 with RNase free water. To evaluate the success of cDNA creation, we performed PCR of the housekeeping gene Gapdh in the sample as well as in a positive control and two negative controls (RNA without the addition of Superscript III during cDNA creation and water). For this, PCR mix was added to 1 µl of the newly generated cDNA (and each of the controls) for a total of 20 µl. PCR was performed with a pre-denaturation step at 95°C for 3 min, followed by 28 cycles of denaturation (95°C for 30 s), annealing (56°C for 30 s), and elongation (72°C for 1 min). After a final elongation step at 72°C for 10 min, the samples were left to cool at 4°C. The resulting products were visualized through gel electrophoresis.

Actual qPCR was performed with the LightCycler Instrument 480 II and its integrated software according to the manufacturer's protocol. In short, 15 µl of Probe mix were added to 1 µl of the sample cDNA diluted in 4 µl of water. Four samples were processed for GM and WM, each, with technical triplicates for each sample. 50 cycles of qPCR amplification were performed, and the resulting CT values were assessed relative to the housekeeping gene Gapdh.

2.2.7 Functional Enrichment Analyses

Five different software tools were used for functional enrichment analyses of our RNA-Seq data: *DAVID* (Huang et al., 2009a; Huang et al., 2009b), *PANTHER* (Thomas et al., 2003; Mi et al., 2013), *GOrilla* (Eden et al., 2007; Eden et al., 2009), *g:Profiler* (Raudvere et al., 2019), and *Gene Set Enrichment Analysis (GSEA)* (Subramanian et al., 2005). Depending on the specific algorithms and requirements of the platform, input files consisted of either a list of all

examined genes and their respective expression values in GM and WM, a list of all DE genes, or separate lists of DE genes in GM and WM. To ensure statistically meaningful observations, we only mentioned GO terms and pathways that were identified as enriched by three or more of the employed tools. Moreover, all functional categories were significant at a p-value < 0.01 adjusted for multiple testing with a false discovery rate ≤ 0.05 using the Benjamini-Hochberg procedure.

2.2.8 Proliferation Assay

EdU was used as a proliferation marker by adding it to the drinking water of mice for 28 days. The animals were then sacrificed, slices were cut on a Cryostat and stained for the desired antibodies. Since EdU assays are not antibody-based, they do not require DNA denaturation for intranuclear signal detection. All further steps were performed according to the manufacturer's protocol (EdU Colorimetric IHC Detection Kit, Thermo Fisher Sc., Waltham, MA, USA). In short, slices were treated with Trypsin-EDTA to aid in antigen retrieval. After washing with PBS, 0.5 ml of the EdU reaction cocktail provided by the manufacturer were added to the slices, and they were left to incubate in a humidified chamber for 30 min. Afterwards, the tissue was washed with EdU wash buffer (also provided by the manufacturer) as well as PBS. 2 drops of streptavidin-peroxidase conjugate were added, and the slices were again left to incubate in a humidified chamber for 30 min. After another washing step, 200 µl of a DAB chromogen/buffer solution were added to the tissue and another 5 min of incubation were allowed to take place. After a final washing step, the tissue sections were mounted using AquaPolymount.

EdU administration and IHC were performed by Dr. Stefanie Ohlig and Manja Thorwirth.

3. Results

3.1 Isolation of Astrocyte-Specific RNA from the Mouse Cerebral Cortex

The purpose of this work was to investigate astrocyte heterogeneity in the GM and WM of the mouse cerebral cortex. Since much of the analysis was to be based on RNA-Seq data, the choice of an appropriate isolation method was crucial to the success of our project. There are a multitude of options available, each with its own strengths and weaknesses. In the end, we opted for the use of two different methods whose respective features complement each other in some ways: MACS and RiboTag.

3.1.1 Purity of Acutely Isolated Astrocytes Using MACS

One of the requirements we had for a good isolation method was that it would guarantee a sufficient level of purity while minimizing mechanical and chemical stress to the cells to interfere with their expression pattern as little as possible. These considerations led to the choice of MACS, a technology established in 1989 by the company Miltenyi Biotec (Miltenyi et al., 1990).

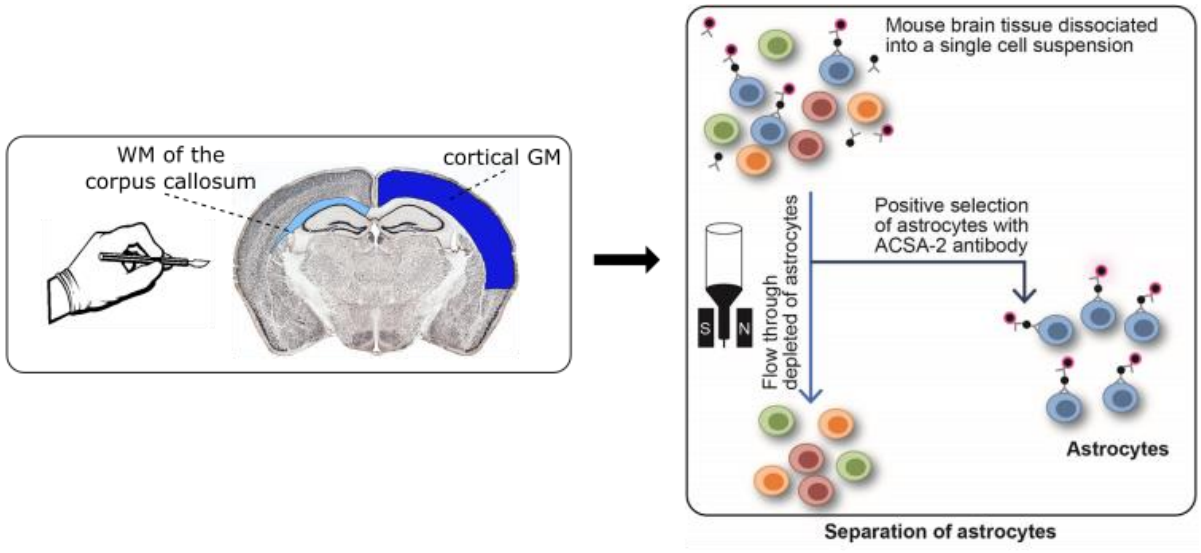


Fig. 12: Isolation of GM and WM astrocytes via MACS using the ACSA-2 antibody
This schematic illustration shows the process of astrocyte isolation from mouse brain using the MACS method. After the initial tissue preparation, astrocytes are enriched for by coupling them to magnetic beads linked to ACSA-2 antibodies. The filled-in dark and light blue brain areas visualize the GM and WM tissue processed for this purpose, respectively (both hemispheres were used, of course). Figure modified from Feldmann et al., 2014.

MACS has since been shown to be a viable option for the isolation of astrocytes both in the neonatal and adult mouse brain, making use of the monoclonal antibody 'anti-astrocyte cell surface antigen-2 (ACSA-2)', developed by Miltenyi to improve upon its earlier ACSA-1 antibody (Fig. 12). ACSA-1 recognizes and binds to an extracellular epitope of GLAST (Jungblut et al., 2012), whereas ACSA-2 was recently found to detect the epitope ATP1B2, a glycosylated surface molecule, which was shown to be expressed in most CNS regions from late embryonic stages to adulthood, although in varying degrees (Kantzer et al., 2017; Batiuk et al., 2017).

We used an Aldh1l1-eGFP reporter mouse line (generated by the GENSAT project, Heintz, 2004) to reliably label the yield of the MACS. Other established astrocyte markers we stained for were GFAP, S100β, ACSBG1, and GS. GFAP/S100β were combined in a single channel to ensure consistent detection of both GM and WM astroglia.

For the GM isolates, the proportion of positive cells in the sorted fraction reproducibly came to a little over 80% for all astrocyte markers tested (82.5 ± 2.3% for Aldh1l1-eGFP, 83.3 ± 1.7% for GFAP/S100β, 80.3 ± 3.2% for ACSBG1, and 82.5 ± 1.5% for GS; n=3 animals with 3 coverslips per animal, 614 cells in total counted for Aldh1l1-eGFP, GFAP/S100β, and ACSBG1, 2161 cells in total counted for GS; Fig. 13A-A''', C-C'', 15). The ACSA-2-negative flow-through was practically devoid of astrocytes, with only very few marker-positive DAPI-cells scattered here or there (2.0 ± 0.4% for Aldh1l1-eGFP, 1.7 ± 0.2% for GFAP/S100β, 1.8 ± 0.5% for ACSBG1, and 2.8 ± 0.3% for GS; n=3 animals with 3 coverslips per animal, 1705 cells in total counted for Aldh1l1-eGFP, GFAP/S100β, and ACSBG1, 1440 cells in total counted for GS; Fig. 14A-A''', C-C'', 15).

The sorted cells from the cortical WM showed marked discrepancies in their individual IHC signatures, ranging from 10% to 80% positive cells depending on the antibodies used (9.8 ± 1.1% for Aldh1l1-eGFP, 51.7 ± 2.4% for GFAP/S100β, 9.7 ± 2.1% for ACSBG1, and 80.2 ± 2.1% for GS; n=3 animals with 3 coverslips per animal, 474 cells in total counted for Aldh1l1-eGFP, GFAP/S100β, and ACSBG1, 618 cells in total counted for GS; Fig. 13B-B''', D-D'', 15). A similar tendency was noticeable in the ACSA-2-negative fraction, where more astroglial cells seemed to be lost overall compared to GM numbers (2.6 ± 0.6% for Aldh1l1-eGFP, 9.7 ± 1.6% for GFAP/S100β, 9.7 ± 2.1% for ACSBG1, and 15.8 ± 2.6% for GS; n=3 animals with 3 coverslips per animal, 479 cells in total counted for Aldh1l1-eGFP, GFAP/S100β, and ACSBG1, 475 cells in total counted for GS; Fig. 14B-B''', D-D'', 15).

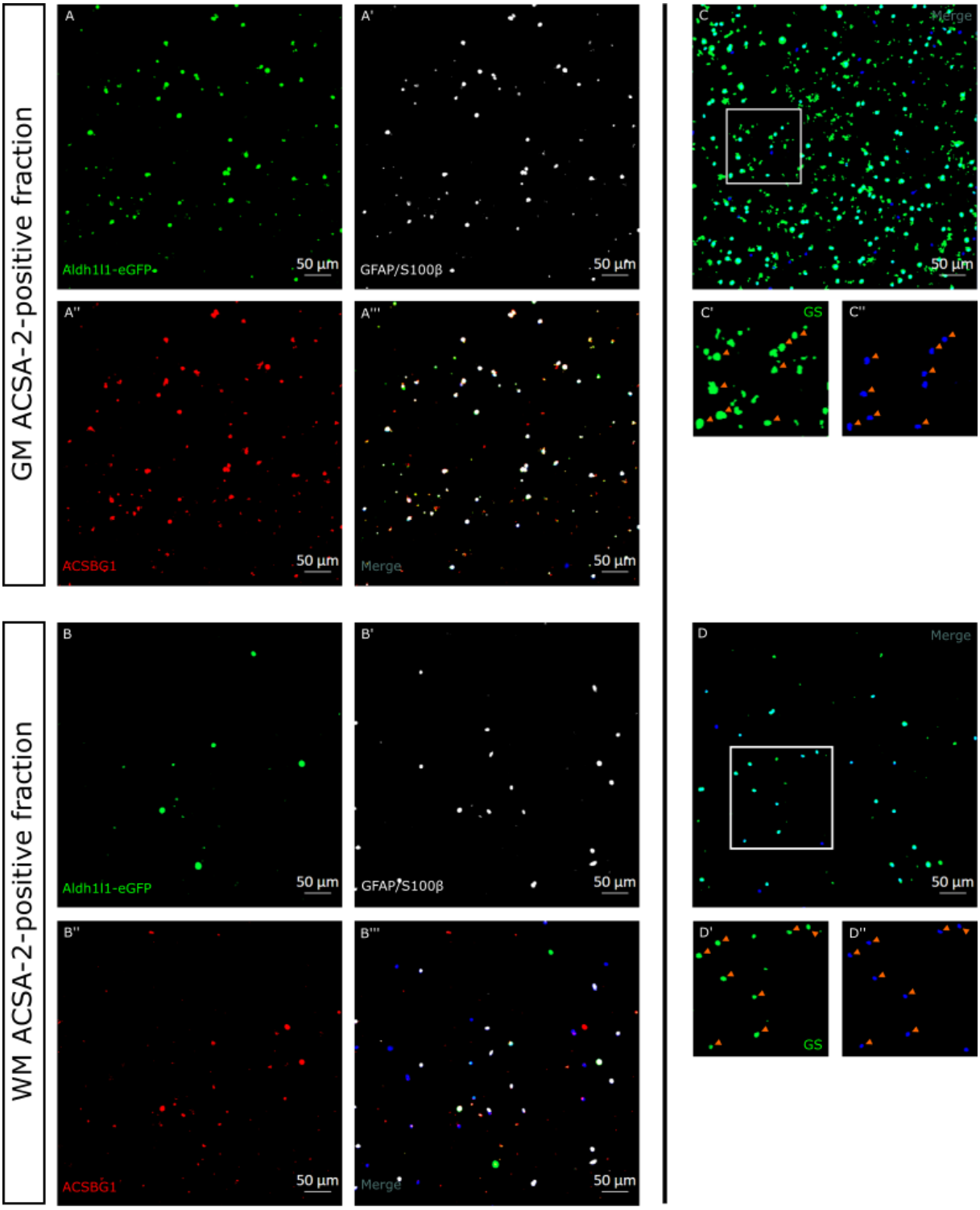


Fig. 13: Staining of plated ACSA-2-MACS-isolated GM and WM cells for astrocyte-specific markers

Representative micrographs are shown. Marker expression in GM (A-A''') and WM (B-B''') cells. GS as a representative marker in GM (C-C'') and WM (D-D'') cells with enlarged detail. Blue staining shows DAPI for all panels. Orange arrowheads indicate overlap of DAPI and GS.

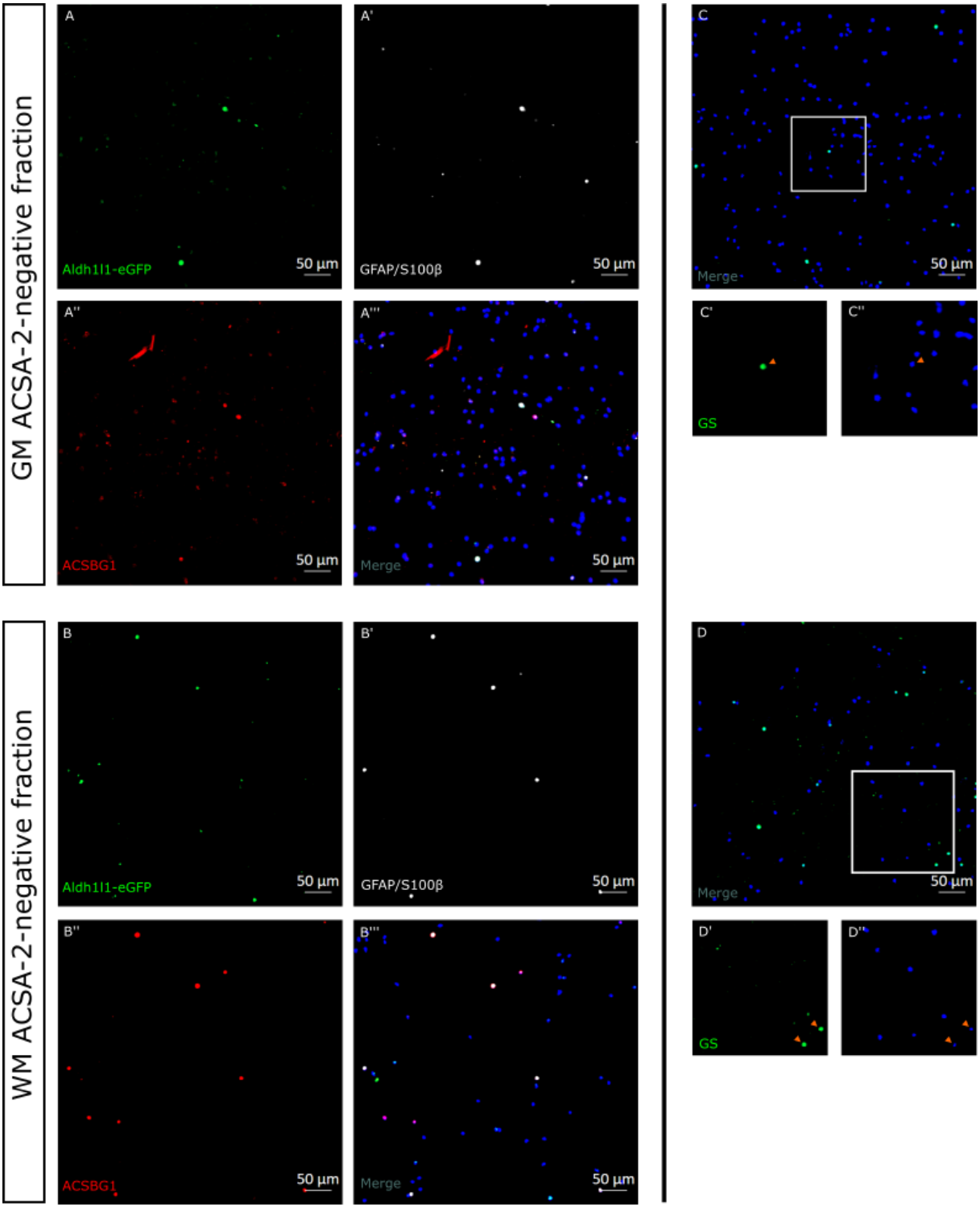


Fig. 14: Staining of plated GM and WM cells from the discarded fraction after ACSA-2-MACS for astrocyte-specific markers

Representative micrographs are shown. Marker expression in GM (A-A''') and WM (B-B''') cells. GS as a representative marker in GM (C-C'') and WM (D-D'') cells with enlarged detail. Blue staining shows DAPI for all panels. Orange arrowheads indicate overlap of DAPI and GS.

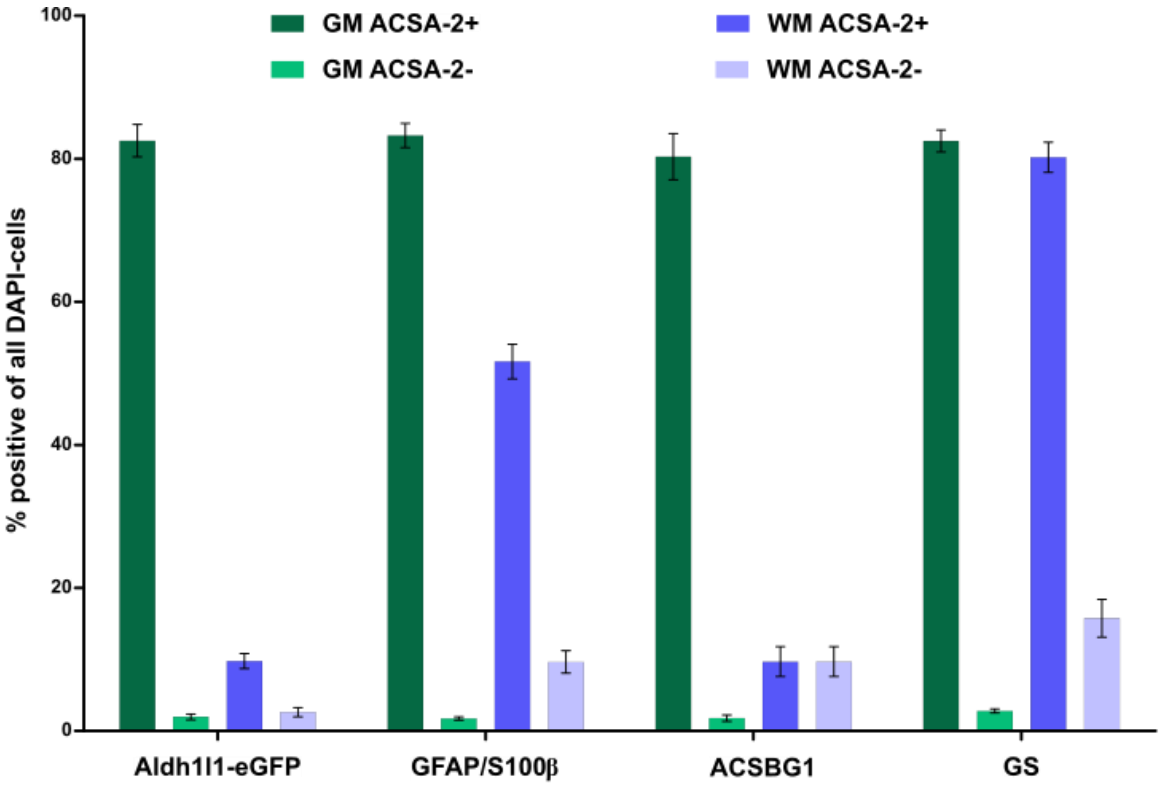


Fig. 15: Percentage expression of astrocyte markers in GM and WM after ACSA-2-MACS
Quantification by counting the number of marker-positive cells among all plated, DAPI-positive cells. Values are given as mean ± SEM, n=3 animals with 3 coverslips per animal.

To determine the nature of the astrocyte marker-negative cells in the ACSA-2+ fraction, we stained for some routinely employed oligodendrocyte markers. As this type of glial cell is thought to share a common developmental ancestry with astrocytes, we considered it the most likely candidate to account for any non-astroglial cells found in the sorts (Raff et al., 1983; Liu et al., 2002; Cai et al., 2007; Li et al., 2021). Besides, staining for Ionized calcium-binding adapter molecule 1 (IBA1) ruled out any potential microglia contamination (data not shown), and neuronal cells were highly unlikely to survive the MACS procedure in the first place. In addition to antibodies against the Oligodendrocyte 4 surface marker (O4) (Sommer and Schachner, 1981; Schachner et al., 1981) and the π form of the Glutathione-S-Transferase enzyme (GST-π) (Cammer et al., 1989; Tansey and Cammer, 1991), we also used the PLPdsRed reporter mouse line (Agrawal et al., 1977; Hartman et al., 1982; for the transgenic mouse cf. Hirrlinger et al., 2005).

Of the ACSA-2 sorted GM cells, 10.3 ± 0.8% were positive for PLPdsRed, 7.9 ± 0.7% for O4, and 10.8 ± 1.1% for GST-π. However, while PLPdsRed and GST-π shared a virtually identical distribution pattern, the fraction of O4-positive cells conformed only partially to it, so that

taken together, the percentage of PLPdsRed and/or O4-positive DAPI-cells was 14.8 ± 1.3% (n=3 animals with 3 coverslips per animal, 1995 cells in total counted for PLPdsRed and O4, 1558 cells in total counted for GST-π; Fig. 16 A-A'', B-B'').

The numbers for the WM isolates were comparable if slightly higher, with PLPdsRed-positives making up 13.9 ± 1.2% of all DAPI-cells, O4 accounting for 8.0 ± 0.6%, and GST-π for 15.6 ± 1.6%. Taking into consideration the distribution patterns of the three markers with O4 again being the odd one out, the percentage of PLPdsRed and/or O4-positive DAPI-cells was 17.2 ± 1.9% (n=3 animals with 3 coverslips per animal, 557 cells in total counted for PLPdsRed and O4, 679 cells in total counted for GST-π; Fig. 16 C-C'', D-D'').

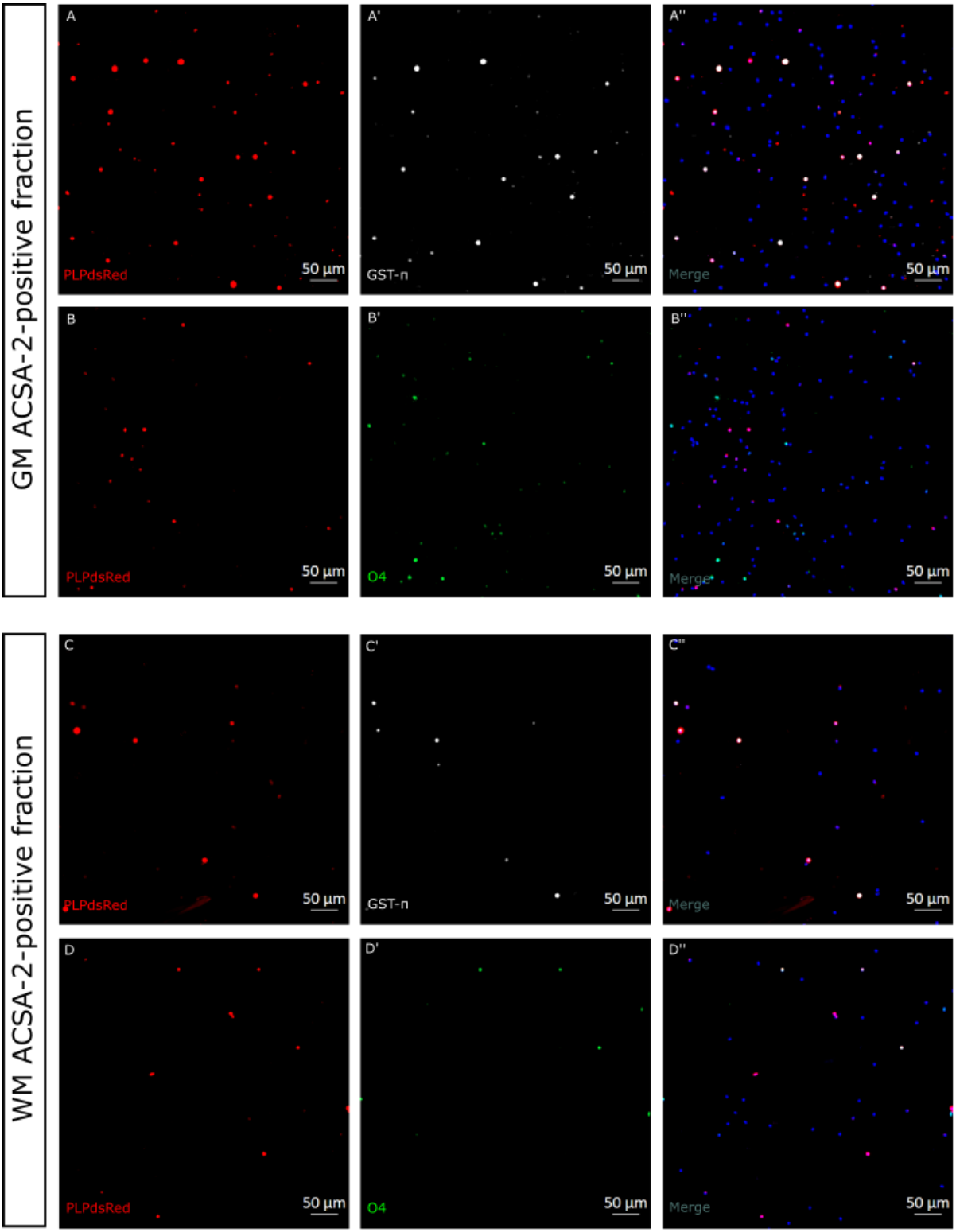


Fig. 16: Staining of plated ACSA-2-MACS-isolated GM and WM cells for oligodendrocyte-specific markers

Representative micrographs are shown. Marker expression in GM (A-A''+B-B'') and WM (C-C''+D-D'') cells. Blue staining shows DAPI for all panels.

These data show that while the oligodendrocyte fraction was roughly equivalent in number between both GM and WM isolates, the percentages of individual astrocyte marker positives revealed considerable variations. We took this as a first indication of certain intrinsic

differences between astroglia from both brain regions, already manifesting at the level of epitope expression. GS seemed to be the only astrocyte marker tested applicable to both GM and WM in the context of cells plated on PDL-coated coverslips. Interestingly, when investigated via IHC, GS had a very similar expression pattern to the other astroglial markers both in GM and WM, and we found no cells positive for GS but negative for Aldh1l1-eGFP, GFAP/S100β, and ACSBG1 (Fig. 17).

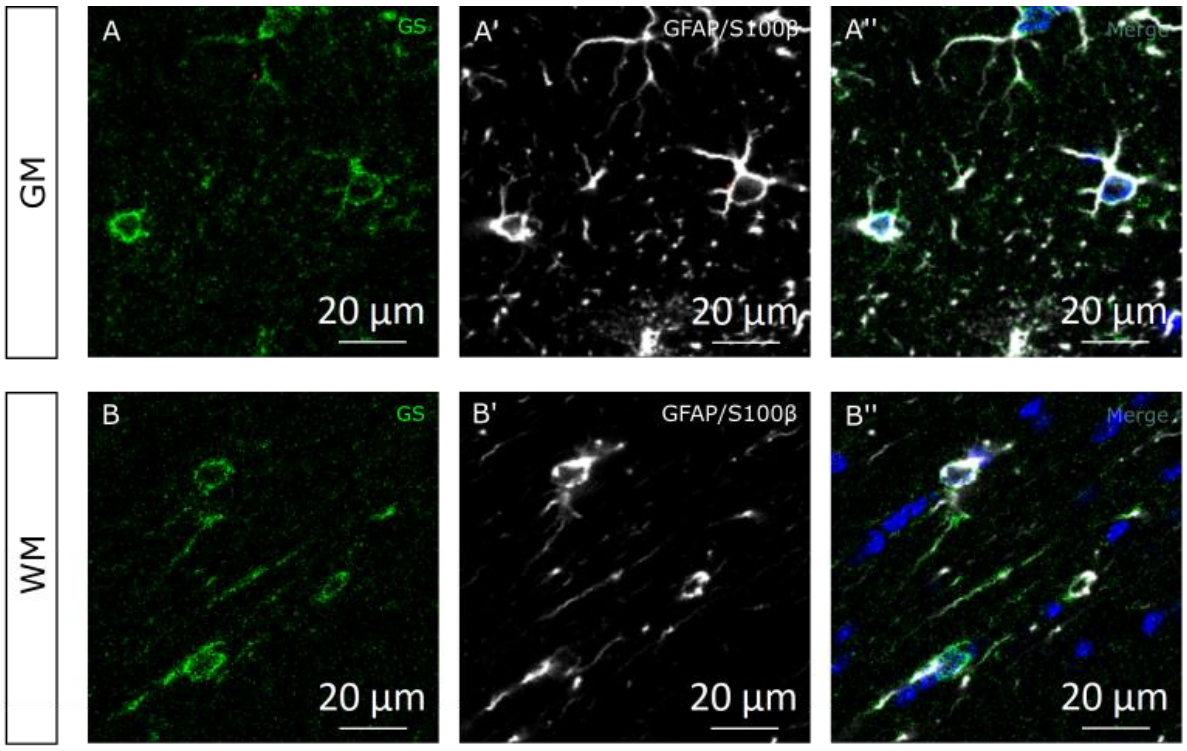


Fig. 17: The same cells express GS and GFAP/S100β in cortical GM and WM of the corpus callosum
Representative micrographs are shown. Expression patterns of GS and GFAP/S100β are comparable in cortical GM (A-A'') and WM of the corpus callosum (B-B'').

3.1.2 Isolation of Ribosome-Associated RNA from Astrocytes Using RiboTag

When specific cells – in our case astrocytes – are targeted through MACS, the resulting RNA material that is prepared for sequencing consists of all the different types of RNA present at the time of isolation. While this kind of analysis certainly promised some intriguing insights into the transcriptomic profile of GM and WM astrocytes, another aspect we considered worth investigating was which RNAs are actually associated with the ribosomal complex and end up getting translated into proteins. For that purpose, we decided to expand the scope of our analyses by including samples for sequencing obtained via RiboTag, a relatively recent

isolation method that specifically pulls down actively translated mRNA from the ribosome (Sanz et al., 2009).

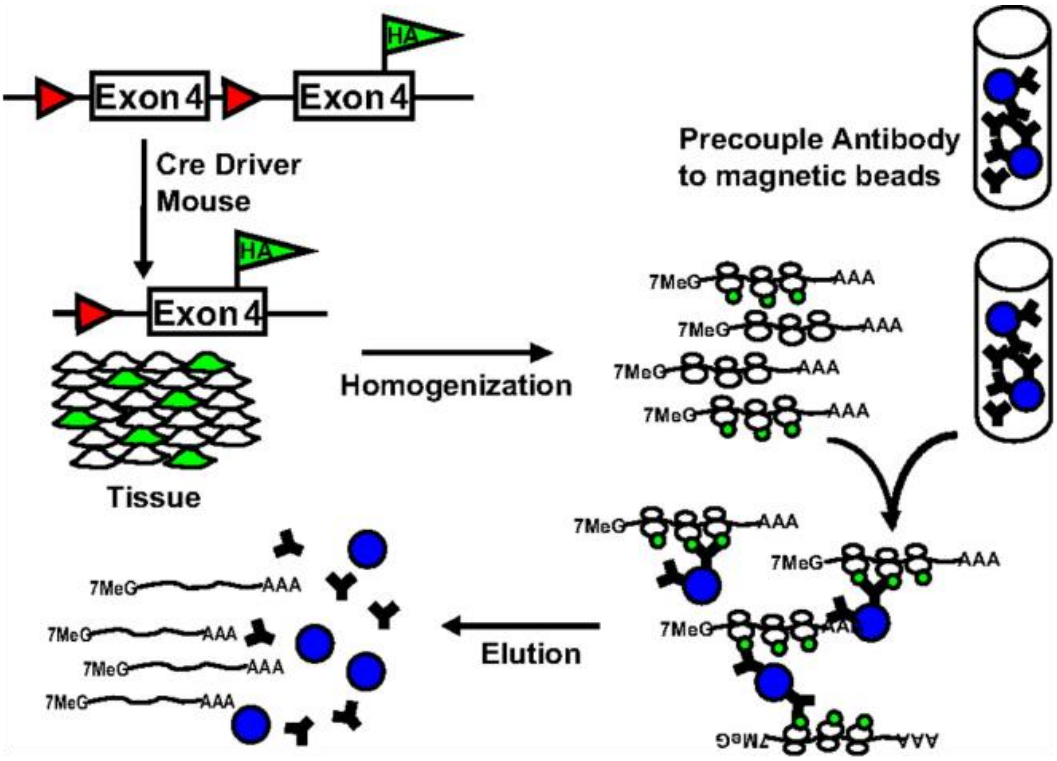


Fig. 18: Isolation of cell-type-specific RNA using RiboTag
This schematic illustration shows how ribosome-associated RNA is obtained from mouse tissue via the RiboTag method. A mouse carrying a floxed duplicate exon of a ribosomal protein gene tagged with HA is crossed to a mouse expressing a cell-type-specific Cre recombinase. The transcriptome of the chosen cell type can then be isolated through immunoprecipitation using an antibody against HA. Figure from Sanz et al., 2009.

In short, a commercially available RiboTag mouse carrying a floxed duplicate exon of a ribosomal protein gene tagged with hemagglutinin (HA) is crossed to a mouse expressing a cell-type-specific Cre recombinase. We chose the GLAST^{CreERT2} mouse for our purposes (Mori et al., 2006; Buffo et al., 2008). That way, the expression of the HA-tag in the ribosomal protein is ideally restricted to cells expressing GLAST, i.e. astrocytes. Their (ribosome-associated) transcriptome can subsequently be obtained by immunoprecipitation using a monoclonal antibody against HA (Fig. 18). Cells from both the GM and WM of sacrificed GLAST^{CreERT2} mice were stained against GLAST and HA as well as the astroglial markers GFAP and ACSBG1 to show colocalization of the proteins (Fig. 19).

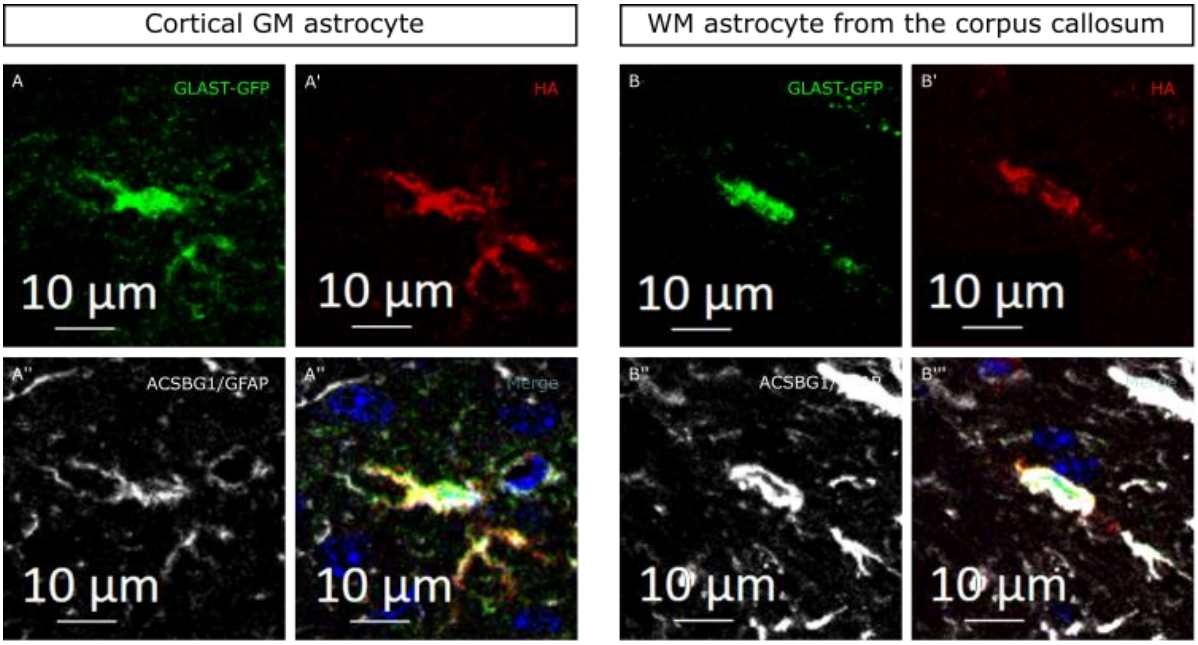


Fig. 19: Expression of HA in GM and WM astroglial cells of GLAST^{CreERT2} x RiboTag mouse as proof of concept for our implementation of the RiboTag method
Representative micrographs are shown. A cortical GM cell (A-A''') and WM cell from the corpus callosum (B-B''') both express astrocyte markers as well as HA. Blue staining shows DAPI for all panels.

3.2 Generating Transcriptomic Profiles of GM and WM Astrocytes Using RNA-Seq

After performing the isolation of RNA material as detailed above, the samples were prepared for RNA-Seq. Due to the mechanism of RNA extraction using the RiboTag method, there was no sure way to estimate the number of astrocytes processed, so we pooled two brains for each sample of GM and WM (n=3 in both cases) to make sure our yield was sufficient.

For the MACS samples, the GM and WM pooled from six brains constituted one n (with total n=4). Although the amount of GM astrocytes we could reliably isolate in this manner was around 200000 cells and thus more than sufficient for our purposes, we opted for six brains per sample because WM numbers tended to be considerably lower and subject to greater variability. We were able to achieve a minimum amount of 40000 to 50000 WM astrocytes this way.

3.2.1 Exploratory Analysis

To assess the overall degree of similarity between our samples, we performed PCA. Here, we plotted the RiboTag and MACS data separately as well as combined to better visualize differences on the level of biological replicates and isolation technique (Fig. 20).

The samples obtained via MACS clustered according to their region of origin, with principal component (PC) 1 accounting for 85.6% of inter-sample variance. Biological diversity of the replicates was reflected in PC 2 (explaining 10.9% of the differences) along the y-axis, where both the GM and WM isolates of animal 4 were closer to each other in value than they were to the other samples of the same brain region.

While the GM of the RiboTag isolates showed strong clustering in respect to both PC 1 and PC 2 (accounting for 67.4% and 16.6% of the variance, respectively), the WM samples were not as distinctly grouped. They spread out more along the x-axis, i.e. PC 1, and did not form a cluster among themselves in regard to PC 2.

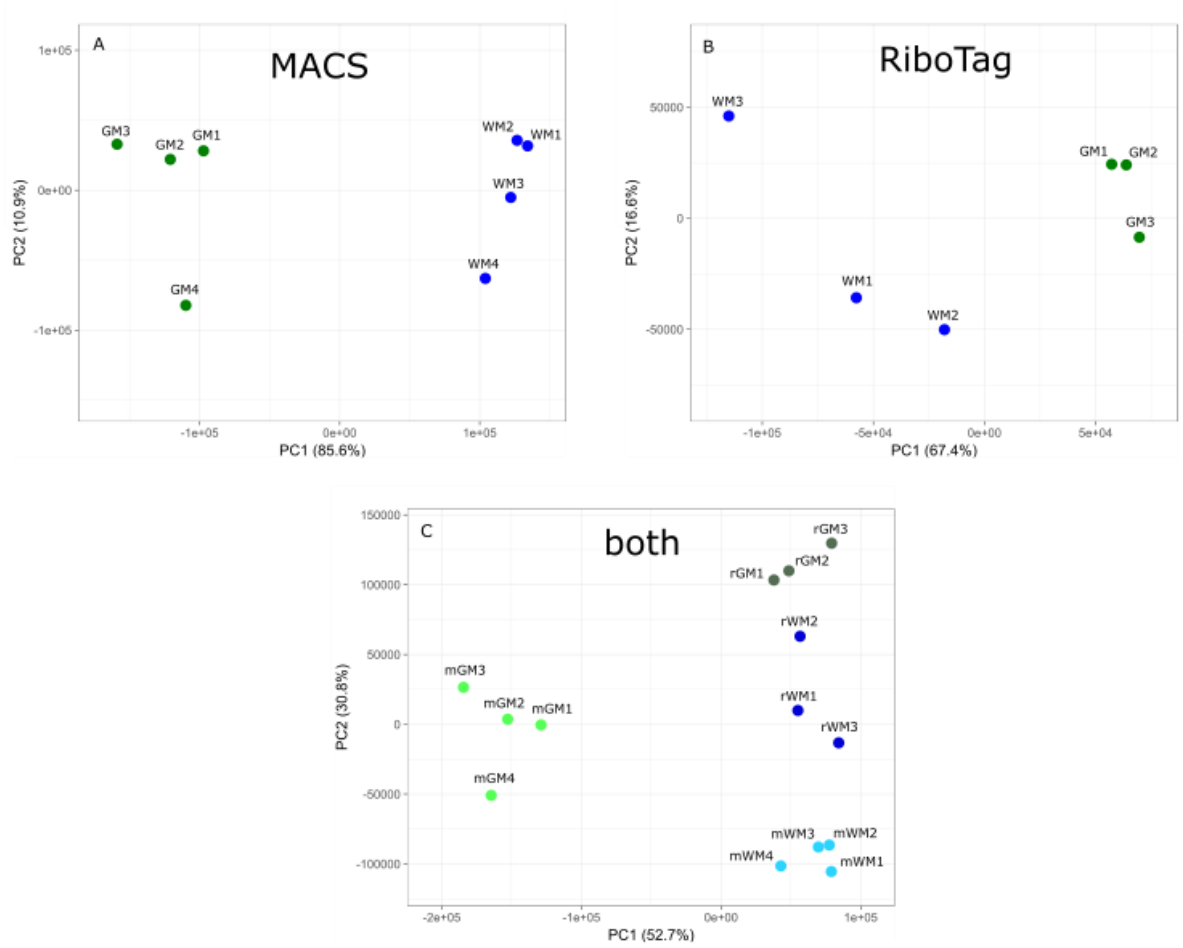


Fig. 20: PCA plots showing intra- and intermethod sample distance for MACS and RiboTag

PCA was performed three times using ClustVis (Metsalu and Vilo, 2015), once seperately for the MACS (A) and the RiboTag samples (B), then comparing all samples across both methods (C). The percentages given in brackets indicate the degree of difference the respective PC accounts for.

Finally, when plotting the RNA-Seq samples from both MACS and RiboTag together, four clusters depending on region of interest and isolation method emerged. Interestingly, the MACS-derived GM samples were the only group clearly separated from the rest when viewed against PC 1 (explaining 52.7% of the differences), while the others mainly clustered along the y-axis (with PC 2 accounting for 30.8% of variance).

3.2.2 Differential Expression Analysis

Two conditions were defined for a gene to be classified as differentially expressed:

1. Its $\log_2$ fold change (i.e. normalized expression value divided by average expression across both regions) had to be either ≥ 2 or ≤ -2.

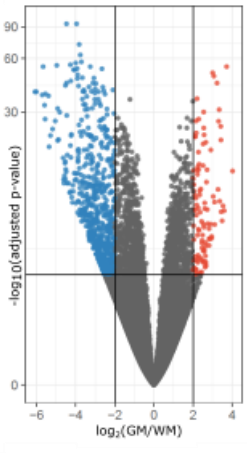
2. The adjusted p-value (corrected for multiple testing by taking into account the false discovery rate) had to be < 0.05.

Applying these criteria to our data, we found 903 genes to be differentially expressed in the RiboTag samples (4.3% of all genes covered), 126 of which showed higher expression in GM and 777 in WM. Analyses of the MACS isolates yielded 1738 genes in total that were differentially expressed (7.9% of all genes covered). 682 of these had stronger expression values in GM, 1056 in WM. Finally, 253 genes showed differential expression in both methods, 30 of which were significantly higher in the GM and 221 in the WM samples (Fig. 21A). The remaining two genes showed opposite tendencies of expression when compared between the MACS and RiboTag isolates.

B

Top 30 RiboTag DE genes ranked

up in GM		up in WM	
by fold change	by p-value	by fold change	by p-value
Gm20649	Sptan1	Gm21671	Mobp
Sptan1	Sptbn2	Gm21698	Plekhh1
Gm6644	Neurod1	5031410I06Rik	Plp1
Fgfbp1	Dbn1	Fam216b	Myrf
RP23-4F16.12	Stard8	Gm21663	Fa2h
Igfn1	Grm2	Gm10220	Ugt8a
Chrdl1	Picxd2	Gm21680	Anln
Fbxo34	Eps8	Speer4a	1700047M11Rik
Gm12371	Ptn	Gm13905	Fam216b
Grm2	Myh10	Gm21655	Pls1
Myh10	Fbxo34	Gm1979	Mbp
Dmp1	Mprip	Gm10471	2610203C20Rik
Dbn1	Luzp2	1700047M11Rik	Mog
Gm42641	Myo5a	Ccdc170	Tmem88b
Gm11128	Gm4735	Dnah12	Dnah6
Neurod1	Gjb6	Gm26747	Tspan2
Rspo1	Chrdl1	Spag17	Mob3b
Sptbn2	Gins-ps1	Haplln2	St18
Gm5617	Fam19a1	Gm44109	Stoml3
Gm6457	Ppp1r9a	Gm38062	Hydin
Ppp1r9a	Slc25a18	Plet1	Elovl7
Dmd	Csl	Gm7361	Fam167a
Ptgfr	Bmp7	Gm43169	Neat1
Lag3	Car8	Gm44235	Aspa
Fam180a	Coch	Neat1	Prr18
Gm4477	1110002E22Rik	Gm42993	Dubr
Spp1	Actn1	Gm37111	Pdeda
Igfbp2	Gstm2-ps1	Mobp	Chd7
1110002E22Rik	Gm20649	Hydin	Tmem98
Prdm8	Car7	Slc43a3	Snrnp70



Top 30 MACS DE genes ranked

up in GM		up in WM	
by fold change	by p-value	by fold change	by p-value
Chrdl1	Mfap3l	Dmrt3	Rsph1
Cldeb	S1pr1	Nek5	Cfap44
Erv3	Chrdl1	Ttll6	Ccdc108
Dio2	Plpp3	Dlec1	Dnah10
Ppp1r3g	Mcc	Cfap52	Rsph4a
Gcnt4	Haplln1	Krt15	Cfap61
Pgr	Slc1a2	Dnah12	Ak7
Aldh1a7	Glul	Ccdc108	Cfap54
Luzp2	Tril	Dnah10	Dnah5
Slc22a6	Gpm6a	Crygn	Ccdc146
Tfap2b	Mras	Ttc21a	Stoml3
Klri2	Sparcl1	Odf3b	Lrriq1
Gm10862	Fjx1	Mapk15	Cdhr3
Kcng4	Lcat	Cfap126	Dnah12
Haplln1	Acsbg1	Ccdc153	Dnah11
Pdzph1	F3	Lrrc36	Dnah9
Htr6	Mfge8	Slc39a4	Cfap161
Ptges	Ttyh1	Capsl	Mns1
Vav3	Kcnk1	Lrrc10b	Ccdc153
Ip6k3	Atp1a2	Musk	Cfap43
Nat8f6	Lgr4	Rsph1	2410004P03Rik
Ranbp3l	Ntm	Tmem212	Dnah2
Higf	Abcd2	Uox	Gm626
Rpe65	Luzp2	Crocc2	Spag6l
Kcnk1	Tspan7	Tsnaxip1	Foxj1
Adra1b	Slc30a10	Cdh26	Iqcg
Ptchd4	Mertk	Dnah5	Dsg2
Nat8f7	Stk17b	Sap30bpos	Spef2
Cxcl14	Scg3	Dnah11	Cfap45
Car8	Ncan	Spag16	Dnah1

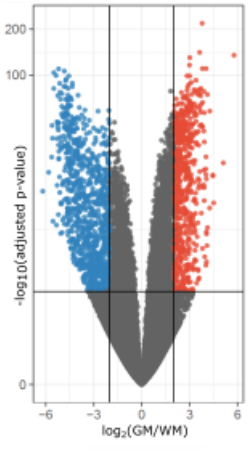


Fig. 21: Genes fulfilling the conditions for differential expression among the GM and WM MACS and RiboTag samples

Venn diagrams showing the amount of differentially expressed (DE) genes between the various isolates. The numbers in the middle of each circle represent the genes that are uniquely overexpressed for the respective brain area and method. Numbers in the intersection of two circles give the overlap of overexpressed genes between the two methods (A). Tables listing the top 30 overxpressed genes for each method and brain area, ranked by fold change and p-value. The volcano plots arrange all genes analyzed according to difference in expression and the adjusted p-value of this difference for both methods. Coloured dots indicate genes that are DE (red for up in GM, blue for up in WM) (B).

Since the overall number of sequenced genes was roughly comparable between the two approaches (21876 for MACS vs. 21116 for RiboTag), the fact that almost twice as many genes were differentially expressed in the MACS data may be attributable to the difference in

method, and the entirety of cellular RNA may be more distinct between GM and WM astrocytes than the ribosome-associated fraction.

3.2.2.1 Validation of RNA Sequencing Technique

To validate our method of RNA-Seq, we randomly selected four genes that were found to be differentially expressed in both the MACS and RiboTag samples: Dmp1, Igfbp2, Fam216b, and Ttll6 – the first two significantly higher in GM, the other two in WM. We then performed quantitative PCR (qPCR) on samples acquired via MACS (n=4 for GM and WM). Each of the four examined genes could be confirmed in its expression pattern (Fig. 22).

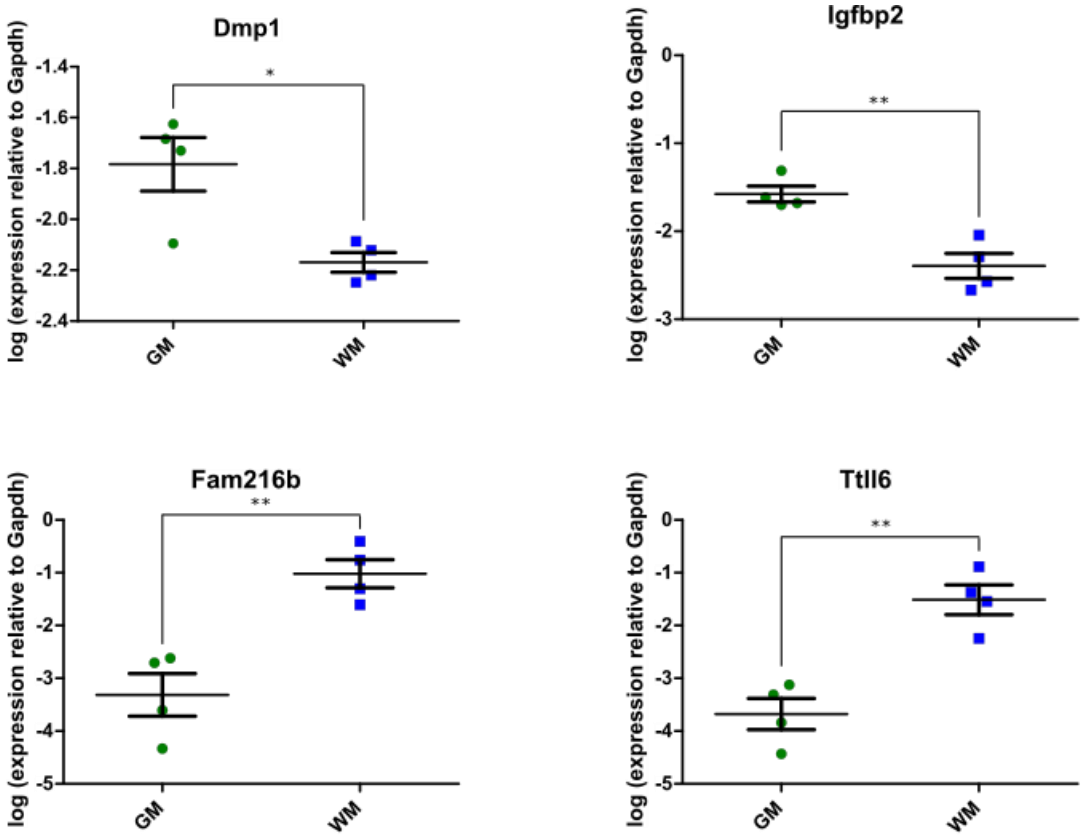


Fig. 22: qPCR evaluation of four randomly selected genes differentially expressed in both the MACS and RiboTag samples

qPCR was performed on four differentially expressed genes from our RNA-Seq data, two higher in GM (Dmp1 and Igfbp2), two higher in WM (Fam216b and Ttll6). Expression was assessed relative to Gapdh levels with n = 4 animals. Values are given as ± SEM. * $p < 0.05$, ** $p < 0.005$.

3.2.2.2 Reassessing Sample Purity

As a further measure towards assessing the purity of our isolates, we looked at the expression levels of five well-described markers for each major cell type of the CNS (Fig. 23). A caveat is due at this point: Without data from different cell types or whole brain tissue to contrast

against, a purely quantitative comparison of RNA-Seq reads is certainly problematic. However, since our samples were obtained from two brain regions using two distinct methods, we expected to at least see a general pattern of expression and, perhaps more importantly, gain some insight into any location- and approach-based differences that might exist.

Read counts for the selected astrocyte-specific genes (GS, Sox9, Aldoc, AQP4, Slc1a3) were high in all samples, with a notable tendency towards stronger expression in both the RiboTag and MACS GM isolates. Neither markers for microglia (Cd83, Ccl2, Il1a, Tlr2, Aif1) nor endothelial cells (Esam, Apold1, Nostrin, Cldn5, Adgrl4) were present at a significant level, practically ruling out any major contamination. Similarly low read counts were found for neuron-specific genes (Eno2, Tubb3, Sst, Gad1, Stmn2) in the MACS samples. The RiboTag values, however, were notably elevated in comparison, which is probably due to the previously described GLAST expression by a subset of neuronal cells (Rothstein et al., 1994; Chaudhry et al., 1995).

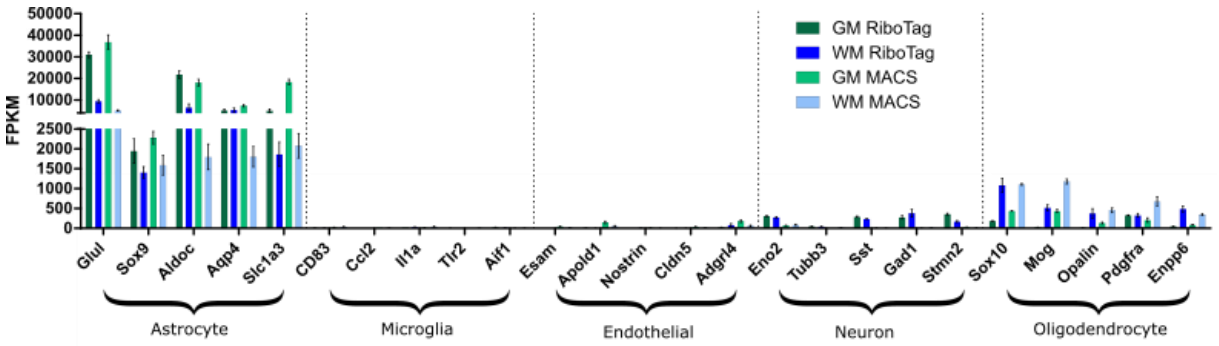


Fig. 23: Expression values of various cell type-specific neural markers in the RNA-Seq data for MACS and RiboTag GM and WM isolates
Discontinuos y-axis chosen to better display smaller but not negligible expression. Values are given as mean ± SEM.

The expression of oligodendrocyte-related genes (Sox10, Mog, Opalin, Pdgfrα, Enpp6) in our samples, finally, was not negligible and especially pronounced in tissue of WM origin. This observation may be partly explained by the likely presence of a small oligodendroglial fraction in our sorts, as noted above. However, this non-astroglial fraction was also detected in the plated cells from our GM MACS isolates, which showed a similar if slightly smaller amount of contamination than the WM MACS isolates. Consequently, it might be argued that the discrepancy in RNA expression could point to a more fundamental difference between astrocytes from both CNS regions.

3.2.2.3 Expression of Previously Established Astrocyte Marker Genes

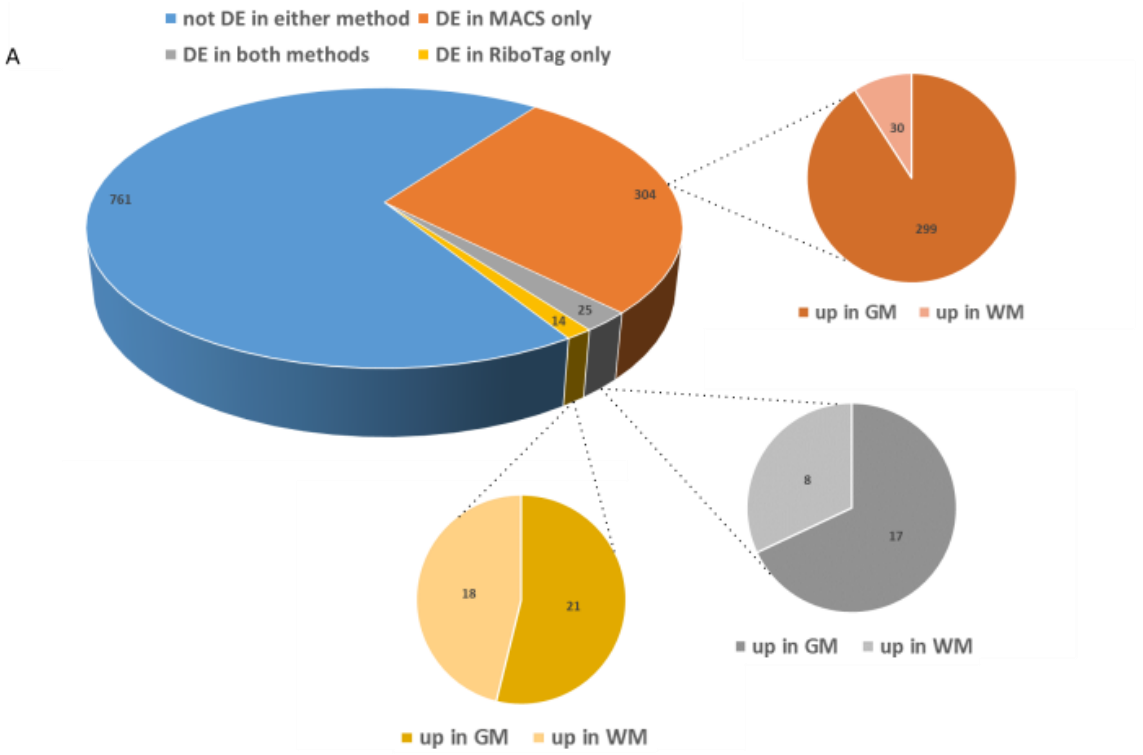
We also looked through recent literature for previously described astrocyte markers and determined their expression values in our samples. To this end, we took the top differentially expressed astrocyte genes from the following papers which include transcriptomic and proteomic data: Lein et al., 2007; Lovatt et al., 2007; Cahoy et al., 2008; Zhang et al., 2014; Sharma et al., 2015; Zhang et al., 2016; McKenzie et al., 2018. A list of 1104 genes was compiled, excluding 206 duplicates between the different publications.

39 of these 1104 astroglial genes (3.5%) were found to be differentially expressed in our RiboTag samples, with 21 significantly higher in GM and 18 in WM isolates. This number was considerably larger for the MACS samples, where we counted 329 differentially expressed genes among the astroglial markers from literature (29.8%). Bearing in mind once again the different method in which RNA is extracted by both approaches, this can be seen as a further indication that GM and WM astroglial differences seem to primarily manifest on the level of total cellular RNA rather than the ribosome-associated fraction.

Interestingly, there was again a notable trend towards higher numbers in the MACS GM isolates, where 299 among the 329 differentially expressed marker genes exhibited a $\log_2$ fold change ≥ 2 against only 30 such genes in the WM samples. If we included expression values below the $\log_2$ fold change-cutoff but with an adjusted p-value < 0.05, this tendency was present in the RiboTag data as well, although not quite as pronounced (340 genes higher in GM vs. 141 higher in WM).

One explanation for this observation is the fact that most genetic profiling studies of neural cells, including the ones from which we compiled our list of astroglial markers, tend to focus on astrocytes from predominantly GM regions. Thus, established astrocyte markers like S100β, Aldoc, GLAST, GS, Aldh1l1, or ACSBG1 all show significantly higher expression in GM isolated by RiboTag and MACS, many of them even past the threshold of differential expression in the latter case, although there are some exceptions, like GFAP (1042 FPKM in RiboTag GM vs. 1364 FPKM in RiboTag WM, 318 FPKM in MACS GM vs. 270 FPKM in MACS WM).

A



B

up in GM RiboTag	up in WM RiboTag	up in GM MACS	up in WM MACS	up in GM both methods	up in WM both methods
Chrdl1	NEAT1	Chrdl1	VIT	Chrdl1	VIT
DMP1	Ugt8a	Dio2	IQCA	RPE65	IQCA
Igfbp2	MYH6	Ppp1r3g	IQUB	DMP1	IQUB
NAT8	FAM167A	GCNT4	FAM167A	LUZP2	FAM167A
CMBL	TTLL8	LUZP2	DNAH7A	NAT8	ADGRV1
FAM163A	Dock10	Hapln1	DCHS2	PYGM	BBOX1
RPE65	Malat1	VAV3	MIAT	Gjb6	TTLL8
BMP7	IQUB	RANBP3L	ADGRV1	Igfbp2	THBS4
PYGM	IQCA	Hgf	PLIN5	BMP7	
Gli1	THBS4	RPE65	Elmod1	HSD11B1	
HSD11B1	NRBP2	PTCHD4	ACOX2	Cyp4f15	
Slc25a18	VIT	CXCL14	Trim67	SLC13A5	
Cyp4f15	ADGRV1	Slc1a2	BBOX1	FAM163A	
LUZP2	LENG8	MFAP3L	STOX1	Nwd2	
PTN	P2RY1	ST6GALNAC5	SNCAIP	MYH15	
SLC13A5	Chd7	GABRG1	TTLL8	CPQ	
Gjb6	MIR99AHG	LGR6	GRAMD1C	PTN	
CPQ	BBOX1	Lcat	AGBL2		
MYH15		Mertk	THBS4		
EFEMP1		ELOVL2	SHROOM3		

Differentially Expressed Genes (ranked by fold-change)

Fig. 24: A subset of astrocyte markers collated from literature is differentially expressed in the RiboTag and MACS samples

A list of 1104 astrocyte markers identified by both transcriptomic and proteomic studies was compiled from the following sources: Lein et al., 2007; Lovatt et al., 2007; Cahoy et al., 2008; Zhang et al., 2014; Sharma et al., 2015, Zhang et al., 2016; McKenzie et al., 2018. A subset of them was differentially expressed in our samples (A). The top 20 of those genes (or all, if total number was smaller) for each modality ranked by fold-change (B).

Finally, 25 among the list of 1104 astrocyte-enriched genes (2.3%) from literature were differentially expressed in both the RiboTag and the MACS samples, 17 of which were higher in GM and 8 in WM isolates (Fig. 24A).

Chrdl1, an antagonist of BMP4 that plays a crucial role in neuronal patterning during development (Nakayama et al., 2001; Sakuta et al. 2001), had the highest relative GM

expression in both the RiboTag and MACS isolates. More recently, its cortical astrocyte-specific localization has been shown, and it has been found to drive synapse maturation and limit synaptic plasticity (Blanco-Suarez et al., 2018). Also significantly GM-enriched was Igfbp2, a protein that has long been known as an important element in the development and progression of various types of glioma (Godard et al., 2003; Mehrian-Shai et al., 2007; Rorive et al., 2008; Huang et al., 2017; Liu et al., 2019a; Liu et al., 2019b; Shen et al., 2019).

Other differentially expressed astrocyte-enriched genes higher in RiboTag and MACS GM include Dmp1, a proteoglycan essential for astroglia maturation and BBB integrity (Jing et al., 2018), Gjb6 (i.e. Cx30), a Connexin exclusively expressed by protoplasmic astroglia (Nagy et al., 1999), and BMP7, found to play an important role in reactive astrogliosis (Fuller et al., 2007; Narantuya et al., 2010; Shin et al., 2012), the switch from neurogenesis to gliogenesis (Yanagisawa et al., 2001; Ortega and Alcántara, 2010), and promoting dendritic growth of neurons *in vitro* (Withers et al., 2000).

An interesting gene from our literature list of astrocyte markers with significantly higher expression in both the RiboTag and MACS WM samples was THBS4. Multiple characteristics have been assigned to this glycoprotein, ranging from association with certain types of astrocytoma (Rorive et al., 2006) to synaptogenic properties (Gan and Südhof, 2019) and important protective functions during post-injury astrogliosis (Benner et al., 2013; Laug et al., 2019).

Two of the astroglia-enriched genes with the highest relative expression in the RiboTag WM data were Neat1 and Malat1, both of them long non-coding RNAs. Malat1 has been implicated in cell cycle regulation (Tripathi et al., 2013; Yang et al., 2013) and changes in expression have been linked to glioma progression (Ma et al., 2015; Chen et al., 2017, Voce et al., 2019), similar to Neat1 (Gong et al., 2016; Zhen et al., 2016; Chen et al., 2018; Zhou et al., 2018). Moreover, Malat1 overexpression led to improved outcome after traumatic brain injury (Patel et al., 2018; Zhang et al., 2019) and stroke (Zhang et al., 2017; Ruan et al., 2019; Fig. 24B). The fact that non-coding RNAs are detected by RiboTag may seem counterintuitive but has been described before, so that ribosome association per se does not necessarily indicate that a given RNA is being actively translated (Jackson et al., 2018).

3.2.2.4 Expression of Genes Related to Cell Maturity and Differentiation

We finally looked at a number of genes that had previously been described as differentially expressed when comparing astrocytes from fetal (Zhang et al., 2016) or early developmental stages (Cahoy et al., 2008) to their mature counterparts; the same was done for astroglia from healthy brain tissue vs. glioblastoma cells (Zhang et al., 2016). A total of 1926 genes were differentially expressed between mature and immature astrocytes (in the two combined data sets), 891 of them upregulated among the first group, 1035 among the latter. 256 genes had significantly higher read counts in the glioblastoma cells vs. 182 in healthy tissue, for a total of 438 differentially expressed genes.

When determining the expression values of these genes in our own RNA-Seq data, an intriguing trend became apparent: many genes associated with cellular immaturity and low levels of differentiation were upregulated in WM astrocytes, while genes that had been found highly expressed in mature and differentiated cells were predominantly enriched in our GM data.

More specifically, 131 of the 1035 marker genes for immaturity (12.7%) had significantly higher read counts in either the RiboTag or MACS WM samples, whereas only 25 of them (2.4%) were enriched in GM astroglia. Conversely, 180 among the 891 maturity-associated genes from literature (20.2%) exhibited differential expression favoring protoplasmic astrocytes isolated via both methods, and only 28 (3.1%) were overexpressed in fibrous astroglial cells.

A similar tendency was present in the data for genes associated with glioblastoma cells, which are characterized by a comparatively low degree of differentiation: our WM astroglia isolates expressed 24 of the 256 tumor genes from literature (9.4%) at a significantly higher level compared to GM, where only 11 genes from the list (4.3 %) showed enrichment. Again, the trend was reversed for healthy brain tissue with nearly a third of the associated genes (58 of the 182 total genes or 31.9%) upregulated in GM vs. a mere 4 genes (2.2%) in the WM samples (Fig. 25).

Although these trends were equally apparent in the RiboTag and the MACS data, there was again a notable discrepancy between the two methods in regard to the number of genes from literature that were differentially expressed. For the GM samples, the ratio was consistently

around 10:1 in favor of the MACS isolates. Similarly consistent, although less pronounced, this ration was roughly 3:1 for the WM astrocytes.

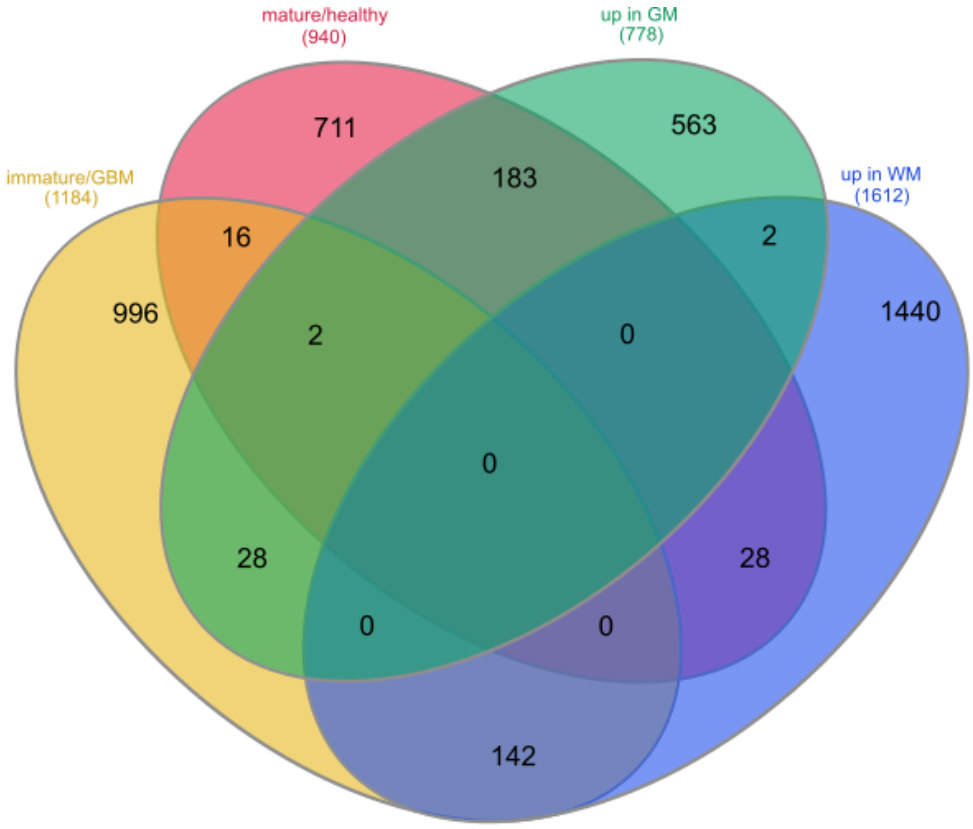


Fig. 25: Many genes associated with immature/neoplastic and mature/healthy neural cells are overexpressed in WM and GM, respectively
A list of genes asscociated with immature vs. mature and neoplastic vs. healthy cells was compiled from Cahoy et al., 2008 and Zhang et al., 2016. The Venn diagramm shows the number of genes from this list that are overexpressed in either the GM or WM samples. For the sake of visual accessibility, immature/neoplastic genes an mature/healthy genes have been grouped together. Similarly, no distinction between DE genes in RiboTag and MACS has been made (which accounts for the small amount of genes shared between opposite categories).

Of the genes significantly associated with an immature cell type that were upregulated in both the RiboTag and MACS WM samples, a large portion coded for proteins involved in brain development and cell cycle, e.g. Tpx2 (Neumayer et al., 2014), Casc5 (Javed et al., 2018), Bub1 (Basu et al., 1999; Miyamoto et al., 2011), Aspm (Buchman et al., 2011; Gai et al., 2016; Jayaraman et al., 2016), Ube2c (Rape and Kirschner, 2004; Chang et al., 2014), Pbk (Gaudet et al., 2000), Ccna2 (Liu et al., 2014), and others. Among the genes listed was also Mki67, encoding a well-established marker for cell proliferation in any biological tissue.

Since many of these genes serve essential functions in cellular mitosis and its control, their deregulation is associated with neoplastic transformation, more specifically the development and progression of glioblastoma. Some examples include Bub1 (Reis et al., 2001; Bie et al.,

2011), Kif11 (Venere et al., 2015; Liu et al., 2016), Ube2c (Ma et al., 2016), Pbk (Joel et al., 2015; Dong et al., 2020), Tpx2 (Neumayer et al., 2014), Top2a (Hong et al., 2012), and Emp1 (Miao et al., 2019; Wang et al., 2019). The latter three consequently also appear on the list with genes enriched in glioma cells vs. healthy tissue.

Among the protein-coding genes associated with mature astroglia and upregulated in both the RiboTag and MACS GM isolates were some that have already been mentioned, like Gjb6 (i.e. Cx30), Dmp1, or Chrdl1. Others figure prominently in various metabolic pathways, e.g. PYGM in glycogenolysis (Müller et al., 2015), Slc13a5 in citrate transport (Inoue et al., 2002; Hardies et al., 2015), Hsd11b1 in steroid hormone metabolism (Yau and Seckl, 2001; Verma et al., 2018), SOD3 in anti-oxidation (Markus et al., 2018), and Cyp4f15 in detoxification (Sehgal et al., 2012). Chrdl1, Gjb6, and Slc13a5 are also among the genes enriched in healthy astrocytes vs. tumor cells.

3.2.3 Functional Analysis

So far, we have looked more generally at various statistics of differential expression in our RNA-Seq data and shortly focused on a handful of interesting genes. In order to gain a better understanding of any cellular or functional differences that might exist between GM and WM astroglia, our next step was to perform enrichment analysis on our samples. Multiple online and offline tools are available for this purpose, similar in many regards but each with its own algorithms. We decided to take a broad approach and use five different gene enrichment programs, reasoning that in this way genuinely interesting details would become more apparent since observations made across different platforms tend to carry more statistical weight. The tools we used were *DAVID* (Huang et al., 2009a; Huang et al., 2009b), *PANTHER* (Thomas et al., 2003; Mi et al., 2013), *GOrilla* (Eden et al., 2007; Eden et al., 2009), *g:Profiler* (Raudvere et al., 2019), and *Gene Set Enrichment Analysis (GSEA)* (Subramanian et al., 2005). In the end, the general concordance between the outcomes of the various analyses proved to be very high, so that any biological terms or numbers mentioned can be assumed as equally or similarly present across multiple platforms, unless otherwise stated. Since the actual numbers vary from software to software, depending on the respective algorithm, no exact p-values for the categories are given in the text. However, all functional terms stated below are significant at a p-value < 0.01 adjusted for multiple testing with a false discovery rate ≤ 0.05

using the Benjamini-Hochberg procedure to ensure a more conservative evaluation of significance.

3.2.3.1 Gene Sets Enriched in GM

Several transcriptomic studies have found pathways involved in cellular metabolism enriched in astrocytes when contrasted with other cell types of the CNS (Bachoo et al., 2004; Lovatt et al., 2007; Cahoy et al., 2008; Zhang et al., 2014, McKenzie et al., 2018). Intriguingly, our data revealed a similar tendency when comparing GM to WM astroglia. 21.1% of all genes enriched in MACS GM and 16.9% of those in RiboTag GM are classified under the PANTHER Gene Ontology (GO) term 'metabolic process', and the term 'metabolic pathways' showed significant enrichment in the MACS GM isolates.

Past publications have especially focused on energy metabolism (Lovatt et al., 2007; Cahoy et al., 2008), where a number of prominent genes were found to be significantly overexpressed in astrocytes. This trend was also apparent in our data: terms like 'oxidative phosphorylation', 'glycolysis and gluconeogenesis', 'glycogen metabolism', 'lipid metabolism', 'citrate cycle', or 'amino acid metabolic process' were all enriched in our GM samples, both RiboTag and MACS.

Along with this observation, many genes encoding proteins crucially involved in these pathways were highly expressed in GM astroglia, e.g. Aldoc, Eno1, PYGM, Ldhb, Pfkm, or Pgm2, to name only a few (Fig. 26C). And although more genes among the different categories met the first criterium for differential expression in the MACS than the RiboTag isolates (i.e. $\log_2$ fold change ≥ 2), the general tendency of higher expression at a significant p-value was equally present in both data sets. This goes along with our earlier observation that the total number of differentially expressed genes is markedly greater for the MACS samples.

In a similar vein, other GO terms enriched in GM included categories like 'nucleotide metabolic process', 'retinol metabolic process', hormone metabolic process', 'organic acid metabolic process', 'drug metabolic process' and 'small molecule metabolic process'. In line with what has been said about the antioxidant capacities of astrocytes in the introduction, the GO terms 'oxidation-reduction process' and 'glutathione metabolic process' appeared on both the RiboTag and MACS GM list. Several genes encoding cytochrome enzymes contributed to the first category, while the astrocyte-specific cystine-glutamate exchanger Slc7a11 (Ottestad-

Hansen et al., 2018) and Gst5, an isoform of glutathione S-transferase found in the brain (Rowe et al., 1997), were two of the upregulated genes involved in glutathione metabolism.

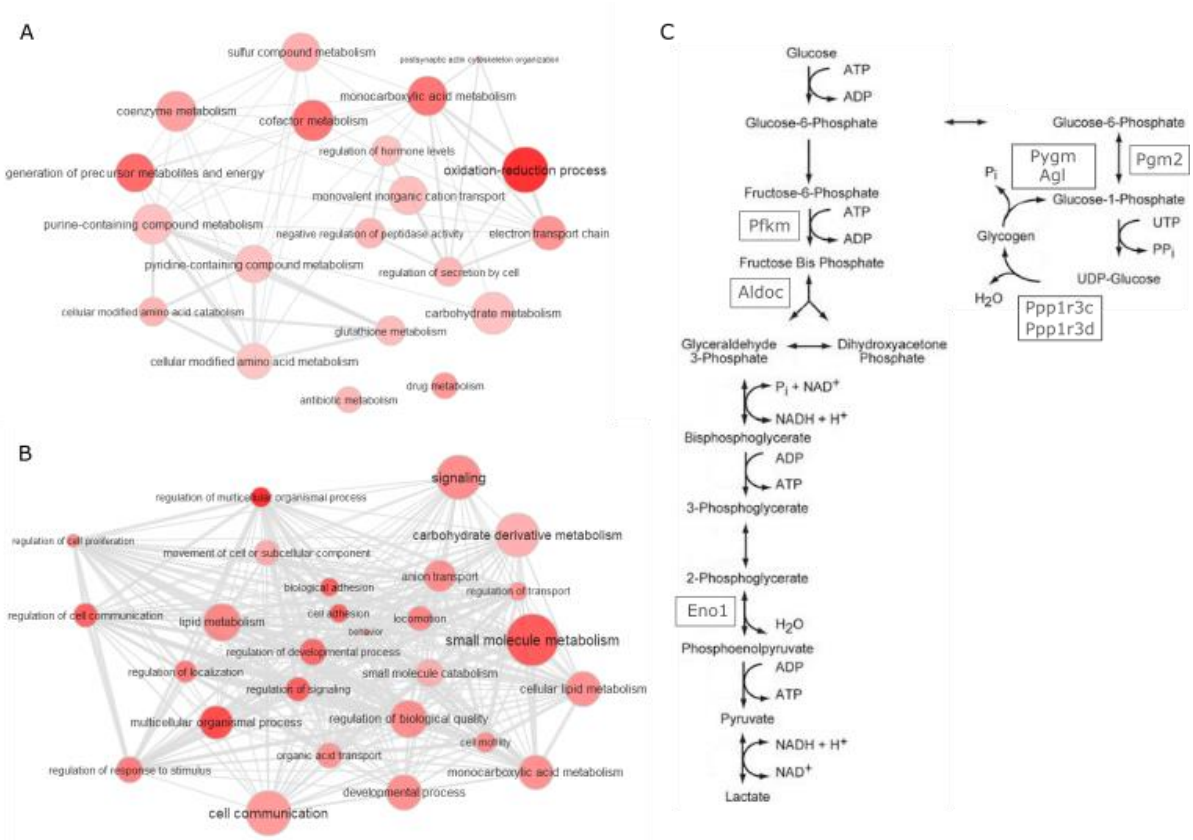


Fig. 26: GO terms enriched in RiboTag and MACS GM are often associated with various metabolic processes

A list of GM enriched GO terms was obtained from GOrilla and subsequently processed through REVIGO (Supek et al., 2011) in order to condense and better visualize the outcome. Connection maps displaying the top GO terms for the RiboTag (A) and MACS (B) isolates. Circle colour indicates the p-value of a category (darker = smaller p-value), circle size indicates the frequency of a category in the underlying database (larger = more general GO term). (C) Pathways for glycolysis and glycogen metabolism with GM enriched genes (in rectangles) from our samples.

Besides GO terms related to various metabolic processes, there were a number of cellular signaling pathways that showed enrichment in our GM data: Among them were ‘regulation of BMP/TGF-β signaling’, ‘PPAR signaling’, ‘Wnt/β-catenin signaling’, and ‘ERK/MAPK signaling’, all of which had previously been found overexpressed in astrocytes when compared to neurons and oligodendrocytes (Cahoy et al., 2008). Interestingly, some of the components figuring in these pathways like Fzd1/2/10, FGFR3, Znrf3, Ndp, Wnt7a/b, Sfrp1, Egfr, Chrdl1, or Gli1/2/3 appeared on our compiled list of astroglial marker genes, suggesting once again that a majority of astrocyte-specific genes described in literature might actually more correctly be defined as protoplasmic astroglial markers.

Another term enriched in GM was ‘Calcium signaling’, reflecting the importance of this mode of cellular communication for astrocytes, as mentioned in the introduction. Here, category-defining genes included Pdgfrβ, a positive regulator of reactive gliosis and scar formation (Pei et al., 2017), Egfr, Ednra (Hamby et al., 2012), and Chrna7, a gene coding for a nicotinic receptor subunit on astrocytes (Shen and Yakel, 2012), that has been shown to play a role in the recruitment of AMPA receptors to glutamatergic synapses (Wang et al., 2013).

In recent years, the essential contribution of astrocytes to synaptic transmission has become increasingly apparent. GO terms like ‘synapse assembly’, ‘regulation of synaptic transmission/plasticity/organization’, or ‘postsynaptic cytoskeleton organization’ – enriched among many similar ones in both the RiboTag and MACS GM data – bear witness to this functional aspect of astroglia. Several interesting genes were comprised by these categories, such as different (subunits of) neurotransmitter receptors like Gabbr2, which is known to regulate the subcellular stability of GS (Huyghe et al., 2014), Gabrg1, Grm3, and Grin2c, whose astroglial expression was shown to be region-specific (Ravikrishnan et al., 2018).

Furthermore, a number of genes crucially involved in synaptogenesis were differentially expressed in our GM data, e.g. Bdnf, Nrxn1 (Zeng et al., 2013), and Sparcl1 (a.k.a. Hevin) (Weimer et al., 2008; Risher et al., 2014; Buosi et al., 2018), the latter two actually interacting on a molecular level (Singh et al., 2016). Ptn, a protein encoded by another gene enriched in this context, seems to more specifically be important for axonal repair after injury (Takeda et al., 1995; Yeh et al., 1998; Iseki et al., 2002).

Finally, synaptic organization by phagocytic activity was another aspect of astroglial involvement reflected in the gene list for the respective GO terms. Most notably among them was Mertk, whose contribution to synaptic pruning has already been outlined in the introduction (Chung et al., 2013), and whose expression along with that of the above-mentioned Sparcl1 has recently been shown to change in an age-dependent manner (Boisvert et al., 2018). However, astrocytes have also been identified as important components of cellular engulfment and phagocytosis in a more general sense. One well-established pathway in this context is the receptor tyrosine kinase and αvβ5 integrin pathway (Finnemann and Nandrot, 2006), the molecular components of which have previously been described as enriched in the astrocyte transcriptome (Cahoy et al., 2008). Intriguingly, we found the

relevant genes (namely Mertk, Axl, and Mfge8) upregulated in our GM data, suggesting that phagocytic activity might more specifically be characteristic for protoplasmic astroglia.

Closely connected to the function of astrocytes in synaptic connectivity is their role in neurotransmitter metabolism, to which the enrichment of categories like 'regulation of neurotransmitter levels' and 'astrocytic glutamate-glutamine uptake and metabolism' testifies. Genes that are relevantly overexpressed in this regard comprise multiple members of the solute carrier family (Slc1a2, Slc1a3, Slc6a1, Slc6a11, Slc7a2, Slc7a11, Slc17a7) and enzymes involved in amino acid metabolism, such as Glul (i.e. GS), a well-established astrocyte marker, Prodh, Ggt1, or Glud1, the astroglial isoform of glutamate dehydrogenase, a central player in CNS neurotransmitter and energy metabolism (Frigerio et al., 2012; Karaca et al., 2015).

Interestingly and in direct contrast to what will be seen for the WM data below, one GO term coming up multiple times in our analyses of the GM isolates was 'negative regulation of cell proliferation', based on the expression of genes such as Gjb6 (i.e. Cx30), BMP7, Tfap2b, Gpc3, Nupr1, Ptn, and Aldh1a2. The latter also plays a central role in the astroglial regulation of retinoic acid levels in the brain (Shearer et al., 2012) (cf. Fig. 26A+B for an overview of enriched GO terms).

3.2.3.2 Gene Sets Enriched in WM

Most striking in the WM gene enrichment analysis was the large number of GO terms having to do with cell proliferation for both the RiboTag and MACS samples. The following list only gives a few examples: 'cell cycle', '(mitotic) cell division', 'regulation of G2/M transition of mitotic cell cycle', 'regulation of chromosome segregation', 'chromosome condensation', 'mitotic metaphase plate congression', 'spindle assembly', 'DNA replication', etc.

Other biological processes that were found to be upregulated in our WM isolates reflect their proliferative activity on a metabolic level, with terms like 'pyrimidine metabolism', 'purine metabolism', or 'nucleoside-triphosphatase activity' and associated genes like Nme5 or Rrm2 (Rasmussen et al., 2016, Sun et al., 2019). In a similar vein, mechanisms of DNA repair, including 'homologous recombination', 'base excision repair', and 'mismatch repair', also made an appearance on the list of WM enriched GO terms.

Many differentially expressed genes from our list of immature astroglia markers compiled from literature were again encountered in this context, some of which have already been mentioned above, namely Tpx2, Aspm, Mki67, Ube2c, Bub1, Ccna2, Casc5, and Pbk. Others also falling under the general category of cell cycle were Kif2c/4/14 (Bie et al., 2012; Wang et al., 2013; Huang et al., 2015; Liang et al., 2016), Nuf2 (Huang et al., 2014), Cenpe (Bie et al., 2011; Liang et al., 2016), Cenpf (Boom et al., 2003), and Myb (Zhang et al., 2013), all of which are further known to be associated with glioma formation and progression. Consequently, 'pathways in cancer' and 'p53 signaling' were two more terms overexpressed in WM.

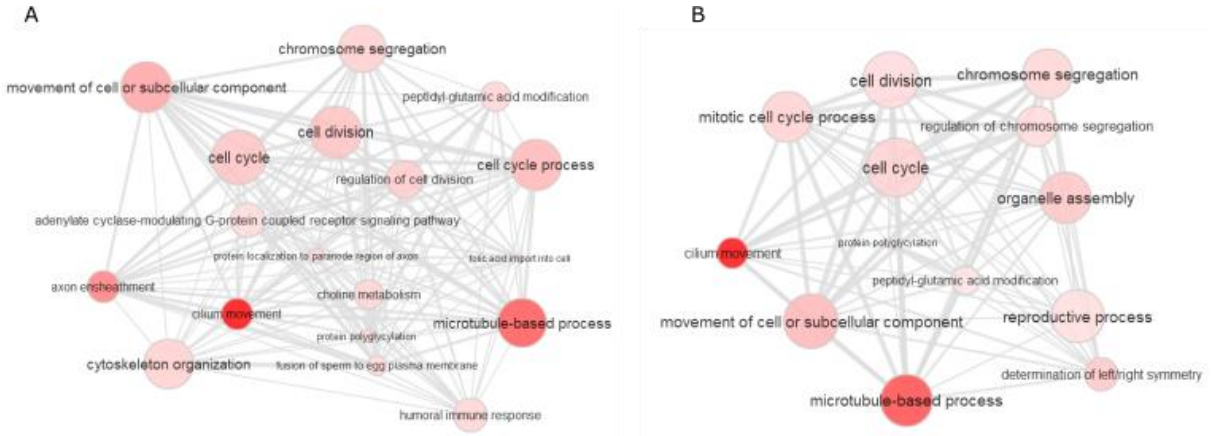


Fig. 27: The mitotic cell cycle is a common denominator of many genes enriched in RiboTag and MACS WM

A list of WM enriched GO terms was obtained from GOrilla and subsequently processed through REVIGO in order to condense and better visualize the outcome. Connection maps displaying the top GO terms for the RiboTag (A) and MACS (B) isolates. Circle colour indicates the p-value of a category (darker = smaller p-value), circle size indicates the frequency of a category in the underlying database (larger = more general GO term).

The upregulation of several GO terms having to do with cellular differentiation and CNS development (e.g. 'apoptotic process involved in development', 'cellular component assembly involved in morphogenesis', 'nervous system development', or 'gliogenesis') is another interesting aspect of WM astroglia. Here again, genes from literature known to participate prominently in early brain development, such as Efhc1 (Nijs et al., 2009) and Cdca8 (Zhang et al., 2009), figured among those differentially expressed.

In fact, when comparing the GO terms significantly associated with astrocyte precursor cells vs. mature astrocytes (Zhang et al., 2016), they mirrored many of the terms discussed here for WM vs. GM astroglia. Among the top 15 maturity-related categories were 'oxidation-reduction process', 'generation of precursor metabolites and energy', 'fatty acid metabolism', 'glucose metabolic process', 'cell-cell signaling', 'ion homeostasis', and 'hexose metabolic

process', all of which were also yielded by our GM enrichment analyses. Conversely, terms like 'chromosome organization', 'cell cycle phase', 'cell cycle', 'cell cycle process', and 'mitotic cell cycle' from our WM analyses were enriched in astrocyte precursors as well.

Complementing the picture of fibrous astroglia as a more proliferative and immature type of neural cell are two signaling pathways enriched in WM. The first of these, ErbB signaling, is known to be involved in a broad spectrum of CNS functions (Mei and Nave, 2014), and there is evidence for its region-specific activation in astrocytes (Sharif et al., 2009). More specifically, its regulation has proven essential for the developmental maintenance of RG and its timely transformation into astrocytes (Schmid et al., 2003), and the reintroduction of one of its components, ErbB2, in the adult cerebral cortex has been shown to promote RG identity (Ghashghaei et al., 2007). ErbB signaling has further emerged as an important inductor of reactive gliosis, a cellular state defined by increased proliferation and loss of differentiation (Chen et al., 2017). Among the genes enriched in WM astrocytes and prominently involved in ErbB signaling are ErbB3 and TGF-α, both of which are known for their neuroprotective properties under certain pathological conditions (Tokita et al., 2001; Karki et al., 2017).

Another GO term overexpressed in WM astroglia when compared to its GM counterpart was the PI3K/Akt/mTOR pathway, an important regulator of the cell cycle and a downstream target of ErbB signaling (Li et al., 2016). Various protective effects have been ascribed to its expression in astrocytes, including a pivotal role in WM homeostasis by secretion of oligodendrocyte-supportive factors (Arai and Lo, 2010) or the alleviation of early brain injury after subarachnoid hemorrhage (Feng et al., 2014). Yet the PI3K/Akt/mTOR pathway is also a central element in astrocytoma/glioblastoma progression (Sick et al., 2011; Li et al., 2016), and its pharmacological inhibition might prove a promising avenue of neuro-oncology (Gaelzer et al., 2016). Mutations in the pathway have been found to cause a whole spectrum of brain malformations (Jansen et al., 2015), emphasizing its role in CNS development (cf. Fig. 27A+B for an overview of enriched GO terms).

3.2.3.3 Astroglial Proliferation Assay

To test whether the predominance of cell cycle related genes in our WM sequencing data was reflected in the actual cellular proliferation rate of the respective astroglia (especially when compared to GM astrocytes), we performed a proliferation assay. 5-ethynyl-2'-deoxyuridine

(EdU), a marker for DNA synthesis and cellular mitosis, was given to Aldh1l1-eGFP mice in their drinking water for 28 days. Afterwards, the animals were sacrificed, and brain slices were stained for Aldh1l1-eGFP and Sox9.

The proliferating fraction of WM cells was comparable between the two astrocyte markers, with 3.9 ± 1.4% of all Aldh1l1-eGFP$^+$ cells and 3.2 ± 0.5% of all Sox9$^+$ cells positive for EdU (n=3 animals with 3 slices per animal). Interestingly, almost every Aldh1l1-eGFP$^+$/EdU$^+$ cell was also positive for Sox9, whereas there were a number of Sox9$^+$/EdU$^+$ cells that did not show a clear Aldh1l1-eGFP signal (Fig. 28). A practical explanation for this observed discrepancy might be furnished by the different staining behavior of the chosen markers: While the antibody against Sox9 produced a very robust nuclear signal throughout the whole z-stack, Aldh1l1-eGFP was more difficult to distinctly align with a given cell and tended to weaken towards the inner slices of the z-stack. This was also reflected in the total number of cells counted for both markers (560 for Sox9 vs. 197 for Aldh1l1-eGFP).

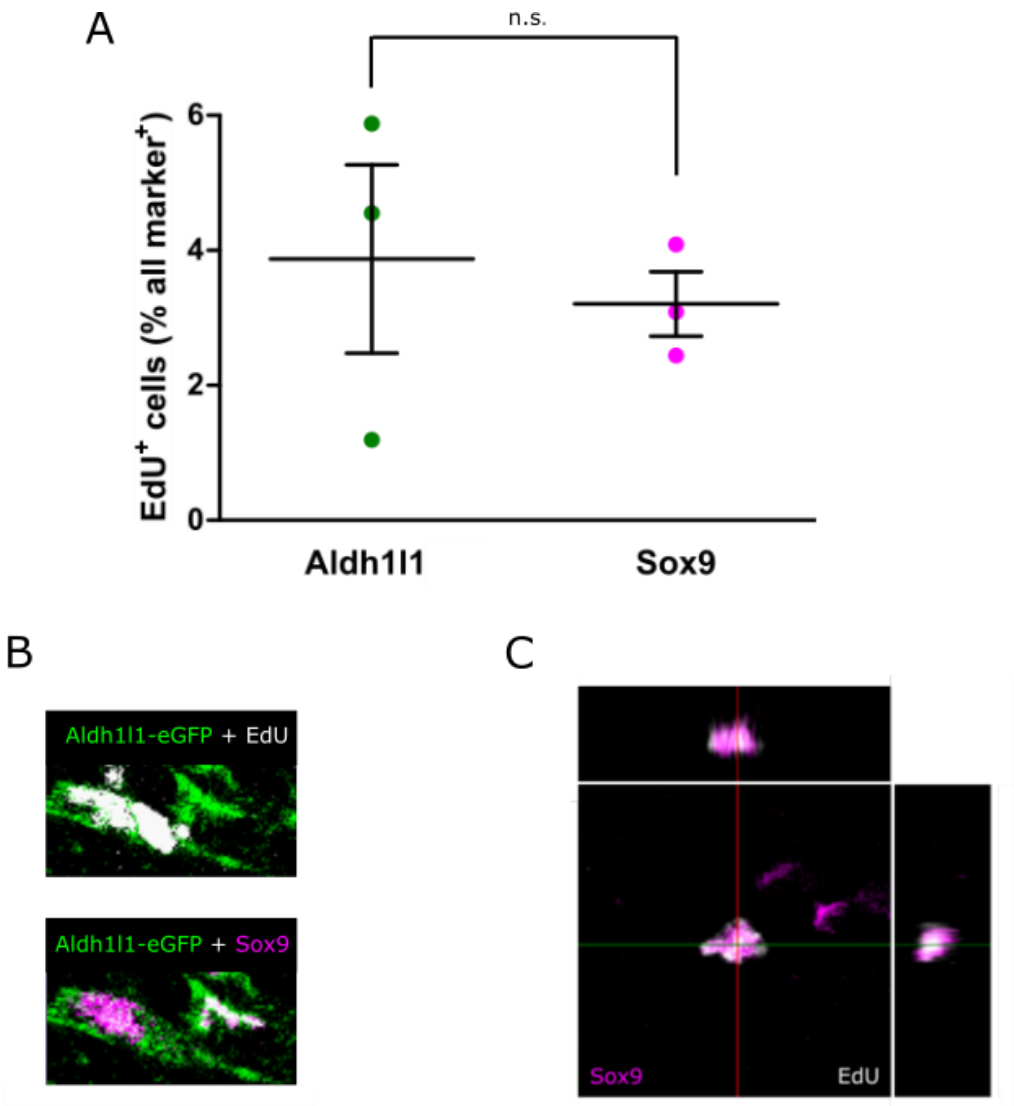


Fig. 28: Proliferation rates of WM astroglia are similarly high assessed by two different astrocyte markers

WM astrocytes of the corpus callosum were identified by using Aldh1l1-eGFP and Sox9. Proliferation was measured by counting the fraction of EdU/Aldh1l1-eGFP and EdU/Sox9 double positives among all marker positive cells. Proliferation rates were comparable when using either Aldh1l1 or Sox9 as an astroglial marker; n=3, 3 brain slices counted per animal, values are given as mean ± SEM (A). Some EdU$^+$ cells showed immunoreactivity for both markers (B). Spatial analysis demonstrating the clear colocalization of Sox9 and EdU signals inside the cell body (this particular cell was negative for Aldh1l1-eGFP) (C).

Intriguingly, not a single cell was found among the cortical GM in our samples that showed a colocalization of either astroglial protein and EdU, although the areas analyzed were similar for GM and WM, and signals for both markers as well as EdU could clearly be distinguished.

3.2.4 FGFR2 as a Potential Marker for WM Astrocytes

There have been many candidates for astrocyte marker genes throughout the years, as mentioned above, each with its own strengths and weaknesses. However, little effort has been made to find proteins that reliably label different astroglial subpopulations. Using the data from our RNA-Seq, we looked for potential marker genes specifically expressed in WM astrocytes when compared to GM or other cell types of the CNS. One promising candidate in this respect was Fibroblast Growth Factor Receptor 2 (FGFR2).

Since staining behavior is dictated by actual epitope expression rather than intracellular RNA levels, we relied on our RiboTag data for the evaluation of potential marker proteins. Although its $\log_2$ fold change of 1.48 in RiboTag WM compared to GM did not meet the ≥ 2 criterium for differential expression defined for our analyses above, the overexpression of FGFR2 was still highly significant with an adjusted p-value of 1.03×10^{-12}.

FGFR2 has previously been found to be astrocyte-enriched in a transcriptomic analysis of astroglia, neurons, oligodendrocytes, microglia, endothelial cells, and pericytes from the murine cerebral cortex (Zhang et al., 2014). Furthermore, there is IHC evidence for its colocalization with GFAP, with which it also shares a notable upregulation around lesion sites (Chadashvili and Peterson, 2006). Another study has identified the corpus callosum as a region of major FGFR2 expression, especially when contrasted with the GM of the cerebral cortex (Yazaki et al., 1994).

IHC showed FGFR2 colocalize very closely with Aldh1l1-eGFP and S100β/GFAP in cortical WM (Fig. 29A-A’’’). No astroglia, however, was labeled in GM (Fig. 29B-B’’’), emphasizing the suitability of FGFR2 as a potential marker specifically for WM astroglia.

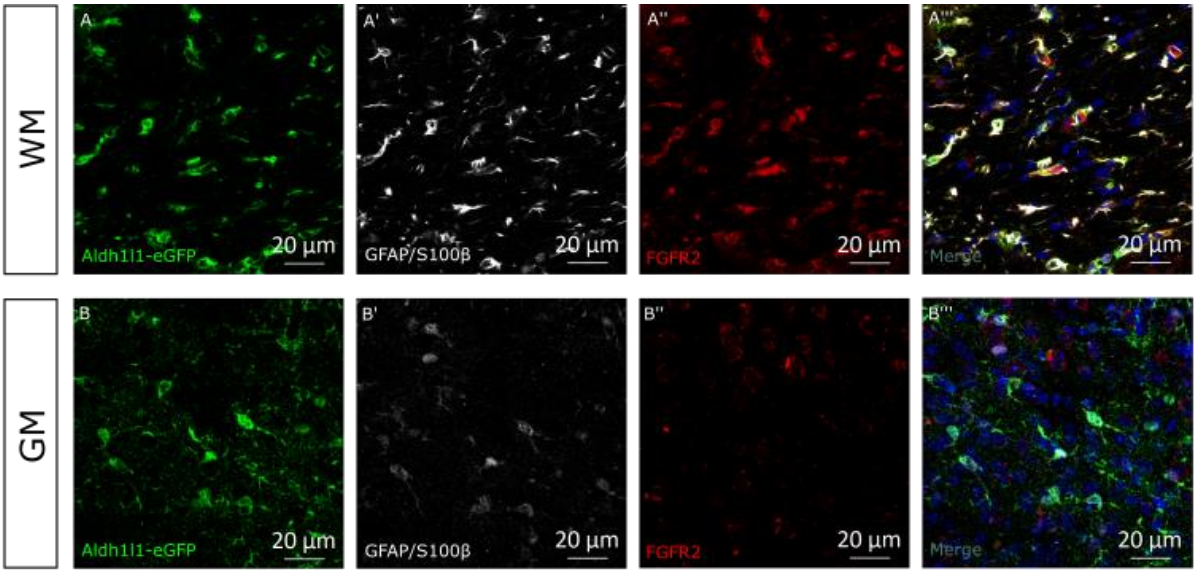


Fig. 29: FGFR2-staining is detected in WM astrocytes from the corpus callosum but not in cortical GM astrocytes

Representative micrographs are shown. Expression of established astrocyte markers and FGFR2 in WM of the corpus callosum (A-A''') and cortical GM (B-B'''). FGFR2 shows clear colocalization with Aldh1l1-GFP and GFAP/S100β in WM tissue but has no discernible astrocytic expression in GM.

4. Discussion

4.1 Purity of Acutely Isolated Astroglia from Murine GM and WM

The specificity of RiboTag as a means of harvesting astroglial RNA could only be assessed indirectly via whole tissue staining or expression analysis of various marker genes after performing RNA-Seq. The purity of the cellular yield obtained by the MACS method, on the other hand, was investigated more directly. Here, we were confronted with a conundrum: While well over 80% of the cells isolated from cortical GM stained positively for each of the chosen astroglial marker proteins, the staining behavior of our WM isolates proved much more inconsistent. GS was expressed in roughly 80% of all counted cells, similar to the numbers seen in GM, but the other markers we stained for showed significantly lower percentages in comparison, with GFAP/S100β detectable in only half the plated cells, and both Aldh1l1-eGFP and ACSBG1 hardly reaching expression values of 10%.

ACSBG1 has only been identified as astrocyte-enriched rather recently (Cahoy et al., 2008; Zhang et al., 2016) and is not routinely used as a marker gene in IHC or ICC, so its low detectability in our WM sorts might be interpreted as its being unsuitable for the purpose. Aldh1l1, however, is a well-established pan-astrocyte marker by now (Cahoy et al., 2008; Yang et al., 2011) and has proven viable for both GM and WM tissue in IHC approaches. Similarly, GFAP and S100β are both widely employed astroglia markers, and their combination should therefore be expected to encompass the majority of the astrocyte family.

The fact that detectable expression of astrocyte markers can vary enormously depending on CNS region, cellular subpopulation, and labeling technique was demonstrated in a number of recent papers each introducing a new transgenic Aldh1l1^{CreERT2} mouse line (Srinivasan et al., 2016; Winchenbach et al., 2016; Hu et al., 2020). Some area-specific astroglia showed almost complete colocalization of Aldh1l1 and GFAP while no expression of S100β could be detected at all (and vice versa), although these extremes were mainly encountered in the spinal cord (Hu et al., 2020). Even among cortical astrocytes, labeling with Aldh1l1 and GLT-1, both widely accepted glial marker genes, was found to differ considerably (Morel et al., 2019). Interestingly, not all cells of the extended astroglial family are reliably labeled by Aldh1l1, as has been demonstrated for enteric glial cells (Boesmans et al., 2014).

Development might provide an explanation for the vast differences in levels of marker expression we noted in our MACS isolates. Whereas the common ancestry of astro- and oligodendroglia remains a matter of debate (cf. Huang et al., 2021 for a brief summary and Li et al., 2021 for a recent point in favor of the thesis), there is striking evidence that distinct developmental mechanisms are at play in the genesis of protoplasmic and fibrous astrocytes. Experimental loss of the TF Olig2 (used as an oligodendrocyte marker in the adult brain) was found to severely compromise astrocyte formation in areas of WM tissue but have no discernible effects on the development of cortical GM astroglia (Cai et al., 2007). These findings are supported by an earlier observation made *in vitro*, where a glial progenitor cell differentiated into either a fibrous astrocyte or an oligodendrocyte depending on whether fetal calf serum was added to the dish (Raff et al., 1983). The molecular kinship between oligodendroglia and (particularly fibrous) astroglia might also account for the fraction of cells among our sorts – slightly higher in the WM isolates – that expressed detectable levels of typical oligodendrocyte marker proteins.

Furthermore, any form of manipulation is bound to have some effect on cell biology and protein expression. There are numerous studies to this effect for astroglia in cultured conditions (Barres et al., 1989; Landis et al., 1990; Kimelberg et al., 1992; Pappas and Ransom, 1994, Kimelberg et al., 1997). Although we chose MACS with the express purpose of subjecting the cells we extracted RNA from to as little mechanical and chemical stress as possible, a certain amount of artificial influencing is inevitable. The consequences of this may be different for GM and WM astrocytes, which could also explain why the markers we tested colocalized almost perfectly in IHC of WM tissue despite the huge discrepancies in our plated isolates.

Finally, it is possible that ACSA-2, the antibody used for astroglial enrichment in MACS, is not equally suitable for WM and GM tissue. A paper examining the general viability of MACS for neural cells used tissue from the whole cortex without explicitly differentiating between GM and WM (Holt and Olsen, 2016). ATP1B2 was recently identified as the epitope targeted by the ACSA-2 antibody (Batiuk et al., 2017), and while its expression was demonstrated for astroglial cells in both cortical GM and the corpus callosum (Kantzer et al., 2017), its IHC pattern might not translate seamlessly to cell sorting.

There is perhaps a more general discussion to be had at this point: The goal of this work was to gain some insight into the virtually unexplored molecular heterogeneity between

protoplasmic and fibrous astroglia. The vast majority of studies concerned with astrocyte biology have either lumped together various subpopulations without differentiating between them or have solely relied on cortical GM astroglia (and its respective marker genes) for their experiments. So any attempt at defining the specifics of WM astrocytes constitutes a petitio principii in some sense – making use of a number of characteristics (like protein expression) the applicability of which it sets out to investigate in the first place. This “problem of astrocyte identity” was more generally recognized in an essay by glia researcher Harold Kimelberg, which fittingly closes by offering the reader a number of “confusing conclusions” (Kimelberg, 2003).

These considerations led us to proceed towards transcriptomics, especially since the numbers we observed were reproducible. Additional procedures, like a separate myelin removal step using pre-manufactured magnetic beads or multiple cycles of the magnetic cell separation step had only very minor effects on the overall composition of the sorts, while at the same time disproportionally increasing the number of astrocytes lost in the discarded fraction.

4.2 Expression Analyses of RNA-Seq Data from GM and WM Astrocytes

After finishing RNA-Seq, we proceeded with a preliminary analysis of gene expression in our RiboTag and MACS samples. This was done with several goals in mind: First, we wanted to get a general understanding of how our samples differed from each other, both in regard to brain region and isolation method, which was accomplished by principal component and DE analysis. We then looked at a number of genes specifically enriched in each of the five major cell types of the CNS and determined their expression values in our data as a means of reassessing the purity of our samples. Finally, we collated a list of astrocyte-enriched genes from literature and investigated whether any of them were DE between our GM and WM isolates. We repeated this process with a second list collecting a number of genes from various studies found to be DE when comparing either immature and mature astroglia or healthy astrocytes and glioblastoma cells.

4.2.1 Differentially Expressed Genes Between Protoplasmic and Fibrous Astroglia

On defining the criteria for differential gene expression, we found them fulfilled by more than 4% of all genes examined in our RiboTag samples and by almost 8% of the genes in the MACS isolates. To put these percentages into perspective: Transcriptomic analyses of GLT-1$^+$/GFAP$^-$ and GLT-1$^+$/GFAP$^+$ cells yielded only 0.96% of differentially expressed genes between these two purported astroglial subpopulations (Lovatt et al., 2007). Microarray performed on astrocytes from cortex, cerebellum, and brainstem showed that 1.2% of 34000 total genes were differentially expressed across the three CNS regions (Yeh et al., 2009). To be fair, the studies mentioned both relied on microarray analysis, which generally tends to identify fewer DE genes than RNA-Seq (Zhang et al., 2014). However, even when comparing human and mouse astroglia from the temporal cortex using RNA-Seq, no more than 0.30% of all examined genes satisfied the criteria for differential expression (Zhang et al., 2016). And differential expression between cortical GM and diencephalic astroglia following RNA-Seq amounted to only around 1.5% of all genes (unpublished data from our lab).

The only transcriptomic study we came across that had similar numbers for DE genes between glial subpopulations was comparing hippocampal and striatal astrocytes and found 4.1% of all examined genes enriched in one of the two groups (Chai et al., 2017; the authors themselves calculate a DE fraction of more than 10% – their cut-off, however, is chosen much less conservatively). There are a handful of studies performing transcriptomics across various cell types of the CNS. Unsurprisingly, their numbers for DE genes between astrocytes and other neural cells are significantly higher than those encountered for analyses of glial subpopulations (Cahoy et al., 2008 perform microarray and find 12.5% of all examined genes DE in astrocytes; Zhang et al., 2014 report 14.2% of all genes enriched in astroglia using RNA-Seq). Comparing these percentages with our own data, it seems that the molecular difference between protoplasmic and fibrous astroglia is more pronounced than has been noted for other subpopulations. The fact that the MACS samples yielded almost twice as many DE genes as the RiboTag samples may be attributed to the methodical difference between both approaches: cells isolated via MACS contribute their entire cellular RNA content towards sequencing, whereas in RiboTag, only ribosome-associated RNA is pulled down for further analysis. Of course, the possibility of a disproportion in purity must also be considered, increasing the DE fraction by adding a certain amount of non-astroglial cells to the mix.

4.2.2 Expression of Neural Cell Type-Specific Marker Genes in the RNA-Seq Data

Strictly speaking, a quantitative assessment of absolute expression values in RNA-Seq data is not viable without some form of normalization (e.g. contrasting against whole brain tissue, different cell types, etc.). It is, however, common practice to determine read counts of several cell type-enriched genes to identify potential sources of contamination and to gain a general impression of any location-based and – in our case – approach-based differences that might exist. We looked at the expression of genes specific for each of the five major CNS cell types (astrocytes, microglia, endothelial cells, neurons, and oligodendrocytes) and found robust read counts for all astroglial marker genes in our samples, although with a notable tendency towards higher values in the GM isolates. A possible explanation for this circumstance is that most glia-enriched genes have been identified in tissue exclusively containing or at least dominated by protoplasmic astrocytes.

Expression of microglia and endothelial genes was all but absent in our data, yet RNA of neuronal genes was detectable in the RiboTag isolates to some extent. Most likely, this can be traced to the known expression of GLAST by a subpopulation of neurons (Rothstein et al., 1994; Chaudhry et al., 1995). An interesting observation was the expression of typical oligodendrocyte genes in our samples, most notable in tissue of WM origin; there are several possibilities to be discussed in this respect.

First, and perhaps most obviously, we know from our ICC controls following MACS that there was a fraction of cells staining positively for various oligodendrocyte marker proteins. This fraction was slightly higher in the WM than in the GM sorts (approx. 17% vs. 15%). While this presumed oligodendroglial contamination might well account for the low but non-negligible expression of genes like Sox10 and Mog in our samples, the fact that the expression was significantly more pronounced in the WM and that, moreover, it was almost equally apparent in the MACS and RiboTag isolates is not readily explained by this interpretation.

There is also a possibility that the low-level expression of genes commonly assumed to be oligodendrocyte-specific is an intrinsic property of fibrous astroglia. A recent paper has identified three subpopulations among cortical astrocytes via expression of GLT-1 and Aldh1l1 using reporter mouse lines. Intriguingly, the group of astrocytes defined by a lack of detectable GLT-1 shows a very similar pattern of oligodendrocyte gene expression to our own WM data.

Moreover, the top genes found to be enriched in this cortical subpopulation (e.g. Dynlrb2, Fam183b, Tmem212, Ttr, Enpp2, Sostdc1, Rsph1) were almost invariably overexpressed in both our WM transcriptomes, most of them significantly so (Morel et al., 2019).

Finally, the presence of typical oligodendroglial genes in our WM isolates might conceivably be caused by a subpopulation with stem cell properties. The common developmental ancestry of fibrous astrocytes and oligodendrocytes has been described by a number of studies, as previously mentioned, and genes like Sox10 or Pdgfrα are expressed by glial progenitor cells as well (Kuhlbrodt et al., 1998; Zhang et al., 2014). While we took great care to avoid any contribution of the known stem cell niches to our tissue samples when dissecting the brains, the presence of precursor cells inside the adult cortical WM cannot be ruled out. The proliferative nature of WM astroglia – especially when compared to its more inert GM counterpart – has been variously observed (Dalton et al., 1968; Emsley and Macklis, 2006) and was also apparent in our transcriptomic analyses. Whether this tendency is due to the biology of WM astroglia as a whole or rather the existence of a proliferative subpopulation remains to be examined.

4.2.3 Fibrous Astrocytes Express Genes Related to Cellular Immaturity/Malignancy

The question of a potential stem cell population among cortical WM astrocytes becomes even more relevant when the enrichment of genes associated with cellular immaturity in our isolates is taken into account. Many of the genes DE by fibrous astroglia were found to be involved in CNS development and cellular mitosis. Since control of the cell cycle is crucially reliant on the exact interplay of multiple genes from this group, their deregulation is associated with neoplastic transformation, more specifically the development and progression of gliomas.

One of the major problems in neuro-oncology consist in determining the cell of origin for malignant gliomas. While early research in the field – mainly based on tumor pathology – considered mature glial cells a likely candidate for the genesis of gliomas (hence the name), this view has shifted over the last decades (Jiang and Uhrbom, 2012; Zong et al., 2015). It now seems increasingly probable that a more immature neural cell underlies the formation of the most common primary CNS tumor, and there is a good amount of evidence for both NSCs (Sanai et al., 2005, Alcantara Llaguno et al., 2009, Alcantara Llaguno et al., 2015, Lee et al.,

2018, Altmann et al., 2019) and oligodendrocyte progenitor cells (OPCs) (Shoshan et al., 1999; Lindberg et al., 2009; Persson et al., 2010; Liu et al., 2011) in this context. In fact, it might not be a question of 'either-or', as it has been shown that the presence of specific extracellular signals can induce the reversion of OPCs to NSCs (Kondo and Raff, 2000), and that even mature astrocytes or neurons can undergo dedifferentiation upon defined genetic alterations and can consequently initiate glioma formation (Friedmann-Morvinski et al., 2012).

Anatomical studies have found that a majority of human gliomas are located in the cerebral hemispheres with a particular emphasis on the frontal lobes (Larjavaara et al., 2007; Wijnenga et al., 2019; Mandal et al., 2020). In line with previous remarks, there seems to be a marked preference for areas expected to be populated by NSCs and OPCs when it comes to glioma formation (Mandal, et al., 2020), especially so for the SVZ (Barami et al., 2009). Furthermore, gliomas are typically located subcortically (i.e. in WM areas of the brain) (Larjavaara et al., 2007). Combining these observations with our RNA-Seq data, one might hypothesize that glioma formation outside of the known stem cell niches could possibly be traceable to fibrous astrocytes or a proliferative subpopulation among them, though these connections are merely speculative at this point and experimental confirmation is certainly needed.

4.3 Implications for Functional Differences between GM and WM Astroglia

The individual investigation of genes enriched in sequencing data can provide a first overview of potential differences between the samples analyzed, but it risks overemphasizing gene-specific observations that may not be relevant on a whole genome level. To avoid this sort of bias and gain some insights into the actual biology of protoplasmic and fibrous astroglia, we proceeded towards functional analysis using software for transcriptomic gene enrichment. The choice to compare the output from multiple tools for this purpose rather than relying on any single one was made in an attempt at, again, generalizing the resulting data and identifying potential outliers. The fact that our RiboTag and MACS samples – two methods with fundamentally different isolation mechanisms – yielded very similar enrichment patterns across all algorithms may be taken as a further indicator that the RNA we harvested for sequencing was indeed of mainly astroglial origin.

4.3.1 Functional Enrichment Analysis of GM and WM Astroglia

As has already been mentioned, many genes typically associated with astrocytes in general seem to be more specifically overexpressed by protoplasmic astroglia. This observation held equally true for our functional analyses where GO terms often defined as astroglial in nature were actually significantly enriched in GM astrocytes. There is one study that explicitly looked at the transcriptome of protoplasmic astrocytes from the murine cortex in comparison to GLT-1^{-} and GFAP^{-} cells and found that more than one third of all genes overexpressed among the former were related to cellular metabolism (Lovatt et al., 2007). GO terms related to metabolism were also very prominent in our GM samples when contrasted with the WM isolates.

Literature suggests that this conspicuous difference between protoplasmic and fibrous astroglia can be accounted for by the way metabolic support is rendered to neurons in the GM and WM, respectively. Synaptic transmission in the hippocampus is crucially dependent on the trafficking of glucose and its metabolites through networks of astrocytes coupled via Cx43- and Cx30-mediated gap junctions (Rouach et al., 2008). In the WM of the corpus callosum, however, axonal function is maintained by glucose delivery from oligodendrocytes, with astroglia apparently playing a subordinate role. Consequently, mice deficient for the oligodendroglial connexin Cx47 were severely compromised in axonal energy metabolism (Meyer et al., 2018).

Difference in anatomical surroundings may similarly explain the multitude of synapse-related GO terms enriched in the GM samples. While fibrous astroglia extend their processes along the axon tracts of the WM (where synapses are rather sparse), astrocytes in the cortical GM are in close contact with sites of synaptic transmission. This spatial as well as functional association underlies the concept of the tripartite synapse (axon – dendrite – astrocyte). Unsurprisingly then, observations concerning the phagocytic properties of astrocytes necessary for synaptic pruning (Chung et al., 2013) and the interaction of astroglial proteins involved in synaptogenesis (Singh et al., 2016) were made in cortical areas of the brain.

Perhaps the most interesting result of our functional analyses, however, was the marked enrichment of GO terms denoting proliferation and cell division in our WM samples (with ‘negative regulation of cell proliferation’ being one of the GM enriched categories). The

proliferative nature of WM astrocytes in the adult brain has been noticed before, with early autoradiographic experiments dating back well over half a century (Altman 1963; Altman and Das, 1964; Noetzel and Rox, 1964; Hommes and Leblond, 1967; Dalton et al., 1968). While the actual percentages of proliferating cells diverge considerably depending on the type of labeling agent and time of application, they are invariably higher for fibrous than for protoplasmic astroglia (cf. Emsley and Macklis, 2006 or Ge et al., 2012 for more recent quantifications). It is interesting to note that a transcriptomic comparison between astrocyte precursor cells and mature astroglia mirrors the differences we saw between WM and GM astroglia in terms of enriched categories (Zhang et al., 2016). Here, we are once again confronted with the question if this proliferative behavior is an inherent property of WM astroglia in general or whether it is due to a more immature subpopulation.

4.3.2 Fibrous Astroglia Is Actively Proliferating *in vivo*

In a next step, we explored whether the enrichment of mitosis-related GO terms in our WM samples could actually be quantified via a higher proliferation rate *in vivo*. EdU expression was used as a surrogate marker for cell division while Aldh1l1-eGFP and Sox9 served to identify astroglia. And indeed, around 3–5% of fibrous astrocytes showed signs of active proliferation between the two markers whereas we did not count a single EdU^+ astrocyte in the cortical GM. These numbers lie somewhat in the middle of what we know from literature, where the values range from 0.445% (Dalton et al., 1968, intraperitoneal injection of ^{3}H-thymidin 9 hours before sacrificing the mice) to around 15% of proliferating astroglia in the corpus callosum (Emsley and Macklis, 2006, BrdU administration for 7 days before sacrificing the mice). It is somewhat surprising that the latter group saw a much larger fraction of proliferating WM astrocytes than we did while at the same time administering their marker molecule for only a fourth of the total time (BrdU for 7 days vs. EdU for 28 days). However, besides differences in the choice of proliferation marker and its dosage, another reason for the discrepancy might be the fact that these values were observed upon quantification with hGFAP-GFP, which is known to have a problem with unspecific expression (Hu et al., 2020). Consequently, when using an antibody against GFAP to label fibrous astroglia, the percentage of proliferating cells was very close to what we observed (Emsley and Macklis, 2006).

Although the numbers of double positive cells were similar for Aldh1l1-eGFP and Sox9, their staining behavior was not identical. Technical limitations certainly played a role in this context, as Sox9 is a nuclear TF and as such showed robust and easily quantifiable expression throughout the z-stack. Aldh1l1-eGFP, on the other hand, could not always be unequivocally aligned with a given nucleus and weakened in its signal strength towards the inner slices of the z-stack. This led to almost three times as many $Sox9^+$ cells being counted as Aldh1l1-$eGFP^+$ ones, with virtually all cells positive for Aldh1l1-eGFP also positive for Sox9.

There might, however, be another aspect to this observation besides mere technical reasons. Sox9 has been identified as a central element for glial fate choice during development of both the spinal cord (Stolt et al., 2003; Kang et al., 2012; Molofsky et al., 2013) and the cortex (Nagao et al., 2016). Moreover, its expression in the neurogenic niche of the SVZ was found to keep NSCs in their precursor state, which was abolished upon Sox9-knockdown (Cheng et al., 2009). These functions of Sox9 in gliogenesis and NSC maintenance are relevant to the point at hand, as they might again hint at a subpopulation of more immature cells among WM astroglia, possibly defined as $Sox9^+$/Aldh1l1-$eGFP^-$ in our quantifications. And indeed, the study that originally identified Sox9 as an astrocyte marker in the adult mouse brain found its otherwise almost perfect colocalization with established marker proteins like GLT-1 to be noticeably lower in the neurogenic areas and in the corpus callosum (Sun et al., 2017).

4.4 FGFR2 Is a Potential Marker Protein Specific to Fibrous Astrocytes

There is a considerable number of astrocyte markers these days, each with its own advantages and disadvantages, as covered in more detail in the introduction to this work. What is missing, however, are genes specifically expressed by glial subpopulations, that can be reliably used for antibody labeling. Part of the problem is that many of these purported subpopulations are still rather ill-defined, so finding proteins more or less exclusively present in the cells of interest poses no small challenge. The distinction between protoplasmic and fibrous astroglia, however, is over a century old by now, and the morphological differences between the two are well-established.

We looked through our RNA-Seq data for potential candidates that might prove suitable for the purpose of specifically labeling WM astrocytes. Several aspects have to be considered in this respect, as simply going through the top 10 of the DE genes list and selecting any one

among them will most likely not yield the desired results. While a nuclear location of the protein in question is not a problem per se and can work quite well in some cases (e.g. Sox9), it usually requires some extra steps during antibody treatment, which can hinder its general applicability. Keeping this in mind, we narrowed our search to genes encoding cell surface proteins. We further limited ourselves to our RiboTag data as we deemed ribosome-associated RNA to most closely reflect actual protein translation. In order to be detectable through antibody staining, moreover, the actual expression value of a gene (measured in FPKM) cannot be too low. At the same time, the difference of expression between GM and WM has to be marked enough for the labeling to be specific to fibrous astroglia. Finally, we consulted the literature to see whether any among our list of potential markers had been described as predominantly expressed by astrocytes before.

Taking all the aforementioned points into consideration, we were left with FGFR2 as the most promising candidate. FGFR2 is one in a family of five distinct cell surface receptors known to fulfill a variety of functions in the developing CNS (Ford-Perriss et al., 2001; Furusho et al., 2011). In the adult brain, FGFR2 is primarily found in WM tissue (Asai et al., 1993; Yazaki et al., 1994), although it is still a point of contention which type of neural cell it is mainly expressed by. There is some evidence for astrocytes as the principal source of FGFR2 expression: Southern blot revealed its presence in low-grade astrocytomas (Yamaguchi et al., 1994), it was shown to overlap largely with GFAP in IHC approaches (Chadashvili and Peterson, 2006), and a transcriptomic comparison of various CNS cells found it significantly enriched in astrocytes when contrasted against oligodendrocytes, microglia, neurons, endothelial cells, and pericytes (Zhang et al., 2014). On the other hand, a similar number of studies argue for a primarily oligodendroglial expression of FGFR2 (Miyake et al., 1996; Messersmith et al., 2000; Bansal et al., 2003; Bryant et al., 2009).

Interestingly, another member of the FGFR family, namely FGFR3, has been described as specifically expressed by astrocytes (Pringle et al., 2003), an observation, that was replicated in a transcriptomic approach (Zhang et al., 2014). Transgenic mouse lines using FGFR3 as a reporter have been created (Young et al., 2010), although there are reports of problems with leakage (Hu et al., 2020). In our RNA-Seq data, FGFR3 was predominantly expressed in the GM samples, serving as another instance of the previously mentioned observation, that many ‘general’ astrocyte markers seem to be above all expressed by protoplasmic astrocytes.

We performed IHC and found that the signal of FGFR2 was robust and colocalized very closely with established astrocyte markers (Aldh1l1-eGFP, S100β/GFAP) in cortical WM, whereas no astroglia was labeled in GM tissue. The reports of its expression by mature oligodendrocytes could not be confirmed upon immunostaining. Our observations qualify FGFR2 for use as a marker protein for fibrous astrocytes, especially in contrast to their anatomical GM neighbors. It would be interesting for future studies to examine the expression of FGFR2 in different glial subpopulations of the CNS.

5. Summary

The present work investigated cellular heterogeneity between protoplasmic and fibrous astroglia using a transcriptomic approach, which, to our knowledge, constitutes a first in the field.

To begin with, we isolated astrocytes from the murine cerebral GM and WM via two different methods, RiboTag (using the GLAST^{CreERT2} mouse line) and MACS. To assess the purity of our MACS isolated cells (relying on the ACSA-2 antibody), we plated both the sorted and the discounted fraction on PDL-coated coverslips and stained for routinely used astrocyte markers. This revealed a first major discrepancy between the two populations, as all tested antibodies showed robust expression in more than 80% of the sorted GM cells, whereas GS was the only marker equally expressed in the WM fraction. GFAP/S100β was detectable in only half the cells, Aldh1l1-eGFP and ACSBG1 in less than 10%. The number of sorted cells exhibiting expression of typical oligodendrocyte markers (PLPdsRed, O4, and GST-π) was comparable between both regions (around 15% for GM vs. 17% for WM).

We then proceeded towards RNA-Seq. To get a first estimate of the overall similarities and differences between our isolates, we performed PCA. Here, the samples tended to form clusters according to region of interest and isolation method, with biological diversity accounting for a certain amount of variance. Criteria for differential gene expression were defined ($\log_2$ fold change ≥ |2| AND adjusted p-value < 0.05), according to which we found 2388 total genes that were differentially expressed between the two methods. In both cases, the WM samples yielded more DE genes with higher read counts, and there were almost twice as many DE genes in our MACS data when compared to the RiboTag samples. qPCR of four randomly selected genes differentially expressed in both the MACS and RiboTag isolates confirmed the validity of our RNA-Seq approach. As a rough estimate of sample purity, expression levels of marker genes for astroglia, oligodendroglia, microglia, neurons, and endothelial cells were investigated. Astrocyte markers were highly expressed in all our samples, although a trend for higher expression in the GM isolates from both methods was apparent. A non-negligible expression of oligodendrocyte-specific genes was especially notable in the WM samples (MACS and RiboTag), possibly suggesting a certain amount of oligodendroglial contamination.

There have been several studies, both transcriptomic and proteomic, that looked at astrocyte-enriched genes in comparison to other cell types of the CNS. We compiled a list of these genes, drawing from multiple papers published over the last two decades, and analyzed their expression in our RNA-Seq data. Some showed differential expression, and at this point, an interesting trend became apparent: The majority of DE genes from our samples that have previously been described as enriched in astroglia seemed more specifically to be enriched in protoplasmic astroglia, most likely owing to the fact that purely or at least predominantly GM tissue has hitherto been used for sequencing purposes. A similar relation was noted on investigating expression levels of various genes from literature that have been associated with either immature/neoplastic (higher in WM) or mature/healthy astroglia (higher in GM).

To gain a better understanding of the functional characteristics of protoplasmic and fibrous astroglia that might underly the DE genes we extracted from our data, we performed gene enrichment analysis using five different software tools. Metabolic activity, broadly defined, was one of the major terms enriched in GM astrocytes. Various exponents of cellular energy metabolism (e.g. glycolysis and gluconeogenesis, citrate cycle, or lipid metabolism) fell into this category, but also terms connected to the metabolism of neurotransmitters, hormones, or nucleotides. Given the differences in micro-anatomical environment between GM and WM astrocytes, it is perhaps not surprising that another functional group enriched in protoplasmic astroglia comprised several GO terms associated with the regulation of synaptic transmission (including synaptogenesis and synaptic pruning/phagocytosis). Once more, it became apparent that observations hitherto made for astrocytes in general applied more specifically to protoplasmic astroglia in our data.

Enrichment analyses for our WM samples were dominated by a number of GO terms related to cellular proliferation. This included genes directly involved in mitosis as well as others associated with DNA repair or deregulation of the cell cycle (i.e. glioma formation and progression). Many of the genes that showed up earlier when investigating the expression of immaturity markers in our isolates also figured prominently in this context. To test whether this tendency of fibrous astroglia towards cell division could be confirmed *in vivo*, we performed a proliferation assay using EdU uptake as a means of quantification: between 3 to 5% of all counted WM astroglia showed proof of proliferation within a span of 28 days, while not a single GM astrocyte was positive for EdU.

In view of harnessing our RNA-Seq data towards a more practical application, the attempt of identifying a potential marker protein specifically labeling fibrous astrocytes was made. Here, FGFR2 was chosen as a promising candidate, due to its being a cell surface molecule, and since its astroglial expression had been described before. The gene was found to be significantly enriched for WM astrocytes in our RiboTag data (which, in the end, reflects actual gene expression more directly than MACS). And indeed, IHC showed a clear colocalization of FGFR2 with GFAP/S100β and Aldh1l1-eGFP, establishing the protein as a possible marker for reliably identifying the subpopulation of cortical WM astrocytes in future applications.

6. Zusammenfassung

Die vorliegende Arbeit untersuchte – unseres Wissens nach erstmalig – die zelluläre Heterogenität zwischen protoplasmischen und fibrösen Astrozyten.

Zunächst isolierten wir Astrozyten aus der zerebralen grauen und weißen Substanz der Maus über zwei unterschiedliche Methoden: RiboTag (unter Verwendung der GLAST^{CreERT2} Mauslinie) und MACS. Um die Reinheit der via MACS (über den ACSA-2 Antikörper) isolierten Zellen zu beurteilen, trugen wir sowohl die selektierte als auch die verworfene Fraktion auf PDL-beschichtete Coverslips auf und färbten diese mit Antikörpern gegen verschiedene Astrozytenmarker. Hier kam eine erste größere Diskrepanz zwischen den beiden Populationen zum Vorschein: Während über 80% der aus der grauen Substanz selektierten Zellen alle getesteten Marker nachweisbar exprimierten, war GS der einzige Marker mit vergleichbaren Expressionswerten in den aus der weißen Substanz selektierten Zellen. Die Kombination aus GFAP/S100β war nur in rund der Hälfte der Zellen nachweisbar, Aldh1l1-eGFP und ACSBG1 in weniger als 10%. Der Anteil an selektierten Zellen, welche typische Oligodendrozyten-Marker (PLPdsRed, O4, and GST-π) exprimierten, war zwischen grauer und weißer Substanz vergleichbar (rund 15% in der grauen Substanz vs. 17% in der weißen Substanz).

Als nächstes folgte eine RNA-Sequenzierung der gewonnenen Materialien. Um einen ersten Eindruck der grundsätzlichen Ähnlichkeit bzw. Unterschiedlichkeit zwischen den Isolaten zu gewinnen, führten wir eine Hauptkomponentenanalyse durch. Hier gruppierten sich die Proben nach Gewebeursprung und Isolationsmethode, wobei die biologische Diversität einen gewissen Anteil an Varianz beisteuerte. Wir definierten Kriterien für differentielle Genexpression ($\log_2$ relativer Unterschied ≥ |2| UND angepasster p-Wert < 0.05), nach denen insgesamt 2388 Gene in beiden Methoden zusammen differentiell exprimiert waren. In beiden Fällen lieferten die aus der weißen Substanz gewonnen Proben mehr differentiell exprimierte Gene mit einer höheren Anzahl an Genkopien, und die Analyse der MACS-Daten lieferte knapp doppelt so viele differentiell exprimierte Gene wie die RiboTag-Isolate. Eine qPCR von vier zufällig ausgewählten Genen, welche sowohl in den MACS- als auch den RiboTag-Proben differentiell eprimiert waren, bestätigte die Validität unserer Sequenzierungsmethode. Zur erneuten, groben Abschätzung der zellulären Reinheit unserer Proben verglichen wir Expressionswerte von typischen Markergenen für Astrozyten, Oligodendrozyten, Mikroglia, Neuronen und Endothelzellen. Astrozytenmarker waren in all

unseren Isolaten hoch exprimiert, allerdings zeigte sich eine Tendenz zur höheren Expression in der grauen Substanz (beider Methoden). Eine nicht zu vernachlässigende Expression oligodendrozytenspezifischer Gene war vor allem in den aus der weißen Substanz gewonnen Proben nachweisbar, möglicherweise als Hinweis auf eine gewisse Beimischung dieses Zelltyps.

Es gibt mehrere Studien, sowohl mit transkriptomischem als auch proteomischem Ansatz, welche Gene identifiziert haben, die im Vergleich zu anderen Zellarten des ZNS in Astrozyten überrepräsentiert sind. Wir stellten eine Liste dieser Gene zusammen, wobei wir uns auf verschiedene Veröffentlichungen der letzten zwei Jahrzehnte stützten, und analysierten deren Expression in unseren Daten. Einige davon waren in der Tat diferentiell exprimiert und hier zeichnete sich ein interessanter Trend ab: Der Großteil an differentiell exprimierten Genen in unseren Proben, die zu irgendeinem Zeitpunkt als in Astrozyten überrepräsentiert beschrieben worden waren, schienen speziell in protoplasmischer Astroglia überrepräsentiert zu sein. Dies lässt sich vermutlich durch den Umstand erklären, dass bis dato im Wesentlichen Gewebe mit ausschließlich oder zumindest überwiegend grauer Substanz für Sequenzierungszwecke benutzt worden war. Ähnlich stellte sich ein Vergleich der Expressionswerte von der Literatur entnommenen Genen dar, welche mit unreifer/neoplastischer (in weißer Substanz überexprimiert) oder reifer/physiologischer Astroglia (in grauer Substanz überexprimiert) assoziiert sind.

Um ein besseres Verständnis der funktionellen Charakteristika von protoplasmischen und fibrösen Astrozyten zu entwickeln, welche den differentiell exprimierten Genen in unseren Daten zugrundeliegen könnten, führten wir Genüberrepräsentationsanalysen durch und nutzten hierzu fünf verschiedene Software Tools. Einer der wesentlichen in Astrozyten der grauen Substanz überrepräsentierten Begriffe war hierbei metabolische Aktivität, im weitesten Sinne. In diese Kategorie fielen verschiedene Exponenten des zellulären Energiehaushalts (z.B. Glykolyse und Glukoneogenese, Zitratzyklus oder Fettstoffwechsel), daneben auch der Metabolismus von Neurotransmittern, Hormonen und Nukleotiden. Wenn man die unterschiedlichen mikroanatomischen Gegebenheiten zwischen Astrozyten der grauen und weißen Substanz bedenkt, so mag es nicht verwundern, dass eine weitere in protoplasmischer Astroglia überrepräsentierte Kategorie mehrere Begriffe umfasste, die mit der Regulation von synaptischer Transmission verbunden sind (darunter Synaptogenese und

Phagozytose von Synapsen). Einmal mehr drängte sich der Verdacht auf, dass viele der bislang in Bezug auf Astroglia im Allgemeinen gemachten Beobachtungen in unseren Daten speziell auf protoplasmische Astrozyten anwendbar waren.

Die funktionellen Analysen unserer Proben der weißen Substanz zeigten sich dominiert von Begriffen, welche mit Zellteilung in Zusammenhang stehen. Dem zugrundeliegend war die differentielle Expression von Genen, die entweder direkter Bestandteil der Mitose sind, oder aber mit der DNA-Reparatur oder der Deregulierung des Zellzyklus in Verbindung stehen (i.e. Entstehung und Progression von Gliomen). Viele der Gene, welche uns bereits bei der Untersuchung von Markern für unreife Zellen begegnet waren, waren auch in diesem Kontext relevant. Um diese vermeintliche Zellteilungstendenz von Astroglia der weißen Substanz experimentell zu bestätigen, führten wir eine Proliferationsuntersuchung durch, wobei die Quantifizierung anhand der Aufnahme von EdU in die Zellen erfolgte: Zwischen 3 und 5% aller fibrösen Astrozyten waren innerhalb von 28 Tagen positiv für den getesteten Proliferationsmarker, während sich dieser in keinem einzigen Astrozyten der grauen Substanz nachweisen ließ.

Mit der Absicht einer praktischen Anwendung unserer RNA-Sequenzierungsergebnisse versuchten wir, anhand der Daten ein spezifisches Marker-Gen für fibrose Astrozyten zu identifizeren. FGFR2 erwies sich hierbei als vielversprechend, da es ein Zelloberflächenmolekül ist und seine Expression durch Astrozyten bereits beschrieben wurde. Das entsprechende Gen zeigte sich in unseren aus der weißen Substanz stammenden RiboTag-Proben signifikant überrepräsentiert, welche die tatsächliche Genexpression letztlich unmittelbarer wiedergeben. Tatsächlich zeigte sich in der immunhistochemischen Färbung eine klare Kolokalisation von FGFR2 mit GFAP/S100β und Aldh1l1-eGFP, so dass das Protein als vielversprechende Option eines Markers für fibrose Astrozyten in zukünftigen Anwendungen gesehen werden darf.

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8. List of Abbreviations

Abbreviations from chemicals and reagents given in the Materials and Methods section are not listed here. If an abbreviation is used three times or more in the text, the associated full term is given the first time. Upper- and lower-case letters are used according to the most common usage. Since the differentiation between the gene and its product is seldom adhered to in any strict sense, it was not implemented in this thesis, either.

Aβ	Amyloid beta
ACSA	Astrocyte cell surface antigen
ACSBG1	Acyl-CoA synthetase bubblegum family member 1
AD	Alzheimer's Disease
Adgrl4	Adhesion G protein-coupled receptor L4
ADK	Adenylate kinase
Aif1	Allograft inflammatory factor 1
Aldh	Aldehyde dehydrogenase
Aldoc	Fructose-bisphosphate aldolase C
ALS	Amyotrophic lateral sclerosis
AMP	Adenosine monophosphate
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANLS	Astrocyte-neuron lactate shuttle
Apold1	Apolipoprotein L domain containing
AQP4	Aquaporin 4
Ascl1	Achaete-scute homolog 1
Aspm	Abnormal spindle-like microcephaly-associated protein
ATP	Adenosine triphosphate
ATP1B2	ATPase Na+/K+ transporting subunit beta 2
Axl	Tyrosine-protein kinase receptor UFO
BAC	Bacterial artificial chromosome
BBB	Blood–brain barrier
Bdnf	Brain-derived neurotrophic factor
BG	Bergmann glia
bHLH	Basic helix–loop–helix

BMP	Bone morphogenetic protein
BrdU	5-bromo-2'-deoxyuridine
Brn2a	Brain-2(a)
Bub1	Budding uninhibited by benzimidazoles 1 homolog
Casc5	Cancer susceptibility candidate 5
Ccl2	CC-chemokine ligand 2
Ccna2	Cyclin A2
Cdca8	Cell division cycle associated 8
Cenp	Centromeric protein
Chrdl1	Chordin-like 1
Chrna7	Cholinergic receptor nicotinic alpha 7 subunit
Cldn5	Claudin 5
CNS	Central nervous system
CNTF	Ciliary neurotrophic factor
CR	Complement receptor
Cre	Cyclization recombination
CSF	Cerebrospinal fluid
CT-1	Cardiotrophin-1
Cx	Connexin
Cyp4f15	Cytochrome P450 family 4 subfamily F member 15
DAPI	4’,6-diamidino-2-phenylindole
DE	Differentially expressed
Dlx2	Distal-less homeobox 2
Dmp1	Dentin matrix acidic phosphoprotein 1
DNMT1	DNA methyltransferase 1
Dynlrb2	Dynein light chain roadblock-type 2
EAAT	Excitatory amino acid transporter
EAE	Experimental autoimmune encephalomyelitis
Ednra	Endothelin receptor type A
EdU	5-ethynyl-2’-deoxyuridine
Efhc1	EF-hand domain-containing protein 1
Egfr	Epidermal growth factor receptor

Emp1	Epithelial membrane protein 1
Emx2	Empty spiracles homeobox 2
Eno	Enolase
Enpp	Ectonucleotide pyrophosphatase/phosphodiesterase
EPSC	Excitatory postsynaptic current
ER	Endoplasmic reticulum
ErbB4-NCoR	Erb-b2 receptor tyrosine kinase 4 – nuclear receptor corepressor
ERK/MAPK	Extracellular signal-regulated/mitogen-activated protein kinases
ERT2	Estrogen receptor 2
Esam	Endothelial cell-selective adhesion molecule
Exx	Embryonal day xx
FABP7	Fatty acid binding protein 7
FACS	Fluorescence-activated cell sorting
Fam	Family with sequence similarity
FGF2	fibroblast growth factor 2
FGFR	fibroblast growth factor receptor
fMRI	Functional magnetic resonance imaging
FPKM	Fragments per kilo base per million mapped reads
Fzd	Frizzled
GABA	Gamma-aminobutyric acid
Gabbr2	Gamma-aminobutyric acid type B receptor subunit 2
Gabrg1	Gamma-aminobutyric acid receptor subunit gamma 1
Gad1	Glutamate decarboxylase 1
GAT	Glycerol-3-phosphate acyltransferase
GFAP	Glial fibrillary acidic protein
(e)GFP	(Enhanced) green fluorescent protein
Ggt1	Gamma-glutamyltransferase 1
Gj	Gap junction
GLAST	L-glutamate/L-aspartate transporter
Gli	Glioma-associated oncogene
GLT-1	Glutamate transporter 1
Glud1	Glutamate dehydrogenase 1

Glul	Glutamate-ammonia ligase (= Glutamine synthetase)
GM	Gray matter
GO	Gene ontology
Gpc3	Glypican-3
Grin2c	Glutamate ionotropic receptor NMDA type subunit 2C
Grm3	Glutamate metabotropic receptor 3
GS	Glutamine synthetase
GSEA	Gene set enrichment analysis
GST-π	Glutathione S-transferase pi
Gst5	Glutathione S-transferase mu 5
HA	Hemagglutinin
HD	Huntington's Disease
HDAC1	Histone deacetylase 1
Hes	Hairy and enhancer of split
Hsd11b1	11β-hydroxysteroid dehydrogenase type 1
IBA1	Ionized calcium-binding adapter molecule 1
ICC	Immunocytochemistry
Igfbp2	Insulin-like growth factor-binding protein 2
IHC	Immunohistochemistry
Il1a	Interleukin 1 alpha
IP3R2	Inositol 1,4,5-triphosphate type-2 receptor
IPSC	Inhibitory postsynaptic current
JAK/STAT	Janus kinase/signal transducers and activators of transcription
Kif	Kinesin superfamily protein
Kir	K^+ inwardly rectifying
Ldhb	Lactate dehydrogenase B
Lhx2	LIM/homeobox protein
LIF	Leukemia inhibitory factor
LTP	Long-term potentiation
LV	Lentivirus
MACS	Magnetic-activated cell sorting
MAO	Monoamine oxidase

Mash1	Mammalian achaete-scute homolog 1
MEGF10	Multiple epidermal growth factor-like domains protein 10
MERTK/Mertk	MER receptor tyrosine kinase
Mfge8	Milk fat globule-EGF factor 8
mGluR	Metabotropic glutamate receptor
MHCI	Major histocompatibility complex I
Mki67	Marker of proliferation Ki-67
Mog	Myelin-oligodendrocyte glycoprotein
MS	Multiple sclerosis
Myb	Myeloblastosis viral oncogene homolog
Myt1l	Myelin transcription factor 1 like
NA	Noradrenaline
Ndp	Norrin
Neat1	Nuclear enriched abundant transcript 1
NeuroD1	Neurogenic differentiation 1
NF1	Neurofibromin 1
NF1A	Nuclear factor 1 A-type
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NG2	Neuron-glial antigen 2
Ngn1	Neurogenin 1
NKCC1	$Na^+/K^+/Cl^-$ cotransporter 1
Nkx6.1	NK6 homeobox 1
NL1	Neuroligin 1
NMDA	N-methyl-D-aspartate
Nme5	Non-metastatic cells 5
NO	Nitric oxide
NP1	Non-structural protein 1
Nrxn	Neurexin
NSC	Neural stem cell
Nupr1	Nuclear protein 1
O4	Oligodendrocyte marker 4
Oasis-Gcm1	Old astrocyte specifically-induced substance – glial cells missing 1

Oct4	Octamer-binding transcription factor 4
Olig2	Oligodendrocyte transcription factor 2
OPC	Oligodendrocyte precursor cell
p300/CBP	p300/cAMP-response element-binding protein-binding protein
Pax6	Paired box protein 6
Pbk	PDZ-binding kinase
PC	Principal component
PCA	Principal component analysis
PcG	Polycomb group
PCR	Polymerase chain reaction
Pdgfr	Platelet-derived growth factor receptor
Pfkm	6-phosphofructokinase, muscle type
Pgm2	Phosphoglucomutase 2
PI3K/Akt/mTOR	Phosphoinositide 3-kinase/Akt/mechanistic target of rapamycin
PLP	Proteolipid protein 1
PPAR	Peroxisome proliferator-activated receptor
Prodh	Proline dehydrogenase
Ptn	Pleiotrophin
PYGM	Glycogen phosphorylase, muscle associated
Pxx	Postnatal day xx
qPCR	Quantitative real-time PCR
RG	Radial glia
RNA	Ribonucleic acid
RNA-Seq	RNA sequencing
ROS	Reactive oxygen species
RPTPδ	Transmembrane receptor-type protein tyrosine phosphatase delta
Rrm2	Ribonucleotide reductase regulatory subunit M2
Rsph1	Radial spoke head 1 homolog
S100β	S100 calcium-binding protein β
SCI	Spinal cord injury
Scl	Stem cell leukemia
SEM	Standard error of the mean

Sema3a	Semaphorin-3A
Sfrp	Secreted frizzled related protein
SGZ	Subgranular zone
Shh	Sonic hedgehog
SLC/Slc	Solute carrier
Slit1	Slit guidance ligand 1
SMAD1	Mothers against decapentaplegic homolog 1
SOD	Superoxide dismutase
Sostdc1	Sclerostin domain-containing protein 1
Sox	SRY-box transcription factor
SPARC	Secreted protein acidic and rich in cysteine
Sparcl1	SPARC-like 1
Sst	Somatostatin
Stmn2	Stathmin 2
SVZ	Subventricular zone
TF	Transcription factor
Tfap2b	Transcription factor AP-2 beta
TGF	Transforming growth factor
THBS4	Thrombospondin 4
Tlr2	Toll-like receptor 2
Tmem212	Transmembrane protein 212
TNFα	Tumor necrosis factor alpha
Top2a	DNA topoisomerase II alpha
Tpx2	Targeting protein for Xklp2
TRAP	Translating ribosome affinity purification
TrkA	Tropomyosin receptor kinase A
Ttll6	Tubulin tyrosine ligase-like 6
Ttr	Transthyretin
Tubb3	Tubulin beta 3 class III
Ube2c	Ubiquitin-conjugating enzyme E2 C
VZ	Ventricular zone
WM	White matter

Wnt	Wingless-type
WT	Wild type
Znrf3	Zinc and ring finger 3

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