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# Role of extracellular matrix in neurodevelopment and neurodegeneration

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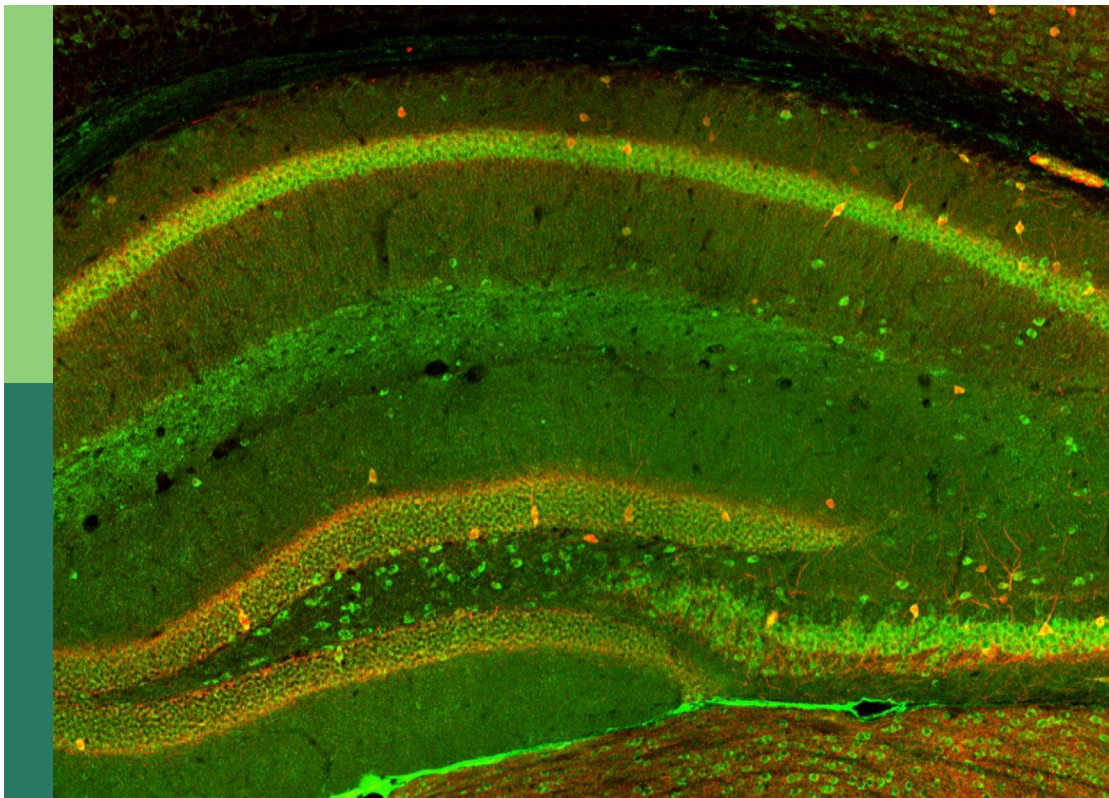

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# Role of extracellular matrix in neurodevelopment and neurodegeneration

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# Editorial: Role of extracellular matrix in neurodevelopment and neurodegeneration

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Editorial on the Research Topic
Role of extracellular matrix in neurodevelopment and neurodegeneration

Extracellular matrix (ECM) is a dense and dynamic network of proteins and sugars embedding various types of cells of the nervous system. It is composed of numerous macromolecules like collagen, elastin, fibronectin, laminin, glycoproteins like tenascin, glycosaminoglycans (GAGs), and proteoglycans. These components are secreted by both neurons and glial cells. It constitutes around 20% of brain volume, yet it has not received the required attention from neuroscience research community. So far, a majority of research focus has been on either neuron or glial cellular components. The role of extracellular system on the etiology and progression of brain disorders and vice versa, how neurological disorders affect the extracellular matrix remains largely unexplored.

The ECM is known to play multiple roles during neurodevelopment, however its role in development of human brain is not fully understood. Condensed ECM, comprised of the perineuronal net (PNN), forms a mesh-like structure surrounding the cell body and proximal neurites of neurons (Sigal et al., 2019). During nervous system development ECM modulates neural progenitor cell proliferation and differentiation. It also governs the cellular morphology including axonal and dendritic elongation regulating their connectivity and cortical folding. Additionally, ECM stores signaling factors that create a microdomain to regulate neuronal migration and synaptic plasticity (Dityatev et al., 2010; Dick et al., 2013). The PNN is thought to act as a molecular brake to close and regulate the critical period of synaptic plasticity (Dityatev et al., 2010; Wang and Fawcett, 2012). Thus, ECM dysfunction, especially PNN impairment has been linked to several neurodevelopmental disorders like autism spectrum disorders, schizophrenia, bipolar disorder, Fragile X syndrome and epilepsy (Reinhard et al., 2015; Rogers et al., 2018; Wen et al., 2018).

Several decades of research on neurodegenerative diseases has indicated increased neuron death but the mechanism behind the ill health of neurons is still far from clear. The function and capabilities of extra cellular matrix around the dying cell has not been investigated in detail. Recently, an interplay between neurodegeneration, extra cellular space and matrix is reported in Parkinson's disease rodent model shedding light upon a neglected compartment for the diffusion of aggregated α-synuclein seeds (Soria et al., 2020). As reviewed recently by Pinter and Alpar, selective ECM components can either proactively trigger the disease-specific toxicants, or reactively accumulate them in ECM (Pinter and Alpar, 2022). Several studies have linked

ECM to neurodegeneration in Alzheimer's, Parkinson's, amyotrophic lateral sclerosis (ALS) and Huntington diseases but there are still huge knowledge gaps regarding the role of ECM and therapeutic potential of its components.

This special issue comprises a collection of original research and review articles which enhance our understanding on the diverse role of ECM in brain development and neurodegeneration.

A well-illustrated article by Long and Huttner reviewed the role of ECM in human neocortex development and compared it to non-human primates. They focused on the neurodevelopmental processes like neural progenitor proliferation and differentiation, elongation and connectivity of neurites, neuronal migration, cortical folding, and neurodevelopmental disorders. With long lasting molecular composition and highly robust nature, ECM is often seen as a stable structure required to maintain neural network in shape. However, in order to support synaptic changes in the adult brain, ECM has the abilities to be remodeled at synapses. ECM remodeling includes regulated secretion of proteolytic enzymes at the synapse along with synthesis of new ECM molecules. Using beautiful figure depictions Dankovich and Rizzoli wonderfully reviewed the existing paradigm for ECM remodeling and recycling that allows synapse to be plastic. The review article by Chen et al. has discussed how sensory activities impact multiple neuronal and glial structures along with the extracellular components within the brain. Sensory deprivation has been shown to impact dendrites and their associated spines, particularly focusing on the cerebral cortex using the rodent whisker-to-barrel system as an illustrative model. This review provided a better understanding of structural plasticity, encompassing multiple aspects of neuro-glial cells, and extra-cellular domain interactions as a system. ECM is required to be remodeled for efficient synaptic plasticity and brain functioning.

Mood disorders and anxiety resulting from environmental stress is a big concern for all of us nowadays, and have caught the interest of Laham and Gould as well. They have contributed a mini-review article to this special issue on ECM, and provided a general overview of studies linking the ECM to brain function during development and adulthood. They have discussed and tabulated the effect of stress on both diffuse and structured ECM, and its functional consequences on emotion processing, learning and memory.

In addition to neurodevelopmental disorders, the functional deficit of neural networks following injury can result from poor regeneration of damaged axons and insufficient target innervation due to faulty ECM. Using a rat contusion model of severe spinal cord injury Kabdesh et al. investigated the spatial and temporal changes in the neuron-glial antigen 2 (NG2) proteoglycan. They identified the change in NG2 expressing cell numbers and the molecular shifts in the ECM of the areas distant from the injury site potentially affecting extended axonal growth and synaptic condition. Through their original research article, they reported elevation in NG2 levels in the segments surrounding the injury along with its comprehensive characterization and distribution around the boundaries of scar formation.

Another original research article on ECM proteoglycans, Brevican, and Neurocan by Hußler et al. measured the levels of these signature proteoglycans in the cerebrospinal fluid (CSF) and serum of 96 neurological patients including ALS, epilepsy and small vessel disease cases. They concluded that monitoring the proteolytic cleavage products of brain-derived perineuronal ECM molecules, such as neurocan fragments, may allow insights into the integrity of the brain's extracellular environment and assist as fluid biomarkers for neurological disorders. With more precise detection techniques, and advancement of methods to restore and maintain myelin functions at early disease states hold great potential for alleviating neurodegeneration. White matter abnormalities due to myelin damage have been associated with multiple neurodegenerative conditions. The original research article by Abi-Ghanem et al. examined the selective homing of cysteine-alanine-lysine glutamine (CAQK), tetrapeptide to sites of myelin damage in three different mouse models of acute, immune-mediated, and toxic demyelination. They assessed the homing by administering fluorescein amine (FAM)-labeled CAQK peptides into the bloodstream of mice and analyzing the sites of demyelination in comparison with healthy brain or spinal cord tissue. The labeled peptides were primarily associated with the fibrous ECM deposited in interstitial spaces proximal to reactive astrocytes at the lesion sites. Thus, CAQK peptide targeting can be developed as diagnostic and therapeutic tool aimed at localized myelin repair in multiple sclerosis and associated disorders.

Further with an interest to identify druggable therapeutic targets for neurodegenerative diseases, Moretto et al. presented an overview of the documented roles of ECM components in the spreading of pathological protein aggregates and recognized the lacunas in the field which needs to be addressed. This review underscored the requirement of extensive *in vivo* experiments in animal models of neurodegeneration, which retain the complex ECM web in its native state, and improved cell culture systems to better recapitulate the ECM under neurodegeneration.

Glial cells, predominantly astrocytes, are one of the major sources of ECM molecules during development and in the adult CNS, hence they play a critical role in ECM mediated brain functions. They also release cleavage proteases, the main effectors of ECM and PNN remodeling during development, adulthood, aging, and diseases. The review article by Tewari et al. discussed the recent advances and understandings, how glial cells are central to ECM and PNN remodeling in normal and disease states of the brain. The pivotal contribution of glial cells to the ECM remodeling process encourages further discussion of glia-centric approaches in addition to the focus on neurons for effective treatment modalities.

Taken together, the insightful review and original research articles complied in this Research Topic on the role of extra cellular matrix in neurodevelopment and neurodegeneration will improve our understanding and stimulate further research ideas on ECM. Further research is warranted to explore the potential of ECM as a diagnostic biomarker and therapeutic target for neurodevelopmental disorders and neurodegenerative diseases.

## Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

## Conflict of interest

CE was employed by Annexon Biosciences.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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REVIEW
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# Sensory Experience as a Regulator of Structural Plasticity in the Developing Whisker-to-Barrel System

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Cellular structures provide the physical foundation for the functionality of the nervous system, and their developmental trajectory can be influenced by the characteristics of the external environment that an organism interacts with. Historical and recent works have determined that sensory experiences, particularly during developmental critical periods, are crucial for information processing in the brain, which in turn profoundly influence neuronal and non-neuronal cortical structures that subsequently impact the animals' behavioral and cognitive outputs. In this review, we focus on how altering sensory experience influences normal/healthy development of the central nervous system, particularly focusing on the cerebral cortex using the rodent whisker-to-barrel system as an illustrative model. A better understanding of structural plasticity, encompassing multiple aspects such as neuronal, glial, and extra-cellular domains, provides a more integrative view allowing for a deeper appreciation of how all aspects of the brain work together as a whole.

**Keywords: microglia, perineuronal net (PNN), barrel cortex, whiskers, develoment**

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## INTRODUCTION

A fundamental question of modern neuroscience centers on how sensory experience shapes the development of the brain and its composite circuitry. Classic works demonstrated that influencing a neocortical neuron's activity by means of peripheral manipulation such as finger amputation (Merzenich et al., 1984) or dark rearing (Blakemore and Van Sluyters, 1975) can affect many features of neuronal structure and function. Early studies suggested that suturing one eye alters the development of structures within the primary visual cortex (Hubel et al., 1979). Within the somatosensory cortex (S1), studies have found that ablating a whisker follicle at birth prevents the development of that whisker's cortical representation (Van der Loos and Woolsey, 1973). Within these many models of manipulating sensory-activities a commonality is that early sensory experience, or lack of it, has profound impacts on the developing brain. The rodent whisker-to-barrel system provides a valuable model for exploring this question of experience-dependent plasticity due to its well-defined local circuits, which develop postnatally, and the ease of peripheral manipulation of afferent activity (for review see Erzurumlu and Gaspar, 2020) to this system.

Woolsey and Van Der Loos discovered the cellular conglomerates aggregated in layer 4 of primary somatosensory (S1) in rodents and reported such cellular clusters as "barrels" that

represent the contralateral whiskers of the mystacial pad in a one-to-one fashion (Van der Loos and Woolsey, 1973; referred to as S1BF hereafter). They then demonstrated that ablating the vibrissae follicles on the contralateral mystacial pad soon after the rodents' birth resulted in a large-scale, dramatic reorganization of barrel patterning (Van der Loos and Woolsey, 1973). However, such a large-scale alteration of areal patterning of barrels was reduced following a developmental critical period after the animals have matured (Weller and Johnson, 1975; Woolsey and Wann, 1975) such that after 5 days post birth, the same manipulations no longer impacted the organization of the barrel cortex. The behavioral and physiological consequences of removing vibrissae from birth further suggested that sensory experiences play a critical role in shaping the proper development of neuronal circuitry and its governed behavior (Simons and Land, 1987; Carvell and Simons, 1996). Whisker removal in rodents caused them to no longer distinguish subtle changes in surface texture even after whisker regrowth (Carvell and Simons, 1996), and the neurons corresponding to clipped whiskers become more excitable (Simons and Land, 1987). These seminal works provided the foundation for subsequent research to explore the structural basis of how sensory deprivation affects circuits in the neocortex in general.

Over recent years, a branch of research has been focusing on the structural basis of the observed physiological and behavioral plasticity that resulted from chronic sensory deprivation (Zuo et al., 2005; Yang et al., 2009; Briner et al., 2010; Chen et al., 2015a). Such structural basis is not limited to neuronal aspects only but also extends to glial as well as extracellular components as well (McRae et al., 2007; Barrera et al., 2013; Chen et al., 2015b; Chu et al., 2018; Kalambogias et al., 2019), indicating that the impact of chronic sensory deprivation far exceeds the conventional assumption that only neurons are affected. These findings illustrate that multiple systems in the brain are affected simultaneously, further suggesting that neurons, glia, and extracellular components operate in concert with one another, coordinating a symphony that denotes how activities shape the proper development of the brain as a whole. From this multi-faceted perspective, it appears that the extent of experience-dependent structural plasticity in the brain has multiple components, all interacting with one another (see **Figure 1**), and which may be the structural foundation that ultimately serves as the basis of behavioral and, or cognitive processes.

This review focuses on how sensory deprivation impacts these multiple aspects of extracellular and cellular structure within the brain with a particular emphasis on the S1BF, similar findings have been observed in other sensory systems (Berardi et al., 2003; Persic et al., 2020; Baroncelli and Lunghi, 2021) but are beyond the focus of the current review. In characterizing structural plasticity, researchers traditionally focused on fixed tissue preparations. However, recent advancements in technology have provided new windows of opportunity to peek into the living brain, with the breakthrough of multi-photon *in vivo* imaging techniques. Understanding how cellular structural alterations in general and structural dynamics in specific correlate with anatomical and physiological features of neural circuits is crucial to understanding their role in information processing and functioning in the brain.

## BASIC NEUROANATOMY OF THE WHISKER-TO-BARREL SYSTEM AND EARLIER WORKS OF SENSORY DEPRIVATION

The barrel cortex is a specialized region of the primary somatosensory cortex (S1) devoted to processing whisker-related information. At the start, the whiskers are perturbed from rest which activates nerve endings embedded in the whisker's associated follicle, and this tactile based information is transmitted in a mono-synaptic connection through the infraorbital nerve, a sub-portion of the trigeminal nerve, to the trigeminal nuclei, where the first synapse is found. Within the brainstem therein lies the principal nucleus and three spinal nuclei (interpolaris, oralis, and caudalis). The principal nucleus of V (PrV) gives rise to the lemniscal pathway, which targets the ventro-posterior medial (VPM) nucleus of the thalamus on the contralateral side, while the three spinal nuclei give rise to the para-lemniscal pathway, which targets the medial portion of the contralateral posterior thalamic nuclei (POm). From the thalamus, VPM predominantly targets the ipsilateral layers 4 and 6 of the barrel cortical column, while POm innervates layers 1, 2 and 5A (Meyer et al., 2010; Wimmer et al., 2010; Oberlaender et al., 2012; Constantinople and Bruno, 2013; also see Deschênes and Urbain, 2009 for a review). At layer 4 where distinct "barrels" (aggregates of cells) can be observed, VPM innervates the barrel centers, whereas the POm targets the septa, or the inter-barrel space (Wimmer et al., 2010; Oberlaender et al., 2012).

The pioneering work of Van der Loos and Woolsey (1973) was the first to demonstrate how ablation of vibrissae follicles after birth can disrupt the formation of the barrel cortex topographic pattern. However, once the animals develop past a critical period (postnatal day 4), the same follicle ablations no longer impacted barrel pattern formation (Weller and Johnson, 1975; Woolsey and Wann, 1975). Chronically depriving sensory experience after birth impacts many aspects of whisker-to-barrel system, but not the overall topography of the barrel cortex. Behaviorally, it alters whisker-related behavior (Carvell and Simons, 1996) in which after whisker regrowth to full length, animals still cannot discern two different gradients of rough surfaces. Sensory deprivation also alters the physiological properties of barrel cortical neurons (Simons and Land, 1987; Lee et al., 2007), resulting in an enlargement in the size of their receptive field, as well as an increase in the strength of their responses to whisker deflections (Keller and Carlson, 1999; Knott et al., 2002). These earlier works provided a solid foundation that sensory activities are paramount for shaping the cellular environment necessary for the proper

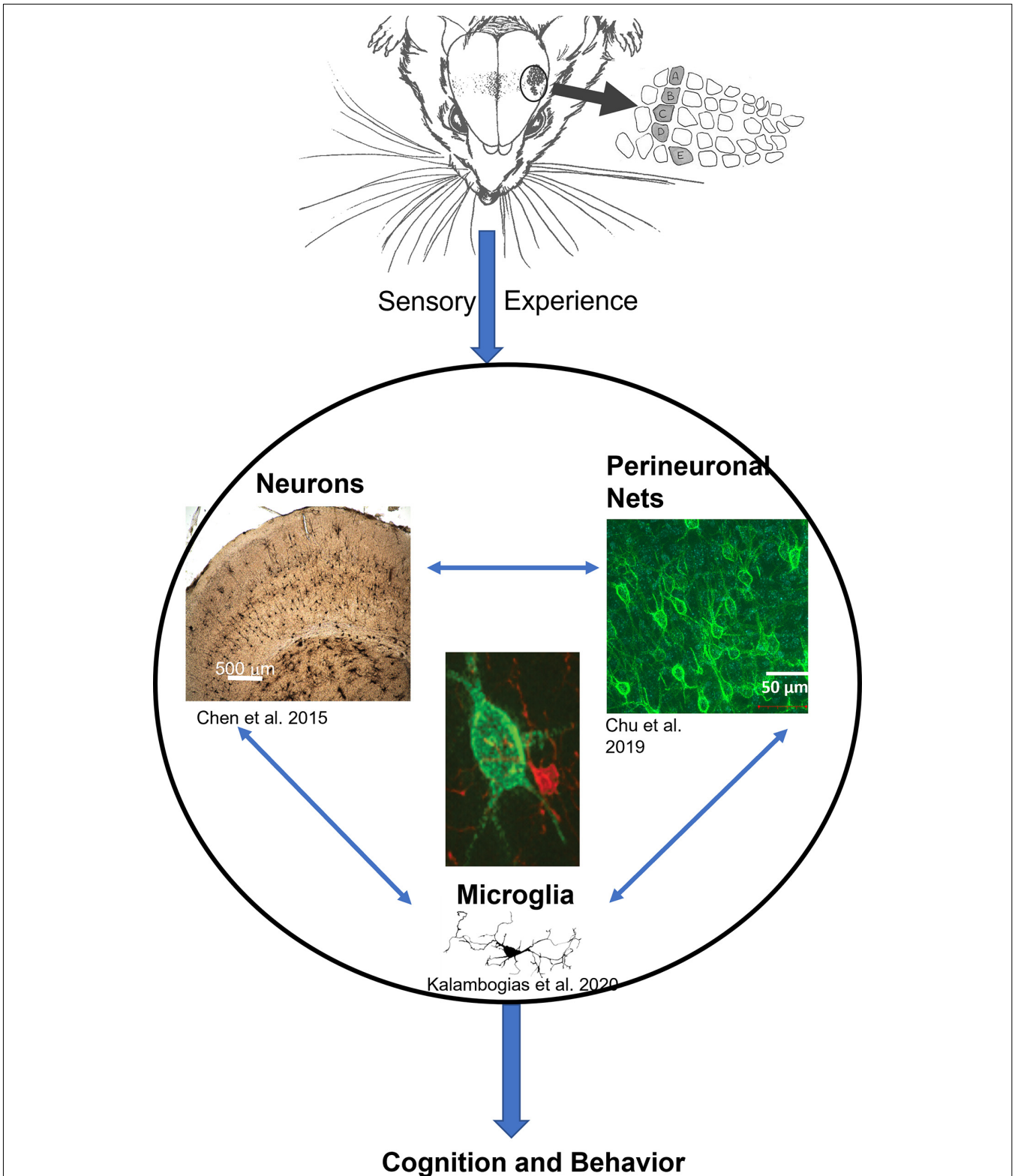


**FIGURE 1 |** The general model of neuron-glia-ECM triad relationship. Sensory experience conveyed through the animal's whiskers impacts neuronal morphology (Chen et al., 2012) and spine density (Chen et al., 2015a), at the same time microglia morphology is impacted (Kalambogias et al., 2019) and perineuronal net density is decreased as well (McRae et al., 2007). We hypothesize that changes in sensory experience can influence microglia (center image in red) to interact with the perineuronal net (center image in green) to change their integrity which in turn impacts cellular functioning (Chu et al., 2018). The result would be changes in behavior. Illustration by A. Barrientos.

functioning of whisker-related behavior and its underlying cerebral circuitries.

## METHODOLOGY OF DEPRIVING SENSORY INPUTS ON WHISKER-TO-BARREL SYSTEM

Sensory deprivation in the whisker-to-barrel system comes in many forms, with the commonality that they impact sensory transduction. We will review the most common means to induce sensory deprivation in the whisker-to-barrel pathway.

### Infraorbital Nerve (ION) Lesion

The ION is a branch of the trigeminal nerve, which gives rise to the starting point of the whisker-to-barrels system. It contains the axons that convey information from the follicle embedded within the mystacial pad into the trigeminal ganglion and then on to the Principal Sensory Nucleus of V and the Spinal Trigeminal nucleus, both located within the caudal brainstem. Peripheral transection of the ION completely blocks the sensory information coming from the mystacial pad to the brainstem, preventing any sensory-related information from reaching higher order processing centers. Hence, this is an extreme form of sensory deprivation. It has been documented that mice which receive ION lesions postnatally (prior to 4 days of age) will not develop the barrel pattern in cortical layer 4. However, ION lesions as a means of sensory deprivation have raised some concerns, as this model has been also used as a model for orofacial pain (Xu et al., 2008), and there is a recent surge of research groups using partial or complete transection of ION as a paradigm to induce neuropathic pain as well as allodynia. This may complicate the validity of ION lesion as a pure form of depriving sensory activities.

### Cauterization

Electrocauterization refers to a surgical technique which utilizes electricity to induce heat to destroy tissue, and in this case, the destruction of whisker follicles located on the mystacial pad. Similar to ION, cauterization is irreversible and has a long-lasting impact on the whisker-to-barrel system. Furthermore, electrocauterization causes skin damage to the mystacial pad, which damages the nerve endings responsible for sensory transmission, thus may complicate the interpretation of "pure sensory deprivation" for the whisker-to-barrel system. Among the most popular uses of electrocauterization is to cauterize one row of whiskers on the mystacial pad, because such manipulation when done early enough in development, causes the corresponding cortical barrels within layer 4 of S1BF to disappear, while the immediately surrounding barrels expand to "fill in" and take their places. However, it has been well documented that this rearrangement of whisker representation within the cortex is developmentally time-sensitive: electrocauterization must be conducted prior to postnatal day 4, otherwise such rearrangement of barrel formation does not occur (Van der Loos and Woolsey, 1973; Belford and Killackey, 1980). This suggests that there is a developmental critical period for peripheral manipulation: after the barrel patterns have formed approximately at postnatal day 4, ION as well as electrocauterization no longer have an impact on the barrel pattern. Both ION and electrocauterization methods may also raise concerns of the validity of micro-level (dendrite morphology, dendritic spines) investigation, as it is not clear to the researchers if the cells being investigated still belong to the macro environment (barrels), as barrel formation is absent, because of dysgranulation of cortical patterning due to such dramatic peripheral manipulations.

### Whisker Trimming

Whisker trimming is considered a milder form of sensory deprivation; some may refer to it as a means of "sensory reduction" rather than sensory deprivation. However, the advantage of whisker trimming over ION or electrocauterization is that trimming does not damage the nerve endings embedded within the mystacial pad. Furthermore, whisker trimming, even from birth, does not disrupt the formation of the barrel cortex pattern in layer 4 of S1BF (McRae et al., 2007; Barrera et al., 2013); this bestows confidence to the researchers that the investigated cells do indeed belong to S1BF. Moreover, the effect of whisker trimming has a profound impact on numerous aspects of neuronal, glial, and extracellular structures. For example, whisker trimming broadens the receptive field and increases neuronal excitability (Simons and Land, 1987; Lee et al., 2007), causes reorganization of dendritic arborizations (Chen et al., 2012), greatly impacts dendritic spine morphology, density, and dynamics (Zuo et al., 2005; Yang et al., 2009; Chen et al., 2015a), decrease perineuronal nets surrounding inhibitory neurons (McRae et al., 2007; Nakamura et al., 2009), and, alters microglia morphology (Kalambogias et al., 2019). Whisker trimming as a means of sensory deprivation has the advantage of reversibility compared to ION lesions and electrocauterization. However, it also has several disadvantages, such as intensive manual labor, due to the constant regrowth of whiskers on the mystacial pad. Another disadvantage of whisker trimming is that starting from approximately when spontaneous movement of the whiskers, whisking, initiates (postnatal day 10–12), mice move their heads frequently as well as spontaneously moving their whiskers, and consequently, require artificial sedation (e.g., isoflurane) in order to trim the whiskers effectively. Furthermore, some delicate trimming methods (e.g., checkerboard trimming, see below) require a more careful and experienced researcher. Overall, whisker trimming requires much more patience compared to ION transection or whisker follicle electrocauterization.

#### Bilateral Trimming vs. Unilateral Trimming

Bilateral trimming refers to trimming all the whiskers on both sides of the face, while unilateral trimming refers to trimming all the whiskers on one side only. Bilateral trimming reduces uniformly all the sensory inputs to both cortical hemispheres, while the unilateral trimming decreases the sensory inputs to the contralateral cortical hemisphere, sparing the ipsilateral sensory input. In the case of unilaterally trimmed animals, some researchers may be tempted to categorize the ipsilateral (the spared side) as the equivalent to the control side, while some do not view them as equal, arguing that trimming one side of

the whiskers may cause the rodent to compensate behaviorally and overuse the spared side, leading to increased activities on the spared side (Whitaker et al., 2007). Furthermore, even in the side that corresponds to sensory deprivation, bilateral and unilateral trimming produce different results. For example, it has been documented that both physiology (Popescu and Ebner, 2010) and spine morphologies (Chen et al., 2015a) are slightly different in the affected side following bilateral vs. unilateral trimming, perhaps due to input that is coming from the opposite hemisphere through corpus callosum connectivities (Ramos et al., 2008).

### Checkerboard Trimming

Checkerboard trimming refers to trimming every other whisker on the mystacial pad, rather than trimming every whisker. Checkerboard trimming is postulated to increase the difference in sensory experience coming from adjacent whiskers and therefore provides competition for the spared whisker to innervate the deprived barrels. This, in turn, produces a novel sensation for the rodents rather than just an overall reduced sensation. Indeed, checkerboard trimming has been shown to increase dendritic spine (an anatomical indicator of excitatory postsynaptic structure) formation over a period of days in the barrel cortex (Fox, 2002; Holtmaat et al., 2006), whereas uniform trimming of whiskers (unilaterally) only decreased spine elimination rate in the contralateral barrel cortex without affecting spine formation rate (Zuo et al., 2005; Yang et al., 2009; Yu et al., 2013; Park et al., 2018).

### Trimming All but One Whisker

The single-whisker experience (SWE) is achieved by trimming all but one whisker. The affected rodent then must rely solely on sensory information coming from that one spared whisker. This type of novel sensory experience causes Hebbian expansion of the spared corresponding barrel within the S1BF, widening its receptive field (Fox, 2002; Feldman and Brecht, 2005), and enhancing the magnitude of neuronal responses to the deflection of the spared whisker (Glazewski et al., 2007). This is also a popular method for awake behavioral studies that train mice to discriminate different textures while simultaneously performing electrophysiological recordings or optical imaging *in vivo* (O'Connor et al., 2010; Sofroniew et al., 2014; Kwon et al., 2016).

## EFFECT OF SENSORY DEPRIVATION ON DENDRITIC MORPHOLOGY

Neurons come in different shapes and forms, but they all share similar structural subcomponents consisting of soma (cell body), dendrites, and axons. Morphologically, mammalian cortical neurons can be separated into two main categories: pyramidal and non-pyramidal neurons. Pyramidal neurons possess triangular somata, along with apical dendrites that typically project towards the pial surface. Non-pyramidal neurons, in contrast, lack apical dendritic features and are predominantly GABAergic neurons that exhibit smooth dendrites lacking dendritic spines (see White, 1989). Non-pyramidal neurons are extremely heterogeneous, the more common types are basket cells (Somogyi et al., 1983), chandelier cells (Lewis and Lund, 1990), double bouquet cells (Somogyi and Cowey, 1981), Martinotti cells (Fanselow et al., 2008; Xu and Callaway, 2009), neurogliaform (Ferrer et al., 1986), and spiny and aspinous stellate cells (Jones, 1975). Interestingly, morphological heterogeneity of cortical neurons can be found even within a single cortical lamina (Tsiola et al., 2003; Chen et al., 2009). Functionally, pyramidal and spiny stellate cells are the excitatory glutamatergic regular-spiking units (RSUs) in the neocortex (Simons and Land, 1987; McCormick et al., 1985). By contrast, the basket, chandelier, double bouquet, and Martinotti cells are inhibitory GABAergic neurons displaying a variety of physiological properties (Tremblay et al., 2016). Histochemically, neocortical interneurons exhibit a wide range of protein expression, including parvalbumin, somatostatin, 5HT-3a, neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), cholecystokinin (CCK), calretinin, and calbindinD28K (Cauli et al., 1997; Lee et al., 2010; Tremblay et al., 2016). How alterations in sensory experience impact specific phenotypes is not well understood, but its differential impact on pyramidal vs. non-pyramidal neurons has been noted (Chen et al., 2012).

In normal development, dendritogenesis follows a simple-to-complex trajectory, in which postnatal neurons exhibit simple structural patterning, and, as development progresses, the dendritic fanning becomes more elaborate (Maravall et al., 2004; Nakazawa et al., 2018). On the other hand, how sensory activities modulate such development of dendritogenesis depends on the duration, onset, and method of sensory deprivation. In general, early onset of sensory deprivation (e.g., before PND4; the time point where the barrel pattern emerges in S1BF) has a more dramatic effect on dendritic fanning than late-onset of sensory deprivation. If sensory deprivation occurs during adulthood or late juvenile developmental stages, it can still cause alterations in other dendritic parameters such as the number and density of spines (see below), it does not lead to dramatic changes in dendritic morphology (Cheetham et al., 2008). For example, trimming whiskers starting at PND 9 led to increased secondary dendritic branching points in layer 2/3 pyramidal neurons' basilar dendrites, but this effect was lost when trimming commenced at PND 15 (Maravall et al., 2004). Even in the extreme case of electrocauterization in the mature cortex, the overall dendritic length does not appear to be affected (Tailby et al., 2005). Qualitatively, however, the growth polarization pattern of layer 2/3 and layer 4 pyramidal basilar dendrites becomes directed away from the barrel center (Tailby et al., 2005), indicating that sensory activities derived from thalamocortical afferents still play a significant role in guiding the direction of dendritic growth patterns in mature cerebral cortex.

How early developmental (i.e., neonatal) sensory deprivation influences the dendritic morphology seems to be cortical layer specific. Early whisker trimming, even for a short duration such as during just the first 3 days of life, results in a larger dendritic span (area encapsulated by dendrites) in layer 4 spiny stellate cells in S1BF (Lee et al., 2009), consistent with findings following early-stage sciatic nerve transection in gerbils' S1 layer 3 pyramidal neurons (Macharadze et al., 2019). Interestingly, Sholl analysis (which investigates the complexity of neuronal

structures as a function of distance away from the somata; Sholl, 1953) showed a double dissociation relationship: sensory deprived neurons exhibit lower complexity at proximal regions (closer to soma), but higher complexity at distal ending regions. The degree of alteration, however, is greater at distal than proximal, resulting in overall larger dendritic complexity in sensory deprived neurons (Lee et al., 2009). Longer duration of whisker trimming from birth for 1 month also results in expansion of basilar dendrites in layer 6 pyramidal neurons but has the opposite effect on apical dendrites of the same cells, which typically extend to layer 5 and even reach as far as layer 4 in some subgroups (Chen et al., 2009, 2012). The net result is that the total dendritic length remains unchanged. Rather, chronic sensory deprivation leads to a re-distribution of dendritic material, suggesting some sort of structural homeostasis that the neuron is attempting to maintain.

Layer 6 pyramidal cells are not the only neuronal population that show this apical-basilar discrepancy in response to sensory deprivation. Layer 5 pyramidal neurons also share this similarity. Sensory deprivation starting from PND 7 for 1 week leads to lower apical dendritic processes, but higher basilar dendritic processes in Layer 5 pyramidal neurons (Zhang et al., 2013). GABAergic neurons, on the other hand, are less studied in the context of sensory deprivation. Studies have shown that Layer 5 GABAergic neurons exhibit less change in their primary and no change in their secondary dendritic processes following 1 week of whisker trimming (Zhang et al., 2013). By contrast, Layer 6 non-pyramidal neurons, which are presumably GABAergic, have a greater dendritic field and longer dendritic length following 1 month of whisker trimming from birth (Chen et al., 2012). Similarly, sensory deprivation has been shown to change in the density of markers associated with interneurons (Ueno et al., 2015) and as well as a decrease in GABAergic neurons within layer IV (Micheva and Beaulieu, 1995). Within the visual system, sensory loss caused a decrease in spines on a subset of inhibitory interneurons (Keck et al., 2011). Future studies may explore a systematic investigation on how whisker trimming may impact the GABAergic neuronal structure by varying sensory deprivation onset time, duration, and cortical laminae for a comprehensive understanding of how this very critical subset of the neuronal population is influenced by sensory activities. Researchers should avoid a general rule of how sensory activities may impact the development of neurons, because each neuronal class, even in different cortical layers, may respond very differently to the same sensory deprivation method.

## EFFECT OF SENSORY ACTIVITIES ON SYNAPTIC STRUCTURES

### Effect on Dendritic Spines

Dendritic spines have fascinated generations of neuroscientists since their initial description by Santiago Ramón y Cajal more than a century ago (Ramon y Cajal, 1888). These delicate protrusions emanate from the dendritic shaft and resemble "bristling thorns or short spines" as described by Cajal. They are the postsynaptic sites of the great majority (>90%) of excitatory glutamatergic synapses in the mammalian brain and contain essential molecular components for postsynaptic signaling and plasticity (see Chen et al., 2014). Therefore, spines and their structural dynamics may serve as indicators for synaptic connectivity and modifications thereof (Segal, 2005; Tada and Sheng, 2006; Harms and Dunaevsky, 2007).

How sensory information potentially modifies the density and functioning of synaptic structures is widely studied in modern neurobiology. Early works have examined fixed tissue samples (e.g., Golgi stain or electron microscopic level examinations) to observe how sensory information/activity impacts dendritic spines. The densities and morphologies of these dendritic protrusions seem to follow a consistent principle as the brain matures postnatally: there is a general peak in the number and density of dendritic spines (synaptogenesis period), followed by a wane (synaptic pruning period), and both events may be shaped by sensory activities (**Figure 2**). Overall, sensory/environmental enrichment seems to enhance the growth of dendritic spines (Landers et al., 2011; Jung and Herms, 2014), while sensory deprivation seems to impact synaptic pruning, delaying the maturation of synaptic refinement (Zuo et al., 2005; Chen et al., 2015a). Paradoxically, sensory deprivation and environmental enrichment both lead to increased spine density, but the mechanism of such increase in spine density is most likely different, due to differences in spine elimination and formation rates. Environmental enrichment causes the increased formation of dendritic spines that are in need, while sensory deprivation stunts the elimination of unnecessary spines. Thus, both increased and decreased experience result in increased spine density, which has been demonstrated in fixed-tissue preparations. Furthermore, sensory restoration following

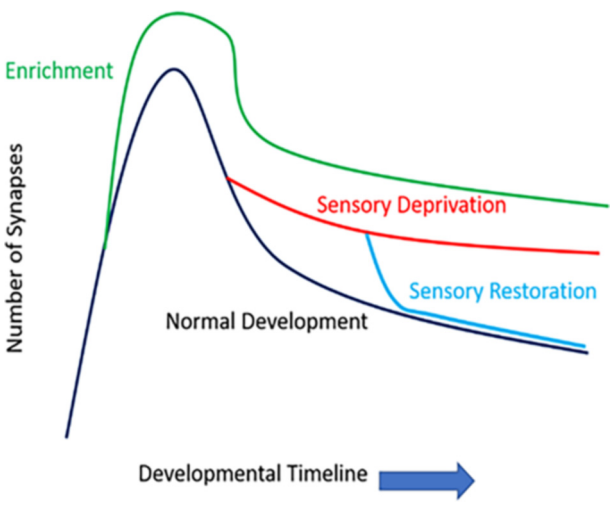


**FIGURE 2 |** The number of synapses of function development and sensory experience. Rapid spinogenesis in early postnatal is followed by a gradual spine pruning in adolescence. Environmental enrichment generally results in more numerous synapses. Sensory deprivation stunts the synaptic refinement stage, delaying the spine pruning process; whereas restoration of sensory activities can accelerate such activity-dependent spine pruning, even in later developmental stages such as late adolescence.

deprivation, depending on the age of onset and cortical areas (or layers) investigated, may accelerate the previously stunted synaptic refinement caused by deprivation (Zuo et al., 2005). It has been shown previously in many systems that different sets of dendritic spines likely encode different types of experiences and the storage of memories. For example, in the visual system, when animals re-experienced a second period of monocular deprivation, no additional dendritic remodeling occurred as it did in the first period of monocular deprivation (Hofer et al., 2009). Similarly in the barrel system, when animals re-experienced the same environmental enrichment (EE), there were no additional gain or loss of dendritic spines, but novel EE induced higher spine formation and elimination rates (Yang et al., 2009). In the motor system, animals that were re-trained with a previously learned task showed comparable spine gain/loss rates to controls, but when these animals were trained with a new motor task, their spine remodeling rate once again increased (Xu et al., 2009; Yang et al., 2009). However, it is important to note that in some cases, gaining a behavioral function is not always associated with the formation of new spines, but the elimination of pre-existing spines. For example, in the frontal association cortex, gaining a fear-related memory is associated with the elimination of dendritic spines, while fear extinction is associated with the re-appearance of previously lost spines (Lai et al., 2012). Similarly, in the barrel cortex, acquisition of a tactile variant of trace eyeblink conditioning is associated with the elimination of dendritic spines (Joachimsthaler et al., 2015). Nevertheless, these studies pinpoint that prior experiences leave a lasting trace in the cortical circuits, and specific dendritic segments and their hosted spines likely encode for each specific experience/memory.

Morphologically, dendritic spines come in many shapes and forms (Arellano et al., 2007a,b; Chen et al., 2015a). Overall, they can be categorized into several morphological classes such as mushroom, thin, stubby, branched, as well as some other classes based on morphological appearances (Comery et al., 1997; Irwin et al., 2002; Arellano et al., 2007a; Yu et al., 2013; Chen et al., 2015a; Tjia et al., 2017; Park et al., 2018). Some researchers may also categorize filopodia (thin long headless protrusions) as dendritic spines, while some may disagree since they do not seem to consistently form synapses with presynaptic boutons' (Arellano et al., 2007b). Nevertheless, morphologies of spines also follow a very consistent principle during development: at younger ages, the brain exhibits higher proportions of "immature" dendritic protrusions consisting of filopodia and thin spines. As animals mature into adulthood, the emergence of "mature" mushroom spines starts to outpace the thin spines and filopodia (Orner et al., 2014; Chen et al., 2015a).

Early studies on the dendritic spine examined fixed neural tissue with light or electron microscopy (Lund et al., 1977; Woolley et al., 1990; Harris and Kater, 1994; Lippman and Dunaevsky, 2005). Although they provided fundamental information about spine morphology and distribution, these fixed tissue examinations only captured static "snapshots" of spines. Over the past decade, fluorescent labeling techniques and multi-photon microscopy enabled time-lapse imaging of dendritic spines in the living brain. A dynamic picture of spines has emerged from such longitudinal studies: spines form, enlarge, shrink, and retract throughout the animal's lifespan. Furthermore, their morphology and dynamics vary among neuronal types, across developmental stages, and in response to experiences such as sensory stimulation and deprivation, environmental enrichment, and various paradigms of learning (Holtmaat and Svoboda, 2009; Fu and Zuo, 2011; Chen et al., 2014; Joachimsthaler et al., 2015).

Similar to their parent dendrites, dendritic spine dynamics and morphology are impacted by sensory deprivation. During normal development, spine density varies significantly across diverse populations of neurons, likely reflecting the diversity of neuronal morphology and function (Nimchinsky et al., 2002; Ballesteros-Yanez et al., 2006). The balance between spine formation and elimination determines the change in spine density: a surplus of spine formation over elimination along a dendritic segment increases spine density thereon, and *vice versa*. In the cerebral cortex, the rates of spine formation and elimination change over time, resulting in non-monotonic alteration in spine density. For example, spines on the apical dendrites of layer 2/3 pyramidal neurons in the rodent barrel cortex exhibit gradually decreasing motility (elongation and shortening of spines) and turnover rate (defined as the total amount of gains and losses of spines) between postnatal day 7 and 24 (P7–24). Nevertheless, spine density continuously increases over this period of time (Lendvai et al., 2000; Cruz-Martin et al., 2010). After this initial phase of net spine gain, spine elimination starts to outpace formation, leading to an overall reduction of spine density (Zuo et al., 2005; Holtmaat et al., 2006; Yang et al., 2009). Between P28 and P42, 17% of spines are eliminated along the apical dendrites of layer 5 pyramidal neurons in the mouse barrel cortex, while only 5% of new spines are formed during the same time period. Importantly, not all spines are equally susceptible to elimination: those with large heads are more stable than thin ones. As spine head size correlates with synaptic strength, this phenomenon suggests that stronger synapses are more stable (Holtmaat et al., 2006). Furthermore, newly formed spines are more likely to be eliminated than pre-existing spines (Xu et al., 2009), and the majority of stable spines formed before adolescence remain incorporated in the adult neuronal circuit (Zuo et al., 2005; Yang et al., 2009; Yu et al., 2013). Finally, in adult animals, spine formation and elimination reach equilibrium; spine density remains roughly constant until the onset of aging (Zuo et al., 2005; Mostany et al., 2013).

During the early postnatal period, sensory inputs play instructive roles in the stabilization and maturation of spines. In the mouse visual cortex, depriving visual input prevented the decrease in spine motility and maturation of spine morphology (Majewska and Sur, 2003; Tropea et al., 2010). In mice that had been subjected to visual deprivation previously, light-induced spine maturation could be partially mimicked by pharmacological activation of the GABAergic system, suggesting an important role of inhibitory circuits in the maturation of excitatory synapses (Tropea et al., 2010). Later, sensory experience drives spine pruning (defined as the net loss of spines). Unilateral trimming of all whiskers in 1-month-old mice for 4 or 14 days dramatically reduced spine elimination in the barrel cortex but left spine formation

largely unperturbed (Zuo et al., 2005; Yu et al., 2013). However, pyramidal neurons from different layers may respond differently to the same whisker-trimming manipulation (Tjia et al., 2017). Pharmacological blockade of NMDA receptors mimicked the effect of whisker trimming, indicating the involvement of the NMDA receptor pathway in such activity-dependent spine elimination (Zuo et al., 2005). In addition, astrocytes have been shown to participate in spine pruning by influencing synaptic glutamate uptake (Yu et al., 2013).

While complete whisker trimming removes sensory input globally, trimming every other whisker ("checkerboard trimming") presumably amplifies any difference in activity levels and patterns of neighboring barrels, thereby introducing a novel sensory experience. Such paradigm has been shown to promote spine turnover and to stabilize newly formed spines selectively in a subclass of cortical neurons (Trachtenberg et al., 2002; Holtmaat et al., 2006). New spines were preferentially added onto layer 5 pyramidal neurons with complex apical tufts, rather than those with simple tufts (Holtmaat et al., 2006). Analogously, brief monocular deprivation (MD) increases the disparity between the inputs from two eyes. Thus, similar to checkerboard trimming, MD has been found to increase spine formation along the apical dendritic tufts of layer 5 pyramidal neurons in the binocular zone of the mouse visual cortex. However, this effect was not observed in layer 2/3 neurons, or in the monocular zone (Hofer et al., 2009), again indicating a cell type-specific and layer-specific synapse remodeling similar to the barrel system (Tjia et al., 2017). Interestingly, a second MD failed to increase spine formation further, but selectively enlarged the spines formed during the initial MD, suggesting that new spines formed during the initial MD had functional synapses that were reactivated during the second MD (Hofer et al., 2009).

While the exact molecular mechanisms of sensory deprivation induced alterations of spine dynamics remain elusive, there are several studies providing potential clues. For example, Retinoid acid (RA), which is involved in transcriptional regulation of neurodevelopmental processes mediated by nuclear RA receptors, seems to regulate dendritic spine dynamics. Transcranial two-photon imaging revealed a significant increase in dendritic spine elimination on apical dendrites of somatosensory cortical layer 5 pyramidal neurons in these mice. Interestingly, the enhancement of spine elimination is experience-dependent as whisker trimming rescued the spine elimination phenotype. Furthermore, the conditional KO mice that lacked RA exhibited increased elimination of mature mushroom spines, in which whisker trimming was rescued (Park et al., 2018). These findings suggest that RA may be more preferentially expressed on these mature, stable types of spines. Another molecular mechanism that is involved in sensory development are ephrins, which are guidance molecules. Particularly, the ephrin A2 receptor seems to play a pivotal role in stabilizing existing dendritic spines, as the ephrin A2 KO mice exhibited accelerated dendritic spine elimination in the barrel cortex, but ephrin A3 KO mice exhibited normal spine dynamics. Whisker trimming in ephrin A2 KO mice, similar to the RA KO mice, also rescued this increased spine elimination phenotype. In addition, this mechanism seems to be NMDA-receptor mediated, because MK801 (non-competitive blocker of NMDA-R) *in vivo* injection can also rescue such accelerated spine elimination phenotype in ephrin A2 KO mice (Yu et al., 2013). Unlike the RA KO mice that exhibited preferential elimination of mushroom spines, ephrin A2 KO mice preferentially eliminated the thin spines instead. This suggests that different spine morphologies may harbor specific types of molecular signals/receptors. Fragile X mental retardation protein (FMRP) may also play a role in sensory-experience-dependent plasticity of dendritic spines. Non-deprived *Fmr1* (the gene responsible for producing FMRP) KO mice exhibits both higher dendritic spine formation and elimination (Pan et al., 2010). Normally, 2 weeks of trimming stunts spine elimination while not affecting formation in WT mice, in contrast, spine elimination rates are not impacted by trimming in *Fmr1* KO mice, as both spine elimination and formation remain high in the KO mice even in whisker-trimmed conditions. Lastly, a recent study found that layer 2/3 pyramidal neurons' basilar dendrites respond functionally to chessboard trimming, but not to all-whisker deprivation, by increasing the production of new dendritic spines. These experience-dependent plasticities were absent in αCaMKII70 T286A mutants that lack LTP, suggesting αCaMKII auto-phosphorylation is an important element for the enlargement of spines, production of new spines, and LTP, thus providing a tighter link between the structural and physiological plasticity (Seaton et al., 2020). As transcranial 2-photon microscope is gaining popularity in the neuroscience field, in addition to the advancement of transgenic mouse technologies, there will be more valuable studies like these to help us gain a more complete knowledge regarding the mechanisms that are responsible for experience-dependent plasticities of dendritic spines in the living brain.

## Effect on Axons and Boutons

Sensory experience does not only impact dendritic and spine development but also impacts the other aspects of the neuron: the soma, its axonal branches, and boutons. Developmentally, the dynamics of axonal boutons are somewhat similar to that of dendritic spines, in which young mice exhibit higher turnover rates and higher net-loss of boutons than adult mice, and these boutons are stabilized during adulthood (Qiao et al., 2016). Whisker trimming in rats from PND 7 for 2 weeks resulted in a reduction of overall axonal length of layer 2/3 to layer 5 corticocortical connections in rats (Bruno et al., 2009). Thalamocortical (TC) afferents in adult rats for 13–27 days also resulted in the reduction of overall axonal length and TC synapses but did not alter the density of TC synapses. Partial whisker trimming (trimming either the upper or lower rows) starting from PND 19 for 13–41 days results in strengthening of the axonal components in the spared cortex when compared to the control cortex. Specifically, the size of axonal varicosities, as well as the contact zones (between boutons and spines), were larger in the spared cortex. In the same partial deprivation paradigm, there is also a rapid reorganization of the axons in both excitatory and inhibitory cells, with a transient increase in axonal bouton density. In the horizontally projecting axons (e.g., layer 2/3 to layer

2/3 connections) there is a net increase of axonal projections from non-deprived whisker barrel columns into the deprived barrel columns. The axons from the inhibitory neurons located in the deprived whisker barrel over-reached in their long–range projections towards the non-deprived whisker barrel columns (Marik et al., 2010). In a separate study using a similar partial sensory deprivation paradigm, it was found that the axonal boutons corresponding to the spared whiskers exhibited larger volume than trimmed whiskers. Furthermore, the bouton volume augmentation occurs only in the en-passant boutons but not terminal boutons. Most importantly, it was found that the sensory-deprived synapses are much more unreliable in which they exhibited significantly higher failure probability to elicit an action potential. Other key physiological features of these deprived synapses are decreased maximal inducible EPSP, correlating with decreased volume of dendritic spine heads (Cheetham et al., 2014). Lastly, sensory activities can also drive homeostatic plasticity in the axonal initial segments: long-term sensory deprivation-induced length increase of axonal initial segments, accompanied with an increase in neuronal excitability, while enrichment caused the axonal initial segment to shorten, with an accompanying decrease in action potentials (Jamann et al., 2021).

## EFFECT OF SENSORY ACTIVITIES ON EXTRACELLULAR COMPONENTS

The formation, stabilization, and refinement of CNS synaptic connections involves the complex interplay between the developing neuronal cell surface and the molecules in the extracellular space. Perhaps, one of the most influential components is the extracellular matrix (ECM), which is thought to play the role of the scaffolding, or the "glue" that supports the cellular structural frame. The perineuronal net (PNN), a neuron-specific type of ECM, is often found ensheathing cortical parvalbumin (PV)-positive inhibitory neurons and their proximal dendrites in a lattice-like structure and has been postulated to play important roles in neural development, regulation of plasticity, as well as the proper functioning of the CNS (Hockfield et al., 1990; Celio and Blümcke, 1994). There are many subcomponents of the PNNs, including aggrecan, hyaluronan, neurocan, versican, brevican, phosphacan, and chondroitin sulfate proteoglycans (CSPGs). The detailed molecular composition of the ECM is out of the scope of this review (for detailed reference see Sorg et al., 2016; Fawcett et al., 2019). The distribution in the barrel cortex for these subcomponents is not uniform. Rather, the immunoreactivity of chondroitin-6-sulfate containing proteoglycan (CS-6-PG), phosphacan, and neurocan are stronger at barrel septa as compared with barrel hollows and surrounding cortex, while the labeling of Wisteria floribunda agglutinin (WFA) was observed to be strongest in the barrel hollows (Nakamura et al., 2009). In the adult visual system, degradation of the CSPGs has been shown to increase dendritic spine motility as little as 3 h *in vivo* (de Vivo et al., 2013), consistent with previously observed in CA1 neurons *via* organotypic hippocampal slices preparation (Orlando et al., 2012), suggesting that CSPGs restricts morphological changes of synapses. Whether these effects may be generalized to the whisker-to-barrel system is yet to be determined. Following whisker trimming from neonates in both mice and rats, there is a reduction in aggrecan expression and WFA expression (McRae et al., 2007), with the WFA expression in the septa being unaltered (Nakamura et al., 2009). Univibrissa rearing (trimming all but one whisker) resulted in an increase of PNN density in the deprived barrels, but only those that immediately neighbor the non-deprived barrel. Although checkerboard pattern trimming impacts dendritic spines, surprisingly, it had no effect on the density of the PNNs (Nowicka et al., 2009). Overall, sensory experiences seem to regulate the formation of ECM, particularly PNNs, which directly impacts the maturation profile of PV+ interneurons, a critical player of neurodevelopment in the cerebral cortex.

There are other components that can impact the extracellular matrix as well. One such well-studied component is the serine protease tissue-type plasminogen activator (tPA). tPA is a well-known treatment that dissolves blood clots in stroke patients. However, it is also present in the nervous system endogenously. In the visual system, tPA is developmentally regulated, with its levels peaking around the critical period, and decreasing progressively into adulthood (Zheng et al., 2008). Furthermore, tPA levels are also activity-dependent; monocular deprivation spanning the developmental critical period is associated with a significant increase in tPA activity, and such increase is regulated by glutamate decarboxylase-65 (GAD65; Mataga et al., 2002). Mice lacking the gene encoded for GAD65 (GAD65-KO) failed to show such monocular deprivation-induced increase of tPA activities. Additionally, tPA has also been implicated in the regulation of synaptic plasticity. Application of tPA accelerates the dynamics of dendritic spines (Oray et al., 2004), whereas the genetic deletion of tPA prevented monocular deprivation-induced changes of dendritic spine density in the visual cortex (Mataga et al., 2004), and prevented stress-induced loss of dendritic spines in the hippocampus (Pawlak et al., 2005). In the barrel system, it has been shown that tPA is present in microglia, excitatory neurons, and parvalbumin+ neurons, while absent in GIN+ somatostatin interneurons (Chu et al., 2015). A month of whisker trimming results in elevated tPA expression in all layers of the barrel cortex, with some subtle difference between bilateral vs. unilateral trimming in the affected contralateral side (Chen et al., 2015b). Interestingly, in the spared barrel cortex (ipsilateral to the trimmed side), tPA expression is decreased compared to the control animals. Such change may be attributed to mice using the spared whiskers significantly more, compensating for not having one side of the whiskers. So far, it is not known if the changed level of tPA is the reason that PNNs are impacted by sensory deprivation, or if there are other extracellular matrix factors such as matrix metalloproteinase-9 (MMP-9) that may partake in such regulation of sensory-deprivation induced PNN changes, as recently demonstrated in the auditory cortex (Wen et al., 2018). It may be especially rewarding for future studies to

systematically explore such possibilities, in order to further elucidate the mechanisms behind sensory activity induced PNN alterations.

## MICROGLIA RESPONSE TO CHANGES IN SENSORY ACTIVITIES

Microglia are the central nervous system's principal immune cells which are mostly derived from the mesodermal yolk sac in addition to a few specific circumstances in which microglia can be derived from bone marrow. Microglia are primarily self-sustained through cell division with an average renewal rate of 4 years (Réu et al., 2017). In the traditional view, the primary function of microglia is to protect and maintain CNS integrity (Banati and Graeber, 1994), which is mediated through surveying the extracellular environment and scavenging for foreign bodies or injured tissue (Kreutzberg, 1996; Batchelor et al., 1999; Aloisi, 2001), phagocytosis (Morsch et al., 2015), antigen presentation (Gehrmann et al., 1995; Aloisi et al., 1998; Aloisi, 2001) and cytotoxicity (Banati and Graeber, 1994; Gehrmann et al., 1995; Medzhitov and Janeway, 2000). While microglia were classically thought to be a homogeneous population with little function in the healthy brain, recent work indicates that they are a largely heterogeneous population (Hammond et al., 2019) which dynamically responds to changes in the extracellular environment. For example, in contrast to homeostatic conditions in which microglia exhibit a complex and ramified morphology, during brain pathogenesis, microglia have been shown to exhibit an altered phagocytic-like phenotype (Morsch et al., 2015). Recent work has continued to elucidate the morphological distinctions in microglial morphology in response to various environmental stimuli. Moreover, human research into aging-associated brain dysfunctions has indicated alterations in microglial morphology (Mosher and Wyss-Coray, 2014; Bachstetter et al., 2015), but interestingly, dystrophic microglial morphology are not associated with healthy aging in humans (Shahidehpour et al., 2021). Taken together, these studies provide evidence to suggest that morphological changes in microglia can be used as a proxy to assess associated changes in the micro-environment of the brain.

Over recent years, electron microscopy work assessing the role of microglia within focal cortical inflammation found an increase in microglial density around the legion site and observed that microglial processes make direct contact with neuronal perikaryal and apical dendrites, which was followed by the displacement of a large portion of axosomatic synapses (Trapp et al., 2007). This microglia-neuron interaction indicated a potential role of microglia in neuroplasticity which was later supported by research showing that microglia exhibit experience-dependent participation of the elimination of postsynaptic structures in the visual system (Tremblay et al., 2010). In the motor cortex, microglia depletion *via* diphtheria toxin administration caused a reduction of motor-learning-related dendritic spine remodeling, associated with deflective motor learning skills (Parkhurst et al., 2013). In developing primary somatosensory cortex, both ablation of microglia and minocycline (inhibitor of microglia) administration led to a decreased dendritic spine density with accompanied reduction of mEPSC frequency, without affecting the amplitude (Miyamoto et al., 2016).

In the barrel system, there is some evidence that microglia respond to sensory deprivation. It is known that while chronic sensory deprivation does not alter the cellular density of microglia in the barrel cortex, it dramatically alters the morphological characteristics of microglia. Following sensory deprivation, somatic size is greatly increased while the number of microglial processes and their length are reduced, indicating a morphological shift from the ramified state towards a more "activated" state (Kalambogias et al., 2019). The increase in somatic size may be evidence of increased engulfment of synaptic components by the microglial lysosome, such as vesicular glutamate transporter 2 (vGlut2), a presynaptic component of thalamocortical (TC) afferents (Gunner et al., 2019). Furthermore, it is known that microglia also participate in whisker lesion-induced synapse elimination, as knock out mice lacking the microglial fractalkine receptor, CX3CR1, were not affected by whisker lesions. *ADAM10*, a metalloprotease known to cleave CX3CL1 (ligand for CX3CR1) into a secreted form, is increased specifically in layer IV neurons and microglia following cauterization. Indeed, pharmacological inhibition of ADAM10 successfully blocked the effect of whisker-lesioning-induced TC elimination in the barrel cortex (Gunner et al., 2019). This suggests microglia not only respond to changes in sensory experience but also play a role in dendritic and synaptic remodeling. The direction of microglia-neuronal communication in the context of sensory activity remains unclear. However, it is known that microglia express glutamate receptors including AMPA, NMDA, and mGluRs (Noda et al., 2000; Taylor et al., 2002, 2003; Byrnes et al., 2009; Murugan et al., 2011; for a review see Liu et al., 2016) as well as GABA receptors (Kuhn et al., 2004; Mead et al., 2012), and the activation/inhibition of these receptors on microglia can directly influence their morphological phenotype (for a review see Liu et al., 2016). It is known that the density of GABA-immunoreactive cells is reduced following chronic whisker trimming (Micheva and Beaulieu, 1995), so this decreased GABA expression in the barrel cortex may be responsible for the altered morphological phenotype observed in the chronically deprived animals. It may be rewarding for future studies to systematically explore the bi-directional nature of microglia-neuronal communication, perhaps through the use of specific Cre-lines and chemogenetic/optogenetic methods.

## ASTROCYTIC RESPONSE TO CHANGES IN SENSORY ACTIVITIES

Astrocytes are a subtype of glial cells within the nervous system. Our understanding of the role astrocytes play has evolved from the traditional supportive cells of neurons to a key player of brain functioning, and may play important roles in neuropathologies (for reviews see Santello et al., 2019; Siracusa et al., 2019). Astrocytes participate in synaptic functioning, particularly with

glutamate signaling, forming the "tripartite synapse" consisting of the pre-synaptic axonal bouton, post-synaptic dendrite/spine, and peri-synaptic astrocytic process (Araque, 1999). It has been demonstrated in the visual system that astrocytes are influential in closing the developmental critical period, and transplanting immature astrocytes into an adult cortex reopens the critical period in the visual system, and this process is guided by connexin-30, a subunit of gap junction channels. It was shown that astroglial connexin-30 works to inhibit the expression of MMP-9 (matrix metalloproteinase-9) *via* the RhoA-ROCK (Rho-associated coiled-coil-containing protein kinase 2) pathway (Ribot et al., 2021). Also in the visual system, both monocular and binocular deprivation induced alterations of astrocytes, in which binocular deprivation showed a stronger impact on reactive astrocytes, while concomitantly strengthening the gap junctions between astrocytes in V1 (Wang et al., 2021). These findings suggest that astrocytes may work in concert with neuronal activity in order to shape the circuit remodeling of the brain. Interestingly, in the visual cortex of rats exposed to dark-rearing, there were lower expression levels of S100beta (a calcium-binding protein primarily expressed in a subset of astrocytes; Argandona et al., 2009). Such a reduced level of S100beta can be partially rescued by increased physical exercise, and full recovery can be induced by multisensory environmental enrichment (Bengoetxea et al., 2013) suggesting that astrocytes are influenced by neuronal activity. In the whisker-to-barrel system, a very limited number of published work is available on the effect of sensory deprivation on astrocytes. It is known that whisker deprivation by means of electrocauterization does not change the density of Sox2+GFAP+ radial-glia-like astrocytes (Gonzalez-Perez et al., 2018), which is consistent with the previous finding that chronic whisker trimming from birth does not impact the overall number of Nissl+ cells in barrel cortex (Barrera et al., 2013). However, gross measurements such as cell counts may not be a sensitive enough means to decipher the fine nuances of microcellular alteration on a synaptic level, which is most likely where and how the astrocytes are being modified following prolonged changes in sensory activities. Future studies may emphasize on this particularly unknown area of neuroplasticity. Other glial components may also play a role in circuit reorganization following sensory deprivation (e.g., Barrera et al., 2013), but are beyond the present discussion.

## FUTURE DIRECTIONS

Sensory activities impact multiple structures at the cellular level within the brain, including neuronal, glial, and extracellular components. As detailed above, sensory deprivation has been shown to impact dendrites and their associated spines in the whisker-to-barrel system. Similar observations have been made in the visual (Hofer et al., 2009) and auditory (Clemo et al., 2016) systems wherein prolonged sensory deprivation alters the underlying circuitry of the primary sensory cortex. Given the easy ability to target specific areas (whiskers) for sensory deprivation (or enrichment), the insights gained from the whisker-to-barrel cortex system can help inform the mechanism(s) that sensory deprivation engages to alter cortical circuitry more generally.

Although these studies have significantly advanced our understanding of how sensory experiences may be the initial trigger of structural plasticity, many questions remain on various fronts. In essence, the interactions of neurons, glia, and ECM in this context are a derivation of the familiar reframe of what came first: the chicken or the egg? For example, in the complex interplay of neuronal-glial-ECM interactions, which alteration following sensory experience/deprivation occurs the earliest? Are all these changes happening simultaneously, or is there a "leading" cause that the other components are compensating for? Alternatively, does one component serve as a permissive role for another to function properly? It is worth noting that almost all works discussed here have focused on a uni-readout approach, i.e., investigating only one dependent variable at a time. Future studies should focus on technical manipulation of neuronal/glial/ECM activities in combination with sensory experience alterations to gain a clearer perspective on how one domain (e.g., neuronal) may influence other domains (e.g., glial/ECM). With technological advances of chemo/optogenetic control of neuronal and/or glial activities, as well as ECM/glial mouse KO/I models, the manifestations of such studies may soon be on the horizon, thus will provide further elucidation of the cellular mechanisms governing experience-dependent plasticities in the brain.

## AUTHOR CONTRIBUTIONS

C-CC and JB contributed equally to this work, discussed concepts, wrote and edited in tandem. All authors contributed to the article and approved the submitted version.

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REVIEW
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# The Role of the Extracellular Matrix in Neural Progenitor Cell Proliferation and Cortical Folding During Human Neocortex Development

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Extracellular matrix (ECM) has long been known to regulate many aspects of neural development in many different species. However, the role of the ECM in the development of the human neocortex is not yet fully understood. In this review we discuss the role of the ECM in human neocortex development and the different model systems that can be used to investigate this. In particular, we will focus on how the ECM regulates human neural stem and progenitor cell proliferation and differentiation, how the ECM regulates the architecture of the developing human neocortex and the effect of mutations in ECM and ECM-associated genes in neurodevelopmental disorders.

**Keywords: extracelluar matrix, human, neocortex, development, neural progenitor**

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## INTRODUCTION

The human brain has long been known to contain a large amount of extracellular matrix (ECM) and ECM-associated molecules (Sanes, 1983, 1989). In the adult, this equates to roughly 20% of the total brain volume, but during fetal development, it is double that amount, at roughly 40% (Jovanov Milošević et al., 2014). Given its abundance, it is a little surprising that we do not fully understand the role of the ECM in the development of the neocortex. In fact, we do not yet know exactly which ECM components are expressed throughout development. We have learnt a lot about the role of ECM in neural development from many different animal and *in vitro* model systems (expertly reviewed elsewhere; Bandtlow and Zimmermann, 2000; Zimmermann and Dours-Zimmermann, 2008; Marthiens et al., 2010; Barros et al., 2011; Franco and Müller, 2011; Long and Huttner, 2019; Amin and Borrell, 2020). This includes roles in neural progenitor proliferation, differentiation, morphology, axonal and dendritic elongation and connectivity, neuronal migration and cortical folding. It has also been suggested that the ECM can create functional microdomains within the developing neocortex, for example by regulating the diffusion of signalling factors, restricting them to a discrete area, or by altering the migration route of newborn neurons (Dityatev et al., 2010). However, much of this data has been generated in animal models and it remains unclear how much can be extrapolated to human neocortex development. Major differences have been reported for human and non-human primate neocortex development in comparison to mouse development. For example, RNA-sequencing studies have revealed important differences in ECM expression between the developing human neocortex compared to the embryonic mouse neocortex (Fietz et al., 2012; Miller et al., 2014; Florio et al., 2015). One of the most striking of these differences is that significantly more ECM is expressed in the human fetal neocortex (Fietz et al., 2012).

This raises the question as to whether this ECM has played an important role in the evolution of the human neocortex.

In this review, we will discuss the role of the ECM in human and non-human primate neocortex development, focusing on neural progenitors, neocortical tissue architecture and neurodevelopmental disorders.

## ECM AND NEURAL PROGENITORS

ECM and ECM receptors have long been known to regulate the behaviour of neural progenitor cells (NPCs; Loulier et al., 2009; Radakovits et al., 2009; Fietz et al., 2010; Barros et al., 2011; Long et al., 2016; Long and Huttner, 2019; Amin and Borrell, 2020). However, compared to the large volume of evidence of ECM function in animal models, the data in human and non-human primate neocortex is limited.

### ECM Expression and Function

The RNA sequencing studies described above have suggested that many ECM components and receptors are expressed in the developing human neocortex (**Figure 1**; Fietz et al., 2012; Miller et al., 2014; Florio et al., 2015). This includes the major family of ECM receptors, the integrins (Hynes, 2002). Downstream of the integrins lie many important signalling pathways already known to regulate aspects of neural development, including PI3-kinase and Akt for cell survival, ERK and CyclinD1 for proliferation, and cdc42 and rac for cell migration (Hynes, 2002). The role of these factors, and others such as integrin-linked kinase and focal adhesion kinase, have been well studied in animal models (Beggs et al., 2003; Niewmierzycka et al., 2005; Tsuda et al., 2010; Valiente et al., 2011; Long et al., 2016), but less is known about their function in the developing human neocortex. However, more is known about the integrins themselves. In particular, integrins alpha 6 and beta 1 are known to be markers of human neural stem cells (NSCs; Hall et al., 2006). This integrin combination is able to bind to two of the main families of ECM components, the laminins and the collagens.

The most well-known of the laminins is laminin-111, which is highly expressed in both early mouse and human neocortex but is down-regulated later in development (Barros et al., 2020). The roles of laminins in mouse neocortex development are highly varied (Haubst et al., 2006; Loulier et al., 2009; Radakovits et al., 2009; Güven et al., 2020), but their roles in human neocortex development are yet to be fully investigated. Current evidence has shown that plating human NSCs onto laminin *in vitro* promotes both neurogenesis of the NSCs and then neurite outgrowth from the newborn neurons (Flanagan et al., 2006; Ma et al., 2008). However, in contrast, laminin has also been shown to promote the proliferation of human NSCs (Flanagan et al., 2006; Hall et al., 2008). Indeed, laminin-derived peptides, such as RGD, can promote both NSC proliferation and differentiation *in vitro* (Li et al., 2014), suggesting the function of laminins is highly complex and may be context-dependent, as seen in the mouse neocortex (Haubst et al., 2006; Loulier et al., 2009; Radakovits et al., 2009; Güven et al., 2020).

Other ECM components have also been reported to promote proliferation of human NSCs/NPCs, such as collagen IV (Hubert et al., 2009) and heparan sulphate proteoglycans (HSPGs; Oikari et al., 2016; Yu et al., 2017). However, as with laminins, these ECM components have also been implicated in neural differentiation (Oikari et al., 2016; Okolicsanyi et al., 2018). HSPGs are part of the proteoglycan family, which also includes the chondroitin sulphate proteoglycans, syndecans and glypicans. They have been shown to regulate several aspects of neural development, particularly neuronal migration (Bandtlow and Zimmermann, 2000; Ishii and Maeda, 2008; Maeda, 2015). The majority of these findings have been reported in 2D *in vitro* experiments using human NSCs/NPCs, but very few studies have investigated their function within an intact tissue environment. As ECM components often interact with each other and the surrounding tissue, their functions may differ between these simplified 2D environments and the complex 3D environment of the neocortex.

To address this, several studies have focused on understanding the specific network of ECM components that are important for human neocortex development. Fietz and colleagues showed that multiple ECM components were more highly expressed in the developing human neocortex compared to mouse (**Figure 1**; Fietz et al., 2012). This was particularly so in regions of the cortical wall that contained highly proliferative progenitors. In the mouse neocortex, this was the ventricular zone, which contains proliferative apical progenitors (APs). Within these APs, ECM was more highly expressed in proliferative progenitors in comparison to progenitors about to undergo differentiative divisions (Arai et al., 2011). However, in the human neocortex, ECM expression was not only high in the ventricular zone but was also high in both the inner and outer subventricular zones, which contain proliferative basal progenitors (BPs; Fietz et al., 2012). This suggested a pro-proliferative role of the ECM in the developing neocortex. Further analysis indicated the regulation of this ECM could be controlled by the transcription factor SOX9. SOX9 was found to only be expressed in mouse APs but was expressed in both APs and BPs in the human fetal neocortex (Güven et al., 2020). Additionally, SOX9 over-expression in the embryonic mouse neocortex resulted in an increase in BP proliferation and increased ECM expression (Güven et al., 2020).

This proliferative role of the ECM is further supported by the expression and activation of integrins in BPs. Integrin alpha v beta 3 is more highly expressed in the proliferative human BPs compared to the less proliferative mouse BPs (Stenzel et al., 2014), consistent with the above reports of higher ECM expression in the former cells (Fietz et al., 2012; Miller et al., 2014; Florio et al., 2015). When integrin alpha v beta 3 was activated in the embryonic mouse neocortex, by addition of a function-activating antibody, this increased BP proliferation (Stenzel et al., 2014). Integrin beta 1 activation is also important for human BP proliferation, as blocking integrin beta 1 function in human fetal neocortex slice cultures decreased BP proliferation (Kalebic et al., 2019). The expression and function of integrins in human BPs is thought to be important for the interaction of these cells with their pro-proliferative ECM environment, enabling their increased proliferative capacity compared to BPs in the mouse. Human BPs have a more complex morphology in comparison

to mouse, with more variation and a higher number of radial processes. These processes contain integrins that are able to interact with the surrounding ECM. Therefore, the more radial processes, the more pro-proliferative signals the BP can receive from the ECM environment (Kalebic et al., 2019; Kalebic and Huttner, 2020).

## Models to Study ECM

A major reason for our limited knowledge of the role of the ECM in human and non-human primate neocortex development is the difficulty in modelling primate neocortex development. There are several options currently available, including the use of primary tissue or the generation of 2D and 3D models, notably brain organoids, from embryonic and induced pluripotent stem cells (ESCs and iPSCS). Primary tissue studies have provided valuable insight, as described above (Kalebic et al., 2019) and in the following section in more detail (Long et al., 2018). This includes the use of primary tissue to study ECM expression patterns (Fietz et al., 2012; Jovanov Milošević et al., 2014; Miller et al., 2014; Florio et al., 2015; Long et al., 2018) and the use of *ex vivo* tissue culture techniques to study the role of the ECM in an intact human tissue environment (Long et al., 2018; Kalebic et al., 2019; Eigel et al., 2021). However, access to this tissue is limited, making the 2D and 3D models from ESCs and iPSCs an attractive alternative for many researchers.

Using these 2D and 3D models comes with many clear advantages. They allow the study of human and non-human primate cells, and recent protocols have shown early neocortex development can be recapitulated relatively well in these *in vitro* systems (Mason and Price, 2016; Jabaudon and Lancaster, 2018; Heide et al., 2018; Kanton et al., 2019; Kyrousi and Cappello, 2019; Molnár et al., 2019). However, how these cells are cultured may have a significant effect on the investigation of ECM function in these models. For example, the ECM generated *in vitro* by human iPSC-derived NPCs is different in 2D cultures compared to 3D culture systems (**Figure 2**). The ECM is more similar to the *in vivo* composition when iPSC-derived NPCs are culture in 3D neurospheres than 2D systems, containing more proteoglycans and fewer basement membrane components (**Figure 2**; Simão et al., 2018). When these cells are grown as more complex cerebral organoids, the ECM composition expressed by the APs is indeed more similar to those of APs in the human fetal neocortex (Camp et al., 2015). This includes the ECM components collagen IV alpha 5, laminin alpha 1 and brevican, and the ECM receptors integrin alpha 6 and beta 8.

Additionally, the non-neuronal cells within the organoids also express ECM found in the fetal neocortex, including decorin, lumican, and collagen I alpha 2 (Camp et al., 2015). This data would suggest that 3D *in vitro* models, such as cerebral organoids, are better suited for the study of the role of ECM in neocortex development. However, although these 3D culture systems promote expression of some ECM components, there are still many that are not expressed that may play important roles. These include those secreted from cell types not present in these organoids, such as the blood vessels and meninges. It would be interesting to investigate how the addition of the ECM generated by these cells affects the development of such cerebral organoids.

One way to study the entire ECM network in 3D cell-based models is to use ECM extracted from primary tissue. Human iPSCs grown on ECM secreted by NPCs increased the expression of neuronal genes, such as tubulin beta 3, in comparison to ECM secreted by ESCs (Yan et al., 2015). ECM extracted from adult human brain tissue promoted increased laminin deposition in cerebral organoids, increased proliferation of APs and increased neural production (Cho et al., 2021). It would be interesting to investigate the effect of ECM extracted from fetal neocortex tissue using this model, as it is highly likely to differ in composition to the adult ECM extract used in this study.

How the ECM composition can direct the neurogenesis of iPSC-derived NPCs is yet to be determined. In addition to the known signalling functions of ECM, such as activation of integrins, the stiffness and elasticity of the matrix can also alter neurogenesis. Human iPSCs grown in softer ECM matrices differentiate into neuroectoderm and generate neurons, unlike iPSCs grown on stiffer matrices (Keung et al., 2012). This raises an interesting issue when comparing 2D and 3D *in vitro* models, as 3D models will inherently generate environments that are softer than 2D cultures grown on glass or plastic. How stiffness changes in these culture systems and how this compares to tissue will be important to determine the different roles of distinct ECM compositions in human neocortex development.

# ECM AND NEOCORTEX TISSUE ARCHITECTURE

The expression and function of ECM have also been studied in human fetal neocortex tissue. ECM expression appears to be dynamic, with changes in the cortical wall over the course of development. For example, hyaluronic acid expression levels change with the laminar structure of the subplate between 13 and 21 post-conception weeks (Kostović et al., 2019). Temporal and spatial changes in ECM expression could have a major impact on the development of the neocortex and has been hypothesised to affect the folding of the cortical plate (van Essen, 2020). This could occur in several ways, including mechanical forces and neuronal migration.

ECM composition has already been shown to affect the stiffness of human fetal neocortex slice cultures, as removing specific ECM components (hyaluronic acid) using enzymatic degradation (by hyaluronidases) resulted in reduced stiffness (Long et al., 2018). In addition, cortical folding could be induced in these human fetal neocortex slice cultures by adding the ECM components Hyaluronan And Proteoglycan Link Protein 1 (HAPLN1), lumican, and collagen I (**Figure 3**; Long et al., 2018). This ECM-induced folding was also replicated in sliced neocortical organoids (Qian et al., 2020). In the primary tissue, the ECM-induced folding required an increase in ECM stiffness prior to the fold formation, resulting in a specific pattern of stiffer ECM at the gyrus of the fold and softer ECM in the sulcus (**Figure 3**; Long et al., 2018). Although tissue and ECM mechanics are hypothesised to regulate cortical folding (Karlinski and Reiner, 2018), this ECM-induced folding also required ERK signalling downstream of the hyaluronic acid receptor CD168 (Long et al., 2018),

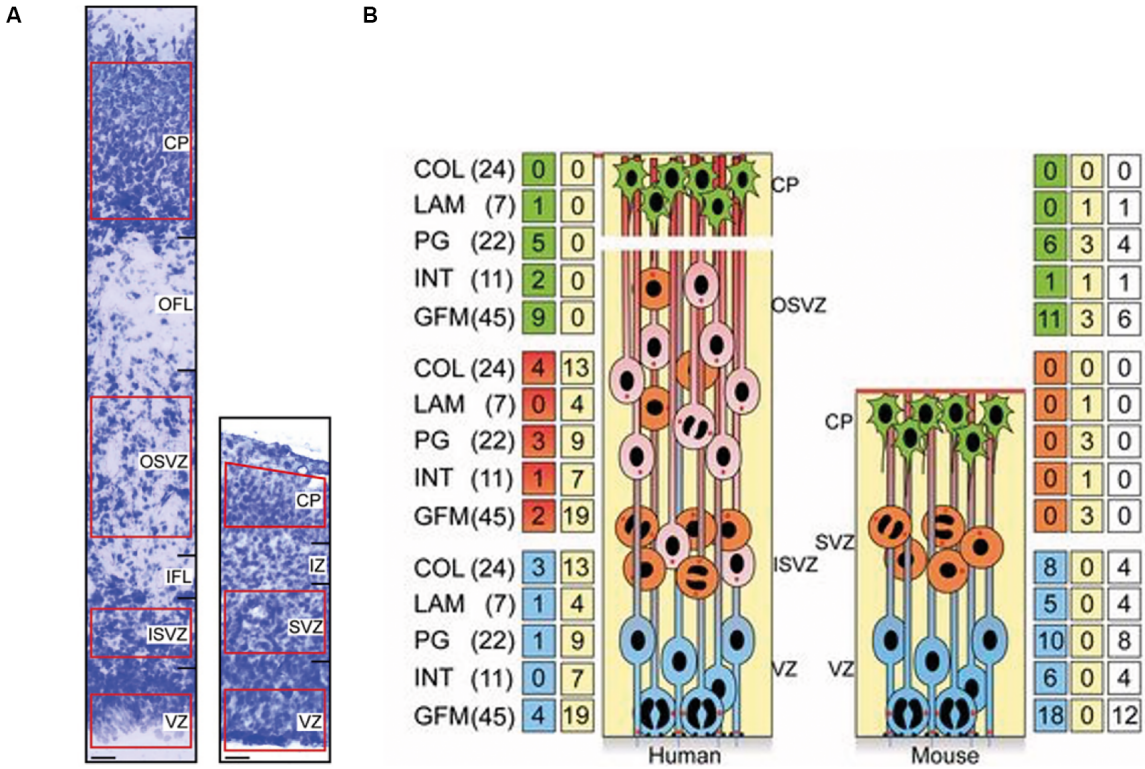


**FIGURE 1 |** Increased expression of extracellular matrix (ECM) components in the proliferative zones of the human fetal neocortex compared to the embryonic mouse neocortex. **(A)** Nissl staining of the cortical wall of a 15 post-conception week (pcw) human fetal neocortex (left) and of an embryonic day (E) 14.5 embryonic mouse neocortex (right). Scale bars represent 20 μm. The areas used for transcriptomic analysis are highlighted in red. The different zones of the cortical wall are labelled as ventricular zone (VZ), subventricular zone (SVZ), inner SVZ (ISVZ), outer SVZ (OSVZ), inner fibre layer (IFL), outer fibre layer (OFL), intermediate zone (IZ) and cortical plate (CP). **(B)** Schematic illustrating the major cell types within the developing cortical wall (APs = blue, BPs = orange and red, neurons = green) and the distribution of ECM and ECM-associated components within these areas. The major families of ECM components are the collagens (COL), laminins (LAM), proteoglycans (PG), integrins (INT) and growth factors/morphogens (GFM). Numbers in parentheses indicate the total numbers of genes within each family. Numbers in the green/red/blue boxes indicate the number of genes overexpressed in the adjacent area. Numbers in the yellow boxes indicate the number of genes that are overexpressed in more than one human or mouse zone. Numbers in the white boxes indicate the number of genes that are overexpressed in both humans and mice. The cells within the zones are: APs are located within the VZ in both mice and humans, BPs are located in the SVZ in mice, and within the ISVZ and OSVZ in humans, neurons are located in the CP in both species. Adapted from Fietz et al. (2012).

suggesting the role of ECM in cortical folding and tissue morphology will require both mechanical changes and signalling *via* ECM receptors.

ECM composition was also hypothesised to affect the migration of neurons. In the mouse, the majority of glutamatergic neuronal migration in the cortex is thought to occur radially, with neurons migrating along the radial glia scaffold up to the cortical plate. However, tangential migration of glutamatergic neurons has been observed in the developing macaque cortex (Cortay et al., 2020) and is thought to be important for cortical folding (van Essen, 2020). It has been hypothesised that ECM could direct this migration by providing directional cues or a scaffold for the neurons to migrate along. ECM components and receptors could also alter the morphology of the newborn neurons, as over-expression of integrin alpha 9 in human NPCs increased the outgrowth of neurites *in vitro* when grown on tenascin-C and when grafted into newborn rat cortex (Forbes and Andrews, 2019). Increased processes may be important for the interaction with the ECM environment, as discussed above for BPs (Kalebic et al., 2019).

ECM, once expressed, can be present in tissues for a long period of time (Brückner et al., 1998; Zimmermann and Dours-Zimmermann, 2008; Ewald, 2020). Therefore, the removal of ECM components at the right time is also important for neocortex development. Modulation of the ECM, such as degradation by metalloproteases (MMPs), has been reported throughout neocortex development. MMP2 has been shown to modulate the ECM deposited by growing microvessels within the early fetal human neocortex (Girolamo et al., 2004). This could impact neuronal migration, as BPs are reported to attach their basal process to blood vessel ECM later in neocortex development (Nowakowski et al., 2016), altering the scaffold of cell processes newborn neurons are able to migrate along. This builds a complex picture of ECM deposition and modulation that may help direct the migration of neurons and eventually determine the shape of the developing neocortex.

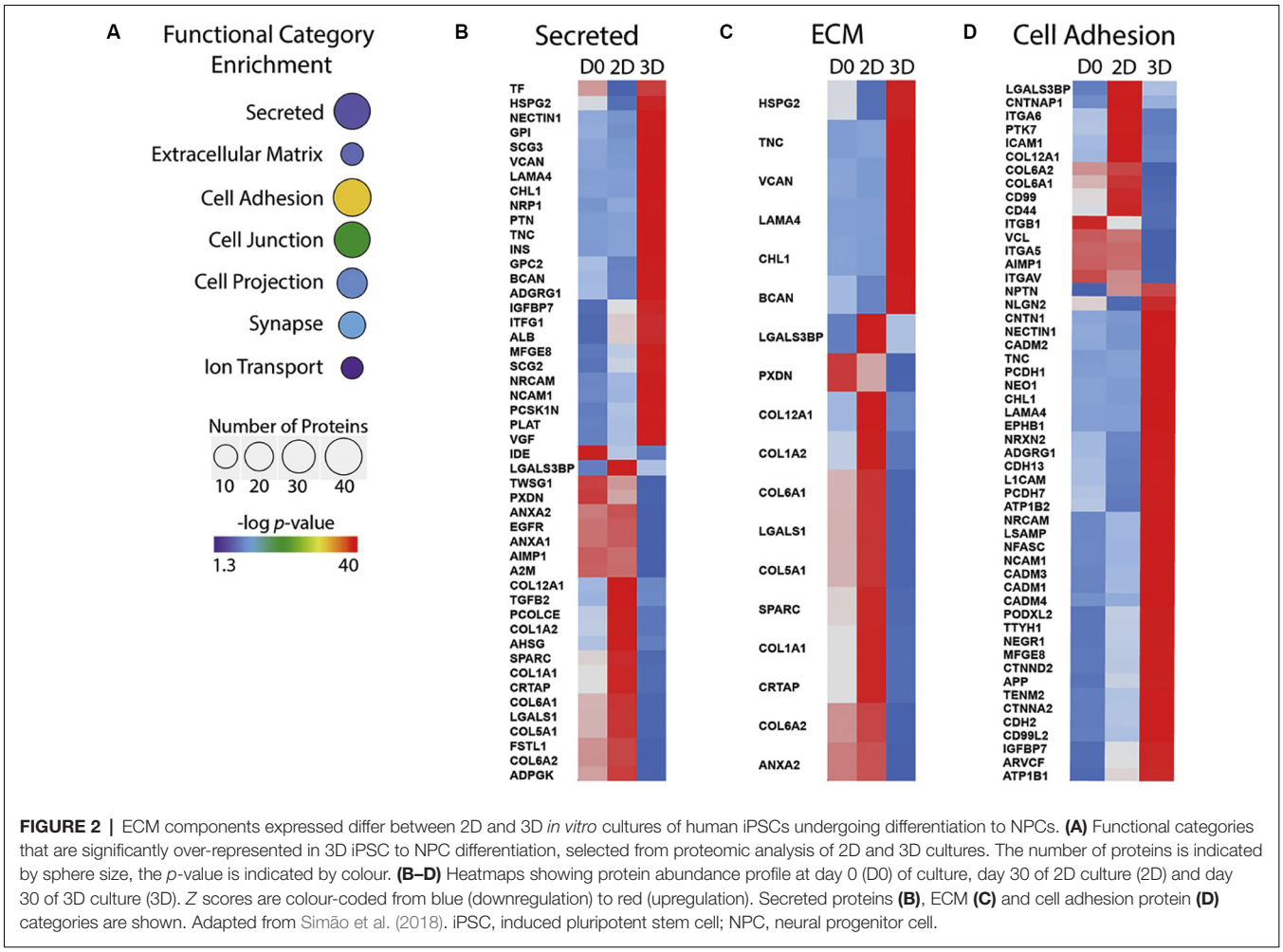


**FIGURE 2 |** ECM components expressed differ between 2D and 3D *in vitro* cultures of human iPSCs undergoing differentiation to NPCs. **(A)** Functional categories that are significantly over-represented in 3D iPSC to NPC differentiation, selected from proteomic analysis of 2D and 3D cultures. The number of proteins is indicated by sphere size, the *p*-value is indicated by colour. **(B–D)** Heatmaps showing protein abundance profile at day 0 (D0) of culture, day 30 of 2D culture (2D) and day 30 of 3D culture (3D). *Z* scores are colour-coded from blue (downregulation) to red (upregulation). Secreted proteins **(B)**, ECM **(C)** and cell adhesion protein **(D)** categories are shown. Adapted from Simão et al. (2018). iPSC, induced pluripotent stem cell; NPC, neural progenitor cell.

## ECM AND NEURODEVELOPMENTAL DISORDERS

It can be difficult to study the function of the ECM in human neurodevelopment, despite the recent advances in human cell- and tissue-based culture models. However, a lot has been learnt about the normal function of the ECM in neocortex development from various neurodevelopmental disorders. There are many mutations in ECM genes, or genes affecting ECM function, that lead to neurodevelopmental disorders, with RELN (for the protein Reelin) being one the most well-known examples.

Reelin is an ECM glycoprotein (Hynes and Naba, 2012) secreted by several cell types, including the Cajal Retzius cells, early in the human fetal cortex (D'Arcangelo et al., 1995; Meyer et al., 2002). Mutations in the human gene RELN are linked to various neurodevelopmental disorders, including schizophrenia, bipolar disorder, and autism spectrum disorder (Ishii and Maeda, 2008; Lakatosova and Ostatnikova, 2012). RELN mutations are also known to cause type I lissencephaly, with abnormal lamination of the cortical plate and a loss of cortical folding (**Figure 4A**; Hong et al., 2000). The mechanisms underlying this malformation have been elucidated in mouse models (Hong et al., 2000; Fatemi, 2001; Rice and Curran, 2001; Pérez-Martínez et al., 2012; Sekine et al., 2012; Hirota and Nakajima, 2017; Mizukami et al., 2018), which show that Reelin is important for directing the migration of newborn neurons and the correct positioning of these neurons in the cortical plate.

The role of ECM in neuronal migration disorders is relatively common. Patients with another type of lissencephaly, cobblestone lissencephaly, also have mutations in genes that alter ECM function. In particular, the enzymes POMT1/2 that glycosylate the ECM receptor alpha-dystroglycan have been found in patients with cobblestone lissencephaly (**Figure 4B**; Devisme et al., 2012) and in Walker-Warburg syndrome (De Bernabé et al., 2002; Van Reeuwijk et al., 2005). Mutations in the genes that encode for the laminin subunits beta 2 and gamma 2 (LAMB2 and LAMC2) have also been associated with disorganisation of the cortical layers, similar to cobblestone lissencephaly (Radner et al., 2013), as have mutations in the laminin subunit beta 1 (LAMB1; Radmanesh et al., 2013). These mutations are thought to lead to these disorders *via* disruption of the pial basement membrane, which lies at the top of the cortical plate. This disruption enables migrating neurons to bypass their usual stop signals, migrating above the cortical

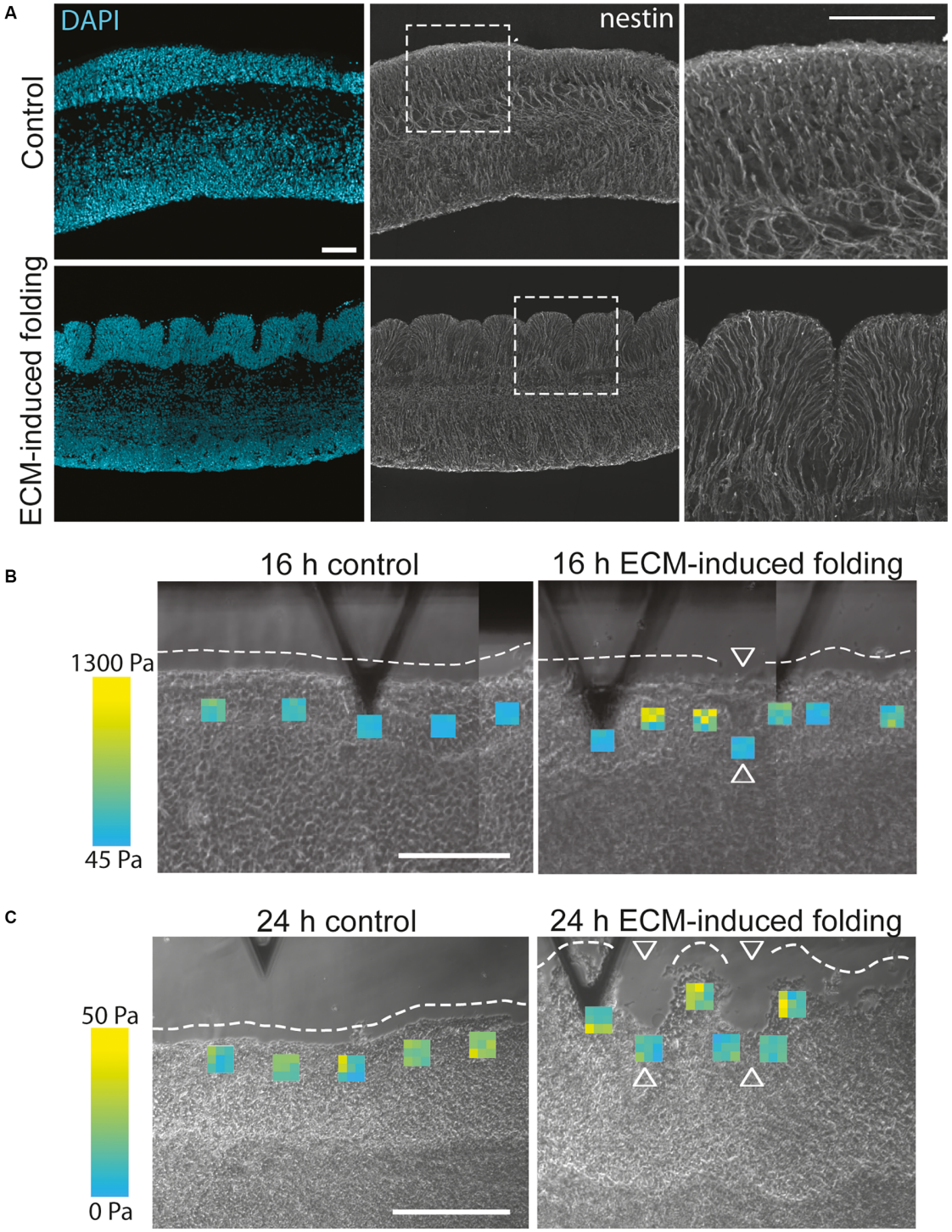


**FIGURE 3 |** ECM can induce folding and changes in ECM stiffness in human fetal neocortex *ex vivo* cultures. **(A)** Images showing a 13 gestation week (GW) human fetal neocortex after 24 h of *ex vivo* culture in the control condition (upper panels) or after the addition of the ECM components HAPLN1, lumican and collagen I (lower panels), with DAPI staining (blue) and immunofluorescence for the radial glial process marker nestin (grey). The addition of these ECM components induced the folding of the cortical plate. White dashed boxes delineate areas shown in the panels on the right. Scale bar represents 50 μm. **(B,C)** Images showing atomic force microscopy of 13 GW human fetal neocortex after 16 h (**B**, folding ongoing) or 24 h (**C**, folding complete) of *ex vivo* culture in control conditions (left panels) or after the addition of the ECM components HAPLN1, lumican and collagen I (right panels, ECM-induced folding). Each heatmap square shows nine Young's modulus values in the position the measurement was taken (see the range of values from blue (lowest) to yellow (highest)). White dashed lines outline the unfolded or folded CP surface; arrowheads indicate sulci. The stiffest measurements (yellow) are found in the forming gyri compared to the softer measurements (blue) in the forming sulci. Scale bar represents 200 μm. Adapted from Long et al. (2018).

plate and creating a bumpy, cobblestone-like appearance (Radner et al., 2013).

Mutations in laminin subunits are also linked to another folding disorder, polymicrogyria, characterised by an excess of shallower folds on the cortical surface. LAMC3 mutations have been found in patients with a developmental delay in the occipital cortex and focal areas of polymicrogyria (Barak et al., 2011). This laminin subunit was found to be highly expressed in the cortical plate at 20 post-conception weeks (pcw) human fetal cortex, with lower expression levels in the ventricular and inner and outer subventricular zones. This expression peaked around mid-gestation and was maintained until 12 months postnatally, coinciding with the peak of dendritogenesis and the generation of the majority of the cortical folds (Barak et al., 2011).

ECM has also been linked to other aspects of cortex shape during development and neurodevelopmental disorders, with several mutations in the collagen family found in patients. Mutations in collagen IV alpha 1 (COL4A1) have been linked to porencephaly, the development of a cavity in the cortex filled with cerebrospinal fluid (Hubert et al., 2009). COL18A1 mutations have also been found in patients with Knobloch syndrome and are associated with malformations of cortical development including heterotopias and pachygyria (Hubert et al., 2009). Both of these malformations are the result of abnormal neuronal migration, resulting in neurons clustering in abnormal layers in the cortical wall. Finally, COL1A1 and COL2A1 mutations have been associated with hydrocephaly, characterised by increased cerebrospinal fluid pressure and enlarged ventricles (Hubert et al., 2009). Together, this suggests an important role of ECM in shaping the developing human neocortex.

Many neurodevelopmental abnormalities are linked to much more focal malformations of cortical development, such as the abnormal lamination of neurons or dysmorphic neuronal cells, called balloon cells (Zamecnik et al., 2012). These malformations can be associated with epilepsy and are therefore often found in resected tissue from these patients. When tissue from such resections was examined, changes in the extracellular space were observed. These included an increase in the expression of the ECM components tenascin-C, tenascin-R, versican, and a decrease in brevican expression (Zamecnik et al., 2012).

Finally, there is also evidence that the modulation of the ECM in the developing neocortex is important for its development. Mutations in the enzyme neurotrypsin, a serine protease that degrades agrin, are linked to intellectual disability in a small number of patients (Molinari et al., 2002). Agrin is expressed during fetal neocortex development and is thought to help regulate synaptic function and be important for memory later on in life (Molinari et al., 2002). Several other ECM components are also known to have a vital role in perineuronal nets, which are also important for synaptic function (Barros et al., 2011; De Luca and Papa, 2016; Sorg et al., 2016; Miyata and Kitagawa, 2017). Perineuronal nets are lattice-like ECM structures that surround the synapses of certain neurons, including several interneurons (Sorg et al., 2016). They are predominately made up of chondroitin sulfate proteoglycans, tenascin, hyaluronan, and proteoglycan link proteins and hyaluronic acid (Sorg et al., 2016).

Impairment of perineuronal nets, in particular the enzyme that modifies them (matrix metalloproteinase-9), has been linked to several neurodevelopmental disorders, including fragile X syndrome, autism spectrum disorder, and epilepsy (Reinhard et al., 2015; Rogers et al., 2018; Wen et al., 2018). It has also been suggested that neuronal activity could feed back and modulate ECM expression surrounding neurons too (Lazarevich et al., 2020). As synaptic pruning occurs over the first few years of life in humans and non-human primates (Hensch, 2004), the ability to modulate the ECM that regulates synapse formation and stabilisation is vital to ensure this happens correctly (Gundelfinger et al., 2010).

There is growing evidence for the role of ECM in neurodevelopmental disorders, with more and more mutations in genes affecting ECM function discovered in patients. However, whilst we can associate the ECM with the cortical abnormalities detected, we still do not understand the mechanisms underlying them. Understanding the normal function of ECM in human neocortex development is an important step that would enable us to uncover how altering its functions leads to the malformations observed in these patients, potentially opening up new therapeutic avenues.

## WHAT ELSE CAN WE LEARN?

Many of the functions of the ECM in neocortex development have been uncovered using animal models (Barros et al., 2011; Faissner and Reinhard, 2015; Long and Huttner, 2019), but it remains unclear how many of these functions are conserved in humans. However, as we described above in the "ECM and Neural Progenitors" section, RNA sequencing studies have revealed key differences in ECM expression between these models and the developing human neocortex. As ECM components often interact with each other, functioning as a network, this change in ECM expression is likely to affect its function. This is one of the reasons why it is important we understand the role of the ECM in the development of the human neocortex.

Another reason is that many of the findings from development can be useful for understanding the role of the ECM in injury and neurodegeneration. For example, how ECM expression changes upon transplantation of iPSC-derived cells into the adult brain, how ECM composition is altered upon injury, and how this change may impact inflammation and repair (Roll and Faissner, 2014), which are all key issues for future research. ECM and ECM modulators are known to be expressed by reactive astrocytes but can also be expressed at lower levels by neurons, oligodendrocytes, microglia, and the cells of the vasculature. These include chondroitin sulphate proteoglycans, associated with the inhibition of axonal outgrowth, and heparan sulphate proteoglycans, associated with the promotion of axonal outgrowth. The balance of these two ECM families is vital for the repair of the adult neocortex (Roll and Faissner, 2014). Understanding their roles in human neocortex development could provide important information about this balance in the adult, informing and potentially improving current avenues for therapeutic options.

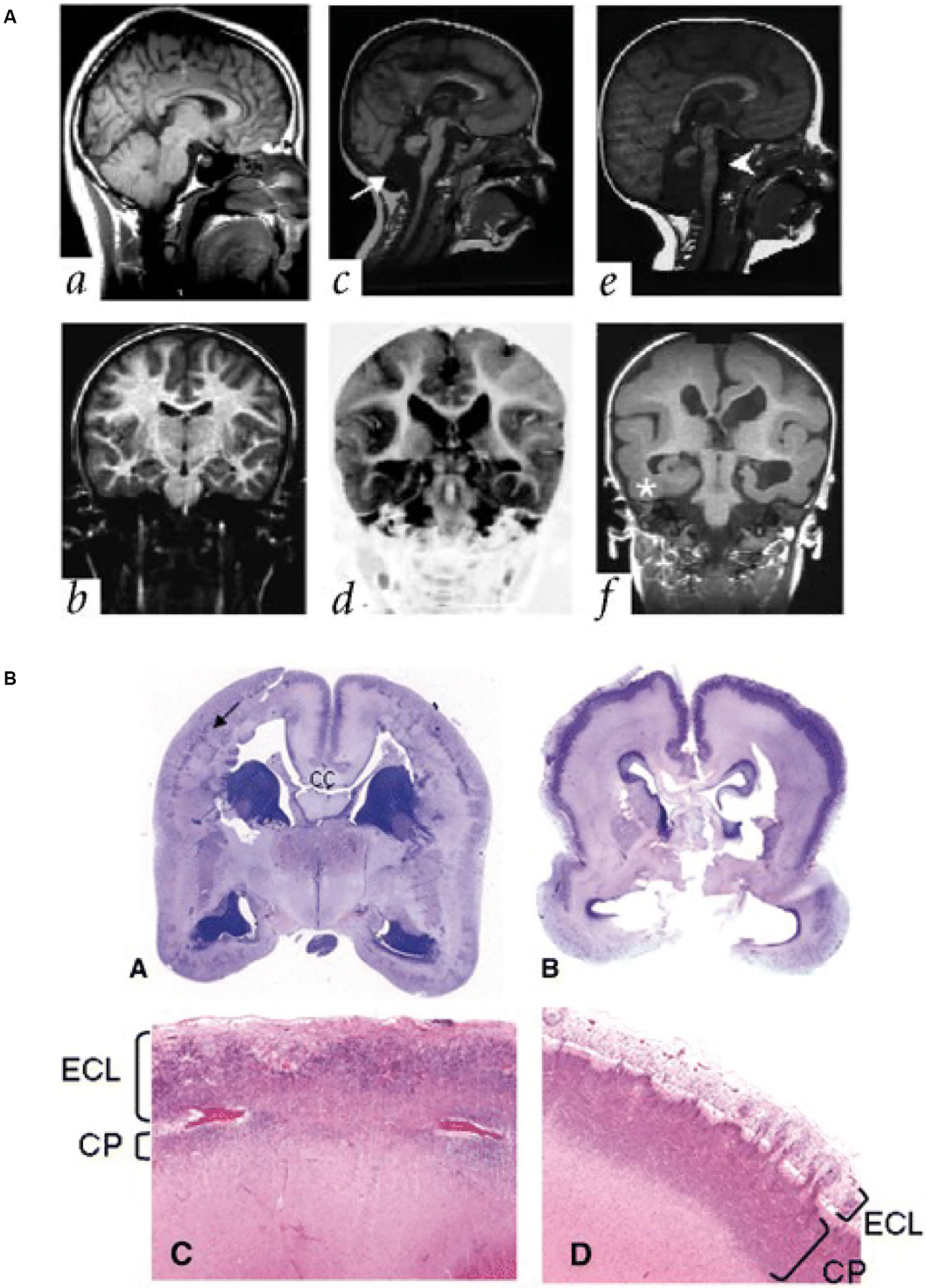


**FIGURE 4 |** Lissencephaly in patients with mutations in ECM and ECM-associated genes. **(A)** Parasagittal (upper panels) and coronal (lower panels) MRI from patients with a normally developing brain (*a,b*), and two patients with lissencephaly associated with RELN mutations (*c,d*; *e,f*). Note the thickened cortex and reduced folding present. Adapted from Hong et al. (2000). **(B)** Images showing examples of cobblestone lissencephaly in a 19 pcw fetus with a POMT1 mutation (**A,C**, left panels) and 26 pcw fetus with a POMGNT1 mutation (**B,D**, right panels). The top panels show whole-mount sections of the brain. Arrow indicates impoverished cortical plate in the top left panel. The lower panels show higher magnification images of the cortical plate from these fetuses. Note the reduced size of the cortical plate (CP) and the thickened extracortical layer (ECL) in the lower left panel, but the thin ECL in the lower right panel. CC indicates the corpus callosum. Adapted from Devisme et al. (2012).

We therefore still have a lot to learn about the role of the ECM in human and non-human primate neocortex development. It is clear that ECM components are involved in almost every aspect of neocortex development, but exactly what ECM is expressed when and where is not yet known. Understanding these dynamics and the differences between species will be an important step forward in understanding how ECM shapes human neocortex development and the role it has played in the evolution of the human brain.

## AUTHOR CONTRIBUTIONS

KL and WH wrote the manuscript. All authors contributed to the article and approved the submitted version.

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MINI REVIEW
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# How Stress Influences the Dynamic Plasticity of the Brain's Extracellular Matrix

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Diffuse and structured extracellular matrix (ECM) comprise ∼20% of the brain's volume and play important roles in development and adult plasticity. Perineuronal nets (PNNs), specialized ECM structures that surround certain types of neurons in the brain, emerge during the postnatal period, making their development and maintenance potentially sensitive to experience. Recent studies have shown that stress affects diffuse ECM as well as PNNs, and that such effects are dependent on life stage and brain region. Given that the ECM participates in synaptic plasticity, the generation of neuronal oscillations, and synchronous firing across brain regions, all of which have been linked to cognition and emotional regulation, ECM components may be candidate therapeutic targets for stress-induced neuropsychiatric disease. This review considers the influence of stress over diffuse and structured ECM during postnatal life with a focus on functional outcomes and the potential for translational relevance.

**Keywords: perineuronal net, extracellular matrix, stress, hippocampus, prefrontal cortex, amygdala**

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## INTRODUCTION

In recent decades, it has been recognized that a comprehensive view of brain function requires considering the entirety of the brain's microenvironment, including neurons, non-neuronal cells, the vasculature, and the extracellular matrix (ECM). Compelling evidence now suggests that each of these entities contributes not just to the brain's structure, but to its function and its ability to respond to experience with adaptive changes (Song and Dityatev, 2018; Cope and Gould, 2019). Perhaps because of their malleability, each of these constituents has also been linked to neuropsychiatric disease. A growing literature suggests that the ECM is particularly important for a wide range of processes that are critical components of experience-dependent change in brain function, including synaptic plasticity (De Luca et al., 2020), neuronal oscillations (Carceller et al., 2020; Wingert and Sorg, 2021), and network connectivity (Bucher et al., 2021; Christensen et al., 2021). Since plasticity at the synapse, neuronal synchrony within a brain region, and coherence across brain regions are critical for healthy brain function, understanding ECM involvement in these processes may also elucidate its role in brain pathology.

The ECM includes two main categories: diffuse and structured (Nicholson and Syková, 1998; Krishnaswamy et al., 2019). Diffuse ECM fills the spaces among neurons, glia, and the brain's microvasculature, and consists of polysaccharides, proteins, glycoproteins, and glycosaminoglycans. Although the ECM was initially thought to function primarily as a supportive substrate, essentially holding brain cells together (Celio, 1999; Cope and Gould, 2019), it is now known to play major roles in both the developing and adult brain, including the guidance of migrating neurons and growing axons, attracting and repelling astrocytes and microglia, and

regulating neurotransmitter receptor availability at the synapse (Franco and Müller, 2011; Lubbers et al., 2014). Structured ECM has a similar general chemical composition to diffuse ECM, but due to a high concentration of chondroitin sulfate proteoglycans (CSPGs), it forms a lattice-like assembly around certain types of neurons, primarily inhibitory interneurons. These structures, known as perineuronal nets (PNNs), typically surround the cell bodies and proximal dendrites of neurons throughout the brain. Multiple functions have been ascribed to PNNs, including to protect neurons from free radical damage, to limit the formation of unnecessary synapses, and to functionally stabilize neuronal systems by increasing inhibitory tone after development has ended (Sorg et al., 2016; Bucher et al., 2021; Burket et al., 2021). Perhaps not surprisingly given the number of important neural processes linked to diffuse and structured ECM, studies suggest that both types are associated with key brain functions, including learning and memory, as well as emotional processing.

Stress is known to affect cognitive processes, as well as mood regulation (McEwen, 2010), raising questions about whether it does so, at least in part, by impacting the ECM. Indeed, a growing literature indicates that stress impacts the ECM both during development and in adulthood (**Tables 1**, **2**). This mini-review first provides a general overview of studies linking the ECM to brain function during development and adulthood, and then focuses on how stress may influence both diffuse and structured ECM, including discussion about potential functional consequences.

**TABLE 1 |** Stress effects on diffuse ECM components.

| Stressor and timing | Species/strain/sex | Brain region | ECM component effect* | References |
|---|---|---|---|---|
| Maternal separation P2-14 (3 h daily) | Rat/Wistar/male | HIP | Reelin decrease during development, increase in adulthood | Zhang et al., 2013 |
| Maternal separation P1-14 (3 h daily) | Rat/SD/male | HIP | Reelin increase in adulthood | Wang et al., 2018 |
| Corticosterone injection in adulthood | Rat/SD/male | HIP, PFC | Reelin decrease in adulthood | Lebedeva et al., 2020 |
| Chronic unpredictable mild stress for 6 weeks in adulthood | Mice/apoE/male | HIP, PFC | Reelin decrease in adulthood | Zhang et al., 2021 |
| Chronic unpredictable stress for 6 weeks in adulthood | Rat/SD/male | HIP, FC | Laminin decrease | Laifenfeld et al., 2005a |
| Social defeat during adolescence | Mice/C57/male | HIP, NAc | Laminin decrease | Rodríguez-Arias et al., 2017 |

**Compared to unstressed controls.*
*SD, Sprague Dawley; apoE, apolipoprotein E; FC, frontal cortex; HIP, hippocampus; PFC, prefrontal cortex; NAc, nucleus accumbens.*

## EXTRACELLULAR MATRIX FUNCTION DURING DEVELOPMENT AND IN ADULTHOOD

Numerous studies have shown that diffuse ECM plays both subtle and vital roles in brain development. With regard to the latter, the glycoprotein laminin is critical for neural tube closure, making its knockout lethal (Miner et al., 1998). After the basic structure of the brain is formed, other ECM molecules, such as the glycoprotein reelin and the glycosaminoglycan hyaluronan, help to coordinate neuronal migration, axon guidance, and synaptogenesis (Borrell et al., 2007; Honda et al., 2011; Vaswani and Blaess, 2016). ECM molecules also sequester growth factors, chemokines, and additional molecules with attractant and repellent properties, all of which work to coordinate brain development. Diffuse CSPGs also play a role in multiple cellular events during development (Sirko et al., 2007; Zimmer et al., 2010), and their sulfation patterns are known to influence their involvement in events such as neuronal migration and maturation (Maeda et al., 2010, 2011).

As development proceeds, molecules of the diffuse ECM, such as reelin, undergo changes in abundance and function. In adulthood, reelin takes on a new function of enhancing synaptic plasticity at excitatory synapses. Reelin facilitates this function *via* its binding to the apolipoprotein E (apoE) receptor 2, which forms a complex with NMDA receptors (Beffert et al., 2005; Korwek et al., 2009). Reelin also functions in stimulating adult neurogenesis and dendritic spine formation in the hippocampus (Pujadas et al., 2010; Sibbe et al., 2015). Hyaluronan seems to have the opposite effect of reelin on adult neurogenesis in that its binding to the CD44 receptor reduces the production of new neurons in the hippocampus. Hyaluronan increases in the aging brain and may play a causal role in age-related reductions in adult neurogenesis (Su et al., 2017). Hyaluronan also participates in synaptic plasticity by regulating dendritic calcium channels (Kochlamazashvili et al., 2010).

During the postnatal period, the ECM surrounding a subset of neurons condense and forms PNNs, with CSPGs as major components. The lattice-like shape PNNs comes from the organization of CSPGs that bind the base of PNNs, which is hyaluronan, also a component of the diffuse ECM. The main CSPGs in the brain include aggrecan, neurocan, brevican, versican, and phosphocan, and their expression amounts are region-specific (Dauth et al., 2016; Pantazopoulos et al., 2021). Although speculative, the heterogeneous combination of PNN components may provide specialized function (Dauth et al., 2016). In addition, PNNs may have different functions depending on the sulfation patterns of their CSPGs, with some patterns conferring greater plasticity than others (Miyata et al., 2012; Yang et al., 2017).

The developmental appearance of PNNs in some brain regions coincides with the closure of critical periods, including the emergence of binocular vision in the visual cortex (Hensch and Quinlan, 2018) and leptin sensitivity in the hypothalamus (Mirzadeh et al., 2019). In adulthood, PNNs are thought to

**TABLE 2 |** Stress effects on PNNs.

| Stressor and timing | Species/strain/sex | Brain region | PNN effect* | References |
|---|---|---|---|---|
| Scarcity/adversity P8-P12 | Rat/SD/male | BLA | Lower PNN intensity at P23 | Santiago et al., 2018 |
| Scarcity/adversity P4-P10 | Rat/SD/male, female | BLA | Higher PNN number in right male BLA at P28 | Guadagno et al., 2020 |
| Maternal separation P2-P20 (4 h daily) | Rat/SD/male, female | BLA, PFC | Lower PFC PNN number at P20; higher PNN intensity in adult male PFC, adult female BLA | Gildawie et al., 2020 |
| Maternal separation P2-P14 (3 h daily) | Rat/SD/male, female | BLA, PFC | No change in PNN number in BLA or PFC at P18 or P28 | Richardson et al., 2021 |
| Maternal separation P2-P14 (3 h daily) | Rat/SD/male | HIP | Higher PNN CSPGs in adults | Dimatelis et al., 2013 |
| Maternal separation and early weaning P2-P16 (4–8 h daily) | Mice/C57/male | HIP | Higher PNN intensity in adults | Murthy et al., 2019 |
| Maternal separation P2-P20, social isolation P21-P35 | Rat/SD/male, female | PFC | Lower PNN PV number, PNN intensity in females | Gildawie et al., 2021 |
| Social isolation P21-P56 | Mice/C57/male | HIP, PFC | Lower PNN number PFC | Ueno et al., 2017 |
| Unpredictable chronic mild stress P28–42 | Mice/C57/male, female | PFC | No change in PNN number | Page and Coutellier, 2018 |
| Social isolation P21-P90 | Mice/FVB/male | HIP, RC | Lower PNN number in HIP, RC | Klimczak et al., 2021 |
| Social defeat (daily for 5 days) in adults, social isolation (3–60 days) | Rat/LE/male | HIP | Lower PNN CSPGs at 3 days, higher at 60 days post-stress | Koskinen et al., 2020 |
| Restraint (6 h/day for 10 days) in adults | Rat/SD/male | HIP, BLA, HAB, RT, PFC | Higher PNN number in PFC; lower in HIP; increased intensity in RT, HAB | Pesarico et al., 2019 |
| Chronic mild stress (10, 20, 30 days) in adults | Rat/SD/male | PFC | Lower PNN number at 20 days | Yu et al., 2020 |
| Social defeat (daily for 5 days) in adults; social isolation (30–60 days) | Rat/Wistar/male | HIP | Higher PNN number | Riga et al., 2017 |

**Compared to unstressed controls.*
*SD, Sprague Dawley; FVB, Friend Leukemia Virus, strain B; LE, Long Evans; PV, parvalbumin; PNN, perineuronal net; BLA, basolateral amygdala; HIP, hippocampus; RC, retrosplenial cortex; HAB, habenula; RT, reticular thalamic nucleus; PFC, prefrontal cortex.*

restrict plasticity, both by preventing the ingrowth of axons and the mobilization of neurotransmitter receptors at the synapse. In some cases, PNNs have been associated with increased firing of neurons they surround, which are primarily parvalbumin+ inhibitory interneurons in the neocortex and hippocampus (Sorg et al., 2016; Wingert and Sorg, 2021). The functional consequences of PNNs seem to differ depending on brain region, cell type, and behavioral task, with some studies showing that PNNs facilitate learning and memory, while others show they have an inhibitory effect on these processes (Paylor et al., 2018; Anderson et al., 2020; Carulli et al., 2020; Cope et al., 2021; Wingert and Sorg, 2021). The overall picture that is emerging is one that is common in biology—an inverted U-shaped curve exists where atypically low or high PNNs impair function. In addition to effects on behavior, PNNs have been shown to regulate neural correlates of cognitive function, including synaptic plasticity, neuronal oscillations, and neuronal synchrony across brain regions (Sorg et al., 2016; Bucher et al., 2021; Wingert and Sorg, 2021). These electrophysiological phenomena are also critical for behaviors associated with emotional regulation, and have been shown to be stress-sensitive (Murthy and Gould, 2020; Tomar et al., 2021), raising the possibility that stress-induced changes in brain function and behavior might occur through changes in the ECM. A growing body of evidence suggests that stress impacts both diffuse and structured ECM during development and in adulthood. The majority of studies investigating the effects of stress on the ECM have focused on stress-susceptible brain regions, including the hippocampus, the prefrontal cortex, and the amygdala, which play crucial roles in cognitive function and emotional processing (McEwen et al., 2015; Smith and Pollak, 2020).

## STRESS EFFECTS ON DIFFUSE EXTRACELLULAR MATRIX

Developmental stress exhibits different effects on reelin signaling depending on the age at which the brains are investigated (**Table 1**). Postnatal stress has been shown to first reduce reelin expression in the hippocampus, and then show a compensatory rebound and overshoot as animals reach adulthood (Zhang et al., 2013). The postnatal stress-induced increase in adult hippocampal reelin expression can be augmented by exposure to a stressful learning paradigm, such as contextual fear conditioning. This effect is accompanied by enhanced hippocampal LTP and dendritic spine density (Wang et al., 2018). These findings suggest that postnatal stress-induced latent increases in reelin expression may serve an adaptive function. In contrast, chronic stress or chronic glucocorticoid treatment in adulthood decrease reelin expression in the hippocampus and prefrontal cortex (Lebedeva et al., 2020; Zhang et al., 2021). Studies additionally suggest that reelin signaling through the apoE receptor is important for mitigating stress-induced behavioral dysfunction, especially in older mice (Zhang et al., 2021). These findings suggest that developmental and acute adult stress may produce adaptive stress responses through augmented reelin signaling, while chronic adult stress may lead

to dysfunction *via* a reduction in reelin expression. Along these lines, it may be relevant that antidepressant drug treatment reverses stress-induced decreases in reelin expression (Fenton et al., 2015; Johnston et al., 2020), and that hippocampal reelin infusions can reverse stress-induced behavioral dysfunction (Brymer et al., 2020).

Laminin expression is also decreased after chronic stress in both the adult hippocampus and frontal cortex (Laifenfeld et al., 2005a; Rodríguez-Arias et al., 2017). Similar to what has been observed with reelin, the stress-induced decrease in laminin can be reversed with antidepressant treatment (Laifenfeld et al., 2005a). Hyaluronan signaling also seems to play a protective role in mediating stress effects, as mice lacking the hyaluronan receptor CD44 exhibit exacerbated stress-induced behavioral dysfunction, as well as reduced brain levels of the neuromodulators serotonin and dopamine (Barzilay et al., 2016). Collectively, the overall picture suggests that components of diffuse ECM are stress-sensitive and potentially involved in adaptive mechanisms enhancing the ability to appropriately respond to stress and buffer against stress-induced pathology.

## STRESS EFFECTS ON PERINEURONAL NETS

Several studies have investigated the effects of postnatal and adult stress on PNNs (**Table 2**). These studies have produced mixed results, likely due to differences in the developmental stage of stress exposure, the type of stressor, as well as the duration of time between stress and brain examination. An additional reason for potential discrepancies may be due to measures used to assess PNNs, which most commonly rely on the binding of an exogenous fluorophore-conjugated plant lectin Wisteria floribunda agglutinin (WFA). Using this approach, researchers most often quantify numbers of WFA+ cells or the intensity of WFA+ cells to assess whether PNNs have changed. WFA is not an endogenous component of PNNs, and although its binding site is known (Nadanaka et al., 2020), it does not label all PNNs (Yamada and Jinno, 2017; Ueno et al., 2018). Thus, changes in WFA labeling may be open to multiple interpretations. One study showed that postnatal stress using a scarcity/adversity model led to reduced PNN intensity in a subregion of the basolateral amygdala (Santiago et al., 2018), while another study using the same model reported increased PNN cell numbers but only on the right side of the amygdala in males (Guadagno et al., 2020). Two additional studies using maternal separation have reported either increased PNN intensity, but only in females (Gildawie et al., 2020), or no differences in PNN measures (Richardson et al., 2021). Although all of these studies used a similar method for identifying PNNs, they did not all use the same measure (WFA+ cell number vs. WFA intensity), and none used a label of a specific CSPG, such as aggrecan. It may be relevant that these studies also examined different time points after postnatal stress (**Table 2**), making it difficult to determine whether the effects would be more similar if the same time point had been examined.

Available evidence suggests a complex set of results from stress studies in the hippocampus and prefrontal cortex, with some studies showing decreased number or intensity of PNNs, others an increase, and others reporting no effects at all (**Table 2**). Examining the papers as a group, however, suggests that stress may have variable effects on PNNs depending on the duration of time after stress exposure. Several studies show decreased PNNs immediately after stress (Ueno et al., 2017; Pesarico et al., 2019; Gildawie et al., 2020, 2021; Koskinen et al., 2020; Yu et al., 2020; Klimczak et al., 2021), and a rebound increase in PNNs as time passes following stress cessation (Dimatelis et al., 2013; Riga et al., 2017; Murthy et al., 2019; Koskinen et al., 2020; Gildawie et al., 2021). Although some findings do not fit with this summary (Page and Coutellier, 2018; Richardson et al., 2021), the majority of studies suggest a stress-induced trajectory involving first suppression followed by an overshooting rebound, similar to what has been observed for reelin signaling (Zhang et al., 2013). This is in line with other theories of stress effects on the ECM, particularly the idea that depressive-like symptoms emerge after an "incubation period," which involves latent increases in PNNs and accompanying plasticity reduction (Koskinen et al., 2020; Spijker et al., 2020). It should be recognized that outside of critical periods, PNNs are capable of rapid remodeling, as has been recently demonstrated in several studies (Marchand and Schwartz, 2020; Pantazopoulos et al., 2020; Uriarte et al., 2020). Evidence suggests that changes in PNNs can occur through alterations in neuronal activity both during development and in adulthood (Dityatev et al., 2007; Carstens et al., 2021; Devienne et al., 2021). Thus, stress-induced changes in neuronal activity (Della Valle et al., 2019; Murthy et al., 2019; Del Arco et al., 2020; Fee et al., 2020) could be a mechanism by which alterations in PNNs occur.

The extent to which stress-induced changes in PNNs represent adaptive or dysfunctional effects remains uncertain. Several studies show that reduced or increased PNN measures in the hippocampus, prefrontal cortex, and amygdala are associated with behavioral changes that are thought to reflect increased avoidance/threat and reduced stress coping behavior (Santiago et al., 2018; Murthy et al., 2019; Koskinen et al., 2020; Yu et al., 2020), as well as impaired cognitive function (Riga et al., 2017; Koskinen et al., 2020). Few studies have addressed causal relationships by including experimental manipulations of PNNs that restore healthy function after stress (Riga et al., 2017). Additional studies in non-stressed rodents have shown that reducing PNNs either by genetic manipulations or by enzymatic degradation can alter stress-susceptible behaviors in rodents, including avoidance, stress coping, cognitive function and substance use. For example, genetic deletion of neuronal membrane linking protein ankyrin-R or the transcription factor OTX2 reduces PNN expression and decreases avoidance of the open arms in an elevated plus maze task (Stevens et al., 2021; Vincent et al., 2021). Furthermore, degradation of PNNs using chondroitinase ABC facilitates extinction of drug-seeking behavior (Xue et al., 2014) and prevents both fear conditioning (Hylin et al., 2013) and cocaine-induced place preference (Slaker et al., 2015). However, studies have also shown that diminished PNNs can produce effects that mimic those of chronic stress, including increased threat responses (Santiago et al., 2018) and diminished cognitive function (Paylor et al., 2018). Since atypical behavioral states have been associated with both reduced or increased PNN measures in several brain regions, it seems likely

that an optimal level of PNNs within a brain region may exist, which when disrupted produces behavioral dysfunction. Along these lines, it has been shown that antidepressant action on behavior and neuronal oscillations both require the presence of PNNs in the hippocampus (Donegan and Lodge, 2017), as well as the degradation of PNNs through the antidepressant-induced release of proteolytic enzymes by microglia (Alaiyed et al., 2019, 2020). Clearly, additional research is needed to better understand links among stress, PNNs, and behavior.

## BRIDGING THE GAP BETWEEN STRESS-INDUCED CHANGES IN EXTRACELLULAR MATRIX AND BEHAVIORAL OUTCOMES

Despite the variability of outcomes regarding stress effects on diffuse and structured ECM, both forms are sensitive to stress and their changes have been linked to alterations in behaviors associated with cognition and emotional processing. These findings raise questions about the mechanisms by which stress-induced ECM remodeling give rise to behavioral change. It is likely that the larger literature on the role of the ECM in electrophysiological function of relevant brain regions may provide clues. As mentioned earlier, both diffuse ECM and PNNs regulate synaptic plasticity (Sorg et al., 2016; Jakob et al., 2017), which has clear links to cognitive function (Dringenberg, 2020), raising a scenario whereby stress produces cognitive dysfunction by disrupting ECM components associated with optimal synaptic plasticity. ECM has also been linked to neuronal oscillations in both the gamma and theta frequency ranges (Murthy and Gould, 2020), which are important for both cognitive function (Mably and Colgin, 2018; Zielinski et al., 2020) and behaviors associated with emotional processing, including avoidance behavior (Padilla-Coreano et al., 2019). These results suggest that stress may produce cognitive dysfunction and enhance avoidance behavior through ECM-induced changes in neuronal oscillations. The ECM has also been linked to synchrony in rhythmic signaling across brain regions (Bucher et al., 2021), which has been shown to play crucial roles in healthy brain function in terms of learning and memory, as well as stress responsivity (Adhikari et al., 2010; Del Arco et al., 2020). It is conceivable that stress-induced ECM changes in these electrophysiological properties are responsible for stress-induced behavioral outcomes. Since antidepressant treatment restores stress-induced ECM changes and behavioral dysfunction in rodents, it seems plausible that this occurs through reversal of atypical synaptic plasticity, neuronal oscillations and/or circuit-level coherence. Antidepressant treatment has been shown to influence all of these electrophysiological measures (Law et al., 2016; Alaiyed et al., 2019; Logue et al., 2021).

## FUTURE STUDIES

Future studies should directly investigate whether stress-induced changes in ECM lead to alterations in behavior through influences on electrophysiological properties at the synapse, among populations of neurons within a brain region, and across brain regions in the broader circuitry. Although these studies are likely to be informative, they will not provide a complete picture without considering other aspects of the brain's microenvironment, such as glia, which are also known to be stress-responsive (Kaul et al., 2021) and participate in ECM remodeling (Strackeljan et al., 2021). Some studies have begun to test the involvement of microglia in regulating ECM in the context of both cognitive function (Alaiyed et al., 2019; Nguyen et al., 2020; Venturino et al., 2021) and emotional processing (Alaiyed et al., 2019; Venturino et al., 2021). Expanding these approaches to other brain regions, as well as other stress paradigms, should be illuminating. Lastly, finding ways to connect the experimental animal literature to humans will be important. Along these lines, it is relevant to note that the components of diffuse and structured ECM described in the rodent brain exist in the human brain (Fatemi et al., 2000; Laifenfeld et al., 2005b; Mauney et al., 2013). Furthermore, major depressive disorder and bipolar disorder, conditions linked to stress, have been associated with altered levels of diffuse ECM molecules, such as reelin, hyaluronan, and laminin (Fatemi et al., 2000; Guidotti et al., 2000; Laifenfeld et al., 2005b; Lubbers et al., 2014; Ventorp et al., 2016), and increased PNNs have been reported in the prefrontal cortex of suicide victims previously exposed to childhood maltreatment (Tanti et al., 2020). The similarities between rodent and human studies increase confidence that a better understanding of the connections between the ECM and stress-induced behavioral dysfunction in rodents may provide a window into stress-induced neuropsychiatric disease in humans.

## AUTHOR CONTRIBUTIONS

BL and EG wrote and edited the manuscript. Both authors contributed to the article and approved the submitted version.

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# Cellular and Molecular Gradients in the Ventral Horns With Increasing Distance From the Injury Site After Spinal Cord Contusion

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To identify cellular and molecular gradients following spinal cord injury (SCI), a rat contusion model of severe SCI was used to investigate the expression of NG2 and molecules that identify astrocytes and axons of the ventral horns (VH) at different distances on 7 and 30 days post-injury (dpi). A gradient of expression of $NG2^+$/$Olig2^+$ cells was determined, with the highest concentrations focused close to the injury site. A decrease in NG2 mean intensity correlates with a decrease in the number of $NG2^+$ cells more distally. Immunoelectron microscopy subsequently revealed the presence of NG2 in connection with the membrane and within the cytoplasm of $NG2^+$ glial cells and in large amounts within myelin membranes. Analysis of the astrocyte marker GFAP showed increased expression local to injury site from 7 dpi, this increase in expression spread more distally from the injury site by 30 dpi. Paradoxically, astrocyte perisynaptic processes marker GLT-1 was only increased in expression in areas remote from the epicenter, which was traced both at 7 and 30 dpi. Confocal microscopy showed a significant decrease in the number of $5\text{-HT}^+$ axons at a distance from the epicenter in the caudal direction, which is consistent with a decrease in β3-tubulin in these areas. The results indicate significant cellular and molecular reactions not only in the area of the gray matter damage but also in adjacent and remote areas, which is important for assessing the possibility of long-distance axonal growth.

**Keywords: NG2, astrocyte, axon-associated proteins, spinal cord injury, ventral horns**

## INTRODUCTION

Research that studies the cellular and molecular mechanisms in spinal cord injury has mainly focused on analyzing the injury area. After primary damage of the spinal cord, remote secondary damage usually occurs. A clinical neurophysiological and MRI studies indicated the development of these events in distant areas of patients' spinal cord after spinal cord injury. Distinct spatiotemporal

dynamics of tissue-specific neurodegeneration were found above and below spinal cord injury (David et al., 2021). Spatiotemporal experimental analysis of the gray and white matter of the spinal cord at a distance from the epicenter of injury shows the differential pattern and severity of pathological reactions, gene expression, and molecular regulation in the remote rostral and caudal regions (Ek et al., 2010; Yu et al., 2019). However, the main cause and mechanism of distant reactions, in contrast to shifts in the epicenter of damage, remain poorly understood. Meanwhile, this effect in distant regions seems to be important to take into account to assess the prospects for outcomes in pathology.

The functional deficit of neural networks in spinal cord injury (SCI) is a consequence of poor regeneration of damaged axons and insufficient target reinnervation. Axonal growth is inhibited by numerous inhibitory molecules of the extracellular matrix, such as chondroitin sulfate proteoglycans (CSPGs) (Ohtake and Li, 2015), as well as molecules associated with oligodendrocytes and myelin (Geoffroy and Zheng, 2014; Baldwin and Giger, 2015; McKerracher and Rosen, 2015). Under the influence of these inhibitors, dystrophic axons in the damaged area terminate growth and undergo retraction (Ramón y Cajal, 1928).

The idea that the expression level of neuron-glial antigen 2 (NG2) proteoglycan, also known as CSPG4, is elevated in the segments surrounding the SCI site in rodents is not new (Lemons et al., 1999; McTigue et al., 2006; Iaci et al., 2007). However, a detailed comparison of the expression levels and distribution of NG2 proteoglycan has not been performed in contusion SCI models. As mechanistic studies within these models begin to identify signaling pathways caused by the activation of CSPGs (Monnier et al., 2003; Duan and Giger, 2010; Coles et al., 2011), it becomes increasingly important to fully characterize the composition and distribution of CSPGs within the boundaries of scar formation.

In SCI, the area of damage contains numerous $NG2^+$ glial cells (NG2 oligodendrocyte precursor cells, NG2/OPC) (Jones et al., 2002; Anderson et al., 2016) and is encased by a compact layer of reactive astrocytes that form a glial scar (Cregg et al., 2014; Hesp et al., 2018) hypothesize that formation of the glial scar depends on proliferating $NG2^+$ cells, which include not only glia but also pericytes. They categorized $NG2^+$ cells as glia or pericytes based on branched versus crescent-shaped morphology, respectively. Dividing $NG2^+$ glia outnumber dividing $NG2^+$ pericytes up to 30-fold, but are restricted to the glial scar and spared tissue, whereas dividing $NG2^+$ pericytes enter lesions concomitant with angiogenesis (Hesp et al., 2018).

$NG2^+$ cells rapidly proliferate in the area of damage, fix the axon growth cones on their surface, and prevent the dieback also known as axonal retraction of dystrophic axons, but at the same time inhibit their further growth (Filous and Schwab, 2018). This phenomenon is consistent with numerous data on the formation of synapse-like structures (synaptoid) "neuron – $NG2^+$ cell" by $NG2^+$ cells (Paukert and Bergles, 2006; Sakry et al., 2011; Kula et al., 2019). There is no unambiguous opinion about the influence of such structures on axon regeneration (Chelyshev et al., 2020). However, there are indications that $NG2^+$ cells may enhance, facilitate, and support axon regeneration (de Castro et al., 2005; McTigue et al., 2006; Yang et al., 2006), as opposed to only influencing (Hossain-Ibrahim et al., 2007) or inhibiting growth (Dou and Levine, 1994; Tan et al., 2005, 2006; Donnelly et al., 2010).

Previous research has focused on how the state of cells and the extracellular matrix in the glial scar and the area immediately adjacent act to inhibit axonal growth. However, to provide extended axon growth and find targets far removed from the damaged area, it is important to characterize the expression of potential inhibitor molecules in morphologically intact tissue adjacent to the injury. Typical signs of degeneration are poorly manifested or completely absent in it, such as cell death, reorganization of the extracellular matrix, a decrease in tissue preservation, and pathological cavities. We wish to ascertain how the expression phenotype of glial cells changes, and if molecular shifts in the extracellular matrix occur in the areas potentially affecting extended axonal growth and synaptic condition.

There is evidence that glial cells located at a distance from the area of SCI, are involved in the pathological changes observed in neural networks. Activation of microglia and pro-inflammatory cytokines at a distance from the injury site predict the onset and severity of neuropathic pain after SCI (Zhao et al., 2007; Detloff et al., 2008; Gwak and Hulsebosch, 2009; Gwak et al., 2017). In this regard, data on the contributions of other types of glial cells, such as astrocytes and $NG2^+$ cells, are still unclear. An increase in reactive astrogliosis and CSPGs, inhibitors of axon growth and plasticity, have been reported at sites distant from the lesion after severe mid-thoracic spinal contusion (Andrews et al., 2012). However, little is known about the signals that trigger such a remote cellular response.

Taking into account the special role of $NG2^+$ glia in the control of neuronal plasticity, it would appear important to assess the reaction of these cells, as well as changes in the expression of NG2 proteoglycan in the area remote from the injury site, to determine the potential of extended axon growth. In this regard, we studied the spatial and temporal changes in the number of NG2 proteoglycan expressing cells, primarily $NG2^+$ glia, as well as the expression of NG2 proteoglycan and markers of astrocytes and axons in a distance from the SCI epicenter in rat spinal cord contusion injury model.

## MATERIALS AND METHODS

### Animals

Adult female Wistar rats (250–300 g, $n = 45$) were used for all experiments. Animals were randomly allocated to intact control ($n = 15$) and experimental ($n = 30$) groups. All animal protocols were approved by the Kazan Federal University Animal Care and Use Committee (No 2, May 5, 2015). Rats were housed under standard conditions (12 h light/dark cycle) with food and water available *ad libitum*.

### Surgery

After intramuscular injection of Zoletil (20 mg/kg, Virbac Sante Animale) experimental rats were deeply anesthetized under general anesthesia with isofluran. The fascia and paraspinous muscles were incised and after that laminectomy was performed

at the Th8 vertebral level. SCI was induced using Infinite Horizon Impactor (Precision Systems and Instrumentation, LLC) and appropriate software (PSI IH Spinal Cord Impactor, version 5.0.3). The force of contusion was 300 kdyn (severe SCI; $n = 30$). After SCI gentamicin (25 mg/kg, Microgen) was injected intramuscularly once per day for 7 consecutive days post-injury (dpi). The urinary bladders were manually emptied twice each day until voiding commenced.

## Tissue Processing

Intact control ($n = 5$) and experimental rats at 7 and 30 dpi ($n = 5$ rats at each time-point; 7 and 30 dpi) were anesthetized and subjected to intracardiac perfusion with cold (4°C) 4% buffered formalin (BioVitrum). After perfusion, 30 mm segment centered around the injury site/Th8 was carefully isolated from the vertebral column, then fixed again in the 4% buffered formalin overnight. Distances 3–5, 6–8, and 10–12 mm from the visualized epicenter of the injury were determined on the isolated fragment of the spinal cord according to the data of our previous studies (Mukhamedshina et al., 2019). The next day, spinal cords were cryopreserved in a sucrose gradient (15 and 30%). For the analysis of the VH, we used longitudinal sections (20 μm thick) of the spinal cord in areas remote from the site of injury/Th8, obtained using a Tissue-Tek Cryo3 DM (Sakura) cryostat. The choice of VH as the object of research is based on the presence of key modules of the spinal locomotor circuit in this area.

## Transmission Electron Microscopy

For transmission electron microscopy, samples were fixed in 10% formaldehyde mixed with 0.2% phosphate-buffered glutaraldehyde solution (Alfa Aesar by Thermo Fisher Scientific, Germany) at 4°C for 12 h, and post-fixed in a 0.5% $OsO_4$ (Sigma–Aldrich, United States) for 1 h. Following fixation, samples were dehydrated in an ethanol gradient and embedded in LR White (Electron Microscopy Sciences, Hatfield). The 0.1 μm ultrathin sections were mounted on Formvar-coated Ni grids. For Immune gold cytochemistry the sections were blocked with TBS-NDS-BSA-TX100 [Tris-buffered saline (Tris 0.01 M, NaCl 0.15 M pH = 8.2), normal donkey serum 10%, bovine serum albumin 0.2%, and Triton X-100 0.1%] for 1 h. After washing in TBS, the sections were incubated overnight at 4°C with anti-NG2 and anti-ALDH1L1 (aldehyde dehydrogenase 1 family member L1) (**Table 1**) antibodies (Abs) and then with secondary Abs conjugated to colloidal gold (5 nm particles to evaluate ALDH1L1 and 10 nm particles to evaluate NG2 proteoglycan) (Sigma–Aldrich, United States) for 2 h at room temperature (RT).

Then sections were stained with uranyl acetate and lead citrate and evaluated using a transmission electron microscope (Hitachi HT7700, Tokyo, Japan). To measure the size of gold nanoparticles, as well as the thickness of the filaments, a ZEN blue Lite program and software supplied with the Hitachi 7700 transmission electron microscope were used.

## Immunohistochemistry

Longitudinal spinal cord sections were processed for immunohistochemistry as previously described (Mukhamedshina et al., 2019). Primary and fluorescence secondary Abs are described in **Table 1**. Nuclear stain *via* 4′,6-diamidino-2-phenylindole (DAPI) (10 μg/mL, Sigma). Coverslips were mounted on slides using ImmunoHistoMount medium (ab104131, Abcam). Sections incubated only with secondary Abs (without primary Abs) were used as a reaction control. The slides were examined and photographed using LSM 700 confocal microscopy (Carl Zeiss). Three-dimensional reconstruction and cell quantification/semi-quantitative analyses were performed with Zen 2012 software (Carl Zeiss).

## Cell Quantification

Five sections per rat in the VH area at distances of 3–5, 6–8, and 10–12 mm in the caudal direction were analyzed in intact control ($n = 5$) and experimental ($n = 5$ rats at each time-point; 7 and 30 dpi) groups. In the confocal images of this area, the arithmetic mean intensities of fluorescence (MIF units, semi-quantitative analysis) of NG2 proteoglycan was measured as previously described (Povysheva et al., 2018). In each VH area we examined 3 zones with $S = 0.02$ mm$^2$, for each channel, the lowest intensity signals were removed to minimize the background. For semi-quantitative analysis, all sections were imaged using identical confocal settings (laser intensity, gain, offset).

**TABLE 1 |** Primary and secondary antibodies used in Western blotting (WB), immunohistochemistry (IHC), and immunoelectron microscopy (IEM) tests.

| Antibody | Host | Dilution | Source |
|---|---|---|---|
| GFAP | mouse | 1:1,000 (WB) | Santa Cruz (sc-33673) |
| GLT-1 | rabbit | 1:1,000 (WB) | Abcam (ab41621) |
| β-III-tubulin | mouse | 1:500 (WB) | Santa Cruz (sc-5274) |
| NG2 | mouse | 1:100 (IHC)<br>1:100 (IEM) | Sigma–Aldrich (N8912) |
| ALDH1L1 | rabbit | 1:100 (IEM) | Abcam (ab87117) |
| 5-HT | goat | 1:400 (IHC) | Abcam (ab66047) |
| NeuN | rabbit | 1:100 (IHC) | Sigma-Aldrich (SAB4300883) |
| Olig2 | rabbit | 1:75 (IHC) | Santa Cruz (sc-48817) |
| Anti-goat IgG conjugated to Alexa 488 | donkey | 1:200 (IHC) | Thermo Fisher (A11055) |
| Anti-mouse IgG conjugated to Alexa 555 | donkey | 1:200 (IHC) | Thermo Fisher (A31570) |
| Anti-rabbit IgG conjugated to Alexa 647 | donkey | 1:200 (IHC) | Thermo Fisher (A31573) |
| HRP-conjugated anti-rabbit IgG | goat | 1:2,000 (WB) | Cell Signaling (7074P2) |
| HRP-conjugated anti-mouse IgG | goat | 1:1,000 (WB) | Sigma–Aldrich (A4416) |

For quantification analyses, 4 types of cells were selected and examined in $S = 0.04$ mm$^2$: NG2$^-$/Olig2$^+$ cells, NG2$^+$ pericyte with crescent morphology (Hesp et al., 2018), branched NG2$^+$ oval shape cells, and NG2$^+$/Olig2$^+$ cells (double-positive). 5-Hydroxytryptamine (5-HT)$^+$ axon profiles (>0.5 μm in length) were counted within VH area containing at least two neurons (NeuN were used for visualization neurons) as previously described (Freria et al., 2017). For 5-HT puncta analysis ImageJ software (version 1.52a, National Institutes of Health) and Puncta Analyzer v. 2.0 plug-in were used. The Z-stack ($S = 0.01$ mm$^2$) was used for more reliable counting of the number of 5-HT$^+$ axon profiles. Investigations were validated by two observers to ensure the correct identification of the immunoreactivity patterns. Both investigators were blinded to the experimental group.

## Western Blotting

The spinal cord for western blotting was isolated from intact controls ($n = 5$) and experimental rats ($n = 5$ rats at each timepoint; 7 and 30 dpi) and washed with sterile phosphate-buffered saline (PBS) twice. Using microdissection technique, including micromanipulations with fragments of the spinal cord, controlled through stereomicroscope with LED illumination (Carl Zeiss) and aimed at separating white and gray matter with subsequent isolation of VH using microinstrumentation, the VH (3–5, 6–8, and 10–12 mm caudally from the injury site/Th8) were isolated and stored at –80°C. For VH total protein isolation samples were dissolved in radioimmunoprecipitation assay (RIPA) lysis buffer (Cell Signaling Technology) with protease inhibitor cocktail (Thermo Fisher Scientific), then incubated on multi-rotator (Biosan) for 1 h at 4°C, homogenized *via* FastPrep-24 Classic bead beating grinder and lysis system (MP Biomedicals) at a speed 4 m/s for 20 s, sonicated in ultrasonic bath for 10 min and finally centrifuged at 15,000 rpm for 30 min at 4°C. Total protein concentrations were measured using the BCA Protein Assay Kit (Thermo Fisher Scientific). 2 × SDS (sodium dodecyl sulfate) sample buffer was added to each sample before incubating at 95°C for 5 min. A 5 μg of protein sample was loaded to each lane of 4–12% gradient SDS-PAGE gel and then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad Laboratories) after electrophoresis. The PVDF membrane was blocked with 5% non-fat dry milk diluted in PBS with Tween-20 (PBS-T) (pH 7.4) for 1 h at RT. The primary Abs (**Table 1**) were diluted and incubated with membranes overnight at 4°C. The next day, after washing in PBS-T the membranes it was incubated with HRP-conjugated secondary Abs in 2 h at RT and visualized with the Clarity Western ECL Substrate kit (Bio-Rad Laboratories). Primary and secondary Abs were diluted in 5% non-fat dry milk in PBS-T. Analysis of the western blotting results with total protein normalization was performed using Image Lab software (Bio-Rad Laboratories).

## Statistical Analysis

Obtained data analysis was performed using R 3.6.3 software (R Foundation for Statistical Computing, Vienna, Austria). Descriptive statistics are presented as mean ± standard deviation (median, first, and third quartile). Sample distributions are visually represented as boxplots. Repeated measures for one animal were averaged. To analyze differences between samples, the Kruskal–Wallis test was used and Dunn's test was implemented as *post hoc* test with Holm adjustment for multiple comparisons. Mann–Whitney *U* test, using Origin 8.0 software, was applied for the statistical analysis of Western blot data.

# RESULTS

## Assessment of NG2 Proteoglycan Expressing Cells With Increasing Distance From the Injury Site in the Caudal Direction

Evaluation of the mean fluorescence intensity of NG2 proteoglycan in the VH showed the highest expression by 7 dpi at a distance of 3–5 mm from the epicenter when compared with 10–12 mm distance at the same timepoint [454.5 (387.7–590.0) at 3–5 mm vs. 207.2 (195.6–256.4) at 10–12 mm, $P < 0.05$]. While expression changed across these distances, we did not find any significant differences between the intact control and experimental groups (**Figure 1A**).

We performed a quantitative analysis of three populations of NG2$^+$ cells: NG2$^+$/Olig2$^+$, branched-shaped, and pericyte-shaped NG2$^+$ cells. In the VH, with the distance from the SCI epicenter, the number of branched-shaped and pericyte-shaped NG2$^+$ cells did not differ significantly. These cells may be absent, or only present in very small numbers not present in the fields of view analyzed (**Figures 1B,C**).

The population of NG2$^+$/Olig2$^+$ cells in the VH was also relatively small. However, at 7 dpi and a distance of 3–5 mm from the SCI epicenter, a larger number of these cells were found in comparison with intact controls and in the later 30 dpi timepoint [0.65 (0.62–0.69) at 7 dpi vs. 0.35 (0.00–0.37) at intact control and 0.47 (0.32–0.57) at 30 dpi, $P < 0.05$] (**Figures 1D,F**). At 7 dpi, the number of NG2$^+$/Olig2$^+$ cells decreased ($P < 0.05$) at a distance of 6–8 mm when compared with the area closer (3–5 mm) or more distant (10–12 mm) to the epicenter of the injury. At the same time, in the VH, the number of NG2$^-$/Olig2$^+$ cells not expressing NG2 proteoglycan was more than 15 times higher than the number of NG2$^+$/Olig2$^+$ cells in all studied groups in the corresponding zones (**Figures 1E,F**).

## Assessment of Astrocyte Markers

Following analysis of all selected distances from the SCI epicenter, it was found that glial fibrillary acidic protein (GFAP) expression in the VH increased by ∼ 2-fold at 7 dpi, a trend that continued to 30 dpi (**Figure 2A**). These results indicate the development of reactive astrogliosis following a spinal cord injury across a wide area that spans a considerable distance from the epicenter of the injury. In addition, these data indicate a sufficient duration of reactive astrogliosis both near the epicenter and at a distance from it.

Conversely, there was no detectable change in the expression of glutamate transporter 1 (GLT-1), which is mainly localized in astrocytes and their perisynaptic processes, within regions close

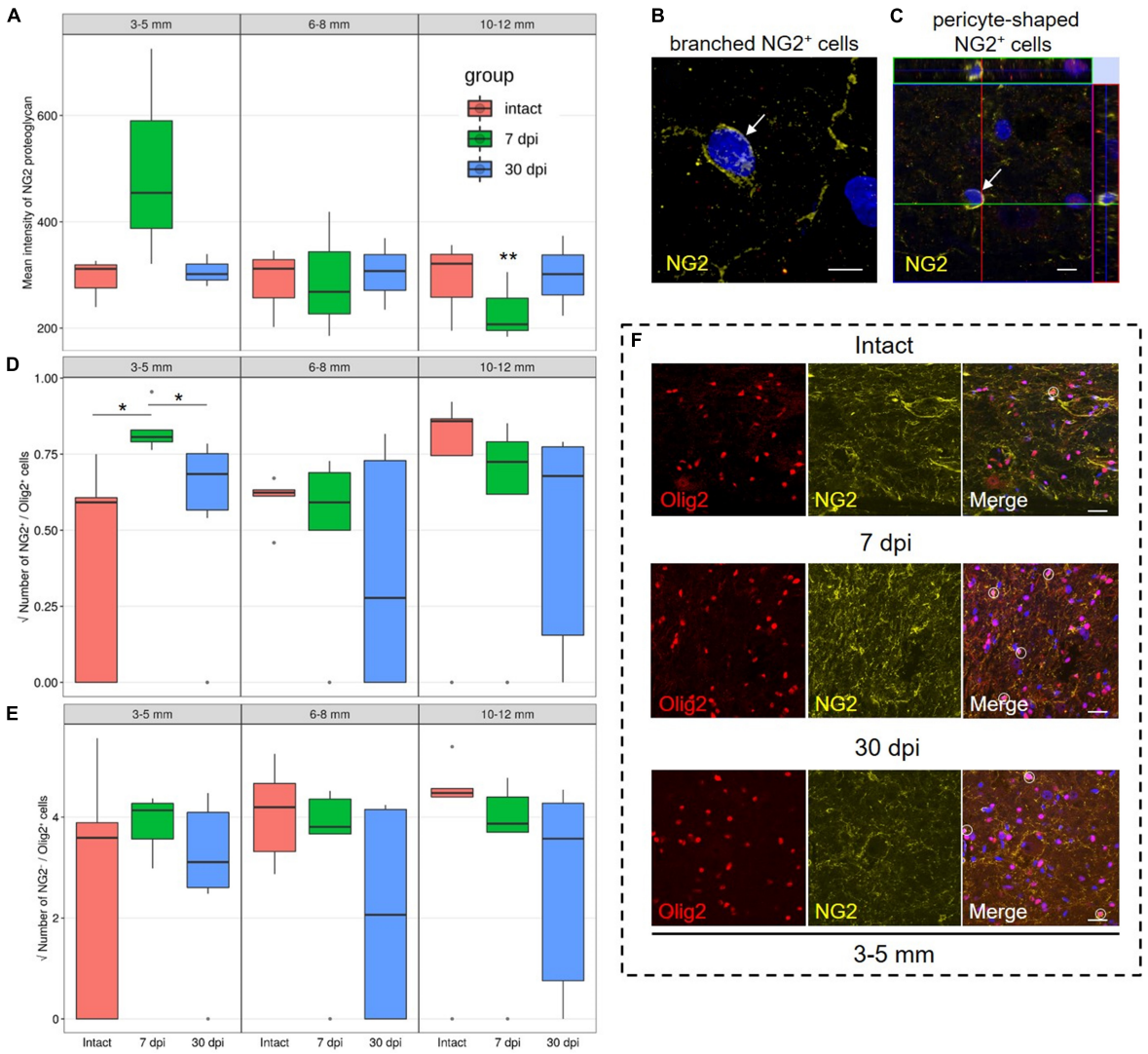


**FIGURE 1 |** Assessment of NG2 expressing cells and NG2$^-$/Olig2$^+$ cells identification. **(A)** The mean intensity of NG2 (MIF units, *Y*-axis) in the intact spinal cord (red column), 7 (green column), and 30 (blue column) dpi in the ventral horns (VH) 3–5, 6–8, and 10–12 mm caudally from the injury epicenter. $^{**}P < 0.05$ – compared with 7 dpi group and distance of 3–5 mm from the epicenter. Visualization of the branched-shaped **(B)** and pericyte-shaped **(C)** NG2$^+$ cells (arrows). Three-dimensional confocal microscopy images are shown. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). The square root of NG2$^+$/Olig2$^+$ **(D)** and NG2$^-$/Olig2$^+$ **(E)** cells number in the examined regions, $^*P < 0.05$. **(F)** Visualization of the NG2$^-$/Olig2$^+$ (red channel) and NG2$^+$/Olig2$^+$ cells (merge channel, circle) in intact spinal cord, 7 and 30 dpi in VH, 3–5 mm caudally from the injury epicenter. Scale bar = 5 **(B,C)** and 20 **(F)** μm.

to (3–8 mm) the injury epicenter in all studied groups. However, more distally (10–12 mm) from the epicenter of damage, GLT-1 expression increased (∼2.4 times) by 7 dpi ($P < 0.05$) and increased (∼3.7 times) further by 30 dpi, when compared with intact controls (**Figure 2B**).

## Astrocyte and NG2 Proteoglycan Expressing Cells in Ventral Horns: Immunoelectron Microscopy

In the VH of the intact spinal cord, NG2 proteoglycan was visible in the cytoplasm of oligodendrocytes and myelin membranes (**Figure 3A**). The perisynaptic region also contained another population of NG2$^+$/ALDH1L1$^-$ cells with thick and short processes and irregular cell bodies with polymorphic nuclei. Astrocytes surrounding neurons exhibited weak immunoreactivity of NG2 proteoglycan along the plasma membranes, while also demonstrating immunoreactivity of ALDH1L1, predominantly in the cytoplasm, as well as on the surface membrane of the processes (**Figure 3A**). Astrocytic morphology of these cells was confirmed by the presence of distinctive lumps of average electron density and intermediate filaments 15 nm in diameter (**Figure 3B**) in the cytoplasm.

In the spinal cord at 30 dpi, in areas remote from the epicenter of the injury in the caudal direction on longitudinal sections of the VH, three zones can be distinguished in the

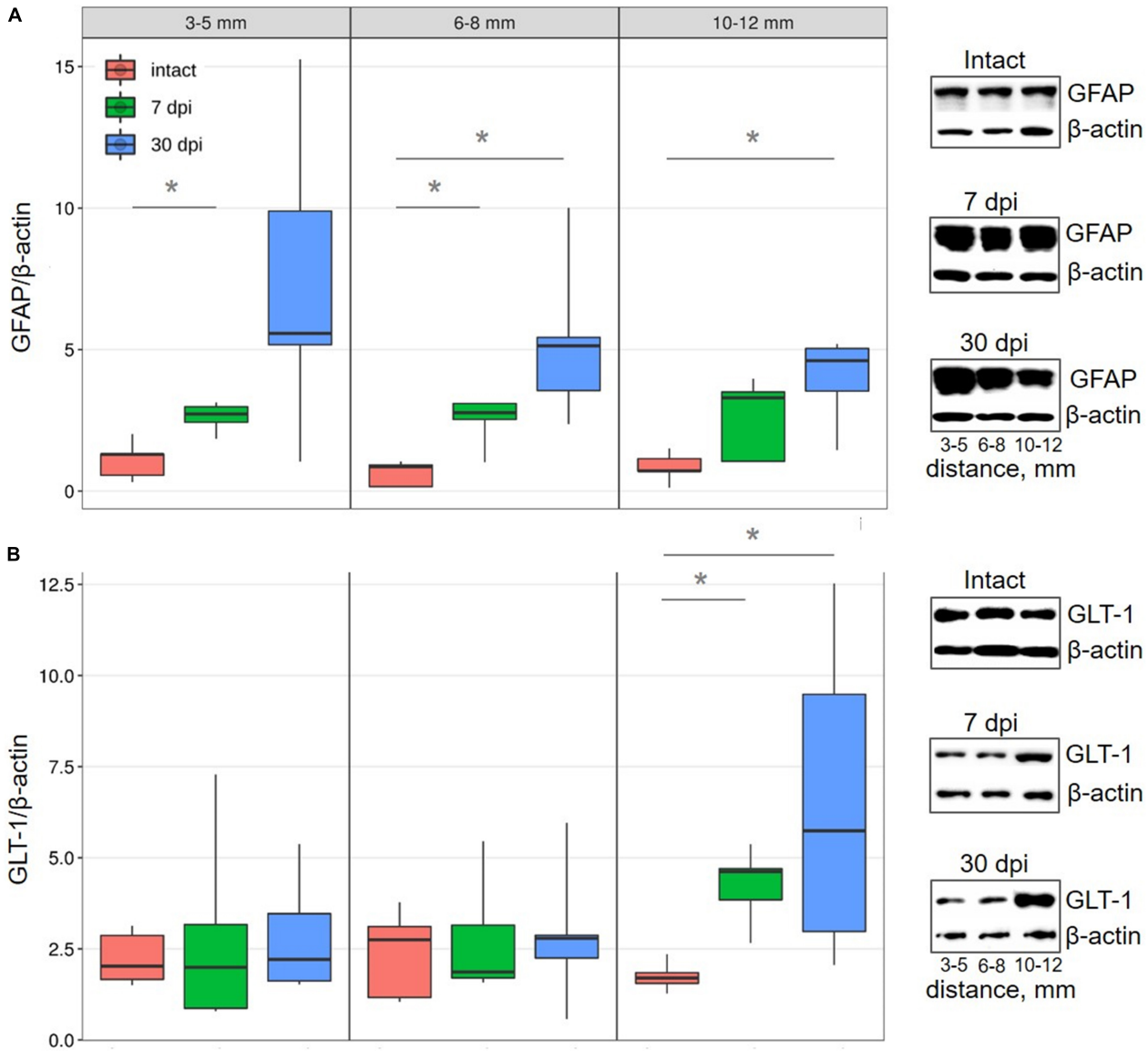


**FIGURE 2 |** Western blot analysis of astrocytes markers in the VH with increasing distance from the injury site in the caudal direction. Representative images of western blots showing GFAP **(A)** and GLT-1 **(B)** and their corresponding control – β-actin. Data are presented as the ratio of the number of target proteins to the reference protein (β-actin), the average value ($n$ = 5 rats per group). *$P < 0.05$, Mann–Whitney test.

dorsoventral direction: the damaged degenerated substance, the area of the glial barrier (there is a strong interlacing of processes of glial and nerve cells, as intact and degenerating) and the area of intact tissue. At the distance farthest from the epicenter of the injury, there is no discernable zone of degenerated substance and the expression of NG2 proteoglycan and ALDH1L1 in the above-mentioned cells, as well as their morphology, was similar to the intact spinal cord. At the same time, the expression of NG2 proteoglycan in $NG2^+$ glial cells, oligodendrocytes, and reactive astrocytes was visually more intense (**Figures 3C,C',D**). In the zone of destruction and glial scar, activated astrocytes had characteristic (specific) ultrastructural features of the cytoplasm structure and more pronounced expression of NG2 proteoglycan and ALDH1L1 when compared with the intact control group or intact tissues far from the lesion epicenter (**Figure 3C**).

$NG2^+$ cells and their processes were found in large numbers and intimately associated with reactive astrocytes. Whilst these cells were present at different distances from the epicenter, they were mainly in the area of the glial scar. Processes of reactive astrocytes with electron-transparent cytoplasm were found between the more electron-dense granular processes of $NG2^+$ cells. In astrocytes processes, à mild immunopositive reaction to NG2 proteoglycan was found mainly on membranes, whilst an intense immunopositive reaction to ALDH1L1 in the cytoplasm was visualized (**Figure 3D**).

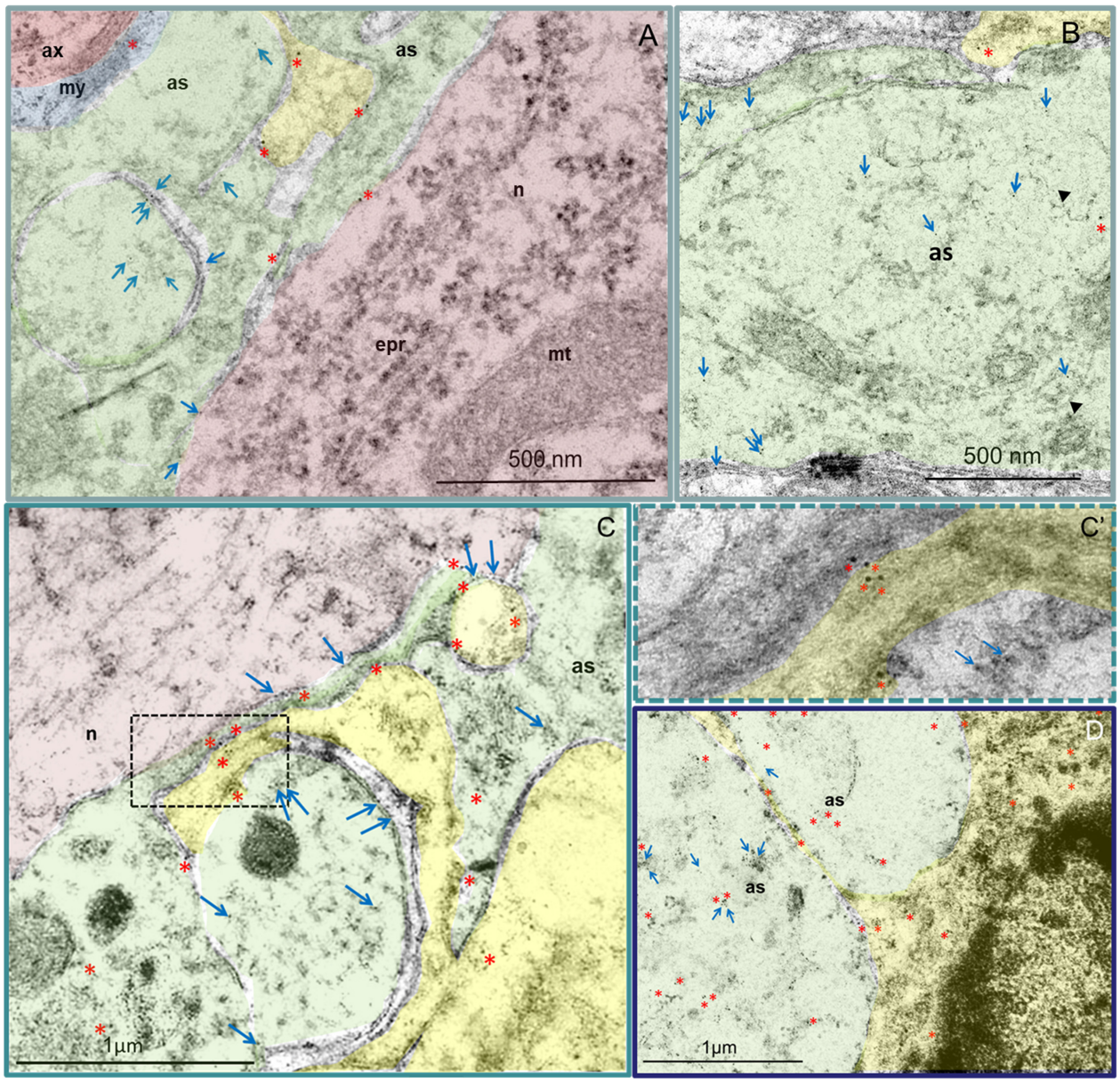


**FIGURE 3 |** Astrocytes (green) and NG2$^+$ (yellow) cells in the VH. Immunoelectron microscopic images of intact **(A,B)** and injured spinal cord 30 dpi 3–5 mm **(C)** and 6–8 mm **(D)** caudally from the epicenter. Asterisks denote 10 nm golden nanoparticles (anti-NG2 antibody) and arrows highlight 5 nm golden nanoparticles (anti-ALDH1L1 antibody). **(A)** In the VH of the intact spinal cord, a positive reaction to NG2 proteoglycan was detected in the cytoplasm of oligodendrocytes and myelin membranes, as well as in a small amount along the plasma membranes in the processes of astrocytes, including those adjacent to the neuron. In NG2$^+$ glia, the ALDH1L1 immunopositive reaction was not observed. **(B)** High ALDH1L1 immunoreactivity was found in the cytoplasm of astrocytes. The belonging of cells to astrocytes can be estimated by the presence in the cytoplasm of characteristic lumps of average electron density and filaments 15 nm in diameter (head arrow). **(C)** At 30 dpi, the distributions of NG2 proteoglycan and ALDH1L1 were similar to those of the intact spinal cord. At the same time, the expression of NG2 proteoglycan in NG2$^+$ glial cells, oligodendrocytes **(C)**, and reactive astrocytes **(D)** was visually more intense. Higher-magnification view of the dashed boxed area in the **(C')**. as – astrocyte; ax – axon; epr – endoplasmic reticulum; mt – mitochondria; my – myelin; n – neuron. Scale bar: 500 nm **(A,B)** and 1 μm **(C,D)**.

## Expression of Axon-Associated Proteins

Expression of β3-tubulin, to determine the microtubules of neurons in the VH showed decreased expression ($P < 0.05$) at a distance of 3–5, 6–8, and 10–12 mm from the injury epicenter by 30 dpi when compared with the intact control groups (**Figure 4A**). At 7 dpi, at all distances from the epicenter, β3-tubulin content also decreases, but the significance of these differences when compared with intact controls was not confirmed.

A significant decrease in the number of 5-HT$^+$ axons in the VH by 30 dpi in comparison with intact control at distances of 6–8 and 10–12 mm from the epicenter of the injury was also found

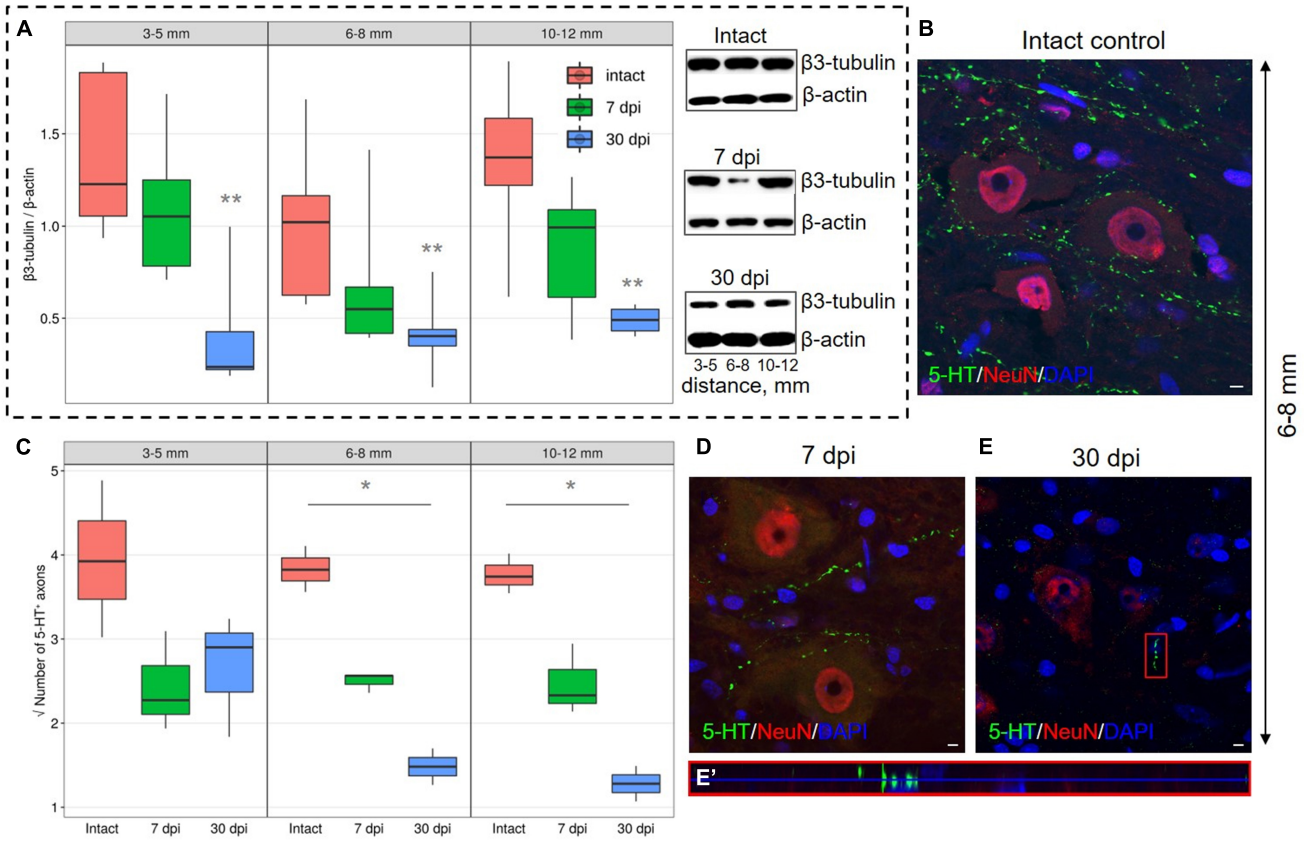


**FIGURE 4 |** Estimation of axon-associated proteins in the ventral horns (VH) with increasing distance from the injury site in the caudal direction. **(A)** Western blot analysis of β3-tubulin, β-actin was used as a loading control. Data are presented as the ratio of the quantity of target protein (β3-tubulin) to the reference protein (β-actin), the average value ($n$ = 5 rats per group). $^{**}P < 0.05$ – compared with the intact group at an appropriate distance from the epicenter, Mann–Whitney $U$ test. Visualization of 5-HT$^+$ axons in the intact spinal cord **(B)**, 7 **(D)**, and 30 **(E)** dpi in VH 6–8 mm caudally from the injury epicenter. 5-HT$^+$ axons at 30 dpi are marked with red boxed area and are shown enlarged in another X, Y, and Z positions in **(E′)**. Three-dimensional confocal microscopy images are shown. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI). **(C)** Square root of 5-HT$^+$ axons ($Y$-axis) number in the intact spinal cord (red column), 7 (green column), and 30 (blue column) dpi in VH 3–5, 6–8, and 10–12 mm caudally from the injury epicenter, $^*P < 0.05$.

(**Figures 4B–E′**). By 30 dpi at a distance of 3–5 mm from the SCI epicenter, the amount of 5-HT$^+$ axons in comparison with 7 dpi remained the same, but the number decreased at distances of 6–8 and 10–12 mm caudally for the same time points.

It should be noted that all our results are presented in this study only for areas in the caudal direction from the injury site.

## DISCUSSION

In traumatic injury, axons degenerate not only in the caudal region but also proximal to the site of spinal cord injury (Freund et al., 2012). Proteomic analysis revealed differences in the spectrum of bioactive molecules when comparing the rostral and caudal regions in relation to the epicenter of injury, with a predominance of molecular markers of cell death in the caudal site (Cizkova et al., 2014). In spinal cord injury, pathological shifts in the caudal region lead to severe locomotor deficits. We focused on the analysis of the ventral horns in the perilesional area along the rostrocaudal axis because of the importance of cellular and molecular shifts in this region for the restoration of locomotor function.

In severe contusion SCI, an increase in the mean fluorescence intensity of NG2 proteoglycan in the VH at a distance of 3–5 mm from the epicenter at 7 dpi was confirmed, this is accompanied by a decrease in more distal regions (10–12 mm). Since the number of NG2$^+$ cells does not change significantly at a distance of 10–12 mm, this effect can be associated with a decrease in the expression of NG2 proteoglycan in this remote zone. By 30 dpi, the expression of NG2 proteoglycan at all the studied distances from the epicenter does not differ from that in intact animals in the VH of the corresponding anatomical regions. The observed dynamics of expression of NG2 proteoglycan can be associated primarily with the population of NG2$^+$ glial cells, although this proteoglycan is also expressed by cells of other types, such as pericytes (Hesp et al., 2018), meningeal fibroblasts, and macrophages (Bu et al., 2001; Jones et al., 2002). The observed increase in NG2 proteoglycan content at a term of 7 dpi in the area close to the lesion epicenter, may be associated with the well-known rapid increase in the amount of

$NG2^+$ glia and subsequent NG2 proteoglycan expression in this area (Filous and Schwab, 2018). This proteoglycan binds FGF2 in a glycosaminoglycan-independent, core protein-mediated manner (Cattaruzza et al., 2013), retaining this neurotrophic and angiogenic molecule on the cell membrane for subsequent receptor presentation.

In the VH with distance from the SCI epicenter, the dynamics of the populations of $NG2^+/Olig2^+$ cells and branched $NG2^+$ cells differ, which may indirectly indicate their heterogeneity. To a greater extent, as it turned out, $NG2^+/Olig2^+$ cells respond to SCI in areas close to the damage epicenter, in contrast to $NG2^-/Olig2^+$ cells, the population of which, judging by our data, significantly exceeds the population of $NG2^+/Olig2^+$ cells. $NG2^+/Olig2^+$ cells react rapidly to SCI and this reaction is characterized by cell body swelling, retraction of cell processes and increased expression of NG2 proteoglycan (Levine, 2016; Hesp et al., 2018) showed that in mice $NG2^+/Olig2^+$ cells were observed in large numbers in the area of the glial scar, while absent in the area of the epicenter of the injury after SCI (Hesp et al., 2018).

It is assumed that when damaged in the central nervous system (CNS), $NG2^+$ glia actively comes into contact with axons, inhibiting their retraction (Filous and Schwab, 2018). However, it remains unclear how long axons in which retraction is prevented and are thwarted by contact with NG2 cells can maintain their regenerative potential and continue to grow out to their targets (Chelyshev et al., 2020). It can also be assumed that thwarting axon growth as a result of their fixation on glial cell surfaces is important not only to prevent their dieback but also as a signal to start sprouting (Geoffroy and Zheng, 2014). NG2 proteoglycan has been shown to enhance serotonergic axon sprouting. It has been shown on NG2 proteoglycan knockouts in SCI that this proteoglycan enhances the penetration of serotonergic fibers into the scar tissue (de Castro et al., 2005). The number of 5-$HT^+$ axons, according to our data, significantly decreases at distances farther from the SCI epicenter by 30 dpi, in contrast to areas close to the epicenter of the injury and when compared with the intact spinal cord. We found that this was not caused by the inhibitory effect of cells expressing proteoglycan NG2, since we did not find quantitative or qualitative changes in the populations of these cells at longer distances (6–8, 10–12 mm) from the SCI epicenter. However, at a distance of 3–5 mm caudal to the lesion area, the decrease in the number of serotonergic axons is consistent with an increase in the number of $NG2^+/Olig2^+$ cells, which may indicate the prevention of axon dieback by this cell population. It is assumed that the effect of NG2 proteoglycan on axonal growth is mediated by the activation of protein kinase C zeta (PKCζ), an atypical molecule for immobilizing dystrophic axons, and this activation is both necessary and sufficient to inhibit axonal growth (Lee et al., 2013). It has been suggested that the immobilization of dystrophic growth cones involves the PTPσ molecule, which, like LARs, but not NgRs, accumulates in the penumbra region during SCI (Lang et al., 2015).

We also found a decrease in the content of β3-tubulin to the same extent in all studied removal zones, which can be explained by a decrease in the number of nerve fibers due to their degeneration, a decrease in axonal transport in preserved fibers, or both of these processes. The observed dynamics of this axon marker in the VH may be associated with a decrease in its synthesis in neurons, inhibition of anterograde transport in preserved descending axons, or with a decrease in the number of the same axons.

The possibility of expression of NG2 proteoglycan by reactive astrocytes of the spinal cord has been shown earlier (Anderson et al., 2016). At the same time, using the method of electron microscopic immunocytochemistry, we clarified the possibility of NG2 expression in reactive astrocytes located near the epicenter of damage, and those astrocytes that are located at a distance from the injury area. It was found that NG2 proteoglycan expressing astrocytes make up approximately 25% of the total number of $NG2^+$ cells in the glial scar by 4 weeks after SCI (Hackett et al., 2018). $NG2^+$ cells also display wide differentiation potential and give rise to reactive astrocytes during ischemic lesion under the influence of Shh signaling activation (Honsa et al., 2016). We found that NG2 proteoglycan expression was visible not only in post-traumatic reactive astrocytes but also in astrocytes of the VH in the intact spinal cord. This observation is consistent with the idea that $NG2^+$ cells can differentiate into $GFAP^+$ astrocytes, which has been shown in several models of CNS injury (Sellers et al., 2009; Tripathi et al., 2010; Komitova et al., 2011).

Judging by the expression of GFAP, reactive astrogliosis at a period of 30 dpi is observed at a considerable distance from the epicenter of the contusion injury and does not grow in the caudal direction from a distance of 6–8 mm. In connection with astrocytes, we stated a paradoxical reaction of an increase in GLT-1 expression at the maximum studied distance at 30 dpi. The absence of shifts in this indicator in the zone close to the damaged area may be associated with the disintegration of synapses, and in the remote area, where synapses are preserved, astrocytes increase the expression of GLT-1 to maintain the functioning of glutamatergic synapses. The increase in GLT-1 that we found in the gray matter area remote from the epicenter may also be associated with the growth and branching of regenerating axons and their participation in the restoration of synaptic contacts, which is not observed near the lesion focus, where reactive astrocytes are present that are not involved in the formation of synapses. This observation is the first indication of differences in the reactions of astrocytes located near and far from the area of tissue damage and destruction.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by the Kazan Federal University Animal Care and Use Committee (No 2, 5 May 2015).

## AUTHOR CONTRIBUTIONS

YC and YM contributed to the conceptualization, methodology, and supervision. IK, DS, and SA contributed to the investigation. IK, DS, MK, YM, and SA contributed to the formal analysis. YC, AV, and AR contributed to obtaining the resources. YC, YM, and IK contributed to writing the original draft. YC, YM, IK, SA, and VJ contributed to writing, reviewing, and editing the manuscript. IK, MK, YM, and SA visualization. YC and AR contributed toward funding acquisition. All authors had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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REVIEW
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# Extracellular Matrix Recycling as a Novel Plasticity Mechanism With a Potential Role in Disease

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The extracellular matrix (ECM) stabilizes neural circuits and synapses in the healthy brain, while also retaining the ability to be remodeled, to allow synapses to be plastic. A well-described mechanism for ECM remodeling is through the regulated secretion of proteolytic enzymes at the synapse, together with the synthesis of new ECM molecules. The importance of this process is evidenced by the large number of brain disorders that are associated with a dysregulation of ECM-cleaving protease activity. While most of the brain ECM molecules are indeed stable for remarkable time periods, evidence in other cell types, as cancer cells, suggests that at least a proportion of the ECM molecules may be endocytosed regularly, and could even be recycled back to the ECM. In this review, we discuss the involvement of such a mechanism in the brain, under physiological activity conditions and in relation to synapse and brain disease.

**Keywords: ECM, synapse, tenascin, recycling, neurodegeneration**

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## INTRODUCTION

In the adult brain, neurons are surrounded by a lattice of extracellular matrix (ECM) molecules that coat the surfaces of neurons and fill the spaces in between synapses (Ferrer-Ferrer and Dityatev, 2018). Since ECM molecules are especially long-lived (Dörrbaum et al., 2018; Fornasiero et al., 2018), these lattices are highly robust, and are believed to stabilize neural circuits and restrict synaptic plasticity (Dansie and Ethell, 2011; Wang and Fawcett, 2012). Therefore, it is not surprising that changes to the expression and organization of various ECM molecules are associated with a plethora of psychiatric and neurodegenerative diseases (Bonneh-Barkay and Wiley, 2009; Lemarchant et al., 2013; Wen et al., 2018). The ECM could be therefore seen as a stable structure, designed to keep neuronal networks in shape, limiting their dynamics. Nonetheless, although the ECM is indeed largely restrictive to plasticity, it still retains the flexibility to be remodeled at synapses, in order to support synapse changes in the adult brain. The mechanism through which this remodeling occurs is not fully understood, but is assumed to involve a local secretion of ECM-cleaving proteases, alongside a synthesis of new ECM molecules (Dityatev et al., 2010; Krishnaswamy et al., 2019). However, while such a mechanism is likely to be sufficient for relatively infrequent events of ECM remodeling at synapses, it is arguably too metabolically expensive for synaptic changes with faster or more frequent dynamics. Recent findings propose an alternative mechanism, whereby the components of the ECM can be continually recycled at synapses (Dankovich et al., 2021). Here, we briefly review the existing paradigm for ECM

remodeling and then discuss these recent findings on ECM recycling at the synapse. Since evidence also suggests that this mechanism is indispensable for synaptic function, we consider how this process may be dysregulated in brain disorders.

## THE EXTRACELLULAR MATRIX AS A STABILIZING FORCE IN THE HEALTHY BRAIN

During the final stages of brain development, the brain ECM undergoes a profound change in its molecular and spatial composition. A central aspect of this change is the appearance of dense ECM coats named "perineuronal nets" (PNNs) around the somas and proximal dendrites of a subset of neurons, in particular around parvalbumin-expressing inhibitory neurons (Härtig et al., 1992; Ruoslahti, 1996; Yamada and Jinno, 2013). These coats are composed of hyaluronic acid chains that exude from the surface of the neurons and, in turn, bind a family of secreted proteoglycans called lecticans (that includes neurocan, brevican, versican, and aggrecan). These lecticans are cross-linked by their binding partner tenascin-R, and their association with hyaluronan is further stabilized through multiple interactions with hyaluronan and proteoglycan link proteins (HAPLNs) (Ruoslahti, 1996; Sorg et al., 2016). The appearance of PNNs coincides with the closure of the critical period of plasticity, when neuronal circuits are highly sensitive to experience (Fawcett et al., 2019). As a result, it is widely believed that PNNs regulate the switch from juvenile to adult plasticity by restricting the reorganization of neural circuits (Gundelfinger et al., 2010). In line with this notion, destroying PNNs by injecting ECM-cleaving enzymes into specific brain regions can revive juvenile forms of plasticity in rodents. One such example is the rejuvenation of ocular dominance plasticity, where deprivation of visual input into one eye leads to a weakening of the neural responses it evokes, alongside an increase in the responses evoked by the non-deprived eye (Pizzorusso, 2002). Similar treatments have also rendered drug addiction and fear memories susceptible to erasure by extinction, a quality that is unique to juvenile animals (Gogolla et al., 2009; Xue et al., 2014).

The stabilizing effect conferred by the PNNs should be essential to normal brain function, since the role of PNNs in stabilizing neural circuits contributes critically to the maintenance of a balance between neuronal excitation and inhibition. In addition, the nets probably also act as a physical and chemical barrier that protects the neurons from oxidative stress and other toxic molecules (Miyata et al., 2007; Suttkus et al., 2012, 2016). Expectedly, alterations in the structure of PNNs have been observed in a number of CNS diseases, several examples being schizophrenia, epilepsy, Alzheimer's disease and amyotrophic lateral sclerosis (ALS) (Bonneh-Barkay and Wiley, 2009; Lemarchant et al., 2013; Bitanihirwe and Woo, 2014; Wen et al., 2018; Kaushik et al., 2021).

Besides the dense PNN formations, advances in imaging resolution have revealed that the ECM is ubiquitous at the neuronal surface, albeit in a looser configuration, and that ECM molecules can also be found in tight proximity to synapses (Dityatev et al., 2006). As with PNNs, this "loose" perisynaptic ECM appears to be equally important for maintaining the stability of mature synapses. For example, enzymatic cleavage of hyaluronan, the structural backbone of neural ECM lattices (Ruoslahti, 1996), was shown to increase the lateral mobility of synaptic AMPA-type glutamate receptors (Frischknecht et al., 2009), which suggests that the appearance of ECM at mature synapses assists in retaining neurotransmitter receptors in the synaptic membrane. In addition, enzymatic cleavage of lecticans was shown to enhance the motility of dendritic spines, as well as the outgrowth of dendritic spine heads, suggesting that the ECM is also restrictive to structural changes to the synapse (Orlando et al., 2012; de Vivo et al., 2013). Besides acting as a physical barrier, evidence also suggests that ECM molecules can interact with the synaptic transmission machinery and potentially promote the organization of these proteins at the synapse. This includes direct interactions with ion channels and neurotransmitter receptors, as well as indirect interactions through synaptic ECM receptors such as integrins (Dityatev and Schachner, 2003). It is therefore to be expected that a deficiency in various ECM molecules or their receptors manifests in dysfunctional synaptic transmission and plasticity (e.g., Qiu et al., 2006; Bukalo et al., 2007; Roszkowska et al., 2016).

## REMODELING OF THE EXTRACELLULAR MATRIX PERMITS SYNAPTIC PLASTICITY

The discussions in the previous section suggest that the ECM predominantly restricts synaptic plasticity. However, since synapses undergo structural changes long after maturity (Yang et al., 2019), the ECM at synapses needs to be susceptible to transient remodeling during such events. The dominant paradigm for ECM remodeling during synaptic plasticity is through proteolytic cleavage, and this process is described roughly as follows: a surge in synaptic activity results in localized activation of ECM-cleaving enzymes (e.g., through translation from mRNA that is locally present at the synapse). The resulting cleavage permits the synapse to undergo structural changes (e.g., the growth of the postsynaptic head), and may also expose latent sequences in the ECM molecules that activate synaptic receptors to further boost plasticity-related changes. Finally, proteolytic activity is inhibited, and *de novo* synthesized ECM molecules are secreted to replace the previously cleaved molecules, thus allowing the structural changes to the synapse to persist (**Figure 1**).

The best-studied example of proteolysis-mediated plasticity involves the activity of the ECM-cleaving enzyme matrix metalloproteinase 9 (MMP9). The activity-dependent increase in MMP9 expression was shown to be necessary for long-term potentiation (LTP) maintenance in the CA1 region of the hippocampus (Nagy et al., 2006; Wang et al., 2008). Upon increased neuronal activity, MMP9 mRNA is translated and

secreted locally at synapses (Zagulska-Szymczak et al., 2001; Gawlak et al., 2009; Dziembowska et al., 2012). Once activated, MMP9 cleaves a variety of targets in the synaptic extracellular space, some examples being the synaptic adhesion molecules neuroligin-1 and intercellular adhesion molecule-5 (ICAM-5) (Peixoto et al., 2012; Kelly et al., 2014), as well as the ECM molecule aggrecan (Mercuri et al., 2000). One effect of MMP9 activity at synapses is an enlargement of the dendritic spine head, which is a structural hallmark of LTP (Yuste and Bonhoeffer, 2001). Besides cleaving synaptic ECM to presumably facilitate spine enlargement, MMP9 activity also results in the activation of postsynaptic β1 integrin receptors, whose signaling further promotes remodeling of the actin cytoskeleton (Wang et al., 2008; Michaluk et al., 2011). Finally, once MMP9 has completed its role, it is presumably inhibited by TIMP1, a member of the tissue inhibitors of metalloproteinases (TIMP) family (Okulski et al., 2007; Magnowska et al., 2016). Lastly, the cessation of plasticity is expected to be accompanied by the secretion of newly synthesized ECM molecules. Indeed, studies have demonstrated that the expression of various ECM molecules is transiently upregulated by increased neuronal activity (Heck et al., 2004; Niekisch et al., 2019; Rao-Ruiz et al., 2019).

Though comparatively less studied, additional enzymes with roles in synaptic plasticity are also emerging. For example, two members of the MMP family, MMP3 and MMP7, have both been shown to drive activity-dependent changes in synaptic structure (Bilousova et al., 2006; Wójtowicz and Mozrzymas, 2014; Aerts et al., 2015; Brzdąk et al., 2017). Recently, the protease cathepsin-S was suggested to remodel PNNs in a circadian manner, which would presumably allow synapses to be modified during sleep (Pantazopoulos et al., 2020; Delorme et al., 2021; Harkness et al., 2021). Additional proteases, including neurotrypsin and members of the "a disintegrin and metalloproteinase with TSP motifs" (ADAMTS) family, may also be involved in mediating synaptic plasticity. Since this topic is beyond our scope, we refer the reader to excellent reviews on the involvement of ECM-cleaving enzymes in plasticity (Dityatev et al., 2010; Gottschall and Howell, 2015; Beroun et al., 2019).

## CAN PROTEOLYSIS SUPPORT THE PHYSIOLOGICAL FREQUENCY OF EXTRACELLULAR MATRIX REMODELING AT SYNAPSES?

If structural changes to synapses were rare events, it would be reasonable to assume that synaptic ECM is destroyed and resynthesized with every change. However, a growing number of studies are revealing that synapse size and morphology are constantly fluctuating, on a timescale of minutes to hours (e.g., Berning et al., 2012; Willig et al., 2014; Wegner et al., 2018). Such studies have taken advantage of super-resolution tools to investigate the dynamics of synapses in live brain tissue, and have shown frequent changes in synaptic position and morphology that had remained hidden for previous studies performed by conventional microscopy tools with resolutions substantially above the synapse size. This correction in our perception of synapse changes *in vivo* implies that one now needs to wonder about how the ECM can cope with the normal physiology of the synapses every day, and not just with rare plasticity events. The notion that the ECM is turned over (by enzymatic degradation and new synthesis) at an equally rapid rate to the synapse changes is not consistent with the fact that its components are some of the longest-lived molecules in the brain (ECM components have, on average, a half-life of over 1 month in rodents *in vivo*) (Toyama et al., 2014; Dörrbaum et al., 2018; Fornasiero et al., 2018; Heo et al., 2018). Hence, while proteolysis may accompany infrequent events of structural synaptic plasticity, an additional remodeling mechanism is needed to account for the more frequent changes to synapses that take place even in the absence of plasticity-triggering stimuli. A compelling possibility, discussed in the next section, is that the ECM at synapses can be remodeled through the recycling of its components, without the need for proteolysis and *de novo* synthesis.

## RECYCLING AS AN ALTERNATIVE MECHANISM FOR EXTRACELLULAR MATRIX REMODELING

The concept of ECM re-internalization from the extracellular space is not entirely unprecedented. In non-neural cells, multiple ECM molecules have been shown to undergo endocytosis after binding cell-surface receptors such as integrin, dystroglycan and CD44 (Coopman et al., 1996; Tammi et al., 2001; Shi and Sottile, 2008; Lobert et al., 2010; Leonoudakis et al., 2014). The common belief among these studies was that these internalized molecules were targeted for degradation, and so they typically did not examine whether a subset of these molecules might eventually be secreted once again to the extracellular space. A number of years ago, a study by Varadaraj and colleagues demonstrated a complete recycling loop for the ECM molecule fibronectin in fibroblasts and in epithelial cells. Using an acidic buffer treatment to strip away cell-surface molecules (thus enabling the investigators to discern the intracellular population), the authors showed that stimulation with TGF-beta induces uptake of fluorescently labeled fibronectin. Subsequently, these molecules were recycled back to the surface, where they were successfully incorporated into fibrils (**Figure 2**). The internalization of fibronectin was found to be dependent on both α5β1 integrin receptors and the type II TGF-β receptor (Varadaraj et al., 2017). To our knowledge, this is the first establishment of a link between cellular dynamics (i.e., fibrillogenesis, a process involved in migration and proliferation: Schwarzbauer and DeSimone, 2011) and ECM recycling.

Recently, we reported that the neuronal ECM is similarly capable of being recycled. More specifically, we showed that the ECM glycoprotein tenascin-R (TNR) is internalized from the synaptic extracellular space, and eventually resurfaces at synapses. The study largely relied on an immunostaining-based assay for labeling TNR molecules that recently emerged at the

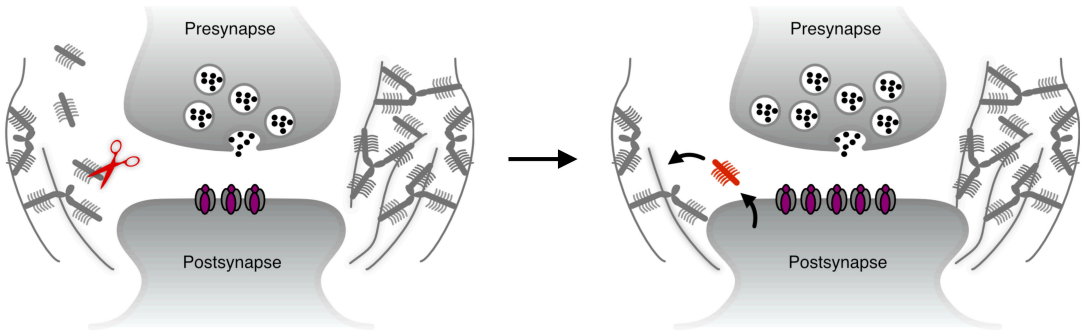


**FIGURE 1 |** The dominant paradigm for ECM remodeling – proteolytic cleavage. Left: when synaptic plasticity is initiated, ECM cleaving proteases such as MMP9 (Michaluk et al., 2011) may be translated and/or secreted locally at the synapse. Right: the synapse undergoes structural plasticity, and new molecules are secreted to re-stabilize the perisynaptic ECM.

surface of live neurons. Using this approach, it was demonstrated that these dynamic molecules are endocytosed by neurons, undergo retrograde trafficking to the soma, and eventually reappear at the neuronal surface (**Figure 3**). Further experiments relying on super-resolution imaging demonstrated that this process occurs preferentially at synapses (Dankovich et al., 2021).

Our observation that TNR recycling lasts ∼3 days is particularly surprising, considering that most surface molecules are known to recycle within minutes to hours (e.g., Bretscher, 1989; Koenig and Edwardson, 1997; Bridgewater et al., 2012). Further immunostaining experiments revealed that the internalized TNRs colocalize with markers for both the Golgi apparatus and endoplasmic reticulum in the neuronal somas. In addition, metabolic labeling of glycoproteins demonstrated that the recycling TNR molecules appear to incorporate new glycans (i.e., become re-glycosylated) following their trafficking through these organelles. Taken together, these findings may provide a partial explanation for this exceptionally long recycling loop.

Interestingly, we also observed that neurons effectively maintain two separate pools of TNR molecules: a stable pool that remains embedded in the ECM and a recycling pool that shuttles back and forth between the neuronal intra- and extracellular space. As mentioned, this recycling pool was found to be enriched at synapses, but in addition, it was also found to be significantly more abundant at highly active synapses. This latter finding strongly supports the notion that ECM recycling constitutes a mechanism for ECM remodeling during synaptic plasticity. Importantly, it was found that disrupting this recycling process also seriously modified synaptic structure and transmission (Dankovich et al., 2021). The findings of this study are summarized in **Figure 4**, below.

In addition to the metabolic benefits of ECM recycling, which frees the cells from the need to repeatedly synthesize both the ECM molecules and the ECM-cleaving enzymes, this mechanism could also serve an additional function, besides remodeling the extracellular space around synapses. As mentioned above, ECM remodeling through proteolysis may reveal latent sequences on ECM molecules that activate synaptic receptors and trigger plasticity-related intracellular signaling cascades (Dityatev et al., 2010). In a parallel fashion, it is possible to imagine that the internalization of *intact* ECM molecules by neurons is necessary for the activation of intracellular signaling cascades, perhaps through the interaction of these ECM molecules with co-trafficked proteins. Furthermore, if it is revealed that recycling ECM molecules are secreted and internalized by different neurons (e.g., from the pre- to the post synapse), this mechanism may constitute a novel form of *trans*-neuronal communication, an interesting possibility that may be explored in further studies.

## POTENTIAL IMPLICATIONS FOR BRAIN DISEASES

Since changes to the expression of ECM molecules are known to accompany a wide variety of brain diseases, it would be expected that perturbations to ECM recycling similarly manifest themselves in disease. In agreement with this notion, the proportion of somatic intracellular TNR molecules was increased in a mouse model of epilepsy (Dankovich et al., 2021). This accumulation of intracellular TNR is unlikely to be solely the result of neuronal damage, since this effect was not observed in a model of familial Alzheimer's disease, where neuronal damage is also prominent (Oakley et al., 2006). Interestingly, seizures have also been shown to upregulate the expression of a number of ECM molecules, including TNR (Ulbrich et al., 2021). While it is possible that these outcomes are causally linked, future studies should investigate this in greater detail and attempt to pinpoint dysregulated processes that modulate TNR recycling (as well as other ECM molecules). Other disease models that involve known modifications of mature ECM and thus warrant further investigation include multiple sclerosis, Alzheimer's disease, ALS, Parkinson's disease, schizophrenia and bipolar disorder (Bonneh-Barkay and Wiley, 2009; Lemarchant et al., 2013; Pantazopoulos and Berretta, 2016; Wen et al., 2018).

Besides its involvement in maintaining mature neural circuits, the ECM also plays an important developmental role, including the support of neurite extension, neuronal migration and cortical folding (Long and Huttner, 2019; Amin and Borrell, 2020). As for mature ECM, it is probable that some of these early roles may be supported by a putative recycling of ECM molecules

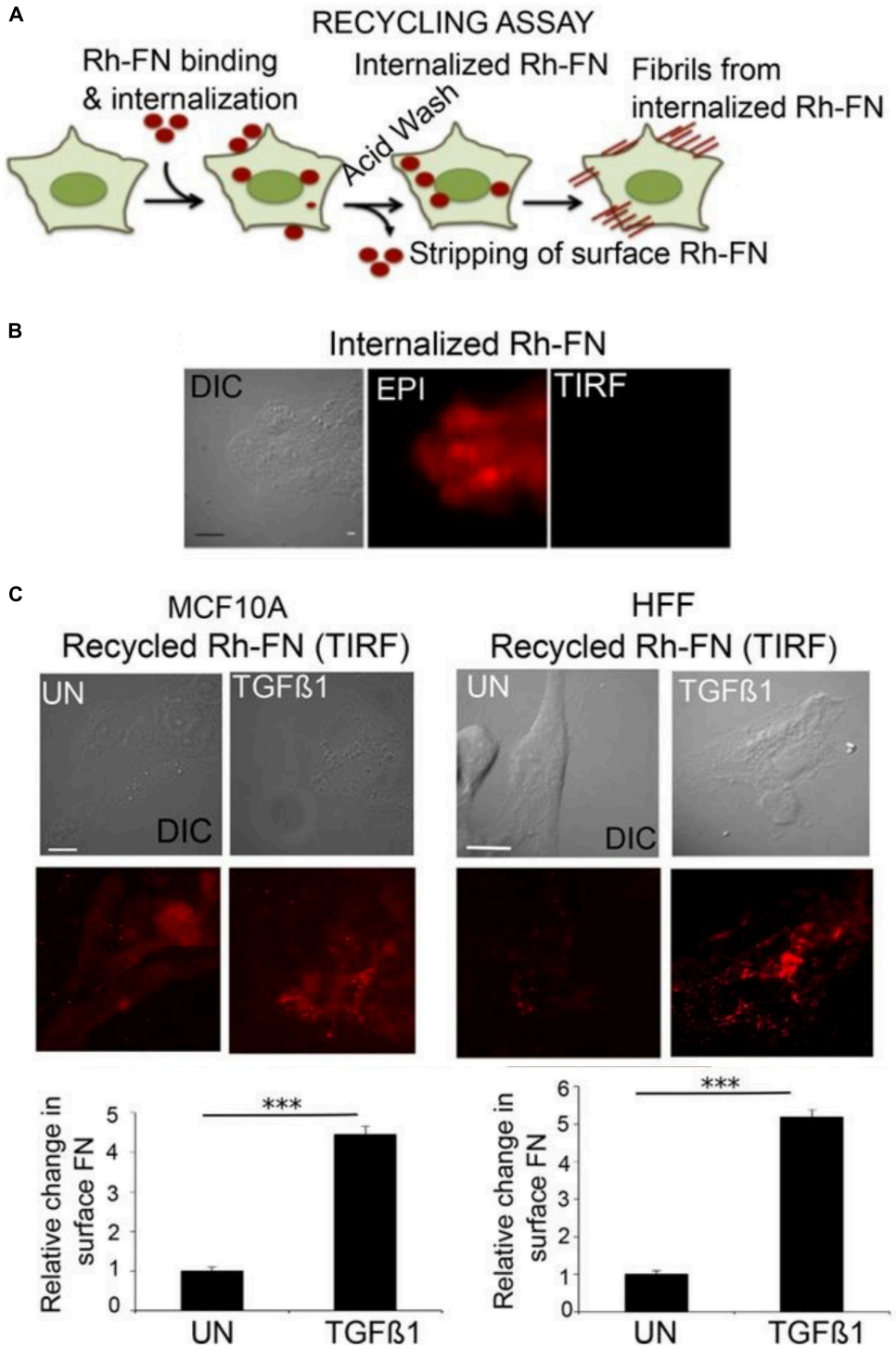


**FIGURE 2 |** Fibronectin recycling in epithelial cells. **(A)** Schematic of the assay used to assess fibronectin recycling. Live cells were incubated with Rh-FN (rhodamine-labeled fibronectin) for 30 min at 37°C. The cells were then stripped by acid washing to remove surface-bound molecules, so that only signal from internalized Rh-FN remains. Afterward, the cells were incubated in medium containing TGF-β1 for 1 h at 37°C, and then imaged using total internal reflection (TIRF) microscopy to assess the appearance of the internalized Rh-FN at the cell surface. **(B)** Following the incubation with Rh-FN, the cells were imaged both in TIRF mode or with epifluorescence to verify that the molecules had internalized. **(C)** The amount of resurfacing Rh-FN was assessed in TGF-β1-treated MCF10A breast epithelial cells or human foreskin fibroblasts (HFF), demonstrating that TGF-β1 induces significant recycling of FN in both cell types. Scale bar = 5 μm. The quantifications below the images were performed using the ImageJ 3D Object Counter plug-in. $N = 10$ cells per condition from at least three independent experiments. ***$p < 0.001$. Adapted from Varadaraj et al. (2017) with permission from the American Society for Cell Biology.

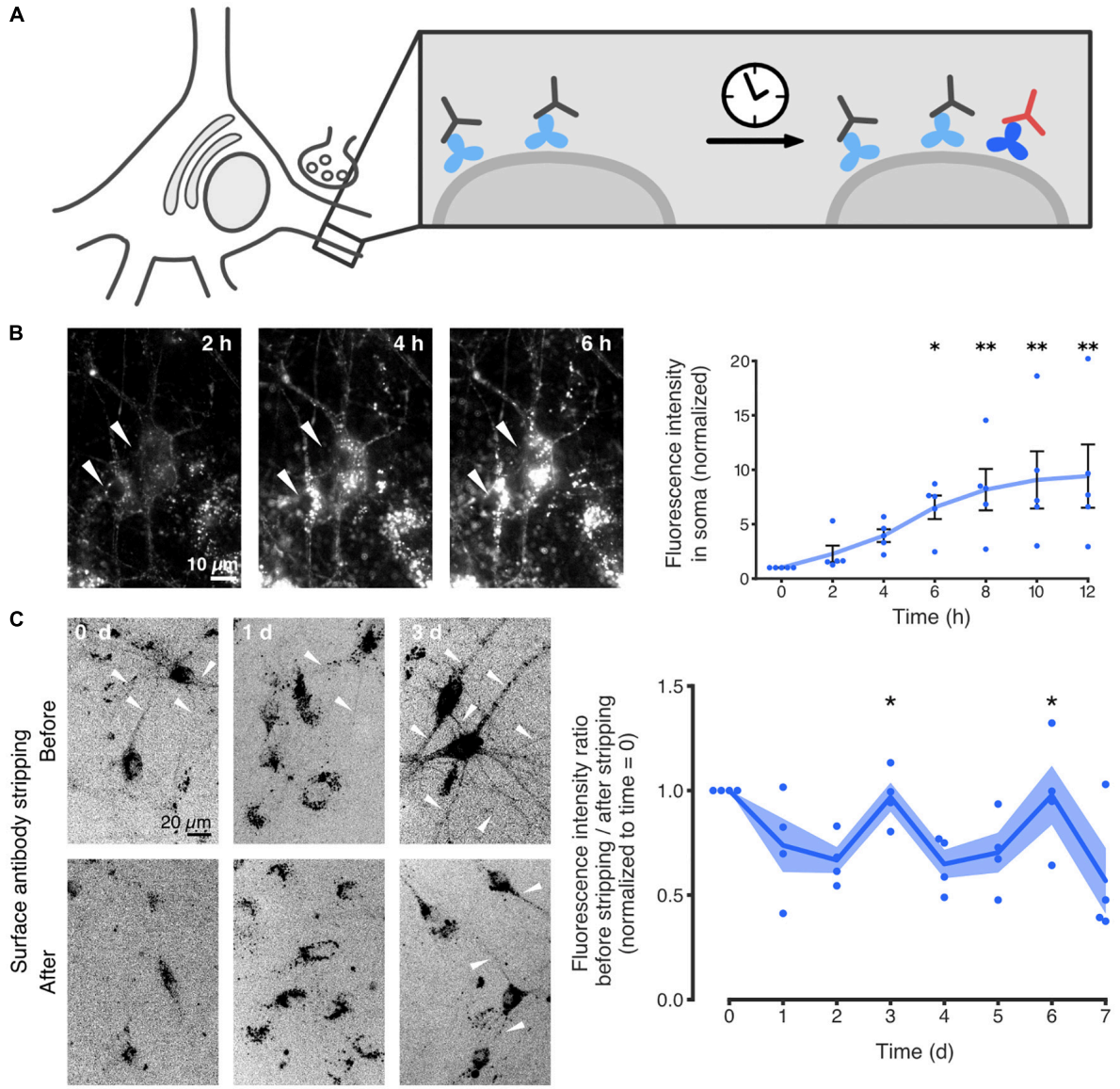


**FIGURE 3 |** TNR internalization and recycling in hippocampal neurons. **(A)** Schematic of the experimental assay for the labeling of recycling molecules. Live cultured neurons were incubated with non-fluorescent antibodies (gray) to block all of the surface TNR epitopes (light blue). After a period of time (4–6 h), the neurons were incubated with fluorophore-conjugated TNR antibodies (red) to label any "newly emerged" TNR epitopes which were not present at the neuronal surface beforehand (dark blue). Since the half-life of TNR in these cultures is ∼7 days (Dörrbaum et al., 2018), the amount of newly synthesized TNR molecules that emerge at the surface should not be significant. The fluorescently labeled TNRs can be subsequently followed in imaging experiments. **(B)** Time-lapse imaging of newly emerged TNR epitopes over 12 h. It is evident that the TNR epitopes are accumulating in the neuronal somas (two examples are indicated by the white arrowheads), demonstrating a significant internalization of these molecules. Scale bar = 10 µm. The plot shows a quantification of the mean TNR fluorescence intensity in multiple neuronal somas, normalized to the intensity at $t = 0$ h. A visible increase over 12 h is observed, confirming the observation that the molecules are internalized. $N = 5$ independent experiments, with 1–4 neurons each. Statistical significance was evaluated using the Friedman test ($\chi^2_6 = 25.46$, $^{***}p < 0.001$), followed by Dunn's multiple comparisons test ($^{*}p = 0.033$, $^{**}p = 0.005$, $^{**}p = 0.005$ and $^{**}p = 0.002$ for the 6, 8, 10, and 12-h timepoints, respectively). **(C)** The proportion of newly emerged TNR epitopes at the neuronal surface was measured over 6 days, by imaging before and after a treatment with proteinase K to strip away cell-surface molecules. Immediately after labeling ("0 days"), virtually no neurites were visible after stripping, indicating that the majority of the newly emerged TNR molecules are at the surface. On day 1, the stripping had little effect, indicating that many TNR molecules had internalized. On day 3, neurites were once again visible before but not after stripping, indicating that a large amount of TNR molecules had returned to the neuronal surface. Scale bar = 20 µm. The plot shows a quantification of the fluorescence ratio before/after stripping, normalized to $t = 0$ days. The peaks at days 3 and 6 indicate that TNR recycles with a periodicity of ∼3 days. $N = 4$ independent experiments. Statistical significance was evaluated with the Kruskal-Wallis test (days 2–4: $H_2 = 8.29$, $^{*}p = 0.016$, days 4 – 6: $H_2 = 6.74$, $^{*}p = 0.036$), followed by Fisher's LSD ("3d" vs. "2d": $^{*}p = 0.046$; "3d" vs. "4d": $^{**}p = 0.005$; "6d" vs. "5d": $^{*}p = 0.022$; "6d" vs. "7d": $^{*}p = 0.028$). All data represent the mean (lines) ± SEM (panel **B**: whiskers; panel **C**: shaded regions), with dots indicating individual experiments. Adapted from Dankovich et al. (2021) with permission from Springer Nature (http://creativecommons.org/licenses/by/4.0/).

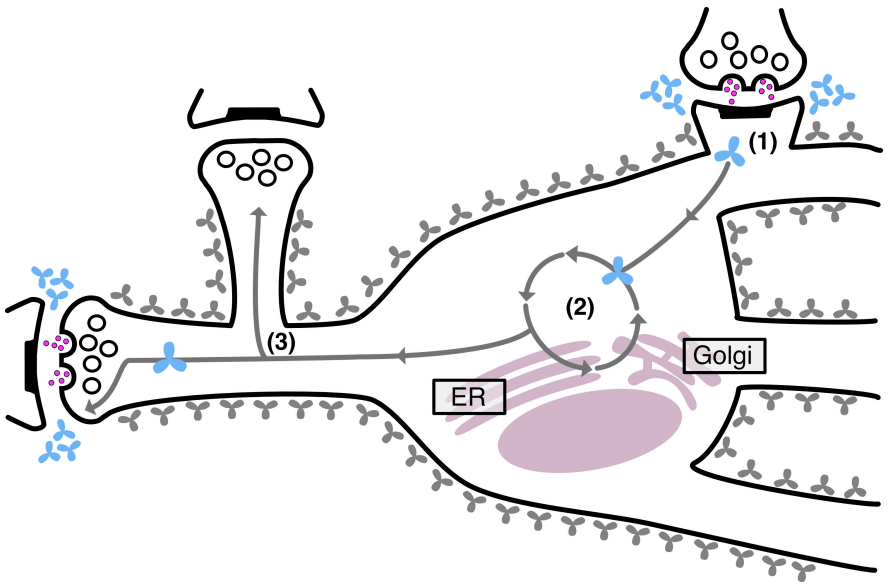


**FIGURE 4 |** TNR recycling mechanism and function in neurons. Neurons contain two pools of TNR molecules: a stable pool (gray molecules) and a recycling pool, which is enriched at synapses (blue molecules). After their internalization at synapses (1), the recycling TNR molecules are trafficked to the Golgi apparatus and the endoplasmic reticulum, where they appear to undergo a re-glycosylation (2). At the end of their route, these molecules are once again trafficked to synapses (3). Stronger synapses (with a larger pool of actively recycling presynaptic vesicles or with larger postsynaptic spine heads) have more recycling TNR molecules in their vicinity.

expressed in the developing brain. For example, dysregulations of the ECM protein reelin, which plays an important role in neuronal migration, have been linked to various disorders such as autism spectrum disorder (ASD), schizophrenia and bipolar disorder (Ishii and Maeda, 2008; Lakatosova and Ostatnikova, 2012). The observation that reelin molecules can be internalized through an interaction with their receptors VLDLR and ApoER2 hints to the possibility that a portion of these molecules can be recycled back to the membrane. The ECM protein laminin is similarly known to be dysregulated in neuronal migration disorders, and was also shown to be internalized in non-neuronal cells (Barak et al., 2011; Radmanesh et al., 2013; Radner et al., 2013; Leonoudakis et al., 2014). The prospect of ECM recycling in early development should be investigated in greater depth in the future, including the possible involvement of such perturbations in various neurodevelopmental disorders.

## CONCLUSION AND FUTURE DIRECTIONS

Synaptic plasticity in the adult brain is believed to be accompanied by a remodeling of the local ECM, presumably through proteolysis and *de novo* synthesis of ECM molecules (Dityatev et al., 2010; Krishnaswamy et al., 2019). However, while this form of remodeling may account for infrequent instances of plasticity, it is likely to be too metabolically costly to support regular fluctuations to synaptic structure, as have been demonstrated to occur by numerous studies (Berning et al., 2012; Willig et al., 2014; Wegner et al., 2018). Importantly, such a mechanism would not be in line with the long lifetimes of ECM molecules (Toyama et al., 2014; Dörrbaum et al., 2018; Fornasiero et al., 2018; Heo et al., 2018). A possible solution to this problem arose from a recent study describing a novel mechanism of ECM remodeling at synapses through the recycling of the ECM molecule TNR (Dankovich et al., 2021). It was shown that a targeted disruption of this mechanism severely modified synaptic function, suggesting that dysregulated recycling *in vivo* is highly likely to play a role in disease.

An interesting line of future research would be to assess potential dysfunctions that are a direct outcome of perturbations to ECM recycling. This could be assessed by treating animals with large aggregates of antibodies directed against ECM molecules, as we performed in our study *in vitro* (Dankovich et al., 2021). Since it was found that interrupting TNR recycling modified evoked synaptic transmission as well as dendritic spine head size, it is likely that potential dysfunctions would involve similar phenotypes. It is possible to imagine, for example, that disrupting ECM recycling would reduce neuronal excitability. In principle, if certain diseases are found to have augmented recycling, inhibiting this process may have potential therapeutic benefits, albeit one would need to develop more suitable inhibitors than antibody aggregates, which would probably have major difficulties in penetrating into the brain, and would probably also have severe inflammation-inducing effects. For the reverse situation, it would be beneficial to develop techniques to enhance ECM recycling, for example, by interfering with the interaction of these molecules with extracellular binding partners. Such a treatment may act to reduce neuronal excitability and could therefore have therapeutic potential in disorders such as epilepsy.

Once sufficient tools are developed for probing ECM recycling in animals *in vivo*, such investigations will be an exciting possibility for future studies.

In conclusion, while further experiments are needed to establish whether ECM recycling is a widespread mechanism among multiple molecules, the discovery of a novel constitutive process in neurons opens up an exciting new avenue of research in models of brain disease. We expect that future studies investigate the involvement of ECM recycling in disease in greater detail, as well as potential therapeutic treatments that target this process.

## AUTHOR CONTRIBUTIONS

TD and SR conceived the manuscript. TD wrote the manuscript. SR revised the manuscript. Both authors contributed to the article and approved the submitted version.

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# Brevican and Neurocan Cleavage Products in the Cerebrospinal Fluid - Differential Occurrence in ALS, Epilepsy and Small Vessel Disease

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The neural extracellular matrix (ECM) composition shapes the neuronal microenvironment and undergoes substantial changes upon development and aging, but also due to cerebral pathologies. In search for potential biomarkers, cerebrospinal fluid (CSF) and serum concentrations of brain ECM molecules have been determined recently to assess ECM changes during neurological conditions including Alzheimer's disease or vascular dementia. Here, we measured the levels of two signature proteoglycans of brain ECM, neurocan and brevican, in the CSF and serum of 96 neurological patients currently understudied regarding ECM alterations: 16 cases with amyotrophic lateral sclerosis (ALS), 26 epilepsy cases, 23 cerebral small vessel disease (CSVD) patients and 31 controls. Analysis of total brevican and neurocan was performed *via* sandwich Enzyme-linked immunosorbent assays (ELISAs). Major brevican and neurocan cleavage products were measured in the CSF using semiquantitative immunoblotting. Total brevican and neurocan concentrations in serum and CSF did not differ between groups. The 60 kDa brevican fragment resulting from cleavage by the protease ADAMTS-4 was also found unchanged among groups. The presumably intracellularly generated 150 kDa C-terminal neurocan fragment, however, was significantly increased in ALS as compared to all other groups. This group also shows the highest correlation between cleaved and total neurocan in the CSF. Brevican and neurocan levels strongly correlated with each other across all groups, arguing for a joint but yet unknown transport mechanism from the brain parenchyma into CSF. Conclusively our findings suggest an ALS-specific pattern of brain ECM remodeling and may thus contribute to new diagnostic approaches for this disorder.

**Keywords: cerebral small vessel disease (CSVD), amyotrophic lateral sclerosis (ALS), extracellular matrix (ECM), biomarker, CNS, serum**

## INTRODUCTION

In the healthy adult human brain approximately 20-22% of the neural parenchyma represent extracellular space (Vargova et al., 2011; Nicholson and Hrabìtová, 2017). This space is not occupied by neural cells but filled with interstitial fluid and with components of the extracellular matrix (ECM) produced by neurons and glial cells. Typical components of the brain ECM are hyaluronic acid, heparan sulfate and chondroitin sulfate proteoglycans (CSPGs), link proteins and glycoproteins like tenascins. Among the CSPGs the lectican family including aggrecan, brevican, neurocan and versican is of utmost importance (Frischknecht et al., 2014). After secretion of ECM building blocks into the extracellular space, these molecules form structurally and functionally diverse three-dimensional meshworks surrounding and insulating neuronal somata and neurites, including synapses or – as perinodal ECM – the nodes of Ranvier and axon initial segments. Thus, they generate defined extracellular compartments. The most peculiar forms of neural ECM are the perineuronal nets (PNN) largely found on parvalbumin-positive inhibitory neurons, but there is also a more diffuse form of ECM filling the entire extracellular space and a laminin/collagen-based perivascular ECM associated with brain microvessels (for review see Ulbrich et al. (2021)).

ECM structures create microcompartments for stem cell migration, diffusion processes, for the presentation of trophic factors to their cognate receptors and for synaptic and volume transmission (summarized in Dityatev et al. (2010)). As demonstrated by a plethora of studies, physiological plasticity processes in the brain like ocular dominance plasticity, remote fear memory or cognitive flexibility are affected by ECM-disintegrating enzymatic treatments (Pizzorusso et al., 2002; Happel et al., 2014; Thompson et al., 2018), and vice versa synaptic activity can actively induce controlled ECM proteolysis, thus locally shaping ECM subcompartments (Mitlöhner et al., 2020).

Indeed, hyaluronan-binding proteoglycans neurocan and brevican are substrates for controlled proteolytic cleavage by matrix metalloproteases (MMPs) or 'A Disintegrin and Metalloproteinase with Thrombospondin motifs' (ADAMTS) enzymes. Like all lecticans, they have in common a dumbbell-like three-dimensional protein structure with hyaluronan-binding domains localized in the N-terminal globular parts and cell-surface- or glycoprotein-interacting domains in the C-terminus, such that proteolytic events within the central rod-like domain separate proteoglycan parts and loosen the ECM integrity (Rauch et al., 2001; Frischknecht and Seidenbecher, 2012; **Figure 1**). The major neurocan fragments of 150 and 130 kDa are thought to be generated intracellularly and independent of ADAMTS activity (Asher et al., 2000). In contrast to neurocan, brevican occurs also as a glycosylphosphatidylinositol (GPI)-anchored minor isoform (Seidenbecher et al., 1995), which constitutes a substrate for ADAMTS-4 cleavage as well, giving rise to the same 60 kDa N-terminal fragment as the soluble isoform. Thus, proteolytic cleavage separates ECM-binding from cell surface-binding entities, leading to disintegration of the cell-ECM-meshwork. This is a dynamic and tightly controlled process to maintain the extracellular homeostasis of the brain.

Under aging and pathophysiological conditions the integrity and composition of the brain ECM undergo extensive changes, leading to a disturbed equilibrium of ECM production and deposition on one hand and ECM cleavage and clearance on the other hand (Ulbrich et al., 2021). In human epilepsy as well as in rodent epilepsy models manifold ECM remodeling phenotypes have been reported in the brain tissue, with a particular focus on PNN disruption due to increased proteolysis, which may contribute to epileptogenesis (summarized in Dityatev et al. (2010), Chaunsali et al. (2021)). In histochemical analyses of postsurgical specimen from focal cortical dysplasia patients, known for a longstanding epilepsy with a high seizure burden, a complete reorganization of the ECM was reported with reduced brevican expression and disturbance of extracellular space diffusion parameters (Zamecnik et al., 2012).

For amyotrophic lateral sclerosis (ALS), an adult-onset neurodegeneration of motor neurons, the picture is less clear, but studies in animal models revealed that in the surrounding of affected motor neurons a non-permissive microenvironment for regeneration is formed. In the lesion area in the adult spinal cord of a rat ALS model this microenvironment was shown to contain accumulations of chondroitin sulphate proteoglycans neurocan and versican (Mizuno et al., 2008). In the superoxide dismutase 1 (SOD1) model of ALS also a dramatic reduction of neuron-protecting PNNs (Forostyak et al., 2014) and a shift in CSPG receptor expression from neurons to glial cells was shown (Shijo et al., 2018). In the serum and CSF of ALS patients MMPs 2 and 9 and their inhibitors were found to be dysregulated (Niebroj-Dobosz et al., 2010) and a quantitative proteomic study of ALS patients' CSF samples revealed significant disturbance in ECM regulatory protein networks (Collins et al., 2015).

Sporadic and familial Cerebral Small Vessel Diseases (CSVDs), a spectrum disorder affecting blood-brain barrier and small vessel wall integrity, are also hypothesized to be linked to dysregulated ECM and its proteolytic fragmentation (Vilar-Bergua et al., 2016; Benveniste and Nedergaard, 2021). CSVDs are considered a major risk factor for vascular cognitive impairment (Schreiber et al., 2020). Dysregulated ECM in the brain tissue of human CSVDs comprises heparan sulfate proteoglycans (van Horssen et al., 2001), Tissue Inhibitor of Matrix Metalloproteases TIMP1 or TIMP3 and the ECM receptor CD44 (Monet-Leprêtre et al., 2013; Manousopoulou et al., 2017; Grand Moursel et al., 2018), overall arguing for a dysbalance of controlled proteolytic ECM dynamics along the CSVD spectrum.

Just recently, perineuronal ECM components, like brevican and neurocan, have been detected in the serum and CSF of humans, providing the opportunity to study their concentration *in vivo* and establish new biomarkers as a proxy for neural ECM reorganization. Thus far, serum and CSF biomarker studies have focused on traumatic brain injury (TBI), on vascular and neurodegenerative dementia and have shown lower CSF brevican, comprising its N-terminal peptides, and neurocan levels as well as decreased total brevican, but an increase of its C-terminal fragments in the patients' serum (Minta et al., 2019a, 2021a,b; Jonesco et al., 2020).

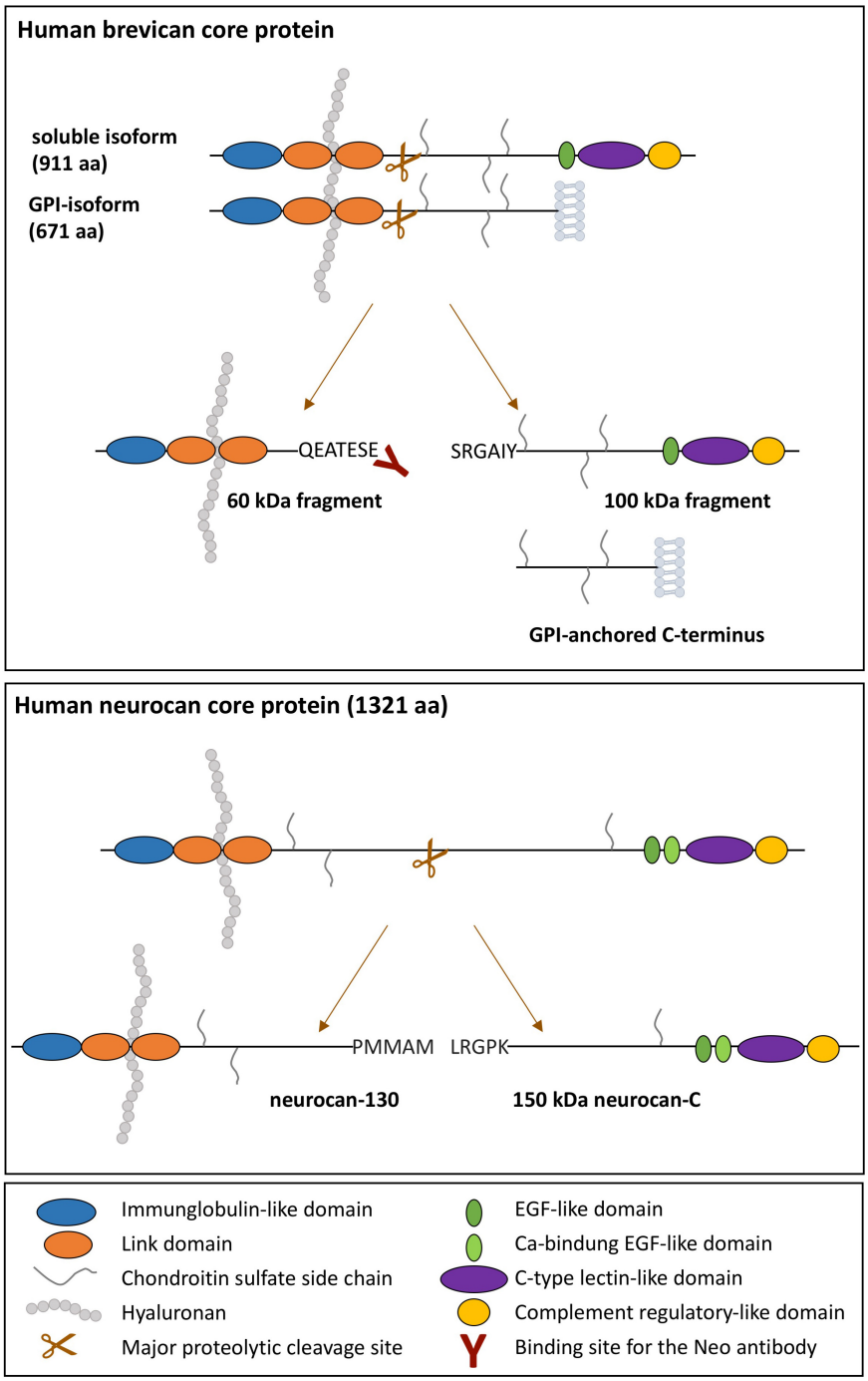


**FIGURE 1 |** Schematic illustration of the domain structure of human soluble and GPI-anchored brevican and neurocan and their major proteolytic fragments. Domains are color-coded. The hyaluronan-binding regions and the binding site of the neoepitope-specific brevican antibody are indicated.

Based on the fact, that in CSVDs, epilepsy, and ALS, three typical neurological disorders in the Western population, ECM dysregulations have been reported, we focused on these disorders in our study. We compared the CSF and serum concentrations of brevican, neurocan and their major cleavage products between the disease groups and a control group, and searched for correlations with further demographic and serological factors as confounders. Our main objective was to detect group differences, i.e., disease-specific ECM signatures in the CSF, focusing on cleaved brevican and neurocan, as a proxy for dysregulation in neural ECM turnover.

# MATERIALS AND METHODS

## Patient Cohort, Neurological Assessment

The study included a cohort of 96 patients comprising 16 with ALS, 26 with focal epilepsy, 23 with CSVD and 31 controls (**Table 1**). Note that subgroups differed slightly in n-numbers due to a few missing samples. Exact n numbers are given in all figure captions.

Patients were recruited from the Department of Neurology at the Otto-von-Guericke University Magdeburg between 02/2012 and 03/2020. ALS diagnosis was based on the revised El Escorial criteria (Brooks et al., 2000; Carvalho and Swash, 2009). According to these, 3 patients suffered from possible, 8 from probable and 5 from definite ALS within the ALS cohort. Epilepsy patients were diagnosed with focal epilepsy according to the International League Against Epilepsy (ILAE) (Scheffer et al., 2017). The CSVD cohort comprised 11 cases with probable CAA diagnosed according to the modified Boston criteria; four of them fulfilled a concomitant clinical diagnosis of Alzheimer's disease (AD) according to the NINDCS/ADRDA criteria (Linn et al., 2010; McKhann et al., 1984). Another 12 CSVD patients had HA and diagnosis was based on the existence of deep and mixed, i.e., deep and lobar hemorrhages, detected on T2*-weighted magnetic resonance imaging (MRI) sequences (Pasi et al., 2018; Scheumann et al., 2020).

A hospital-based cohort of neurologic patients, comprising cases with non-specific complaints who underwent lumbar puncture in terms of a diagnostic workup to rule out any neurologic condition served as controls. None of those disease controls suffered from any neurological disorder, especially not from ALS, epilepsy or seizures, CAA or AD, and HA.

## Sample Taking and Biomarker Measurements

In all patients and controls lumbar puncture (LP) and venous puncture (VP) have been conducted for diagnostic workup. For lumbar puncture patients were seated and 9 ml CSF were taken. In all samples, relevant CSF blood contamination was excluded visually/macroscopically and through microscopy of non-centrifugated CSF aliquots. After centrifugation, the pellet was separated to avoid the presence of cells in the final analysis samples. Within 20 min of LP and VP, CSF and serum samples were centrifuged at 4°C, aliquoted and stored at −80°C until brevican and neurocan analysis (see below). In ALS and CSVD, CSF markers of neuroaxonal damage and neurodegeneration were determined immediately after sample collection; they were not available in the epilepsy group. Neurofilament light chain (NF-L) was measured with commercially available ELISA (NF-light® ELISA, IBL International GmbH, Hamburg, Germany) and total Tau (tTau) was determined either through ELISA (Innotest hTauAg, Innogenetics, Ghent, Belgium (until 12/2019)) or with an automated immunoassay (LUMIPULSE® G600 II, Fujirebio lnc., Japan (from 01/2020 on)), following the manufacturer's instructions (see also Schreiber et al. (2018a,b)). CSF Abeta 1-40 was assessed with the Innotest β-Amyloid(1-40) ELISA (until 12/2019) and after 1/2020 also with the automated immunoassay. CSF and serum albumin as well as Immunoglobulin G were determined by rate nephelometry (Nephelometer Immage 800, Beckman Coulter, Fullerton, CA, United States). The CSF albumin/serum albumin ratio ($Q_{alb}$ x $10^{-3}$) was calculated to assess blood-brain barrier (BBB) integrity, and the IgG-Index ($Q_{alb}/Q_{IgG}$) was calculated to assess intrathecal immunoglobulin synthesis as a predictor for neuroinflammatory processes.

## Ethics and Informed Consent

The study was approved by the Ethics Committee of the Otto-von-Guericke-University Magdeburg (Sign 07/17). Patients donating biological material gave written informed consent in accordance with the Declaration of Helsinki. Data and material were handled in a coded fashion guaranteeing patient anonymity.

## Enzyme-Linked Immunosorbent Assay Protocols

For the quantitative measurement of total brevican and neurocan we used commercial anti-human brevican und anti-human neurocan ELISA kits, respectively (RayBiotech Norcross, GA, United States) (ELH-BCAN-1 und ELH-NCAN-1). Serum samples were diluted 1:2 for brevican ELISAs and used undiluted for neurocan ELISAs. The dilution factor for CSF was 1:200 to 1:300 for anti-brevican ELISAs (to optimize the dilution for the detection range of the ELISA batches) and 1:10 for anti-neurocan ELISAs. ELISA measurements were essentially conducted according to the manufacturer's instructions. In brief, pre-diluted CSF and serum samples were incubated in 96-well strip microplates pre-coated with monoclonal mouse anti-brevican (immunogen: aa 23-911) or polyclonal sheep anti-neurocan (immunogen: aa 23-1321). Samples were analyzed as triplicates or duplicates.

To compensate for sensitivity differences in ELISA batches the calibrator concentration of the recombinant brevican or neurocan protein for the standard curve was adjusted according to OD values obtained (highest standard concentrations were set to 3 ng/ml in brevican ELISAs and 25 ng/ml in neurocan ELISAs; standard dilution series: 6 × 1:2 dilution steps).

After 2.5 h incubation, 1:80 diluted biotinylated antibody against the target protein was added and incubated for 1 h, followed by a 45 min incubation with HRP-streptavidin (1:200 for brevican and 1:500 for neurocan) and a 30 min incubation with 3,3′,5,5′-tetramethylbenzidine in the dark. The reaction was stopped by adding 0.2 M $H_2SO_4$. OD values were measured in a VersaMax™ Tunable Microplate Reader (Molecular Devices, San Jose, United States) at 450 nm and at 650 nm for unspecific background subtraction. According to the manufacturer's information intra- and inter-assay variability is below 10 and 12%, respectively.

## Antibodies

Following primary antibodies were used: rabbit anti-brevican "neo" (Rb399) (custom-made; rat neo-epitope QEAVESE; see Valenzuela et al., 2014; 1:500), polyclonal sheep anti-human/rat brevican (AF4009, R&D Systems, Minneapolis, United States,

**TABLE 1 |** Demographic data of the patient cohorts.

| Patients | | CSF samples | | | Serum samples | | |
|---|---|---|---|---|---|---|---|
| **N** | **Group** | **n** | **Male/Female (%)** | **Mean Age ± SD** | **n** | **Male/Female (%)** | **Mean Age ± SD** |
| **31** | **Controls** | **26** | 46.2/53.8 | 60.5 ± 15.5 | **26** | 42.3/57.7 | 62.3 ± 13.6 |
| **16** | **Amyotrophic Lateral Sclerosis (ALS)** | **16** | 43.8/56.2 | 64.4 ± 13.2 | **10** | 40.0/60.0 | 63.0 ± 14.9 |
| **26** | **Epilepsy** | **25** | 44.0/56.0 | 54.7 ± 18.6 | **26** | 46.2/53.8 | 54.3 ± 18.4 |
| **23** | **Cerebral Small Vessel Disease (CSVD)** | **22** | 68.2/31.8 | 72.6 ± 9.4 | **22** | 72.7/27.3 | 72.8 ± 9.5 |
| 11 | Cerebral Amyloid Angiopathy (CAA) | 11 | 54.5/45.5 | 74.0 ± 4.9 | 10 | 60.0/40.0 | 73.9 ± 5.2 |
| 12 | Hypertensive Arteriopathy (HA) | 11 | 81.8/18.2 | 71.2 ± 12.6 | 12 | 83.3/16.7 | 71.8 ± 12.2 |
| **96** | **Total cases** | **89** | **50/50** | **62.6 ± 16.1** | **84** | **51.2/48.8** | **62.7 ± 15.9** |

*Bolded numbers indicates sum up to the total. CAA and HA are subcategories of CSVD.*

1:1000), polyclonal sheep anti-rat/mouse neurocan (AF5800, R&D Systems, Minneapolis, United States, 1:1000), and the antibodies from the ELISA kits (see above).

To prove if the neo-antibody, which was originally produced against the neo-epitope of the rat N-terminal brevican fragment, is also detecting the human brevican fragment neo-epitope EATESE we performed a competition assay with pre-incubation of the primary antibody with a 100-fold molar excess of the peptide Biotin-Ahx-CGQEAVESE containing the immunogen for 1 h at RT. As shown in **Supplementary Figure 1**, the 60 kDa band detected by this antibody in human CSF samples is clearly diminished in the peptide competition condition, demonstrating its specificity.

As secondary antibodies we used peroxidase-conjugated AffiniPure donkey anti-rabbit IgG (H + L) and peroxidase-conjugated AffiniPure donkey anti-sheep IgG (H + L) (Jackson ImmunoResearch, Cambridgeshire, United Kingdom, 1:2000).

## Immunoblotting

For semiquantitative assessment of brevican and neurocan fragments undiluted CSF samples were incubated with chondroitinase ABC (Sigma-Aldrich, St. Louis, United States) at 1U/mg at 37°C for 30 min to digest chondroitin sulfate side chains and afterward solubilized in 5 × SDS loading buffer (250 mM Tris/HCl, pH 8, 50% glycerol, 10% SDS, 0.25% bromophenol blue, 0.5 M DTT) and boiled at 95°C for 10 min. Samples were separated on 2,2,2-trichloroethanol (TCE)-containing stain-free 5–20% Tris-glycine SDS polyacrylamide gels under reducing conditions. Before Western blotting proteins were activated under UV light for 5 min. Protein transfer onto PVDF membranes (Merck Millipore, Burlington, MA, United States) was performed according to standard protocols. To control for proper protein transfer UV images of the membranes were captured. After blocking for 1 h at RT in 5% horse serum (Sigma-Aldrich, St. Louis, United States) in TBS-T (150 mM sodium chloride, 50 mM Tris, 0.1% (v/v) Tween20, pH 7.6) membranes were incubated in primary antibodies overnight at 4°C. After washing three times with TBS-T for 10 min, secondary antibodies were added and incubated for 60 min at RT. After washing membranes again three times with TBS-T for 10 min, immunodetection was performed using an ECL Chemocam Imager (INTAS Science Imaging Instruments GmbH, Göttingen, Germany). To improve semiquantitative comparability of optical density data from immunoblots we used a standard sample loaded on all gels as a reference to calibrate individual blot data and compensate for gel differences. Quantification of band intensities was done using NHI ImageJ software version 1.52a (US National Institutes of Health, Bethesda, MD, United States).

## Statistical Analysis of Data

For the analysis of brevican and neurocan levels non-parametric testing was used due to the sample characteristics, particularly the relatively small sample sizes of the ALS and CSVD groups. Because the overall groups were not matched for age and sex, a rank analysis of covariance was performed on brevican and neurocan levels following the protocol of Quade (1967). To this end, the data were transformed into rank coefficients and sex and age were partialled out of the respective dependent variable (brevican and neurocan levels) using a multiple regression. The resulting residuals of the dependent variable were z-standardized and entered a one-way ANOVA including the factor group (control, ALS, epilepsy, and CSVD). Given a significant group effect, *post hoc* comparisons were conducted using Tukey's multiple comparison tests (Tukey's HSD). For statistical comparisons within the CSVD (HA versus CAA) group, t-tests were calculated on age- and sex-corrected brevican and neurocan levels. For analyses of data uncorrected for sex and age, please see **Supplementary Tables 1**, **2**. For assessment of correlations on the uncorrected data, Spearman's rank correlation ($r_\rho$) was used and p values were reported with Bonferroni correction for multiple testing. The level of significance was defined as $p \leq 0.05$ and all tests were conducted two-tailed. Results were reported with Cohen's d and partial eta squared ($\eta_p^2$) as effect sizes. Statistical analysis was done using GraphPad Prism version 9 (GraphPad Software, Inc., San Diego. CA, United States) and SPSS Version 28 (IBM Corp., Armonk, NY, United States).

# RESULTS

## Brevican and Neurocan Levels in the Cerebrospinal Fluid and Serum of Patient Groups

The concentrations of total neurocan and total brevican were quantitatively assessed in patients' body fluids with commercially available ELISAs. For all patient groups the mean values ± SD

and the median values of raw data are given in **Table 2**. These levels are in the same concentration range as documented in previous reports (Minta et al., 2019a; Jonesco et al., 2020). It has to be noted, though, that serum concentrations of neurocan were at the lower detection limit of the assay in many cases. The rank analysis of covariance including the factor group (control, ALS, epilepsy, & CSVD) did not reach statistically significance on both, brevican, $F(3,85) = 1.04$, $p = 0.38$, $\eta_p^2 = 0.04$, and neurocan total CSF concentration levels, $F(3,83) = 0.03$, $p = 0.99$, $\eta_p^2 < 0.01$; see **Figures 2A-D**. The effect of group was also not significant for brevican total serum levels, $F(3,75) = 0.75$, $p = 0.53$, $\eta_p^2 = 0.03$, and for neurocan total serum levels, $F(3,80) = 1.84$, $p = 0.15$, $\eta_p^2 = 0.07$. No significant differences were measured within the CSVD subgroups (CAA versus HA) for brevican and neurocan total CSF concentration levels (all $ts \leq 1.40$, $ps \geq 0.18$) and total serum concentration levels (all $ts \leq 0.86$, $ps \geq 0.40$).

Interestingly, the levels of neurocan and brevican in the CSF showed a significant positive correlation ($r_\rho = 0.641$, $p < 0.001$) which was observable in all sample groups (**Figure 2E**). There was also a significant correlation between the serum total neurocan and total brevican concentrations ($r_\rho = 0.34$, $p < 0.05$; not shown as a separate plot, but indicated in **Figure 3**).

## Levels of Brevican and Neurocan Fragments in the Cerebrospinal Fluid

To assess the relative abundance of major proteoglycan fragments, i.e., the 60 kDa N-terminal brevican fragment derived from ADAMTS-4 cleavage and the 150 kDa neurocan-C fragment produced independently from ADAMTS activity, we used immunoblots with chondroitinase ABC-digested CSF samples and a reference sample for calibration. Since brevican and neurocan fragment concentrations in the serum samples were too low to be reliably detected on immunoblots, we focused on CSF samples.

### Cleaved Brevican in the Cerebrospinal Fluid

The neo-specific brevican antibody (Valenzuela et al., 2014) detected the human 60kDa brevican fragment in the CSF (see example blot in **Figure 4A** and in **Supplementary Figure 2A**). The rank analysis of covariance including the factor group (control, ALS, epilepsy, & CSVD) was neither significant for cleaved brevican ($F(3,82) = 1.31$, $p = 0.28$, $\eta_p^2 = 0.05$) nor for the ratio of cleaved versus total brevican ($F(3,82) = 1.81$, $p = 0.15$, $\eta_p^2 = 0.06$) (**Figures 4B,C**). Also, for the CSVD subgroups (HA versus CAA) there was no significant difference in cleaved brevican as well as cleaved normalized to total brevican (all $ts \leq 1.80$, $ps \geq 0.09$). Across all groups, cleaved brevican fragment levels significantly correlated with total brevican concentrations ($r_\rho = 0.3460$, $p < 0.05$; **Figure 5A**).

### Cleaved Neurocan in the Cerebrospinal Fluid

Human neurocan fragments in the CSF appeared as 150 kDa C-terminal product and approx. 130kDa N-terminal fragment, which is in agreement with previous work (Rauch, 2012; see example blot in **Figure 4D** and in the **Supplementary Figure 2B**). The rank analysis of covariance with the factor group (control, ALS, epilepsy, and CSVD) on C-terminal neurocan fragment

**TABLE 2** | CSF and serum concentrations of total brevican and neurocan in all groups.

| Group | CSF samples | | | | | | Serum samples | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Neurocan | | | Brevican | | | Neurocan | | | Brevican | | |
| | n | Mean ± SD (ng/ml) | Median | n | Mean ± SD (ng/ml) | Median | n | Mean ± SD (ng/ml) | Median | n | Mean ± SD (ng/ml) | Median |
| **Controls** | **26** | 29.26 ± 14.12 | 24.25 | **26** | 190.4 ± 83.98 | 196.2 | **26** | 0.59 ± 0.079 | 0.39 | **23** | 1.766 ± 0.693 | 1.712 |
| **Amyotrophic Lateral Sclerosis (ALS)** | **15** | 29.16 ± 13.06 | 29.27 | **16** | 208.5 ± 91.88 | 198.2 | **10** | 0.7 ± 1.63 | 0.14 | **10** | 1.665 ± 0.568 | 1.557 |
| **Epilepsy** | **24** | 25.83 ± 6.18 | 25.10 | **25** | 208.9 ± 73.33 | 202.8 | **26** | 0.92 ± 1.61 | 0.46 | **24** | 1.55 ± 0.76 | 1.377 |
| **Cerebral Small Vessel Disease (CSVD)** | **22** | 31.20 ± 15.51 | 28.79 | **22** | 200.9 ± 81.98 | 176.8 | **22** | 0.44 ± 0.53 | 0.29 | **22** | 2.05 ± 2.365 | 1.644 |
| Cerebral Amyloid Angiopathy (CAA) | 11 | 28.04 ± 8.234 | 29.90 | 11 | 178.8 ± 54.54 | 173.1 | 10 | 0.49 ± 0.73 | 0.19 | 10 | 2.559 ± 3.501 | 1.553 |
| Hypertensive Arteriopathy (HA) | 11 | 34.37 ± 20.37 | 26.00 | 11 | 223.0 ± 100.3 | 208.4 | 12 | 0.40 ± 0.31 | 0.33 | 12 | 1.626 ± 0.47 | 1.644 |
| **Total cases** | **87** | **28.79 ± 12.6** | **25.45** | **89** | **201.5 ± 81.08** | **193.7** | **84** | **0.67 ± 1.17** | **0.33** | **79** | **1.77 ± 1.37** | **1.636** |

*Bolded numbers indicates sum up to the total. CAA and HA are subcategories of CSVD.*

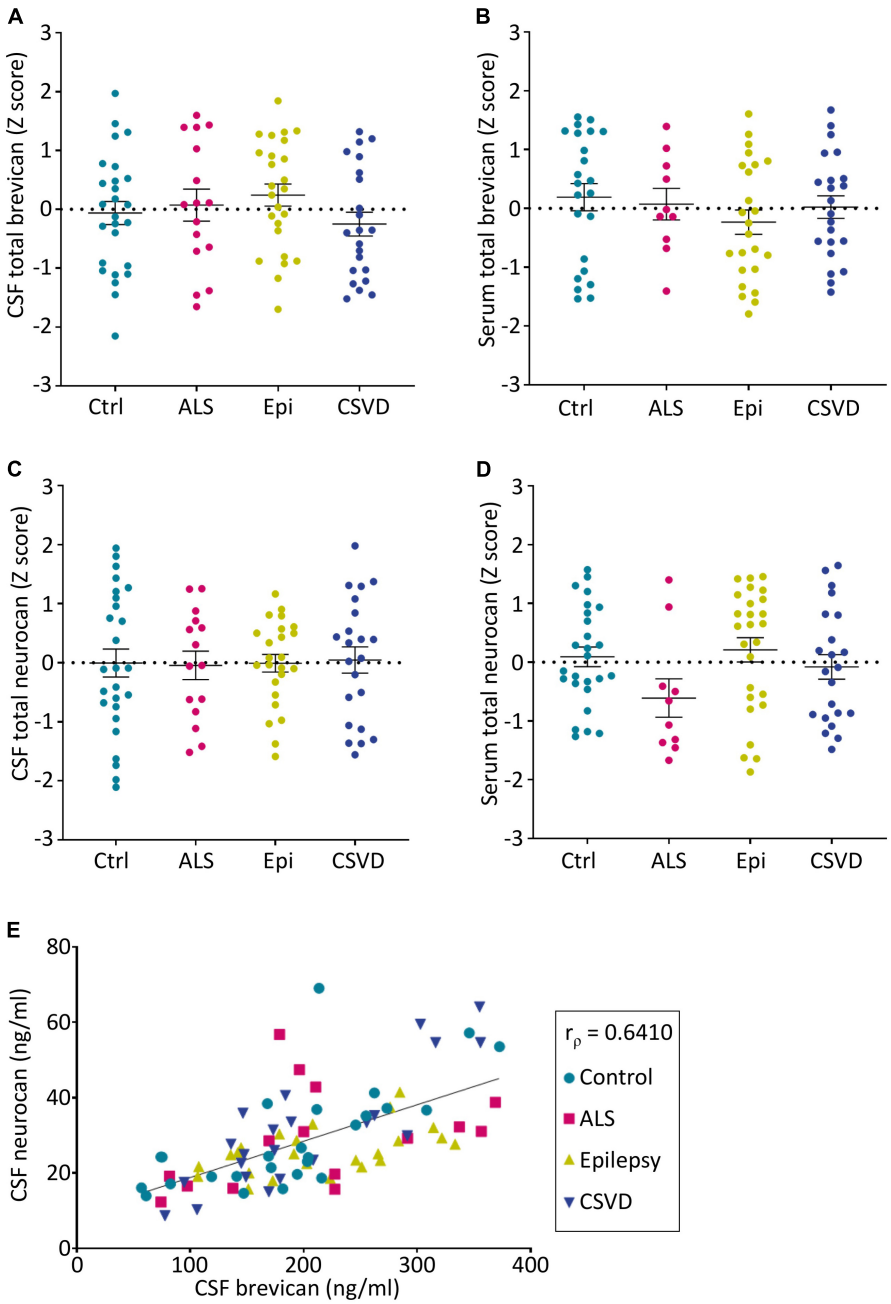


**FIGURE 2 |** Group-wise comparison of z-scores from age- and sex-corrected total levels of brevican **(A,B)** and neurocan **(C,D)** as measured *via* ELISAs in CSF **(A,C)** and serum **(B,D)** samples (CSF BCAN: Ctr. $n = 26$, ALS $n = 16$, Epi $n = 25$, CSVD $n = 22$; CSF NCAN: Ctr. $n = 26$, ALS $n = 15$, Epi $n = 24$, CSVD $n = 22$; Serum BCAN: Ctr. $n = 23$, ALS $n = 10$, Epi $n = 24$, CSVD $n = 22$; Serum NCAN: Ctr. $n = 26$, ALS $n = 10$, Epi $n = 26$, CSVD $n = 22$). No statistical significances were detected between groups. **(E)** The concentrations of total brevican and total neurocan in the CSF correlate with each other ($r_\rho = 0.6211$, $p < 0.0001$). CSF, cerebrospinal fluid; Ctrl, control; ALS, amyotrophic lateral sclerosis; Epi, epilepsy; CSVD, cerebral small vessel disease.

levels reached statistical significance, $F(3,78) = 4.35$, $p < 0.01$, $\eta_p^2 = 0.14$. As shown in **Figure 4E**, Tukey's HSD tests indicated significantly increased mean scores in the ALS versus all other groups (all ps $\leq 0.02$). The rank analysis of covariance on cleaved normalized to total neurocan levels revealed a large significant effect of group (control, ALS, epilepsy, and CSVD), $F(3,78) = 8.48$, $p < 0.001$, $\eta_p^2 = 0.25$, with *post hoc* Tukey's HSD tests again indicating significantly increased mean scores in the ALS compared to all other groups (all ps $< 0.001$; see **Figure 4F**). In the ALS group, we also found the descriptively strongest correlation between total neurocan and its cleaved C-terminal fragment (all groups: $r_\rho = 0.659$, $p < 0.001$; ALS:

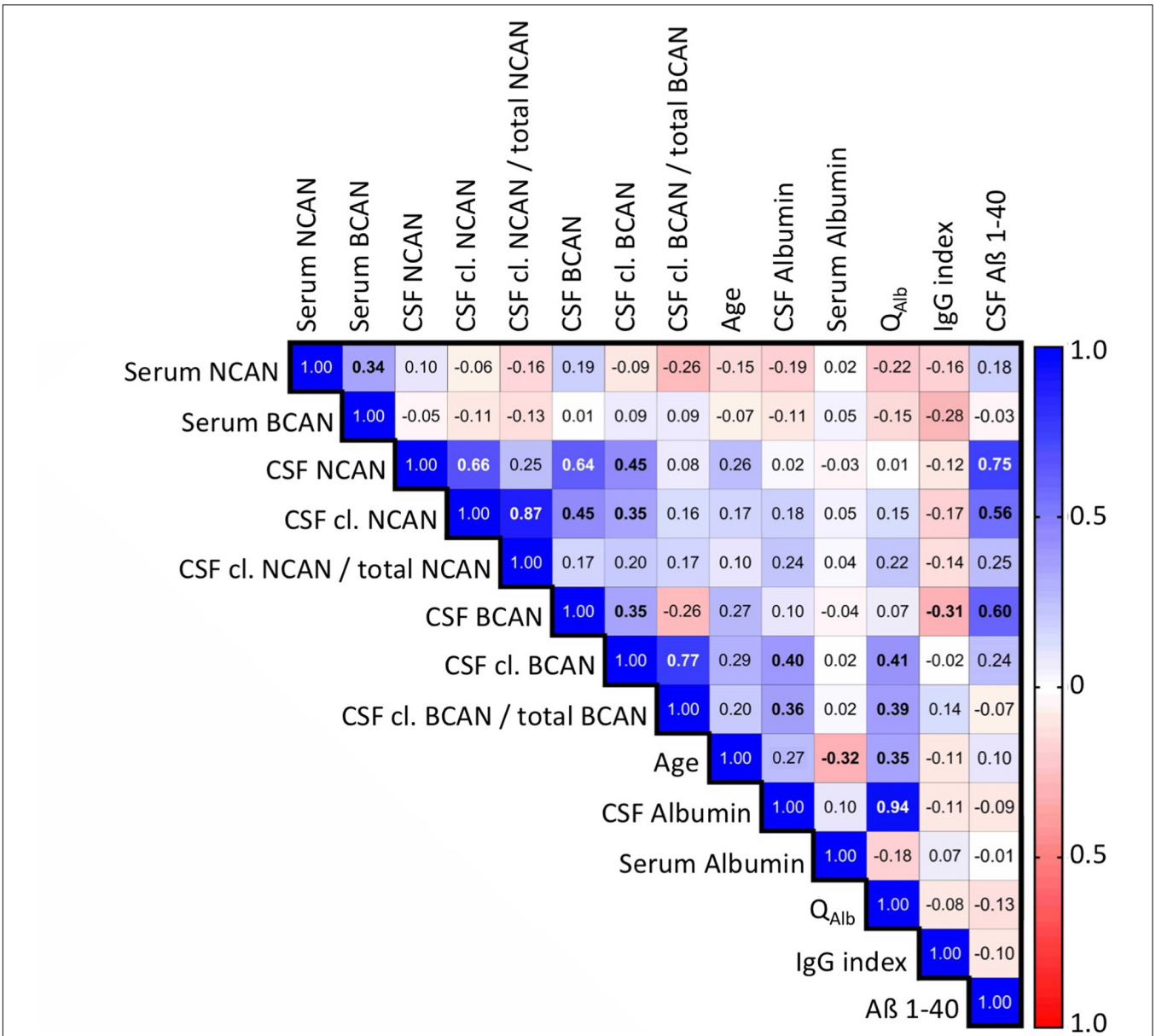


**FIGURE 3 |** Spearman's correlation matrix of the concentrations of ECM proteoglycans brevican and neurocan in the CSF and serum of all patients including controls with demographic and serological factors measured during diagnostic work up. Values show the Spearman rank results (significant correlations are indicated in bold). R values are color-coded with blue colors showing positive correlations, red showing negative correlations, and white showing insignificant correlations. BCAN, brevican; NCAN, neurocan; cl., cleaved; $Q_{Alb}$, albumin quotient; IgG index, Immunoglobulin G index.

$r_\rho = 0.90$, $p < 0.001$; **Figure 5B**). No significant differences were found within the CSVD subgroups (CAA versus HA) for cleaved neurocan and cleaved normalized to total neurocan levels (all $ts \leq 0.64$, $ps \geq 0.53$).

## Elucidation of Further Diagnostic Parameters as Confounders

To detect correlations of the estimated CNS-derived proteoglycan concentrations in the patients' body fluids with each other and with established biomarkers we performed a Spearman's correlation analysis covering relevant diagnostic parameters obtained in the clinical work up (**Figure 3**). The serum concentrations of brevican and neurocan did not show any correlations with other parameters.

For the total ($r_\rho = 0.272$, $p = 0.01$) and cleaved brevican levels ($r_\rho = 0.288$, $p = 0.007$) as well as for total neurocan ($r_\rho = 0.262$, $p = 0.014$) in the CSF we detected significant positive correlations with the age of the patients, which did not survive Bonferroni correction. Interestingly, CSF proteoglycan levels, except for cleaved brevican, also correlated strongly

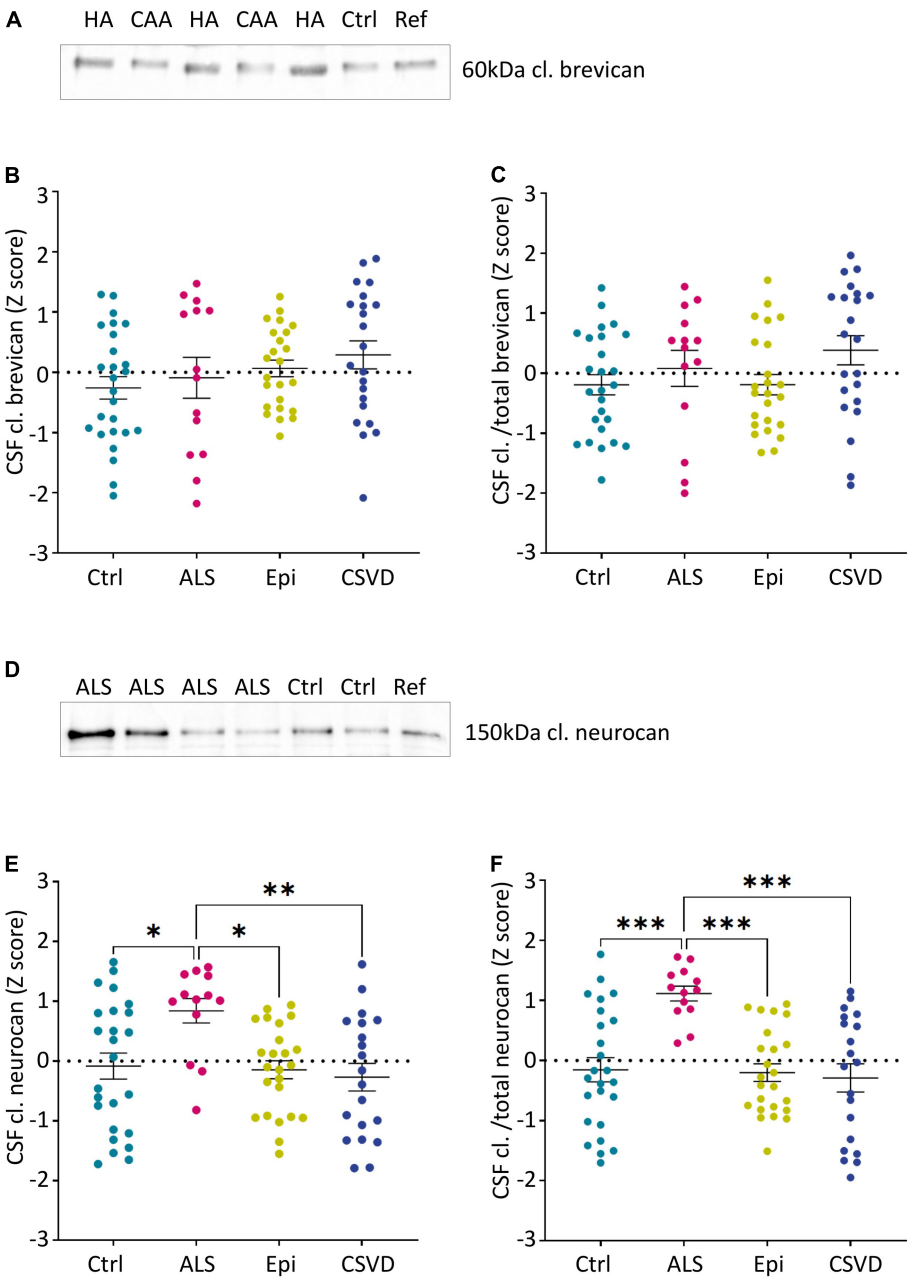


**FIGURE 4 |** Group-wise comparison of z-scores from age- and sex-corrected CSF levels of the 60 kDa brevican fragment **(A–C)** and the 150 kDa neurocan-C fragment **(D–F)** as measured *via* immunoblots (normalized to controls). Cutouts of example western blots of cleaved brevican **(A)** and neurocan **(D)** immunoreactivity in individual CSF samples and a reference sample loaded on all gels for calibration. **(C)** and **(F)** show the ratios of cleaved brevican **(B)** and cleaved neurocan **(E)** to the total immunoreactivity determined for these proteoglycans (CSF cl.BCAN and cl./total BCAN: Ctr. $n = 26$, ALS $n = 14$, Epi $n = 24$, CSVD $n = 22$; CSF cl. NCAN and CSF cl./total NCAN: Ctr. $n = 25$, ALS $n = 13$, Epi $n = 24$, CSVD $n = 20$). Statistical significance is indicated (*$p \leq 0.05$, **$p \leq 0.005$, ***$p \leq 0.0005$, ****$p < 0.0001$). Ctrl, control; ALS, amyotrophic lateral sclerosis; Epi, epilepsy; CSVD, cerebral small vessel disease; HA, hypertensive arteriopathy; CAA, cerebral amyloid angiopathy; BCAN, brevican; NCAN, neurocan; CSF, cerebrospinal fluid; cl., cleaved.

with the Alzheimer precursor protein fragment Abeta 1-40, a physiological brain-derived extracellular polypeptide (total neurocan $r_\rho = 0{,}747$, $p < 0.001$, total brevican $r_\rho = 0{,}6$, $p < 0.001$, cleaved neurocan $r_\rho = 0{,}56$, $p < 0.001$). For cleaved as well as for cleaved/total brevican in the CSF, we observed significant correlations with CSF albumin and with the albumin quotient ($r_\rho = 0.41$ and 0.39, respectively, $p < 0.01$).

Since ALS and CSVD are neurodegenerative disorders, we also explored the correlations with neurodegeneration markers NF-L and total Tau. As shown in **Figure 6**, there were positive

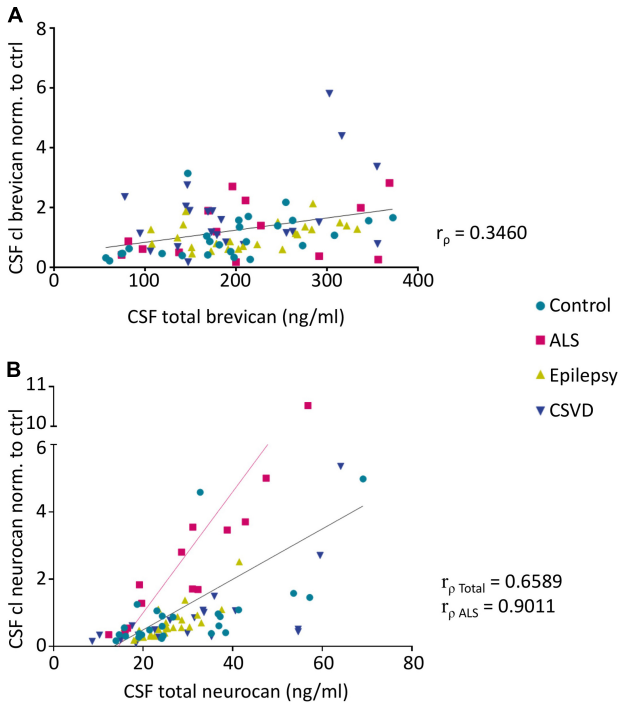


**FIGURE 5 |** Spearman's correlation analysis reveals significant correlations between CSF levels of cleaved brevican (normalized to controls) and total brevican ($r_\rho$ = 0.346, $p < 0.05$) **(A)** (Pairs: Ctr. $n$ = 26, ALS $n$ = 14, Epi $n$ = 24, CSVD $n$ = 22) and also between the CSF levels of cleaved neurocan (normalized to controls) and total neurocan ($r_\rho$ = 0.6589, $p < 0.001$) **(B)** (Pairs: Ctr. $n$ = 25, ALS $n$ = 13, Epi $n$ = 24, CSVD $n$ = 20) over all groups. Within the ALS group this correlation is strongest ($r_\rho$ = 0.9011, $p < 0.0001$). CSF, cerebrospinal fluid, ALS, amyotrophic lateral sclerosis, CSVD, cerebral small vessel disease.

correlations with t-Tau for all neurocan measures analyzed in CSF of the ALS samples (in A for total neurocan $r_\rho$ = 0.82, $p < 0.001$; in B for cleaved neurocan-C fragment $r_\rho$ = 0.90, $p < 0.001$; and in C for the ratio of cleaved to total neurocan $r_\rho$ = 0.73, $p < 0.01$). In the CSVD sample, only total neurocan was significantly correlated with t-Tau values (**Figure 6A**; $r_\rho$ = 0.52, $p < 0.05$), and contrary to our expectations we did not find any correlation with NF-L levels in the patients' CSF samples.

As epilepsy can be caused by neuroinflammatory processes we checked our epilepsy cohort for the occurrence of encephalitis. There were no cases with acute, but 5 cases with putative subacute encephalitis. A comparison between these two subgroups revealed significantly lower amounts of CSF neurocan and neurocan-C in the encephalitis cases (**Supplementary Figure 3**), however, we did not find a correlation of neurocan levels with the IgG index across groups (**Figure 3**).

## DISCUSSION

Here we show in a cohort of neurological patients that the CSF levels of the major proteolytic fragment of the perineuronal ECM proteoglycan neurocan, neurocan-C, are significantly higher in the ALS group as compared to controls, but also to epilepsy and CSVD patients. This effect became even more evident after normalizing concentrations of the cleaved to total core proteoglycan. The lack of significant findings from serum samples may be due to the more distal relation of serum to brain processes, but mostly due to the large dynamic range of plasma proteins and the huge intra- and inter-individual variability in serum protein composition. The low abundance of neurocan is another factor contributing to the lack of significant findings in serum samples.

### Proteoglycan Fragments as Readout for Proteolytic Activity

The neural ECM has a relatively low turnover rate (i.e., within days; see Fawcett et al. (2019)), with its biosynthesis and degradation being in equilibrium under healthy conditions keeping homeostasis (Dityatev et al., 2010). Nevertheless, the composition and integrity of the brain ECM can be dynamically adapted to the activity state of neural networks (i.e., within minutes to hours), mostly *via* the action of ECM-cleaving matrix metalloproteinases (Frischknecht et al., 2014). Thus, strictly controlled proteolytic events yield a more loosened ECM which allows structural changes in the synaptic wiring and functional connectivity alterations. Importantly, dysregulation of these ECM-shaping processes can be a hallmark for several neurological disorders (Gottschall and Howell, 2015), making glycoprotein or proteoglycan fragments in body fluids of patients valuable readouts for the general ECM proteolytic activity. This activity may vary largely with disease stages and severity and may therefore be an additional proxy for the disease progression. For neurocan it was shown that the major proteolytic event yielding the neurocan-C fragment is affected by cytokines like Transforming Growth Factor beta (TGFbeta), Platelet-Derived Growth Factor (PDGF), and Epidermal Growth Factor (EGF) (Asher et al., 2000).

Furthermore, next to the proteolytic activity by itself (both in the brain parenchyma and in the CSF) also the passing rate across the BBB resp. the blood CSF barrier may affect the levels measured in body fluids. This assumption is supported by the observed correlation of cleaved versus total brevican in the CSF with the albumin quotient (**Figure 3**), arguing for increased barrier leakage driving the occurrence of the proteoglycan fragment (which is in the same size range as albumin with 66 kDa) in the CSF.

Finally, the glymphatic flux rate inside the brain parenchyma is supposedly a major factor in the regulation of the CSF composition. This assumption is supported by our finding that CSF proteoglycan levels largely correlate with the levels of Abeta 1-40, another neural polypeptide found in the extracellular space of the CNS and cleared by the glymphatic system (Iliff et al., 2012), indicating common efflux mechanisms to the CSF. However, cleaved neurocan shows also an effect of the disease group (ALS), pointing to

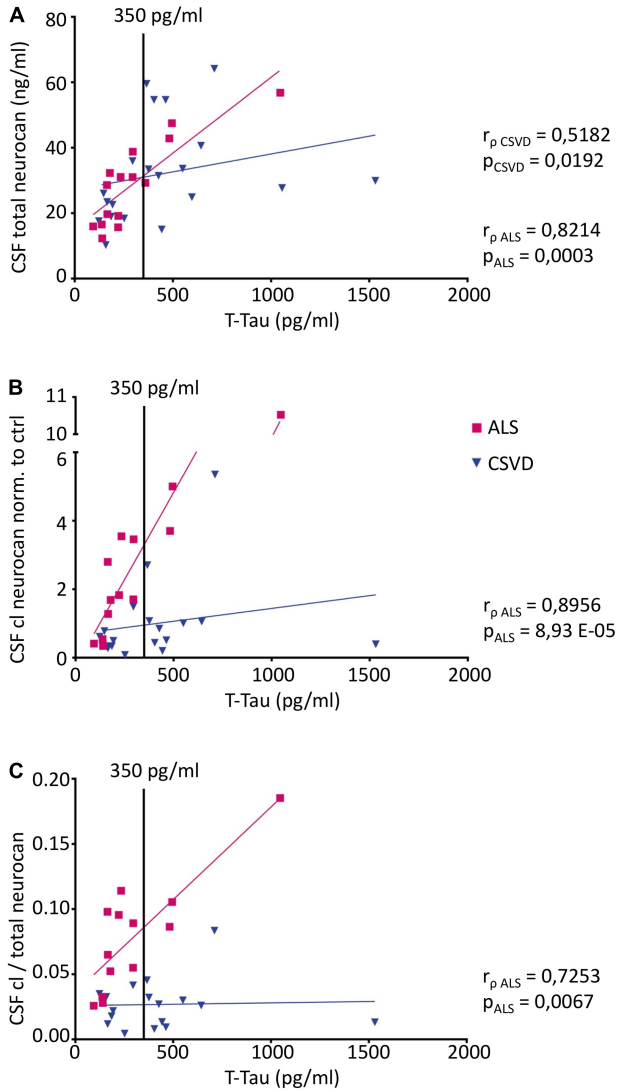


**FIGURE 6 |** Correlation analysis of CSF neurocan levels with the neurodegeneration marker total Tau (t-Tau) (Pairs CSF NCAN: ALS $n$ = 15, CSVD $n$ = 20; Pairs CSF cl. NCAN: ALS $n$ = 13, CSVD $n$ = 18; Pairs CSF cl./total NCAN: ALS $n$ = 13, CSVD $n$ = 18) **(A)** Total neurocan correlates with t-Tau levels both in the ALS and the CSVD group (ALS: $r_\rho$ = 0.8214, $p$ = 0.0003; CSVD: $r_\rho$ = 0.5182, $p$ = 0.0192). For cleaved neurocan **(B)** and for the ratio of cleaved to total neurocan **(C)** correlations with t-Tau were found in the ALS group only (CSF cl. NCAN: $r_\rho$ = 0.8956, $p < 0.0001$; CSF cl./total NCAN: $r_\rho$ = 0.7253, $p$ = 0.0067). The t-Tau threshold value of 350 pg/ml is indicated with a black line.

a disease-specific mechanism not explained by physiological clearance rates.

## Links to Neurological Disorders

The neural ECM is a dynamic molecular filter for all cellular secretomes in the brain. It undergoes constant homeostatic remodeling and appears disorganized in several pathologies. So far, to our knowledge no other study investigated brevican and neurocan fragments as potential biomarkers for epilepsy, CSVD or ALS. However, in a series of biochemical or proteomic studies a Swedish group investigated brevican and neurocan already in a spectrum of other neurological conditions. In 2018 they demonstrated both brevican and neurocan CSF levels being lower after prophylactic cerebral radiotherapy of lung cancer patients, but only in cases without brain metastases (Fernström et al., 2018). In TBI patients the same group showed that CSF levels of brevican and neurocan as well as of their binding partners tenascin-C and tenascin-R change over time after the injury – again arguing for a disease stage dependence of ECM modifications - and that lower values correlated with a favorable outcome for the TBI patients (Minta et al., 2019a). For neurocan they found a reduction in the serum of TBI cases. In a follow-up study, they demonstrated that mostly N-terminal brevican peptides (present on the 60 kDa fragment investigated here) were reduced in TBI as compared to idiopathic normal pressure hydrocephalus (iNPH) CSF samples as contrast group, and that ADAMTS-like proteolytic activity in the CSF of TBI patients was increased (Minta et al., 2021b). Interestingly, in their study the rise in ADAMTS-like activity correlated also with matrix metalloprotease (MMPs 1, 2, 3, 10) concentrations in CSF, suggesting a general increase in extracellular proteolytic activity. In iNPH patients the same group compared lumbar and ventricular CSF concentrations of proteoglycans and MMPs demonstrating that neither brevican nor neurocan differed between CSF compartments, however, MMPs −1, −2, −10 and the tissue inhibitor of metalloproteases TIMP-1 were found increased in lumbar as compared to ventricular CSF (Minta et al., 2021c).

For different types of dementias specific correlations with neural proteoglycans have been reported: In AD patients the CSF and serum levels of brevican or neurocan were concurringly found to be unchanged as compared to non-demented controls and there was no correlation with the $Abeta_{42/40}$ values but a significant correlation between brevican, neurocan, and the brevican-interacting ECM glycoprotein tenascin-R (rho = 0.68–0.77, $p < 0.05$) (Begcevic et al., 2018; Brinkmalm et al., 2018; Minta et al., 2019b, 2021a; Jonesco et al., 2020).

### Vascular Dementia and Cerebral Small Vessel Disease

With the help of a fragment-specific anti-brevican antibody, Jonesco and colleagues found in the serum of a highly heterogeneous group of patients with other dementias (including mostly vascular and fronto-temporal lobar dementia, but also Lewy body dementia, Parkinson's disease, normal pressure hydrocephalus, depressive pseudo-dementia, apoplexia, aphasia, paraneoplasia, epilepsy, hippocampal atrophy, and pick's disease cases) slightly reduced amounts of total brevican but increased levels of the C-terminal brevican fragment derived from ADAMTS-4 cleavage, yielding a clearly increased ratio of cleaved over total brevican (Jonesco et al., 2020). In our clinically more homogeneous CSVD cohort we did not find any differences to the other groups (**Figure 4B**) and also not between the two subgroups HA and CAA. In contrast to Jonesco et al. and to

our data, Minta and coworkers tested for the abundance of neurocan- and brevican-derived peptides in the CSF of two small cohorts of vascular dementia patients and found in the 1st cohort all peptides significantly reduced as compared to control subjects and AD patients, and in their 2nd cohort two N-terminal brevican peptides remained significantly diminished (Minta et al., 2021a), demonstrating the need for detailed analysis of demographic as well as disease-specific confounding factors in the cohorts.

### Amyotrophic Lateral Sclerosis

This motoneuron disease is considered as non-cell autonomous, i.e., beyond the motor neurons also the surrounding glial cells are affected (Galbiati et al., 2020). To come closer to the mechanisms behind elevated levels of neurocan-C in our ALS group we looked for correlations with clinical measures like ALS diagnosis according to El Escorial criteria, disease severity (as expressed by ALSFRS-R score and ECAS score, placement of a percutaneous endoscopic gastrostomy (PEG) or non-invasive ventilation (NIV)) and disease duration in our sample. However, we did not find any significant correlations with cleaved neurocan levels, probably because our sample size is actually too low to form instructive subgroups. Data from a larger cohort need to be collected which will allow a reliable stratification of ALS patients according to El Escorial criteria.

Interestingly, in ALS conditions two of the factors known to stimulate neurocan cleavage, namely EGF and TGFbeta (Asher et al., 2000) are affected: at later stages of ALS, the TGFbeta pathway is persistently upregulated, leading, e.g., to excessive activation of microglia (Galbiati et al., 2020), and inhibitors of the EGF receptor have proven to be beneficial in the SOD1 mouse model of ALS (Le Pichon et al., 2013). These findings make it tempting to speculate that dysregulated cytokine levels may cause the observed effect, a hypothesis which could be tested in larger patient groups.

A proteomic study in ALS patients showed no differences for brevican but a significant downregulation of total neurocan ($p$ = 0,0067; −0,39-fold change) in the CSF samples as compared to healthy control samples (Bereman et al., 2018). The difference to our findings may be due to sample size differences (33 ALS patients in Bereman et al. (2018) versus 15 cases in our sample), age differences (40-55 yrs. versus 64 yrs.) or to the different methodology (proteomics versus immunoblot).

### Epilepsy

A study in mesial temporal lobe epilepsy patients (Perosa et al., 2002) showed upregulated chondroitin sulfates and hyaluronic acid in the post mortem hippocampus, but no differences in the CSF. Conflicting data have been published for total brevican levels in epilepsy. While one study found *via* immunoblot of post mortem human epilepsy hippocampus samples a reduction of total brevican immunoreactivity (Favuzzi et al., 2017) a more recent study reported upregulated brevican levels in human post mortem frontal cortex samples from epilepsy patients as compared to controls (Pires et al., 2021).

Inflammatory mechanisms, which are quite common in epilepsy cases, may severely affect both ECM cleavage and clearance from brain tissue. In our sample we had no cases with acute inflammation, but a few cases with putative subacute encephalitis. Indeed, in these cases CSF neurocan levels were significantly lower than in patients without inflammation, but we did not find a correlation with the IgG index as proxy for intrathecal immunoglobulin synthesis. Only CSF brevican levels correlate with the IgG index, suggesting that there may be a disease-independent effect of neuroinflammation is of potential diagnostic and also mechanistic interest. However, it is early times to formulate a specific hypothesis about the particular role of neuroinflammatory mechanisms and the specific occurrence of proteoglycan fragments in the CSF, and further studies with larger case numbers with specified epilepsy syndromes are needed.

## Proteoglycan Fragments as Matrikines

Fragments produced by proteolytic cleavage of CNS proteoglycans may not only be an indicator for the deconstruction of the intact ECM, but they could provide a gain of function by acting as matrikines. This term describes bioactive proteolytic (or glycosidic) fragments exerting signaling functions which may be different from the role of the uncleaved ECM components (Ricard-Blum and Salza, 2014; Fontanil et al., 2021).

Although transport modalities for brain-derived matrikines into the CSF and into the serum are still fully unclear, to study them under pathophysiological conditions could be of interest both from a clinical but also from a cell biological perspective.

## Limitations of This Study and Outlook

Further experiments are necessary to confirm these findings. in an independent sample and to clarify the clinical value.

The statistical power from the relatively small numbers of individuals involved was felt to be too low to draw firm conclusions with regard to the suitability and disease specificity as bona fide biomarkers, however, our results are promising and deserve further validation in larger cohorts to unravel the full potential in the clinical work up, not least to come closer to the pathophysiological mechanisms behind our observations.

Taken together, our experiments suggest that – beyond association with clinical phenotypes – the CSF levels of the neural proteoglycans investigated here seem to be differentially regulated: while total brevican and neurocan as well as the C-terminal fragment of neurocan appear to be largely controlled by the general glymphatic flux, the 60kDa brevican fragment seems to be rather controlled by the BBB function.

From our work we conclude that proteolytic cleavage products of brain-derived perineuronal ECM molecules, such as neurocan fragments, may allow insights into the integrity of the brain's extracellular environment, being potent CSF indicators for the actual proteolytic ECM fragmentation activity in the CNS tissue, especially if normalized to the concentration of total core proteoglycans. In the clinic, their correlation with established markers for neurodegeneration or BBB leakage may pave the way to a more differentiated diagnosis of neurological disorders, based on body fluids. Our results suggest to include the ratio of cleaved to total neurocan into informative biomarker panels to improve the discriminatory power between disorders.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

## ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Otto von Guericke University Magdeburg. The patients/participants provided their written informed consent to participate in this study.

## AUTHOR CONTRIBUTIONS

CIS, EG and SS conceptualized the study. SS, CG, FS and SV assessed the clinical phenotypes and provided patient samples. CG provided the clinical data. WH and LH performed the experiments and designed the figures. CS and WH performed data analysis. CIS and EG designed the experiments and wrote the draft. All authors revised and finalized the manuscript and approved the submitted version.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2022.838432/full#supplementary-material

**Supplementary Figure 1 |** The neo antibody recognizes the human brevican fragment in the CSF. Western blot showing the result of the antigen competition experiment to prove specificity of the antibody directed against the C-terminal neoepitope of the N-terminal brevican fragment. In human CSF the antibody shows reactivity with a 60kDa protein band (lane A, arrow) which is clearly reduced after preincubating the antibody with a 100fold molar excess of the rat brevican peptide used for immunization (lane D). The band at 70kDa results from cross-reactivity with albumin (asterisk), which is not affected by the competition experiment. In soluble fractions of human temporal (tCtx; B) and frontal (fCtx; C) cortex the antibody does not show any blockable specificity in the 60kDa range (lanes E, F). Protein marker (M) sizes are indicated (in kDa).

**Supplementary Figure 2 |** Example immunoblots of cleaved brevican **(A)** and neurocan **(B)** immunoreactivity in individual CSF samples and a reference sample loaded on all gels for calibration. Protein marker (M) sizes are indicated (in kDa). Ctrl – control; HA – hypertensive arteriopathy; CAA – cerebral amyloid angiopathy; ALS – amyotrophic lateral sclerosis; Ref – reference sample. Quantified bands are indicated with arrows. Asterisk marks the nonspecifically labelled albumin band.

**Supplementary Figure 3 |** Neurocan levels in the CSF of epilepsy patients with or without subacute encephalitis. Group-wise comparison of z-scores from age- and sex-corrected total concentrations of neurocan as measured *via* ELISA **(A)**, and of the cleaved neurocan-C fragment as measured *via* immunoblot **(B)**. Statistical significance is indicated.

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REVIEW
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# The Role of Extracellular Matrix Components in the Spreading of Pathological Protein Aggregates

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Several neurodegenerative diseases are characterized by the accumulation of aggregated misfolded proteins. These pathological agents have been suggested to propagate in the brain *via* mechanisms similar to that observed for the prion protein, where a misfolded variant is transferred from an affected brain region to a healthy one, thereby inducing the misfolding and/or aggregation of correctly folded copies. This process has been characterized for several proteins, such as α-synuclein, tau, amyloid beta (Aβ) and less extensively for huntingtin and TDP-43. α-synuclein, tau, TDP-43 and huntingtin are intracellular proteins, and their aggregates are located in the cytosol or nucleus of neurons. They have been shown to spread between cells and this event occurs, at least partially, via secretion of these protein aggregates in the extracellular space followed by re-uptake. Conversely, Aβ aggregates are found mainly extracellularly, and their spreading occurs in the extracellular space between brain regions. Due to the inherent nature of their spreading modalities, these proteins are exposed to components of the extracellular matrix (ECM), including glycans, proteases and core matrix proteins. These ECM components can interact with or process pathological misfolded proteins, potentially changing their properties and thus regulating their spreading capabilities. Here, we present an overview of the documented roles of ECM components in the spreading of pathological protein aggregates in neurodegenerative diseases with the objective of identifying the current gaps in knowledge and stimulating further research in the field. This could potentially lead to the identification of druggable targets to slow down the spreading and/or progression of these pathologies.

**Keywords: tau, alpha synuclein, amyloid beta, TDP-43, huntingtin, extracellular matrix, HSPG, proteases**

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## INTRODUCTION

Most neurodegenerative diseases are characterized by the accumulation of misfolded protein aggregates. These include tau in tauopathies, α-synuclein in synucleinopathies, such as Parkinson's disease (PD), TDP-43 in frontotemporal dementia (FTD) and amyotrophic lateral sclerosis (ALS), huntingtin in Hungtinton's disease (HD) and amyloid beta (Aβ) in Alzheimer's disease (AD) (Ross and Poirier, 2004). These aggregates exert varying degrees of toxicity on neurons and glial cells, ultimately driving their degeneration. Although each of these diseases originates in and affects

different regions in the brain, all show some level of spreading of pathology over time. Each disease also exhibits characteristic rates and routes of distribution of pathological protein aggregates to other brain regions (reviewed in Brettschneider et al., 2015). For example, tau aggregation in AD initiates in the locus coeruleus and entorhinal cortex and subsequently spreads to the hippocampus, reaching the neocortex only at later stages of the disease. Aβ plaques instead are first observed in the neocortex, only afterward reaching deeper brain structures in later stages of AD. α-synuclein deposits, on the other hand, are first observed in the olfactory bulb and the dorsal motor nucleus of the vagus nerve, subsequently spreading to the midbrain and later, to the neocortex.

These pathological aggregates have the ability to induce downstream aggregation of natively folded proteins similarly to the prion protein (Vaquer-Alicea and Diamond, 2019). This finding, along with the observation that patterns of diffusion across brain regions are conserved between patients, suggests that some form of seed exist, which have the ability to travel across the brain and spread pathology. This spreading activity is distinguishable in two main classes, one involving intracellular proteins and the other, extracellular aggregates. Tau, α-synuclein, TDP-43 and huntingtin all have intracellular localization, and their aggregates also form intracellularly, whereas Aβ aggregates are found predominantly extracellularly. For intracellular proteins, aggregation is believed to start in a subset of cells from which seeds are then released, either by active secretion or passively due to cell death. Such proteopathic seeds would then be endocytosed by other cells and act as a template for the misfolding of endogenous proteins, thus causing further aggregation. Extracellular aggregation-prone Aβ, on the other hand, could move to different brain regions by simple diffusion in extracellular fluids and nucleate aggregation of locally generated Aβ .

These pathological proteins have been suggested to be present in the extracellular space in free forms, although at least some of them have also been found in extracellular vesicles (e.g., exosomes) or in tunneling nanotubes (Lee et al., 2011). It is thus clear that both classes of proteins will, at some point, be in the extracellular space and therefore enter into contact with components of the extracellular matrix (ECM). The ECM is a ubiquitous and complex protein network present in the space between cells of solid tissues, including the nervous system (Ruoslahti, 1996; Novak and Kaye, 2000; Krishnaswamy et al., 2019). Primarily consisting of laminins, collagens, glycoproteins and proteoglycans, the ECM undergoes regulated remodeling by virtue of extracellular proteases. All of these ECM components are secreted by neurons as well as glia and, in addition to providing physical support for these cells, play pivotal roles in regulating cell division, differentiation and migration, among other functions (Dityatev and Schachner, 2003; Dityatev et al., 2010). Since ECM components form an extracellular meshwork, they regulate the diffusion of molecules in the brain. Thus, rather unsurprisingly, components of the ECM can modulate the properties and spreading of these proteopathic seeds in neurodegenerative diseases. Interestingly, a growing body of evidence suggests that the levels of ECM components are severely affected in several neurodegenerative diseases, such as AD, PD, and ALS (Wong and Venkatachalam, 2019; NeuroLINCS Consortium et al., 2021; Downs et al., 2022; Johnson et al., 2022).

In this review, we summarize the known roles of ECM components in the spreading process, with the largest body of evidence existing for proteoglycans and extracellular proteases. Proteoglycans, which are either found in secreted form or bound to the plasma membrane, are glycosylated proteins which are post-translationally modified by the addition of glycosaminoglycans. The most abundant proteoglycans are heparan sulfate proteoglycans (HSPGs) and chondroitin sulfate proteoglycans (CSPGs), both of which are involved in different steps of the spreading process, including endocytosis of seeds, promoting aggregation and protecting aggregates from degradation (Holmes et al., 2013; Maïza et al., 2018). In addition to typical ECM proteases such as zinc-dependent matrix metalloproteinases (MMPs), additional proteases have been found in the extracellular space and altogether regulate ECM functions (Wang et al., 2008; Krishnaswamy et al., 2019). As detailed below, many of these enzymes cleave proteins that aggregate in neurodegenerative diseases, generating fragments with reported pro- and anti-aggregating effects. Although some of these proteases, such as calpains and cathepsins, mainly localize intracellularly and their activity on proteopathic proteins has been investigated in this context, their presence has also been reported in the extracellular space. Here, we discuss the possibility that cathepsins and calpains retain their activity on protein aggregates in the extracellular milieu. Emerging evidence suggests that this area of research requires further attention, especially as understanding the regulatory roles of ECM components on spreading may identify potential therapeutic targets that could reduce the progression of these devastating diseases.

## TAU

Tau is a highly expressed neuronal protein that, through its microtubule binding region (MTBR), binds to and stabilizes axonal microtubules. The MTBR tends to form β-sheets which drive protein aggregation (Wang and Mandelkow, 2016). In pathological conditions, collectively named tauopathies, tau loses its affinity for microtubules, becomes hyper-phosphorylated, and aggregates into oligomers, fibrils and neurofibrillary tangles (NFT) (Braak et al., 1994). Pathological tau aggregation and propagation follows a characteristic spatiotemporal sequence between functionally connected brain regions (Braak and Braak, 1991). This has led to the hypothesis that pathological tau is secreted by a "donor" neuron into the extracellular space before being internalized by "acceptor" neurons (Holmes and Diamond, 2014). This hypothesis implies that tau is exposed to the extracellular environment where different

proteases and ECM components could affect its ability to propagate pathology.

## Proteoglycans

Heparan sulfate proteoglycans play an important role in the spreading of tau pathology (**Figures 1Ai**,**Bi**). HSPGs are proteoglycans characterized by one or more heparan sulfate (HS) groups linked to a protein core. HS is composed of disaccharide chains consisting mainly of glucuronic acid and *N*-acetyl-D-glucosamine (Sarrazin et al., 2011). HSPGs exist in different classes: transmembrane HSPGs, including glypicans and syndecans; serglycins, found in extracellular vesicles; and secreted HSPGs, such as perlecans and agrins (Sarrazin et al., 2011; Condomitti and de Wit, 2018). HSPGs bind to tau and have been shown to act at all stages of its spreading: secretion into the extracellular space (Zehe et al., 2006; Katsinelos et al., 2018), cellular uptake (Holmes et al., 2013; De La-Rocque et al., 2021) and self-assembly into higher-order states (Zhao et al., 2021). Tau binds to heparin, a densely sulfated form of HSPG, through both the N-terminus and the MTBR (Goedert et al., 1996). The sulfate moieties on HSPGs appear to be crucial for tau binding, requiring both 3-*O*- and 6-*O*-sulfation (Zhao et al., 2017, 2020; Stopschinski et al., 2018). Heparin induces a conformational change in the MTBR and its flanking region that exposes previously masked tau phosphorylation sites and can induce oligomerization (Paudel and Li, 1999; Sibille et al., 2006; Elbaum-Garfinkle and Rhoades, 2012). However, heparin and related molecules inhibit tau uptake (Zhao et al., 2017; Weisová et al., 2019; Puangmalai et al., 2020), thus preventing tau fibrils from driving intracellular aggregation (Holmes et al., 2013), as shown by the heparin mimetic F6 (Holmes et al., 2013). These results suggest that an excess of extracellular HSPGs could have similar beneficial effects. However, a synthetic heparinoid with nanomolar affinity for tau failed to show any effect on tau pathology after chronic administration *in vivo* (Stopschinski et al., 2020). It is important to note that most studies have concluded that cell surface-bound HSPGs are involved in tau transfer without assessing the possible contribution of extracellular HSPGs.

Agrin, a major extracellular HSPG, accumulates in AD brains and more specifically, in NFTs (Verbeek et al., 1999; Kroger and Schroder, 2002; Smith and Hilgenberg, 2002; Del Campo Milan et al., 2015). Since agrin contains protease-inhibiting domains, it has been suggested to protect protein aggregates against extracellular proteolysis, leading to the accumulation of these deposits (Verbeek et al., 1999). In addition, agrin may participate in tangle formation because sulfated glycosaminoglycans have been shown to stimulate tau phosphorylation, thus promoting the formation of paired helical filaments (PHFs) (Goedert et al., 1996; Wang et al., 1996; Hasegawa et al., 1997). However, whether or not agrin displays these activities and how it interacts with tau tangles remains to be elucidated.

The CSPG aggrecan forms perineuronal nets predominantly ensheathing fast spiking interneurons (Morawski et al., 2012; Suttkus et al., 2014; van't Spijker and Kwok, 2017). These nets were proposed to be neuroprotective against pathological tau, since areas with high densities of perineuronal nets in post-mortem AD brains were largely spared of tangles, even at late disease stages (Brückner et al., 1999, 2008; Diamandis et al., 2000; Morawski et al., 2010a,b). Follow-up studies confirmed that the protective action of perineuronal nets was, in fact, mainly mediated by aggrecan. Most interestingly, aggrecan was shown to generate an external barrier restricting internalization and distribution of exogenous tau in organotypic slices (Nowicka et al., 2009; Suttkus et al., 2014, 2016). Indeed, aggrecan knock-out mice presented elevated tau uptake (Suttkus et al., 2016). Based on these results, it was hypothesized that these CSPGs may act by inhibiting the interaction of tau with HSPGs. However, in a recent study, mice expressing human tau carrying the FTD-linked mutation P301L crossed with heterozygous aggrecan KO mice, displayed changes in the expression and phosphorylation of tau but unaltered distribution of tau aggregates (Schmidt et al., 2021).

## Proteases

Several proteases have been shown to cleave tau (**Table 1**). Multiple studies have shown elevated levels of several MMPs in tauopathies as well as demonstrated functional links between different MMPs and tau. For example, upregulated MMP-3 levels were found in the cortex of AD-like amyloidosis transgenic rat models (Pentz et al., 2021), and the concentration of MMP-3 and levels of total and phosphorylated tau positively correlate in the cerebrospinal fluid (CSF) of AD patients (Stomrud et al., 2010; Hanzel et al., 2014). Although MMP-9 is enriched in AD patients' brains and co-localizes with tau, it is sparsely present in extracellular NFTs (Hernandes-Alejandro et al., 2020). Nübling et al. (2012) tested the effect of MMP-3 and MMP-9 on tau oligomer formation and aggregation. While MMP-3 mildly reduced tau aggregation, MMP-9 processing promoted oligomerization (Nübling et al., 2012; Wang et al., 2014). Multiple potential MMP-3 cleavage sites were identified within the MTBR of tau (Nübling et al., 2012), suggesting this protease could inhibit tau aggregation by degrading regions crucial for oligomer formation. In contrast, MMP-9 cleavage sites were mainly located either in the N-terminal region or close to the C-terminus (Nübling et al., 2012), thus sparing the MTBR and facilitating the generation of tau oligomers. More recently, docking simulations have predicted that a high-affinity complex can be formed between MMP-9 and full-length tau (Hernandes-Alejandro et al., 2020). This binding involves the catalytic domain of MMP-9, suggesting that this interaction could be initiated when MMP-9 is active. Interestingly, MMP-9 can be directly activated by MMP-3 (Ogata et al., 1992; Okada et al., 1992; Shapiro et al., 1995), implying that elevated MMP-3 levels might result in increased MMP-9 activity, indirectly facilitating tau aggregation. MMP-2 also has the capacity to cleave recombinant tau *in vitro* but fails to process its hyper-phosphorylated forms in NFTs (Terni and Ferrer, 2015). It is thus possible that the accumulation of MMP-2 around NFTs found in the entorhinal cortex at the early stages of AD (Terni and Ferrer, 2015) is a potential response aimed at eliminating the production of toxic fragments in AD brains. The cleavage sites of MMP-2 on tau, however, are yet to be determined. In conclusion, MMP-2, MMP-3 and MMP-9 show differential actions on tau

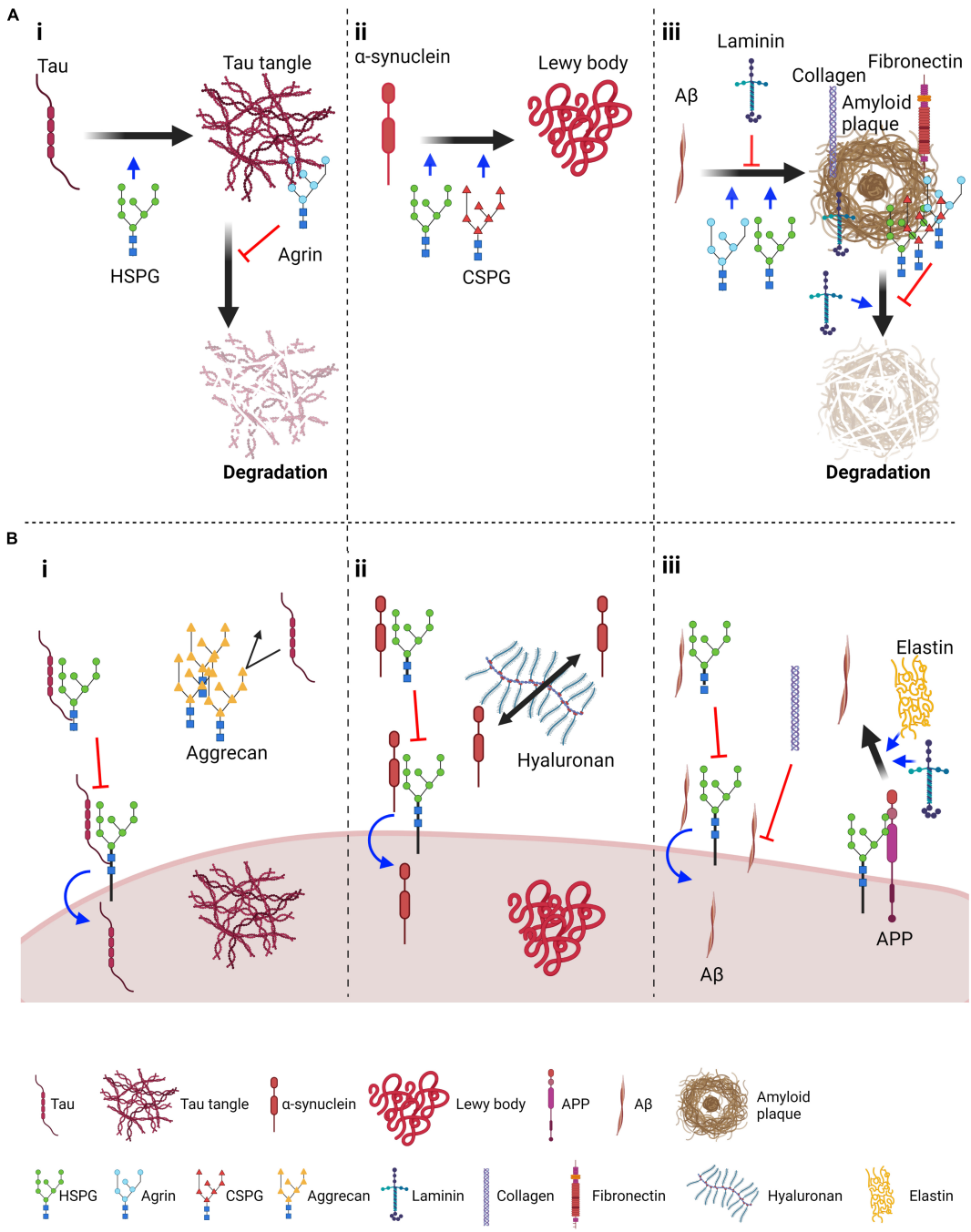


**FIGURE 1 |** Roles of proteoglycans and ECM components in the aggregation and spreading of tau, α-synuclein and amyloid β. **(A)** Suggested roles of proteoglycans and core ECM components in aggregation and degradation of pathological protein aggregates: (i) Tau panel: HSPGs promote tau aggregation; Agrin, an extracellular HSPG, localizes on neurofibrillary tangles and prevents their degradation; (ii) α-synuclein panel: HSPGs and CSPGs promote aggregation of α-synuclein; (iii) Aβ panel: HSPGs and agrin can promote Aβ aggregation whereas laminin has been suggested to impair this process. Several proteoglycans, including HSPGs, agrin and CSPGs, and ECM components such as laminin, collagen and fibronectin associate with amyloid plaques. Of these, laminin enhances plaque degradation whereas proteoglycans inhibit this process. **(B)** Suggested role of proteoglycans and ECM components in endocytosis and spreading: (i) Tau panel: HSPGs on the plasma membrane favor tau seed endocytosis. In contrast, if tau is bound to extracellular HSPGs, its interaction with surface HSPGs might be inhibited, thus reducing tau endocytosis. Aggrecan perineuronal nets have been shown to reduce propagation of tau pathology, possibly by acting as a barrier; (ii) α-synuclein endocytosis is also promoted by HSPGs and similarly extracellular HSPGs can inhibit endocytosis. In the extracellular space, hyaluronan was found to promote spreading of α-synuclein pathology; (iii) Aβ panel: as for tau and α-synuclein, transmembrane HSPGs can promote internalization of Aβ, and this might be inhibited by extracellular interactions. Collagen has been found to reduce the interaction of Aβ peptides with the cell surface. HSPGs have also been found to promote Aβ production from APP, a process that is also promoted by ECM components such as elastin and laminin. Figure was prepared with Biorender (Biorender.com).

**TABLE 1 |** This table summarizes the known extracellular proteases of prion-like proteins, their region and site of cleavage, the resulting fragment and the effect of their activity on spreading and aggregation of the pathological proteins.

| Tau | | | | | |
|---|---|---|---|---|---|
| **Protease** | **Cleavage region** | **Cleavage site** | **Fragments produced** | **Effect on spreading/aggregation** | **References** |
| MMP-2 | Potentially C-terminus* | Unknown | Potentially N-terminus* | Unknown | Terni and Ferrer, 2015 |
| MMP-3 | MTBR | Multiple potential cleavage sites | Unknown | Reduces aggregation | Nübling et al., 2012 |
| MMP-9 | N- or C-terminus | Multiple potential cleavage sites | Unknown | Enhances aggregation | Nübling et al., 2012 |
| Cathepsin D | Multiple | Phe8, Met419, Leu436, Thr427, Leu428 Additional sites detected between Asp34-Gly161, Pro200-Lys257, and Lys267-Asp358 | Contains MTBR | Potential to enhance aggregation* *In vivo,* reduces neurodegeneration | Kenessey et al., 1997; Khurana et al., 2010 |
| Cathepsin L | MTBR | Lys257 | 258–372 | Unknown | Wang et al., 2009 |
| | MTBR | Ile360 | 258–360 | Enhances aggregation | Wang et al., 2009 |
| | MTBR | Val363 | 258–363 | Enhances aggregation | Wang et al., 2009 |
| Cathepsin S | Unknown | Unknown | Approximately 34 and 24 kDa | Enhances aggregation | Nübling et al., 2017 |
| Calpain-1 | C-terminus | Arg242 | 243–441 (24 kDa) | *In vitro,* accelerates aggregation Enhance seeding activity | Matsumoto et al., 2015 |
| | N-terminus to mid-domain | Lys44 | 45–230 (17 kDa) | *In vivo* and *in vitro,* accelerates degeneration and synaptic defects *In vitro,* reduces aggregation | Yang and Ksiezak-Reding, 1995; Amadoro et al., 2006; Park et al., 2007; Ferreira and Bigio, 2011; Reinecke et al., 2011; Lang et al., 2014; Chen et al., 2021 |
| | N-terminus to mid-domain | Arg230 | 45–230 (17 kDa) | *In vitro,* neurotoxic | Park and Ferreira, 2005 |
| | N-terminus to mid-domain | Arg230 | 26-230 | *In vitro,* neurotoxic | Park and Ferreira, 2005 |
| | N-terminus | Gln124 | 125–230 | No alterations to cell health | Garg et al., 2011 |
| Calpain-2 | N-terminus to mid-domain | Arg230 | *45*–230 (17 kDa) | No alterations to cell health | Garg et al., 2011 |
| | N-terminus | Lys224 | Unknown | Unknown | Cicognola et al., 2019 |
| | N-terminus | Gln124 | 125–230 | No alterations to cell health | Garg et al., 2011 |
| Thrombin | Proline-rich and MTBR | Arg155, Arg209, Arg230, Lys257, and Lys340 | Unknown | Unknown | Arai et al., 2005 |
| | N-terminus | Gln124 | 125–230 | No alterations to cell health | Garg et al., 2011 |

*(Continued)*

**TABLE 1 |** (Continued)

| α -synuclein | | | | | |
|---|---|---|---|---|---|
| **Protease** | **Cleavage region** | **Cleavage site** | **Fragments produced** | **Effect on spreading/aggregation** | **References** |
| MMP-1 | Multiple | Ala19, Lys21, Gly41, Gly47, Thr72, Gln79, Ala91, Asp98, Tyr133 | Unknown | *In vitro,* enhances aggregation | Levin et al., 2009 |
| MMP-2 | Unknown | Unknown | Unknown | *In vivo,* reduces aggregation and spreading | Oh et al., 2017 |
| MMP-3 | N-terminus | Between Thr54-Glu57 | Unknown | Potential to enhance aggregation* | Sung et al., 2005 |
| | NAC domain | Ala78 | Unknown | Enhances aggregation and increases toxicity | Choi et al., 2011 |
| | NAC domain | Gly93 | 1-93 | *In vivo*, no change in aggregation, toxicity and spreading | Choi et al., 2011 |
| MMP-9 | Unknown | Met5, Leu8, Ala19, Val66, Val70, Val74, Ala78, Gln79 | Multiple | *In vitro,* reduces aggregation | Levin et al., 2009 |
| Plasmin | N-terminus and NAC | Ala11, Thr33, Thr44, Thr59, Thr81, Asp98 | Unknown | *In vitro*, reduces aggregation and spreading | Kim et al., 2012 |
| Neurosin | NAC domain | Lys80, Lys97, Glu114, Asp121 | Unknown | *In vitro and in vivo,* reduces aggregation | Tatebe et al., 2010; Pampalakis et al., 2017 |
| Cathepsin B | Unknown | Gly14 and Ala90 | Several fragments, most prominent at 10 kDa | No effect on seeding capabilities | Tsujimura et al., 2015; McGlinchey et al., 2020 |
| Cathepsin D | C-terminus | Primarily Ala124 and Gly132 Additional site Met116 | 10–13 kDa | Potentially aggregating fragments* | Hossain et al., 2001; Sevlever et al., 2008; McGlinchey et al., 2020 |
| Cathepsins E | Unknown | Unknown | Fragments between 5 and 13 kDa | Unknown | McGlinchey et al., 2020 |
| Cathepsins G | Unknown | Unknown | Most prominent at 10 kDa | Unknown | McGlinchey et al., 2020 |
| Cathepsin K | Spans the whole protein | Monomer: Ser9, Ala27, Ala53, Gly68, Thr75, Glu114 | Multiple | Unknown | McGlinchey et al., 2020 |
| | N and C-terminus | Fibrils: Ser9, Gln109, Glu114, and Ser129 | 10–140, 10–129, 10–114, and 10–109 | Potential to enhance aggregation* | McGlinchey et al., 2020 |
| Cathepsin L | Spans the whole protein | Monomer: Met5, Ser9, Ala17, Ala27, Val40, Gly41, His50, Ala53, Gly67, Thr75, and Asn103 | 1–17, 18–140, 1–103, 104–140, 1–41, 42–140, 1–50, 51–140, 1–53, 54–140, 1–75, 76–140, 6–140, 10–140, 28–140, 1–40, and 68–140 | Unknown | McGlinchey et al., 2017 |
| | N and C-terminus | Fibrils: Met5, Gly101, Asn103, Glu114, Asn122, Glu126 and Gln134 | 1–134, 1–126, 1–122, 1–114, 1–103, 1–101, 6-114 | Potential to enhance aggregation* | McGlinchey et al., 2017 |
| Cathepsins S | Unknown | Unknown | 10kDa | Unknown | McGlinchey et al., 2020 |
| Cathepsins V | Unknown | Unknown | 10 kDa | Unknown | McGlinchey et al., 2020 |

*(Continued)*

**TABLE 1 |** (Continued)

| α -synuclein | | | | | |
|---|---|---|---|---|---|
| **Protease** | **Cleavage region** | **Cleavage site** | **Fragments produced** | **Effect on spreading/aggregation** | **References** |
| Cathepsin B | Unknown | Gly14 and Ala90 | Several fragments, most prominent at 10 kDa | No effect on seeding capabilities | Tsujimura et al., 2015; McGlinchey et al., 2020 |
| Calpain-1 | N-terminus or central region | Ala18, Gly31, Tyr39, Glu57, Gly73, Thr75 and Glu83 | Unknown | Controversial *in vitro* results on aggregation potential. *In vivo,* enhances aggregation | Greenbaum et al., 2005; Mishizen-Eberz et al., 2005; Kim H. J. et al., 2006; Dufty et al., 2007; Diepenbroek et al., 2014 |
| | C-terminus | Glu114 and Asn122 | Unknown | Potential to enhance aggregation* | Mishizen-Eberz et al., 2005; Diepenbroek et al., 2014 |
| **Huntingtin** | | | | | |
| **Protease** | **Cleavage region** | **Cleavage site** | **Fragments produced** | **Effect on spreading/aggregation** | **References** |
| MMP-10 | C-terminus | Gly402 | 45 and 55 kDa | Potential to enhance aggregation* | Miller et al., 2010 |
| MMP-14 and -23B | Unknown | Unknown | 55 kDa | Potential to enhance aggregation* | Miller et al., 2010 |
| Calpain | C-terminus | Val347 or Ile494 | 1-347 and 1-494 fragment | Potential to enhance aggregation* | Gafni et al., 2004; Landles et al., 2010 |
| | C-terminus | Ala468-Val470 and Ser535-Val537 | 45 and 72 kDa | In cells, enhances aggregation | Kim et al., 2001; Gafni and Ellerby, 2002; Lunkes et al., 2002; Kim et al., 2003; Gafni et al., 2004 |
| | C-terminus | Unknown | ∼100 kDa | *In vitro and in vivo,* enhances aggregation | Menzies et al., 2015; Weber et al., 2018 |
| Cathepsin B | C-terminus | Unknown | 55–60 kDa | Potential to enhance aggregation* | Kim J. et al., 2006 |
| Cathepsin L | C-terminus | Unknown | 55–60 kDa | Potential to enhance aggregation* | Kim J. et al., 2006 |
| Cathepsin D | C-terminus | Unknown | 45–60 kDa | Potential to enhance aggregation* | Kim J. et al., 2006 |
| **TDP-43** | | | | | |
| **Protease** | **Cleavage region** | **Cleavage site** | **Fragments produced** | **Effect on spreading/aggregation** | **References** |
| Calpain | Unknown | Leu229, Glu246, Gln286, Gly295, Ala297, Met323 | Unknown | Neurotoxic | Yang et al., 2014 |
| **Amyloid beta** | | | | | |
| **Protease** | **Cleavage region** | **Cleavage site** | **Fragments produced** | **Effect on spreading/aggregation** | **References** |
| Neprilysin | Unknown | Unknown | Unknown | *In vivo,* reduces Aβ peptide levels and plaque burden | Yasojima et al., 2001; Marr et al., 2004; Saido and Leissring, 2012 |
| Neprilysin 2 | Unknown | Unknown | Unknown | *In vivo*, decreases Aβ peptide levels | Saido and Leissring, 2012 |
| Insulin-degrading enzyme | Unknown | Unknown | Unknown | Decreases Aβ peptide levels | Farris et al., 2003 |
| MMP-2, MMP-9 and MMP-14, Cathepsin B and Plasmin | Unknown | Unknown | Unknown | Decreases Aβ fibrils | Saido and Leissring, 2012; Hernandez-Guillamon et al., 2015 |

**Not yet experimentally addressed. Aβ, amyloid-beta; MMP, matrix metalloproteinases; MTBR, microtubule binding region; NAC, non-amyloid component; PHF, paired helical filament.*

aggregating behavior, suggesting their differential contribution to tau pathology.

Thrombin was also found to cleave tau at multiple arginine and lysine residues in the MTBR and proline-rich domains (Arai et al., 2005; Quinn et al., 2018; Zhang et al., 2021). In AD brains, thrombin was found to be upregulated and co-localized with amyloid plaques, microglia and NFTs (Arai et al., 2006). However, PHFs of tau extracted from AD brains were more resistant to thrombin cleavage compared to dephosphorylated PHFs (Arai et al., 2005). The seeding potential of tau fragments produced by thrombin is largely unknown, but tau 125–230 generated by thrombin-mediated cleavage at Gln124-Ala125 was shown to be non-toxic (Garg et al., 2011). This site is also cleaved by calpain-1 and calpain-2 (Garg et al., 2011). However, it is important to note that thrombin-cleaved tau fragments are yet to be identified in tauopathy brains, thereby questioning the pathophysiological relevance of this process.

Several cathepsins have been shown to interact with and modulate tau spreading. As previously mentioned, these proteases are mainly localized to lysosomes, but they can also be secreted in the extracellular space (Boonen et al., 2016; Vidak et al., 2019; Niemeyer et al., 2020). Whilst the majority of data on the action of cathepsins on tau mostly reflects their activity in lysosomes, independent experiments have been conducted either *in vitro* using recombinant proteins or using cathepsin knockout animals. Therefore, a role for extracellular cathepsins cannot be ruled out. Cathepsins B, D, L, and S have all been proposed to cleave tau. Although cathepsin B accumulates in close proximity to NFTs and amyloid plaques (Ii et al., 1993), there is no direct evidence that this protease can cleave tau. In contrast, cathepsin D cleaves recombinant tau at several sites, mostly sparing the MTBR (Kenessey et al., 1997), and as such these fragments could retain the ability to generate higher-order PHFs. At least one of these fragments was produced at neutral pH *in vitro*, suggesting that this cleavage could also occur in the extracellular space (Kenessey et al., 1997). Furthermore, cathepsin D upregulation was detected in an aging *Drosophila* model of tauopathy. The same group observed enhanced tau-induced neurodegeneration in cathepsin D-deficient flies which suggests that this protease may have a neuroprotective effect (Khurana et al., 2010). Cathepsin L has also been found to cleave tau at neutral pH. This activity targets sites within the MTBR, generating several fragments, such as tau 258–360 and tau 258–363, which enhance the aggregation of the full-length protein (García-Sierra et al., 2008; Wang et al., 2009; Zhang et al., 2021). Cathepsin S has been seen to associate with NFTs and its levels are elevated in the brain of AD patients (Lemere et al., 1995; Munger et al., 1995; Nübling et al., 2017). Treatment with cathepsin S *in vitro* yields a distinct cleavage pattern of tau, in which the MTBR seems to remain intact, allowing its association to NFTs. However, this study did not specify whether cleavage occurred at an acidic or neutral pH (Nübling et al., 2017). It is important to note that cathepsin-cleaved tau fragments have yet to be identified in AD brains, highlighting the need for further investigations to unravel the pathophysiological relevance of this protease in AD.

Calpains are proteases mainly found in the cell cytosol (Goll et al., 2003), albeit secretion of calpain has also been observed (Frangié et al., 2006; Letavernier et al., 2012; McDougall et al., 2021), including from isolated rat brain synaptosomes (Pestereva et al., 2021). Calpain has been detected in the CSF, though it remains unclear whether its presence is due to leakage from dying cells (Laske et al., 2015). This dual localization makes investigations on the role of calpain in the context of tau spreading and seeding challenging and as such, the action of extracellular calpains has not been directly investigated as yet. Both calpain-1 and -2 cleave tau (Chesser et al., 2013), and play opposing roles in inducing neurodegeneration (Baudry and Bi, 2016). Calpain-1 activity is significantly upregulated in AD cortical brain tissue from Braak stage III (Kurbatskaya et al., 2016). Much of the interest surrounding the role of calpain in tau pathology has centered on the 17 kDa N-terminal to mid-domain tau fragment (45–230), which is generated through cleavage by calpain-1 at Lys44-Glu45 (Yang and Ksiezak-Reding, 1995) and by calpain-1 (Park and Ferreira, 2005) or -2 (Garg et al., 2011) at Arg230-Thr231. These events have been observed *in vitro*, suggesting calpain-1 and -2 could also exert their activity in the extracellular space, although direct evidence is still lacking. This fragment has been observed in patients affected by AD and other tauopathies, such as progressive supranuclear palsy (PSP) and corticobasal degeneration (CBD) (Ferreira and Bigio, 2011; Garg et al., 2011). However, the specific effect of this tau fragment remains controversial as it was found to induce apoptosis (Park and Ferreira, 2005; Amadoro et al., 2006; Park et al., 2007; Reinecke et al., 2011; Lang et al., 2014) or morphological changes in cell lines and primary neurons (Chen et al., 2021), whereas other groups have observed no such alterations (Garg et al., 2011). Nonetheless, both *Drosophila* and mouse models overexpressing tau 45–230 showed accelerated hippocampal degeneration and synaptic defects (Reinecke et al., 2011; Lang et al., 2014). However, when this 17 kDa fragment was incubated with full-length tau, it significantly lowered aggregate formation, suggesting a possible protective effect (Ferreira and Bigio, 2011). Fascinatingly, aggregation and phosphorylation of full-length tau protected it from calpain cleavage (Ferreira and Bigio, 2011), which is in line with previous data indicating aggregated tau is less susceptible to protease cleavage (Yang and Ksiezak-Reding, 1995; Yang et al., 1997). In a recent study investigating stroke biomarkers, this 17 kDa tau fragment accumulated in primary neurons and their media upon hypoxic treatment, and was decreased by calpain inhibitors (Chen et al., 2021). These findings suggest that calpain-mediated cleavage is specific and can be initiated under different pathological conditions. Similarly, the AD-relevant fragment, tau 26–230, is generated by calpain-1 (Park and Ferreira, 2005) and calpain-2 (Garg et al., 2011) cleavage at Arg230-Thr231. In contrast, Matsumoto and colleagues (Matsumoto et al., 2015) have demonstrated that tau is cleaved *in vitro* by calpain-1 at Arg242-Lys243 producing a 24 kDa C-terminal fragment lacking the N-terminal projection domain (aa 243–441; CTF24). This truncated tau accelerated heparin-induced aggregation and was unable to support microtubule assembly. Furthermore, CTF24 efficiently

propagated to other tau-expressing cells, where it displayed higher aggregation and seeding activity than full-length tau (Matsumoto et al., 2015). Interestingly, active calpain-2 co-localizes with tau filaments in AD, Down syndrome and FTD brains (Adamec et al., 2002a,b). A recent study by Cicognola et al. (2020) identified that calpain-2, but not calpain-1, cleaves tau at Lys224, generating an N-terminal fragment previously found to be enriched in CSF in tauopathies (Cicognola et al., 2019). Knockdown of the calpain-2 catalytic subunit gene caused a significant reduction of this N-244 tau fragment in cell-conditioned media (Cicognola et al., 2019). Overall, calpain cleavage appears to promote tau aggregation, thus potentially enhancing its spreading.

## α-SYNUCLEIN

α-Synuclein is a small cytosolic protein highly abundant in neurons, and is predominately present as a soluble monomer in physiological conditions (Lee and Trojanowski, 2006). It is composed of 140 amino acids forming three main regions: the N-terminus (aa 1–60) which contains apolipoprotein binding motifs, the central non-amyloid component (NAC) (aa 61–95), which has the propensity to fold into beta sheets, and a negatively charged mostly unstructured C-terminus (aa 96–140) (Stefanis, 2012). The function of α-synuclein remains elusive, although reports on its presynaptic localization and ability to interact with lipids suggest it might have a role in neurotransmitter release (Lee and Trojanowski, 2006; Bernal-Conde et al., 2019). The presence of misfolded, aggregated α-synuclein in Lewy bodies is the molecular hallmark of PD and other neurological conditions termed synucleinopathies, and mutations in its coding gene, *SNCA*, are causative of PD (Stefanis, 2012). α-synuclein was the first pathological protein shown to behave in a manner similar to the prion protein (Karpowicz et al., 2019) and is found extracellularly in the brain and in extracellular fluids, such as CSF (El-Agnaf et al., 2003; Lee et al., 2005). Injection of anti-α-synuclein antibodies in the mouse brain parenchyma halts propagation of α-synuclein pathology (Tran et al., 2014), supporting the possibility of its interneuronal transfer as a free protein. Similar to tau, α-synuclein endocytosis has been shown to occur through interaction with HSPGs and CSPGs (**Figures 1A**ii,**B**ii), and it is similarly susceptible to cleavage by extracellular proteases (**Table 1**).

### Proteoglycans

In C17.2 mouse-derived neural stem cells, internalized α-synuclein fibrils colocalize with HSPGs (Holmes et al., 2013). Similar to tau, α-synuclein endocytosis in cell lines is inhibited by co-application of heparin in a dose-dependent manner (Holmes et al., 2013; Ihse et al., 2017). Heparin acts competitively by binding α-synuclein at sites responsible for its interaction with HSPGs, thus blocking its internalization. However, heparin has been found to be less efficient in blocking internalization of monomeric and oligomeric forms of α-synuclein (Ihse et al., 2017). A neuroblastoma cell line exposed to heparin lyases I, II, and III showed reduced endocytosis of α-synuclein fibrils and similarly, cells lacking enzymes responsible for HPSGs biogenesis fail to internalize α-synuclein (Ihse et al., 2017). Additionally, HSPG sulfation was found to be important, as treatment with chlorate, which inhibits sulfation, reduces α-synuclein fibril internalization (Holmes et al., 2013; Hudák et al., 2019). Further analyses on the sulfation requirements of HSPGs was carried out using differentially sulfated heparin. When applied to C17.2 cells, 2-O, 6-O and *N*-desulphated heparin showed lower efficiency in inhibiting α-synuclein uptake and seeded aggregation compared to standard heparin. Shorter heparin chains are also less efficient in inhibiting α-synuclein uptake and seeding. Accordingly, a CRISPR/Cas9 genetic screen in HEK293 cells identified *EXT1, 2,* and *3*, which mediate the initiation and elongation of the glycosaminoglycan chain in HSPGs, to be involved in α-synuclein uptake and seeding. Similarly, *NDTS1*, which encodes an enzyme responsible for *N*-deacetylation and *N*-sulfation of HSPGs, also appeared to play a role, as its knockout reduced uptake and seeding of α-synuclein. Although the ablation of *HS6ST2*, an enzyme involved in the 2-O sulfation of HSPGs, did not show overt effects on α-synuclein fibril uptake, its overexpression decreased α-synuclein internalization but increased seeding. This suggests a more complex role of 2-O sulfation on α-synuclein spreading (Stopschinski et al., 2018). Zhang et al. (2020) recently analyzed the interaction between α-synuclein and HSPGs by molecular modeling, suggesting that α-synuclein fibrils display more stable binding to HSPGs compared to its monomeric or dimeric forms, possibly explaining the stronger inhibitory effect of heparin on fibril internalization (Zhang et al., 2020).

In a follow up study, treatment of neuroblastoma cells or primary neurons with heparinase failed to inhibit internalization of N-terminal acetylated α-synuclein monomers or fibrils. Since this modification is present at high levels *in vivo* (Burré et al., 2013), this result questions the physiological importance of HSPGs in this process. In contrast, peptide-*N*-glycosidase F (PNGase F), which cleaves complex N-linked glycans from glycoproteins, reduced endocytosis of both fibrillar and monomeric *N*-acetylated α-synuclein but did not affect non-acetylated α-synuclein. Interestingly, acetylated α-synuclein was found to interact with glycans in the absence of their protein core (Birol et al., 2019). Of note, surface membrane proteins as well as secreted proteins and ECM components are heavily glycosylated (Scott and Panin, 2014), creating an ideal multivalent binding environment for pathological α-synuclein. These data indicate that HSPGs are fundamental players in the internalization of α-synuclein fibrils.

CSPGs have also been implicated in α-synuclein aggregation and spreading (Lehri-Boufala et al., 2015; Mehra et al., 2018). Accordingly, incubation of α-synuclein with chondroitin sulfate A and B *in vitro* enhances the formation of aggregates able to enter SH-SY5Y cells (Mehra et al., 2018). Interestingly, chondroitin sulfate, and glycosaminoglycans in general, inhibit cathepsin D, suggesting that the endocytosis of an α-synuclein-chondroitin sulfate complex could induce higher seeding effects due to lysosomal inhibition (Lehri-Boufala et al., 2015). However, co-injection of α-synuclein

aggregates with chondroitinase in mice did not change the spread of pathology. On the other hand, degradation of hyaluronan, another major component of the ECM, reduced α-synuclein pathology in the same model, although the mechanism at the basis of this effect is currently unclear (Soria et al., 2020).

## Proteases

It is generally assumed that fragments of α-synuclein must contain the NAC region to propagate pathology. *In vitro* studies using recombinant α-synuclein found that negative charges at the α-synuclein C-terminus counteracts the aggregation propensity of the NAC domain (Izawa et al., 2012). As such, cleavage of the C-terminus by proteases increases aggregation and seeding (Sorrentino et al., 2018, 2020; Chakroun et al., 2020; Sorrentino and Giasson, 2020). Interestingly, α-synuclein carrying PD-linked mutations is more efficiently cleaved at the C-terminus than the wildtype protein (Li et al., 2005).

Conversely, the effect of N-terminal truncation is less clear. Most studies report no change (Luk et al., 2009; Volpicelli-Daley et al., 2011) or a decrease (Luk et al., 2016) in seeded aggregation, even though removal of the first two apolipoprotein binding motifs can lead to an increase (Kessler et al., 2003; Sorrentino and Giasson, 2020). Increased propagation of α-synuclein pathology after brain injection of recombinant α-synuclein lacking the first 10 or 30 residues, compared to full length fibrils has been observed (Terada et al., 2018), suggesting that the N-terminus of α-synuclein can also modulate aggregation. Therefore, the action of extracellular proteases has the potential to both negatively and positively modulate α-synuclein propagation.

Different MMPs such as MMP-1, -2, -3, -9, and -14 cleave α-synuclein, with MMP-3 proven to be the most effective (Levin et al., 2009). *In vitro* tests have shown cleavage at multiple sites at the N-terminus of the NAC (Sung et al., 2005). These cleavage products were observed in the extracellular media of neuroblastoma SK-N-BE cells overexpressing human α-synuclein. MMP-3-cleaved α-synuclein had an elevated propensity to aggregate *in vitro* and displayed increased toxicity compared to full length α-synuclein when applied to SK-N-BE cells (Sung et al., 2005). Interestingly, MMP-3 is elevated in rat brains exposed to 6-hydroxydopamine or 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, two classical models of parkinsonism (Sung et al., 2005; Leem et al., 2020). Accordingly, MMP-3 localization in Lewy bodies was detected in the substantia nigra of PD brains (Choi et al., 2011). Choi et al. (2011) investigated the MMP-3-dependent cleavage of α-synuclein harboring mutations associated with PD, and observed that *in vitro,* the A53T mutant is processed more efficiently than the wildtype and A30P mutant proteins, especially at sites 78–79 and 91–93. In contrast, the overexpression of MMP-3 and α-synuclein in COS cells produced lower levels of insoluble α-synuclein aggregates. However, overexpression of the A53T-containing 1–93 α-synuclein fragment in mice induced increased toxicity and formation of Lewy body-like structures in dopaminergic neurons of the substantia nigra, both at the site of injection of the α-synuclein-encoding adeno-associated virus and contralateral side (Choi et al., 2011). These results imply that the 1–93 fragment, which contains the full NAC domain and lacks the C-terminus, is prone to aggregation and spreading. Nevertheless, this is not the only fragment produced by MMP-3 cleavage and the overall effect of MMP-3-cleaved α-synuclein has yet to be conclusively evaluated *in vivo*.

MMP-2 has also been suggested to degrade α-synuclein fibrils. Injection of MMP-2 into animals inoculated with α-synuclein in the cortex and striatum led to reduced levels of insoluble and oligomeric α-synuclein. In addition, human α-synuclein positivity was limited to the injection site in MMP-2 treated animals, suggesting reduced spreading (Oh et al., 2017).

Plasmin is a serine protease that degrades fibrin blood clots, and is also involved in inflammation, collagenase activation and synaptic plasticity (Pang et al., 2004). Application of recombinant plasmin to α-synuclein monomers, oligomers or fibrils resulted in their degradation, including when they harbored several PD-linked mutations, including A53T. Interestingly, α-synuclein found in the media of SH-SY5Y cells was also cleavable by plasmin at different sites spanning the N-terminal domain and the NAC. Using a propagation model utilizing SH-SY5Y cells expressing α-synuclein, co-cultured with the microglia-like cell line BV2 lacking α-synuclein, Kim et al. (2012) showed that exogenous plasmin added to the media could reduce spreading between these two cell types. However, further studies are necessary to confirm the importance of plasmin cleavage on α-synuclein aggregation and spreading *in vivo*.

α-Synuclein has been found to be degraded by neurosin, also called kallikrein 6. Similar to other members of the kallikrein family, neurosin is a secreted trypsin-like serine protease that is activated extracellularly by sequential cleavage (Yoon et al., 2008) and can be found in human CSF (Diamandis et al., 2000). *In vitro* treatment of recombinant α-synuclein with neurosin generates several fragments by cleavage within and in the proximity of the NAC domain. Neurosin digestion was found to inhibit α-synuclein aggregation *in vitro* (Iwata et al., 2003), and treatment of α-synuclein oligomers with neurosin induced their almost complete degradation (Spencer et al., 2013). Pro-aggregation variants such as α-synuclein phosphorylated at Ser129 (Kasai et al., 2008) or carrying the PD-linked mutations A30P, A53T or E46K showed reduced cleavage by neurosin (Iwata et al., 2003; Kasai et al., 2008; Spencer et al., 2013). Of note, neurosin levels are lower in brains of patients affected by dementia with Lewy bodies and in α-synuclein transgenic mouse models (Spencer et al., 2013). Furthermore, overexpression of neurosin in HEK293 cells or in cortical neurons induced degradation of α-synuclein in the culture media, whereas primary neurons from neurosin knockout mice showed increased α-synuclein internalization and aggregation (Tatebe et al., 2010; Pampalakis et al., 2017). In addition, neurosin can also activate proMMP-2 (Pampalakis et al., 2017) and an unidentified extracellular protease (Ximerakis et al., 2014), further promoting α-synuclein degradation. Although it is unclear whether neurosin acts directly or through activation of a downstream protease, its activity

appears to strongly reduce both aggregated and monomeric forms of α -synuclein.

Cathepsins B, D, E, G, K, L, S and V have been shown to cleave α-synuclein, both in its monomeric and fibrillar forms. In particular, both cathepsins L and K were shown to completely ablate α-synuclein fibrils, whereas cathepsins B, D, E, G, S and V generate small α-synuclein fragments (McGlinchey and Lee, 2015; McGlinchey et al., 2017, 2020). Importantly, degradation of α-synuclein by cathepsins L and K was achieved upon long incubation (16 h) and at an acidic pH, which would be typically found in lysosomes. Shorter incubation times or treatment at a neutral pH mimicking that of the extracellular space, generated fragments truncated at the N- and C-termini (McGlinchey et al., 2017, 2020). As discussed above, these fragments display an increased aggregation propensity, albeit this was not tested experimentally. Cathepsin B was found to cleave both α-synuclein monomers and fibrils *in vitro* upon incubation at low pH. It is thus unclear whether this activity could be retained at neutral pH in the extracellular space (McGlinchey and Lee, 2015; Tsujimura et al., 2015). C-terminal cleaved fragments were also observed upon addition of recombinant cathepsin B to lysates of 3D5 cells expressing human α-synuclein, or to mouse and human brain extracts (Sevlever et al., 2008; Tsujimura et al., 2015). Cathepsin D was also found to cleave recombinant α-synuclein *in vitro* at residues 116, 124 and 132, thus potentially generating aggregating fragments (Hossain et al., 2001; Sevlever et al., 2008). Since the proteolytic activity of both cathepsin B and D on α-synuclein was only observed at acidic pH, it is questionable whether these events occur in the extracellular space. However, cathepsin D knockout mice display insoluble α-synuclein in brain extracts, even in the absence of overexpression. A similar result was observed in human post-mortem brains affected by mutations in the *CTSD* gene (Cullen et al., 2009). Accordingly, knockdown of the *C. elegans Ctsd* ortholog caused increased aggregation of overexpressed human α-synuclein, whereas overexpression of *Ctsd* in α-synuclein expressing worms increased survival of DA neurons, an effect that was not present upon expression of cathepsins B and L (Qiao et al., 2008).

The effects of calpain-1 on α-synuclein have been studied in detail. However, similar to the action of calpain on tau, its activity on α-synuclein has been assumed to occur intracellularly. Calpain cleaves monomeric α-synuclein mostly within the N-terminal or central region (Mishizen-Eberz et al., 2003; Greenbaum et al., 2005; Kim H. J. et al., 2006; Diepenbroek et al., 2014). The aggregation potential of monomeric α-synuclein treated with calpain is still controversial (Mishizen-Eberz et al., 2005; Dufty et al., 2007). *In vitro,* calpain-1 cleaves both wildtype and PD mutant forms of fibrillar α-synuclein within the C-terminus (Mishizen-Eberz et al., 2003; Diepenbroek et al., 2014), which may increase the aggregating potential of α-synuclein. Diepenbroek and colleagues evaluated the role of calpain *in vivo* by crossing mice expressing human α-synuclein carrying the A30P mutation with a calpastatin knockout or overexpressing mouse model. Overexpression of calpastatin, which acts as a calpain inhibitor, reduced α-synuclein aggregates in the brain, whereas calpastatin knockout mice showed the opposite effect (Diepenbroek et al., 2014). Similar findings were obtained by treating mice expressing human wildtype α-synuclein with the calpain inhibitors gabadur and neurodur. This treatment decreased insoluble α-synuclein aggregates with a concomitant reduction in gliosis, neurodegeneration and hyperactivity (Hassen et al., 2018). Therefore, calpain processing of α-synuclein appears to increase its aggregation potential.

## HUNTINGTIN

Huntingtin is a large 384 kDa protein that contains an N-terminal region, which functions as a nuclear export signal, followed by a stretch of glutamines that contains between 9 and 35 residues in healthy subjects. Higher numbers of CAG repeats, which encode for these glutamines, in the huntingtin gene are associated with aggregation and HD. The remaining portion of this protein is far less characterized, although it presents several HEAT repeats that are important for protein–protein interactions. Huntingtin is involved in several functions from vesicular trafficking to translation and autophagy (Saudou and Humbert, 2016). The neuronal spread of huntingtin pathology has been described; however, the evidence is less definitive compared to other proteins discussed in this review (Pecho-Vrieseling et al., 2014). Aggregates of recombinant polyQ peptides are internalized and induce further aggregation in HEK293 cells (Ren et al., 2009). Moreover, huntingtin aggregates extracted from the brain of R6/2 mice, which overexpress human huntingtin exon1 carrying ∼115 CAG repeats, were able to increase aggregation of overexpressed huntingtin in Neuro2a cells (Nekooki-Machida et al., 2009). However, in HD patients that received fetal neural allografts, aggregated huntingtin was found in the graft region in the extracellular space but not in neurons, arguing against the ability of aggregated huntingtin to spread between cells *in vivo* (Cicchetti et al., 2014). In this light, further research is required to confirm the relevance of huntingtin spreading in the progression of HD.

Aggregation of huntingtin is driven by polyQ expansion and, as such, this region needs to be retained for huntingtin to act as a seed. In addition, N-terminal fragments of huntingtin have been shown to be more toxic than the full-length protein. For example, shorter versions of huntingtin terminating at residue 145 or 650 are more aggregation-prone in a polyQ length-dependent manner and induce increased sensitivity to oxidative stress in primary neurons compared to the full-length protein. Interestingly, these fragments recruit endogenous huntingtin into aggregates (Martindale et al., 1998). Further studies confirmed that the shorter the huntingtin fragment, the more aggregation prone it is, as long as the polyQ region is retained (Hackam et al., 1998). This observation therefore suggests that extracellular proteolytic cleavage could modulate disease progression (Graham et al., 2006; Ratovitski et al., 2011; O'Brien et al., 2015). C-terminal fragments have been less extensively studied. Work from El-Daher et al. (2015) found that the 587–3144 fragment could cause toxicity on its own, but

had a protective effect against fragment 1–167–mediated toxicity, whereas it potentiates 1–586 toxicity in both striatal cells and *Drosophila*.

## Proteases

Truncated huntingtin species are generated through cleavage by different proteases that can be found in the extracellular space such as MMPs, calpains and cathepsins (**Table 1**). MMP-10, -14 and -23B were identified in an shRNA-based knockdown screen in HEK293 cells looking for a reduction in huntingtin fragments. Knockdown or pharmacological inhibition of these MMPs reduced toxicity in a striatal cell line expressing huntingtin with 111 glutamines, as well as in *Drosophila* lines expressing the first 336 amino acids of the human huntingtin protein with 128 glutamines. MMP-10 was confirmed to cleave recombinant huntingtin both *in vitro* and in cell lysates, mostly producing a 45 kDa fragment which could also be observed in brain extracts from HD patients. MMP-10 and -14 activity was increased in a striatal cell line expressing huntingtin with 111 glutamines compared to a non-pathogenic variant with only 7 (Miller et al., 2010). Furthermore, increased MMP-2, -3 and -10 activity was observed in neural stem cells from HD patients compared with their isogenic lines, where the polyQ expansion was corrected. In contrast to this, the activities of MMP-9 and -14 were found to be reduced in HD (Naphade et al., 2017).

Calpain also mediates proteolytic processing of huntingtin. Application of recombinant calpain-1 to recombinant huntingtin or to mouse brain extracts caused the appearance of fragments with molecular weights between 45 and 72 kDa, which could also be observed in post-mortem brain samples of both healthy and HD-affected subjects (Kim et al., 2001, 2003; Gafni and Ellerby, 2002; Lunkes et al., 2002). However, high-polyQ huntingtin appeared to be more resistant to calpain cleavage compared to lower repeat mutants *in vitro* (Gafni and Ellerby, 2002). Calpain-resistant variants showed reduced aggregation and toxicity in HEK293T cells compared to calpain-cleavable huntingtin (Gafni et al., 2004). In addition, calpain-derived fragments have been observed both in mouse models expressing human huntingtin with 150 polyQ (Gafni et al., 2004; Landles et al., 2010), and in post-mortem caudate samples from HD patients which also presented increased calpain levels (Gafni and Ellerby, 2002; Gafni et al., 2004). These results were confirmed in *Drosophila*, where knocking out calpain reverted the toxic effects of huntingtin overexpression, although this effect appeared to be due to the intracellular activity of calpain, since a double knockout for calpain and the autophagic protein Atg8 failed to show the same rescue effects (Menzies et al., 2015). Furthermore, crossing calpastatin knockout mice with HD mice with 111 polyQ showed increased production of huntingtin N-terminal fragments and subsequent aggregation (Weber et al., 2018). Similar to tau and α-synuclein, a direct demonstration of calpain-mediated cleavage of huntingtin in the extracellular space is still lacking.

Cathepsins also cleave huntingtin. In particular, cathepsins B, D and L were found to generate two 55–60 kDa fragments when incubated with striatal cell lysates. Interestingly, cathepsin D only generated an N-terminal fragment from wildtype huntingtin, whereas up to four different fragments were detected when huntingtin containing 100 polyQ was exposed to cathepsin D. This cleavage was carried out at a neutral pH, suggesting that it could occur in the extracellular space (Kim J. et al., 2006).

## TDP-43

Transactive response (TAR) DNA binding protein 43 (TDP-43) is a protein involved in transcriptional regulation and processing of thousands of different RNAs (Prasad et al., 2019). Under physiological conditions, it is localized in the nucleus but redistributes to the cytosol in several neuropathologies. TDP-43 aggregation is a hallmark of FTD and amyotrophic lateral sclerosis (ALS) and mutations in its coding gene are causative for these diseases (Chhangani et al., 2021). Evidence of its prion-like activity has also been described. For example, injection of brain extracts from FTD patients induced formation of aggregates in neuronal cell lines (Nonaka et al., 2013) and mouse models (Porta et al., 2018; Peng et al., 2020). TDP-43 seeds were also able to transfer between cultured neurons grown in microfluidic devices both as a free protein and inside exosomes (Feiler et al., 2015).

TDP-43 is composed of an N-terminal domain, two RNA recognition motifs and a C-terminal domain. Cryo-electron microscopy studies have identified the core of TDP-43 aggregates to be formed of residues 282–360 (Cao et al., 2019; Arseni et al., 2021). Several TDP-43 fragments have been observed in human samples from ALS-FTD patients (Smethurst et al., 2016) and C-terminal fragments appear to retain aggregation properties (Igaz et al., 2009; Nonaka et al., 2009; Furukawa et al., 2011; Shimonaka et al., 2016). Several studies suggest that a 25 kDa C-terminal fragment, possibly generated by cleavage at residue 216, (Caccamo et al., 2015) promotes seeded aggregation. This fragment is more abundant in the brain than in spinal cord samples isolated from the same FTD patient and, in accordance, brain extracts showed increased seeding capabilities compared to spinal cord extracts (Smethurst et al., 2016). However, other studies have found that expression of this C-terminal fragment in mice does not cause aggregate formation and drives limited behavioral defects (Caccamo et al., 2012, 2015; Akamatsu et al., 2013; Dayton et al., 2013; Walker et al., 2015; Porta et al., 2018). Other C-terminal fragments have also been identified (CT35kDa and CT27kDa), but their aggregation and seeding properties are still controversial (Zhang et al., 2009; Suzuki et al., 2011). In contrast, a recent study showed that injection of aggregates of an N-terminal 1–265 fragment was sufficient to induce formation of TDP-43-positive cytosolic stress granules and cause neuronal death (Pirie et al., 2021).

To the best of our knowledge, there is no evidence directly linking ECM core components with TDP-43 spreading. However, calpains have been found to process TDP-43, and as mentioned for other prion-like aggregates, it is possible

that this protease could modify the seeding properties of TDP-43 in the extracellular milieu. Calpains cleave TDP-43 *in vitro* at multiple sites in mouse and rat brain extracts (**Table 1**). These fragments retained toxicity when applied to cultured primary neurons. Similar fragments were observed in mouse models of traumatic brain injury (TBI) and in the CSF of TBI patients (Yang et al., 2014). Interestingly, phosphorylated TDP-43 is resistant to calpain cleavage *in vitro* (Yamashita et al., 2016), whereas the A315T and M337V TDP-43 mutants showed increased processing (Yamashita et al., 2012). TDP-43 fragments corresponding to calpain cleavage events have been observed in extracts from the motor cortex and spinal cord of FTD-ALS patients with a concomitant increase in activated calpain-1 and -2. Interestingly, both fragments and activated calpain were higher in spinal cord samples compared to the motor cortex (Yamashita et al., 2012).

## AMYLOID BETA

Amyloid precursor protein (APP) is an integral transmembrane protein consisting of a large extracellular domain, a transmembrane domain and a short intracellular tail (Chen et al., 2017). APP is widely expressed in neuronal and non-neuronal cells, and its physiological function is still unclear, although it is believed to function as a cell adhesion molecule (Müller et al., 2017). APP is sequentially cleaved by β- and γ-secretase to produce Aβ peptides (Aβ40 and Aβ42), which form the core of amyloid plaques found in the brain of AD patients (Hardy and Selkoe, 2002; Selkoe, 2002; Haass et al., 2012). In neuronal cells, APP is predominantly found in the secretory pathway, from where it is trafficked to the axon termini and dendrites. Once APP reaches the plasma membrane, it is quickly internalized, leading to only a small fraction being present on the neuronal surface (Haass et al., 2012). Accordingly, Aβ production is likely to occur in the endocytic pathway, since inhibiting the internalization of cell surface APP leads to a significant decrease in Aβ production (Koo and Squazzo, 1994; Koo et al., 1996). The formation of extracellular Aβ aggregates is discussed below, since the involvement of ECM components in this process has been more extensively studied.

Very little is known about the precise molecular mechanisms triggering the conversion of soluble Aβ peptides to oligomers, protofibrils, amyloid fibrils and plaques in the brain (Friesen and Meyer-Luehmann, 2019). Intraneuronal Aβ42 accumulation precedes the deposition of extracellular plaques and has been observed in multivesicular bodies, endosomes and along microtubules (Takahashi et al., 2002, 2004). These intraneuronal Aβ42 clusters are enriched in pyramidal neurons of the hippocampus and entorhinal cortex, both of which are particularly vulnerable to pathology (Gouras et al., 2000). Most Aβ peptides are released from distal axons and synapses, and spread to synaptically connected regions (Friesen and Meyer-Luehmann, 2019); however, no *in vivo* study has demonstrated the transport and spread of Aβ and the roles that ECM might play. Domert et al. (2014) have shown that oligomeric Aβ is transferred between connecting, differentiated SH-SY5Y cells, and have postulated that this is due to impaired degradation of these. In addition, the cellular mechanism(s) leading to plaque spreading is also not fully elucidated. Thus, many fundamental questions remain about Aβ dynamics *in vivo* and its deposition in plaques in the AD brain.

### Proteoglycans

A class of extracellular proteins which is found to play extensive roles in Aβ uptake, aggregation and deposition is the HSPG family (**Figures 1A**iii,**B**iii). Amyloid plaques were first found to contain HSPGs by Snow and colleagues (Snow et al., 1988), followed by the observation that there is a direct interaction between HSPGs and both APP and Aβ (Narindrasorasak et al., 1991; Brunden et al., 1993). Cell lines deficient in HS biosynthesis, or treated with heparin, show a decrease in the binding of Aβ peptides to the cell membrane and subsequent internalization (Kanekiyo et al., 2011). An AD mouse model with a neuron-specific impairment in HS biosynthesis exhibited decreased plaque burden and Aβ oligomerization (Liu et al., 2016). Interestingly, the low-density lipoprotein-related receptor (LRP1)-dependent endocytosis of Aβ42 relies on HSPGs, since the addition of heparin abrogated the increase in Aβ42 uptake observed by the overexpression of an LRP1 mini-receptor (Kanekiyo et al., 2011). In conjunction with this, the Bu group has observed that ApoE-immunoisolated extracellular vesicles, derived from astrocytes, suppress Aβ binding and uptake in mouse cortical neurons, an effect abrogated by heparin (Fu et al., 2016). These findings suggest that HSPGs are involved in Aβ42 internalization. Although mostly localized at the plasma membrane, syndecans and glypican-1 show extensive co-localization with extracellular amyloid plaques in the brain, with the neuron-specific syndecan-3 being most effective at increasing Aβ42 uptake (van Horssen et al., 2002). By virtue of their HS chains, syndecan-3 and -4 also trigger formation of Aβ42 fibrillar assemblies, underscoring their relevance in plaque formation (Letoha et al., 2019). Glypican-1 interacts with higher-order Aβ structures and reduces SH-SY5Y viability when overexpressing APP (Watanabe et al., 2004). However, its function in plaque seeding or aggregation is currently not known.

Apart from their roles in mediating cell surface binding and endocytosis of APP/Aβ, HSPGs, along with CSPGs, also play a role in clearance and degradation of Aβ peptides (Gupta-Bansal et al., 1995). While post-mortem AD brain samples contain higher than normal levels of HSPGs, clearance of soluble Aβ in the mouse hippocampus is increased upon HSPG depletion (Liu et al., 2016). It is currently postulated that HSPGs function to promote Aβ fibrillation or act as protective chaperones, thus inhibiting Aβ degradation (van Horssen et al., 2003). Both agrin and perlecan, like other HSPGs, bind to Aβ40 fibrils through glycosaminoglycan side chains, protecting them from degradation and accelerating the fibrillation of monomeric Aβ40 (Castillo et al., 1997; Cotman et al., 2000). This role

of agrin was recapitulated in an AD mouse model crossed with a conditional *Agrn* KO line where deletion was driven by an endothelial cell-specific promoter. These mice exhibited increased Aβ generation and deposition (Rauch et al., 2011). However, this effect was not observed when neuronal agrin was deleted, pointing to a non-cell autonomous effect on plaque formation.

## Proteases

Proteolytic degradation contributes to the regulation of extracellular Aβ levels and deposition in amyloid plaques. Several extracellular proteases have been implicated in the degradation of Aβ40/42, including neprilysin (NEP) and the insulin-degrading enzyme (IDE) (**Table 1**; Saido and Leissring, 2012).

Neprilysin is a zinc metalloprotease with broad substrate specificity and cell surface localization. Demonstrating an inverse correlation with age, it is reported to cleave only Aβ monomers along the axon and at synaptic sites, and has negligible enzymatic activity for higher-order Aβ structures (Fukami et al., 2002; Saido and Leissring, 2012). Consistent with its role in the reduction of Aβ42 levels, brain tissues of AD patients exhibit a decrease in NEP expression in AD-vulnerable areas, including the hippocampus, cortex and temporal gyrus (Yasojima et al., 2001). The genetic deletion of NEP or its pharmacological inhibition results in a two-fold increase in Aβ levels in the brain as well as increased hippocampal plaque burden (Marr et al., 2004; Farris et al., 2007). Conversely, overexpression of neuronal NEP results in reduced Aβ levels and deposition, along with a reduction in associated pathology (Leissring et al., 2003). Additionally, its homolog NEP2 also contributes to decreasing Aβ levels (Saido and Leissring, 2012).

The zinc metalloprotease IDE also displays a broad distribution in the extracellular space, cytosol and mitochondria. Similar to NEP, IDE catalyzes the degradation of monomeric Aβ in the brain and its ablation causes an increase in Aβ levels in primary neurons as well as *in vivo* (Farris et al., 2003). Other extracellular proteases processing Aβ include MMP-2, MMP-9, and MMP-14, cathepsin B and plasmin (Saido and Leissring, 2012; Hernandez-Guillamon et al., 2015). Interestingly, these enzymes are capable of catabolizing Aβ fibrils, in contrast to NEP and IDE (Saido and Leissring, 2012). Their investigation *in vivo* remains limited, though cathepsin B has been observed in amyloid plaques (Mueller-Steiner et al., 2006).

## Other Extracellular Matrix Components

In contrast to HSPGs and extracellular proteases, the role of ECM core proteins in Aβ production, seeding or plaque deposition is less clear. Several ECM proteins, including laminin, collagens and fibronectin co-localize with senile plaques in AD brains (Perlmutter et al., 1991). Proteomic profiling of AD hippocampi has revealed an upregulation of several ECM proteins during all disease stages (Hondius et al., 2016). However, whether these observations are causative, or correlative of amyloid formation, is currently unknown.

Laminin1 binds to soluble Aβ40 through its α-chain and inhibits its fibrillogenesis in a time- and dose-dependent manner, thus influencing the survival of cortical neurons (Drouet et al., 1999). While it increases the amyloidogenic fragment production, laminin1 also promotes Aβ40 depolymerization when incubated with pre-formed fibrils *in vitro* (Bronfman et al., 1996; Castillo et al., 2000).

Total collagen levels are upregulated in AD; however, the roles of individual isoforms are currently unclear. The Mucke group has shown that synthetic Aβ42 oligomers induce the transcription of the *Col6a1* gene in hippocampal and cortical neurons and that collagen VI decreases the interaction of Aβ42 oligomers with the neuronal cell surface, ultimately leading to a reduction in Aβ42 neurotoxicity (Cheng et al., 2009).

In addition, when the 7PA2 cell model of AD is treated with peptides derived from elastin, it shows an elevation in Aβ40 and Aβ42 production. This effect was dependent on the length of the individual peptides, since longer peptides were more potent in Aβ generation and led to AD-related behavior in mice. Significantly, these changes were pinned down to an increased expression of β- and γ-secretase mRNAs (Ma et al., 2019).

# CONCLUDING REMARKS

In this review we have presented evidence supporting the role of different ECM components in modulating the spread and aggregation of pathological, misfolded proteins. Although the available evidence remains limited, predominately due to the complexity of the ECM and its partial reconstitution observed in cell cultures, available data provide the rationale for further exploration of this area of research. In addition to the proteins discussed in this review, other proteins have been shown to possess the ability of transcellular spread, such as superoxide dismutase 1 (SOD1) (Ayers et al., 2014) and C9orf72 (Westergard et al., 2016) in ALS-FTD. However, to the best of our knowledge, we could not identify studies exploring the contribution of ECM components in mutant SOD1 and C9orf72 spreading.

The findings presented here highlight a high level of overlap between pathways modulating aggregation properties and/or propensity of spreading that affect most of these protein aggregates. In particular, proteoglycans and proteases are shared players in several of these diseases. HSPGs appear to participate in several steps of the spreading process of tau, α-synuclein and Aβ (**Figures 1A,B**), as well as the prion protein (Horonchik et al., 2005). Furthermore, direct binding to HSPGs has been shown for all three proteins. As mentioned above, HSPGs represent a heterogenous class of molecules. The presence of HSPGs on both the plasma membrane and in the extracellular space further complicates the experimental dissection of the underlying mechanism. Plasma membrane-exposed HSPGs have been clearly linked to internalization of aggregation-prone forms of tau and α-synuclein, a property shared with the prion

protein (Horonchik et al., 2005). This suggests that HSPGs represent a common node for the endocytosis of pathological protein aggregates. As discussed above, the interaction appears to be mediated by the glycan groups, rather than the protein core, the contribution of which remains unclear. As such, it is likely that glycans may have some predisposition for binding to aggregated proteins, although the biochemical determinants of these interactions need to be further elucidated. In addition, heparin is routinely used to induce aggregation of recombinant tau and α-synuclein *in vitro* (Goedert et al., 1996; Cohlberg et al., 2002). This points to a potential role of extracellular HSPGs in inducing aggregation of these proteins, thus facilitating their internalization and as such, favoring the spread of pathology.

However, exogenous application of heparin can inhibit internalization of protein aggregates. This suggests that extracellular HSPGs could also act in a similar manner. However, the only study that has directly addressed extracellular HSPGs in this context has demonstrated that agrin promotes aggregation of Aβ and protects aggregates from degradation (Cotman et al., 2000). A similar function has also been proposed for agrin on tau, given the presence of agrin in NFTs (Verbeek et al., 1999). This multi-pronged action of HSPGs on several steps of the spreading process complicates predictions as to whether HSPGs could be targeted to slow down disease progression. Of note, chronic application of heparin mimetics failed to show significant effects on tau pathology in mice (Stopschinski et al., 2020). Interestingly, a role of HSPGs in the internalization of huntingtin aggregates was excluded (Holmes et al., 2013), suggesting a certain level of specificity. An in-depth characterization of this process is therefore required to understand the role of each HSPG species on the different steps of spreading, and to identify the biochemical determinants driving each of these effects. This could potentially lead to the identification of an endogenous HSPG, the levels of which could be modulated to reduce spreading. Alternatively, synthetic molecules that mimic HSPG protective effects could also be designed. Other ECM components, such as CSPGs, laminin, collagen and elastin, have been found to participate in the spreading of protein aggregates (**Figure 1**), but their role remains less characterized.

Proteases have emerged as major regulators of prion-like pathological spreading. In addition to a general clearing effect on aggregates, specific proteases can process monomers or oligomers of pathological proteins, generating fragments which display altered aggregation propensity (**Table 1**). Less is known about how these fragments behave with regard to their internalization properties. This area should be further investigated to conclusively establish the contribution of these proteases to the spreading of protein aggregates. MMPs are active against tau, α-synuclein, huntingtin and Aβ, with each MMP performing distinctive roles. MMP-3, for example, has been shown to reduce the propensity of tau to aggregate, whereas it was suggested to increase α-synuclein aggregation. However, MMP-3 is elevated in AD and PD models (Sung et al., 2005; Leem et al., 2020; Pentz et al., 2021) and in patients (Stomrud et al., 2010; Choi et al., 2011; Hanzel et al., 2014). This suggests that MMP-3 expression might be increased in an attempt to reduce pathology, however with opposite outcomes on tau and α-synuclein. Similarly, MMP-9 levels are increased in AD brains (Hernandes-Alejandro et al., 2020), again as a potential compensatory measure to reduce both Aβ and tau pathology. However, not only does MMP-9 degrade Aβ plaques (Hernandez-Guillamon et al., 2015), but it also induces the production of tau fragments with enhanced aggregation properties (Nübling et al., 2012). Conversely, MMP-2 reduces α-synuclein pathology and Aβ plaque burden *in vivo*, but was suggested to increase tau aggregation. Similar to other MMPs, its levels are increased in AD (Terni and Ferrer, 2015). In contrast, MMP-2 is reduced in synucleinopathies (Lorenzl et al., 2002), suggesting its causal role in these diseases. With this exception, these observations indicate that MMPs are upregulated in response to pathology, possibly in an attempt to degrade protein aggregates. However, in several cases, protease activity has proven detrimental, potentiating the aggregation of seeds.

Cathepsins and calpains are key intracellular proteases, the roles of which have been poorly characterized in the extracellular space. This makes the interpretation of the data difficult, as most studies do not specifically investigate whether their action is solely exerted intracellularly, or also extracellularly. Therefore, future experiments should be carried out to clarify whether these proteases would retain activity against proteopathic proteins in the extracellular compartment. Despite this, cathepsins seem to have a protective role by reducing the aggregation of tau, Aβ and α-synuclein. As described above, several cathepsins appear to be upregulated in AD suggesting that lysosomal activity is increased in an attempt to reduce the aggregate load. Whether this reflects an increase of extracellular cathepsin is still unclear. In contrast, risk variants in cathepsin B and D genes were identified in synucleinopathies (Robak et al., 2017; Blauwendraat et al., 2020), which support the involvement of these proteases in the mechanism of disease. The role of these proteases in HD and TDP-43 pathology has not yet been investigated in sufficient detail. The well-defined role of cathepsins in lysosomal degradation, in addition to their presence in the extracellular space, makes them an interesting pharmacological target to modulate protein aggregation and spreading in a two-pronged manner. Conversely, calpain cleavage was suggested to promote aggregation of α-synuclein, tau, TDP-43 and huntingtin, with increased levels of calpain detected in related pathologies. This strongly suggests that increased calpain activation could be a common pathological mechanism in neurodegenerative disorders, thus making it a promising therapeutic target. Further research should therefore be carried out to characterize the extracellular proteases involved in the proteolytic processing of prion-like proteins during the prodromic and early symptomatic phases of disease.

The findings presented in this review support the need to clarify the contributions of ECM components in neurodegenerative diseases. To do so, research should include *in vivo* experiments in animal models of neurodegeneration, which retain the complex ECM web in its native state, and using culture systems better recapitulating this aspect. For example, three-dimensional (3D) organoids generated from human induced pluripotent stem cells (hIPSCs) may allow easier access to study the ECM compared to animal models, albeit maintaining a greater complexity than 2D cultures (Cho et al., 2021). This would not only help the discovery of novel roles for ECM components but could also drive the development of new therapeutic strategies for neurodegenerative diseases. Although these therapies would not directly target initial pathological events, such as the generation of misfolded proteins, they could halt their spreading between brain regions, therefore slowing down disease progression. In addition, given their extracellular localization, these proteins have the advantage of being more easily targetable and their modulation might have reduced side effects as they would not directly affect neuronal and glial cell function. As such, increased efforts in exploring the role of ECM components in modulating the spreading of pathological protein aggregates should be strongly encouraged.

## AUTHOR CONTRIBUTIONS

EM and GS conceptualized the manuscript. EM, SSt, and SSu wrote the manuscript. EM, SSt, SSu, JV, and GS reviewed and edited the manuscript. GS, JV, and EM acquired funding. All authors contributed to the article and approved the submitted version.

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# CAQK, a peptide associating with extracellular matrix components targets sites of demyelinating injuries

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The destruction of the myelin sheath that encircles axons leads to impairments of nerve conduction and neuronal dysfunctions. A major demyelinating disorder is multiple sclerosis (MS), a progressively disabling disease in which immune cells attack the myelin. To date, there are no therapies to target selectively myelin lesions, repair the myelin or stop MS progression. Small peptides recognizing epitopes selectively exposed at sites of injury show promise for targeting therapeutics in various pathologies. Here we show the selective homing of the four amino acid peptide, cysteine-alanine-lysine glutamine (CAQK), to sites of demyelinating injuries in three different mouse models. Homing was assessed by administering fluorescein amine (FAM)-labeled peptides into the bloodstream of mice and analyzing sites of demyelination in comparison with healthy brain or spinal cord tissue. FAM-CAQK selectively targeted demyelinating areas in all three models and was absent from healthy tissue. At lesion sites, the peptide was primarily associated with the fibrous extracellular matrix (ECM) deposited in interstitial spaces proximal to reactive astrocytes. Association of FAM-CAQK was detected with tenascin-C although tenascin depositions made up only a minor portion of the examined lesion sites. In mice on a 6-week cuprizone diet, FAM-CAQK peptide crossed the nearly intact blood-brain barrier and homed to demyelinating fiber tracts. These results demonstrate the selective

**Abbreviations:** BBB, blood-brain barrier; BSA, bovine serum albumin; CAQK, cysteine-alanine-glutamine-lysine; CFA, complete Freund's adjuvant; CNS, central nervous system; CSPG, chondroitin sulfate proteoglycan; DAPI, 4',6-diamidino-2-phenylindole; EAE, experimental autoimmune encephalomyelitis; ECM, extracellular matrix; FAM, fluoresceinamine; FITC, fluorescein isothiocyanate; GFAP, glial fibrillary acidic protein; Hapln, proteoglycan link protein; Iba1, ionized calcium binding adaptor molecule 1; i.p., intraperitoneally; LPC, lysophophatidylcholine (lysolecithin); MBP, myelin basic protein; MRI, magnetic resonance imaging; MS, multiple sclerosis; NF-κB, nuclear factor kappa B; OCT, optimal cutting temperature compound; OPC, oligodendrocyte progenitor cell; PBS, phosphate-buffered saline; PET, positron emission tomography; PFA, paraformaldehyde; PID, post induction day; PTX, pertussis toxin; TBI, traumatic brain injury; TBST, Tris-buffered saline with 0.02% Triton X100; TNF, tumor necrosis factor; USPIO, ultrasmall super paramagnetic iron oxide nanoparticles.

targeting of CAQK to demyelinating injuries under multiple conditions and confirm the previously reported association with the ECM. This work sets the stage for further developing CAQK peptide targeting for diagnostic and therapeutic applications aimed at localized myelin repair.

KEYWORDS

**peptide targeting, extracellular matrix, mouse demyelination models, tenascins, myelin repair**

## Introduction

Myelin, a multilayered, lipid-rich membrane sheath formed by oligodendrocytes in the central nervous system (CNS), enables rapid nerve impulse conduction and sustains the functional integrity of neurons and their encircled axons. In multiple neurodegenerative diseases, compromised myelin and impaired nerve functions are closely interlinked. In multiple sclerosis (MS), peripherally activated T-cells and macrophages break through the blood-brain barrier and infiltrate the brain parenchyma causing local inflammation (Mapunda et al., 2022), thus activating a host of tissue reactions that proceed to destroying the myelin. Myelin injury and death of oligodendrocytes impair nerve functions and lead to neurodegeneration with progressive patient disability. Although current MS therapies are efficient in altering or suppressing the immune response, there are no treatments to repair damaged myelin and thus protect nerve functions. Oligodendrocyte progenitor cells (OPC) residing near MS lesion sites retain a limited myelin repair capacity during the early stages of the disease, however, they lose this vital ability with disease progression (Franklin and Goldman, 2015). The cause for remyelination failure is multi-factorial and includes lesion-associated factors that impinge on OPC proliferation, inhibit progenitor migration into lesion sites, or halt OPC maturation, and myelin formation (Galloway et al., 2020). Innovative approaches are needed to identify novel therapies that enable OPC-mediated regeneration of myelin during disease advancement or increase the resilience of myelin to damage.

Significant progress has been made *in vitro* and in animal models in identifying molecules and mechanisms that promote or inhibit remyelination (Franklin, 2002; Franklin and Ffrench-Constant, 2017). Amongst the latter, extracellular matrix (ECM) components are dynamically remodeled during injury and their interactions with surrounding cells exposing new epitopes or receptors induce injury-related pathological pathways (Zimmermann and Dours-Zimmermann, 2008; de Jong et al., 2020; Srivastava et al., 2020; Su et al., 2021). Upregulation of ECM components, including chondroitin sulfate proteoglycans (CSPGs), tenascins, and fragmented hyaluronan, block steps in OPC differentiation and myelination and are implicated in contributing to remyelination failure (Czopka et al., 2010; Karus et al., 2016; Srivastava et al., 2020; Su et al., 2021). Accordingly, studies in mouse models show that CSPG synthesis block or pharmacologically induced clearance promotes remyelination (Lau et al., 2012; Karus et al., 2016; Keough et al., 2016). Interventions with inhibitory tenascin-C deposition reduce susceptibility to inflammatory demyelinating experimental autoimmune encephalomyelitis (EAE) and improve remyelination in mice (Momcilovic et al., 2017; Bauch and Faissner, 2022). Thus, approaches to hinder the deposition of pathological ECM components or to neutralize its adverse effects have gained interest in approaching myelin repair.

With better molecular understanding of myelin destruction and repair processes, the next critical step is translating these findings into remyelination therapies. This entails the development of therapeutics effective in stimulating the remyelination processes and concentrating them at effective doses at sites of myelin damage. Systemic administration often requires high therapeutic concentrations that can lead to undesired side effects in patients. A further challenge is drug delivery across the blood-brain barrier (BBB) that limits access to the CNS (Daneman and Prat, 2015). A promising approach to overcome these limitations is the use of small peptides that target epitopes selectively exposed at myelin injuries. Differentially expressed molecules at lesion sites can serve as docking sites for concentrating drug conjugates or cargo-loaded nanoparticles, and thus increase the efficacy of targeted therapeutics while limiting side effects (Ruoslahti et al., 2010). Peptide targeting is emerging as a promising therapy for a variety of diseases (reviewed in Wang et al., 2022). In cancer, peptides deliver agents to unfold local actions that change signaling pathways or serve as agonists or antagonists for cancer-related receptors (Olson et al., 2009; Thundimadathil, 2012; Marqus et al., 2017; Ruoslahti, 2017; Scodeller and Asciutto, 2020). Small peptides are often able to cross the blood-brain barrier raising a potential additional advantage for CNS targeting (Islam et al., 2021).

Aiming to identify peptides for targeting traumatic brain injuries (TBI), Rouslahti and colleagues conducted a systemic *in vivo* peptide phage display library screen that identified a novel four amino acid peptide, cysteine-alanine-glutamine-lysine, referred to as CAQK (Mann et al., 2016).

This peptide selectively associates with focal and impact-induced brain lesions while not interacting with healthy brain and other tissues (Mann et al., 2016). Traumatic brain injury (TBI) lesions feature a myriad of cellular alterations, including the breakdown of the BBB, immune cell infiltration, activation of astrocytes and microglia, axon- and neuron degeneration, extracellular matrix remodeling, demyelination and oligodendrocyte death (George and Geller, 2018; Qin et al., 2021). Many of these key cellular pathologies in TBI are manifest in MS and other diseases associated with myelin destruction (Su et al., 2021). We thus speculated that CAQK peptide may also target sites of demyelination and potentially prove useful in advancing therapies aimed at achieving myelin repair. We here examined the homing of CAQK tetrapeptide to sites of myelin damage in mouse models of acute, immune-mediated, and toxic demyelination. We report selective targeting of CAQK to injured but not intact myelin in the three demyelination models and thus open prospects for exploring the potential utility of CAQK towards myelin repair.

# Materials and methods

## Materials

Peptides were synthesized with an N-terminal fluorescein amine (FAM) tag and provided at >90% purity by (Genscript). Lyophilized aliquots of ∼3–4 mg were stored at −80°C and reconstituted with sterile phosphate-buffered saline (PBS) for use in mice. Lysolecithin was from Sigma (Cat# L4129) and cuprizone from Thermo Fisher Scientific (Cat# 370-81-0).

## Mice and mouse models of demyelination

All animal procedures were conducted in accordance with Institute Animal Care and Use Committee guidelines at Sanford Burnham Prebys Medical Discovery Institute (La Jolla, CA). The C57BL6/J mouse strain was used in all experiments. Mice constitutively expressing the red fluorescent tdTomato reporter in oligodendrocytes (in text CNP1-TdTom mice) were generated by crossing CNP1-Cre transgenic mice (Lappe-Siefke et al., 2003) with the Rosa26-tdTomato reporter (Jax # 007909) and used in some experiments.

## Lysolecithin-induced focal demyelination

Focal demyelination was induced by stereotaxic injection of 1 μl of 1% lysolecithin solution into the right ventral white matter of 8–10-week-old male mouse spinal cords under ketamine/xylazine anesthesia (Arnett et al., 2004; Bielecki et al., 2016). Injections were made after piercing the dura mater with an 18G needle into the space between T12 and T13 and inserting a fine-tipped glass capillary connected to a Hamilton syringe mounted on an infusion pump run at a rate of 0.2 μl per minute over 5 min. The capillary was kept in place for at least 2 min to allow the solution to diffuse before retraction of the capillary and incision closure. Sham-operated mice were injected with vehicle (PBS). Animals were allowed to recover from surgery and were used for experimentation 24 h and 5 days after LPC injection.

## Experimental autoimmune encephalomyelitis (EAE)

Robust EAE was reproducibly established in 8–12-week old female mice by immunizing groups of 10 or 20 animals with 200 μg $MOG_{35–55}$ peptide in combination with 200 mg *Mycobacterium tuberculosis* emulsified in complete Freund's adjuvant (CFA; EAE kit 2110; Hooke Laboratories, Lawrence, MA, United States) according to the manufacturer's protocol. Pertussis toxin (PTX; 80 ng in PBS) was administered intraperitoneally (i.p.) at the time of induction 0 and 24-h later. Mice in the control group were injected with PTX only. Females from the control and the experimental group were housed together in a cage. Starting at post-induction day 5 (PID5) and ending on PID 28, mice were scored daily for clinical symptoms on the established rating scale (Kihara et al., 2005; Jonnalagadda et al., 2021). Mice were weighed and scored daily for signs of progressive paralysis: 0—no abnormalities; 0.5—mild loss of tail tone; 1.0—complete loss of tail tone; 1.5—mildly abnormal gait and difficulty in righting; 2.0—abnormal gait and hindlimb weakness; 2.5—beginning hindlimb paralysis; 3.0—complete or almost complete hindlimb paralysis; 3.5—paralysis and inability to upright body; 4.0—hindlimb paralysis and forelimb weakness or paralysis; 4.5—paralysis without an attempt to move around the cage; 5.0—moribund or dead. The scoring was performed blinded to the experimenter.

## Demyelination with cuprizone

C57BL/6J 8-week-old male mice were maintained for 6 weeks on a ground chow without or supplemented with 0.2% cuprizone (Bis (cyclohexanone) oxaldihydrazone 98%; Thermo Fisher Scientific, Cat# 370-81-0). Mice were weighed every other day and the cuprizone-fed group showed lower body weights compared to control mice on a normal chow consistent with the effectiveness of the supplement (Tagge et al., 2016).

## Peptides

In all experiments, FAM-CAQK or FAM-control scrambled (FAM-ACKQ) peptides were administered at 100 nmoles/100 µl in PBS into the tail vein or the plexus retroborbitalis. After 60 min in the circulation, animals were anesthetized by inhalation of isofluorane (VetOne, Cat#502017). Circulating FAM-peptides were removed from the bloodstream by perfusion with PBS. Spinal cords were isolated and drop fixed in 4% paraformaldehyde (PFA; Sigma Aldrich, Cat#P6148) in PBS overnight. For harvesting brain cortices, animals were perfused with 4% PFA in PBS. Tissue was postfixed overnight at 4°C in 4% PFA. All tissues were cryoprotected by immersion into 30% sucrose solution 48 h before freezing and then embedded in optimal cutting temperature (OCT) compound (Scigen Cat# 4586) on dry ice/isopentane/methyl pentane at −78°C. Tissues were kept frozen at −20°C until use.

## Processing and immunohistochemistry of spinal cord tissue

Spinal cords from LPC injured or sham-operated mice were cut in cross-section at the level of the injury site. Spinal tissue from mice with EAE or controls was partitioned into four pieces and embedded en block for simultaneous cutting at different levels. Crosssections were cut on a cryostat at 12 µm and collected on Fisherbrand™ Superfrost™ Plus microscope slides (Fisher Scientific Cat#12-550-15). Staining was performed essentially as previously described (Colakoglu et al., 2014). Unspecific binding sites were blocked by sequential incubation with 1% glycine and blocking solution (5% BSA, 2% normal donkey serum, 0.02% Triton X100) for 15 min and 1 h, respectively. Primary antibodies diluted in blocking solution were applied overnight at 4°C in a humid chamber. Slides were washed three times with PBS before being incubated with species-specific secondary antibodies coupled to either Alexa-488, Alexa-594, or Alexa-647 in Tris-buffered saline with 0.02% Triton X100 (TBST) for 1 h at room temperature. After three washes in PBS, slides were counterstained with blue-fluorescent nuclear DNA stain (DAPI) and mounted using Fluoromount G. Staining was examined on a Zeiss LSM 710 confocal microscope. The following primary antibody reagents were used: Rabbit anti-fluorescein/Oregon Green (Thermo Fisher Scientific, Cat#A-889, RRID:AB_221561), goat anti-FITC (Thermo Fisher Scientific, Cat# PA1-26793, RRID:AB_794297), rat anti-MBP aa 82-87 (Millipore, Cat# MAB386, RRID:AB_94975), rabbit anti-Olig2 (Millipore, Cat# AB9610, RRID:AB_570666), mouse mAB anti-CC1 (Millipore Cat# OP80, RRID:AB_2057371), rat anti-GFAP monoclonal antibody 2.2B10 (Thermo Fisher Scientific Cat# 13-0300, RRID:AB_2532994), rabbit anti-Iba1 (Wako Cat#019-19741, RRID:AB_839504), rat anti-tenascin-C (R&D Systems, Cat# MAB2138, RRID:AB_2203818), and goat anti-tenascin-R (R&D Systems, Cat# AF3865, RRID:AB_2207009). Cross absorbed secondary antibodies were from Thermo Fisher Scientific: donkey anti-rabbit Alexa 488 (Cat# A21206, RRID:AB_2535792); donkey anti-goat Alexa 488 (Cat# A32814TR; RRID: AB_2866497); donkey anti-rat Alexa 594 (Cat# A-21209; RRID:AB_2535795); goat anti-rabbit Alexa 594 (Cat# A11037; RRID:AB_2534095); goat anti-mouse Alexa 594 (Cat# A11005; RRID:AB_2534073); goat anti-rat Alexa 647 (Cat# A-21247, RRID:AB_141778); donkey anti-rabbit Alexa 647 (Cat# A-31573, RRID:AB_2536183); goat anti-mouse Alexa 647 (Cat# A-21235, RRID:AB_2535804). Negative signals in controls without primary antibodies and the use of primary antibodies of inappropriate species established specificity of the secondary antibodies.

## Staining of brain tissue

Coronal sections (30 µm; Leica cryostat CM3050S) were cut in anterior to posterior direction starting at the level of bregma -1.58 mm. To estimate the status of myelination, brain sections were mounted onto gelatin-coated slides for staining with Black Gold II (Biosensis, Thebarton, SA, Australia; Cat #TR-100-BG). Free floating immunofluorescence staining was performed to detect different neural cell types and extracellular matrix proteins. Briefly, after washing the brain sections in PBS, the FAM signal was amplified with an anti–FITC polyclonal antibody (Thermo Fisher Scientific, Cat# PA1-26793, RRID:AB_794297) or fluorescein/Oregon green polyclonal antibody (Thermo Fisher Scientific, Cat# A-889; RRID:AB_221561). Primary antibodies used were chicken anti-GFAP (Neuromics, Cat#CH22102, RRID:AB_10014322), rabbit anti-MBP (Abcam, Cat# ab-40390, RRID:AB_1141521), rabbit anti-Iba1 (Wako, Cat# 019–19741, RRID:AB_839504), goat anti-tenascin R (R&D Systems, Cat# AF3865, RRID:AB_2207009), and rat anti-tenascin C (R&D systems, Cat# MAB2138, RRID:AB_2203818) in 5% normal donkey serum (Jackson Immuno Research Cat# 017-000-121) at room temperature overnight. The tissue was washed thrice in PBS the next day for 5 min each and incubated with the corresponding secondary antibody (goat anti-chicken Alexa Fluor 568, Thermo Fisher Scientific, Cat#A-11041, RRID:AB_2534098; donkey anti–rabbit Alexa Fluor 568 Invitrogen, Cat#A-10042, RRID:AB_2534017; goat anti-rat IgM Alexa Fluor 647, Thermo Fisher Scientific, Cat# A21248, RRID:AB_2535816; donkey anti-goat Alexa Fluor 647, Thermo Fisher Scientific, Cat# A21447, RRID:AB_2535864) in 0.3% TritonX-100/PBS for 1.5 h at room temperature in the dark. Care was taken to minimize exposure to light from this step onward. After washing thrice in PBS, the sections were mounted onto gelatin-coated slides and coverslipped with a mounting medium containing DAPI (Vectashield, Cat#H-1500).

## Imaging

Sections with fluorescent labels were imaged on the Zeiss LSM 710 NLO Multiphoton microscope. Histological brain sections were analyzed using a Keyence microscope (BZ-X-800 series). ImageJ version 1.52q[1] was used for image processing.

### Data reproducibility

For each time point and condition, 3–5 animals were used, and from each animal 2–4 sections were examined to obtain results.

# Results

## Mouse models of demyelination

To probe the ability of CAQK peptide to associate with sites of demyelination injury, we employed multiple mouse models that reflect different aspects of acute and chronic demyelination (Denic et al., 2011; Ransohoff, 2012). Rapid focal demyelination with immune cell infiltration and a compromised blood-brain barrier was induced by a local lysophosphatidylcholine (LPC; lysolecithin) injection into the spinal cord. In this model, acute spatiotemporally precise damages to the lipid-rich myelin membrane sheaths are observed at the injection site as early as 24 h with continuing demyelination for up to 7 days after injury (Hall, 1972; El Waly et al., 2014). In the toxin-induced cuprizone model, demyelination occurs in mice fed the cuprizone diet with a highly reproducible time course and well-defined mechanisms in the corpus callosum and more variable results in other fiber tracts (Skripuletz et al., 2011; Praet et al., 2014). Cuprizone is a copper chelator that induces mitochondrial toxicity and death of oligodendrocytes. After 6-weeks on the cuprizone diet, mice show strain-reproducible demyelination in the brain with well-characterized spatiotemporal cellular changes in the corpus callosum in the presence of a nearly closed blood-brain barrier (Praet et al., 2014). Finally, chronic inflammatory demyelination that most closely mirrors features of human MS occurs in the EAE model (Constantinescu et al., 2011; Hasselmann et al., 2017). Here, peripheral administration of myelin protein peptides results in the activation of peripheral T-cells that transgress the blood-brain barrier where they induce inflammatory myelin damage accompanied by neurodegeneration and gliosis with simultaneous counteracting anti–inflammatory processes and remyelination. EAE mice develop ascending paralysis after EAE induction with the most severe clinical manifestations in the spinal cord permitting the monitoring of disease severity. Utilizing these models allowed us to assess CAQK peptide association in the demyelinated brain and spinal cord under different pathophysiological conditions.

## CAQK peptide homes to sites of focal demyelination inflicted by acute lysolecithin injection

Acute focal demyelination induced by stereotactic injection of lysolecithin results in a defined injury site with an open blood-brain barrier like in the TBI model plus has high selective toxicity to oligodendrocytes and myelin while leaving neurons and axons largely intact (Blakemore and Franklin, 2008). Demyelination is detectable within 24 h and reaches a maximum by 7 days post LPC injection after which remyelination occurs (El Waly et al., 2014). We employed this rapid model to test the hypothesis that CAQK peptide homes to injured myelin. A unilateral injection of 1% lysolecithin was made into the ventral funiculus of the mouse spinal cord to induce the lesion. FAM-CAQK was administered intravenously at a concentration of 100 nmoles/100 μl at 24 h or 5 days post-injury and allowed to circulate for 1 h before perfusion and tissue harvesting. Sites of demyelinating injury correlate with the accumulation of $DAPI^+$ stained mononuclear cells (Luo et al., 2014; Bielecki et al., 2016; Lozinski et al., 2021). We utilized this characteristic to localize the lesion area and detect the FAM-CAQK label in the ventral spinal cord white matter near the injection site. As illustrated for the 5-day post lesion time point, we observed the accumulation of FAM-CAQK peptide at the injury site identified by an increased density of mononucleated $DAPI^+$ cells and fragmented myelin stained for myelin basic protein (MBP; **Figures 1A–D**). The peptide was not detected in the spinal cords of control sham-operated (PBS injected) mice showing regular densities of $DAPI^+$ cells (**Figures 1E–H**), and secondary antibody controls at a DAPI-dense lesion site showed no signals (**Figures 1I–L**). Similar results were obtained for the 24 h post lesion time point (not shown). These observations supported the ability of CAQK to home to lesion sites in an established mouse demyelination model and encouraged further studies in long-term mouse models of demyelination.

## CAQK homes to the demyelinated corpus callosum in the cuprizone model

To attain further evidence for the targeting of CAQK peptides to sites of demyelinating injury, we employed additional mouse models of established demyelination. First, since acute LPC-induced demyelination is induced by inflicting an open wound that disrupts the blood-brain barrier and leads to immune cell accumulation, we sought to investigate CAQK

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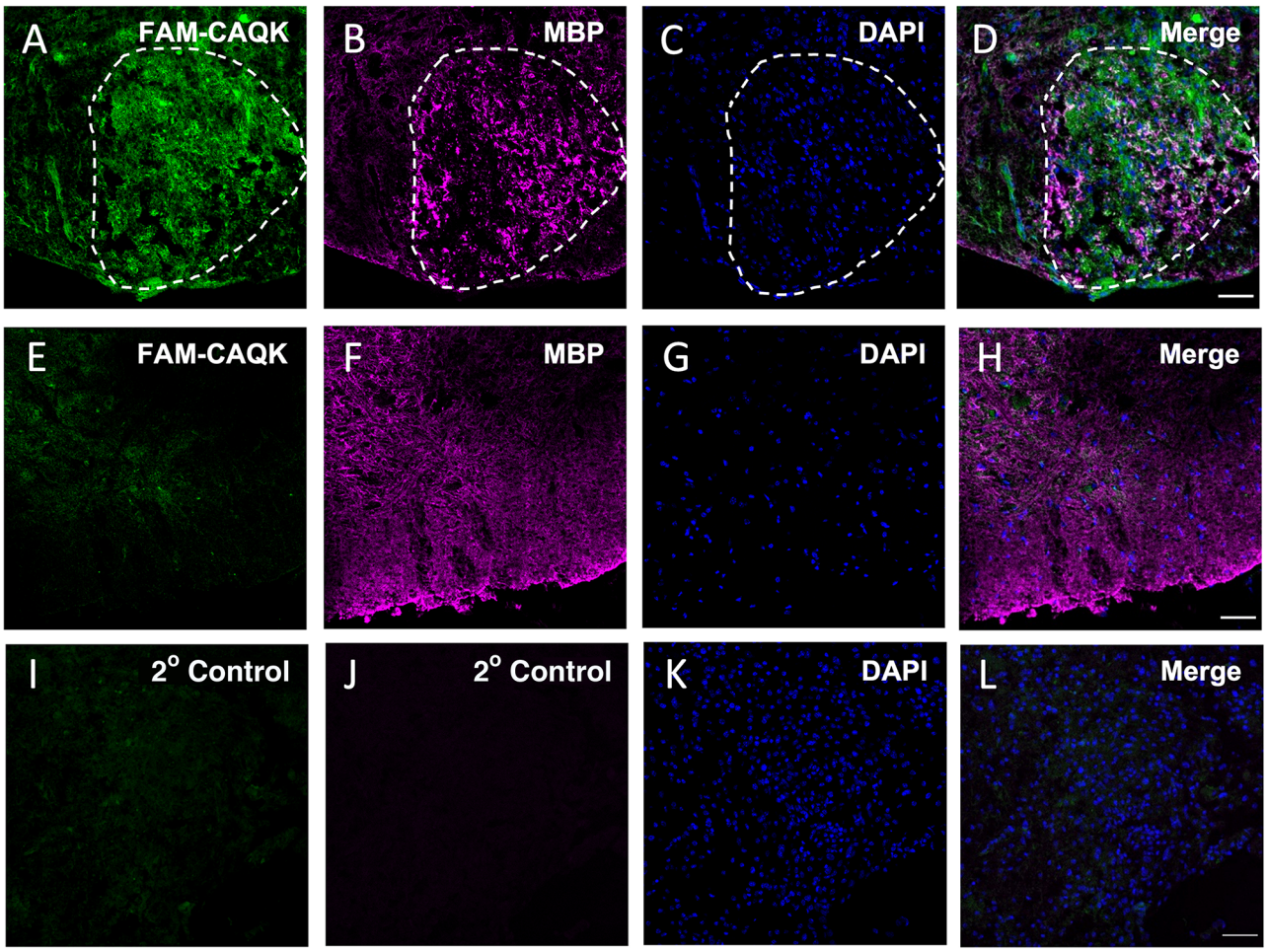


FIGURE 1
CAQK homes to lysolecithin-induced focal lesions in the spinal cord. FAM-CAQK (green) accumulates at lesion sites 5 days after LPC injection (**A–D**, LPC). The lesion area outlined by white dashes was identified by disorganized MBP staining (**B**, magenta) and the increased density of mononucleated DAPI$^{+}$ cells (blue in **C,K**) compared to healthy tissue (blue in **G**). FAM-CAQK peptide **(E)** does not accumulate in white matter (**F**, MBP, magenta) in sham-operated, PBS-injected spinal cord tissue (**E–H**, sham). Panels **(I–L)** show the corresponding secondary antibody controls around a lesion site in LPC-injected tissue. *N* = 3–4 mice per group. Scale bar 50 μm.

targeting in a model of demyelination that is independent of peripheral immune cell invasion. Feeding mice a diet containing the neurotoxin cuprizone leads to myelin loss in central white matter tracks (Praet et al., 2014). Demyelination extends to multiple brain areas, including the cortex and hippocampus, and is well-defined and highly reproducible in the corpus callosum in the C57Bl6 mouse strain (Skripuletz et al., 2008; Schmidt et al., 2013). Although early alterations of the BBB have been noted (Skripuletz et al., 2011; Berghoff et al., 2017), the blood-brain barrier is intact in C57BL/6 mice on the cuprizone diet for 6 weeks allowing studies of demyelination in the absence of a peripheral immune response. Thus, to characterize CAQK peptide targeting under non-inflammatory conditions and an intact (or minimally altered) BBB, we fed 8-week-old mice a 0.2% cuprizone diet for 6 weeks (Praet et al., 2014), and then assessed homing of FAM-CAQK peptide to demyelinating fiber tracts of the corpus callosum.

The cuprizone diet produced pronounced demyelination, most evident by the reduction in Black Gold II staining in the corpus callosum (**Figure 2A**). For comparison, the full extent of myelination is shown in naïve mice fed a normal diet (**Figure 2D**). Consistent with the reported loss of myelin from several brain areas in mice on the 6-week cuprizone diet, FAM-CAQK peptide was detected in cortical and hippocampal areas and the corpus callosum (**Figures 2B,C**). In contrast, the peptide did not associate with normal healthy myelin in the brains of naïve mice (**Figures 2E,F**).

To determine if CAQK associates with specific structures within the demyelinated corpus callosum, brain sections from peptide-treated mice fed the cuprizone diet were stained

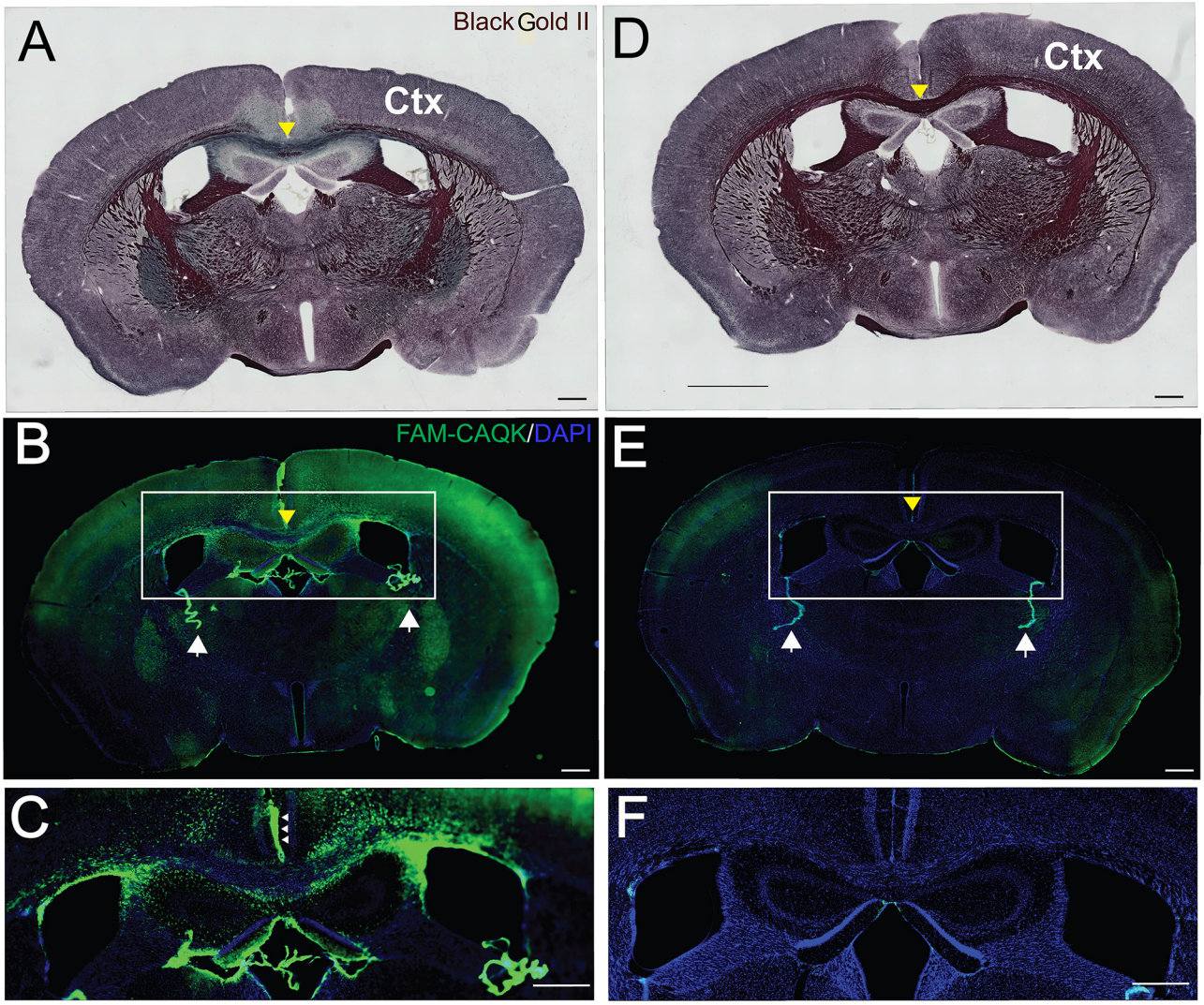


FIGURE 2
CAQK peptide targets areas of demyelination in the cuprizone model. Histological Black Gold II staining detects myelin in coronal brain sections of **(A)** FAM-CAQK peptide-treated cuprizone treated mice and **(D)** naïve mice. Demyelination is indicated by the reduction of Black Gold II labeled myelin in multiple brain areas **(A)** in mice on the cuprizone diet compared to **(D)** control mice on a normal diet. Note in particular the reduced black myelin signal in the corpus callosum in **(A)** (yellow arrowhead) compared to **(D)**; images at 10× magnification. **(B)** FAM-CAQK peptide (green) targets the demyelinated areas including the cortex (Ctx) in cuprizone-treated mice, and in **(E)** is absent from myelin in the naïve brain; images at 20× magnification with identical exposure time. In both conditions, circulating peptide associates with the choroid plexus (white arrows in **B,E**) that separates the blood from cerebral spinal fluid. The framed regions with the centered corpus callosum (yellow arrow) are shown enlarged in **(C,F)**, correspondingly. Ependymal cells facing the cerebral spinal fluid (small arrows in **C**) harbor the peptide. DAPI staining (blue) demarcates cellular nuclei in **(B,C,E,F)**. Scale Bar = 500 μm.

for cell type and myelin specific markers, including glial fibrillary protein (GFAP) for astrocytes (Figure 3B), MBP for myelin (Figure 3E) and ionized calcium binding adaptor molecule 1 (Iba1) for resident microglia (Figure 3H). Images taken from the medial corpus callosum show FAM-CAQK peptide staining (Figures 3A,D,G) on fibrous extracellular material associated with GFAP+ reactive astrocytes that are highly upregulated in this area (Figures 3B,C). FAM-CAQK signal also extended beyond this region to include cells with intermediate to low GFAP expression around fibers of the fornix and the dorsal hippocampal commissure (Figure 3C). Consistent with published work analyzing myelin after 6 weeks on the cuprizone diet, myelin was largely lost as indicated by a modest staining signal for MBP (Figures 3E,I; see Praet et al., 2014). Little if any of the FAM-CAQK signal overlapped with the MBP+ staining. Iba1+ microglia (Figure 3H) that are sparse at the 6-week cuprizone feeding timepoint (Praet et al., 2014)

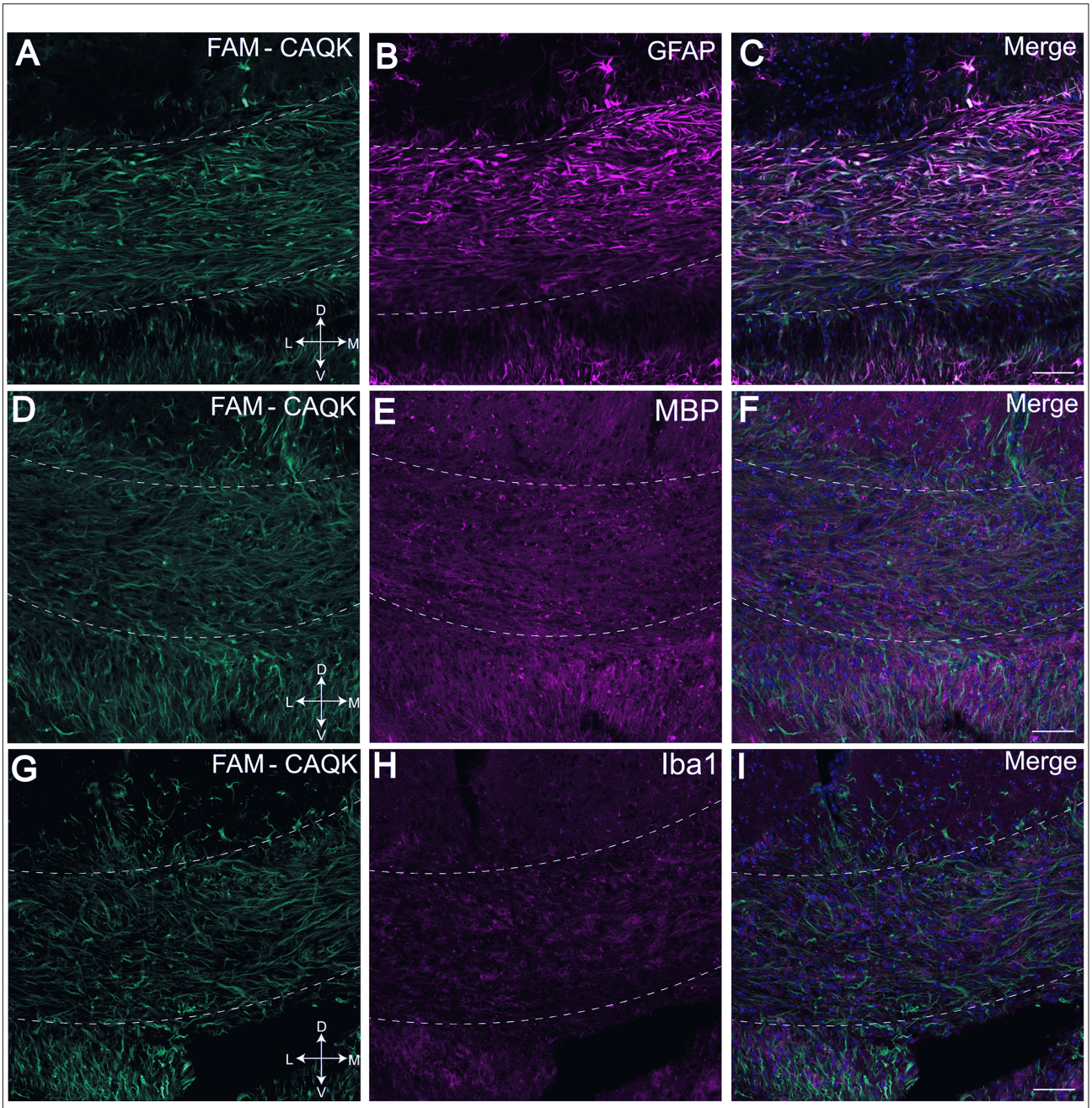


FIGURE 3
FAM-CAQK peptide associates with reactive astrocytes in corpus callosum during cuprizone-mediated demyelination. FAM-CAQK peptide is targeted to the demyelinating crossing fiber tracts in the corpus callosum (hatched lines) in mice after 6 weeks on the cuprizone diet (**A,D,G**, green). Peptide association (in **C**) is prominent with GFAP+ reactive astrocytes (**B**, magenta). Staining for MBP+ myelin (**E**, magenta) and microglial Iba1 (**H**, magenta) is marginal at the 6-week feeding point and no association of FAM-CAQK is evident; merged images **(C,F,I)** respectively. D, dorsal; V, ventral; L, lateral; M, medial. Scale bar = 50 μm.

also did not show FAM-CAQK peptide association. For controls, mice fed a normal diet did not accumulate FAM-CAQK peptide in the normal myelinated corpus callosum (**Supplementary Figure S1**).

Finally, as FAM-CAQK was reported to associate with extracellular matrix components in a mouse model of traumatic brain injury (Mann et al., 2016), we tested its association with extracellular matrix proteins in the cuprizone model. Tenascin-C and tenascin-R, components of the ECM produced at CNS injury sites mainly by reactive astrocytes are dynamically remodeled during demyelinating injury (Zhao et al., 2009) and were chosen for analysis. Examination of FAM-CAQK

association with tenascin-R in the corpus callosum revealed minimal, if any overlap (**Figures 4A–C**). The images indicate that peptide association within the demyelinated corpus callosum was strongest in areas of low tenascin-R deposition (**Figure 4C**). Association of FAM-CAQK (**Figure 4D**) with tenascin-C (**Figure 4E**) that was weakly expressed in the demyelinated corpus callosum at the examined timepoint was restricted to only a few puncta (**Figures 4D–F**). These results illustrate the ability of FAM-CAQK peptide to home from the circulation through a largely intact BBB to areas of demyelination where it strongly associates with reactive astrocytes surrounded by fibrous extracellular material. The timepoint of our study thus did not yield convincing evidence for the association of the peptide with ECMs of the tenascin family and suggested targeting epitopes on yet unidentified molecules.

## CAQK targets demyelinating regions in the spinal cord in the chronic EAE model

The homing of CAQK peptide in toxin-induced demyelination models prompted further investigations of CAQK targeting under pathophysiological conditions that are closely related to human MS. The chronic mouse EAE model and human MS share several critical features: In both conditions, peripherally activated immune cells infiltrate the brain parenchyma where they cause an amplified immune response that results in tissue damage, demyelination, gliosis and ultimately axon loss. In the widely used EAE model, tissue deficits in the spinal cord correlate with the formation of mononucleated cell clusters and progressive paralysis in experimental animals (Voskuhl et al., 2009; Levy-Barazany and Frenkel, 2012; Hasselmann et al., 2017). We reproducibly induced EAE with an average onset of 7–8 days and peak at 14–15 days by injection of $MOG_{35-55}$ peptides in groups of 10 or 20 mice (**Figure 5A**). Focal perivascular lesions in the spinal cords of experimental mice were indicated by clusters of infiltrating DAPI-stained mononucleated cells (**Figures 5B–D**). CAQK homing was assessed by intravenous injection of FAM-CAQK peptide at disease onset (score 1), peak (score 3–3.5), and at late stages (28 days). FAM-CAQK showed robust targeting to lesion clusters of $DAPI^+$ cells indicating immune cell invasion into the outer rim of the spinal cord parenchyma. In **Figure 5**, taken from a mouse at peak score, FAM-CAQK peptide targeting is shown in two lesions identified by accumulation of DAPI-stained nuclei in ventral white matter (**Figures 5B–D**). No peptide is seen in surrounding healthy spinal cord tissue. Similar results were obtained at EAE onset (score 1) and at late stages (28 days post-immunization, data not shown). Control mice injected with PTX only did not develop EAE and did not indicate targeting of FAM-CAQK peptide into the CNS (not shown).

## CAQK associates in proximity of cells located at lesion sites in the EAE model

As CAQK is associated with demyelinating lesions in an amorphous pattern, we next queried by double labeling whether the peptide targets specific cell types. Spinal cord sections from EAE mice treated at disease onset (score 1) with FAM-CAQK peptide were used for analyses. Peptide association with oligodendrocytes was assessed in mice expressing the fluorescent tdTomato reporter in transgenic CNP1-Cre mice (Zhao et al., 2009). In contrast to observations in the TBI model (Mann et al., 2016), oligodendrocytes showed no or only a rare association with CAQK peptide (**Figures 6A–C**). However, we detected CAQK in association with reactive astrocytes and cells that make up the immune response. Examination of spinal cord sections revealed a gross overlap of FAM-CAQK with GPAP+ reactive astrocytes at lesions sites at the tissue rim (**Figures 6D–F**). Staining for microglial cells labeled with anti-Iba1 antibody (**Figures 6G–I**) or for CD3+ immune cells (**Figures 6J–L**) similarly showed partial overlap with the FAM-CAQK peptide signal. These results suggest that in the inflammatory demyelination EAE model, FAM-CAQK peptide targets lesions and localizes in proximity to infiltrating immune cells, microglia, and reactive astrocytes.

## CAQK associations with tenascins in EAE demyelinating lesions

Reactive astrocytes contribute to changing the extracellular milieu at CNS injury sites (Roll and Faissner, 2019). The ECM molecule tenascin-C is absent in the healthy adult CNS but upregulated and deposited by reactive astrocytes at CNS injury sites (Roll and Faissner, 2019). Tenascin-R, expressed by astrocytes and oligodendrocytes in the adult CNS also undergoes extensive remodulation during CNS injury (Zhao et al., 2009). As CAQK was reported to bind components of an ECM complex, including tenascin-R, in the TBI model (Mann et al., 2016) and we consistently observed extracellular fibrous structures harboring the peptide, we next investigated FAM-CAQK association in combination with tenascins. First, staining for MBP showed in the EAE model that FAM-CAQK associates only marginally, if at all, with myelin membranes during demyelination (**Figures 7A–D**) similar to the findings in the cuprizone model (**Figures 3D–F**). Tenascin-R was detected in a uniform distribution over the uninjured spinal cord. In the illustrated example of demyelinating injury, identified by clusters of DAPI-stained mononucleated cells, tenascin-R deposits were mainly observed in healthy spinal cord tissue and appeared downregulated from the lesion area at the rim (**Figures 7F,G**). FAM-CAQK covered the cluster of $DAPI^+$ cells at the lesion rim and accordingly showed no or only

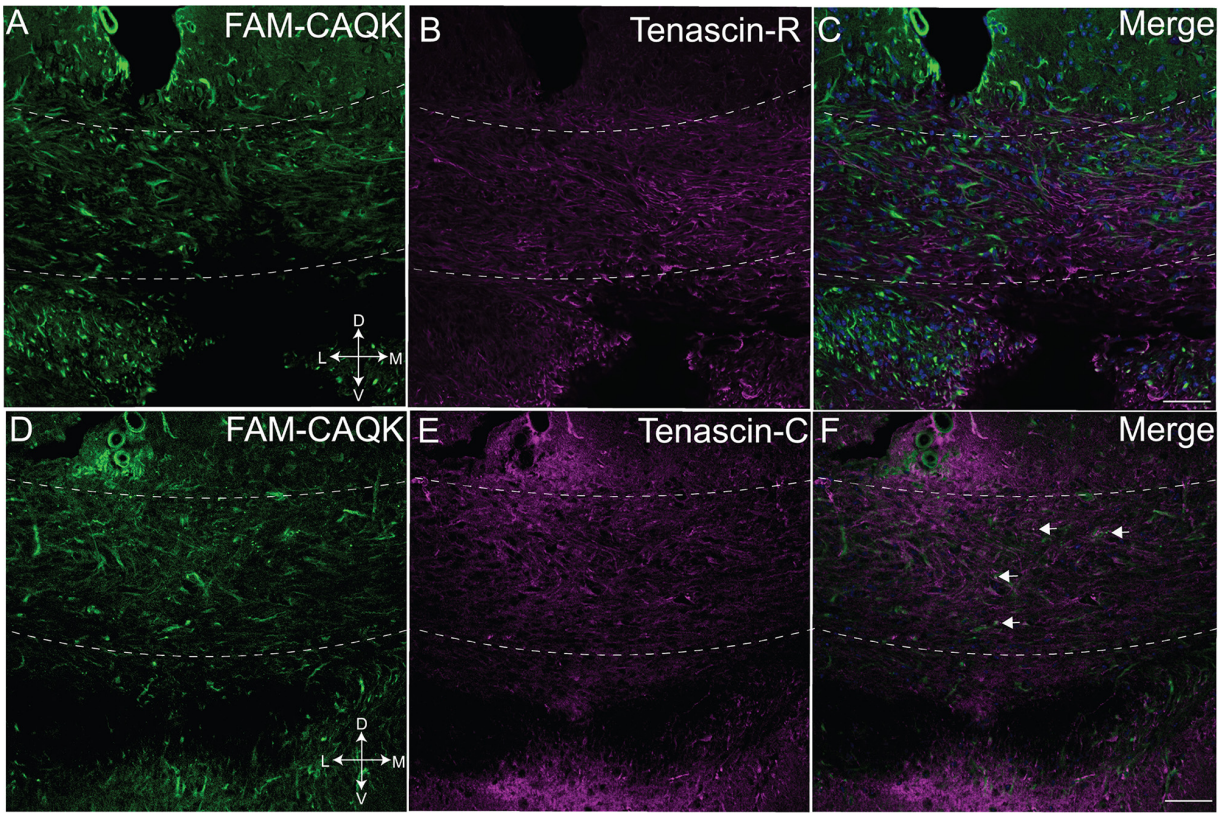


FIGURE 4
Marginal CAQK peptide association with tenascins in the cuprizone model. Confocal images from the demyelinating corpus callosum of mice on a 6-week cuprizone diet show FAM-CAQK peptide (**A,C**, green) in complementary distribution with tenascin-R (**B**, magenta); merged images in **(C)**. FAM-CAQK peptide **(D)** shows marginal association (white puncta pointed out by arrows in **F**) with tenascin-C (**E** magenta). DAPI-staining (blue) indicates cell nuclei in **(C,F)**. D, dorsal; V, ventral; L, lateral; M, medial. Scale bar = 50 μm.

marginal overlap with tenascin-R (Figures 7E–H). Tenascin-C was selectively upregulated in interstitial spaces within $DAPI^+$ EAE lesion sites and absent from surrounding healthy CNS tissue (Figures 7K,L). The FAM-CAQK signal overlapped in a fibrous pattern in regions of high tenascin-C accumulation and also covered a large region within the lesion that was devoid of tenascin depositions (Figures 7I–L). Thus, our data provide evidence for FAM-CAQK association with epitopes codistributing with tenascin-C, and simultaneously suggest additional yet unidentified peptide interactions with binding sites exposed on other cellular or extracellular molecules in demyelinating regions.

## Discussion

In this study, we report that the four amino acid peptide CAQK selectively targets demyelinating lesions. We show that upon systemic administration and 1 h in circulation, CAQK homes specifically to sites of CNS demyelinating injury and accumulates reliably and reproducibly at sites of myelin damage. We document these homing capacities in three different demyelination models, toxic, chemical, and inflammatory/autoimmune. Both cuprizone and lysolecithin-induced demyelination models are highly reproducible in the time course of demyelination with various degrees of BBB opening and neuroinflammation. CNS access of CAQK peptide through the compromised BBB was previously indicated from studies in the TBI model (Mann et al., 2016) and evident in the focal demyelination lysolecithin model. However, the suggestion that FAM-peptide can reach demyelinating lesions through the nearly closed endothelial barrier is novel. In the cuprizone model at the 6-week timepoint analyzed here, a tight BBB was documented in earlier studies (Bakker and Ludwin, 1987; Kondo et al., 1987; McMahon et al., 2002; Praet et al., 2014). Recent reports re-examining the BBB in the cuprizone model at early and late timepoints also confirmed almost intact barrier functions at the 6-week timepoint. These latter reports indicated compromised BBB permeability at the onset and during the first weeks of cuprizone feeding and progressive repair towards a nearly closed BBB at the 6-week timepoint (Berghoff et al., 2017; Shelestak et al., 2020). How CAQK enters the CNS and how

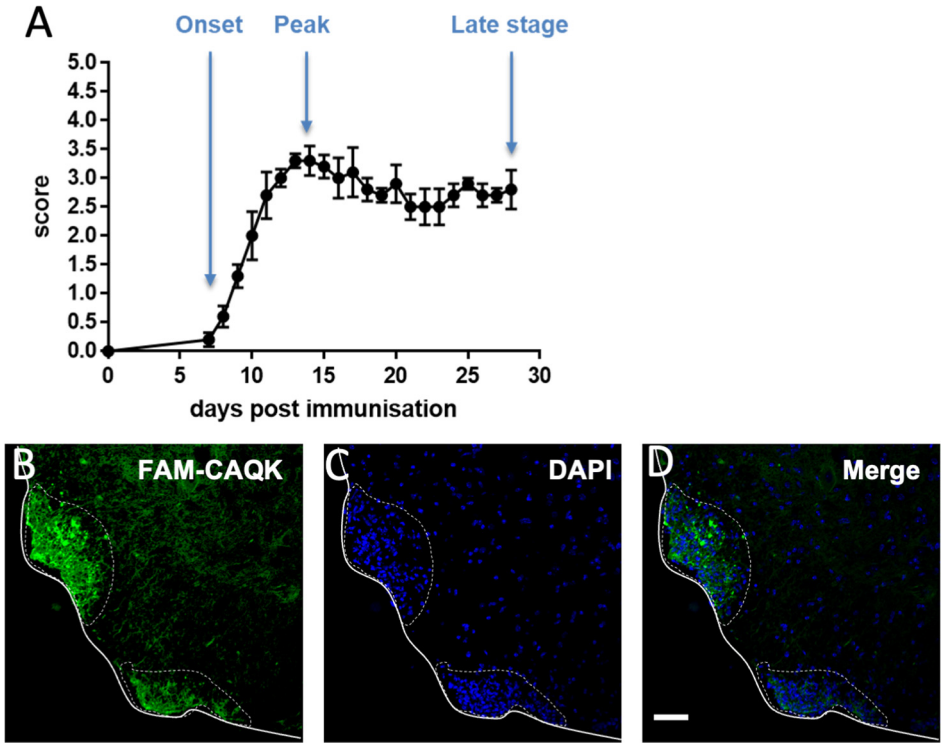


FIGURE 5
CAQK homes to demyelinating lesions in the mouse model of experimental autoimmune encephalomyelitis (EAE). **(A)** Disease scores over a 28-day period show EAE progression with an average 7-day onset and a 14-day peak. Control mice show no disease. **(B)** FAM-CAQK (green) accumulates at lesion sites in the lumbar spinal cords of mice with EAE. Lesions are indicated by clusters of DAPI-stained nuclei (blue) in **(C)** representing invasions of peripheral inflammatory cells, prominently T-cells and macrophages, into the spinal cord parenchyme. Merged images are shown in **(D)**. Continuous line delimitates the spinal cord tissue. The dotted line indicates area of active EAE lesion. Scale bar: 50 μm.

it interacts with endothelial cells comprising the BBB requires detailed future studies that assess mechanisms in various models and coupled to different tags, therapeutic molecules, or targeting particles. The consistency of peptide targeting to lesion sites in three mouse models of demyelination suggests that CAQK is reliable as a targeting vector for demyelinating lesions in the CNS under diverse states of the BBB.

## CAQK targets components of the ECM

The extracellular matrix plays an important role during CNS development, homeostasis, and injury providing cues that regulate cell adhesion, signaling, and motility (Ruoslahti, 1996; Lau et al., 2013; Su et al., 2021). ECM components are secreted by resident cells and assemble into a scaffold in interstitial spaces to regulate a myriad of cellular responses. During white matter injury, the composition of the ECM dynamically changes as hyaluronan is proteolytically fragmented and CSPGs and tenascins are upregulated or remodeled (Lau et al., 2013; de Jong et al., 2020; Srivastava et al., 2020; Diao et al., 2021; Ghorbani and Yong, 2021). The altered ECM is inhibitory to OPC maturation, OPC recruitment to lesion sites, and remyelination (Franklin and Ffrench-Constant, 2008; Czopka et al., 2010).

In this light, it is significant that in the three demyelination models studied here, FAM-CAQK was targeted from the circulation to fibrous extracellular material at lesion sites. Original work from the Ruoslahti laboratory reported CAQK interactions with an extracellular matrix complex composed of versican, hyaluronan, proteoglycan link protein (Hapln), and tenascin-R by affinity purification of injured brain tissue and confirmed peptide colocalization by immunohistochemistry in the TBI model (Mann et al., 2016). Specific CAQK targeted epitopes within the lesioned ECM or its receptors, however, have remained elusive. In the current study, we chose to assess two ECMs, tenascin-R and tenascin-C that are dynamically regulated in white matter lesions and in TBI (George and Geller, 2018). In both the cuprizone and the EAE models at the time points examined, tenascin-R showed lower abundance at lesion sites than in healthy surrounding tissue, and accordingly, no mentionable peptide overlap with tenascin-R was observed. In contrast, tenascin-C

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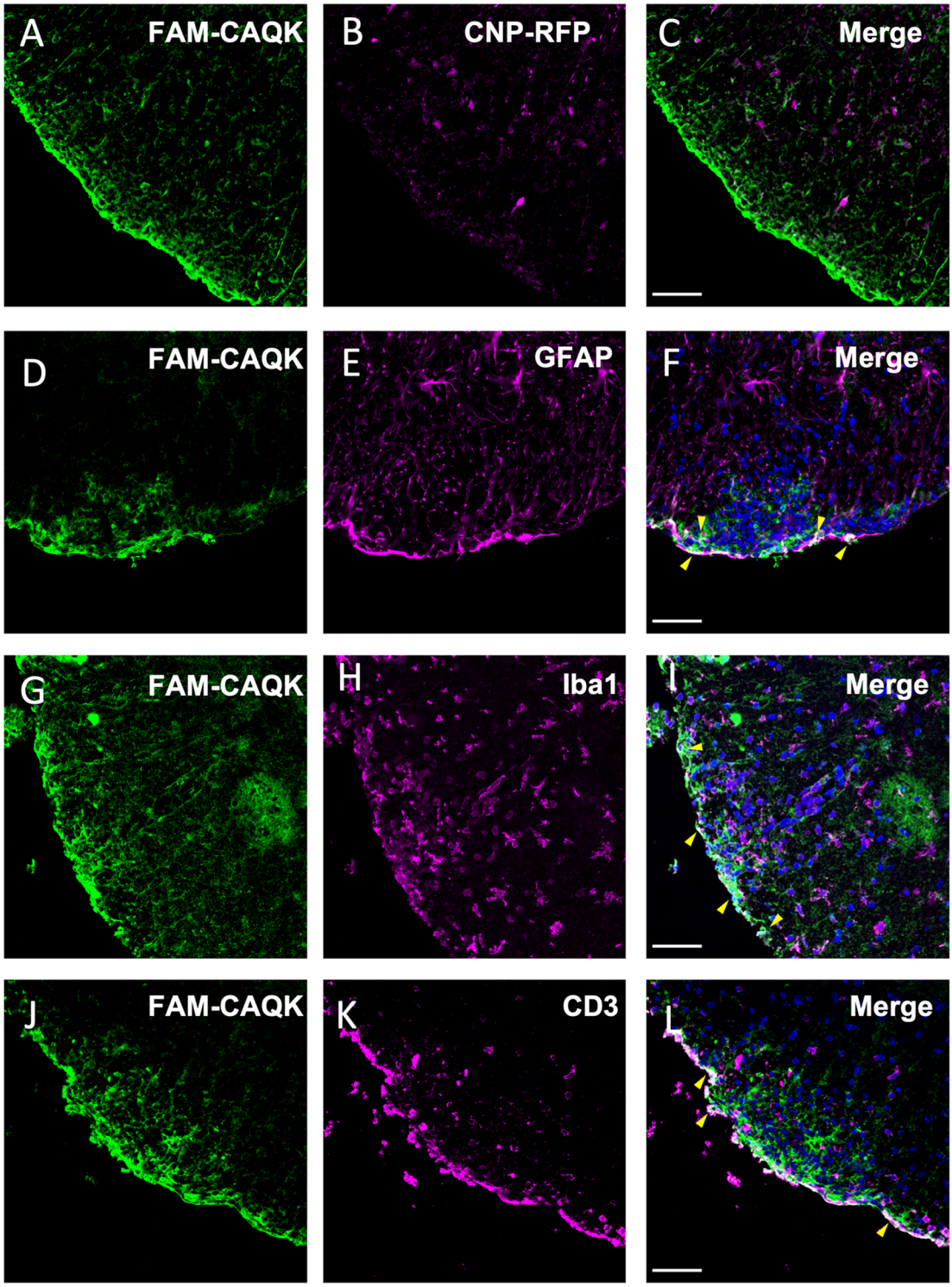


FIGURE 6
Association of CAQK with invading immune cells and reactive astrocytes at lesions in the EAE model. Fluorescent FAM-CAQK (green in **A,D,G,J**) homes to lesion sites identified by $DAPI^{+}$ cell clusters (blue in **C,F,I,L**) after intravenous application at disease peak. The peptide does not associate with oligodendrocytes (**B**, magenta) genetically labeled in CNP1-Cre;Rosa26-tdTomato transgenic mice. Merged images in **(C)**. At the gross level, CAQK peptide associates with cells characterizing lesion sites, including reactive astrocytes (GFAP, **E**, magenta), microglia (Iba1, **H**, magenta) and infiltrating immune cells (CD3, **K**, magenta). Merged images are shown in **(C,F,I,L)**, and overlaps indicated in white color are pointed out with yellow arrowheads in **(F,I,L)**. Scale bars: 75 μm.

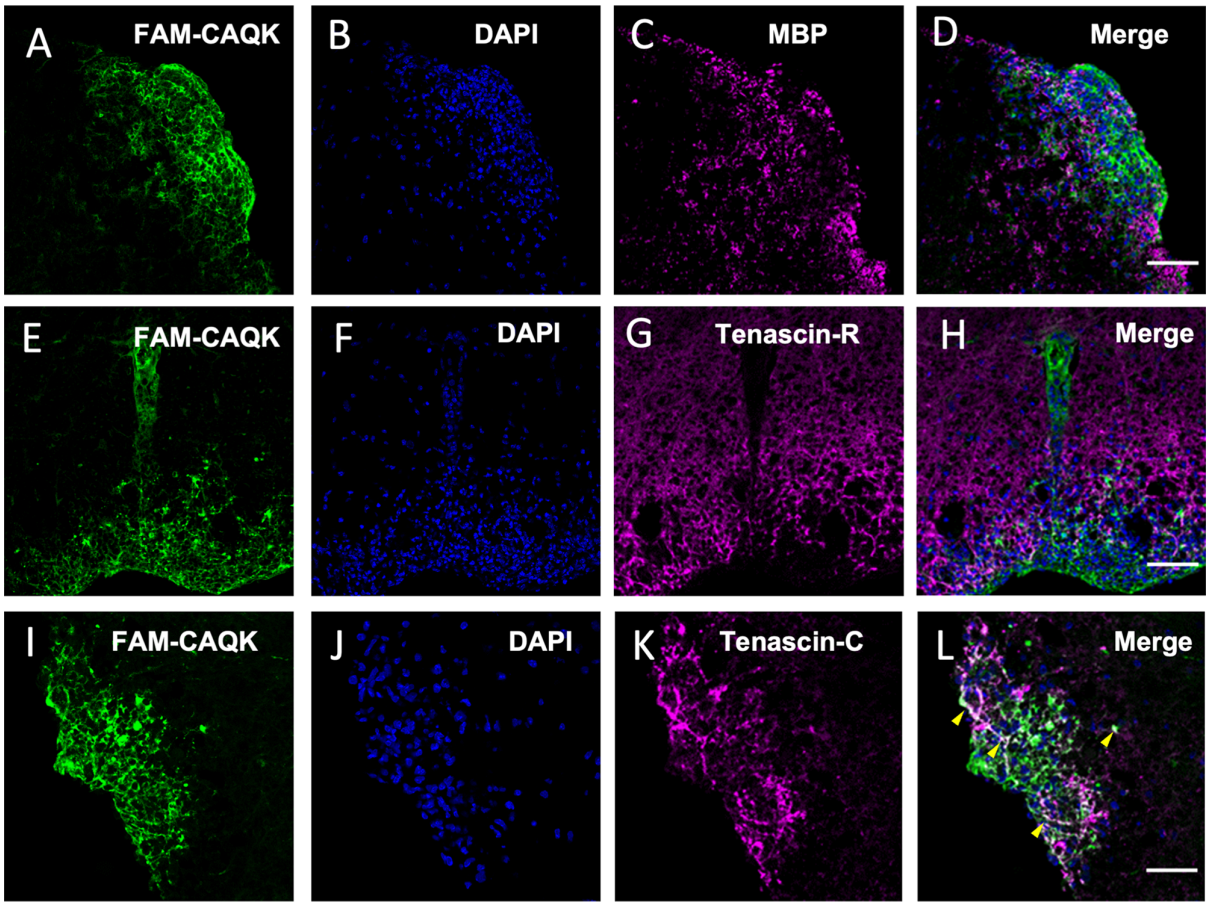


FIGURE 7
CAQK and tenascins in EAE lesions. **(A–D)** Confocal images identify FAM-CAQK peptide (**A,E,I**, green) at DAPI$^{+}$ lesion clusters (**B,F,J**, blue) in the lumbar spinal cord of mice with EAE. The peptide does not associate with damaged myelin stained for MBP (**C**, magenta; merge in **D**) and largely complements staining for tenascin-R (**G**, magenta; merge in **H**). Some overlap is detected between FAM-CAQK and fibrous tenascin-C deposition (**K**, magenta) as indicated by the white signal in the merged image (**L**, arrowheads). Note that the majority of the lesion site covered by the peptide is negative for either tenascin **(H,L)**. Scale bars: 20 μm.

deposits appeared to differing degrees within demyelinating lesions in the two models. In the EAE but not in the cuprizone model, CAQK localized to fibrous tenascin-C deposits suggesting that its target sites include epitopes in the local ECM. It is important to note, however, that most of the peptide targeted lesion area was devoid of tenascin-C expression. This leaves open the nature of the peptide interacting molecules at sites of demyelination and studies assessing peptide targeting during the dynamic progression of de-and remyelination will need to provide additional insights.

Our results are mixed regarding the question whether FAM-CAQK peptide homes to specific cellular targets. In none of the current models, the peptide associated with MBP+ demyelinating fibers at lesion sites, and contrary to findings in the TBI model (Mann et al., 2016) oligodendrocytes did not take up the peptide. At gross view, we find populations of reactive astrocytes associated the peptide in all three demyelination models. While our data cannot distinguish the cellular or pericellular distribution of the peptide with reactive astrocytes in the spinal cord models, FAM-CAQK seemed to reside in the cytoplasm of GFAP+ glia in the non-inflammatory cuprizone model (Figure 3). If confirmed, this could suggest autophagic activity during the 1 h in the circulation. In the inflammatory EAE model, CD3+ immune cells and Iba1+ microglia that enter the spinal cord parenchyma to initiate demyelinating lesions sequestered FAM-CAQK peptide from the circulation. Thus, the CAQK peptide associated with cell populations characterizing sites of injury supporting the conclusion that the peptide targets sites of demyelinating injury. Further work will be required to understand the nature of the peptide's interactions with cells and whether phagocytosis of peptide-associated interstitial components by reactive astrocytic cells and microglia or other parameters are at play.

## Potential use of CAQK peptide to achieve remyelination after injury

Remyelination of demyelinated axons restores function and is important for preventing axonal and neuronal degeneration and thus functional and cognitive decline. The findings from the current study raise the possibility for translational purposes in diagnosis and therapeutic applications in aiding myelin repair. Because of its small size, CAQK is a candidate for biomedical imaging, for example for visualizing demyelinating lesions in MRI or PET scans, with CAQK coupled to ultra small super paramagnetic iron oxide nanoparticles (USPIO), or to radioactive tracers, respectively. These technologies are in development for the diagnosis of cancers (Wu et al., 2011; Novy et al., 2019; Rangger and Haubner, 2020), fibrosis (Belkahla et al., 2020), and detection of amyloid plaques in Alzheimer's models (Wadghiri et al., 2013). Engineering CAQK peptides to adapt to medical imaging might aid earlier diagnosis of white matter lesions and demyelinating diseases.

A highly promising approach, already shown to work in animal models, is to couple the peptide to suitable transport carriers to target and deliver the therapeutic payload. In their original work, Ruoslahti and colleagues provided proof of this principle by targeting siRNA encapsulated in CAQK-coated porous nanoparticles to TBI lesions and locally suppressing gene expression for several days (Mann et al., 2016). Follow-up work in a spinal cord injury model engineered CAQK for exposure on mesenchymal stem cell-derived exosomes to deliver CRISPR/Cas9 plasmids suppressing the pro-inflammatory actions of tumor necrosis factor TNFα. CAQK-mediated targeting the injury site reduced inflammatory TNFα/NFκB signaling pathways, achieved tissue recovery, and restored motor function (Wang et al., 2022). Coupling of CAQK to nanoparticles could similarly enhance the targeting of therapeutic payloads to sites of demyelinating lesions thereby reducing toxicity and off-site effects (Liu et al., 2021). Neutralizing inhibitors of remyelination could enhance OPC recruitment and foster myelin repair, for example by blocking tenascins (Lau et al., 2013; Ghorbani and Yong, 2021; Su et al., 2021), ECM CSPGs (Lau et al., 2012; Keough et al., 2016), Hyaluronan (Back et al., 2005), Lingo-1 (Mi et al., 2008), or components of the Wnt and Notch pathways (John et al., 2002; Williams et al., 2007; Fancy et al., 2009; Syed et al., 2011). Conversely, several drugs including benztropine, clemastin, quetiapine, clobestasol, and minonazole show effects in improving OPC recruitment and remyelination (Deshmukh et al., 2013; Mei et al., 2014; Najm et al., 2015). Developing CAQK as a vector for targeting and concentrating such drugs at lesion sites may overcome the limits of systemic applications. Systemic drug administration often requires high concentrations and results in undesired side effects, thus packaging therapeutic drugs into peptide-coated natural or artificial particles could increase accumulation at lesion sites and improve myelin repair. Importantly, the CAQK binding epitope is preserved in human injured brain tissue (Mann et al., 2016) which provides an incentive to explore further applications for clinical utilization.

Demyelination is a hallmark of multiple sclerosis and loss of myelin leads to neurodegeneration. White matter abnormalities are evident in multiple neurodegenerative disorders, including Alzheimer's and Parkinson's disease and stroke (Chen et al., 2022 for review). With more refined detection methods, maintaining or restoring myelin functions at early stages of the disease may become a promising approach to halt neurodegenerative processes and physical and cognitive decline. By demonstrating CAQK targeting to myelin injuries and sites of progressive demyelination, the work reported here opens new possibilities for potential CAQK applications in early detection and diagnosis of MS and other neurodegenerative disorders and may ultimately prove useful to initiate myelin repair processes to limit neurodegeneration.

## Data availability statement

The original contributions presented in the study are included in the article/**Supplementary material**, further inquiries can be directed to the corresponding author.

## Ethics statement

The animal study was reviewed and approved by Sanford Burnham Prebys Medical Discovery Institute Animal Care and Use Committee (La Jolla, CA). Written informed consent was obtained from the owners for the participation of their animals in this study.

## Author contributions

Conceptualization, overall study design, organization of work and manuscript (BR); design and performance of experiments (CA-G: LPC and EAE models; DJ and YK: cuprizone model); manuscript writing or editing (all authors); manuscript revision (BR, CA-G, and DJ). All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



## Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fncel.2022.908401/full#supplementary-material.

SUPPLEMENTARY FIGURE 1
FAM-CAQK peptide does not target normal myelin. In mice fed a normal diet. **(A,E)** FAM-CAQK peptide (green) does not associate with healthy myelin in corpus callosum (marked by dashed lines). Absence of Iba+ microglia (**B**, magenta) and the presence of myelin basic protein (**F**, MBP, magenta) indicate normal myelin. Merged images are shown in **(D,H)**, respectively. Panels **(C,G)** show DAPI-stained nuclei (blue). Circulating FAM-CAQK peptide reaches ependymal cells (Ep) lining the ventricle. Images were taken on a Zeiss Imager M2.

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# A glial perspective on the extracellular matrix and perineuronal net remodeling in the central nervous system

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A structural scaffold embedding brain cells and vasculature is known as extracellular matrix (ECM). The physical appearance of ECM in the central nervous system (CNS) ranges from a diffused, homogeneous, amorphous, and nearly omnipresent matrix to highly organized distinct morphologies such as basement membranes and perineuronal nets (PNNs). ECM changes its composition and organization during development, adulthood, aging, and in several CNS pathologies. This spatiotemporal dynamic nature of the ECM and PNNs brings a unique versatility to their functions spanning from neurogenesis, cell migration and differentiation, axonal growth, and pathfinding cues, etc., in the developing brain, to stabilizing synapses, neuromodulation, and being an active partner of tetrapartite synapses in the adult brain. The malleability of ECM and PNNs is governed by both intrinsic and extrinsic factors. Glial cells are among the major extrinsic factors that facilitate the remodeling of ECM and PNN, thereby acting as key regulators of diverse functions of ECM and PNN in health and diseases. In this review, we discuss recent advances in our understanding of PNNs and how glial cells are central to ECM and PNN remodeling in normal and pathological states of the CNS.

KEYWORDS

**perineuronal nets (PNNs), astrocytes, extracellular matrix (ECM), microglia, PV neurons, matrix metalloproteinases**

## Introduction

In 1895, Hens Gierke proposed the idea of a homogeneous and amorphous ground substance that embeds neuroglia and forms the structural architecture of the brain (Celio, 1999). Soon after, in 1898 Camillo Golgi described a pericellular coating around specific neurons in his seminal study on the eponymous Golgi complex (Celio et al., 1998; Celio, 1999). After nearly a century of relative obscurity, these structures unambiguously established themselves as different forms of neuroglial-embedding

extracellular matrix (ECM) known as interstitial matrix and perineuronal nets (PNNs). Brain ECM is rich in hyaluronan (HA), chondroitin sulfate proteoglycans (CSPGs), and glycoproteins, with a minor proportion of fibrous proteins. Together in conjunction with water, ions, and secreted molecules, ECM creates a functionally dynamic extracellular milieu that provides structural support and effectuates diverse neuromodulatory functions (Hrabetova et al., 2018; Fawcett et al., 2019).

A large fraction of ECM is homogeneous and amorphous; however, several morphologically distinct forms are distributed throughout the brain (Fawcett et al., 2019; Patel et al., 2019; Chaunsali et al., 2021). A thin sheet-like condensation of ECM molecules on the pial surface and around parenchymal vasculature forms basement membranes (BMs) which carries out structural, signaling, and barrier functions. Another phenotypic specialization of ECM is PNN, which is a lattice-like condensation predominantly juxtaposing the soma, dendrites, and axon initial segment (AIS). A vast majority of PNN-expressing neurons are fast-spiking parvalbumin (PV)-expressing GABAergic neurons; however, several non-PV neurons also express PNNs (Lensjø et al., 2017a; Patel et al., 2019; Chaunsali et al., 2021). Brain ECM, including PNNs, is spatiotemporally malleable and maintains a characteristic composition and structural organization at different stages of pre and postnatal development, adulthood, aging, and central nervous system (CNS) pathologies. The key advantage of the malleability appears to be a functional versatility, owing to which the ECM and PNNs perform diverse functions at specific stages of life. Since functional versatility is predominantly determined by spatiotemporal dynamics, the central question arises; what regulates the ECM and PNN dynamics and thereby critically determines their functions?

Recent studies suggest that the structural organization of ECM and PNNs, and therefore their functions, are regulated by intrinsic mechanisms- driven by neurons- as well as extrinsic-driven primarily by glial cells (Wiese et al., 2012; Rowlands et al., 2018; Crapser et al., 2021; Ribot et al., 2021). CNS glia, including astrocytes, oligodendrocytes, and microglia are capable of producing ECM and PNN components and are significant sources of ECM during development and adulthood (Wiese et al., 2012; Song and Dityatev, 2017). In addition, astrocytes excessively produce ECM molecules under several CNS pathologies, effectuating both protective and detrimental outcomes (Fitch and Silver, 2008; Kim et al., 2016, 2017; George and Geller, 2018). Besides producing ECM molecules of structural and signaling utility, astrocytes release an array of diverse matrix-remodeling proteases and their inhibitors to tightly control the structural integrity of PNNs and ECM (Fitch and Silver, 2008; Patel et al., 2019; Chaunsali et al., 2021).

While astrocytes are mainly engaged with the synthesis and release of ECM and their proteolytic enzymes, it is microglia that contribute significantly to the continuous elimination of the ECM molecules due to their characteristic phagocytic property. Normally, the homeostatic states of ECM and PNNs are maintained by a constitutive expression of ECM and proteases by neurons and astrocytes, as well as clearance by microglia. However, as seen in several recent studies on epilepsy, Alzheimer's disease (AD), Huntington's disease (HD), neuropathic pain, etc., dysfunctional microglia leads to abnormal clearance or accumulation of the ECM and PNNs contributing to the pathology (Tewari et al., 2018; Patel et al., 2019; Crapser et al., 2020b, 2021; Chaunsali et al., 2021; Carceller et al., 2022; Tansley et al., 2022).

In this review, we discuss the classic roles of and recent advances in the functions of ECM and PNNs, followed by the role of glial cells in ECM and PNN remodeling in healthy brain and pathologies. These roles suggest a pivotal contribution of glial cells to this remodeling process and thus encourage a discussion on a glia-centric approach to treatment strategies.

## Structure and functions of extracellular matrix and perineuronal nets in the central nervous system

Extracellular matrix is present in all tissues of the body as a structural framework of amorphous and diffused interstitial matrix; however, brain ECM is unique in its composition and organization. From a composition point of view, a major fraction of the brain ECM consists of glycosaminoglycans (GAGs), proteoglycans, and glycoproteins, with a negligible fraction of fibrous proteins which is contrary to the fibrous protein-rich ECM in a majority of other tissues (McRae and Porter, 2012). Another key feature of the brain ECM is its structural organization into distinct forms such as thin sheets of BMs and highly condensed pericellular coats of PNNs.

### Basement membranes

Basement membrane is an organized ECM assembly in the form of thin sheets that surround the pial surface (meningeal BM) and brain vasculature (vascular BM) (Thomsen et al., 2017). Similar to other forms of ECM, the BMs also show a spatiotemporally dynamic composition which determines their functions at different stages of life. By and large, collagen IV, laminins (1–5), nidogens (1 and 2), and heparin sulfate proteoglycans (HSPGs) (perlecan and agrin) are the most static components (Thomsen et al., 2017). On the other hand, insoluble fibronectin, fibulins, thrombospondins (TSPs), and secreted protein acidic and rich in cysteine (SPARC) are more dynamic and are expressed at specific developmental and pathophysiological states (Thomsen et al., 2017). Besides serving

as a major route *via* which fluids and soluble molecules enter and leave the brain, BMs provide structural support by acting as an adhesive substrate for cells to anchor to and mediate signal transduction *via* integrin and other transmembrane matrix receptors (Baeten and Akassoglou, 2011). Meningeal BM is critical for brain development and the absence of the BM or its constituents causes abnormal brain development (Halfter et al., 2002). The vascular BM plays a critical role in maintaining the blood-brain barrier (BBB), as evidenced by BBB disruption and cerebrovascular defects in the absence of BM components such as laminins (Yao et al., 2014) and collagens (Engelhardt, 2003; Jeanne et al., 2015). In several CNS disorders, predominantly in stroke and traumatic brain injury (TBI), BBB disruption is associated with an altered BM, causing an infiltration of otherwise impermeable serum components and immune cells to trigger inflammation and subsequently neuroglial dysfunctions (Thomsen et al., 2017). Extravasation of blood proteins fibrinogen and albumin trigger molecular changes in astrocytes, transforming them into their reactive state which in turn further remodels the ECM and forms glial scars (Kim et al., 2016, 2017) (discussed later).

## Interstitial matrix

Historically, the idea of ECM was pioneered as a neuroglia-embedding structural framework of a diffused, amorphous, and ubiquitously distributed ground substance in the extracellular space (ECS) (Celio, 1999). This form is now known as interstitial matrix and constitutes the highest fraction of brain ECM. Interstitial matrix fills nearly the entire ECS and embeds other phenotypes of ECM such as perineuronal, perisynaptic, and perinodal matrices (Engelhardt, 2003; Lau et al., 2013; Fawcett et al., 2019). The meshwork of the interstitial matrix consists of hyaluronan, proteoglycans, tenascins, link proteins, glycoproteins such as laminins and fibronectin, and a relatively small fraction of fibrous proteins such as collagens and elastin (Rauch, 2007; Lau et al., 2013; Lei et al., 2017). Several transmembrane and membrane-coupled proteins and receptors including CD44, receptor for hyaluronan-mediated motility (RHAMM), Stabilin-2, TNFIP6, SHAP, TLR-2, and TLR-4 are connected directly with the hyaluronan to anchor and stabilize the ECM (Jiang et al., 2011). Similarly, chondroitin sulfate binds to several transmembrane receptors including RPTPσ, LAR, RPTPδ, and Nogo receptors as well as adhesion molecules including NCAM and integrins (Yu et al., 2018). The interstitial matrix harbors ions, secreted molecules such as growth factors and neuromodulatory agents, and most importantly, provides a high hydration capacity to maintain ECS volume and thereby normal brain activity (Perkins et al., 2017; Hrabetova et al., 2018).

A large fraction of diffused interstitial matrix coats the synapses, forming a perisynaptic matrix, and is involved in synaptogenesis and plasticity often under the regulation of matrix remodeling enzymes (Orlando et al., 2012; Korotchenko et al., 2014; Fawcett et al., 2019). Depletion of perisynaptic HA affects synaptic potentiation by altering the lateral mobility of AMPARs (Frischknecht et al., 2009) as well as the activity of L-type voltage-dependent calcium channels (L-VDCCs) at synaptic terminals (Kochlamazashvili et al., 2010). Similarly, Tenascin-C (Tn-C) deficiency impairs synaptic plasticity by altering L-VDCCs signaling, however, Tenascin-R (Tn-R) deficiency, which is expressed around perisomatic synapses, alters NMDAR-dependent LTP by reducing the perisomatic inhibition (Evers et al., 2002; Hayani et al., 2018). More recently, Tn-R appears to be recycled at the active synapse in an activity-dependent manner influencing the synaptic structure (Dankovich et al., 2021). These studies suggest a pivotal role of interstitial matrix molecules in effectuating the dynamic changes at synapses.

Besides PNNs, few other specialized phenotypes of the ECM are embedded largely within the diffused interstitial matrix. For example, perinodal ECM is a condensed form of ECM around the nodes of Ranvier (Bekku and Oohashi, 2019) consisting of Tn-R, brevican, versican, phosphacan, Bral1, and neurocan (Susuki et al., 2013). Tn-R plays an essential role in axonal functions presumably by acting as an ion diffusion barrier (Bekku and Oohashi, 2019) as evidenced by decreased axonal conduction velocity in the optic nerve in Tn-R deficient condition (Weber et al., 1999). Axonal coats are another phenotypic specialization of ECM which are rich in CSPGs, including aggrecan and brevican; however their functional relevance is elusive (Morawski et al., 2012; Jäger et al., 2013). Recent studies support the presence of brevican and NG2 expressing axonal coats surrounding myelinated axons in human brains and are suggested to aid axonal properties (Pantazopoulos et al., 2022).

## Perineuronal nets

Historically, PNNs have been the most intriguing yet enigmatic ECM structures. PNNs are widely expressed in several brain regions including the cerebral cortex, amygdala, striatum, and hippocampus (Morikawa et al., 2017; van't Spijker and Kwok, 2017; Ulbrich et al., 2021) as well as in the spinal cord (Irvine and Kwok, 2018) of rodents and humans (Chaunsali et al., 2021; Carceller et al., 2022). PNNs are predominantly present on the fast-spiking PV interneurons; however, a small population of other inhibitory and excitatory neurons in brain and spinal cord express PNNs (Irvine and Kwok, 2018; Chaunsali et al., 2021). The typical lattice of PNN is a ternary complex of hyaluronan, link proteins (HAPLNs), proteoglycans of the lectican family or CSPGs including aggrecan, brevican, versican, and neurocan, and

tenascin glycoproteins (Tn-C, Tn-R). PNNs can be visualized by fluorescently labeled antibodies that bind to the core proteins or by lectins such as Wisteria floribunda agglutinin (WFA) that bind the GAGs sidechains (Fawcett et al., 2019; Tewari and Sontheimer, 2019; **Figure 1A**). The cavities of the PNN lattice on the soma, AIS, and dendrites house both excitatory and inhibitory synaptic terminals (Fawcett et al., 2019; Carceller et al., 2020).

The key role of anchoring the extracellular components of PNNs to the cell membrane is performed by HA-producing transmembrane enzymes, hyaluronic acid synthase (HAS 1-3) (Kwok et al., 2011). HAS-associated long chains of HA are connected directly to the link proteins (HAPLN), which in turn bind to the CSPG core proteins. The core proteins of lecticans also form a backbone to which numerous side chains of sulfated GAGs are attached. Lecticans are cross-linked by Tn-R to further secure the assembly (**Figure 1B**) and a loss of aggrecan crosslinking by Tn-R impairs the PNN assembly around dendrites (Morawski et al., 2014). Despite being a multimolecular assembly, WFA-labeled PNNs remain minimally distorted in the absence of single or multiple PNN components including HA (Arranz et al., 2014), neurocan (Zhou et al., 2001), brevican (Brakebusch et al., 2002), Tn-C (Irintchev et al., 2005; Gottschling et al., 2019), Tn-R (Brückner et al., 2000; Gottschling et al., 2019), and link proteins (Carulli et al., 2010). However, as the one indispensable component of the PNN, aggrecan deficiency leads to the absence of PNNs (Giamanco et al., 2010; Rowlands et al., 2018) (See review Carceller et al., 2022).

In different regions of the developing mouse brain, traces of PNN appear at different ages and gradually achieve fully condensed arborization in several weeks. For example, immature PNNs can be identified in brainstem by postnatal day 4 (Brückner et al., 2000); however in the cerebral cortex and amygdala by postnatal days 14 and 21, respectively (Brückner et al., 2000; Horii-Hayashi et al., 2015). In humans, PNNs appear near 8 weeks in medial prefrontal cortex and mature around 8 years of age (Rogers et al., 2018). By and large, this developmental trajectory of PNN formation coincides with the critical periods of heightened neuroplasticity, in which neuronal circuits are highly responsive to sensory inputs and brain connections are established and strengthened in an activity-dependent manner (Pizzorusso et al., 2002). Sensory deprivation within the critical period permanently disrupts the normal development of the brain circuits; however, sensory deprivation outside the critical period or in adults does not affect neuronal circuits and brain functioning (Hubel and Wiesel, 1965, 1970; Reh et al., 2020). Pioneering studies have shown that preventing PNN formation in the developing brain prolongs the critical period of plasticity, and that disruption of PNNs outside the critical period using a bacterial-derived enzyme Chondroitinase ABC (ChABC) reinstates neuroplasticity similar to that of the critical period, suggesting PNNs as the primary regulator of the critical period plasticity (Pizzorusso et al., 2002; Wang and Fawcett, 2012; Rowlands et al., 2018; Fawcett et al., 2019).

Despite a progressive condensation of CSPGs into PNNs in the developing brain, the total CSPG content remains largely unchanged- which complicates the question of mechanisms whereby PNN CSPGs are inhibitory to neuroplasticity (Miyata et al., 2012). Intriguingly, the plasticity-permissive nature of the developing brain is attributed to a characteristic sulfation pattern of the CSPGs. The developing brain exhibiting immature PNNs and high neuroplasticity possesses a higher C6S proportion than C4S to maintain a low C4S/C6S ratio. Over the developmental period, the ratio changes to a higher C4S/C6S, which not only promotes PNN maturation but also suppresses neuroplasticity (Miyata et al., 2012; Miyata and Kitagawa, 2016; Foscarin et al., 2017). Nevertheless, the downstream cellular and molecular mechanism behind the inhibitory nature of a higher C4S/C6S ratio remains elusive.

Developmental formation of PNNs is activity-dependent, and several brain regions including barrel cortex, thalamus, visual cortex, and vocal center in songbirds show underdeveloped PNNs if deprived of activity, suggesting a high malleability of PNNs (Guimarães et al., 1990; Lander et al., 1997; Pizzorusso et al., 2002; McRae et al., 2007; Nakamura et al., 2009). Although PNNs in the mature CNS appear to be largely stable in a normal physiological state, emerging evidence suggests bidirectional changes in the structure and numerical density of PNNs on a cyclic basis as well as under specific conditions such as drug addiction, maternal hormone fluctuations, and chronic pain (Lasek et al., 2018; Pantazopoulos et al., 2020; Uriarte et al., 2020; Harkness et al., 2021; Mascio et al., 2022).

## Functions of perineuronal nets

Chondroitin sulfate proteoglycans are critical constituents of the PNNs and several signaling functions of CSPGs are independent of their phenotypic appearance as PNNs or interstitial matrix as evidenced in the following studies. In the extensively studied visual system, a high density of CSPGs repels the growing retinal axons to navigate them to their target areas in the developing brain. Conversely, depleting CSPGs with ChABC is disruptive to the axonal guidance and misleads the axons to non-target areas (Brittis et al., 1992; Laabs et al., 2005). Since axonal growth and guidance is a developmental phenomenon, the inhibitory role of CSPGs appears extraneous in adult CNS physiology. However, in CNS injury and trauma, the damaged axons fail to regenerate due to the CSPG-rich glial scar at the injury site and ChABC-mediated removal of CSPGs improves the repair and regeneration and to a certain extent, functional recovery (Silver and Miller, 2004).

Subsequently, in adolescence, CSPGs are condensed as PNNs, which are largely known to lock the synapses to

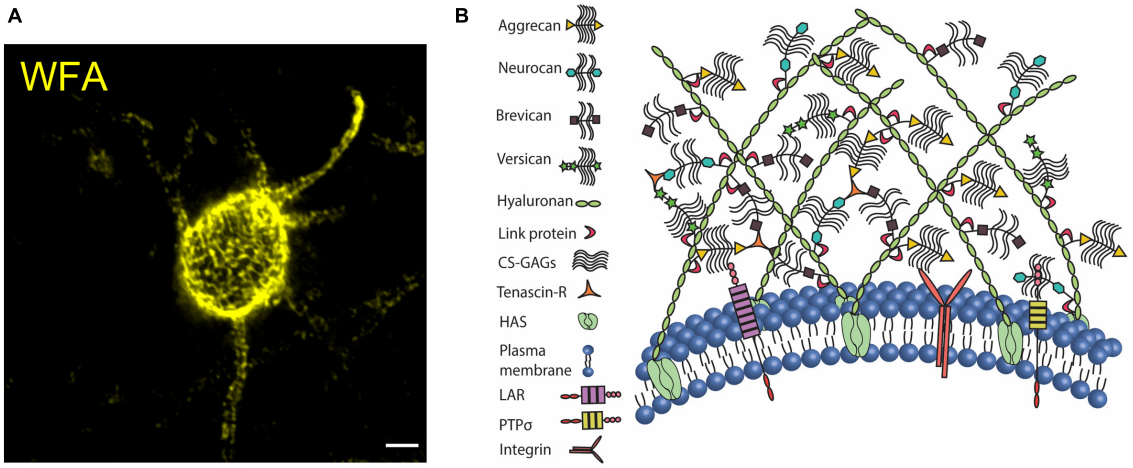


FIGURE 1
Perineuronal net in mouse cerebral cortex. **(A)** Confocal micrograph of WFA-labeled PNN in the mouse cerebral cortex. PNN coats cell soma, dendrites, and axon initial segment. **(B)** The organization of CSPGs, hyaluronan, link proteins, and tenascin on the plasma membrane forms the assembly of PNN. CSPGs interact with their cell surface receptors to modulate intracellular signaling cascades. Scale bar 5 μm.

prevent further modifications and close the critical period of heightened neuroplasticity as discussed in the previous section. Intriguingly, the plasticity reinstates in the adult CNS when PNNs are disrupted. Mechanistically, disruption of PNN or its constituents triggers several short and long-term cellular and molecular changes which can promote neuroplasticity. For example, PNN depletion induces synaptic potentiation in otherwise plasticity-resistant CA2 synapses (Carstens et al., 2016). Brevican, a CSPG in the PNNs, is suggested to regulate the localization of potassium channels and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors expression on PV cells (Favuzzi et al., 2017). At the network level, PNN depletion affects gamma oscillations (30–80 Hz) (Lensjø et al., 2017b) and sharp wave ripples (SWRs) (Sun et al., 2018). Since PV neuron activity is pivotal for the generation of gamma oscillations and SWRs, it is plausible that neuroplasticity upon PNN depletion is partly effectuated by the altered activity of PV neurons. These functional changes due to PNN disruption are also accompanied by structural changes at synapses, including alterations in the numbers of synaptic contacts, spine dynamics, and expression of ion channels and receptors (Frischknecht et al., 2009; Favuzzi et al., 2017; Carceller et al., 2020). These studies suggest a variety of ways by which PNN disruption can effectuate the synaptic plasticity.

Perineuronal nets and interstitial matrix are by and large composed of the same set of ECM molecules; therefore several functions of PNNs can be considered independent of their structural integrity. However, there are several functions which require the organized PNN assembly with sulfated proteoglycans (Miyata and Kitagawa, 2016; Fawcett et al., 2019). Several signaling proteins including OTX2, Semaphorin 3a, Narp, and reelin are trapped in the PNN lattice and activate intracellular signaling cascades to facilitate the developmental maturation of PV neurons (Fawcett et al., 2019). The condensed PNN has a high density of negative charge which protects PV neurons from extracellular stressors (Suttkus et al., 2014), which is markedly evidenced in schizophrenia (Cabungcal et al., 2013, 2014) and AD wherein PV neurons are relatively spared due to their PNN coats (Morawski et al., 2010, 2012).

The sulfated proteoglycans on the PNNs constitute a high-density cloud of negative charges around the PV cells which can attract a high concentration of $Na^+$, $K^+$, or $Ca^{++}$ ions. During the fast-spiking activity of PV neurons, a dynamic exchange of $Na^+$ and $K^+$ ions with the stationary negative charges of the PNNs can aid the PV neuron activity (Härtig et al., 1999; Morawski et al., 2015). Besides the ion buffering in the ECS, PNNs can also directly influence the spiking properties of the PV neurons as shown by us (Tewari et al., 2018) and others (Balmer, 2016; Wingert and Sorg, 2021). The pioneering *in vitro* (Dityatev et al., 2007) and more recent studies on hippocampal fast-spiking interneurons *in situ* brain slices (Favuzzi et al., 2017; Hayani et al., 2018) report a lower firing threshold without any changes in passive neuronal properties, however, few other studies show a reduced spiking upon PNN disruption (Balmer, 2016; Tewari et al., 2018). This ambiguity can be attributed to several factors including PNN disruption methods, brain regions, experimental design, resting state, and excitatory/fast-spiking type of PNN-expressing neurons, as discussed in detail by Wingert and Sorg (2021).

In a mouse model of human glioma-associated epilepsy, we observed that disruption of cortical PNNs by glioma-released matrix metalloproteinases (MMPs) increases the membrane capacitance of the PV neurons leading to a reduction in

spike firing activity and consequently reducing the overall inhibitory drive. Experimental disruption of PNNs mimics the increased capacitance and reduced firing activity of PV neurons as shown by PV neurons with disrupted PNNs in glioma (Tewari et al., 2018), suggesting a pivotal role of PNNs in aiding the fast-spiking properties of PV neurons. PNNs seem to determine the activity of excitatory neurons equally well, as evidenced in a recent study in which microglia-mediated degradation of PNNs around excitatory projection neurons in the spinal cord enhances their activity and induces pain-related behavior (Tansley et al., 2022). Another example is the induction of synaptic plasticity in CA2 neurons upon their PNN depletion, which are otherwise resistant to potentiation (Carstens et al., 2016).

The necessity of PNNs in CNS functioning is profoundly evidenced in CNS disorders in which PNN disruption is commonly observed; experimental PNN disruption largely phenocopies the disease characteristics. In acquired forms of epilepsies triggered by injury, stroke, and brain tumors, elevated matrix remodeling proteases disrupt the PNNs (McRae et al., 2012; Rankin-Gee et al., 2015; Kim et al., 2016; Dubey et al., 2017; Tewari et al., 2018; Patel et al., 2019). PNN disruption potentially exposes the PV neurons to heightened oxidative stress (Cabungcal et al., 2013), leading to a reduction in the overall abundance of PV neurons and thereby further lowering the inhibitory drive as shown by us (Tewari et al., 2018) and others (Enwright et al., 2016; Hatcher et al., 2020). Elimination of PNN and its constituents not only increases the propensity of seizure and epileptiform activity in excitatory neurons, but also causes spontaneous seizures (Arranz et al., 2014; Rempe et al., 2018; Tewari et al., 2018; Patel et al., 2019). These studies support the idea that PNN disruption is not only able to generate neuronal hyperexcitability, but that PNN disruption due to CNS insults can contribute to the process of epileptogenesis by PV neuron dysfunction and altered inhibition.

A similar dysfunction of PV neurons accompanied by disrupted PNNs is evidenced in animal models and human subjects of schizophrenia, bipolar disorder, and autism spectrum disorders (Pantazopoulos and Berretta, 2016; Fawcett et al., 2019), wherein preventing PNN disruption by blocking MMP activity largely ameliorates disease symptoms (Levkovitz et al., 2009; Khodaie-Ardakani et al., 2014). Several recent studies on CNS disorders including AD (Abbott and Kepler, 1990; Fawcett et al., 2019; Crapser et al., 2020b), HD (Crapser et al., 2020a, 2021), multiple sclerosis (MS) (Lau et al., 2013), and schizophrenia (Pantazopoulos et al., 2010; Mauney et al., 2013) also show altered PNN in key brain areas. Notably, glial contribution in PNN remodeling is explicitly evidenced in many of these studies as discussed in later sections.

In summary, a large number of studies suggest that the physiological functions of the PNNs and their constituents broadly encompass developmental signaling, regulation of neuroplasticity, modulation of neuronal activity, extracellular ion homeostasis, and neuroprotection. In diseased states increased proteolytic cleavage of PNNs disrupts their structural integrity and reduces the overall abundance. Depending on the brain area/s involved, loss of PNNs can contribute to disease etiology predominantly by PV neuron dysfunction and altered E-I balance, loss of neuroprotection, altered ECS and ionic balance, and maladaptive neuroplasticity (Reichelt et al., 2019). The causal role of PNNs in E-I imbalance and ECM and ionic homeostasis in epilepsy appears to be convincing; meanwhile, the causal role of PNNs in many neuropsychiatric and neurodegenerative disorders is still in its infancy. A vast majority of the studies on the PNNs use ChABC or hyaluronidase enzymes which indiscriminately cleave the GAGs of PNNs and interstitial matrix. This lack of tools to selectively manipulate PNNs is a major limitation in the field.

## Homeostatic regulation of extracellular matrix and perineuronal net by central nervous system glia

One of the classic housekeeping functions of glial cells is the continuous secretion of ECM molecules to maintain the architecture and extracellular milieu of the CNS. In principle, both neurons and glial cells synthesize and secrete ECM molecules; however, glial cells- especially astrocytes and microglia- are the primary regulators of ECM wear and tear in CNS pathophysiology. In this section, we discuss the role of glial cells in the homeostatic regulation of ECM and PNN and consequently the functional outcome.

Astrocytes, the most abundant CNS glia, are involved in a variety of functions including neuronal migration, secretion of growth factors and neuromodulatory molecules, synaptogenesis, synaptic pruning, and water, ion, and neurotransmitter homeostasis (Phatnani and Maniatis, 2015; Patel et al., 2019; Alcoreza et al., 2021). In the developing brain, astrocytes are the predominant source of ECM molecules including CSPGs, HA, and tenascins, which in turn serve both structural and signaling roles (**Figures 2**, **3**). By varying the spatiotemporal expression of CSPGs and Tn-C, astrocytes regulate the proliferation, maintenance, and maturation of neuronal precursor stem cells and oligodendrocyte precursor cells (OPCs) as well as neuronal migration, neurite outgrowth, extension, and guidance (Powell et al., 1997; Powell and Geller, 1999; Wiese et al., 2012; Amin and Borrell, 2020; Somaiya et al., 2022). Tn-C appears to be expressed by radial glia as well as differentiated astrocytes, and additionally regulates the proliferation of astrocyte progenitor cells (Karus et al., 2011). In a quadruple knockout mouse, astrocyte-derived Tn-C, Tn-R,

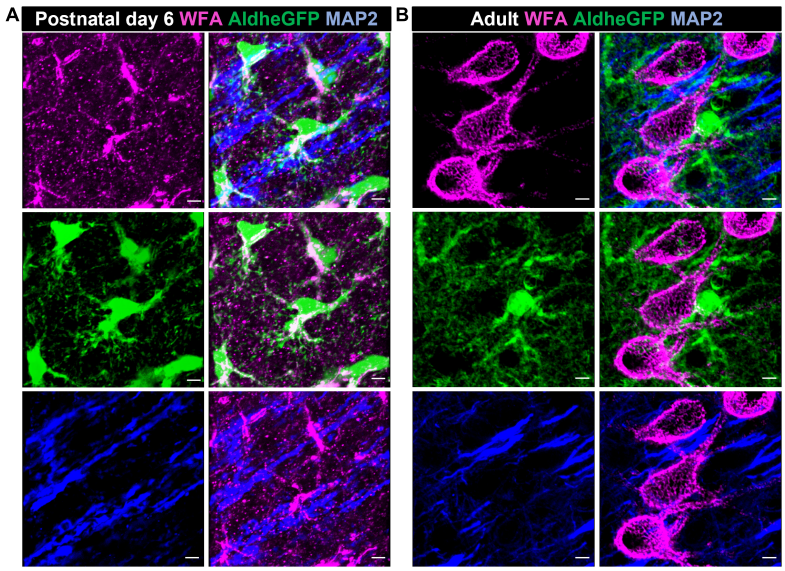


FIGURE 2
Astrocytes produce CSPGs in the developing brain. Confocal micrographs showing WFA reactivity (magenta – top left), GFP-immunolabelled aldheGFP-expressing astrocytes (green – middle left) and neuronal marker MAP2 (blue- bottom left) in postnatal day 6 **(A)**, and adult **(B)** mouse cerebral cortex. In postnatal day 6 **(A)**, WFA reactivity predominantly colocalizes with GFP-labeled astrocytes (**A**, middle right) compared to the MAP-2 labeled neuronal processes (**A**, bottom right). In adult, WFA reactivity is present only in PNNs (**B**, top left); astrocytic (**B**, middle right) and neuronal (**B**, bottom right) processes show no detectable WFA reactivity. Scale bar 5 μm.

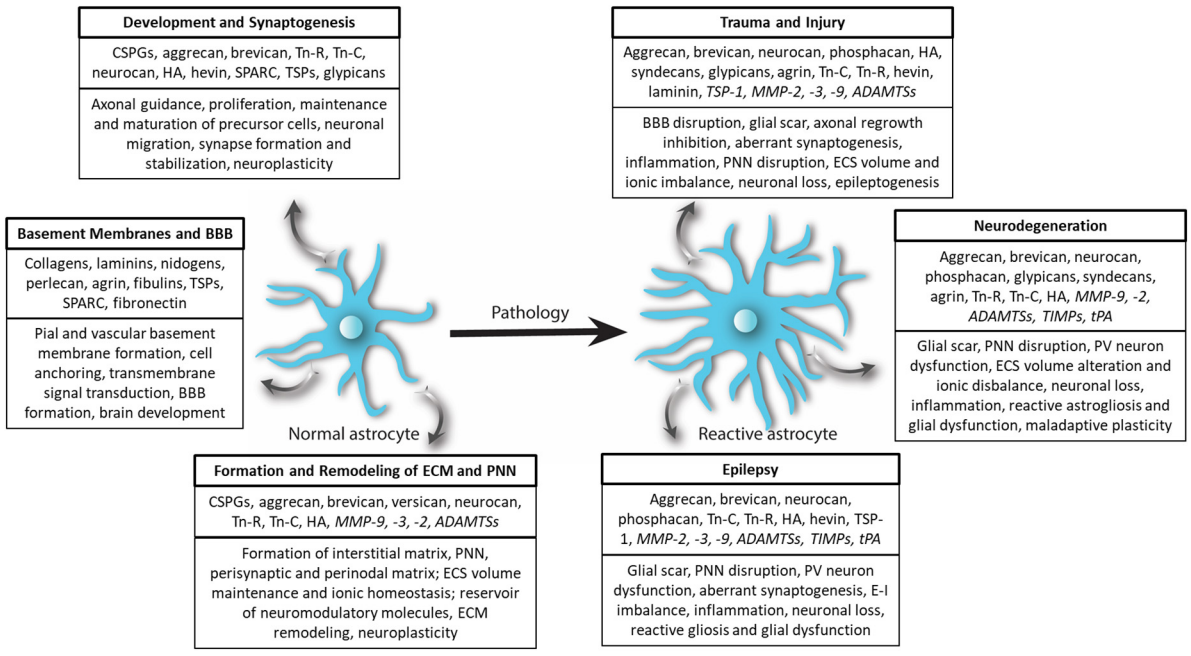


FIGURE 3
Extracellular matrix remodeling by astrocytes in physiology and pathology. In physiological conditions, normal astrocytes release several ECM molecules to govern diverse processes associated with CNS development, synaptogenesis, basement membrane and BBB formation as well as formation of the structural scaffold of brain ECM and PNN. Astrocytes also release several proteases and regulatory molecules (italicized) to remodel ECM and PNNs to maintain homeostatic neuroplasticity. In CNS pathologies, most prominently in trauma and injury, epilepsy, and neurodegenerative diseases, normal astrocytes turn reactive and remodel ECM and PNN by accumulating ECM or degrading ECM and PNN by releasing proteases (italicized) thereby effectuating beneficial and deleterious outcomes.

brevican, and neurocan have been reported to control synapse formation and stabilization (Geissler et al., 2013; **Figure 3**).

Astrocytes are the predominant source of hyaluronan that constitutes the interstitial matrix and PNNs in the gray matter and surrounds the myelinated fibers in white matter (Cargill et al., 2012; Peters and Sherman, 2020). Since hyaluronan chains of the PNN extend from the neuronal surface-bound HAS (Fawcett et al., 2019), neurons appear to be a predominant source of PNN-forming hyaluronan. Generally, hyaluronan is secreted as a high molecular weight (HMW) polymer which interacts with HA receptors such as CD44, RHAMM, or LTR2; and triggers proliferation, differentiation, and migration of the stem cell in developing brain (Lindwall et al., 2013; Peters and Sherman, 2020). In the adult brain, astrocytes produce hyaluronan as well as hyaluronan-cleaving enzyme hyaluronidase in the subventricular zone, where adult stem cells reside. Thus, by regulating the HA catabolism, astrocytes are speculated to keep stem cells in a quiescent state (Lindwall et al., 2013). In pathologies such as trauma and injury, HMW hyaluronan can be cleaved into short fragments of low molecular weight by hyaluronidases and MMPs (Peters and Sherman, 2020), which by and large have distinct and sometimes opposite biological effects. By expressing both hyaluronidase and MMPs, astrocytes play an important role in hyaluronan synthesis and catabolism (Muir et al., 2002; Al'Qteishat et al., 2006). HA in turn regulates the morphology of astrocytes and proper trafficking and function of glutamate transporters by CD44-evoked Rac1 signaling (Hayashi et al., 2019; Peters and Sherman, 2020).

Besides CSPGs, tenascins, and HA, which act as both structural and signaling molecules, astrocytes secrete several ECM glycoproteins known as matricellular proteins primarily for a signaling function. By adjusting the spatiotemporal expression of matricellular proteins such as hevin/SPARC (Kucukdereli et al., 2011), thrombospondin (TSP) (Christopherson et al., 2005), and Glypican 4 and 6 (Allen et al., 2012), astrocytes control excitatory synaptogenesis in the developing CNS (**Figure 3**). The expression of the majority of matricellular proteins decreases during the later phase of postnatal development; however reactive astrocytes upregulate their expression in several pathological conditions, potentially causing aberrant synaptogenesis and maladaptive plasticity (Jones and Bouvier, 2014; Kim et al., 2016).

From a structural point of view, astrocytes, in conjunction with capillary endothelial cells and pericytes, synthesize and assemble ECM components to form the BM (Yao et al., 2014; Thomsen et al., 2017). Subsequently, astrocytic perivascular endfeet- in association with the BM and pericytes- form the structural basis of the BBB, for whose maintenance astrocytic laminin is indispensable (Thomsen et al., 2017). In the parenchymal space, astrocytes secrete hyaluronan, CSPGs-including brevican, neurocan, versican, and aggrecan- and Tn-C in order to form and maintain interstitial matrix (Wiese et al., 2012; Patel et al., 2019).

Although astrocytes and neurons produce aggrecan, which in turn orchestrates PNNs, the contribution of astrocytic aggrecan in PNN formation and maintenance can be questioned (Song and Dityatev, 2017) as evidenced by the absence of PNNs in neuron-specific aggrecan knockout (Rowlands et al., 2018) and formation of ECM coatings *in vitro* without astrocytes (Miyata et al., 2005; Giamanco and Matthews, 2012). However, how critical astrocytes are in maintaining the PNNs *in vivo* is a frontier area of investigation. A recent study suggests that astrocytes are key regulators of PNN maturation and thereby critical period plasticity. During the critical period, immature astrocytes increase connexin 30 levels, which subsequently activates the RhoA-ROCK pathway to suppress MMP-9 expression and allows PNN condensation and PV cell maturation (Ribot et al., 2021). These studies suggest that although astrocytes do not directly synthesize PNNs, they indirectly regulate their developmental formation.

Other CNS glial cells such as OPCs and oligodendrocytes, and microglia are also a source of ECM, but in a more restricted manner (Pu et al., 2018). For example, oligodendrocytes and OPCs deposit a peculiar ECM rich in Tn-C and Tn-R around the node of Ranvier termed the perinodal ECM, which is essential for the clustering of $Na^+$ channels (Susuki et al., 2013; Fawcett et al., 2019). OPCs secrete brevican during myelination and are suggested to form axonal coats around myelinated axons (Pantazopoulos et al., 2022). Besides being a source of CSPGs, oligodendrocyte lineage cells are critically susceptible to CSPGs as evidenced by inhibition of OPCs migration and maturation into oligodendrocytes especially near the demyelinated lesion (Lau et al., 2013). Microglia can, under special circumstances, produce ECM molecules including CSPGs; however, their contribution to the formation of brain ECM and PNNs is not entirely clear (Lau et al., 2013; Pu et al., 2018). These studies suggest that glial cells are major sources of ECM molecules whereby ECM constituents are used as building blocks of brain architecture as well as signaling molecules to regulate brain development. How glial cells remodel ECM in CNS pathologies and the consequences of said remodeling are discussed in the next sections.

## Extracellular matrix and perineuronal net remodeling by astrocytes in central nervous system pathologies

In nearly all CNS pathologies, astrocytes respond by undergoing morphological, molecular, and functional changes that turn them into reactive astrocytes (Escartin et al., 2021). Upregulation of glial fibrillary acidic protein (GFAP), an intermediate filament protein, is the most common molecular change and has been used widely as a marker for reactive astrocytes (Patel et al., 2019; Escartin et al., 2021). In a broader

sense, reactive astrocytes influence ECM and PNN homeostasis by altering the expression levels of ECM as well as the matrix remodeling proteases including MMPs. As a consequence, in several CNS pathologies such as acquired epilepsies, TBI, MS, and glioma, the interstitial matrix is upregulated; however, the PNNs are disrupted or lost (Lau et al., 2013; George and Geller, 2018; Rempe et al., 2018). This contrasting fate can be attributed to the glial upregulation of ECM molecules at the injury and increased expression of matrix-degrading proteases in the surrounded areas. Interestingly, CNS disorders without a focal injury such as schizophrenia also show glial ECM abnormalities in conjunction with PNN disruption in several key brain areas suggesting a potential role of glia in PNN reduction (Pantazopoulos et al., 2010, 2015; Mauney et al., 2013). Considering its widespread prevalence, ECM remodeling appears to be a generic response of reactive astrocytes to a majority of brain disorders (**Figure 3**).

The deleterious consequences of ECM remodeling by glial cells are remarkable in CNS pathologies with a focal lesion such as ischemia, glioma, MS lesion, and brain and spinal cord injury. At the focal lesion or injury site, astrocytes turn reactive (Escartin et al., 2021) and form a barrier or glial scar, aided by infiltration of microglia, macrophages, meningeal cells, and fibroblast (Rhodes et al., 2003). Scar-forming reactive astrocytes accumulate a variety of ECM molecules, including CSPGs, HA, tenascins, fibronectins, and laminins, and isolate the insult from surrounding areas to limit the spread of inflammation and tissue damage (Kim et al., 2016). In the case of traumatic injuries, especially in the spinal cord, reactive astrocytes deposit various CSPGs in the glial scar which inhibits neuronal recovery and axonal regrowth (Silver and Miller, 2004; Lau et al., 2013). Similarly, in cortical injury, reactive astrocytes highly upregulate CSPGs and inhibit cortical axonal regeneration (McKeon et al., 1999; Busch and Silver, 2007; Kim et al., 2016). ChABC application to dissolve CSPGs enhances axonal growth and functional recovery to great extent (Bradbury et al., 2002; Huang et al., 2006; Lee et al., 2010). Interestingly, astrocyte specific ChABC expression in transgenic mice reduced CSPG expression after spinal cord injury and enhanced axonal growth and recovery (Cafferty et al., 2007). These studies provide compelling evidence of inhibitory functions of glial-derived CSPGs and can be harnessed to generate glia-centric therapeutic tools.

Reactive astrocytes also release non-sulfated proteoglycan HA in the glial scar areas in the brain or spinal cord lesions, evoking beneficial and deleterious effects (Sherman et al., 2015). The HMW HA accumulation after spinal cord injury suppresses the activation of astrocytes as well as glial scar formation. On the other hand, low MW hyaluronan accumulation [perhaps as a cleavage product of HMW HA due to increased hyaluronidase activity (Al'Qteishat et al., 2006)] promotes astrocytic activation and proliferation (Struve et al., 2005). Following an ischemic stroke, scar-forming astrocytes upregulate the hyaluronan as well as HA receptor RHAMM, and are speculated to evoke the migration of adult stem cells from the subventricular niche to the ischemic site for repair (Lindwall et al., 2013). Glial upregulation of HA is also evidenced in aging; however, the downstream effects require thorough investigation (Peters and Sherman, 2020).

Similarly to CNS trauma and injury, in MS several ECM components such as hyaluronan, CSPGs (aggrecan, versican, and neurocan), and glycoprotein (fibronectin and vitronectin) are deposited around the lesion, primarily by reactive astrocytes forming the glial scar (Lau et al., 2013). Condensed CSPGs and HA at the lesion prevent OPC growth, differentiation, and migration to the lesion site, thereby making remyelination nearly impossible. Despite providing beneficial effects to the axon growth in spinal cord injury, HA and CSPG disruption strategies using exogenous MMPs, ChABC or hyaluronidase have not proven promising, largely due to indiscriminate disruption of both permissive and non-permissive ECM components and generation of non-permissive cleavage product (Lau et al., 2013). Since reactive astrocytes are the major CSPG and HA-producing cells at the lesion, strategies to manipulate the cellular machinery of CSPG and HA biosynthesis and release can be speculatively proposed as an alternative approach.

Since PNN disruption is commonly observed in several CNS disorders, it is a legitimate question whether astrocytes play any role in PNN disruption in such pathologies. The answer lies partly in the fact that astrocytes are a major source of PNN-disrupting MMPs in both homeostatic and diseased states. After brain injury and trauma, PNN disruption is accompanied by upregulation of MMP-2 and MMP-3 levels by reactive astrocytes (Muir et al., 2002; Falo et al., 2006; Lorenzo Bozzelli et al., 2018). MMP-9 appears to be the major effector of PNN disruption triggered by activation of TGFβ signaling in reactive astrocytes after albumin extravasation due to BBB disruption in various neurological insults (Kim et al., 2017). Notably, microglia, infiltrating immune cells, and neurons are also sources of MMPs after CNS insults; therefore astrocytes may not be solely responsible for PNN disruption (Rosenberg, 2002). This fact is also supported by our study on glioma-associated epilepsy, in which PNN disruption did not spatially correlate with reactive astrogliosis and astrocytes did not show MMP activity (Tewari et al., 2018).

## Extracellular matrix and perineuronal net remodeling by microglia in central nervous system pathologies

Microglia are the resident immune cells in the brain and are known for their classic phagocytic function of eliminating entire cells or subcellular structures- mainly synapses- in healthy and diseased states (Wolf et al., 2017). The developing brain

forms an excessive number of synapses, which are subsequently eliminated by a process known as synaptic pruning, in which microglia play a pivotal role. In a healthy adult brain, microglia also actively prune synapses in an effort to aid neuronal plasticity (Wolf et al., 2017). Besides these well-known functions, recent studies suggest a conversion of normal astrocytes to a detrimental neurotoxic phenotype under the influence of microglia-released cytokines IL-1α, TNF-α, and complement protein C1q in pathological conditions (Liddelow et al., 2017).

Pioneering studies in the recent past implicate microglia equally well in the regulation of homeostatic functions such as ECM and PNN remodeling, eventually governing synaptic plasticity. The classic example is a reversible increase in the abundance and condensation of cortical PNNs (Crapser et al., 2020a; Liu et al., 2021) and increased brevican accumulation around hippocampal synapses upon elimination of microglia in normal CNS (Strackeljan et al., 2021). PNN and ECM disruption is evidenced in CNS pathologies such as AD, wherein microglia appear to effectuate the clearance of the ECM and PNN components either directly, by stripping PNNs from the neuronal surface; or indirectly, by clearing the PNN debris after proteolytic cleavage by MMPs (Fawcett et al., 2019; Crapser et al., 2020b, 2021). Interestingly, eliminating microglia effectively restores the ECM components as well as PNN (Crapser et al., 2020a,b) and can be explored further from a therapeutic angle.

A similar role of microglia has recently been reported in the spinal cord dorsal horn wherein peripheral nerve injury promotes microglial degradation and endocytosis of PNNs around projection neurons. PNN depletion activates the projection neurons to induce pain-related behavior (Tansley et al., 2022). Despite such explicit presentations of microglial involvement, what triggers microglia to start stripping PNNs and ECM components with or without proteolytic cleavage is still an open question. At a minimum, it can be speculated that homeostatic PNN regulation can also be regulated by neuronally released cytokine interleukin-33 (IL-33) which guides microglia to clear ECM to facilitate homeostatic synaptic plasticity; a loss of IL-33 signal leads to ECM accumulation around synapses and dendrites (Nguyen et al., 2020). Similarly, in the developing brain, astrocytes are the main source of IL-33, which in turn instructs microglia to engulf redundant synapses (Vainchtein et al., 2018). Another study revealed the role of microglial protease cathepsin-S in maintaining a diurnal rhythm of the PNN labeling by demonstrating modification in PNNs in a circadian manner that coincides with the rhythmic expression of protease cathepsin-S (Pantazopoulos et al., 2020); however, cyclic rhythm of PNNs is still debated (Barahona et al., 2022). Collectively these and several other recent studies (Venturino et al., 2021) suggest microglia as an integral element of the ECM and PNN remodeling in homeostasis and diseased states; however, the mechanistic insight and therapeutic potential warrant further investigation.

## Metalloproteinases: Main effectors of extracellular matrix and perineuronal net remodeling

Despite a wide range of upstream cellular and molecular events, the mechanisms of ECM and PNN remodeling largely converge onto the metalloproteinases, which serve as an immediate effector to decisively control the ECM and PNN remodeling. Metalloproteinases are zinc-containing endopeptidases, normally expressed by neurons, astrocytes, microglia, and endothelial cells. However, under pathological conditions such as ischemic stroke and brain tumors, non-resident immune cells and tumor cells can also produce metalloproteinases (Tewari et al., 2018; Patel et al., 2019; Chaunsali et al., 2021). Proteolytic remodeling of ECM by metalloproteinase is pivotal in regulating multiple physiological and pathophysiological processes during CNS development and survival, angiogenesis, neurogenesis, axonal growth and regeneration, CNS injury, tumor metastasis, and neuroinflammation (Gottschall and Howell, 2015; Rempe et al., 2016).

Metalloproteinases consist of two families: MMP and a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTSs). In principle, out of 24 MMPs, MMP-1–3, 7–17, 24, and 28 are expressed in the brain under specific pathophysiological circumstances; however, a vast majority of them remain largely undetectable in a normal physiological state (Rempe et al., 2016; Beroun et al., 2019). Based on substrate specificity, major MMPs can be categorized into collagenase (MMP-1, 8, 13); gelatinase (MMP-2, 9) which can degrade collagen IV, fibronectin, laminin, aggrecan, gelatin, elastin, and non-matrix substrates; and stromelysins (MMP-3, 10, 11) which can cleave collagens I, III, IV, V, IX, and X (Nakamura et al., 2009), fibronectin, denatured collagens, laminin, and cartilage proteoglycans (Chang et al., 2016). ADAMTSs are equally diverse with 19 family members, of which ADAMTSs 1, 4, 5, 9, and 15 are expressed in specific brain regions under different circumstances (Gottschall and Howell, 2015). ADAMTSs1, 4, and 5 are known as proteoglycanases or aggrecanases as they can cleave major brain proteoglycans aggrecan, brevican and versican (Kelwick et al., 2015). By and large, ADAMTSs complement the homeostatic and pathological functions of MMPs in developmental ECM remodeling, tissue repair after injury, inflammation, and cancers (Song and Dityatev, 2017; Testa et al., 2019).

Matrix metalloproteinases and ADAMTSs can collectively cleave all elements of the ECM and PNNs as well as several growth factors and membrane proteins (See review

Yong et al., 2001; Lu et al., 2011; Gottschall and Howell, 2015); thus, their upstream and downstream regulations are critically important. Indeed, the activity of metalloproteinase is tightly regulated at multiple levels, which makes them ideal candidates for spatiotemporally controlling the ECM and PNN remodeling. At the transcriptional level, most of the MMPs are expressed non-constitutively and need some form of extrinsic signals to trigger their transcription, such as inflammatory cytokines, growth factors, chemokines, and cell-cell or cell-matrix interactions. Several MMPs are expressed as inactive zymogens and need an activation process; this is predominantly cleavage of a propeptide region by intra- and extracellular proteases, including other MMPs. These post-translation modifications bring another level of MMP activity regulation. Even after MMPs are synthesized, secreted, and activated, tissue inhibitors of metalloproteinases (TIMP1-4) can inactivate them by reversibly binding to their catalytic site and thereby terminating the proteolytic activity (Yong et al., 2001). Among other extrinsic regulators, activation of neurotransmitter receptors for glutamate (Dziembowska et al., 2012), dopamine (Mitlöhner et al., 2020), and serotonin (Bijata et al., 2017) are shown to directly regulate MMP-9 and ADAMTS-4 and -5 activity and subsequently trigger neuroplasticity.

From the brain ECM perspective, MMP-2 and MMP-9, and to a lesser extent MMP-3, are the most studied metalloproteinases, perhaps due to their reliable detection and measurement methods and also their involvement in multiple processes (Dzwonek et al., 2004). MMP-2, or gelatinase A, is expressed by both neurons and glial cells; however, astrocytes appear to be the major source (Dzwonek et al., 2004; Brkic et al., 2015). Functionally, mostly MMP-2, along with MMP-9, is involved in several processes including neurogenesis, migration, dendritic and axonal outgrowth, and guidance in the developing brain as well as migration, regeneration, adult neurogenesis, and angiogenesis in the adult brain (see review Verslegers et al., 2013). In several CNS pathologies in which PNN disruption and ECM remodeling is commonly observed-including ischemia, traumatic brain injury, and seizures-MMP-2 levels, especially in glial cells, are also upregulated (Dzwonek et al., 2004; Kim and Hwang, 2011; Verslegers et al., 2013; Quattromani et al., 2018). Despite such explicit correlation, the decisive contribution of astrocytic MMP-2 in PNN disruption is hard to discern due to a concomitant release of MMP-9 from neurons which can also degrade PNNs (Kim and Hwang, 2011).

MMP-9 is another gelatinase expressed by neurons, astrocytes, and oligodendrocytes (Kamat et al., 2014; Song and Dityatev, 2017) and is the most widely implicated metalloproteinase in CNS homeostasis and pathologies. MMP-9 appears to be the key player in ECM and PNN remodeling-induced neuroplasticity in physiological and pathological conditions. For example, MMP-9 and MMP-2 loosen PNN after enriched environment rearing (Foscarin et al., 2011) or light exposure after monocular deprivation (Wen et al., 2018b) to facilitate neuroplasticity. MMP-9 plays a pivotal role in hippocampal-dependent plasticity, possibly by ECM degradation and inducing surface recruitment of NMDAR between non-synaptic and synaptic compartments, activation of L-VDCCs, and dendritic spine modifications (Kochlamazashvili et al., 2010; Verslegers et al., 2013). A recently reported fundamental role of astrocytes in closing the critical period of visual plasticity is effectuated by MMP-9. Astrocytic connexin 30 signaling suppresses MMP-9 expression to allow maturation of PNNs, thereby closing the critical period of visual plasticity (Ribot et al., 2021). On the other hand, reactive astrocytes in CNS pathologies upregulate MMP-9 *via* inflammatory signaling (Kim et al., 2016). MMP-9 upregulation correlates remarkably with disruption of the PNN in an overwhelming number of studies on epilepsy (McRae and Porter, 2012; Kim et al., 2016, 2017; Tewari et al., 2018; Chaunsali et al., 2021), TBI and stroke (Kim et al., 2017), glioblastoma (Tewari et al., 2018; Hatcher et al., 2020), neurodegenerative (Kamat et al., 2014; Brkic et al., 2015) and neuropsychiatric diseases, and blocking MMP-9 activity generally prevents PNN disruption and improves the disease pathologies (Testa et al., 2019). More recently, inhibition of MMP-2/9 by IPR-179 showed promising antiseizure and antiepileptogenic effects as well as improved cognitive deficits induced by seizures (Broekaart et al., 2021). Similarly, in genetic disorders including Fragile X Syndrome, elevated MMP-9 correlates with PNN disruption, and genetic reduction of MMP-9 promotes PNN formation (Wen et al., 2018a). Overall, MMP-2 and MMP-9 are primary effectors of neuronal and glial cell mediated ECM and PNN remodeling in health and diseases.

MMP-3 or stromelysin-1 is an emerging ECM regulator due to its broad substrate specificity, particularly in its ability to activate pro-MMPs including MMP-2 and MMP-9 (Kim and Hwang, 2011). Perhaps due to this upstream control ability, MMP-3 level in healthy neurons is very low to undetectable. In pathological conditions, neurons, oligodendrocytes, astrocytes, and microglia release MMP-3, which is implicated in neuroinflammation and apoptosis associated with several neurodegenerative disorders (Rempe et al., 2016). Compared to MMP-9, the roles of MMP-3 in context to PNN remodeling and neuroplasticity in adult brains are not explicitly studied. However, MMP-3 has largely been associated with detrimental processes including inflammation, apoptosis, BBB breakdown, neurodegeneration, and demyelination (Kim and Hwang, 2011; Van Hove et al., 2012).

Besides metalloproteinases, CSPGs and PNNs can also be cleaved by tissue-type plasminogen activator (tPA) which is a serine protease and classically known to dissolve blood clots. tPA can degrade PNN directly as its substrate or *via*

activating MMPs and upregulated tPA levels may be associated with disrupted PNNs in ischemia and epilepsy (McRae and Porter, 2012; Quattromani et al., 2018). However, whether the homeostatic tPA expression can remodel PNNs is not known. Endogenous hyaluronidase is another enzyme that can degrade PNNs indirectly by cleaving the hyaluronan backbone of the PNNs. Although hyaluronidase levels are elevated after stroke and brain injury, not much is known about their role in the homeostatic and pathological remodeling of ECM and PNNs (Sherman et al., 2015; Reed et al., 2019).

## Concluding remarks

Extracellular matrix forms the structural scaffold of the brain architecture and serves both structural and signaling functions in the developing and adult brain. PNNs are a highly organized form of ECM and confer several functions including neuroplasticity, ionic homeostasis, neuroprotection, and regulation of neuronal activity. A growing body of evidence causally links PNN disruption with the pathophysiology of several CNS disorders such as epilepsy. Glial cells, predominantly astrocytes, are one of the major sources of ECM molecules in developing and adult CNS, thereby directly influencing brain development and functions. Glial cells also release matrix cleaving proteases which are the main effectors of ECM and PNN remodeling during development, adulthood, aging, and diseases. By regulating the spatiotemporal expression of ECM molecules as well as the ECM remodeling proteases, glial cells play a central role in ECM homeostasis in CNS physiology and pathology. Owing to their multifaceted roles in ECM homeostasis, glial cells appear to be promising targets for therapeutic interventions. However, more studies are required to understand the mechanistic insight of glial regulation of ECM and PNNs to pinpoint the molecular pathways and target molecules.

## Author contributions

BT: literature search, data curation, and manuscript writing, editing, and communication. LC and CP: literature search, data curation, and manuscript editing. HS: literature search, manuscript editing, and funding acquisition. All authors contributed to the article and approved the submitted version.

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## Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

## Publisher's note



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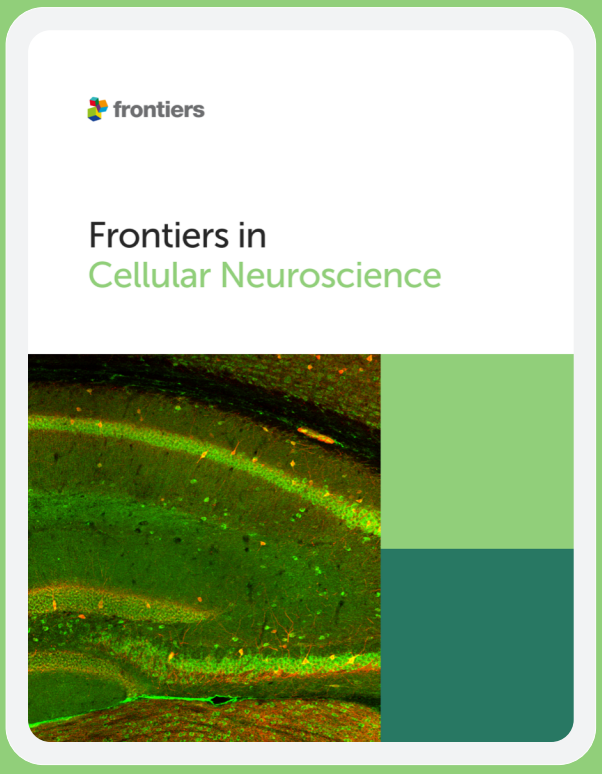


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