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Astrocyte phenotypes: Emphasis on potential markers in neuroinflammation

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Summary. Astrocytes, the most abundant glial cells in the central nervous system (CNS), have numerous integral roles in all CNS functions. They are essential for synaptic transmission and support neurons by providing metabolic substrates, secreting growth factors and regulating extracellular concentrations of ions and neurotransmitters. Astrocytes respond to CNS insults through reactive astrogliosis, in which they go through many functional and molecular changes. In neuroinflammatory conditions reactive astrocytes exert both beneficial and detrimental functions, depending on the context and heterogeneity of astrocytic populations. In this review we profile astrocytic diversity in the context of neuroinflammation; with a specific focus on multiple sclerosis (MS) and its best-described animal model experimental autoimmune encephalomyelitis (EAE). We characterize two main subtypes, protoplasmic and fibrous astrocytes and describe the role of intermediate filaments in the physiology and pathology of these cells. Additionally, we outline a variety of markers that are emerging as important in investigating astrocytic biology in both physiological conditions and neuroinflammation.

Key words: Astrocytes, Intermediate filaments, Experimental autoimmune encephalomyelitis

Introduction

Astrocytes are the most abundant and heterogeneous cells of the mammalian CNS that contribute to the physical and metabolic support to the neurons (Sofroniew and Vinters, 2010). Therefore, astrocytes' innate ability to protect neurons appears to be a promising therapeutic target during neurodegeneration (Liu et al., 2017). Based on cell morphology and anatomical location, two main types have been documented. Fibrous astrocytes reside in the white matter, display many elongated fiber-like processes along with myelinated fibers, whereas star-shape protoplasmic astrocytes occupy the gray matter, creating extensive contacts with neurons and blood vessels (Nair et al., 2008; Correale and Farez, 2015). During neuroinflammation, astrocytes have significant roles, and their response to danger signals may be beneficial or detrimental depending on the stimuli from the inflamed environment (Colombo and Farina, 2016). In terms of acute neuroinflammation, astrocytes can hinder their dysfunction and restrict the dissemination of cytotoxic inflammation (Sofroniew, 2015). However, astrocytes are exposed to a variety of danger signals during chronic neuroinflammation, leading to their increased astrocyte reactivity that contributes to glial scar formation, demyelination, and neurodegeneration in MS/EAE (Liddelow et al., 2017; Wheeler and Quintana, 2018). These reactive astrocytes display considerable heterogeneity at numerous levels (Anderson et al., 2014), including region-specific gene expression (Itoh et al., 2018), cell morphology, signaling, and function (Sofroniew, 2015). It was recently proposed that reactive astrocytes exist in two different states upon neuroinflammation, with neurotoxic and neuroprotective reactive phenotype capabilities (Liddelow and Barres, 2017). The subtype of reactive astrocytes, referred to as A1, possesses the ability to damage neurons and oligodendrocytes, while neuroprotective A2 reactive astrocytes may promote neuron survival and tissue repair (Liddelow et al., 2017).

Origin and development

From their discovery to the present day, the point of view towards astrocytes has evolved from being considered as a homogeneous population of cells simply representing structural support to CNS, to a heterogeneous population with a multitude of functions such as the following: active participation in the neuronal activity by modulating synaptic transmission; neurotransmitter trafficking; production of gliotransmitters; monitoring blood-brain barrier and regional blood flow; energy and antioxidant reservoir for

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neurons; repairment of injured nervous system and many others (Khakh and Sofroniew, 2015; Burda et al., 2016). It became apparent that astrocytes display heterogeneity at multiple levels, concerning their morphology, molecular signature, function, and anatomical location. Since these properties are intertwined, determining astrocyte functional diversity represents the ultimate challenge. However, as experimental techniques in this field become more technically sophisticated, some problems are slowly being elucidated. Thus, the development of astrocytes might be the foundation of their future heterogeneity in adulthood (Clarke et al., 2021; Ben Haim and Rowitch, 2017). Since the theme of astrogenesis has been extensively covered elsewhere (Chaboub and Deneen, 2012; Bayraktar et al., 2014; Molofsky and Deneen, 2015), in this segment, we will briefly discuss the relationship between embryonic patterning and generation of different astrocyte subtypes. Astrocytes share the same origin as neurons and oligodendrocytes, so they are assumed to follow similar developmental stages (neural stem cell specification, migration, proliferation, and differentiation) and spatial and temporal patterning principles. However, unlike neurons and oligodendrocytes, they retain mitotic potential, and their differential endpoint is dynamic in the adult form (Chaboub and Deneen, 2012). It all starts in the ectoderm, which develops into the neuronal tube (NT), whose wall consists of neuroepithelial cells, with cell bodies located around the tube lumen, in the ventricular zone (VZ), and processes sent towards the pial surface. Neuroepithelial cells give rise to primary neural stem cells - radial glial cells (RGCs) that retain apicobasal orientation and have neurogenic and gliogenic potential. Factors (intrinsic and extrinsic) favoring the production of neurons are the first ones to dominate, which means that neurogenesis precedes gliogenesis (Deneen et al., 2006). The root of neuronal diversity lies in the developmental patterning program, enabling progenitor cells to arrange in region-specific compartments. Thus, neuroepithelial cells will receive external signals in the form of following morphogenic proteins: bone morphogenetic proteins (BMPs), wingless (WNT) proteins, and Sonic Hedgehog (SHH) (Jessell, 2000). Gradual concentration of these proteins along a dorsoventral axis in developing spinal cord (BMP and WNT decreasing, SHH increasing) will induce either activation or inhibition of homeodomain and basic Helix-Loop-Helix transcription factors (such as members of the following families: Nkx, Pax, Dbx, Olig2), resulting in different combinations of their expression (Muroyama et al., 2005; Hochstim et al., 2008). Each combination will be specific for a certain population of progenitor cells, reflected in the spinal cord's segmental organization and formation of ventral progenitor domains (p0-most dorsal, p1, p2, pMN and p3-most ventral). From domains p0-p3, three distinct types of ventral interneurons (V0, V1, V2, and V3) will arise, while pMN will produce motor neurons. In the murine embryonic spinal cord, gliogenic input comes around E12.5, from transcription factors Sox9 and NFIA (Deneen et al., 2006; Kang et al., 2012). Upon gliogenic switch, RGCs in domains p1-p3 will generate three distinct types of ventral white matter astrocytes (VA1-VA3), p0 will produce protoplasmatic and fibrous astrocytes, while progenitors in pMN will produce oligodendrocytes. Astrogenesis does not co-occur in the dorsal and ventral segment of the spinal cord; thus, dorsal astrogenesis follows after ventral. Interestingly, gliogenic progenitors will use a similar combinatorial system, like in the case of neuronal diversification, consisting of homeodomain patterning proteins (Pax6, Nkx6.1), but now to produce different subtypes of astrocytes (Hochstim et al., 2008). The first wave of astrocyte proliferation occurs in VZ, where RGSs will asymmetrically divide and produce intermediate astrocyte precursors (IAP) (Tien et al., 2012). These astrocyte precursors will migrate along with the processes of RGCs to their final destination. In the spinal cord that would not be so far away from their progenitor domains in VZ. Upon arriving at their location, IAPs divide symmetrically (second wave of proliferation), to generate more astrocytes, which then fully maturate (Tien et al., 2012). VA1-VA3 astrocytes position along dorsoventral axes of ventral white matter in a manner that corresponds to the dorsoventral arrangement of their progenitors' domains along VZ. Each of these astrocyte subtypes is identified based on the expression of specific markers: Slit1 and Reelin (Hochstim et al., 2008). Furthermore, this astrocyte heterogeneity established by intrinsic mechanisms early during development, in later stages might further upgrade by extrinsic signals coming from local neuronal circuits. Compared to the spinal cord, gliogenic switch in the mouse cortex comes later, around E16-18, also characterized by two periods of astrocyte proliferation (Ge et al., 2012). According to a 3D imaging study of cortical astrocyte clones, it seems that astrocyte precursors colonize the neocortex in a non-ordered manner, and their heterogeneity is guided mostly by environmental factors (Clavreul et al., 2019). The recent large-area spatial transcriptomic study revealed that the mouse cortical gray matter contains three layers of astrocytes (superficial, mid, and deep) distinctive from the six neuronal layers (Bayraktar et al., 2020). These astrocytic domains are established early in postnatal development and maintained into the adult stage (Bayraktar et al., 2020). Therefore, it is plausible that CNS overall applies multiple strategies in the development of astrocyte functional heterogeneity across different regions, covering the spectrum from a combination of embryonic patterning and environmental cues to extrinsic signals solely.

It is crucial to determine the developmental and mature markers of astrocytes to follow the processes of astrogenesis mentioned earlier. Markers used to label astrocyte precursors are Glast, FGFR3, and FABP7 (Owada et al., 1996; Shibata et al., 1997; Hartfuss et al., 2001; Pringle et al., 2003). However, these markers are not exclusive for astrocyte lineage since they label

oligodendrocyte or neuronal precursors, too (Anthony et al., 2004). Regarding mature markers of astrocytes, there is myriad of them: GFAP, S100b, Aldh1L1, CD44, Glt1, GS, AQp4, and Ascgb1, whose features will be addressed later in the text.

Intermediate filament characterization in astrocytes

Intermediate filaments represent the main constituents of the cytoskeleton. These filaments are comprised of a huge family of proteins and spread through the cytoplasm and inner nuclear membrane. Their major role is to maintain the stability of the cells and tissues (Bott and Winckler, 2020). There are 6 known classes of intermediate filaments.

Glial fibrillary acidic protein (GFAP)

Glial fibrillary acidic protein (GFAP)(molecular weight 55kDa) is a typical cytoskeletal intermediate filament (IF) protein that provides mechanical support to the astrocytes in physiological conditions (Pekny et al., 2016). Regardless of their heterogeneity, astrocytes respond to different stimuli by increasing GFAP expression, which leads to the reorganization of the cytoskeletal network (Eng, 1985; Gomes et al., 1999). The existence of different isoforms further enhances the complexity of the astrocytic cytoskeleton. In addition to two IF proteins - vimentin and nestin (Hol and Pekny, 2015), astrocytes express ten different GFAP isoforms (Kamphuis et al., 2012). It was previously shown that GFAP splice variants influence astrocytes' shape and characteristics in physiological and pathological conditions (Middeldorp and Hol, 2011; Moeton et al., 2016b).

It has been proposed that an aberrant IF system is more detrimental to a cell than the complete absence of IFs (Eliasson et al., 1999). Indeed, expression of GFAP appears not to be a prerequisite for the development and/or tissue distribution of astrocytes in the CNS, and the loss of GFAP IFs is not compensated by the up-regulation of other intermediate filament proteins, such as vimentin (Aquino et al., 1988). GFAP-negative mice displayed post-traumatic reactive gliosis, which suggests that GFAP up-regulation, a hallmark of reactive gliosis, is not an obligatory requirement for this process (Pekny et al., 1995; Hol and Pekny, 2015).

GFAP isoforms

The most abundant GFAP isoform is a GFAP-α, a canonical isoform found in CNS (Boyd et al., 2012). The other GFAP isoforms are made from alternative splicing at 3' or 5' ends of its pre mRNA (Boyd et al., 2012). The first alternative spliced form was recognized in the rat and termed GFAP-δ (Condorelli et al., 1999), while its human homologue was entitled GFAP-ε (Nielsen et al., 2002). It was confirmed that Schwann cells express the GFAP-β transcript (Jessen et al., 1984). Additional three splice variants GFAPΔEx6, GFAPΔ164, and GFAPΔ135 were found in the human brain and have been implicated in Alzheimer`s disease and epilepsy (Boer et al., 2010; Kamphuis et al., 2012) Also, GFAP-ζ (zeta) was detected in the mouse developing brain, while γ-transcript for GFAP was identified in mouse spleen (Zelenika et al., 1995). GFAP-ϰ is involved in maintenance of rigidity and stability of GFAP (Blechingberg et al., 2007). Altogether, the role of these isoforms is ambiguous; among them, it seems that only GFAPδ influences astrocyte cytoskeleton in the CNS (Moeton et al., 2016b).

GFAP-δ was first described in rat primary astrocyte cultures (Condorelli et al., 1999). It is expressed in proliferating neurogenic astrocytes of the developing human and mouse brain (Middeldorp et al., 2010; Mamber et al., 2012; Thomsen et al., 2013). The expression of this GFAP isoform was found in several neurodegenerative disorders, including vanishing white matter disease (Bugiani et al., 2011) and epilepsy (Martinian et al., 2009; Miyahara et al., 2011). Likewise, GFAP-δ is overexpressed in different glial tumors (Andreiuolo et al., 2009; Choi et al., 2009; Stassen et al., 2017). It was suggested that GFAP-δ has a substantial impact on the astrocyte cytoskeleton, affecting cell adhesion, morphology, and motility (Martinian et al., 2009; Moeton et al., 2016b). In humans, GFAP-δ expression is associated with neuronal stem cells and immature astrocytes, with an ability to self-renew (Middeldorp et al., 2010), while in the developing and adult mouse brain, GFAP-δ is not a preferential neural stem cell marker (Mamber et al., 2012). The ability of GFAP-δ to alter the cytoskeleton of astrocytes indicates its capacity to affect IFs to form heterodimers with other cytoskeleton proteins or to influence organelle or vesicle position and trafficking (Moeton et al., 2016b). Neurogenesis decreases during MS, but still, GFAP-δ expressing astrocytes are found in the narrow subventricular zone in MS, pointing to its neurogenic potential (Tepavcevic et al., 2011). The predominant expression of GFAP-δ was shown in disassembled astrocytes in vanishing white matter disease (Bugiani et al., 2011), where these astrocytes display an anomalous IF network. Similarly, dysfunctional astrocytes occur in Alexander's disease, which involves immense myelin loss due to heterozygous mutations in the *GFAP* gene, followed by extensive reactive astrogliosis (Perng et al., 2008; Sosunov et al., 2017). Specifically, it was reported that GFAP-δ transcript mutation leads to aberrant GFAP-associated filamentous cytoskeletal network (Melchionda et al., 2013), pointing to the importance of GFAP-δ in proper IF assembly astrocytes.

To date, it is unknown how does high GFAP-δ expression influences whole astrocyte network, but it seems dependent on GFAP-δ/GFAP-α ratio (Nielsen and Jørgensen, 2004). Previous studies by other authors (Roelofs et al., 2005) reported that GFAP-δ expression causes a collapse of IFs near the nucleus, when GFAP-

δ/GFAP-α ratio reaches a critical level (Blechingberg et al., 2007; Moeton et al., 2016a). Hence, it should be highlighted that for normal astrocytic functions, both the quality and quantity of GFAP isoforms are needed.

Vimentin

Vimentin (molecular weight 57kDa) belongs to a class III intermediate filament protein. Vimentin intermediate filaments are generally present in mesenchymal cells, or cells that contain a distinct nucleus, and predominately expressed in developing embryo and in cells (Franke and Moll, 1987; Clarke and Allan, 2002). The main role of vimentin is to preserve the integrity of the cell and stabilize cytoskeleton interactions, thus providing endurance after the cell injury. Also, it is associated with cell migration and signaling (Ivaska et al., 2007; Battaglia et al., 2018). It was shown that vimentin deficient mice show atypical astrocyte morphology that leads to cognitive impairment and compromised motor coordination (Colucci-Guyon et al., 1999; Wilhelmsson et al., 2004). Also, it was reported that vimentin secreted from astrocytes might induce axonal growth (Teshigawara et al., 2013), while activated macrophages secrete vimentin into the extracellular space with a bactericidal effect (Mor-Vaknin et al., 2003). Although it was reported that vimentin expression in adult CNS is low, its expression in the white matter of the rat spinal cord was observed (Jakovljevic et al., 2019). The re-expression of vimentin is seen subsequent to the neuroinflammation or injury of the CNS (Pekny et al., 2016).

Nestin

Nestin (molecular weight ~ 176 kDa) belongs to a type VI intermediate filament protein. It was detected in the multipotent stem/progenitor cells of the central nervous system, mainly in immature neurons, immature oligodendrocytes, in astrocytes of the developing brain and neuronal precursors (Schmidt-Kastner and Humpel, 2002; Cahoy et al., 2008; Bott et al., 2019), though its expression was proved in non-neuronal tissue (Sejersen and Lendahl, 1993; Kachinsky et al., 1995). In the adult brain, nestin is down-regulated in neurons and astrocytes (Bernal and Arranz, 2018; Bott et al., 2019), except for radial glia (Gubert et al., 2009). In the adult brain, nestin is detected in the subependymal cells of the lateral ventricle, and in the subgranular cells of the dentate gyrus, parts of the CNS known to contain neural stem/progenitor cells (Gonzalez-Perez, 2012). It appears that mature astrocytes fail to express nestin immunoreactivity (Bott et al., 2019). However, the presence of nestin is seen in white matter astrocytes, presented with only a few fibers in the rodent spinal cord (Jakovljevic et al., 2017). Nestin is rapidly up-regulated in response to an injury. In general, nestin was barely expressed in the adult CNS, but induction of nestin is used as a typical marker of reactive astrocytes (Pekny et al., 2016; Krishnasamy et al., 2017).

Astrocytes in homeostasis

Astrocytes have a multitude of essential roles in the healthy CNS. Their active role in synaptic transmission has long been recognized and formulated in the concept of the tripartite synapse (Araque et al., 1999). Astrocytic processes envelop neuronal synapses and release neuroactive molecules such as glutamate, adenosine, ATP, D-serine, and GABA, directly influencing synaptic activity. Even though astrocytes are not excitable cells in a classic manner, and do not generate electrical signals across the plasma membrane-like neurons, they increase intracellular Ca^{2+} concentrations in response to certain stimuli, which is their form of electrical excitability (Perea et al., 2009). In addition to this active role via gliotransmission, astrocytes also influence the formation and pruning of the synapses during development, along with synaptic remodeling and pruning in adult CNS (Ullian et al., 2001; Sofroniew and Vinters, 2010). These cells are traditionally known for their supportive role for neurons; they synthesize metabolic substrates such as glycogen, sterols, and lipoproteins (Göritz et al., 2002; Brown and Ransom, 2007; Wang and Bordey, 2008). In hypoglycemic conditions and periods of intense neuronal activity, astrocytes break down glycogen and produce lactate in order to provide energy for neurons (Brown and Ransom, 2007). They also provide trophic support for neurons and support neuronal survival and neurite outgrowth, by secreting growth factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), activity-dependent neurotrophic factor (ADNF), fibroblast growth factor-2 (FGF-2), ciliary neurotrophic factor (CNTF) and leukemia inhibitory factor (LIF) (Kıray et al., 2016). Furthermore, they regulate extracellular concentrations of ions and neurotransmitters and maintain fluid and pH homeostasis in the synaptic cleft. To this end, astrocytic processes have many aquaporin 4 (AQP4) water channels, transporters, and channels for the uptake of K^+, as well as for proton shuttling. Astrocytes also have transporters for uptake of neurotransmitters such as glutamate, GABA, and glycine, which enables their clearance from the synaptic space (Wang and Bordey, 2008; Sofroniew and Vinters, 2010). They are involved in the formation and maintenance of the blood-brain barrier (BBB) (Abbott et al., 2006) and regulation of cerebral blood flow (Koehler et al., 2009; Marina et al., 2020). Astrocytes are also important for myelination and promote maturation and differentiation of oligodendrocyte precursor cells (OPCs) by secreting growth factors including platelet-derived growth factor (PDGF), LIF, CNTF, neurotrophin-3, neurotrophin-4 and insulin-like growth factor 1 (IGF1) (Salem et al., 2016). In recent years, astrocytes have been recognized as a part of the glymphatic system, a functional waste clearance system in the CNS that also helps distribute glucose, amino acids, lipids, growth

factors, and neuromodulators throughout the CNS. Astrocytes are involved in the formation of this system of perivascular tunnels through which compounds are distributed, and toxic waste products are removed (Jessen et al., 2015).

Astrocytes in neuroinflammation

The roles of astrocytes during MS/EAE are emerging. Astrocytes are very dynamic cells in both physiological and pathological settings. Reactive astrogliosis with accumulations of hypertrophic astrocytes expressing IF proteins, such as GFAP, nestin, and vimentin, represents a prominent feature of MS/EAE (Guo et al., 2011). These IFs proteins are fundamental constituents of the cytoskeleton affecting morphology and driving numerous processes, including proliferation and motility. Furthermore, it was reported that GFAP isoforms might influence astrocytic function (Middeldorp and Hol, 2011). It is well-known that astrocyte heterogeneity in terms of morphology and physiology may influence the glial propensity to respond differently to inflammatory stimuli and affects the disease outcome (Colombo and Farina, 2016). Specifically, reactive astrogliosis may be detrimental to neuronal recovery; however, the astrocytic scar may restrict inflammation to the CNS and possibly induce neuroprotection (Sofroniew, 2015). It was hypothesized that the beneficial effects of reactive astrogliosis are found in the acute state of diseases, such as in our EAE model. In MS, abundant GFAP-α expressing astrocytes are usually scattered around demyelinating foci, although a negligible number of these astrocytes express GFAP-δ (Hochstim et al., 2008). Previously, it was postulated that GFAP isoforms might have different functional roles, including modulation of the volume and cellular location of IFs in astrocytes (Hochstim et al., 2008; Chaboub and Deneen, 2012).

It is well-known that immature astrocytes express nestin and vimentin, whereas the only vimentin is found in GFAP mature astrocytes (Middeldorp et al., 2010). Previously, we and others reported that vimentin expression in astrocytes declines with the withdrawal of inflammation, preserving its expression in reactive astrocytes at the end of EAE (Aquino et al., 1988; Lavrnja et al., 2012). Astrocytes are attached to the blood vessels through unambiguously resilient glial processes. The interaction between astrocytes and vessels is found to be crucial for maturation (Ulfig et al., 1999; Li et al., 2019). During pathological conditions, astrocytes and vessels lose contact, leading to the destabilization of the cytoskeleton and re-expression of nestin and vimentin in the formation of hypertrophy of the astrocytes (Sofroniew and Vinters, 2010).

Prompt up-regulation of nestin/vimentin was observed in reactive astrocytes of the adult rodent brain after injury, ischemia, and seizures (Bernal and Arranz, 2018). It is accepted that nestin and vimentin expression in reactive astrocytes has a role in stabilizing the growing cytoskeleton. Indeed, nestin protein expression was up-regulated at the peak of disease and was mainly localized in astrocytes around inflammatory infiltrates, where the axons are most affected (Lavrnja et al., 2009). This may indicate that they belong to neural stem cells, with a role to maintain microtubules that are often disturbed when neurons are insulted (Hendrickson et al., 2011). It is known that in proliferating cells, nestin has a crucial role in the co-assembly of IFs that contributes to astrocytic IF network reorganization and preferential formation of heterodimers with vimentin (Chou et al., 2003). Nestin expression increases toward the end of the disease, implying that the recovery from EAE is related to the increased number of progenitor cells in the spinal cord (Jakovljevic et al., 2017).

Inflammation is an integral part of all pathologies of the CNS, whether it is the cause or an effect of the dysregulation. In injuries and infections of the CNS and other neurological diseases where the integrity of the BBB is disrupted, the main sources of inflammatory events are circulating bone marrow-derived leukocytes. However, resident CNS cells such as microglia and astrocytes have been shown to be fundamental in inflammatory processes (Sofroniew, 2015). Both microglia and astrocytes can have pro-inflammatory and anti-inflammatory functions, and thus be either detrimental or beneficial in neuroinflammation, depending on the underlying signaling mechanisms and complex intercellular interactions (Gertig and Hanisch, 2014; González et al., 2014; Klein and Hunter, 2017; Liddelow and Barres, 2017).

Astrocytes respond to all forms of CNS insults with a range of molecular and functional changes, a process termed astrogliosis. Sofroniew (Sofroniew, 2009) postulated the major characteristics of this process, describing astrogliosis as a spectrum of changes in astrocytes that develop after disruption of CNS homeostasis, however big or small. The extent of these changes corresponds to the severity of the insult and can include changes in molecular expression, cellular hypertrophy, and in extreme cases, the proliferation of astrocytes and formation of the glial scar. Specific signaling molecules regulate these changes in a context-specific process. They can lead to both gain and loss of astrocytic function and pro-inflammatory, as well as anti-inflammatory events (Sofroniew, 2009). Astrogliosis has many features in the injured CNS. Depending on the extent of CNS disruption, reactive astrocytes can secrete neurotrophins to support injured neurons (Lee et al., 1998; Ikeda et al., 2001), phagocytize synapses and clear debris and dead cells (Chung et al., 2013; Tasdemir-Yilmaz and Freeman, 2014), form the glial scar to enclose the damaged area, halt inflammation and help axon regeneration (Sofroniew and Vinters, 2010; Anderson et al., 2016) and can also be involved in the restoration of a compromised BBB (Bush et al., 1999; Michinaga and Koyama, 2019). These beneficial functions of reactive astrocytes were confirmed in animal studies where

genetic ablation of reactive astrocytes or some of their transporters and signaling molecules leads to increased inflammation or neurodegeneration (Rothstein et al., 1996; Bush et al., 1999; Faulkner et al., 2004; Drögemüller et al., 2008; Herrmann et al., 2008; Voskuhl et al., 2009; Wanner et al., 2013). However, animal models also showed detrimental effects of astrogliosis and its potential to exacerbate inflammation and inhibit axon regeneration and neuronal repair (Silver and Miller, 2004; Brambilla et al., 2009; Spence et al., 2011). Studies on astrogliosis show that this is a complex and context-specific process that can improve or impede the CNS's health and homeostasis.

Beneficial roles of astrocytes in CNS insults

In severe disruptions of the CNS reactive astrocytes form glial scars that act as neuroprotective barriers that restrict migration of leukocytes and other inflammatory cells or infectious agents (Bush et al., 1999; Faulkner et al., 2004; Toft-Hansen et al., 2011). Ablation of dividing reactive astrocytes led to more significant cortical degeneration after moderate traumatic brain injury (Myer et al., 2006) and widespread tissue disruption and degeneration after spinal cord injury (Faulkner et al., 2004), showing that the glial scar is necessary for the constraint of the injury and to halt the spread of damage to the adjacent tissue. Studies analyzing signaling mechanisms that regulate astrocyte reactivity showed that deletion of signal transducer and activator of transcription 3 (STAT3) led to widespread infiltration of inflammatory cells and neural disruption, pointing to STAT3 as a crucial regulator of reactive astrocytes and their anti-inflammatory functions after various insults of the CNS (Okada et al., 2006; Herrmann et al., 2008).

Reactive astrocytes up-regulate the expression of several neuroprotective molecules. They produce neurotrophic factors such as ciliary neurotrophic factor (CNTF) (Lee et al., 1998) and brain-derived neurotrophic factor (BDNF) (Ikeda et al., 2001) that support neuronal survival. Glutamate transporters such as GLAST and GLT-1 are up-regulated on reactive astrocytes, and through this mechanism, the healthy neurons are protected from excitotoxicity (Rothstein et al., 1996). Retinoic acid derived from astrocytes has been implicated in anti-inflammatory astrocytic functions, by attenuating oxidative stress and protecting BBB function (Mizee et al., 2014). Astrocytes also produce glutathione, which helps neurons combat oxidative stress and protect them from nitric oxide toxicity (Chen et al., 2001). Further, they produce molecules that have anti-inflammatory effects on microglia and monocytes (Min et al., 2006; Kostianovsky et al., 2008). Even though reactive astrocytes are known for their production of chondroitin sulfate proteoglycans (CSPGs), a major inhibitory component of the glial scar that presents an obstacle for neurite outgrowth (Dow et al., 1994; Canning et al., 1996), they also produce laminin (Frisén et al., 1995) and fibronectin (Tom et al., 2004), extracellular matrix molecules that are permissive and supportive for neurite growth.

Another molecular mechanism that astrocytes use to limit neuroinflammation is through transforming growth factor-beta (TGFβ) signaling. TGFβ is an anti-inflammatory and neuroprotective cytokine that is up-regulated in CNS insults and has been shown to limit inflammation and reduce neuronal damage after stroke and in Toxoplasma infection of the CNS (Cekanaviciute et al., 2014a,b).

Estrogens have also been shown to have a role in mediating neuroprotection by signaling through ERα on astrocytes and leading to decreased expression of the pro-inflammatory chemokines CCL2 and CCL7 by astrocytes in EAE (Tiwari-Woodruff et al., 2007; Spence et al., 2011, 2013).

Detrimental roles of astrocytes in CNS insults

Even though astrogliosis has many beneficial functions, as previously described, reactive astrocytes can also have many harmful effects on neurons and surrounding tissue, depending on the specific context of the CNS insult. The characteristic detrimental effect of the formation of the glial scar is its inhibitory impact on axon regeneration (Silver and Miller, 2004). Besides, reactive astrocytes produce pro-inflammatory cytokines, chemokines, growth factors, and reactive oxygen species. Pro-inflammatory cytokines, among which astrocytes produce TNF-α, IL-1β, IL-6, IL-12, IL-15, IL-17, and IL-23, exacerbate inflammation in the CNS (Jensen et al., 2013). Chemokines that astrocytes produce are also numerous and include CCL2, CCL5, CXCL1, CXCL9, CXCL10, CXCL12, which have a role in recruiting diverse leukocytes, such as monocytes, macrophages, neutrophils, T and B cells (Meeuwsen et al., 2003; Jensen et al., 2013). The inflammatory environment is further aggravated by the production of NO and other reactive oxygen species (Swanson et al., 2004). Additionally, astrocytes release vascular endothelial growth factor, VEGF-A, which leads to BBB disruption and promotes leukocyte extravasation (Argaw et al., 2009, 2012). Astrocytes also express adhesive molecules, including vascular cell adhesion molecule 1 (VCAM-1) that appears to be important for T cell entry into the CNS parenchyma (Gimenez et al., 2004). These cells can cause cytotoxic edema after trauma and stroke through water channel aquaporin-4 (AQP4) (Zador et al., 2009).

Studies using transgenic animal models implicated nuclear factor-κB (NF-κB) and suppressor of cytokine signaling 3 (SOCS3) as the main transcriptional regulators in astrocytes that exert pro-inflammatory functions (Brambilla et al., 2005, 2009; Okada et al., 2006). Inactivation of astroglial NF-κB in transgenic mice led to decreased expression of pro-inflammatory cytokines, chemokines, and CSPGs in the glial scar and conclusively to improved recovery after spinal cord injury (Brambilla et al., 2005). Similarly, inhibition of

NF-κB in the EAE model decreased the expression of pro-inflammatory genes, activated neuroprotective mechanisms, and advanced functional recovery (Brambilla et al., 2009). Act1 signaling in astrocytes leads to activation of IL-17, which is critical for leukocyte recruitment in EAE (Kang et al., 2010).

A1 and A2 subsets of reactive astrocytes

Recently, dual roles of reactive astrocytes have been explained and described in the context of two subset populations: A1 and A2 astrocytes (Liddelow et al., 2017). Neuroinflammatory conditions such as LPS stimulation induce A1 astrocytes that can exacerbate the disease, secrete neurotoxins, and lead to the death of neurons and oligodendrocytes. In contrast, A2 astrocytes are induced by ischemia and promote neuronal survival and tissue repair (Zamanian et al., 2012; Liddelow and Barres, 2017). The terminology is analogous to M1 and M2 denotations that are used to describe activation states of macrophages and microglia, even though it is becoming widely accepted that both macrophages and microglia display more than two polarization states (Gertig and Hanisch, 2014; Martinez and Gordon, 2014; Ransohoff, 2016). In neuroinflammation, activated microglia induces the A1 profile of astrocytes by secreting IL-1α, TNF, and complement component 1q (C1q). In addition to being neurotoxic, A1 astrocytes lose many necessary functions such as the ability to promote neuronal survival and outgrowth, synaptogenesis, and phagocytosis. The presence of A1 astrocytes has been shown in neurodegenerative diseases, including multiple sclerosis, amyotrophic lateral sclerosis, Parkinson's, Alzheimer's, and Huntington's disease (Liddelow et al., 2017). Their induction has also been shown in normal aging, implicating them in age-related cognitive decline (Clarke et al., 2018). Transcriptome analysis showed that both types of astrocytes up-regulate a specific set of genes (Clarke et al., 2018). A1 up-regulated genes are harmful to synapses, while A2 up-regulated neurotrophic factors and thrombospondins promote synapse repair. Complement 3 (C3), complement factor B (CFB), and myxovirus (influenza virus) resistance gene MX dynamin Like GTPase 1 (MX1) were found to be characteristic for the A1 subtype and can be used as markers for these reactive astrocytes. For the A2 subtype, S100 calcium-binding protein A10 (S100A10) was found to be a specific marker (Liddelow et al., 2017). There is an indication that these subtypes are not fixed and could be interchangeable, thus opening a window for therapeutic opportunity. Milk fat globule epidermal growth factor 8 (MFG-E8) was shown to have a regulatory role on A1/A2 conversion through downregulation of nuclear factor-κB and upregulation of PI3K-Akt (Xu et al., 2018).

In light of this classification of reactive astrocytes, it can be hypothesized that these cells' detrimental roles can be attributed to A1 type, while beneficial roles are associated with A2 activation. Indeed, astrocytes are neuroprotective after an ischemic event in the CNS, which has now been associated with A2 activation (Takano et al., 2009, Becerra-Calixto and Cardona-Gómez, 2017). Studies also suggest that in the A2 subtype, the activated transcription factor is the neuroprotective STAT3, while A1 is characterized by activation of the pro-inflammatory NF-κB pathway (Xu et al., 2018). However, the A1/A2 classification may not be sufficient to explain the complexity of astrocyte heterogeneity and reactivity. Additionally, not enough is known about subtypes of reactive astrocytes, and the presence of multiple activation states cannot be excluded (Liddelow et al., 2017).

Since astrocytes are regarded as main regulators of physiological and biophysical functions in the CNS, we will focus on this review on regional differences of these cells in rodents. Specifically, we will profile a regional and developmental influence of astrocytes in the brain and spinal cord, and how these cells respond to neuroinflammation. Also, we will review potential astrocyte markers in the healthy and injured CNS.

Heterogeneity of astrocytes

Astrocytes are an extremely heterogeneous group of cells that differ between regions of the CNS, but also within these regions, termed as local heterogeneity. The most apparent regional differences are in the morphology, molecular expression, and functionality of the protoplasmic and fibrous astrocytes of the gray and white matter (Hewett, 2009; Miller, 2018). However, protoplasmic astrocytes are also diverse between different regions of the gray matter. For example, astrocytes in the hippocampus and Bergmann glia in the cerebellum express high levels of GFAP, unlike protoplasmic astrocytes of the cerebral cortex and striatum (Hewett, 2009). Differences have also been shown in the expression of other molecules, as well as in their functionality (Xu et al., 2018). Local heterogeneity within the same CNS regions, for example, in the expression of glutamate transporters, receptors, and ion channels, is presumably important to allow astrocytes to perform different functions to care for their environment (Miller, 2018). Astrocytes in the mouse somatosensory cortex were shown to have layer-specific differences in morphology and molecular expression. This is in accordance with the hypothesis that there are different subpopulations of astrocytes in different cortical layers, tailored to the needs of diverse neuronal populations (Lanjakornsiripan et al., 2018). John Lin and colleagues recently used fluorescence-activated cell sorting (FACS) to analyze astrocytes and found five distinct subpopulations that show broad molecular diversity and different roles in synaptogenesis (John Lin et al., 2017). It is still a matter of debate whether astrocyte diversity is a consequence of different astrocyte lineages, the divergence of differentiated astrocytes, or a combination of both; thus, astrocyte heterogeneity is an ongoing field

of research (Hewett, 2009). Diversity of astrocytes includes the existence of several subtypes, based on differences related to their origin or morphology, gene expression, or cell plasticity due to injury (Zhang and Barres, 2010).

Further, astrocytes differ in biophysical characteristics, including ion channel expression of calcium signaling (Olsen et al., 2015; Pestana et al., 2020). In the rodent brain and spinal cord, different astrocyte populations were observed in grey and white matter based on gross morphology; we divide them into protoplasmatic and fibrous astrocytes, respectively (Westergard and Rothstein, 2020). In addition, various subtypes of astrocytes in rodents include Müller glia in the retina, Bergmann glia of the cerebellum, tanycytes at the base of the third ventricle, velate glia, ependymal glia, perivascular glia, pituicytes in the neurohypophysis, and cribrosocytes at the optic nerve head (Emsley and Macklis, 2006). The existence of interlaminar astrocytes, varicose projection astrocytes, and polarized astrocytes was found in the human brain (Colombo and Reisin, 2004; Oberheim et al., 2006, 2009). Interlaminar astrocytes are located in the primate cortices. They do not form exclusive domain organizations; however, these cells spread straight, with a few branches, processes to the gray matter (Oberheim et al., 2006).

Similarly, varicose projection astrocytes extend long, straight processes with numerous varicosities, to the neuropil or vasculature (Oberheim et al., 2009). Polarized astrocytes are found to be sporadically present in deep cortical layers in the vicinity of the white matter. These cells are unipolar, and their processes display small bead-like varicosities that project away from the white matter (Oberheim et al., 2009).

The major role of the blood-brain barrier and blood–spinal cord barrier is to prevent infiltration of toxic circulating molecules and cells into the CNS (Zlokovic, 2011). These barriers are not the same in all brain/spinal cord regions. The density of capillaries is higher in GM than in WM (Wilhelm et al., 2016). The differences in the BBB properties differ in the gray and white matter, most likely due to the protoplasmatic or fibrous astrocyte properties. In that regard, we will discuss these two major subtypes of astrocytes.

Protoplasmic astrocytes

Protoplasmic astrocytes are confined primarily to gray matter. These cells are among the most complex cells of the CNS in terms of structure and function. Previously, it was reported that protoplasmic astrocytes support neuronal and synaptic functions (Fig. 1A) (Allen and Eroglu, 2017). Interestingly, in rodents, a single astrocyte domain contacts up to 100,000 synapses, while a human astrocyte covers approximately 2,000,000 synapses (Bushong et al., 2002; Oberheim et al., 2009; Allen and Eroglu, 2017). Protoplasmic astrocytes have a lot of organelles (a number of rough endoplasmic reticulum cisternae and a medium-sized Golgi apparatus) and cytoplasm and are endowed with ample short and thick processes. The nucleus of a protoplasmic astrocyte is large, ellipsoid, with chromatin that is very finely dispersed (Sofroniew and Vinters, 2010). Between the numerous, branched processes of protoplasmic astrocytes, various intercellular junctions mediate ion exchange between these stellate cells (Allen and Eroglu, 2017) (Table 1, Fig. 1A,C). In humans, these cells are abundantly present in deep cortical layers, with cell bodies around 10 μm in diameter and ample processes 50 μm in length. However, human astrocytes are 3 fold larger and have more than 10 times ramified processes compared to rodent astrocytes (Allen and Eroglu, 2017). Also, gene studies on human astrocyte cells imply a different expression profile than in their rodent counterparts (Zhang et al., 2016). In the brain, some of these processes contact end-feet to blood capillary establishing perivascular end-feet, while protoplasmatic astrocytes that send processes to the pial surface form the glial limiting membrane, part of the blood-brain barrier (Fig. 1F,G).

In contrast, fibrous astrocytes in the spinal cord contribute to forming glia limitans (Fig. 1E,G). The arborization of adjacent protoplasmic astrocytes outlines distinct territorial domains and occupies non-overlapping domain organizations Fig. 1C,F (Sofroniew and Vinters, 2010). In general, protoplasmatic astrocytes show insignificant GFAP expression; however, it can be stained using pan astrocytic markers like ALDH1L1, GS, and Cx43. In addition, Cx30, Acsbg1, and EAAT2

Table 1.

Protoplasmatic astrocytes	Fibrous astrocytes
Located in gray matter	Located in white matter
Considerable amount in the CNS	Fewer in number in the CNS
Star-shaped	Relatively straight
Cytoplasm is granular (more organelles, less filaments)	Cytoplasm is less granular (less organelles, more filaments)
Cytoplasmic processes have short, branched and bushy processes	Cytoplasmic processes have long, unbranched, thin processes
Processes end-feet encircle neuronal bodies and synapses	Processes end-feet envelop Ranvier`s node and oligodedroglia.
Some of the processes are attached to the neighboring capillaries forming vascular end-feet	The long processes have vascular feet that enfold the capillaries
Metabolic mediators for the neurons	Repair damaged tissue

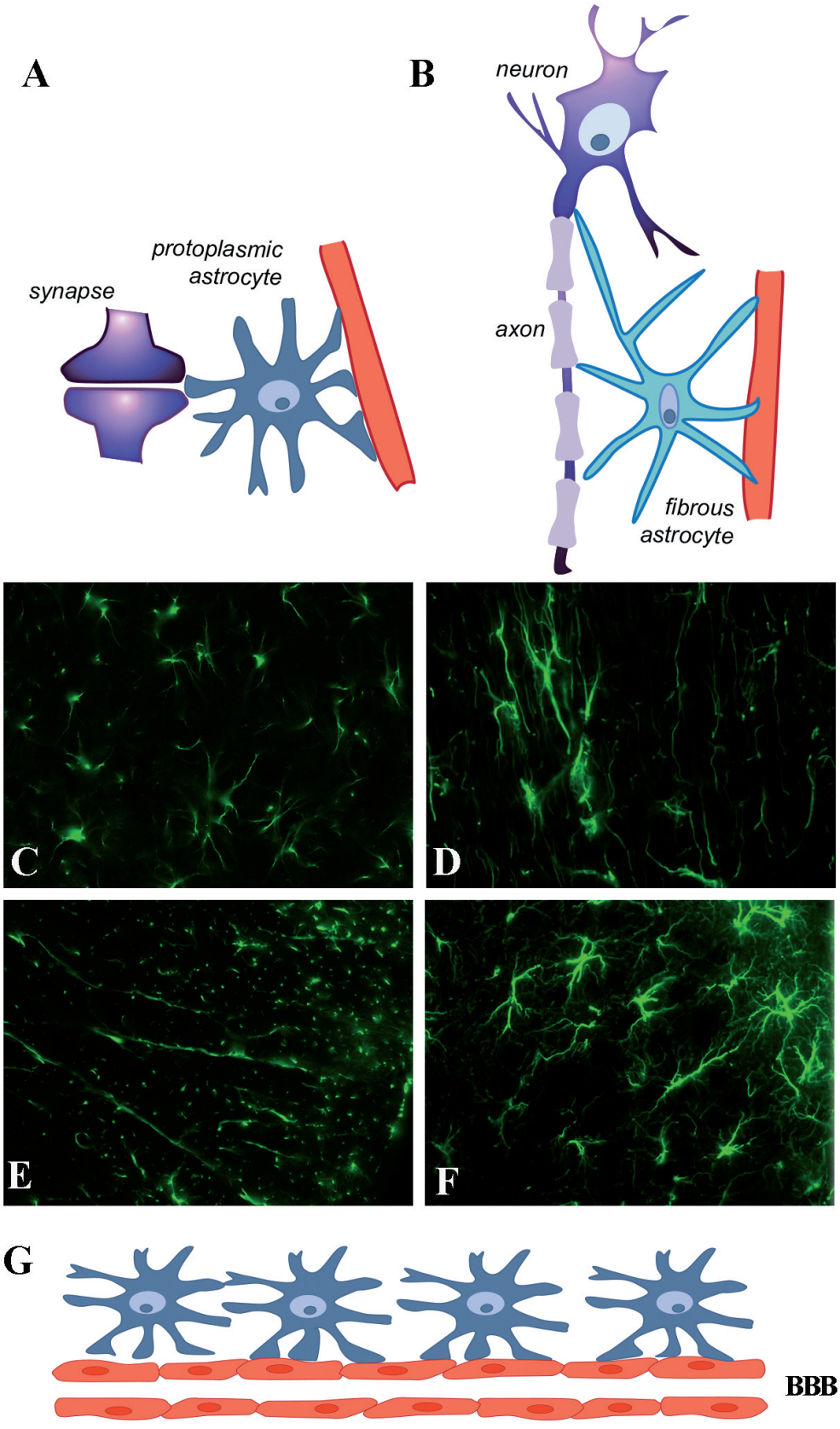

Fig. 1. Protoplasmatic and fibrous astrocytes in healthy spinal cord and brain. Protoplasmic astrocytes are in direct contact with blood vessels through a specific cellular compartment called the end-foot and ensheet (end-sheet? end-feet?) neuronal synapses through their fine perisynaptic processes **(A)**. Fibrous astrocytes are associated with myelinated axonal tracts and are in contact with the nodes of Ranvier **(B)**. The typical star-shaped protoplasmatic phenotype of astrocytes in the gray matter in the healthy spinal cord **(C)** and brain **(F)**. Elongated, unbranched fibrous astrocytes in the white matter of spinal cord **(E)** and brain **(D)**. Fibrous astrocytes in the spinal cord **(E, G)**, while in the brain, protoplasmatic astrocytes **(D, G)** contribute to forming glia limitans.

have a higher expression in this subtype.

Fibrous astrocytes

Other subtypes of astrocytes that reside in the white matter are fibrous astrocytes. They are linked to the myelinated axonal tracts and form intimate contact with the Ranvier's nodes (Fig. 1A). Morphologically, fibrous astrocytes are endowed with fewer, but long, relatively straight and unbranched, thin processes parallel with axons (Table 1, Fig. 1B,D). These processes are highly intermingled, thereby exhibiting overlapping domains. Nonetheless, neighboring cells are equidistant (Sofroniew and Vinters, 2010). The cytoplasm is less granular and contains only a few organelles, while the nucleus is spherical, with a dotted appearance. Previous studies point to white matter astrocytes as a generator of Ca^{2+} signals, which are mainly transmitted via purinergic receptors (Franke et al., 2012), as a response to neurotransmitters or/and axonal activity (Lecca et al., 2012). In general, it is established that electrically active axons may release neurotransmitters alongside and not just at synapses. Thus, it is accepted that neuronal support and synaptic formation, maintenance, and plasticity is the general astroglial function (Hamilton et al., 2008; Franke et al., 2012). It is plausible to think that interaction between fibrous astrocyte processes and the node of Ranvier might buffer the concentration of Na+ and K^+ ions. Indeed, due to neuronal excitability, astrocytes through Kv channel expression play a regulatory role in K^+ clearance (Bélanger and Magistretti, 2009).

Additionally, previously it was shown the expression of Kv1.3 and Kv1.5 on fibrous astrocytes in the rodent spinal cord (Bozic et al., 2018, 2019). The specific role of fibrous astrocytes is to provide metabolic support to the neurons, although their particular functions remain unclear (Sica et al., 2016). However, their function was associated with blood vessels and neuronal activity (Sica et al., 2016). Similar to the protoplasmic astrocytes, the fibrous astrocytes send processes in the white matter to the pial surface, where they form a portion of glia limitans (Lavrnja et al., 2012). Fibrous astrocytes are stained more intensely with GFAP compared to the protoplasmic astrocyte (Oberheim et al., 2009; Lavrnja et al., 2012). During EAE, the protoplasmic and fibrous astrocytes are activated during EAE (Lavrnja et al., 2012); however, in the demyelination areas, only fibrous astrocytes are selectively injured (Jukkola et al., 2013).

Astrocyte markers

Generally, to clarify the specific developmental origin and functional properties of protoplasmatic and fibrous astrocytes, additional research is needed. The detailed description of the markers is as follows:

Glial fibrillary acidic protein (GFAP)

GFAP (molecular weight 50kDa) is a protein encoded by the *GFAP* gene. It belongs to class-III intermediate filament, usually found in the mature and

Table 2. Potential astrocyte markers in health and neuroinflammation during MS/EAE.

Astrocyte marker	Fibrous astrocyte		Protoplasmatic astrocyte		Nuclear expression		Cytoplasmatic expression				Reference
							Cell body		Cell processes		
	healthy	MS/EAE	healthy	MS/EAE	healthy	MS/EAE	healthy	MS/EAE	healthy	MS/EAE	
GFAP	+++	++	+	++			++	++	++	++	Guo et al., 2011; Lavrnja et al., 2012; Jukkola et al., 2013
S100β	++		+++		+	+	++		+		Petzold et al., 2002; Barateiro et al., 2016; Zhang et al., 2019
ALDH1L1	+		++				+++				Cahoy et al., 2008; Waller et al., 2016; Yoon et al., 2017
CD44	+++	+++						+	++	++	Girgrah et al., 1991; Chang et al., 2012; Dzwonek and Wilczynski, 2015
GLAST/ EAAT1	+++	+++					++	+++	++		Swanson et al., 1997; Vallejo-Illarramendi et al., 2006; Köhler et al., 2019
GLT1/ EAAT2	+	+	++	++			+++	+++	++		Maragakis et al., 2004, Vallejo-Illarramendi et al., 2006, Köhler et al., 2019
Glutamine synthetase	+++	++	+	+			++	++	+		Waller et al., 2016; Jakovljevic et al., 2019; Zhang et al., 2019
Aquaporin 4	+		++	++			+	+	++	++	Rash et al., 1998; Jukkola et al., 2013; Jakovljevic et al., 2019
Connexin 30			+++						++		Nagy et al., 1999; Fang et al., 2018; Sánchez et al., 2020
Connexin 43	++		++						++		Brand-Schieber et al., 2005; Roscoe et al., 2007; Sánchez et al., 2020
Sox9			++		+	+					Stolt et al., 2003; Sun and Cornwell, 2017
Lipidosin	+	+	++	++			+	+	++		Song et al., 2007; Cahoy et al., 2008

+++: strong expression, ++: medium expression, +: low expression.

developing astrocytes in the CNS (Sofroniew and Vinters, 2010), non-myelinating Schwann cells in the PNS (Jessen and Mirsky, 2019), enteric glial cells (Sullivan, 2014), in radial glia and ependymal cells, which are derived from radial glia in the developing brain (Liu et al., 2006). It is regarded as the standard marker of mature astrocytes. On the contrary, several analyses have revealed that not all the astrocytes express GFAP. The higher GFAP expression was recorded in fibrous astrocytes, while negligible expression was found in the gray matter in protoplasmatic astrocytes. It was suggested that GFAP expression might be so low within a subset of cells, because it is fundamentally undetectable (Walz, 2000), and only ~15% of the total volume of an astrocyte is stained (Bushong et al., 2002; Oberheim et al., 2006). In contrast, the accurate astrocyte morphology remains challenging to differentiate among various astrocyte subtypes. Technical reasons may underlie this issue. Fixation procedures of the CNS tissue may result in inconsistency in the immunohistological detection of GFAP (Walz, 2000). Namely, GFAP antibodies only recognize the cytoskeleton, leaving the soluble subunits unstained, and provide a false negative result with regard to of GFAP expression in the cell (Sillevis Smitt et al., 1993). In addition, GFAP primarily delineates astrocyte processes, which prevents visualization of the entire cell. Conversely, neuroinflammation, or trauma-induced brain injury, leads to the formation of reactive gliosis, which is characterized by an increase in GFAP immunoreactivity (Sofroniew, 2015). It is interesting to note that a GFAP level is higher in astrocytes that reside in the developing and adult spinal cord in comparison to the astrocytes from the brain. Accordingly, GFAP reactivity in the spinal cord is more pronounced than in the brain due to inflammatory or toxin-induced demyelination (Lavrnja et al., 2012; Yoon et al., 2017).

Vimentin

During CNS development, vimentin (molecular weight: 57kDa) expression gradually decreases in astrocytes to undetectable levels, while GFAP levels increases. However, in Bergmann glia, Müller glia, radial glia and a subset of cortical the expression of vimentin in astrocytes continues in the adult CNS, pointing its functional role (Potokar et al., 2020). Generally, the expression of the cytoskeletal filament marker vimentin has been linked with immature as well as with reactive astrocytes (Hol and Pekny, 2015). In nonreactive astrocytes, GFAP and vimentin (low to intermediate levels) are main components of IFs. The low level of vimentin expression in astrocytes are needed for normal IFs formation, since in astrocytes lacking vimentin, IFs form more compact bundles when compared to control (de Pablo et al., 2019). The levels of vimentin expression in hypertrophic reactive astrocytes increases during neuroinflammation and neurodegeneration, as it was shown in animal models of brain injury, multiple sclerosis etc. (Peković et al., 2005; Voskuhl et al., 2009; Lavrnja et al., 2012, 2015). However, its expression is prominent in A2 reactive astrocytes, while GFAP is upregulated in both the A1 and A2 reactive astrocytes (Liddelow et al., 2017). In spinal cord of EAE animals, vimentin staining was observed in ependymal cells of central canal (Guo et al., 2011). Further, long and thin processes of immunopositive vimentin staining were found throughout white matter during EAE (Guo et al., 2011; Lavrnja et al., 2012; Jakovljevic et al., 2019). In gray matter of spinal cord, the low level expression of vimentin was found in deep gray matter around central canal (Guo et al., 2011; Savic et al., 2012), implying that gray matter consistency was altered during EAE (Huizinga et al., 2008).

S100β

S100β protein (molecular weight 10.7 kDa) belongs to an S100 protein family characterized by their calcium-dependent biological effects and is highly expressed in the brain, especially in astrocytes, and is one of the most abundant soluble proteins in the human brain, constituting 0.5% of them. However, S100β has been shown to label astrocytes, but it is also expressed by oligodendrocytes, their progenitors, and neurons (Hachem et al., 2005). It is commonly used as a marker for mature astrocytes. Expression of S100β was seen in all regions of the brain and spinal cord, where it occupies the nucleus and part of the cytoplasm and processes (Waller et al., 2016; Zhang et al., 2019). In addition, S100β fails to express in astrocytes of the germinal zones (Raponi et al., 2007). Although the exact physiological function of S100β is unknown, it has been attributed to play a crucial role in cell-cell interaction, cell cycle regulation, cytoskeleton organization, and scavenging of toxic substances (ROS, COX-2, cytokines, and other inflammatory markers) at the BBB. Also, intracellular S100β is considered to stimulate cell proliferation, migration, along with inhibition of apoptosis and differentiation (Donato et al., 2009). In astrocytes, extracellular S100β is known to regulate their response to inflammation in a receptor for advanced glycation end products (RAGE) dependent manner (Bongarzone et al., 2017). At high concentrations of S100β in the extracellular milieu, it actively takes part in the pathological condition in neurodegenerative diseases (Michetti et al., 2019), where it can be used as a biomarker of cell damage in the nervous system (Michetti et al., 2012, 2019). During MS, it has been shown that S100β levels correlate to the severity of the disease (Michetti et al., 2012). Indeed, around active and chronic demyelinating foci in the white and gray matter during MS, increased expression of S100 β was noticed (Petzold et al., 2002; Barateiro et al., 2016). A recent study proposes S100β as a potential therapeutic target in MS, where blocking this protein during EAE a significant amelioration of clinical symptoms occurs (Di

Sante et al., 2020).

ALDH1L1

Aldehyde dehydrogenase 1 family, member L1 (ALDH1L1) (molecular weight 99kDa) is found to be expressed in rodent astrocytes (Neymeyer et al., 1997). ALDH1L1, also known as 10- formyltetrahydrofolate dehydrogenase is a folate metabolizing enzyme, with a role in various reactions, including *de novo* nucleotide biosynthesis and the regeneration of methionine, influencing growth and division of the cells (Krupenko, 2009; Horita and Krupenko, 2017). It was reported that ALDH1L1 stains all astrocytes within the brain, preferably cortical astrocytes, while, as mention above, GFAP was predominantly expressed in the white matter astrocytes (Waller et al., 2016). GFAP staining delineates mainly processes, while ALDH1L1 immunoreactivity was restricted to the cell body (Waller et al., 2016). It was suggested that using ALDH1L1 antibody pure astrocytic cultures can be established with FACS analysis (Cahoy et al., 2008), presenting this protein as a highly specific, astrocytic marker. However, it was reported that ALDH1L1 might co-localize oligodendrocyte (Zhang et al., 2019). Astrocyte marker ALDH1L1 occurs earlier during development in the spinal cord astrocytes than in the brain (Yoon et al., 2017); the decrease in ALDH1L1 expression was still accompanied by the maturation of spinal cord astrocytes (Yang et al., 2011). During demyelination, increases in ALDH1L1 were equally increased throughout CNS (Yoon et al., 2017). In active lesions in MS, it was reported that ALDH1L1 expression is increased (Ludwin et al., 2016). However, no specific changes in ALDH1L1 expression in reactive astrocytes associated with brain injury, aging, and/or neurodegenerative disease was observed (Yang et al., 2011).

CD44

CD44 (molecular weight: 85-90 kDa protein) is a cell surface glycoprotein and cell adhesion molecule. As a ubiquitous molecule, CD44 is expressed in various types of lymphoid cells and CNS cells, including astrocytes. In humans, astrocytes that express CD44 might be divided in two populations. Namely, one population presents astrocytes with long processes that are CD44+, where these processes finished on blood vessels. The second population comprises mixed phenotypes of star-shaped cells with tiny processes and cells with elongated processes lacking branches (Sosunov et al., 2014). In rodent astrocytes, the staining by CD44 was found to be expressed only in long, un-branched, pial-based, fibrous-like astrocytes in white matter, while in gray matter CD44 was lacking from protoplasmatic astrocytes (Chang et al., 2012; Dzwonek and Wilczynski, 2015). It is assumed that CD44 recognizes astrocyte-restricted precursor cells (Liu et al., 2004). Also, it was suggested that CD44 influences alternation in the shape of astrocytes (Dzwonek and Wilczynski, 2015). Previously, it was reported that CD44 is upregulated in hypertrophic astrocytes in the vicinity of demyelination foci in MS (Girgrah et al., 1991). The finding that CD44 deletion can lead to the increased severity of EAE implies that CD44 has a role in limiting inflammation (Flynn et al., 2013).

EAAT1 or GLAST

Excitatory amino acid transporter 1 (EAAT1 or sodium-dependent glutamate-aspartate transporter GLAST) (molecular weight: ~ 60kDa, GLAST/EAAT1) is the main glutamate transporter in astrocytes, where it was involved in the control of glutamate concentration and neuronal migration (Šerý et al., 2015). GLAST/EAAT1 is abundantly present in the cerebellar cortex, Muller cells of the retina, cerebellum, and tanycytes in the circumventricular organs. Also, its expression was confirmed at the mammalian neuromuscular junction and in the choroid plexus (Šerý et al., 2015). In the adult CNS, GLAST/EAAT1 has a role in the glutamate clearance from the extracellular space, preventing excitotoxicity. Since the highest level of GLAST/EAAT1 was found in the white matter astrocytes, its leading role is to protect neurons (Köhler et al., 2019). It was shown that GLAST/EAAT1 is found in astrocytes in the vicinity of excitatory synapses (Murphy-Royal et al., 2017). The main localization of GLAST/EAAT1 was observed in astrocytes' cell bodies (Swanson et al., 1997) and the extending delicate processes of astrocytes (Waller et al., 2016).

EAAT2 or GLT1

Excitatory amino acid transporter 2 (EAAT2 or glutamate transporter 1/GLT1) (molecular weight: ~ 62 kDa) is another glutamate transporter found to be expressed in astrocytes late in development, associated with the migration and maturation of neurons (Holmseth et al., 2009; Gegelashvili and Bjerrum, 2019). The main function of this transporter is the clearance of the glutamate. GLT1/EAAT2 shows robust expression and specificity in astrocytes compared to GFAP (Lovatt et al., 2007); however, it is downregulated in the diseases associated with reactive gliosis (Verkhratsky et al., 2016; Dorsett et al., 2017). In the adult brain, GLT1/EAAT2 is expressed in protoplasmatic and fibrous astrocytes. The dominant expression of GLT1/EAAT2is reported in the gray matter astrocytes (Maragakis et al., 2004), while its expression is lower in the white matter astrocytes (Köhler et al., 2019). In rodent spinal cord, GLAST/EAAT1 and GLT1/ EAAT2 are abundantly expressed, mainly on their cell bodies (Regan et al., 2007) and thin processes (Rothstein et al., 1996). The expression of GLAST/EAAT1 and GLT1/ EAAT2 is increased during EAE and MS. However, their expression was observed in microglia and oligodendrocytes, so using these antibodies as markers

for astrocytes is questionable (Vallejo-Illarramendi et al., 2006; Mitosek-Szewczyk et al., 2008).

Glutamine synthetase

Glutamine synthetase (GS) (molecular weight 45 kDa) is accepted as a classical astrocyte marker. Its presence was proved in the brain primarily in astrocytes in the retina in Muller cells, Bergmann glia, and tanycytes. It was reported that GS mRNA is found early in the development in the brain and increases its expression during maturation of astrocytes (Mearow et al., 1989). GS stain high and low GFAP expressing astrocytes. These cells support neurons using glutamate as a neurotransmitter (Anlauf and Derouiche, 2013). This enzyme, as a glutamate catabolizing enzyme, has a role in synthesizing glutamine, using ammonium and glutamate (Suárez et al., 2002). Since GS is involved in the Glutamate-Glutamine cycle, its main role is to prevent excitotoxicity in the neurons (Rose et al., 2013). In the rat spinal cord, GS expression was confined to the astrocytes, oligodendrocytes, and microglia (Cammer, 1990), where GS expression was lower than GFAP levels (Patel et al., 1985). It was shown that GS staining was found in the astrocyte cell body and thick processes (Waller et al., 2016). GS expression was higher in fibrous astrocytes when compared to the protoplasmatic astrocytes in the rodent spinal cord. Accordingly, in fibrous astrocytes, GS staining is uniformly distributed throughout the cytoplasm, however their processes and vascular end-feet are heavily stained (Jakovljevic et al., 2019). The dense GS staining in gray matter is attributed to the oligodendrocytes (Anlauf and Derouiche, 2013). However, the expression of GS was reported in protoplasmic astrocytes of the murine hippocampus (Zhang et al., 2019). During the inflammatory-induced demyelination, GS expression was associated with the neuroprotective phenotype of astrocytes (A2) (Jakovljevic et al., 2019).

Aquaporin 4

Aquaporin 4 (AQP4)(molecular weight: 48kDa), is another important marker for astrocytes, representing a water-selective channel protein responsible for the transport of water. It is abundantly present at the end-feet of astrocytes, where it regulates water transport through the blood-brain barrier, along with extracellular K+ clearance. In addition, AQP4 is involved in the clearance of interstitial fluids having an important role in the brain glymphatic system (Mestre et al., 2018). In the rodent brain, a smaller amount of fibrous astrocytes expressed AQP4 in comparison to a stronger expression in protoplasmic astrocytes, found to be localized to the perivascular endfeet (Rash et al., 1998; Abbott et al., 2006). In MS, the expression of AQP4 is up-regulated in demyelinating lesions and in normal-appearing white matter (Masaki et al., 2013). During EAE, in the spinal cord, AQP4 is highly expressed in the fibrous astrocytes, while its expression in protoplasmatic astrocytes is limited and concentrated in the astrocytic endfeet (Jukkola et al., 2013).

Gap junction alpha-1 protein or Connexin 30 and 43 (Cx30/Cx43)

It was established that astrocytes express Cx30 and 43 (the numbers relate to their molecular weight), where the most abundantly expressed connexin is Cx43. During development, low-level Cx43 expression was found in radial glia, which increases postnatally, mainly in astrocytes. The expression of connexins at astrocyte endfeet maintains the integrity of the blood-brain barrier (Abbott et al., 2006). There are observed differences in the expression of Cx30 and 43 in fibrous and protoplasmatic astrocytes, because of their different coupling properties. Namely, protoplasmatic astrocytes are more coupled by gap junctions than fibrous astrocytes; thus, Cx 30 and 43 are not spread equally in these astrocytic subpopulations (Nagy et al., 1999). It was found that protoplasmic astrocytes strongly express Cx30 and 43. In general, the fibrous astrocytes express Cx43, while Cx30 expression is absent. However, when Cx43 is lacking in white matter astrocytes, the expression in Cx 30 is up-regulated (Sánchez et al., 2020). Indeed, recently, it was reported that Cx30 deficiency provokes some increase of protoplasmatic astrocytic processes in the spinal cord with concomitant reduction of Cx43 expression fibrous astrocytes (Fang et al., 2018). It is important to note that modification of Cx expression has been described in brain tumors and several neurodegenerative diseases, including Alzheimer's, Huntington's, and Parkinson's disease (Sánchez et al., 2020). In MS and MS-associated diseases (neuromyelitis optica and Balo's disease), Cx43 loss in astrocytes was observed (Masaki et al., 2013). In the spinal cord in animals afflicted with EAE, alternation in Cx43 was recorded (Brand-Schieber et al., 2005), and the decrease was noted in the vicinity of inflammatory infiltrates (Roscoe et al., 2007).

Sox9

Sox9 (SRY-Box Transcription Factor 9)(molecular weight: 56kDa) is a member of the high mobility group box (HMG-box) family of transcription factors (Batiuk et al., 2020). It was shown that gene and protein Sox9 expression is conveyed exclusively by astrocytes in the adult murine brain (Lovatt et al., 2007). SOX9 is primarily found in the nucleus and specifically labels astrocytes outside of the neurogenic regions (Sun et al., 2017). Due to its localization, it was proposed that SOX9 could be a useful marker to evaluate astrocytes in various pathologies (Yu et al., 2020). During development, Sox9 has role specification of both astrocytes and oligodendrocyte lineage cells (Tatsumi et al., 2018). In the adult brain, Sox9 might be a useful

marker in gray matter astrocyte diversification (Stolt et al., 2003). It was found that Sox9 did not modify its expression during aging; however, it was up-regulated in reactive astrocytes in mouse models of ALS and stroke (Sun et al., 2017). Also, Sox9 is up-regulated after spinal cord injury (Xia and Zhu, 2015). In general, Sox9 might be a useful marker in the identification of astrocytes, particularly in settings where astrocytes lose their domain organization.

Acyl CoA synthetase bubblegum family member 1 (Acsbg1 or Lipidosin)

Lipidosin (molecular weight:80kDa) is a protein with long-chain fatty acid-activating enzymes expressed in the brain, adrenal gland, and gonads. In the brain, lipidosin is expressed postnatally and is abundantly present in the astrocytic cytoplasm (Cahoy et al., 2008). Also, lipidosin staining was confirmed in the end-foot processes near blood vessels and thick processes adjacent to the neuropil (Song et al., 2007). After brain injury, the levels of lipidosin are increased (Wu et al., 2012). It was described that lipidosin shows higher expression in protoplasmic astrocytes in comparison to fibrous astrocytes. Since lipidosin expression in astrocytes is higher around remyelination areas, it was proposed to have a role in the recovery of myelin (Song et al., 2007).

Astrocytes in MS pathology

MS is a chronic neurological disease characterized by excessive inflammation, demyelination, and axonal degeneration, leading to serious neurological disturbance (Bjelobaba et al., 2017). It is accepted that the main inflammatory culprits in MS pathology are myelin reactive immune cells from the periphery that migrate to the CNS and cause demyelinating lesions and axon damage. However, the immune response by resident CNS cells has also been recognized as a crucial component of inflammatory injury to oligodendrocytes and axons. Indeed, reactive astrocytes have been found in and around demyelinating lesions, and it is now known that these cells have complex roles in MS pathology (Correale and Farez, 2015).

It was previously believed that the role of astrocytes in lesion pathology comes mainly after the inflammatory stage, in the formation of the glial scar. However, it is now known that astrocytes are involved early in the initiation of the lesion formation, as well as in the chronic phases of the lesion pathology, even after the acute inflammation has subsided and immune cells have withdrawn (Ponath et al., 2018). Their number, morphology, and degree of reactivity are heterogeneous and depend on the stage of the disease, how far or close they are from the lesion and whether they are positioned in the white or gray matter. Astrocytes appear to be activated and hypertrophic even at the early stages of the disease, before lesion formation. In active lesions, astrocytes are characterized by hypertrophic cell bodies, reduced number of processes that appear swollen, large nuclei, and elevated expression of GFAP and other intermediate filaments (Ludwin et al., 2016; Brambilla, 2019). They are associated with T cells and oligodendrocytes, which they ingest (Ludwin et al., 2016). It was demonstrated that astrocytes in active lesions contain myelin debris and that this uptake of myelin was an early event, followed by activation of NF-κB, secretion of pro-inflammatory cytokines and chemokines and recruitment of inflammatory cells (Ponath et al., 2017). Reactive astrocytes within lesions that are in close contact with vasculature also show hypertrophic cell bodies, damaged end-feet, and dissociated gap junctions, causing BBB disruption and entry of immune cells into the CNS (Brosnan and Raine, 2013).

Additionally, they express VEGF, promoting angiogenesis in the acute lesion (Ludwin et al., 2016). Reactive astrocytes have also been found in the normal-appearing white and gray matter that is adjacent to the lesions, suggesting their role in early lesion development (Ponath et al., 2017, 2018). In chronic inactive lesions, astrocytes with elevated GFAP levels and differing degrees of reactivity can be found (Ludwin et al., 2016).

Astrocytes directly influence lesion development by secreting chemokines such as CCL2, CCL5, CXCL8, CXCL10, and CXCL12, which attract immune cells from the periphery and microglia to the lesion site. Furthermore, by secreting pro-inflammatory cytokines, reactive oxygen and nitrogen species, as well as glutamate and ATP, astrocytes have an immediate toxic effect on oligodendrocytes and axons (Correale and Farez, 2015). In MS, normal functions of astrocytes are disrupted, including the uptake of glutamate, which leads to increased extracellular concentrations of glutamate that are damaging to neurons and oligodendrocytes (Matute et al., 1997). TNF-α decreases the expression of glutamate transporter GLAST in astrocytes, causing reduced glutamate intake and contributing to excitotoxicity (Korn et al., 2005).

In MS, as in other CNS insults, astrocytes form the glial scar, as evidenced in tissue from MS patients and animals with EAE (Holley et al., 2003). The function of the glial scar is to limit the further entry of leukocytes in the CNS and stop the spread of damage to the neighboring tissue (Voskuhl et al., 2009; Correale and Farez, 2015) . Deletion of the glycoprotein 130 (gp130) in astrocytes, IL-6 receptor, led to apoptosis of astrocytes in inflammatory lesions, which caused further infiltration of T cells and more severe EAE pathology (Haroon et al., 2011). However, apart from valuable functions, glial scar also has an inhibitory effect on axon regeneration and remyelination. It is characterized by the massive production of inhibitory CSPGs (Lau et al., 2012), FGF-2 that prevents maturation of OPCs and consequently restricts remyelination (Goddard et al., 1999), and ephrins that induce the collapse of the axonal

growth cone (Wahl et al., 2000).

In summary, astrogliosis is an early and persistent component of MS pathology that appears to be a good indicator of disease progression (Brambilla, 2019). Considering that astrocytes have dual roles in the pathogenesis of the disease, the net effect of their actions will depend on the stage of the disease, distance from the lesion, degree of reactivity, and their interactions with other cells involved in the process (Williams et al., 2007).

Roles of astrocytes in myelination and remyelination

Astrocytes have an important role in proliferation and maturation of OPC, by producing key growth factors for this process, including PDGF, BDNF, LIF, CNTF, neurotrophin-3, neurotrophin-4 and IGF1 (Salem et al., 2016). Astrocytes also control molecular cues that regulate oligodendrocyte production (Traiffort et al., 2020). For example, in the optic nerve astrocytes express the multiligand megalin receptor that binds Sonic Hedgehog (Shh), which is important for proliferation and migration of the OPCs during the development of the optic nerve (Ortega et al., 2012). They also provide crucial metabolic support, by supplying lactate, important as an energy source, but also as a precursor in lipid biosynthesis. The critical component of myelin is cholesterol, which cannot pass the BBB and is synthesized in the CNS by astrocytes and oligodendrocytes. Cholesterol produced by astrocytes is transported to oligodendrocytes via apolipoprotein E (ApoE) and ATP-binding cassette transporter ABCA1 (Traiffort et al., 2020). The availability of cholesterol for oligodendrocytes appears to be a rate-limiting factor for myelination (Liu et al., 2010). In relation to this, the cholesterol synthesis pathway was found to be down-regulated in astrocytes in EAE, and cholesterol homeostasis seems to be a novel treatment target (Itoh et al., 2018). Astrocytes also provide SREB cleavage activating protein (SCAP), which is crucial in lipid production (Nutma et al., 2020).

Furthermore, GFAP appears to be important for white matter integrity, since GFAP knockout mice exhibit abnormal myelination (Liedtke et al., 1996). Additionally, deletion of oligodendrocytic Cx47 and astrocytic Cx30, which are important for gap junctional communication between astrocytes and oligo-dendrocytes, caused serious myelin pathology (Tress et al., 2012). The search to depict all the molecular mechanisms through which astrocytes contribute to myelination is still ongoing; however, it is clear that astrocytes have many crucial roles in this process (Nutma et al., 2020).

In demyelinating diseases, such as MS, the damaged myelin sheath is one of the components that inhibit remyelination (Kotter et al., 2006). Astrocytes contribute to the clearance of myelin debris by recruiting microglia to the damaged areas, and ablation of astrocytes inhibits regeneration of oligodendrocytes and myelin (Skripuletz et al., 2013). However, they were also directly involved in myelin phagocytosis, as astrocytes in active lesions were shown to contain myelin debris. Myelin uptake by astrocytes appears to be an early event followed by activation of NF-ϰB, secretion of chemokines, and recruitment of immune cells (Ponath et al., 2017). For remyelination to be successful, the OPCs need to migrate to the injury site, differentiate and mature into oligodendrocytes. Astrocytes play critical roles in this process, and it was shown that remyelination is not carried out if they are absent (Talbott et al., 2005). Astrocytes support oligodendrocytes in remyelination by producing CNTF, BDNF, LIF, and other growth factors (Butzkueven et al., 2002; Albrecht et al., 2003; Miyamoto et al., 2015). They also produce anti-inflammatory IL-4 that indirectly protects oligodendrocytes from NO by inhibiting NF-ϰB activation, which leads to the downregulation of iNOS (Paintlia et al., 2006).

Nevertheless, remyelination fails in chronic MS lesions, and astrocytes' destructive functions are a part of this process. They secrete pro-inflammatory cytokines that lead to apoptosis of oligodendrocytes, e.g., TNF-α, which was found in fibrous astrocytes in active MS lesions (Selmaj et al., 1991). Astrocytes also express endothelin-1that inhibits remyelination through Notch activation (Hammond et al., 2014). CSPGs that astrocytes produce in the glial scar also have an inhibitory effect for remyelination, and their degradation leads to an improvement in remyelination (Lau et al., 2012). Specifically, CSPGs inhibit outgrowth and differentiation of OPCs, while treatment with chondroitinase ABC reversed this effect *in vitro* (Siebert and Osterhout, 2011). Furthermore, a novel inhibitor of CSPG synthesis rescued OPCs process outgrowth *in vitro* and improved remyelination in mice with focal demyelination (Keough et al., 2016).

Taken together, these studies point to the dual and complex role of astrocytes, on the one hand, necessary for myelination, yet their functions in remyelination can be both beneficial and detrimental. This could also be a consequence of their heterogeneity and the existence of different subtypes in MS lesions. Indeed, Haindl and colleagues found an A1 subtype in active lesions, while A2 was present in remyelination (Haindl et al., 2019). Thus, finding approaches that inhibit detrimental actions of astrocytes and simultaneously stimulating their beneficial roles could be the needed therapeutic strategy for the treatment of MS and other neurological diseases. The characterization of astrocytes must involve multipoint features to define better cell types that include morphology and protein and gene profiling with methods that involve the determination of functional properties.

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