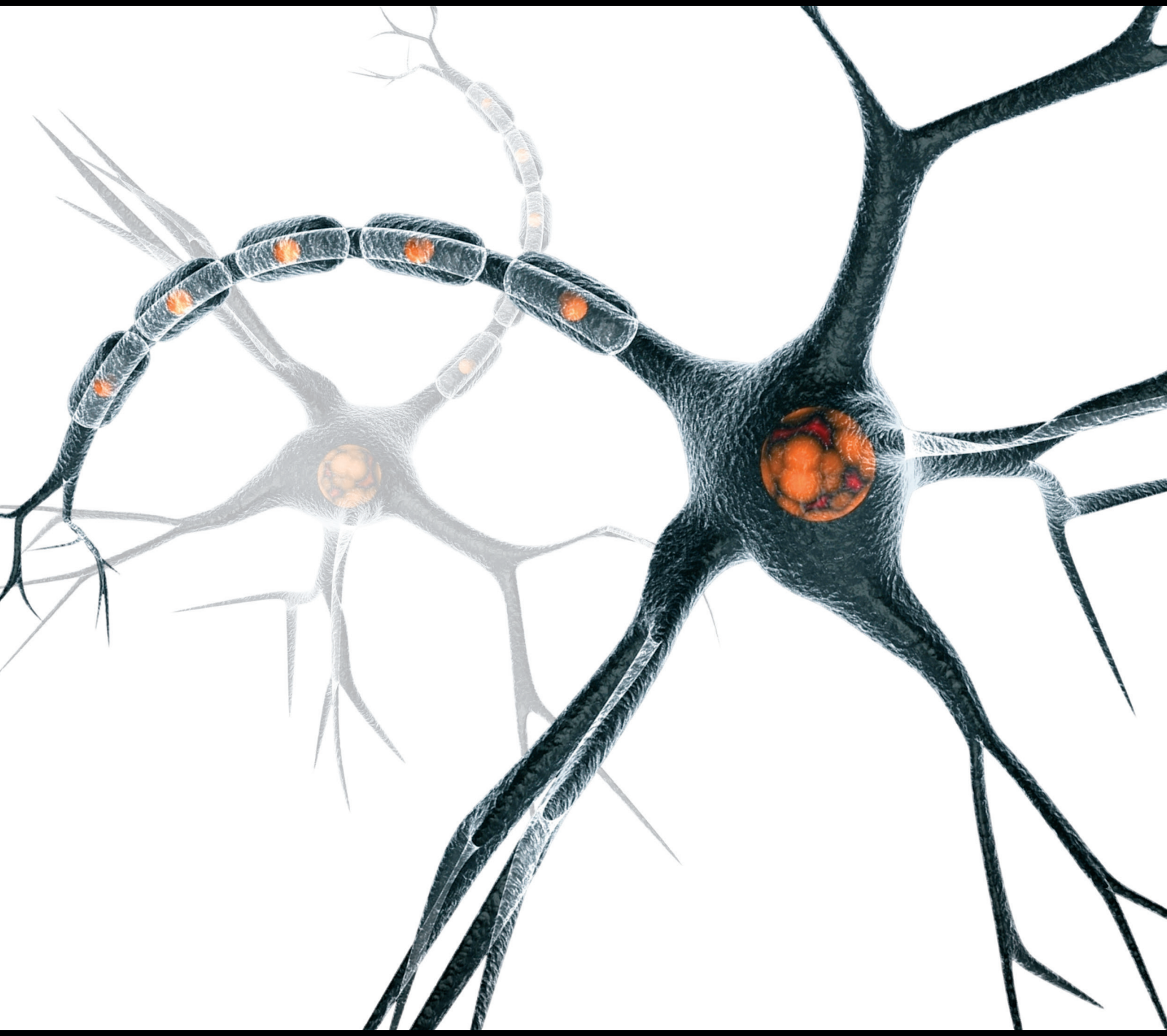
Neural Plasticity

Perineuronal Nets and CNS Plasticity and Repair

Guest Editors: Daniela Carulli, Jessica C. F. Kwok, and Tommaso Pizzorusso



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Editorial Board

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Editorial

Perineuronal Nets and CNS Plasticity and Repair

Daniela Carulli,[1,2,3] Jessica C. F. Kwok,[4,5] and Tommaso Pizzorusso[6,7]

[1]*Department of Neuroscience, Neuroscience Institute of Turin (NIT), University of Turin, 10100 Turin, Italy*
[2]*Neuroscience Institute of the Cavalieri-Ottolenghi Foundation (NICO), Regione Gonzole 10, Orbassano, 10043 Turin, Italy*
[3]*Netherlands Institute for Neuroscience (NIN), 1105 BA Amsterdam, Netherlands*
[4]*John van Geest Centre for Brain Repair, Department of Clinical Neuroscience, University of Cambridge, Forvie Site, Cambridge CB2 0PY, UK*
[5]*School of Biomedical Sciences, Faculty of Biological Sciences, University of Leeds, Leeds LS2 9JT, UK*
[6]*Department of Neuroscience, Psychology, Drug Research, and Child Health (NEUROFARBA), University of Florence, 50134 Florence, Italy*
[7]*Institute of Neuroscience, CNR, Via Moruzzi 1, 56124 Pisa, Italy*

Correspondence should be addressed to Daniela Carulli; daniela.carulli@unito.it

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The extracellular matrix (ECM) of the nervous system regulates numerous events during development, from neurogenesis and gliogenesis to circuitry formation, as well as in adulthood, affecting damage responses, plasticity, and regeneration. Substantial changes in the quantity and the composition of ECM occur during development. At the end of critical periods, that is, temporal windows during development when experience-dependent neuronal plasticity is heightened, a specialised ECM structure called perineuronal net (PNN) deposits around many types of neuron, helping in stabilizing the newly established neuronal connections. In recent years, several other functions of the PNNs have been revealed, including restriction of neuronal plasticity, neuronal protection, and modulation of the pathogenesis of various CNS diseases. Elucidating the mechanisms through which PNNs act is challenging but holds a tremendous therapeutic potential for treating several CNS conditions.

In this special issue, we collected research and review articles that focus on different aspects of PNN structure, development, and function in health and disease.

In the article "Development and Structural Variety of the Chondroitin Sulfate Proteoglycans-Contained Extracellular Matrix in the Mouse Brain," N. Horii-Hayashi et al. provide the first systematic study of PNN formation at the level of the whole brain of the mouse, from postnatal (P) day 3 to 11 weeks. The spatiotemporal distribution of *Wisteria floribunda* agglutinin-binding PNNs is described in several brain regions, including the brainstem, hypothalamus, limbic regions, and cerebral cortex. The period of PNN formation differs among distinct brain areas, supporting the idea that PNN maturation is functionally related to the closure of critical periods for the acquisition of specific functions.

The study by A. L. Mueller and colleagues, entitled "Distribution of N-Acetylgalactosamine-Positive Perineuronal Nets in the Macaque Brain: Anatomy and Implications," addresses the distribution of PNNs and the proportion of neurons surrounded by PNNs in different areas of the rhesus macaque CNS. Highly variable proportions of PNNs characterize the monkey CNS, being most abundant in the cerebellar nuclei and less abundant in the cerebral cortex and midbrain. PNNs were found around parvalbumin-positive as well as parvalbumin-negative neurons. A useful discussion is provided about PNN expression in the primate CNS compared to rodent and human brain, which suggests that PNN prevalence is broadly maintained across taxa.

In the review "Neuron-Glia Interactions in Neural Plasticity: Contributions of Neural Extracellular Matrix and Perineuronal Nets," A. Faissner et al. show recent data on the role of PNNs in the context of astrocyte-neuron interactions and their regulatory function in the establishment, maintenance, and plasticity of synaptic connections. The impact of specific ECM components on the expression of PNNs,

neuronal activity, synaptogenesis, and synapse stabilization is discussed. A comprehensive overview of PNN structure, cellular origin of PNN components, PNN binding partners, and main functions of PNNs in the regulation of plasticity (at the circuit, cellular, and synapse level) is also provided, together with a description of neurological conditions in which PNNs are altered.

The article "Reorganization of Synaptic Connections and Perineuronal Nets in the Deep Cerebellar Nuclei of Purkinje Cell Degeneration Mutant Mice" by M. Blosa et al. addresses the role of PNNs in the regulation of structural plasticity in the adult brain in a deafferentation model. By employing *pcd* mice, which display slow Purkinje cell degeneration during the late postnatal age, the authors show increased sprouting of glutamatergic afferents, paralleled by decreased expression of specific PNN components, in the denervated cerebellar nuclei. Based on their findings, an interesting discussion on the role of neuron-versus astrocyte-released PNN molecules is provided.

The condensation of chondroitin sulfate proteoglycans (CSPGs) into PNNs and, as a consequence, the termination of the critical period for ocular dominance plasticity in the mouse visual cortex depends on a developmental increase in the 4-sulfation/6-sulfation ratio of chondroitin sulfates in the CSPGs (Miyata et al., 2012, Nature Neuroscience). In the article "Chondroitin 6-Sulfation Regulates Perineuronal Net Formation by Controlling the Stability of Aggrecan," S. Miyata and H. Kitagawa further extend our knowledge on this topic, showing that increased 6-sulfation leads to a decreased expression of the CSPG aggrecan, by accelerating ADAMTS-5-mediated aggrecan proteolysis.

Another key evidence demonstrating the significance of CS sulfation in regulating PNN functions is detailed in the review "Otx2-PNN Interaction to Regulate Cortical Plasticity" by C. Bernard and A. Prochiantz. The group has previously demonstrated that sulfation pattern is crucial for the interaction between CSPGs and one of its binding molecules, the homeoprotein Otx2. Otx2 binds to specifically sulfated CS of PNNs enwrapping cortical parvalbumin interneurons. Otx2 is then internalized by the interneurons, where it promotes their maturation and consequently the closure of the critical period. In their current paper, the authors discuss how the PNN interplays with Otx2 to regulate visual cortex plasticity and how interfering with this interaction can reopen windows of plasticity in the adult.

The idea that the concentration of specific plasticity-regulatory factors around neurons is controlled by PNNs may be also true for the repulsive axon guidance molecule Semaphorin 3A (Sema3A), as discussed in the review "The Chemorepulsive Protein Semaphorin 3A and Perineuronal Net-Mediated Plasticity" by F. de Winter et al. In this paper, recent data on Sema3A distribution in PNNs in the adult CNS, interaction of this molecule with specific PNN-CS sugars, and changes in Sema3A expression during brain plasticity are reported. It strongly suggests that Sema3A is an important PNN component for regulation of neuronal plasticity.

Emerging evidence implicates ECM/PNNs in the pathophysiology of several neurodevelopmental, neurological, and psychiatric disorders. In the paper "In Sickness and in Health: Perineuronal Nets and Synaptic Plasticity in Psychiatric Disorders," H. Pantazopoulos and S. Berretta review recent data about PNN abnormalities in psychiatric conditions, with particular focus on schizophrenia, and discuss the hypothesis that ECM/PNN alterations may significantly contribute to synaptic dysfunction, which is a critical pathological component of several brain disorders.

One of the most recent discoveries concerning PNNs is their role in drug addiction and drug-related memories. In the review "Caught in the Net: Perineuronal Nets and Addiction," M. Slaker et al. address this topic by discussing drug-induced changes in PNNs in brain circuitries underlying drug-related motivation, reward, and reinforcement.

We hope that this special issue will stimulate further studies on gaining a deeper understanding of the role of PNNs in brain physiology and pathology. We believe that a better knowledge of the structure and function of PNNs in physiological and pathological conditions and of the consequences of manipulating the PNN has a strong potential for the development of therapies to enhance neuronal plasticity and functional recovery in a number of CNS conditions, from neurodevelopmental disorders to injury and drug addiction.

Daniela Carulli
Jessica C. F. Kwok
Tommaso Pizzorusso

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Review Article

Caught in the Net: Perineuronal Nets and Addiction

Megan Slaker, Jordan M. Blacktop, and Barbara A. Sorg

Department of Integrative Physiology and Neuroscience, Washington State University, Vancouver, WA 98686, USA

Correspondence should be addressed to Barbara A. Sorg; sorg@vetmed.wsu.edu

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Exposure to drugs of abuse induces plasticity in the brain and creates persistent drug-related memories. These changes in plasticity and persistent drug memories are believed to produce aberrant motivation and reinforcement contributing to addiction. Most studies have explored the effect drugs of abuse have on pre- and postsynaptic cells and astrocytes; however, more recently, attention has shifted to explore the effect these drugs have on the extracellular matrix (ECM). Within the ECM are unique structures arranged in a net-like manner, surrounding a subset of neurons called perineuronal nets (PNNs). This review focuses on drug-induced changes in PNNs, the molecules that regulate PNNs, and the expression of PNNs within brain circuitry mediating motivation, reward, and reinforcement as it pertains to addiction.

1. Introduction

Repeated exposure to drugs of abuse creates persistent drug-related memories that may be the foundation of the chronic relapse problem. Memories for the drug, drug-related cues, and drug-related contexts are strengthened over time with repeated drug use. Disruption of these drug-related memories by targeting key neuroplastic events required for memory stabilization may lead to suppression of relapse. Recently, attention has shifted from targets located on the pre- and postsynaptic cells and astrocytes to targets located in the extracellular matrix (ECM) within brain regions important for drug-taking behavior and drug-related memories. This shift has contributed to the advancement from a tripartite synapse theory ((1) presynapse; (2) postsynapse; (3) astrocyte) to a tetrapartite synapse theory, which includes the ECM [1, 2].

Perineuronal nets (PNNs) are net-like structures composed of aggregations of ECM molecules. PNNs surround a subpopulation of neurons, and, in many brain regions, they surround primarily GABAergic, parvalbumin- (PV-) containing, fast-spiking interneurons [3–5]. They are most commonly identified by staining with the plant lectin, *Wisteria floribunda* agglutinin (WFA), although other methods have also been used [3, 6, 7]. PNNs are rich in chondroitin sulfate proteoglycans (CSPGs; primarily of the lectican family, including aggrecan, brevican, and versican) and hyaluronan (HA [8, 9]). Other molecules in the PNN are tenascins and link proteins (HAPLNs [10, 11]). Many plasticity-regulating molecules bind to or interact with the PNN, including semaphorin-3A, brain-derived neurotrophic factor (BDNF), and Otx2 [12–14]. PNNs have been heavily studied for their contributions to critical period plasticity within the visual system, motor system, and somatosensory system [15–17]. Despite the emerging significance of the role of PNNs in a variety of types of plasticity, very little is known about the plasticity of PNNs and PNN-surrounded neurons induced by drugs of abuse. Much less is known about how PNNs regulate function of their underlying neurons within brain regions that contribute to aberrant motivation, reward, and reinforcement underlying addiction. Understanding the contribution of PNNs to plasticity, and eventually harnessing this plasticity, may lead to therapeutic intervention for many pathologies, including addiction. Here we discuss how PNNs may regulate plasticity of brain regions heavily implicated in memory, reward, and addiction.

2. PNN and Memory

The brain regions that are important for acquiring and maintaining a fear-conditioned memory overlap with brain regions that are important for acquiring and maintaining a drug-related memory [18]. Both types of memory consist of

context- and cue-related information and motivated behavior. The most common method to examine a fear-conditioned memory is to train an animal to associate the presentation of a cue, typically a tone, with a footshock. This memory can be extinguished by repeated presentation of the cue without presence of the shock. Interestingly, in rats younger than 3 weeks, extinction training of the fear-conditioned memory is capable of rewriting or potentially erasing the original memory trace, whereas, in adult animals, extinction training creates a new memory but does not erase the original memory [19]. PNNs are important for the acquisition and maintenance of a fear-conditioned memory. Juvenile animals do not have PNNs but adult animals do. Thus, the developmental timetable of PNNs within the basolateral amygdala (BLA), a region of the brain critical for fear conditioning, mirrors the loss in ability of extinction training to override the original memory trace [19]. Not only does the appearance of PNNs coincide with this behavioral change, but removing PNNs from the BLA by local administration of chondroitinase-ABC (Ch-ABC), a bacterial enzyme used to degrade components of the ECM and PNN, reverts the behavior of an adult animal back to the behavior of a juvenile animal [19]. These findings suggest that PNNs contribute to the maintenance of strong fear memories.

PNNs within the hippocampus and medial prefrontal cortex (mPFC) also play a role in fear conditioning [20]. Removal of PNNs within the hippocampus impairs context-induced reinstatement of fear conditioning, whereas removal of PNNs within the mPFC impairs cue-induced reinstatement of fear conditioning [20]. Collectively, these findings suggest that PNNs contribute to the development and maintenance of a fear-conditioned memory; however, the role they play in specific types of memory maintenance (e.g., cue- or context-dependent memory) appears to be brain region-dependent. Each of the studies used a global disruption method to target not only the PNN, but all of the ECM. Future studies will be needed to more specifically target the PNN and verify the effects of these studies.

3. PNNs and Reward-Related Circuitry

Examination of brain regions with relatively high PNN expression may provide insight into the neuroanatomical function of PNNs, including brain regions implicated in the circuitry of motivation, reward, and reinforcement. Brain regions that have been heavily focused on include the amygdala, hippocampus, and regions of the PFC because they are critical for activation of goal-directed behavior, memory, and addiction [21–23]. Significant PNN expression has been reported in over 100 brain regions of the rat, ranging from the rostral cortex to the spinal cord, but few studies have characterized the expression of PNNs in brain regions implicated in addiction: the prefrontal cortex (PFC), amygdala, hippocampus, cerebellum, striatum, ventral pallidum, and hypothalamus [3, 24, 25].

3.1. Prefrontal Cortex. The PFC contains numerous distinct regions that contribute to addiction. In general, the dorsomedial PFC (dmPFC) facilitates drug seeking, while the ventromedial PFC (vmPFC) impedes drug seeking [18, 26–29]. Within both of these regions, PNNs are primarily colocalized with PV-containing, GABAergic interneurons, which are well conserved across species including rat, primate, and human [3, 6, 30–36]. This population of PV-containing interneurons regulates pyramidal neuron output from the PFC and regulates gamma oscillations important for working memory and optimal cognitive processing [37–41]. In addition to the mPFC, the orbitofrontal (OF) region of the PFC has consistently been implicated in the representation of value of a reinforcer and in value-based decision-making [22, 42]. Aberrant OF activity has been observed following drug exposure and is thought to mediate compulsive drive and craving in humans and cue-induced reinstatement in rodents [43, 44]. The OF exhibits relatively robust PNN expression and many PNN components within the OF increase following intermittent binge ethanol exposure [24, 45]. Collectively, PNNs across subregions of the PFC are affected by drug exposure and may provide a target to specifically modify circuitry of motivated behavior.

3.2. Amygdala. The amygdala is critical for emotional processing, reward valuation, and learning [22, 46]. The amygdala is well situated between the PFC and the ventral striatum to provide key neurocircuitry mediating both stress- and cue-induced reinstatement of drug-seeking behavior [22, 47]. Studies on PNN expression differ between species within the amygdala. Early studies examining the amygdala of the *rodent* reported relatively low PNN expression [24, 25]; however, a more recent study examining the BLA of *humans* reported significant PNN expression [48]. Despite these conflicting reports, PNNs within the amygdala of the rodent have been directly implicated in both fear and addiction behavior (see PNNs in memory and addiction sections).

3.3. Hippocampus. The hippocampus is importantly involved in memory and has also been implicated in reward circuitry regulating drug-seeking and drug-taking behavior [49–55]. The hippocampus expresses significant levels of PNNs [24, 25]. Both PV and PNNs colocalize within basket cells in the CA1 and CA3 regions and the granule cell layer of the dentate gyrus [56–58]. This colocalization does not appear within the CA2 region, where PNNs surround cells that are presumed to be non-GABAergic pyramidal neurons [59]. The expression of PNNs within the hippocampus provides an interesting region to study the differences among the subpopulations of PNN-surrounded neurons (PV+, PV−, and pyramidal) and how each of these subpopulations contributes to memory and drug-seeking/taking behavior.

3.4. Cerebellum. Although not traditionally viewed as part of addiction-related circuitry, the cerebellum is activated during episodes of drug craving and exhibits drug-induced plasticity [60–63]. In the cerebellum, PNNs are found primarily around large, excitatory neurons of the deep cerebellar nuclei, while granule cell layer Golgi neurons of the cerebellar cortex express PNNs in comparatively low numbers and do not

colocalize with PV [24, 25, 64, 65]. Exposure to cocaine increases the proportion of PNNs expressing strong WFA staining intensity in the deep cerebellar medial nucleus, which is a PNN-rich region characterized by large projection neurons [63].

3.5. Other Regions. The striatum is heavily implicated in reward and motivated behaviors and consists of the nucleus accumbens, caudate nucleus, and putamen. Low levels of sporadic PNN staining have been reported in all three regions of the striatum in the rat [24, 25]; in contrast, in the mouse, significant PNN expression has been reported throughout the striatum [66].

The ventral pallidum is essential for the integrative component of the limbic system contributing to motivated behavior and drug seeking [47, 67]. The ventral pallidum exhibits robust PNN expression [24], making it a promising, yet greatly understudied, brain region with regard to the role of PNNs in motivated behavior.

Finally, the lateral and anterior hypothalamus are important for motivated behaviors and contain strong PNN expression [24, 68–71]. However, PNNs in the lateral hypothalamus are PV-containing glutamatergic neurons rather than GABAergic neurons, while PNNs in the anterior hypothalamus are calretinin/enkephalin-containing neurons rather than PV-containing neurons [70, 71].

Understanding the functional plasticity of PNNs throughout the circuitry underlying motivated behaviors is in its infancy but shows promise in delineating drug-induced changes in circuit plasticity. Based on regional differences in the expression of PNNs, additional work is needed to address whether drugs of abuse alter expression of PNNs in these different regions and whether modifying PNNs after drug exposure or other behavioral manipulations could mitigate drug-induced plasticity. Next, we examine the current research addressing these questions.

4. PNNs and Addiction

While the expression of PNNs varies across circuitry mediating reward, few studies have investigated the role of PNNs in drug- and reward-related behaviors. In 2010, Van Den Oever and colleagues [27] trained rats on a heroin self-administration task and measured levels of PNN components within the mPFC following cue-induced reinstatement. Protein and mRNA levels of brevican, tenascin-R, and HA were decreased following a period of *forced abstinence* or *extinction training* from animals that self-administered heroin compared to levels from animals that self-administered saline. However, following cue-induced reinstatement of heroin self-administration, brevican returned to control levels. These findings suggest that PNN components dynamically respond to environmental cues, possibly via a reduction in ongoing MMP activity or a redistribution of PNN components [27]. Following cue-induced reinstatement, the frequency of spontaneous inhibitory postsynaptic currents (sIPSC) was increased compared to animals not exposed to the cues; the amplitude and decay-time constant of sIPSC did not change. This finding suggests that the functioning of GABAergic interneurons within the mPFC is increased in the presence of drug-associated cues and may be related to the changes that are occurring within PNNs that envelop these interneurons. Future studies are needed to explore these possibilities.

In the second study examining the role of PNNs in drug-seeking behavior, Xue et al. [72] removed PNNs using Ch-ABC within the BLA or central amygdala prior to extinction training of morphine-induced or cocaine-induced conditioned place preference (CPP). They found that PNN removal enhanced extinction training, resulting in decreased reinstatement of CPP behavior. Additionally, removal of PNNs within the BLA or central amygdala prior to extinction training of heroin self-administration also enhanced extinction training and decreased spontaneous recovery. In addition to behavior, markers of plasticity were examined, including BDNF and GluR1–3. Interestingly, removal of PNNs without extinction training or extinction training alone resulted in a similar increase in protein levels of BDNF, GluR1, and GluR2, whereas removal of PNNs *plus* extinction training resulted in an even greater increase in BDNF, GluR1, and GluR2 levels compared with no extinction training in animals with intact PNNs [72]. This study suggests that PNN removal may augment metaplastic conditions necessary to enhance extinction training of drug-induced CPP. This finding is consistent with previous studies demonstrating that removal of PNNs promotes experience-dependent plasticity [12, 15, 17, 73]. Future studies are needed to explore more specific methods of targeted PNN disruption and exploration of changes that are occurring in the PNN components following drug exposure.

Finally, recent work from our laboratory has examined the role of PNNs within the mPFC on the acquisition and maintenance of a cocaine-induced CPP memory. Removal of PNNs using Ch-ABC within the dmPFC impaired acquisition and reconsolidation of a CPP memory [36]. Interestingly, this effect was specific for the dmPFC because removal of PNNs from the vmPFC had no effect on acquisition of the task. Additionally, removal of PNNs within the dmPFC prior to extinction training had no effect on extinction or subsequent cocaine-induced reinstatement of CPP. In parallel with the observed behavioral decrease following PNN removal, we also found a decrease in the number of PNN-surrounded neurons that were positive for the immediately early gene c-Fos, but only in animals that displayed impaired memory. These results suggest that the acquisition and reconsolidation processes are impaired following PNN removal from the dmPFC, which may be a result of impaired activation of PNN-surrounded neurons. Additionally, we found that removal of PNNs results in hyperexcitability in pyramidal cells within the dmPFC. Removal of PNNs using Ch-ABC decreased the frequency of mIPSCs on pyramidal neurons and the number of action potentials. Interestingly, cocaine-induced CPP decreased both the frequency and amplitude of mIPSCs on pyramidal neurons. These findings suggest that ECM disruption and PNN removal may occlude cocaine-induced adaptations in the dmPFC.

5. Regulation of PNNs by Molecules Involved in Addiction

Under normal physiological circumstances, the ECM and PNNs have many endogenous regulators that can remodel PNNs to potentially allow for drug-induced plasticity to occur. Many molecules regulate components of the PNN and may therefore contribute to drug-induced plasticity. The two molecules we will focus on here are matrix metalloproteinases (MMPs) and BDNF.

5.1. Matrix Metalloproteinases. MMPs are a family of proteolytic enzymes (25+ members) that degrade components of the ECM, including those found within PNNs. MMPs are synthesized in an inactive proform, released from the cell following neuronal stimulation, and cleaved into an active MMP form. Many MMPs can act on PNN components and have been implicated in drug-related plasticity.

Two and 24 hours following the fifth consecutive day of noncontingent methamphetamine exposure, protein and activity levels of MMP-2 and MMP-9 increased within the nucleus accumbens and PFC of rats [74]. Additionally, following reinstatement of cocaine-induced CPP, activity levels of MMP-9 within the mPFC peaked 1–3 hours following reinstatement and returned to normal levels by 24 hours; however, MMP-2, MMP-3, and MMP-9 levels within the dorsal hippocampus and MMP-2 and MMP-3 levels within the mPFC were unchanged [75]. Similarly, a 15 min cue-induced reinstatement of cocaine or nicotine self-administration, a 45 min cocaine-induced reinstatement, or a 2-hour extinction session following cocaine or nicotine self-administration increased activity of MMP-2 and MMP-9 within the nucleus accumbens core [76]. Additionally, a 15 min cue-induced reinstatement of heroin self-administration increased MMP-2 and MMP-9 activity in the nucleus accumbens core [76]. In contrast to stimulant exposure, repeated alcohol exposure decreased MMP-9 activity within the hippocampus and PFC of rats after 2, 4, or 6 days [77]. These studies suggest that drugs of abuse change the activation of MMPs, which may allow for remodeling of the ECM and PNNs. These studies also suggest that the direction of changes is dependent on the type of drug, stimulant or depressant, although it remains unknown whether the duration and dose of drug exposure might also contribute to the opposite outcomes in MMP activation state.

To test the hypothesis that MMP activation is contributing to drug-seeking behavior, the effect of impairing MMPs on this behavior has been investigated. Impairment of MMP-2 and MMP-9 in knockout mice or administration of a nonspecific MMP inhibitor into the PFC reduced sensitization and disrupted methamphetamine-induced CPP [74]. Additionally, administering a nonspecific MMP inhibitor, FN-439, into the lateral ventricles blocked cocaine-induced reinstatement of CPP, reduced alcohol intake following acute withdrawal, and attenuated cue-induced heroin seeking [27, 78, 79]. These studies provide evidence that MMPs are involved in the neural response to drugs of abuse; however, additional studies need to investigate the effect of overexpression of MMPs on drug-seeking behavior or endogenous inhibitors of MMPs, such as tissue inhibitors of MMPs (TIMPs [74]). In addition, other ECM remodeling enzymes need to be investigated, including members of a Disintegrin and Metalloproteinase with Thrombospondin Motifs (ADAMTs) family of enzymes [80].

5.2. Brain-Derived Neurotrophic Factor. BDNF is important for many types of plasticity, including drug-induced plasticity. For example, global upregulation of BDNF enhanced extinction training that in turn decreased drug-seeking behavior [81]. Since BDNF has been implicated in a number of types of plasticity, it is perhaps not surprising that it also affects the expression of PV and PNNs, but the relationship between BDNF and PNN expression is far from clear. Overexpressing BDNF *in vivo* accelerates the appearance of PV+ synapses during development, which is used as a marker for neuronal maturation [82]. As demonstrated by Xue et al. [72], the removal of PNNs increased BDNF levels in the BLA in animals exposed to morphine, which suggests that PNN removal modifies how BDNF responds to this drug of abuse. Future research is required to determine detailed interactions between BDNF and its impact on PNNs. Interestingly, MMP-9 is thought to promote the conversion of pro-BDNF to the biologically active BDNF [83], and BDNF also induces MMP-9 expression *in vitro* [84].

6. Caveats and Future Directions

The role of PNNs in the creation and maintenance of drug-related memories and drug-seeking behavior is in its infancy. A foundation has been formed, but future studies will need to focus on how PNNs are modulated by different drugs of abuse (contingent and noncontingent) within brain regions important for motivation, reward, and reinforcement.

One important issue to consider is the methodology used to study the role of PNNs. Most studies, including our own, have used the bacterial enzyme, Ch-ABC, to degrade CSPG chains found both within the loose ECM and within the PNNs. This manipulation confounds results due to lack of specificity for the PNN. Knockout mice have been created that lack Hapln-1 within the central nervous system. Halpn-1 knockout mice die at birth because a total lack of Halpn-1 leads to cardiac defects [12]. To overcome this issue, Halpn-1 can be expressed under the control of type II collagen cartilage-specific promoter and enhancer, which allows Halpn-1 to be expressed in cartilage but not in the central nervous system [12]. These conditional knockout mice have attenuated PNNs and display similar ocular dominance plasticity as wild-type juvenile mice prior to the appearance or development of PNNs [12]. Additionally, this plasticity is similar to adult animals treated with Ch-ABC [12]. These results suggest that Halpn-1 within the central nervous system is necessary for proper formation of PNNs and for limiting plasticity following the critical period in development. Currently, this knockout exists only in mice. siRNA, shRNA, or morpholinos are other strategies to target Halpn-1 in other species. A second methodology consideration is how PNNs are visualized. Most PNNs are labeled using WFA, although other plant lectins also bind to and label sugar groups present

in PNNs [6]. Using an antibody for a specific component of PNNs, for example, aggrecan, is one method to visualize the aggregation of the CSPGs within the PNN.

A second consideration is the length of time between PNN removal and examination of behavioral or cellular changes. For example, removal of PNNs with Ch-ABC decreases WFA staining of PNNs for a period of days, but the reappearance of PNNs is gradual and is expected to create various stages of plasticity in the intervening period until PNNs are fully restored [36]. Thus, the direction and extent of behavioral and cellular changes are likely to be different depending upon the extent of removal and when they are examined following removal.

A third important consideration is how well conserved PNN expression is across species, from rodents to humans. Subpopulations of PNN-surrounded neurons differ across species and brain regions. In some studies, PNN expression around cortical pyramidal neurons is found in humans and primates but not in rodents [85, 86]. However, in other studies, PNN expression around glutamatergic pyramidal neurons has been reported in rodents [87]. PNN expression in the amygdala has also been found primarily around *astrocytes* in the human, but primarily around *neurons* in the monkey and rat [48]. Taken together, these results suggest that PNN expression within the brain is heterogeneous in both a species- and brain region-specific manner. Further comparative anatomical characterization of PNN expression across mouse, rat, monkey, and human will benefit the translational impact of this research.

7. Conclusions

While the role of PNNs in motivation, reward, and reinforcement is only beginning to be understood, recent findings indicate that exposure to drugs of abuse alters PNNs and that these structures are necessary for creating and/or maintaining drug-related memories. Future studies will be needed to provide a detailed understanding of the dynamic cellular changes occurring in PNNs and PNN-surrounded neurons to determine how these structures and neurons contribute to maintaining drug-related memories. In summary, PNNs and PNN-surrounded neurons may serve as novel targets for diminishing memories that drive relapse to drugs of abuse.

Conflict of Interests

The authors declare no competing financial interests.

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Research Article

Chondroitin 6-Sulfation Regulates Perineuronal Net Formation by Controlling the Stability of Aggrecan

Shinji Miyata[1,2] and Hiroshi Kitagawa[3]

[1]*Institute for Advanced Research, Nagoya University, Furo-cho, Nagoya 464-8601, Japan*
[2]*Bioscience and Biotechnology Center, Nagoya University, Furo-cho, Nagoya 464-8601, Japan*
[3]*Department of Biochemistry, Kobe Pharmaceutical University, 4-19-1 Motoyamakita-machi, Kobe 658-8558, Japan*

Correspondence should be addressed to Hiroshi Kitagawa; kitagawa@kobepharma-u.ac.jp

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Perineuronal nets (PNNs) are lattice-like extracellular matrix structures composed of chondroitin sulfate proteoglycans (CSPGs). The appearance of PNNs parallels the decline of neural plasticity, and disruption of PNNs reactivates neural plasticity in the adult brain. We previously reported that sulfation patterns of chondroitin sulfate (CS) chains on CSPGs influenced the formation of PNNs and neural plasticity. However, the mechanism of PNN formation regulated by CS sulfation remains unknown. Here we found that overexpression of chondroitin 6-sulfotransferase-1 (C6ST-1), which catalyzes 6-sulfation of CS chains, selectively decreased aggrecan, a major CSPG in PNNs, in the aged brain without affecting other PNN components. Both diffuse and PNN-associated aggrecans were reduced by overexpression of C6ST-1. C6ST-1 increased 6-sulfation in both the repeating disaccharide region and linkage region of CS chains. Overexpression of 6-sulfation primarily impaired accumulation of aggrecan in PNNs, whereas condensation of other PNN components was not affected. Finally, we found that increased 6-sulfation accelerated proteolysis of aggrecan by a disintegrin and metalloproteinase domain with thrombospondin motif (ADAMTS) protease. Taken together, our results indicate that sulfation patterns of CS chains on aggrecan influenced the stability of the CSPG, thereby regulating formation of PNNs and neural plasticity.

1. Introduction

Chondroitin sulfate proteoglycans (CSPGs) consist of core proteins with one or more covalently attached chondroitin sulfate (CS) chains and are essential components of the brain extracellular matrix (ECM). During late postnatal development, CSPGs condense around subpopulations of neurons and form lattice-like ECM structures called perineuronal nets (PNNs) that surround synaptic contacts on the soma and dendrites [1, 2]. The appearance of PNNs coincides with the termination of the critical period during which neural circuits are highly plastic [3]. Enzymatic disruption of PNNs by chondroitinase ABC treatment reactivates neural plasticity in the adult cerebral cortex after the critical period has ended, suggesting that formation of PNNs restricts neural plasticity in the adult brain [3]. In several regions of the brain, including the cerebral cortex, PNNs are selectively formed around inhibitory interneurons expressing parvalbumin (PV-cells), which is implicated in many neural processes including regulation of the critical period plasticity [4].

PNNs can modify PV-cell function by facilitating the sequestration of secreted proteins at the neuronal surface via interactions with CS chains. Otx2 homeoprotein produced in the retina and choroid plexus is transported to PV-cells in the cerebral cortex, where it promotes maturation of PV-cells and controls the timing of the critical period [5, 6]. In addition, PNNs capture other secreted molecules, such as semaphorin3A and neuronal activity-regulated pentraxin, to regulate PV-cell function [7, 8]. CS chains in PNNs are required for localization of these molecules at the PV-cell surface [5, 7–9].

CSPGs belonging to the lectican family (aggrecan, versican, neurocan, and brevican) are major components of

PNNs [1, 2]. Lectican family members share an amino-terminal hyaluronan binding domain and a carboxy-terminal tenascin-R binding domain [10]. In PNNs, lecticans bind to hyaluronan, which is tethered to the neuronal surface by transmembrane hyaluronan synthase, and this interaction is enhanced by link proteins [1, 11]. Multimeric forms of tenascin-R cross-link lecticans and stabilize the PNN structure [12].

Formation of PNNs is regulated by spatiotemporal expression of CSPG core proteins and link proteins [10]. In addition, dynamic changes in sulfation patterns of CS side chains are also observed during brain development [9, 13]. CS chains are linear polysaccharides composed of a repeating disaccharide unit consisting of uronic acid (UroA) and *N*-acetylgalactosamine (GalNAc). In the biosynthetic pathways, GalNAc residues of the repeating disaccharide units are sulfated by chondroitin 6-sulfotransferase-1 (C6ST-1) or chondroitin 4-sulfotransferase-1 (C4ST-1), thereby generating 6-sulfation or 4-sulfation, respectively [14, 15]. Subsequently, a small portion of 6- and 4-sulfation are further sulfated to form disulfated disaccharide units. 6-sulfation is abundant in the juvenile brain, whereas 4-sulfation is dominant in the adult brain [9, 13]. We previously reported that transgenic (TG) mice overexpressing C6ST-1 retained juvenile-like CS sulfation throughout life and showed impaired PNN formation [9]. As a result, C6ST-1 TG mice maintained juvenile-like plasticity in adulthood.

PNNs can be labeled with the broad marker *Wisteria floribunda* agglutinin (WFA) lectin. CS chains on aggrecan are proposed to be recognized by WFA because neurons from aggrecan-deficient mice lack staining for WFA [16]. We previously reported that C6ST-1 TG mice showed decreased WFA-positive conventional PNNs and ectopic appearance of 6-sulfation-enriched PNNs, which were stained by CS56 antibody but not by WFA lectin [9]. Incorporation of Otx2 into PV-cells was dependent on CS sulfation pattern of perineuronal nets: Otx2 accumulates in PV-cells surrounded by WFA-positive PNNs, but not observed in PV-cells surrounded by CS56-positive PNNs. Although these results imply the importance of sulfation patterns of CS chains, the mechanism by which the forced expression of 6-sulfation disrupts PNN formation in the adult brain remains unknown. Here we report that overexpression of C6ST-1 selectively decreases aggrecan in the aged brain and inhibits its accumulation into PNNs.

2. Materials and Methods

2.1. Animals. C6ST-1 TG mice were described previously [9]. Mice were housed under specific pathogen-free conditions in an environmentally controlled clean room at the Institute of Laboratory Animals, Kobe Pharmaceutical University. All experiments were conducted according to institutional ethics guidelines for animal experiments and safety guidelines for gene manipulation experiments.

2.2. Developmental Expression of PNN Components. Brains of wild-type (WT) or C6ST-1 TG mice at different postnatal ages were homogenized with a tight-fitting Potter glass homogenizer in HBSS buffer containing 1% Triton X-100 and protease inhibitor cocktail and incubated on ice for 60 min. After centrifugation at 15,000 rpm for 30 min at 4°C, protein concentrations of supernatants were determined using a BCA assay kit (Thermo). For chondroitinase digestion, the brain lysate (600 μg as protein) was digested with 5 milliunits of chondroitinase ABC (Seikagaku Corp.) for 2 h at 37°C. Chondroitinase-digested lysate (40 μg as protein) was separated by 5 or 10% acrylamide gel electrophoresis, transferred onto PVDF membranes (GE Healthcare), and incubated overnight at 4°C with the primary antibodies described in Table 1. Undigested lysate was used for detection by CS56 antibody. The blots were subsequently incubated with the appropriate HRP-labeled secondary antibodies for 1 h at room temperature and developed with the ECL detection system (GE Healthcare).

2.3. Sequential Extraction of CSPGs from Adult Mouse Brain. Fractionation of brain tissue was performed as described previously with minor modifications [11, 17]. Brains from 6-month-old adult WT or C6ST-1 TG mice were homogenized in PBS containing 2 mM EGTA, 2 mM phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (buffer 1). The homogenate was centrifuged at 15,000 rpm for 20 min at 4°C. The supernatant (PBS extract) was collected, and the pellet was further extracted with buffer 2 (buffer 1 containing 0.5% Triton X-100), followed by extraction with buffer 3 (buffer 2 containing 6 M urea). To obtain total brain extract, brains were directly extracted with buffer 3. The 6 M urea and total extracts were dialyzed against PBS, and the protein content of the extracts was quantified by BCA assay kit. 20 μg aliquots pretreated with chondroitinase ABC were subjected to Western blot analysis as described above.

2.4. Aggrecan Degradation Assay by ADAMTS-5. Whole brain lysate was prepared from 6-month-old adult WT or C6ST-1 TG mice as described above, except that protease inhibitors were omitted. Lysate was incubated with 0–500 nM recombinant human ADAMTS-5 (R&D Systems) in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 5 mM $CaCl_2$, and 0.05% Triton X-100 for 4 h at 37°C. Samples were further digested with chondroitinase ABC and subjected to Western blot analysis as described above.

2.5. Quantification of Proteins on Western Blot. Intensity of the bands was determined using CS analyzer (ATTO) and the background was subtracted. For aggrecan, intensity was determined by measuring the signal intensity in the region indicated by the bracket in Figures 1 and 2.

2.6. Immunohistochemistry. Mice were perfused transcardially with PBS followed by 4% paraformaldehyde in PBS. Brains were removed and postfixed overnight with 4% paraformaldehyde in PBS. Coronal sections (40 μm thick) were cut with a vibratome (DSK). Sections were permeabilized with 0.2% Triton X-100 in PBS, blocked with 2% BSA in PBS, and incubated overnight at room temperature with

Table 1: List of the antibodies used in this study.

Antigen	Isotype	Source	Dilution
Aggrecan	Rabbit IgG	Millipore, AB1031	1 : 2000 for WB, 1 : 200 for IHC
Brevican	Mouse IgG1	BD Transduction Laboratories, 610894	1 : 2000 for WB, 1 : 200 for IHC
Versican GAGβ	Rabbit IgG	Millipore, AB1032	1 : 2000 for WB
Neurocan	Sheep IgG	R&D Systems, AF5800	1 : 3000 for WB, 1 : 200 for IHC
Phosphacan	Mouse IgG1	DSHB, 3F8	1 : 200 for WB, 1 : 5 for IHC
Tenascin-R	Mouse IgG1	R&D Systems, MAB1642 (clone 619)	1 : 2000 for WB, 1 : 200 for IHC
Crtl1	Goat IgG	R&D Systems, AF2608	1 : 2000 for WB, 1 : 200 for IHC
CS56	Mouse IgM	Sigma, C8035 (clone cs-56)	1 : 2000 for WB, 1 : 200 for IHC
Δ6S	Mouse IgM	Cosmo Bio, CAC-PRPG-BC-M04 (clone 3B3)	1 : 200 for WB, 1 : 50 for IHC
Δ4S	Mouse IgG1	Millipore, MAB2030 (clone BE-123)	1 : 20000 for WB, 1 : 1000 for IHC
VGlut1	Guinea pig IgG	Millipore, AB5905	1 : 1000 for IHC
WFA	Lectin	EY Laboratories, BA-3101-1	1 : 1000 for IHC

WB, Western blot. IHC, immunohistochemistry.

the primary antibodies described in Table 1. Sections were incubated with the appropriate Alexa488/594/647-labeled secondary antibodies (Invitrogen) for 1 h at room temperature. For WFA lectin staining, sections were incubated with biotinylated WFA followed by secondary labeling with Alexa594/647-conjugated streptavidin. Images were captured with an FV1200 laser scanning confocal microscope (OLYMPUS). For quantification of the number of WFA- and CS56-positive PNNs, labeled cells were counted in a 1.27 × 1.27 mm area spanning all cortical layers of the cerebral cortex. For three-dimensional reconstruction of PNNs, images were acquired at 0.5 μm steps using a ×60 or ×100 objective and processed using FV10 ASW software (OLYMPUS).

2.7. Statistical Analysis. Statistical significance was determined using the unpaired two-tailed Student's t-test. Differences were considered significant at a P value of less than 0.05.

3. Results

3.1. Developmental Expression of PNN Components in C6ST-1 TG Mice. We first examined developmental expression of CSPG core proteins, tenascin-R, and cartilage link protein 1 (Crtl1) in detergent-soluble fractions of the postnatal mouse brain (Figures 1(a) and 1(b)). Western blot analysis showed that expression of aggrecan, brevican, tenascin-R, and Crtl1 increased during postnatal development in both WT and C6ST-1 TG mice. In contrast, expression of neurocan and versican GAGβ was high during early postnatal period and decreased with postnatal development. Phosphacan showed a peak of expression around postnatal day 15 and then decreased in the adult brain. Overall expression patterns of PNN components in C6ST-1 TG mice were similar to those of WT mice between postnatal days 1 and 45. However, we noticed that in the aged brain (>postnatal day 120) C6ST-1 TG mice showed decreased levels of aggrecan. Densitometric analysis revealed that the aggrecan level in C6ST-1 TG mice was significantly decreased compared with age-matched WT mice (Figure 1(c)).

To confirm the reduction of aggrecan in the aged C6ST-1 TG mice, we sequentially extracted PNN components with PBS and 6 M urea. It was reported that tightly associated components of PNNs were PBS-insoluble and can only be extracted in 6 M urea [17]. We found that the total level of aggrecan in the aged C6ST-1 TG mice was significantly decreased compared with controls (Figure 2(a)). In WT mice, aggrecan was much enriched in the 6 M urea-soluble fraction as compared to the PBS-soluble fraction. C6ST-1 TG mice showed slightly reduced aggrecan levels in both the PBS extract and 6 M urea extract, although it did not reach statistically significant difference. In contrast to aggrecan, other CSPGs, including brevican, neurocan, and phosphacan, were mostly extracted by PBS without 6 M urea (Figures 2(b)–2(d)). Considerable amounts of tenascin-R and Crtl1 were found in the 6 M urea-soluble fraction (Figures 2(e) and 2(f)). However, the levels of these molecules were not different between WT and C6ST-1 TG mice neither in the PBS extract nor 6 M urea extract.

3.2. Effects of Overexpression of C6ST-1 on the CS Moiety and the Oligosaccharide Linker to the Core Protein. Overexpression of C6ST-1 may influence the sulfation pattern of the repeating disaccharide region and/or the linkage region of CS chains (Figure 3(a)). CS56 antibody was reported to recognize oligosaccharide structures containing 6-sulfation in the repeating disaccharide region of CS chains and its reactivity is lost after chondroitinase ABC digestion [9, 18]. In contrast, the so-called "anti-stub antibodies" recognize the linkage region oligosaccharide neoepitopes (Δ4S or Δ6S) that are generated after chondroitinase ABC treatment [19]. In WT mice, CS56-reactivity was abundant in neonatal brain and gradually decreased during postnatal development (Figure 3(b)). C6ST-1 TG mice showed higher CS56-reactivity than WT mice until postnatal day 30. However, CS56-reactivity was largely absent in the aged C6ST-1 TG mouse brain, proposing a possibility that CSPGs, which are abnormally modified by 6-sulfation, may be liable to degradation in the aged brain (see Section 3.4). Similar staining intensity was obtained from WT and C6ST-1 TG mice only

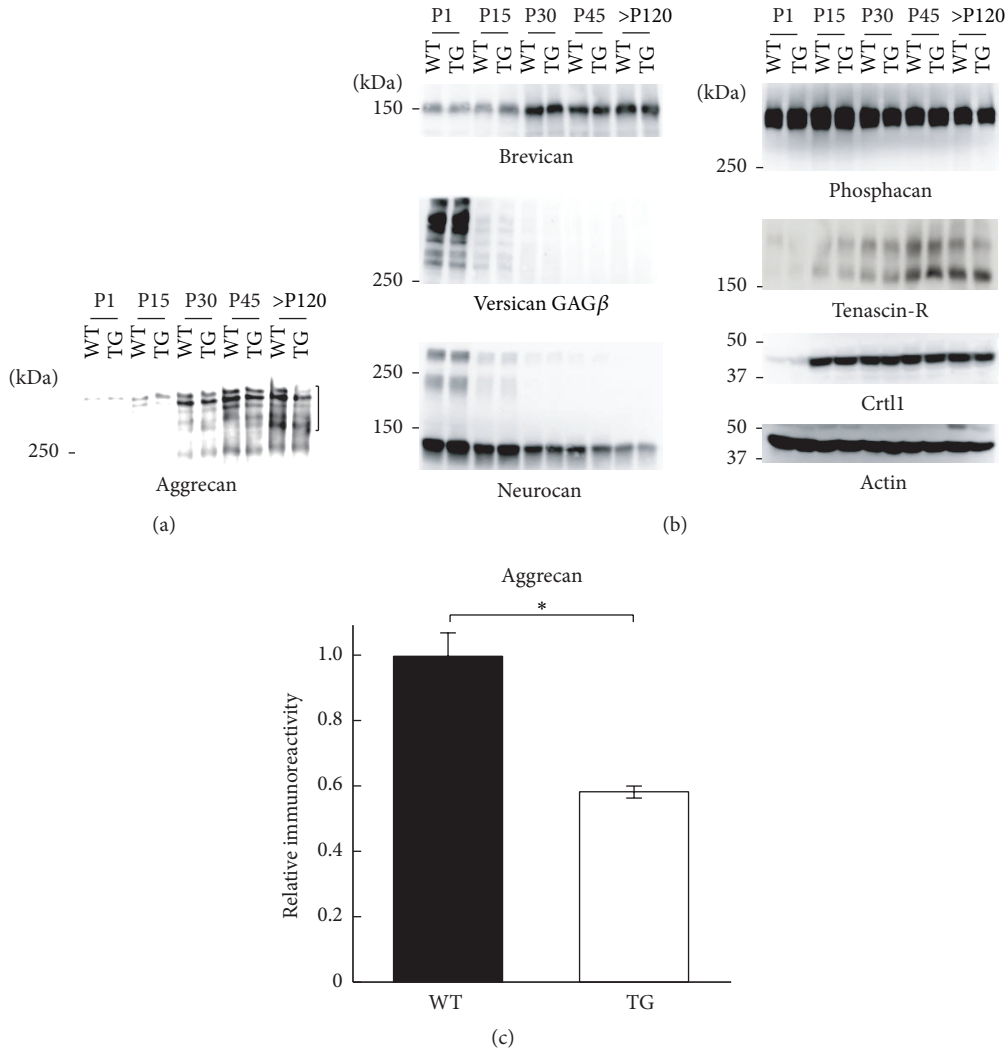
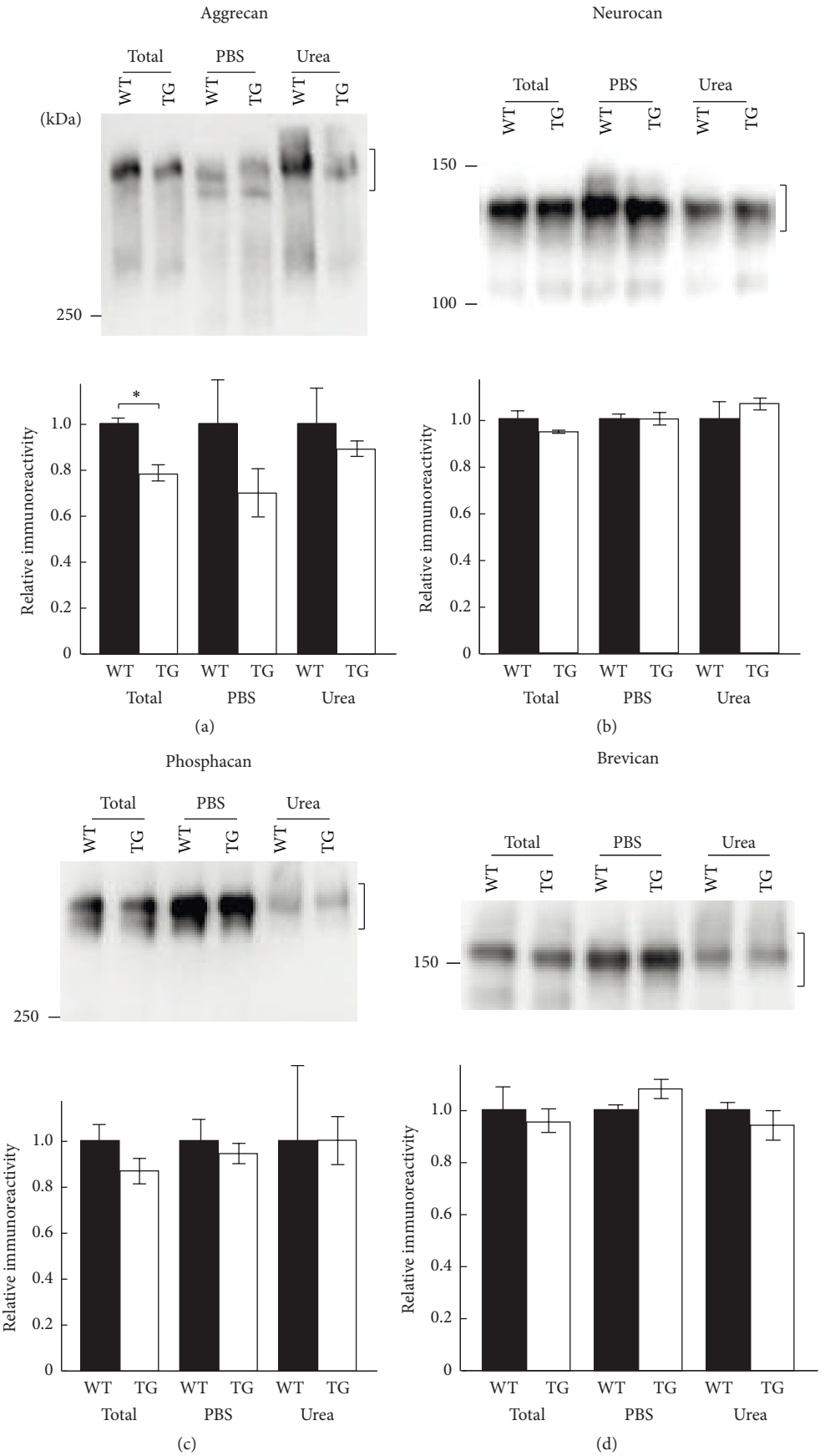


Figure 1: Developmental expression of PNN components in detergent-soluble fractions of WT and C6ST-1 TG mouse brain. (a) Expression of aggrecan in the brain of WT and C6ST-1 TG (TG) mice from postnatal day (P) 1 to >P120. (b) Expression of other PNN components, including brevican, versican GAGβ, neurocan, phosphacan, tenascin-R, and Crtl-1, during development. Actin was detected as loading control. (c) Densitometric quantification of aggrecan in the aged brain (>P120). Intensity values were generated by measuring the signal intensity in the region indicated by the bracket. Asterisks denote significant differences ($P < 0.05$, Student's t-test) between WT and TG mice. Error bars represent SEM; $n = 3$ for each genotype.

after a long exposure time. Δ4S and Δ6S antibodies recognized distinct subsets of CSPGs, which showed differential developmental expression (Figures 3(c) and 3(d)). C6ST-1 TG mice showed slightly higher Δ6S-reactivity than WT mice, especially during early postnatal development (Figure 3(c)). In contrast, there was no substantial difference in Δ4S-reactivity between WT and C6ST-1 TG mice (Figure 3(d)). We also found that Δ6S-reactive CSPGs preferentially localized in PNNs, whereas Δ4S-reactive CSPGs were not limited to PNNs and were widely distributed throughout the cerebral cortex (Figure 3(e)), raising the possibility that localization of CSPGs into PNNs is regulated by the sulfation pattern of the CS chain linkage region. These results suggest that overexpression of C6ST-1 influences CS structures in both the repeating disaccharide and linkage region.

3.3. Condensation of Aggrecan Was Primarily Affected in C6ST-1 TG Mice. We previously reported that, in the cerebral cortex of young adult C6ST-1 TG mice, a small portion of PV-cells was enclosed by 6-sulfation-enriched PNNs, which were labeled by the CS56 antibody, but not the conventional PNN marker WFA lectin (Figure 4(a)) [9]. We found that, during postnatal development of C6ST-1 TG mice, both WFA-positive and CS56-positive PNNs gradually increased until postnatal day 60 (Figures 4(a)–4(c)). Thereafter, the numbers of both PNNs were maintained in the aged animals (>postnatal day 120).

Three-dimensional reconstruction of WFA-positive PNNs displayed condensed lattice-like structures surrounding soma and proximal dendrites in both WT and C6ST-1 TG mice (Figure 4(d)). WFA-positive PNNs enwrapped



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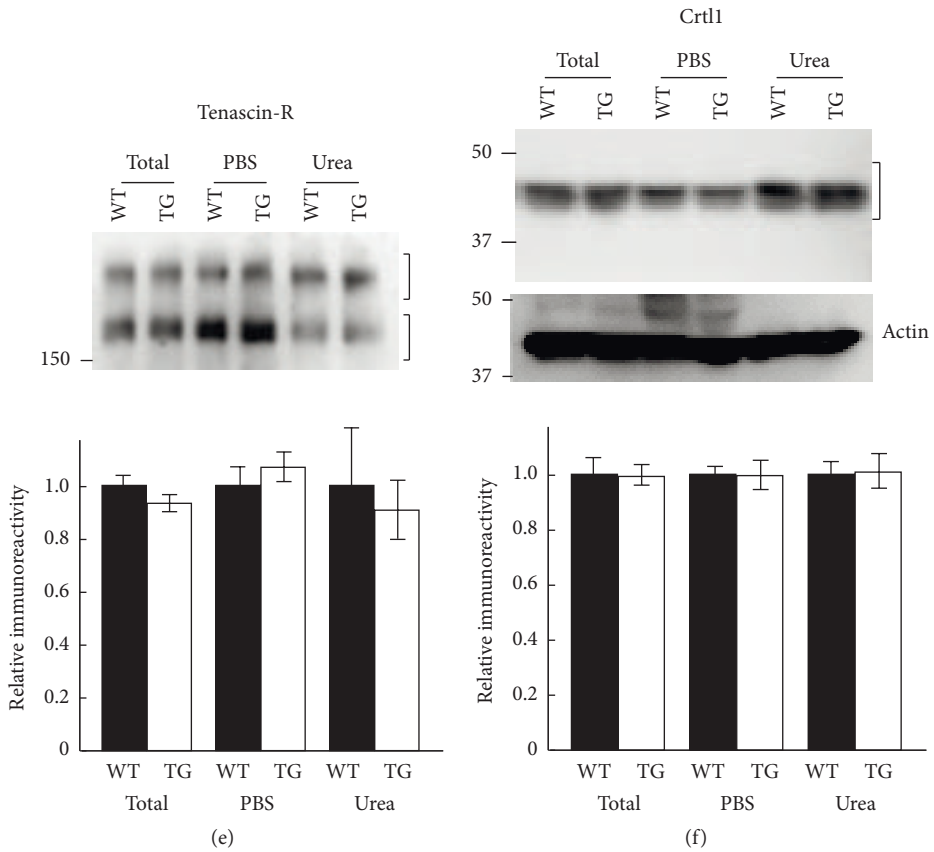


Figure 2: Sequential extraction of PNN components from adult mouse brain. Six-month-old adult WT and C6ST-1 TG mouse brain was sequentially extracted as described in Section 2. The same protein amount of the extracts was analyzed and intensities of the indicated bands were determined as relative values to WT mice for each extract. (a) The total level of aggrecan in C6ST-1 TG mice was significantly decreased compared with WT mice. Aggrecan levels in both the PBS extract and 6 M urea extract slightly reduced in C6ST-1 TG mice although this did not reach statistically significant difference. The levels of neurocan (b), phosphacan (c), brevican (d), tenascin-R (e), and Crtl1 (f) were not different between WT and C6ST-1 TG mice. Actin was detected as loading control. Asterisks denote significant differences ($P < 0.05$, Student's t-test) between WT and C6ST-1 TG mice. Error bars represent SEM; $n = 3$ for each genotype.

presynaptic terminals labeled by antibody to vesicular glutamate transporter 1 (VGlut1). In contrast, CS56-positive PNNs in C6ST-1 TG mice showed sparse dot-like particles rather than a meshwork structure and did not tightly surround presynaptic terminals (Figure 4(d)).

We next examined which PNN components account for the difference between the two distinct PNNs. Staining with antibody recognizing core protein portion of aggrecan revealed a well-formed meshwork and a similar pattern to that with WFA lectin in WT mice (Figures 5(a) and 5(b)). As consistent with previous finding [9], CS56-positive PNNs were not observed even in the aged WT mice. In C6ST-1 TG mice, WFA-positive PNNs showed marked condensation of aggrecan core protein, which is similar to that observed in WT mice (Figures 5(c) and 5(d)). In contrast, aggrecan staining of neighboring CS56-positive PNNs appeared diffuse and less condensed over the soma (Figure 5(e)).

Neurocan and phosphacan were also observed in PNNs but did not show clear lattice-like structures. We observed no difference in staining of these CSPGs between WFA-positive and CS56-positive PNNs, suggesting that condensation of these CSPGs was not influenced by overexpression of 6-sulfation (Figures 5(f) and 5(g)). Brevican did not accumulate in PNNs neither in WT nor in C6ST-1 TG mice (data not shown). In addition, accumulation of tenascin-R and Crtl1 was not affected in CS56-positive PNNs compared with WFA-positive PNNs (Figures 5(h) and 5(i)). These results indicate that overexpression of 6-sulfation primarily impairs condensation of aggrecan into PNNs with little effect on other PNN components.

3.4. Overexpression of 6-Sulfation Rendered Aggrecan More Susceptible for Degradation by ADAMTS-5. Finally, we examined whether aggrecan produced in C6ST-1 TG mice is liable to degradation by ADAMTS-5 (aggrecanase-2), which has been proposed as an aggrecan-degrading enzyme and is expressed in the adult mouse brain [20, 21]. Whole brain lysate was digested with various concentration of recombinant ADAMTS-5 and degradation of aggrecan was compared by Western blotting. We found that high molecular weight aggrecan bands in C6ST-1 TG mice were degraded more efficiently by ADAMTS-5 than those of WT mice

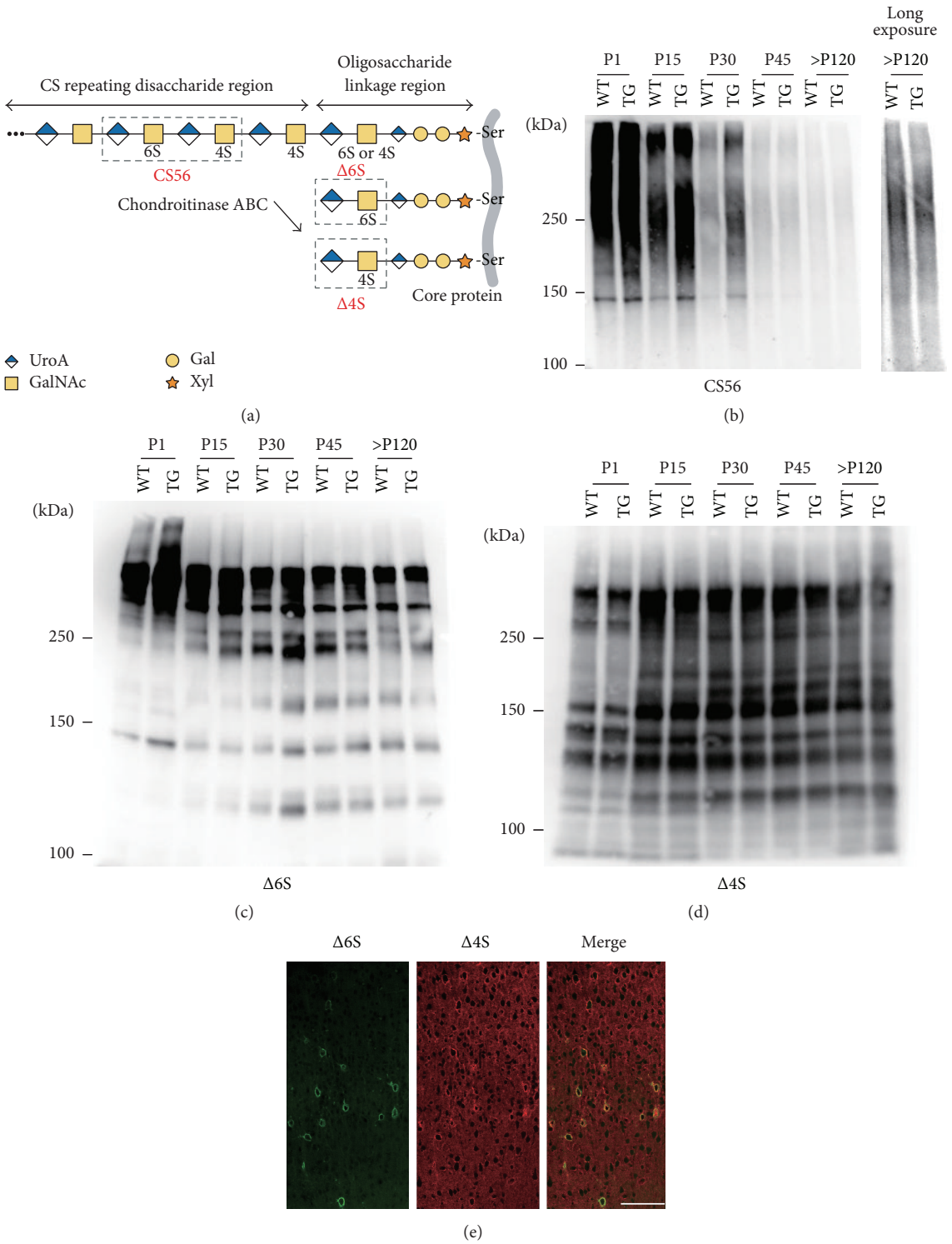


Figure 3: Effects of overexpression of C6ST-1 on the repeating disaccharide region and linkage region of CS chains. (a) Epitopes recognized by antibodies CS56, Δ6S, and Δ4S. 6S and 4S represent 6-sulfation and 4-sulfation, respectively. (b) C6ST-1 TG mice showed a delayed decrease in CS56-reactivity as compared with WT in the developing brain. Δ6S (c) and Δ4S (d) antibodies recognized distinct subsets of CSPGs. During early postnatal development, C6ST-1 TG mice exhibited greater Δ6S-reactivity than WT mice, whereas Δ4S-reactivity was not different between the two groups. (e) Distinct localization of Δ6S- and Δ4S-reactive CSPGs in 6-month-old adult cerebral cortex of WT mice. Scale bar, 100 μm.

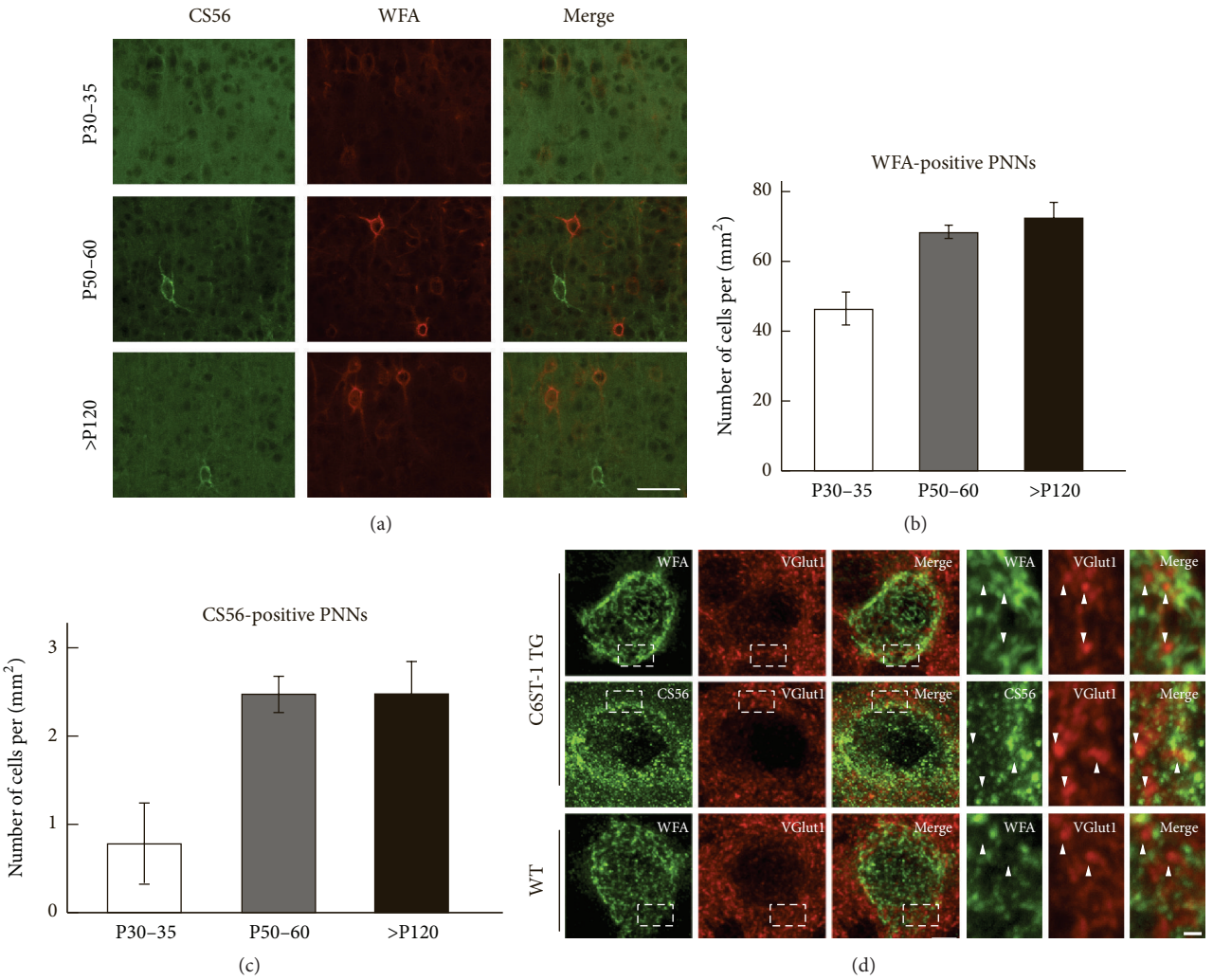


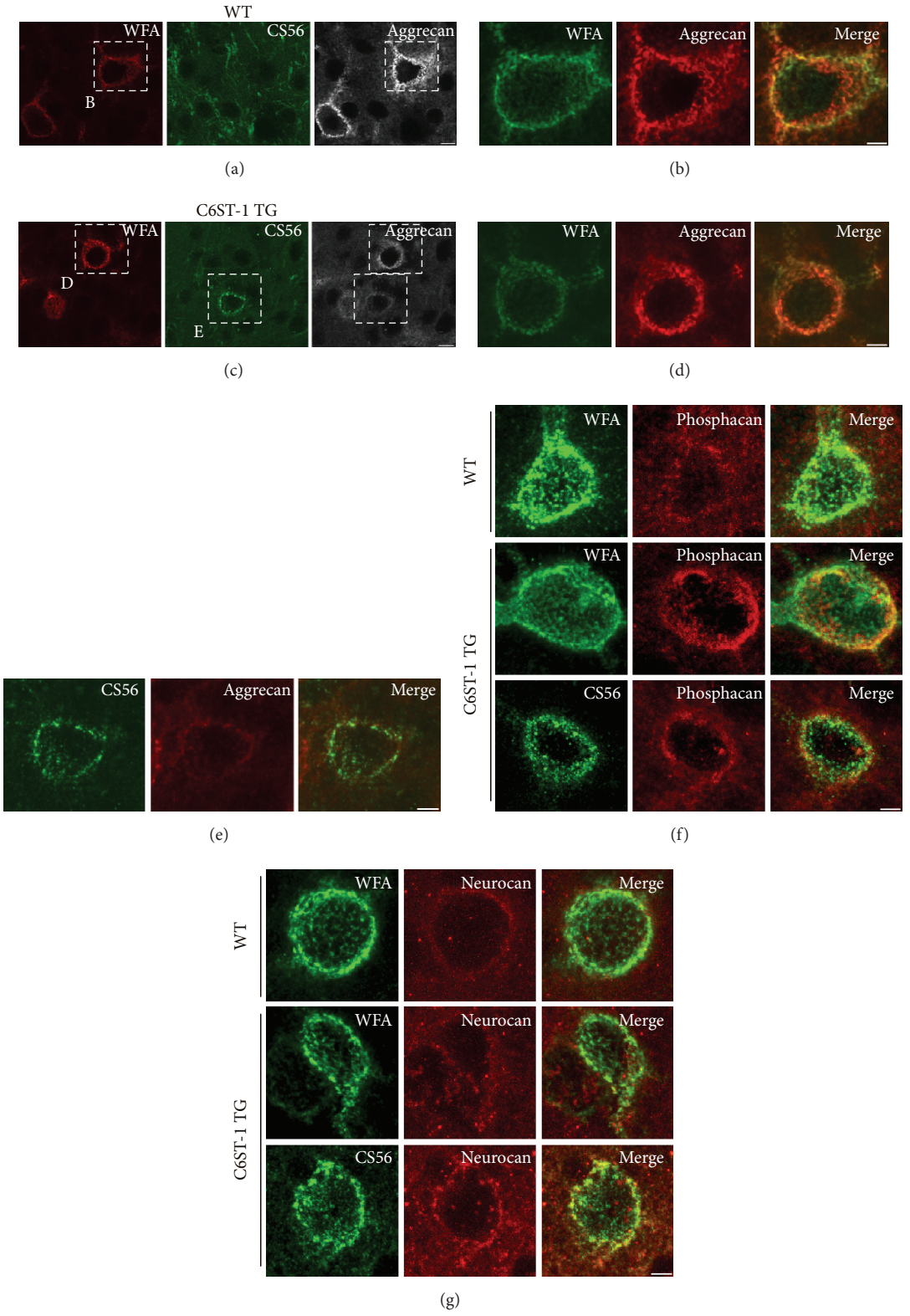
Figure 4: Developmental formation and morphological features of WFA-positive and CS56-positive PNNs. (a) Formation of WFA-positive and CS56-positive PNNs in the cerebral cortex of C6ST-1 TG mice during development. Note that CS56-positive PNNs were not colocalized with conventional WFA-positive PNNs. The numbers of both WFA-positive (b) and CS56-positive PNNs (c) increased until P60 and were maintained in aged C6ST-1 TG mice. Error bars represent SEM; $n = 2$-3. (d) Three-dimensional reconstruction revealed a distinct lattice-like structure of WFA-positive PNNs in both 6-month-old adult WT and C6ST-1 TG mice. In contrast, CS56-positive PNNs in C6ST-1 mice were sparse dot-like structures. Right: magnification of boxed regions in the left panels. VGluT1-labeled presynaptic terminals (arrowheads) were embedded in the meshwork of WFA-positive PNNs, whereas these terminals were not tightly surrounded by CS56-positive PNNs in C6ST-1 mice. Scale bars represent 50 μm (a), 5 μm ((d), left panels), and 1 μm ((d), right panels).

(Figures 6(a) and 6(b)), indicating that increased 6-sulfation accelerated proteolysis of aggrecan by ADAMTS-5.

4. Discussion

Our study revealed that overexpression of 6-sulfation markedly decreased aggrecan in the aged brain. Previous studies have implicated the involvement of CS chains in the metabolism of aggrecan in cartilage. Mice deficient in chondroitin GalNAc transferase-1 (ChGn-1), which catalyzes the initial step of CS biosynthesis, show a decreased amount of CS chains and an accelerated degradation of aggrecan in the cartilage [22, 23]. Together with our data, this indicates that proper sulfation patterns of CS chains play an essential role in the stability of aggrecan, probably by providing protection from aggrecan-degrading enzymes.

Several members of metalloproteinases family and ADAMTS family cleave CSPGs including aggrecan in the ECM [20]. ADAMTS-4 (aggrecanase-1) and ADAMTS-5 (aggrecanase-2) have been proposed to be responsible for aggrecan degradation [20]. However, recent study using ADAMTS-4 and ADAMTS-5 knockout mice suggested the presence of additional aggrecan-degrading enzymes in the spinal cord [24]. In the cerebral cortex, ADAMTS-8 and ADAMTS-15 are exclusively expressed by PV-cells, which are surrounded by PNNs [25, 26], suggesting that these may be novel aggrecan-degrading enzymes specifically involved in turnover and remodeling of PNNs *in vivo*. Our results



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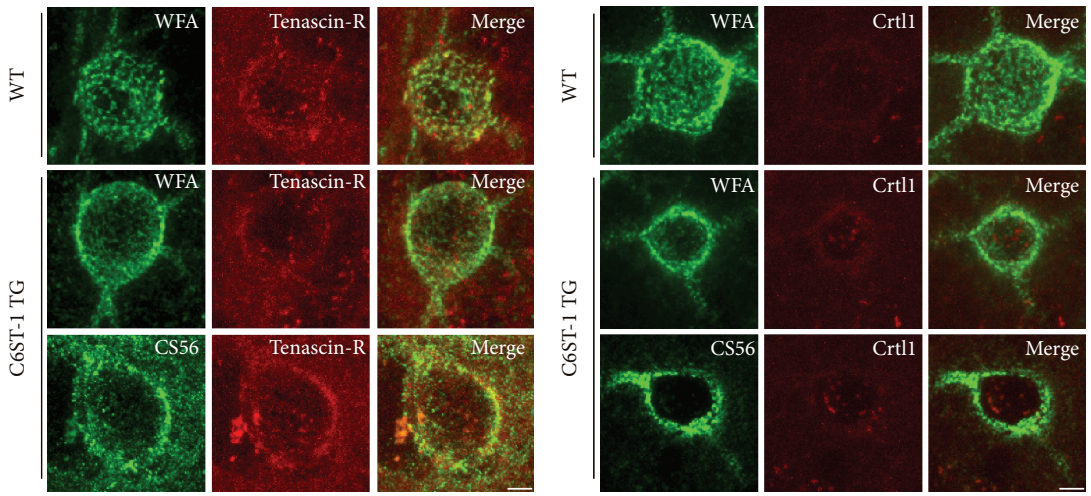

Figure 5: Impaired condensation of aggrecan into PNNs by overexpression of 6-sulfation. ((a), (b)) In 6-month-old adult WT mice, aggrecan showed a well-formed meshwork, which is similar to WFA lectin staining. (b) Magnification of boxed regions in (a). ((c)–(e)) In age-matched C6ST-1 TG mice, WFA-positive PNNs showed marked condensation of aggrecan, whereas neighboring CS56-positive PNNs showed diffuse and less condensed aggrecan staining. ((d), (e)) Magnification of boxed regions in (c). Immunolocalization of other PNN components including phosphacan (f), neurocan (g), tenascin-R (h), and Crtl1 (i) in WFA-positive and CS56-positive PNNs. In contrast to the impaired accumulation of aggrecan in CS56-positive PNNs in C6ST-1 TG mice, condensation of other PNN components was comparable between WT and C6ST-1 TG mice. Scale bars, 5 μm.

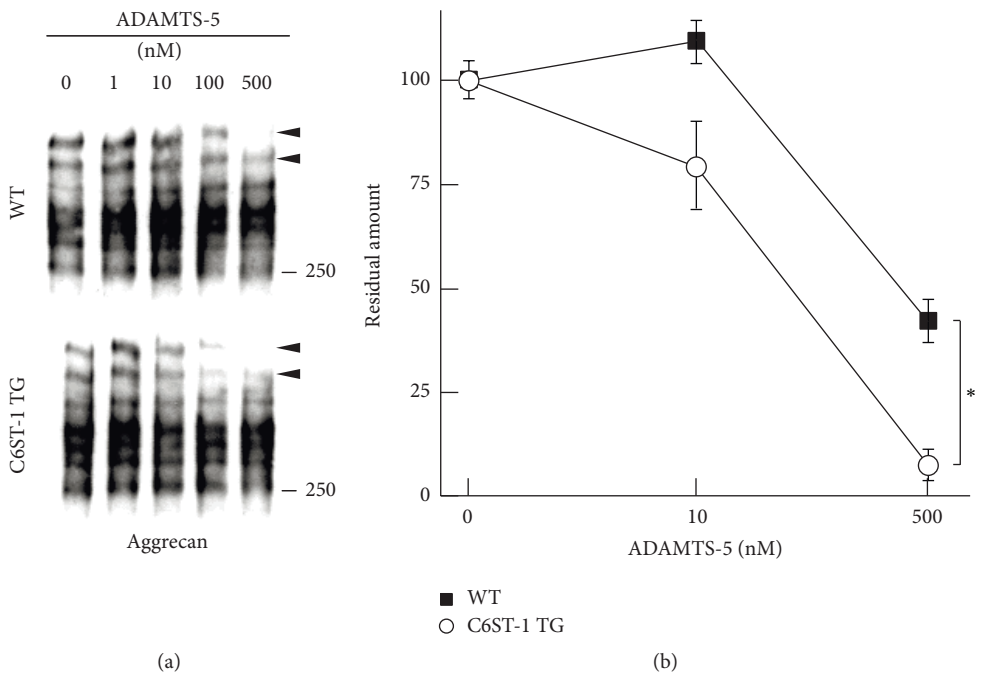

Figure 6: Degradation of aggrecan by ADAMTS-5 *in vitro*. (a) Whole brain lysate of 6-month-old adult WT or C6ST-1 TG mice was digested with increasing amounts of ADAMTS-5. The concentrations of ADAMTS-5 used for digestion were indicated. Degradation of aggrecan was compared by Western blotting. Two high molecular weight aggrecan bands shown by arrowheads in C6ST-1 TG mice were degraded more efficiently by ADAMTS-5 than those of WT mice. (b) The residual amounts of two high molecular weight bands in WT (filled square) and C6ST-1 TG (open circle) mice were expressed as values relative to the no enzyme control. Asterisks denote significant differences ($P < 0.05$, Student's t-test) between WT and C6ST-1 TG mice. Data ± SEM were obtained from the triplicate experiments.

imply that juvenile-type CS sulfation rich in 6-sulfation allows remodeling of PNNs by ADAMTSs, thereby keeping high plasticity during the critical period. The developmental shift of sulfation patterns from 6-sulfation to 4-sulfation may render aggrecan resistant to degradation and stabilize PNNs in adult brain. Some ADAMTSs have been shown to cleave CSPGs in a manner depending on CS chains attached to core proteins [27, 28]. Thus, further studies are needed to determine how CS sulfation patterns on aggrecan are involved in CSPG degradation by ADAMTSs. It is currently unknown why aggrecan is selectively decreased by excess 6-sulfation. Aggrecan is distinctive from other CSPGs in terms of the number of CS chains attached to the core protein, because it contains approximately one hundred CS chains per core protein [29]. Thus aggrecan may be highly susceptible to structural changes in CS chains.

In spite of the disturbed condensation of aggrecan into PNNs in C6ST-1 TG mice, other PNN components appeared to be unaffected, indicating that these components accumulate into PNNs in an aggrecan-independent manner. This is consistent with previous reports in which condensation of Crtl1, tenascin-R, and brevican is unaffected in neuronal cultures prepared from aggrecan-deficient mice [16, 30]. Production of aggrecan and formation of WFA-positive PNNs are dependent on neuronal activity, whereas tenascin-R and brevican are produced in a glia-dependent manner [31–33]. Thus, there may be at least two independent mechanisms of PNN formation: one is neuron-dependent and the other is glia-dependent. However, the contribution of the glia-dependent mechanism seems to be unnecessary for the development of PNNs, because previous reports found that WFA-positive PNNs were formed in dissociated neuronal culture in the absence of glial cells [31, 34]. In addition, we previously demonstrated *in vivo* that PNN formation and Otx2 accumulation were locally manipulated by PV-cell autonomous production of 6-sulfation [9]. These results indicate that CSPGs produced by neurons, most likely aggrecan, are required for the proper function of PNNs.

In this study we found that overexpression of C6ST-1 increases 6-sulfation in the repeating disaccharide region as well as the linkage region of CS chains. Chondroitinase ABC treatment releases Otx2 and semaphorin3A from PNNs, indicating that these molecules bind to the repeating disaccharide region of CS chains [7, 35]. Indeed, it was reported that both Otx2 and semaphorin3A selectively interact with CS chains rich in disulfated E units [GlcA-GalNAc(4,6-*O*-disulfate)] [35–37]. However, the significance of the linkage region of CS chains in PNN formation is unknown. Here we show differential localization of Δ4S- and Δ6S-reactive CSPGs, proposing the possibility that sulfation of the linkage region influences localization of CSPGs into PNNs. Our laboratory previously demonstrated that 4-sulfation of the GalNAc residue in the linkage region catalyzed by C4ST-2 triggers the elongation of chondroitin backbone, which is prerequisite for the formation of sulfation patterns of the repeating disaccharide units [38, 39]. Therefore, it is possible that sulfation of the linkage region affects assembly of CSPGs within the ECM by modulating sulfation patterns of the repeating disaccharide region.

WFA lectin is widely used to label PNNs. However, a small portion of PNNs in C6ST-1 TG mice was devoid of WFA-labeling but was labeled by CS56 antibody recognizing oligosaccharide structures containing 6-sulfation. It has been proposed that WFA recognizes a CS structure on aggrecan because WFA-staining is abolished by either chondroitinase ABC digestion of CS chains or deletion of aggrecan [3, 16]. However, the precise structure recognized by WFA is unknown. During formation of PNNs, there is a progressive decrease in 6-sulfation that is mirrored by an increase in 4-sulfation [9, 13]. Furthermore, C6ST-1 TG mice contain less 4-sulfation and more 6-sulfation than WT mice, which is accompanied by decreased WFA-staining and increased CS56-staining. Thus, WFA may recognize a CS structure on aggrecan consisting of 4-sulfation. Notably, drastic changes in WFA-reactivity have been reported in the brains of patients with schizophrenia. In schizophrenic brain, the number of WFA-positive PNNs around PV-cells is markedly decreased [40–42]. In addition, WFA-labeled astrocytes are increased, whereas CS56-labeled astrocytes are decreased in schizophrenia [43]. Taken together, an abnormal balance of 4-sulfation and 6-sulfation produced by both neurons and astrocytes may contribute to the disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Review Article

The Chemorepulsive Protein Semaphorin 3A and Perineuronal Net-Mediated Plasticity

F. de Winter,[1] J. C. F. Kwok,[2] J. W. Fawcett,[2] T. T. Vo,[1] D. Carulli,[3] and J. Verhaagen[1]

[1]*Netherlands Institute for Neuroscience, Royal Academy of Arts and Sciences, Meibergdreef 47, 1105 BA Amsterdam, Netherlands*
[2]*John van Geest Centre for Brain Repair, University of Cambridge, Cambridge CB2 OPY, UK*
[3]*Department of Neuroscience, Neuroscience Institute of Turin (NIT) and Neuroscience Institute Cavalieri-Ottolenghi (NICO), University of Turin, Orbassano, 10043 Turin, Italy*

Correspondence should be addressed to J. Verhaagen; j.verhaagen@nin.knaw.nl

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During postnatal development, closure of critical periods coincides with the appearance of extracellular matrix structures, called perineuronal nets (PNN), around various neuronal populations throughout the brain. The absence or presence of PNN strongly correlates with neuronal plasticity. It is not clear how PNN regulate plasticity. The repulsive axon guidance proteins Semaphorin (Sema) 3A and Sema3B are also prominently expressed in the postnatal and adult brain. In the neocortex, Sema3A accumulates in the PNN that form around parvalbumin positive inhibitory interneurons during the closure of critical periods. Sema3A interacts with high-affinity with chondroitin sulfate E, a component of PNN. The localization of Sema3A in PNN and its inhibitory effects on developing neurites are intriguing features and may clarify how PNN mediate structural neural plasticity. In the cerebellum, enhanced neuronal plasticity as a result of an enriched environment correlates with reduced Sema3A expression in PNN. Here, we first review the distribution of Sema3A and Sema3B expression in the rat brain and the biochemical interaction of Sema3A with PNN. Subsequently, we review what is known so far about functional correlates of changes in Sema3A expression in PNN. Finally, we propose a model of how Semaphorins in the PNN may influence local connectivity.

1. Introduction

Since the early 1990s, chemorepulsion has been recognized as an effective way to guide growing neurites towards their targets in the developing nervous system [1]. One of the protein families that has been extensively studied in the context of repulsive axon guidance is the Semaphorin family (reviewed in [2, 3]). Although the secreted and transmembrane Semaphorins were initially identified as repulsive axon guidance cues [4], in the past decade they have also been linked to many other cellular processes, including cell migration, proliferation, and polarization. Because of their role in these key cellular functions they also have been implicated in various diseases, including cardiovascular and immunological diseases and cancer [5, 6]. In addition, the repulsive properties of especially the secreted Semaphorins are thought to hamper regenerative processes following nervous system trauma [7–9]. Recently, studies have implicated Semaphorins in synaptic and structural plasticity (reviewed in [10]) and in certain neurological diseases (reviewed in [11]).

Invertebrate, vertebrate, and viral Semaphorins all share a conserved Semaphorin domain. Semaphorins are divided into 8 classes, based on their structure and sequence similarities [12]. All transmembrane and secreted Semaphorins, except for Sema7A, use Plexins as signal transducing receptor. However, many Semaphorins make use of additional receptor components. For instance, all class 3 Semaphorins, except for Sema3E, require Neuropilin as ligand-binding receptor component to achieve repulsive guidance signaling through Plexins [13–18]. Other than Plexins, VEGFR2, ErbB2, and IgCAM can also be part of different Semaphorin receptor complexes (reviewed in [19]). The outcome of Sema-Plexin signaling is, however, highly context dependent. For example, class 5 Semaphorins demonstrate repulsive properties on neurites that express chondroitin sulfate proteoglycans (CSPGs) adjacent to Plexins while these same Semaphorins

turn into an attractive cue if neurites coexpress heparan sulfate proteoglycans (HSPGs) with Plexins on their surface [20]. Binding of Semaphorin to its receptor complex starts an intrinsic signaling pathway that involves GTPases and the PI3K/Akt pathway and eventually leads to reorganization of the cytoskeleton [21–24]. In addition, Semaphorin stimulation leads to an activation of the Plexin interacting protein MICAL (molecule interacting with casL) and to the phosphorylation of intracellular proteins of the CRMP (collapsing responsive mediator protein) family, which in turn affects F-actin and microtubule disassembly and thereby growth cone motility [25–31].

Next to their role in long distance axon guidance during development of the nervous system, Semaphorins also play a role in local target selection (reviewed in [10]). Recent studies have shown that class 3 Semaphorins/Plexins complex regulates connectivity not only at a cellular but also at a subcellular level such as the formation of synapse and spine. In the developing spinal cord reflex circuitry, Sema3E is only expressed by motoneurons that innervate the cutaneous maximus muscle but not by motoneurons that innervate the triceps muscle [32]. PlexinD1 expressing sensory axons of the triceps muscle form direct synaptic contacts with their Sema3E negative motoneurons. The PlexinD1 positive sensory afferents of the cutaneous muscle are however repelled by Sema3E expressing cutaneous maximus motoneurons. As a result, sensory and motor neurons of the cutaneous muscle cannot form direct but only indirect synapses. Knockout of either PlexinD1 in the sensory afferents or Sema3E in the motoneurons does allow the formation of direct synaptic contacts between cutaneous sensory and cutaneous motoneurons. In addition, the Sema3E-PlexinD1 interaction specifically determines the number of glutamatergic connections formed between Sema3E expressing thalamostriatal projection neurons and the PlexinD1 positive direct pathway medium spiny neurons but not the PlexinD1 negative indirect pathway medium spiny neurons [33].

At a subcellular level, several Semaphorin family members have been shown to control multiple aspects of synapse formation during late development of the mammalian nervous system. Sema3A increases the clustering of pre- and postsynaptic proteins in cortical neurons *in vitro* without affecting the number of spines [34, 35] while Sema3F limits the number of dendritic spines distributed along the apical dendrites of pyramidal neurons in the cerebral cortex, thereby locally restricting the options for synapse development on the apical dendrite [36, 37]. In the hippocampus, Sema5A limits the number of spines formed on the dendrites of developmentally and adult born granular neurons in a cell autonomous way. Mutant mice for Sema5A or its receptor PlexinA2 display an increase in glutamatergic synapses [38]. In addition, the transmembrane class 4 Semaphorins, Sema4B and Sema4D, have been shown to regulate GABAergic and glutamatergic synapse numbers in cultured dissociated hippocampal neurons [39–41]. Also in the developing cerebellum, Semaphorins control the number of synapses. Sema3A and Sema7A control the different stages of synapse elimination. In Purkinje cells knockdown Sema3A leads to an acceleration in synapse elimination while a knockdown of Sema7A results in an impairment of synapse elimination [42]. In addition, the diffusible fragments of processed Sema5B have the capacity to induce synapse elimination in cultured hippocampal neurons [43]. Besides their guidance role in neuronal circuit formation during development, Semaphorins can modulate the functional properties of established synapses in the adult brain. Sema3F application to adult hippocampal slices induces an increase in frequency and amplitude of excitatory postsynaptic currents in dentate granule neurons and CA1 pyramidal neurons [44]. However, application of Sema3A to the hippocampus results in a reduction in synaptic efficiency [45].

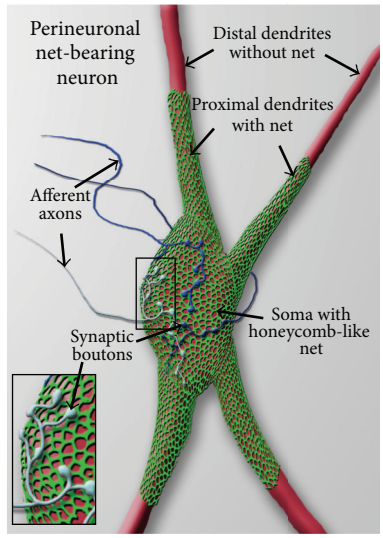

Figure 1: Schematic view of a perineuronal net-bearing neuron. A neuronal cell body (soma) with its proximal dendrites (red) covered by a typical reticular, honeycomb like net (green). More distally, dendrites are devoid of nets. The holes in the perineuronal nets are occupied by synaptic boutons of afferent axons (insert, blue) that synapse on the net-bearing neuron.

The continuous expression of some Semaphorins even into adulthood indicates that Semaphorins may be involved in sculpturing the neuronal circuits beyond the initial development of the nervous system [46]. A structure that is thought to play an important role in adult central nervous system neuronal plasticity is the perineuronal net (PNN; Figure 1) [47]. PNNs are highly condensed extracellular matrix aggregates that form around the cell bodies and proximal dendrites of many populations of neurons during the closure of a critical period [48, 49]. Recent work shows that the chemorepulsive proteins, Semaphorin 3A and Semaphorin 3B, are highly concentrated in the PNNs of distinct neuronal populations in the maturing brain and are therefore in an excellent position to control PNN-mediated neuronal plasticity during and/or beyond the critical period [50, 51]. In the following sections, we summarize the localization of Semaphorin 3A protein in the adult rodent brain [51], review the biochemical data on the interaction of this protein with the extracellular matrix [52], and discuss the possible role of Semaphorin 3A and Semaphorin 3B in PNN-controlled structural plasticity.

2. Semaphorin Expression in the Mature Brain

The persistent expression of Sema3A in the mature rat and human brain was initially shown at the mRNA level [46]. Neurons in the olfactory system, the cerebral cortex, and entorhinal-hippocampal system exhibited high levels of Sema3A mRNA expression. Extensive characterization of recently developed commercial antibodies against Sema3A and Sema3B has led to the identification of several antibodies that allowed reliable protein localization studies of Sema3A and 3B in the rodent nervous system [51]. Immunohistochemistry for Sema3A confirmed the ongoing expression of Sema3A at the protein level in the adult rat and mouse brain [51]. Several specific brain structures along the rostral-caudal axis, including the olfactory system, hippocampus, and cerebral cortex, show distinct patterns of Sema3A-immunoreactivity. In general, the immunoreactivity of Sema3A varied from a diffuse signal in between subsets of neurons, to a highly concentrated signal that appears as a reticular structure around the cell bodies and proximal dendrites of individual neurons (Figure 2). Sema3B-immunoreactivity shows a signal with similar features but its expression throughout the brain is more restricted [51].

In the *olfactory system*, Sema3A is diffusely expressed around some mitral cells and their dendrites in the outer plexiform layer (Figures 2(a) and 2(b)). The inner plexiform and granule cell layers have only weak diffuse Sema3A expression while the deeper portions of the granular cell layer are devoid of any immunoreactivity. Clear Sema3A positive PNN structures are present in the ventral anterior telencephalon, especially in layer III of the taenia tecta and the piriform cortex. Also the axonal projections of mitral cells through the lateral olfactory tract are Sema3A positive. In contrast to Sema3A, Sema3B expression is not detected in the main olfactory bulb [51].

In the *thalamic region*, PNN-like structures in the medial septal nucleus and the reticular nucleus show strong Sema3A immunoreactivity (Figure 2(c)). In addition, an intense but diffuse staining is present in between the cell bodies throughout the entire reticular nucleus. A similar but less intense staining is seen between the neurons in the zona incerta. The zona incerta also contains small groups of neurons with Sema3A positive PNNs. The stria medullaris of the thalamus and the optic chiasm have Sema3A-positive axons. Sema3B-immunoreactivity in the thalamic area is restricted to the reticular nucleus [51].

In the *hippocampal system*, numerous neurons have a PNN-like Sema3A immunoreactivity while in the indusium griseum, fasciola cinerea, and the stratum oriens of CA1, the pyramidal layer of CA3 and in the dentate gyrus granular cell layer only occasional Sema3A positive PNNs are observed (Figures 2(d)–2(f)). The pyramidal cells in CA2 have remarkable strong Sema3A-labelled PNNs and also intense diffuse labeling between the cell bodies. The neurons of the stratum oriens and stratum radiatum of the CA2 and CA3 and the polymorph layer of the dentate gyrus have a more diffuse Sema3A staining. Sema3B expression in the hippocampus is much more restricted than Sema3A. Sema3B positive PNNs are only present in the subiculum and weak diffuse labeling is seen in the CA1, CA2, and CA3 pyramidal cell layer [51].

Both Sema3A and Sema3B are prominently expressed in the PNN of numerous neurons in all subareas of the *neocortex* (Figures 2(g)–2(i)). Neurons with Sema positive PNNs are distributed scatteredly in the infralimbic, cingulated, parietal association, motor, somatosensory, agranular, gustatory, retrosplenial, auditory, and visual cortex. Most prominent expression exists in the cortical layers IV and V, except for the parietal association cortex and the anteromedial visual cortex where most Sema labeling is present in the deeper portion of layer V.

Several nuclei in the *midbrain* express Sema3A in PNN-like structures (Figures 2(j)–2(l)). There are strongly Sema3A-labelled PNNs on numerous neurons in the nucleus of Darkschewitsch and in the deep layers of the superior colliculus, whereas the intermediate layers of the superior colliculus contain moderately Sema3A-labelled PNNs and the superficial layers only contain weakly labelled PNNs. The stratum zonale has only weakly stained PNNs. The magnocellular neurons of the red nucleus have clear dense Sema3A staining in their PNNs while the staining between the neurons is more diffuse. Also in the inferior colliculus the PNNs are strongly Sema3A-labelled with a diffuse signal between neurons. In the tegmental reticular nucleus and in the nuclei of the lateral lemniscus, Sema3A is located around and between subpopulations of neurons. Sema3B-labeling largely overlaps with that of Sema3A. Sema3B stained PNNs are apparent in the deep gray layer of the superior colliculus and many neurons in the red nucleus [51].

In the *hindbrain,* Sema3A and Sema3B positive PNNs are heterogeneously present throughout the lateral, spinal, and medial vestibular nuclei (Figures 2(m)–2(o)). In addition, these nuclei have a strong diffuse immunoreactivity. Also large neurons in the pontine nucleus and in the superior and inferior olive have Sema3A and Sema3B labelled PNNs [51].

Many Purkinje cells in the *cerebellum* are surrounded by thin and granular Sema3A-positive PNNs (Figures 2(p)–2(r)). This is in contrast to the deep cerebellar nuclei, including the interpositus, fastigial, and dentate nuclei, where they all have a very intense Sema3A-signal around their neurons and a moderately diffuse staining throughout the nuclei. Sema3B is also expressed around and between the neurons of the deep cerebellar nuclei (Figure 2(s)).

3. Semaphorin 3A Is a Component of Perineuronal Nets

Double labeling for Sema3A and the classical PNN marker *Wisteria floribunda* agglutinin (WFA) indicates that Sema3A is located in the PNNs surrounding neurons [51]. In the PNN, Sema3A colocalizes with known individual components of PNN, including various CSPGs such as versican, aggrecan, phosphacan, and the glycoprotein tenascin-R. The presence of Sema3A around neurons depends directly on intact PNN. Enzymatic digestion of chondroitin sulfates, a key component in the PNNs, *in vivo* strips the neurons not only from their WFA positive nets but also from Sema3A protein.

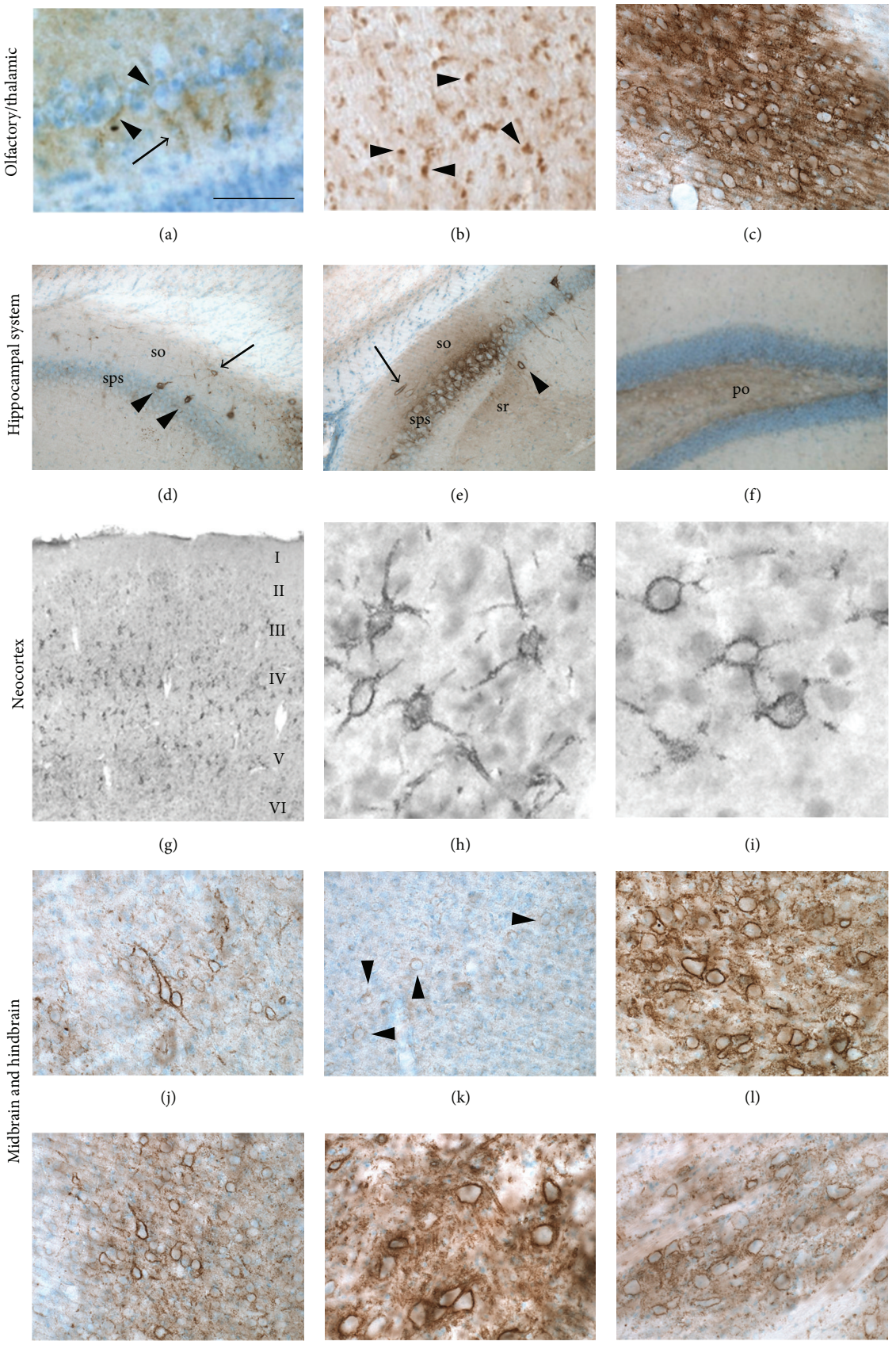

Figure 2: Continued.

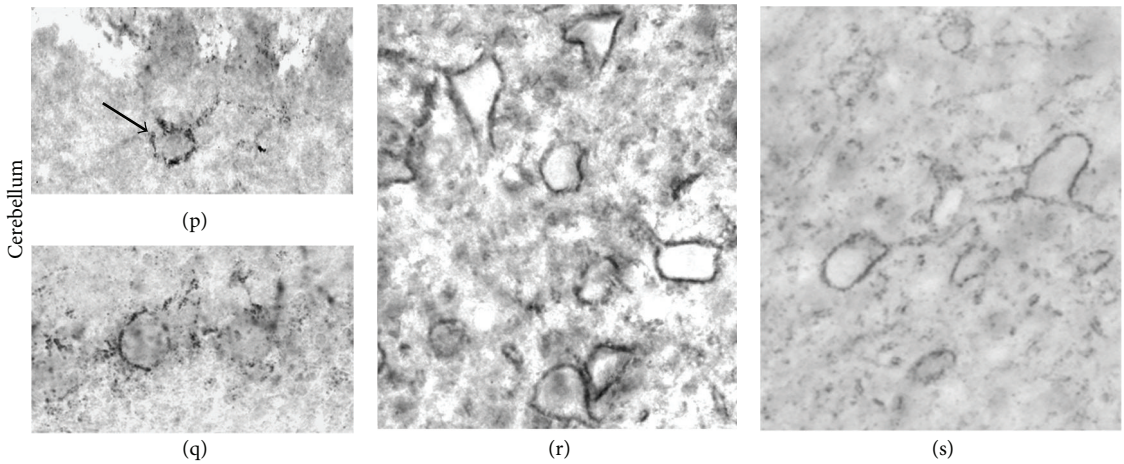

Figure 2: Sema3A expression in the rat brain from rostral to caudal. In the olfactory bulb (a), faint Sema3A-immunoreactive labeling was detected between mitral cells (arrowheads) as well as around the initial segments of the mitral cell dendrites in the external plexiform layer (arrow). A coronal section of the lateral olfactory tract (b) showed strong Sema3A-immunostaining in myelinated axons (arrowheads). In the reticular thalamic nucleus (c), intense diffuse labeling is present throughout the nucleus. The neurons in the reticular thalamic nucleus are surrounded by strongly Sema3A-labelled PNNs. Sema3A immunoreactivity in the hippocampus was observed in various structures. Interneurons in the stratum oriens of CA1 (so, arrow) (d) and in the stratum oriens (so, arrow) and stratum radiatum (sr, arrowhead) of CA2 (e) displayed Sema3A positive PNNs. Only the pyramidal neurons in CA2 and not in CA1 and CA3 displayed striking intense Sema3A-labelled PNNs with strong diffuse labeling between the cell bodies of pyramidal neurons (sps) (f). The polymorph layer (po) of the dentate gyrus displayed a weak diffuse staining for Sema3A. In the neocortex, neurons with a Sema3A-positive PNN are scattered throughout layers II to VI (g). This typical Sema3A labeling in the cortical layers was observed in various anatomical subdivisions of the neocortex, including the somatosensory cortex (h) and the visual cortex (i). Well defined Sema3A-labelled PNNs are present around neurons in the deep gray of the superior colliculus (j); this is in contrast to the weakly labelled PNNs surrounding the neurons of the intermediate gray of the superior colliculus (arrowheads) (k). The red nucleus shows a large number of strongly Sema3A-labelled PNNs around the magnocellular neurons. In addition, an intense diffuse Sema3A staining was observed between neurons (l). Also the external nucleus of the inferior colliculus displayed strong and well-defined Sema3A immunoreactive PNNs and strong diffuse labeling between the neurons throughout the nucleus (m). The vestibular nucleus of the hindbrain contains many large neurons with robust Sema3A-positive PNNs and strong diffuse immunoreactivity between the cells in the nucleus (n). Moderately Sema3A-labelled PNNs were observed around neurons in the inferior olive while throughout the nucleus a diffuse Sema3A labeling was present (o). In the cerebellum, some Golgi neurons bear Sema3A-positive PNNs ((p), arrow). Many Purkinje cells displayed a semiorganized Sema3A-pattern which is typical for the PNNs found around Purkinje cells (q). Deep cerebellar nuclei display well-defined large Sema3A and Sema3B immunoreactive neurons (resp., (r) and (s)). Images (d)–(s) have been reproduced with permission from Vo et al. [51]. Scale bar for (a) and (b) is 25 μm; for (c) and (j)–(o) is 100 μm; for (d)–(f) is 200 μm; for (g) is 300 μm; for (h) and (i) is 33 μm; and for (p)–(s) is 35 μm.

Recent work has shown that hyaluronan and proteoglycan link protein 1 (hapln1; also known with an alternative name as cartilage link protein 1) is necessary for normal PNN formation. Brain specific hapln1-mutant mice form only vestigial PNNs around neurons that normally have well-developed PNN [55]. In these mice with attenuated PNN, virtually no Sema3A-positive PNN are found [50, 51]. The colocalization of Sema3A with WFA and multiple CSPGs and the decline of Sema3A-positive PNN after enzymatic or genetic perturbation of the PNNs demonstrate that this repulsive axon guidance protein is a genuine component of PNN.

In the adult brain, pericellular Sema3A staining was only observed around cells that do also have a WFA positive PNN. However, not all WFA-positive neurons have Sema3A-immunoreactivity in their PNN, indicating that the presence of Sema3A in PNN is restricted to a subset of PNN bearing neurons. In the neocortex, most PNN are found around parvalbumin (PV) and calbindin positive inhibitory interneurons [56]. Double staining for these interneuron markers and Sema3A revealed that, in the rat and mouse visual cortex, Sema3A is only present in the PNNs around PV-positive interneurons. High magnification images showed that Sema3A positive PNNs form a honeycomb-like structure around the soma and proximal dendrites of the PV-cells (Figure 3). The holes in these honeycomb-like PNNs are occupied by presynaptic terminals that synapse on the PV-interneurons. This means that the localization of Sema3A in the PNN brings it not only in close contact with the PV-interneuron itself but also with the presynaptic neurons that form synapses on the somatodendritic compartment of the PV-interneuron. Sema3A receptors have been shown to be expressed by PV-interneurons of the visual cortex. Sema3A in the PNN is closely associated with PlexinA1 and A4 positive microdomains on the PV-interneurons cell membrane. This close contact is suggestive of direct signaling

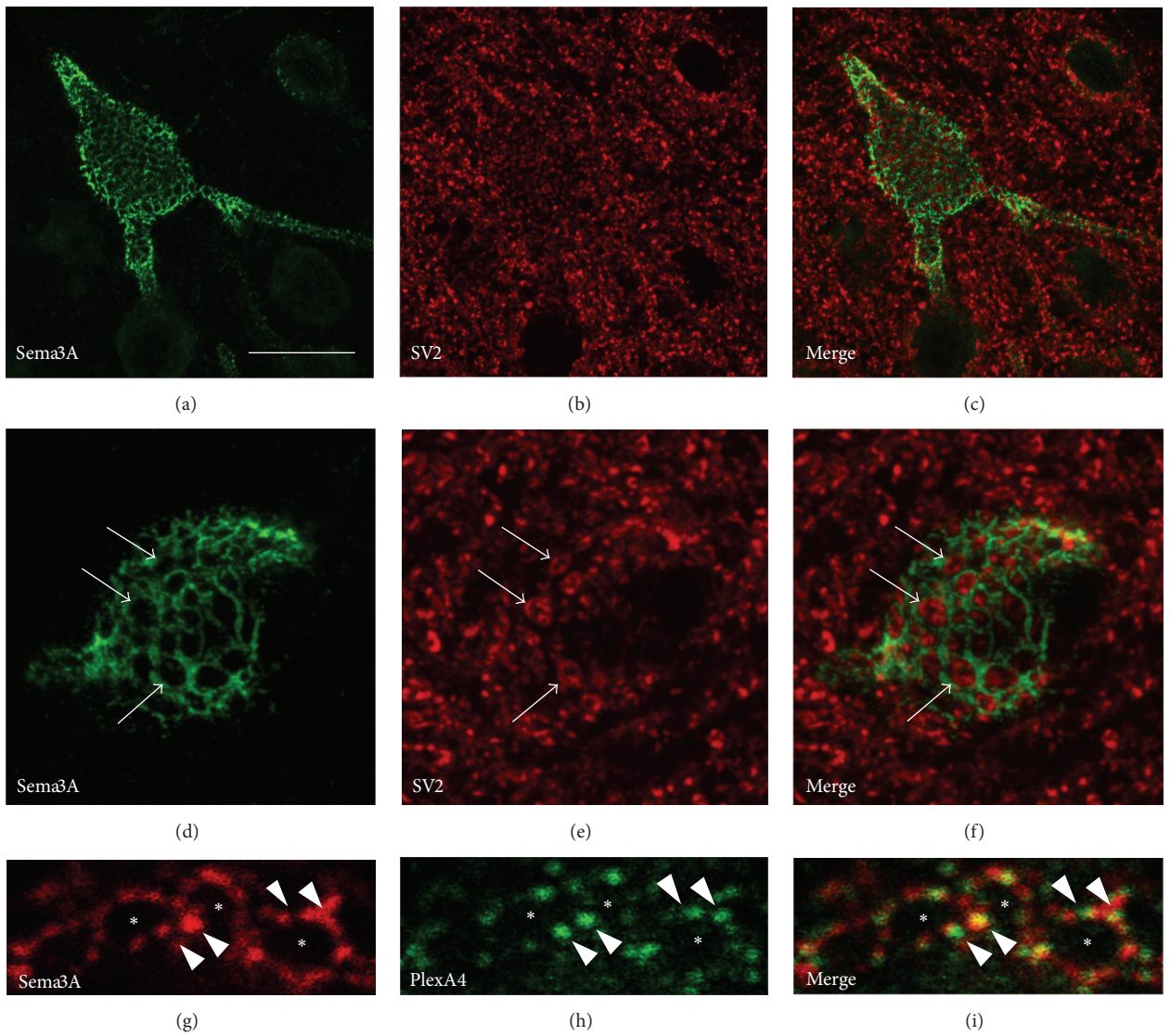

Figure 3: Sema3A expression in PNN. Confocal microscopy shows Sema3A immunoreactivity (green) which surrounds the somatodendritic compartment of an interneuron in the rat neocortex. Sema3A staining displays a honeycomb-like pattern which is typical for the structure of PNNs (a)–(c). Colabeling with the presynaptic marker SV2 (red) reveals that synaptic boutons on the neuron surface fill the holes in the Sema3A-positive honeycomb-like structure of the PNN ((d)–(f), arrows). The Sema3A receptor component PlexinA4 (green) is closely associated with the Sema3A (red) positive net. Like Sema3A, PlexinA4 (arrowheads) is not present in the holes (asterisks) of the honeycomb-like structure of the PNN (g)–(i). Scale bar for (a)–(c) is 20 μm; for (d)–(f) is 8 μm; for (g)–(i) is 3.5 μm.

between Sema3A in the PNN and the PV-interneuron. It is currently not clear if the synaptic terminals on the PV cell body also express Sema3A receptor components.

The cellular source of Sema3A protein in PNN is not known. Sema3A protein might be locally produced or it could be produced elsewhere in the brain and subsequently transported to the PNNs. Comparing results from *in situ* hybridization studies with immunohistochemistry studies for Sema3A reveals that there is no direct correlation between Sema3A mRNA expression and the presence of a Sema3A-positive PNN around a particular neuron. Both Sema3A mRNA expressing and nonexpressing neurons can have a Sema3A-positive PNN. Moreover, neurons that do express Sema3A mRNA but do not have a Sema3A bearing net exist. The absence of a clear overlap between mRNA expression and the presence of Sema3A protein in the PNN suggest that Sema3A is produced elsewhere, being transported and presented to the PNN. Previous studies have shown that in embryonic rat cortical neurons, endogenously or exogenously expressed Sema3A is actively transported in vesicles through both the axon and dendrites of the cell [57, 58]. In axons, Sema3A is almost exclusively transported in an anterograde direction. This transport is activity dependent, whereby depolarizing the cell results in an arrest and action potential blocking in an acceleration of axonal transport of Sema3A. Moreover, depolarization also induced the delivery

of Sema3A at the cell surface as a stable deposit [58]. Taken together, this suggests a model in which presynaptic neurons produce Sema3A, transport the protein to their terminals, and subsequently deposit it in the PNN of PV-interneurons in an activity dependent manner.

Alternatively, Sema3A could be produced and secreted by nonneuronal cells in the brain. Previous studies showed that astrocytes do not express Sema3A and these cells can therefore be excluded as a source of Sema3A [46]. Recently, it has been shown that Orthodenticle homeobox 2 (OTX2), another protein that binds to the PNN, is produced by cells in the choroid plexus and released in the cerebral fluid [54, 59]. From the cerebral spinal fluid, OTX2 is able to travel and bind to the PNN in the neocortex. Conditional removal of OTX2 expression in choroid plexus cells results in PNNs that are devoid of OTX2 in the neocortex [59]. This shows that choroid plexus cells are indeed an unexpected source of OTX2 in PNN. Like OTX2, Sema3A is highly expressed by choroid plexus cells in the ventricles (unpublished observations). In addition, meningeal cells surrounding the brain express high levels of Sema3A [7]. Similar to OTX2 [59], Sema3A may be released by cells of the choroid plexus or the meninges in the cerebral spinal fluid and travel through the brain to bind to the PNNs.

4. Biochemical Properties of Sema3A-PNN Interactions

The interaction of Sema3A with PNNs is mediated through a specific glycan structure called chondroitin sulfate (CS) (Figure 4). In order to understand the molecular interaction between Sema3A and PNNs, a more detailed understanding of PNNs and their molecular composition is crucial. PNNs are stable macromolecular aggregates of brain extracellular matrix molecules tethered on the surface of subpopulations of neurons [60–62]. They are composed of CSPGs, tenascin-R, and members from the Hapln family [63, 64]. Both CS and hyaluronan belong to the family of glycosaminoglycans (GAGs), of which the linear GAG chains are covalently attached to a protein core forming proteoglycan (PG) [65]. The number of disaccharides in a single CS-GAG chain and the number of CS-GAG chains attaching to a core protein are highly variable. The complexity of CS GAGs is further heightened by the addition of sulfate groups at various locations in the disaccharide subunits, resulting in wide heterogeneity of CSs, of which CS-A, -C, -D, and -E are the most common isoforms [66].

The first indication of Sema3A interacting with CSs comes from a study by De Wit et al. [67]. With the use of Neuro2a cells, it has been shown that the addition of excess GAGs including CS-A and CS-B, heparan sulfate and heparin to the culture medium is able to displace the surface bound Sema3A. Enzymatic removal of CSs using chondroitinase ABC (ChABC), but not of heparan sulfate by heparinase III, releases Sema3A-GFP to the culture medium. This suggests Sema3A is indeed bound to CSs on the cell surface. Further evidence for a direct interaction of Sema3A and CS is shown by immunoprecipitation of Sema3A in embryonic brain extract, in which CS is visualized using a pan-CS antibody [68].

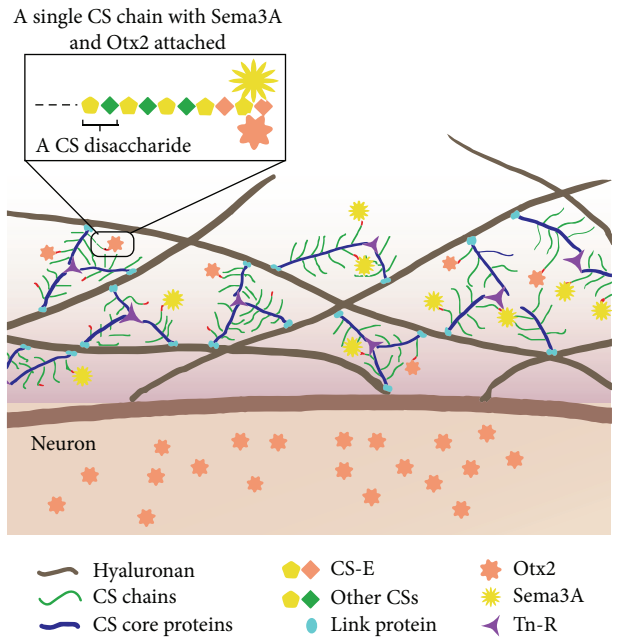

Figure 4: Schematic representation of Sema3A protein localization in PNNs. Hyaluronan molecules attached to the neuron cell membrane contain link proteins that carry the CS chain bearing CS core proteins. Tn-R act as a cross-linking protein between several CS core proteins present on different hyaluronan molecules. Both Sema3A and OTX2 preferentially bind to the CS-E chains attached to the CS core proteins. After binding to the PNN via CS-E, OTX2 can translocate into the neuron [53, 54], whereas Sema3A appears to reside in the PNN using CS-E as "an anchor."

The biological properties of CSPGs are heavily dependent on the attached CS GAG chains. Digestion of the CS GAGs using ChABC in the inhibitory glial scar formed after spinal cord injury has rendered the environment more permissive to axonal growth and regeneration [69–71]. While ChABC removes all CS isoforms, recent study suggests that the different CS isoforms impose distinct effects on neuronal growth [72–74]. Knockout mice with reduced level of 6-sulfated CS (CS-C) demonstrate worse regeneration in nigrostriatal axons when compared with wild type mice [73]. This inferior regeneration ability is, however, not observed in a peripheral nerve injury system due to a compensatory upregulation of 6-sulfates in the PNS. Increased expression of 6-sulphated CS in animals that overexpress the 6-sulfotransferase enzyme is associated with increased plasticity, which persists into adulthood [74]. On the contrary, chondroitin 4-sulfates (CS-A) are strongly upregulated after spinal cord injury and have been shown to inhibit axonal growth in cultured cerebellar granular neurons, suggesting a diversity of functions from various sulfated CSs [72, 75]. An analysis of CS sulfation composition in an adult brain has demonstrated differences between the GAGs recovered from the PNNs or from the loose brain extracellular matrix (ECM) in an adult rat brain [76]. While 4-sulfation (CS-A) is the predominant

CS isoform in an adult brain, the percentage has however dropped from ~90% in the ECM GAGs to ~80% in the PNN GAGs. On the contrary, both the 6-sulfation (CS-C) and the 4,6-disulfation (CS-E) are increased in the PNN GAGs [76]. These experiments prompt a question: could the interaction of Sema3A with PNNs be sulfation specific? The binding of several growth factors and morphogens has been shown to be sulfation pattern dependent [77]. With the use of a modified enzyme-linked immunosorbent assay, carbohydrate microarrays, and proteomic analysis, it has now been confirmed that the interaction between Sema3A and PNNs is sulfation-dependent [52, 78]. Among the four most common CSs and heparan sulfate used in the assay, Sema3A interacts preferentially to CS-E in a concentration dependent manner. This binding is partially blocked by an anti-CS-E antibody [52]. CS-E is a disulfated CS structure, similar to CS-D, bearing two highly negatively charged sulfate groups. The lack of binding of Sema3A to CS-D but only to CS-E suggests that this interaction is specific and is not due to a nonspecific charge interaction.

The binding of homeoprotein OTX2 to the PNNs in PV positive neurons is also mediated through CS-E [54, 79]. Enzymatic digestion of CS or blocking the binding of OTX2 to the PNNs prevents the internalisation of OTX2, changes the expression of PV, and reopens ocular dominance plasticity in an adult visual cortex [54, 59]. A basic motif composed of a 15-amino acid motif enriched in arginine-lysine (RK) doublets at the N-terminal of OTX2 is responsible for OTX2 binding to PNNs [54]. Although a specific CS-binding domain has not yet been identified in Sema3A, Sema3A also contains a long basic domain at its C-terminus. Whether this basic domain is responsible for binding to the PNNs merits further investigation.

5. Functional Implications of Sema3A in PNNs

The effect of Sema3A binding to the PNNs in mature CNS neurons has yet to be elucidated. We could however get a glimpse of the potential function from the work in dorsal root ganglion (DRG) neurons or embryonic cortical neurons *in vitro*. Growth cones of embryonic DRG neurons were exposed to Sema3A collapse and this collapse is potentiated by heparin [67]. Adult DRG neurons cultured on isolated PNN-GAGs project shorter neurites than those being cultured on general brain GAGs, suggesting that PNN-GAGs are more inhibitory than general GAGs. This inhibition is further enhanced by the presence of Sema3A. Blocking Sema3A binding to PNN-GAGs reverses this inhibition [52]. Similar effects are also observed in E13 cortical culture. Cortical neurons avoid the area where CSPGs are present and this repellent property is strengthened in the presence of Sema3A [68]. These results suggest that CS may be involved in regulating the degree of inhibition of Sema3A on neuronal growth.

Based on these studies, it is hypothesized that Sema3A binding to PNN-GAGs may act as an inhibitory cue for presynaptic remodeling and formation of new connections on PNN-bearing neurons. To test this hypothesis, recent studies examined Sema3A expression in conditions associated with plasticity. For example, when adult mice are reared in an enriched environment (a condition known to strongly promote neuronal plasticity (see for reviews [80, 81])), a strong reduction of WFA-positive PNNs occurs in these mice and the Sema3A content of the PNN in the cerebellar nuclei decreases (Figure 5). In parallel, enhanced structural plasticity of Purkinje cell terminals and precerebellar afferents is observed [50, 82]. Sema3A levels in PNNs are also altered in the injured CNS during compensatory sprouting. Partial deprivation of cerebellar nuclei neurons of their main inputs, the Purkinje cells, results in a strong decrease of both CSPG and Sema3A labeling surrounding denervated neurons, in association with structural reorganization of the local connectivity [50]. These studies suggest that Sema3A in PNNs can be actively modulated to facilitate or restrict plasticity according to specific functional requirements. Interestingly, reduced Sema3A expression in PNNs is also observed in the cerebellar nuclei of transgenic mice selectively overexpressing the growth-associated protein GAP-43 in Purkinje cells [50], suggesting that enhanced intrinsic growth properties of Purkinje cells are accompanied by parallel modulation of extrinsic regulatory cues [83, 84]. Mice lacking Hapln1, which have attenuated PNNs with strongly diminished Sema3A levels, display persistent plasticity into adulthood [51, 55]. Collectively, these data suggest that Sema3A is an important functional attribute of PNNs, contributing to their growth-inhibitory properties.

In accordance with the idea that Sema3A may play a role in the regulation of the functioning of neuronal circuits in the adult brain, in an experimental model of temporal lobe epilepsy, which is characterized by robust sprouting of hippocampal mossy fibres in the molecular layer of the dentate gyrus, a transient downregulation of Sema3A mRNA in stellate neurons of the entorhinal cortex occurs. Entorhinal stellate neurons project their axons to the molecular layer of dentate gyrus and thus may secrete Sema3A into this region (which indeed is devoid of Sema3A mRNA) [85]. These data suggest that Sema3A derived from the entorhinal cortex may restrict structural changes in the molecular layer of the dentate gyrus throughout life. Also in the cerebellum, where Sema3A may be released by axon terminals of Purkinje cells, which express Sema3A mRNA, to regulate Purkinje cell specific connection patterns in the cerebellar nuclei. Indeed, Sema3A is tightly associated with Purkinje axons and their terminals and its amount in PNNs around cerebellar nuclei neurons is relation to Purkinje cell innervation of target neurons [50]. Similarly, Sema3A produced by primary sensory neurons regulates the number of sensory innervations of trabecular bone in an autocrine manner [86]. Also during development, Sema3A exerts a cell-autonomous effect on the outgrowth of axons, by regulating the sensitivity of motoneuron growth cones to exogenous Sema3A [87]. Moreover, in cultures of differentiated hippocampal neurons, Sema3A elicits a strong reduction of the size of pre- and postsynaptic structures [45]. Finally, inhibition from Sema3A on axon outgrowth of calcitonin gene related peptide- (CGRP-) positive sensory fibers is shown following injection of adenovirus encoding Sema3A in the spinal cord

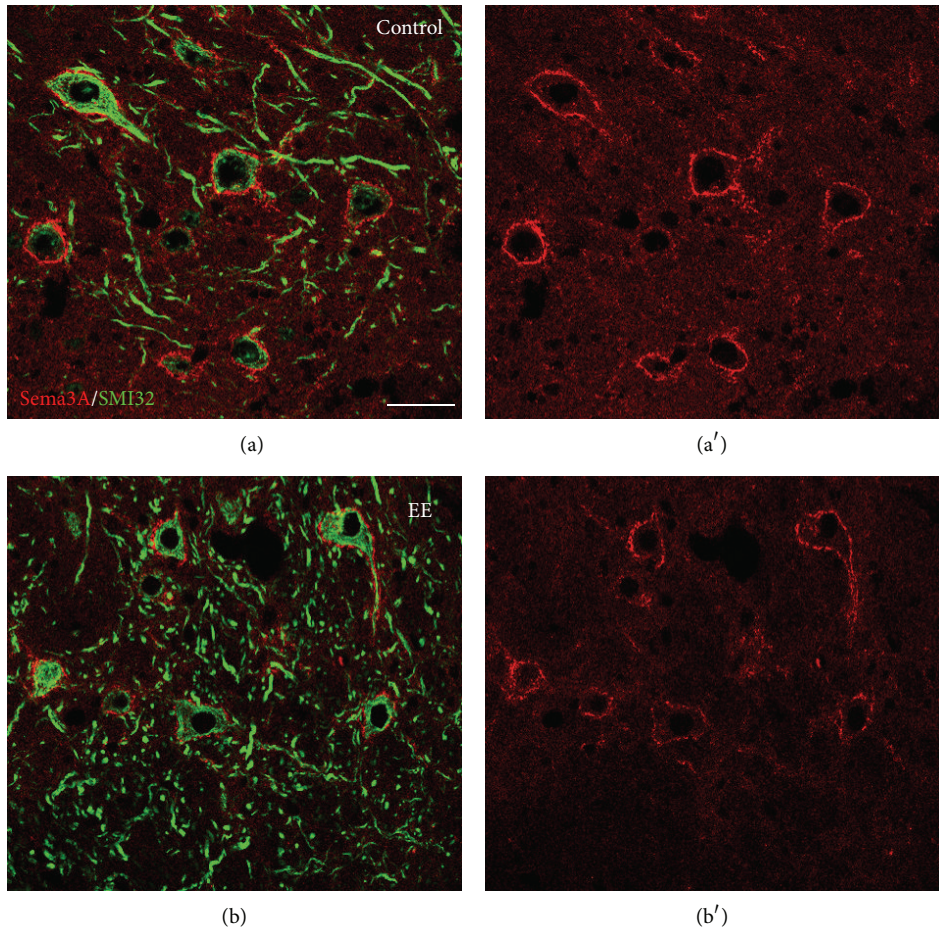

Figure 5: Exposure to enriched environment induces a reduction of Sema3A in the cerebellar nuclei of the adult mouse. (a)–(b′) show Sema3A immunostaining (red) around projection neurons of the cerebellar nuclei (stained by SMI32, green) of control mice (a, a′) and mice exposed to enriched environment (b, b′). A clear reduction of Sema3A levels is observed in enriched animals. EE: enriched environment. Scale bar: 50 μm.

after complete spinal transection in a model of autonomic dysreflexia [88].

To exert an effect on PNN-bearing neurons or synaptic boutons, Sema3A must interact with the NP-1/Plexin receptor complex. In the cerebellar nuclei, where strong perineuronal Sema3A is present, both nuclear neurons and Purkinje cell axons express the Sema3A receptor component PlexinA4 [89]. NP-1 is strongly expressed in the molecular layer of the dentate gyrus, where entorhinal stellate neurons project their axons [85]. In the cerebral cortex, where Sema3A containing PNNs are mainly associated with GABAergic interneurons, PlexinA1 and PlexinA4 are concentrated in globular structures on the plasma membrane of those neurons. These Plexin-positive microdomains are closely associated with Sema3A that is concentrated in the PNN [51], suggestive of Sema3A-Plexin-mediated signalling between the PNN and the soma of the inhibitory neuron. Moreover, interneurons display abundant expression of flotillin-1 [51], a lipid raft protein that is essential for Sema3A induced growth cone turning and endocytosis [90].

The interaction of Sema3A with CSPGs could concentrate these cues at specific sites and/or potentiate or modify their activity. The possible additive effects of Sema3A with PNN-GAGs could be mediated by the independent signalling pathways these two families of molecules are eliciting. Whereas Sema3A signals through the NP-1/Plexin receptor complex, the inhibition from CSs in the PNN-GAGs may be triggered via CSPG receptors, including protein-tyrosine phosphatase-sigma, leukocyte common-related phosphatase, or Nogo receptor-1 or receptor-3 [91–93]. It is therefore possible that the simultaneous presentation of both Sema3A and CSs on the neuronal surface confers stronger inhibitory properties to the PNNs.

6. Conclusions and Perspective

PNNs around inhibitory interneurons play a key role in the regulation of the critical period. In many developing neural systems, the closure of the critical period shows a strong correlation with the appearance of PNNs around inhibitory

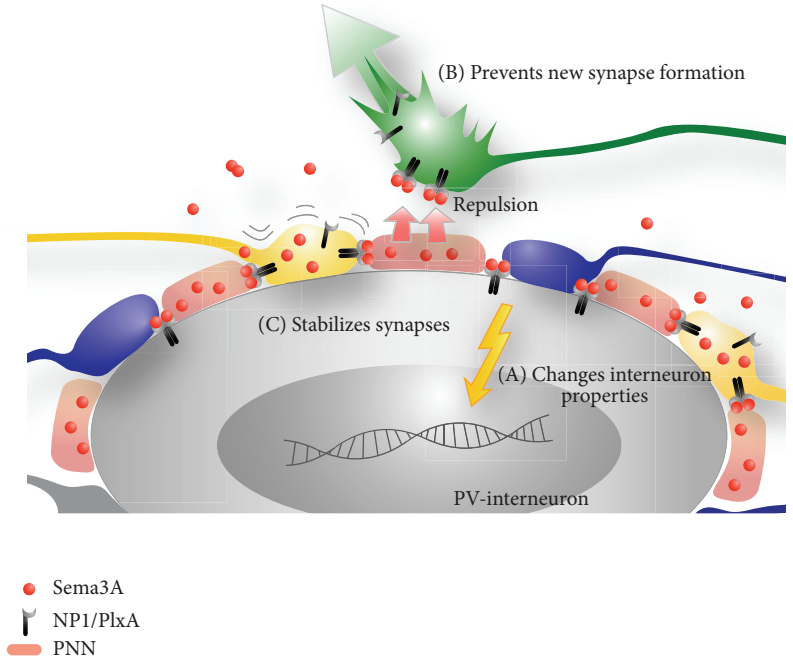

Figure 6: Schematic view of possible roles for Sema3A in PNN related plasticity. Sema3A protein molecules (red spheres) derived from the more distant cells in the environment, that is, meningeal cell or cells of the choroid plexus, or secreted along axons or by presynaptic terminals (yellow) integrate in the PNN surrounding parvalbumin (PV) positive interneurons. PV-interneurons express Sema3A receptor components (NP1/PlxA) which may trigger an internal response upon Sema3A binding that eventually may change the properties of the PV cell (A). Alternatively, Sema3A may act on (new) presynaptic terminals. Sema3A bound to the PNN may repel growing axons (green) away from the PV cell membrane and thereby prevent the formation of new synapses between PV-interneurons and ingrowing axons (B). Sema3A in the PNN may also "stabilize" synaptic contacts on the PV-interneuron surface by preventing local rearrangements of existing synaptic terminals (C).

neurons [94–97]. Preventing sensory input, like dark rearing, prolongs the period of ocular dominance plasticity and delays PNN formation in the visual cortex [94, 95]. Degradation (by chondroitinase ABC) or the formation of strongly attenuated PNN (as in Hapln1 mutant mice) restores or retains plasticity, respectively [55, 94, 96]. How the PNN regulates plasticity is still poorly understood, as it turns out to be very difficult to unravel the mechanistic cellular and molecular basis of PNN function. The discovery that chemorepulsive proteins like Sema3A and Sema3B are molecular components of PNN offers new avenues for research into how the PNN may influence neuroplasticity.

Three conceivable theories which are not mutually exclusive and based on the currently available literature are outlined in Figure 6. First of all, Sema3A captured in PNN can have an effect on the PV-neuron itself by interacting with Sema3A receptors on the PV cell plasma membrane (Figure 6, model A). Secondly, as PNNs form during postnatal development, Sema3A gradually accumulates in PNN and this may repel Sema3A-sensitive fibers from the cell bodies of inhibitory interneurons thus preventing them from forming synapses on the PV-neurons (Figure 6, model B). Third, during adulthood, Sema3A in PNN could restrict the plasticity of (a subtype of) existing synapses on the PV-neuron (Figure 6, model C).

Currently, the origin of Sema3A in the PNN is not known. Studies with (genetically) labelled Sema3A in adult animals may be a first step to identify the Sema3A producing cells. Information of the cellular source is required to design future experiments to interfere with Sema3A expression and study the mechanism(s) by which Sema3A may be involved in PNN regulated plasticity.

More knowledge on the expression of Sema receptor components and their localization in PNN-positive cells and their synaptic partners is needed. The observation that net-bearing cells themselves express some components (PlexA1 and A4) of the Sema3A receptor complex is a first indication that the interneuron itself may be (one of) the target(s) of Sema3A (Figure 6, model A). PV-cells in the cat visual cortex selectively express a downstream effector of Sema3A signalling, collapsin responsive mediator protein-4 (CRMP-4) [98]. Interestingly, reorganization of the cortical projection zone following a binocular retinal lesion is accompanied by an increase in CRMP4 expression [99].

Well in line with its role during development of the nervous system, Sema3A in the PNN could repel or induce pruning of inappropriate fibers that try to establish contacts on PV-interneurons during late postnatal development (Figure 6, model B). Cortical and thalamic axons have abundant synapses on neocortical PV-interneurons but they also

are sensitive to the repulsive activity of Sema3A *in vitro* [100–102]. One could hypothesize that, during development, the accumulating levels of Sema3A in the PNN reach a threshold level which can act as a selective force that only allows the strongest and/or nonreceptor carrying axons to establish or maintain synaptic contacts on the PV-interneuron and that all others are repelled or pruned. Although effects of Sema3A on the cytoskeleton restrict growth cone motility, it is currently unknown if Semaphorins in the PNN also can contribute to the stabilization of existing synapses on the PV-interneuron (Figure 6, model C). However, there is clear evidence that several members of the Semaphorin family, including Sema3A, have a central role in the formation, pruning,y and function of different types of synapses (reviewed in [10]).

NP1 is an obligatory component of the functional Sema3A receptor complex. NP1 binds to Sema3A and to Plexin and is essential for the stabilization of the Sema3A-Plexin interaction [103]. To date, we have not been able to detect NP1 in inhibitory interneurons or in synapses on the cell bodies of these neurons. This questions the validity of the idea that PNN-bound Sema3A has the capacity to act as a repulsive signal through the classical NP/Plexin receptor complex. The specific interaction of Sema3A with CS-E in PNN does raise the intriguing possibility that CS-E may stabilize the interaction of Sema3A with PlexinA which may result in functional Sema3A-PlexinA signalling independent of its interaction with NP1.

An increasing number of studies provides evidence that developmental axon guidance cues also play a significant role in synaptic remodeling and function in the adult nervous system [104]. Dysregulation of these proteins during adulthood may contribute to undesirable changes in synaptic connectivity and lead to neurological dysfunction [11]. If Semaphorins play an important role in PNN regulated neuronal plasticity, then unwanted changes in their expression could have serious consequences for the function of the nervous system. In two experimental models for epilepsy (temporal lobe and status epilepticus), dysregulation of Semaphorins is thought to contribute to the aberrant sprouting observed in the hippocampal system in these models [85, 105]. Taken together, we can also envision that controlled neutralization of Sema3A in PNN may be an important approach to enhance neuronal plasticity and functional repair after injury.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Otx2-PNN Interaction to Regulate Cortical Plasticity

Clémence Bernard and Alain Prochiantz

Center for Interdisciplinary Research in Biology, CNRS UMR 7241/INSERM U1050, Labex Memolife, Collège de France, 11 Place Marcelin Berthelot, 75005 Paris, France

Correspondence should be addressed to Alain Prochiantz; alain.prochiantz@college-de-france.fr

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The ability of the environment to shape cortical function is at its highest during critical periods of postnatal development. In the visual cortex, critical period onset is triggered by the maturation of parvalbumin inhibitory interneurons, which gradually become surrounded by a specialized glycosaminoglycan-rich extracellular matrix: the perineuronal nets. Among the identified factors regulating cortical plasticity in the visual cortex, extracortical homeoprotein Otx2 is transferred specifically into parvalbumin interneurons and this transfer regulates both the onset and the closure of the critical period of plasticity for binocular vision. Here, we review the interaction between the complex sugars of the perineuronal nets and homeoprotein Otx2 and how this interaction regulates cortical plasticity during critical period and in adulthood.

1. Introduction

During postnatal development, anatomical and functional plasticity of neural circuits allows the cerebral cortex to adapt to the environment, as cortical connections can be remodeled by physiological activity. These windows of learning, or critical periods, are needed to establish an optimal neural representation of (and adaptation to) the surrounding environment. Several sensory, motor, linguistic, and psychological abilities can only be acquired during these periods [1], since plasticity is very limited outside them, in particular in the adult when circuits and synapses have been consolidated. Critical periods have been observed in various systems and across species [2], but since the pioneering work of Wiesel and Hubel 50 years ago in cats, the critical period has been primarily studied in the binocular visual cortex. It was shown at the time that the inputs from the two eyes compete when they first converge onto individual neurons in the binocular zone of the primary visual cortex [3]. This leads to a physiological and anatomical cortical representation of the relative inputs contributed by either eye [4]. During the critical period, monocular deprivation, the extended closure of one eye, produces a loss of cortical response to the deprived eye and a gain in the input of the open eye [5]. This sensitivity to monocular deprivation is restricted to the critical period that begins at postnatal day (P) 20 in rodents (about 1 week after eye opening), peaks at P30, and rapidly declines over the next days [6]. In humans, imbalanced inputs during this critical period result in a neurodevelopmental disorder called amblyopia. Indeed, improper timing of critical periods is responsible for many central nervous system pathologies, possibly including certain psychiatric diseases [7].

Many molecular factors have been implicated for the onset and the closure of the critical period. Binocular interactions are detected by the integrated action of local excitatory and inhibitory connections in the visual cortex. This excitatory/inhibitory balance is dynamically adjusted by cortical circuits where inhibitory connections develop later than the excitatory ones [8]. As an optimal excitatory/inhibitory balance is required for plasticity, critical period onset is triggered by the maturation of local inhibitory circuits [4, 9]. More specifically, critical period onset is triggered by the maturation of a subset of GABAergic inhibitory interneurons, the fast-spiking parvalbumin interneurons (PV-cells), located in layer IV of the cerebral cortex [10]. Precocious maturation of GABAergic innervation is prevented during the precritical period by factors such as α-2,8-polysialic acid bound to the neural cell adhesion molecule (PSA-NCAM) [11]. In response to sensory input, critical period onset is triggered when factors such as brain-derived neurotrophic factor (BDNF [12, 13]) or neuronal activity-regulated pentraxin (NARP [14, 15])

promote PV-cell maturation. This triggers a sequence of structural and molecular events that lead to circuit rewiring and physiological consolidation. During the critical period, layer IV PV-cells are gradually enwrapped by a specialized extracellular matrix giving rise to the perineuronal nets (PNNs) that surround the cell soma and proximal dendrites [16, 17]. PNNs are enriched in complex sugars called glycosaminoglycans (GAGs) and constitute a highly organized structure composed of hyaluronic acid, link proteins, proteoglycans, and tenascin-R [18, 19]. Physiologically, PNNs are part of the molecular brakes that progressively decrease plasticity and eventually close the critical period. Indeed, an emerging view is that the brain is intrinsically plastic and that adult plasticity is dampened by molecular brakes that limit excessive rewiring after critical period closure [20]. However, this is a reversible process and plasticity can be reopened after critical period closure, either by reinstalling lower levels of inhibition [21–23] or by lifting the molecular brakes (e.g., by disrupting the PNNs [24]). Opening windows of plasticity in the adult is of therapeutic interest [25], given that it has been used to cure amblyopia in rodents [26–31]. This review discusses how the PNN extracellular matrix interplays with homeoprotein Otx2 to regulate visual cortex plasticity and how interfering with this interaction can reopen windows of plasticity in the adult.

2. Otx2 Homeoprotein Transfer Regulates the Critical Period for Ocular Dominance Plasticity

The transfer of homeoprotein Otx2 in the visual cortex during postnatal development is necessary and sufficient for the onset and closure of the critical period for ocular dominance plasticity in mice [32]. Homeoproteins are well-known transcription factors that play major roles during embryonic development. For instance, several homeoproteins (including Otx2) are fundamental in controlling the specification, maintenance, and regionalization of the vertebrate brain [33]. Homeoprotein transcription factors share a highly conserved DNA-binding domain called homeodomain, but many homeoproteins also share activities that extend beyond their classical transcriptional role. Indeed, they are paracrine signaling factors that transfer between cells due to the presence within the homeodomain of sequences necessary for their unconventional intercellular transfer: a secretion sequence "Δ1" [34] and an internalization sequence "penetratin" [35].

Otx2 is no exception and has noncell autonomous activity in the supragranular layers of the binocular visual cortex [32, 36]. When transferred from extracortical sources into the visual cortex during postnatal development, Otx2 is internalized preferentially by PV-cells: in the visual cortex, a majority of neurons containing Otx2 are GABAergic inhibitory interneurons and over 70% of them are PV-positive [32]. The time course of Otx2 accumulation in PV-cells parallels that of PV-cell maturation: Otx2 protein is barely detected in the primary visual cortex prior to critical period onset, is increasingly concentrated by PV-cells during the critical period, and persists in adulthood. Interestingly, *Otx2* conditional knockdown heterozygous mice have a delayed onset of ocular dominance critical period, suggesting that a 50% reduction in Otx2 protein is sufficient to alter PV-cell maturation [32]. Otx2 therefore not only accumulates in PV-cells but also promotes their maturation and consequently regulates the onset of the critical period of plasticity for binocular vision.

3. Otx2 Binds Sulfated Glycosaminoglycans of the PNNs

The preferential capture of Otx2 protein by PV-cells suggests the existence of Otx2-binding sites at the PV-cell surface. As PV-cells are gradually enwrapped by PNNs during the critical period, a strong association between Otx2 and PNNs is observed in layer IV of the adult visual cortex [30]. PNN hydrolysis with the enzyme chondroitinase ABC (ChABC), which digests the GAG chains and reactivates plasticity in the adult cortex [24], decreases endogenous Otx2 concentration in PV-cells [30]. Another study showed that a decrease in PNN formation due to redox deregulation prevents the internalization of Otx2 by PV-cells [37]. It was thus concluded that complex sugars of the PNNs participate in the specific recognition of Otx2 before its internalization. A short motif within Otx2 sequence (RKQRRERTTFTRAQL), which partially overlaps with the first helix of the homeodomain, possesses consensus traits of a GAG-binding domain [38] and is a requisite for the specific recognition of Otx2 by PNN-surrounded PV-cells. Indeed, while a full-length exogenous Otx2 protein injected in the visual cortex shows a preference towards PNN-enwrapped cells, an Otx2-AA protein, in which the arginine-lysine (RK) doublet is replaced by two alanines (AA), shows less preference for PNNs and is internalized by a wider range of cells [30]. Accordingly, when a synthetic peptide corresponding to this GAG-binding motif (RK-peptide) is infused into the visual cortex of adult mice, it competes with endogenous Otx2 and blocks its transfer into PV-cells. The reduced capture of Otx2 by PV-cells results in a downregulation of PV expression and PNN assembly, as if the maturation status of PV-cells was reversed to a critical period state. This "rejuvenation" was confirmed by the reopening of ocular dominance plasticity following infusion of the RK-peptide in the adult cortex and the ensuing recovery of visual acuity in amblyopic mice [30].

The sulfation pattern of glycan chains is thought to encode specific information for the binding of growth factors and morphogens, such as Wnt, Hedgehog, BMP, and FGF [39]. The sulfation pattern of PNNs differs from that of GAGs of the diffuse matrix and the three main types of GAGs present in the PNNs are the chondroitin sulfates (CS), heparan sulfates, and hyaluronic acid [18, 19]. Isothermal titration calorimetry experiments with commercial subtypes of chondroitin sulfates showed that the RK-peptide binds strongly to disulfated chondroitin sulfates CS-D and CS-E, has a lower affinity for CS-C and heparin, and shows no measurable binding to CS-A [30]. However, the affinities of GAGs for the full-length 32 kD protein may be different from the 15-amino-acid peptide as specificity is expected to be altered by

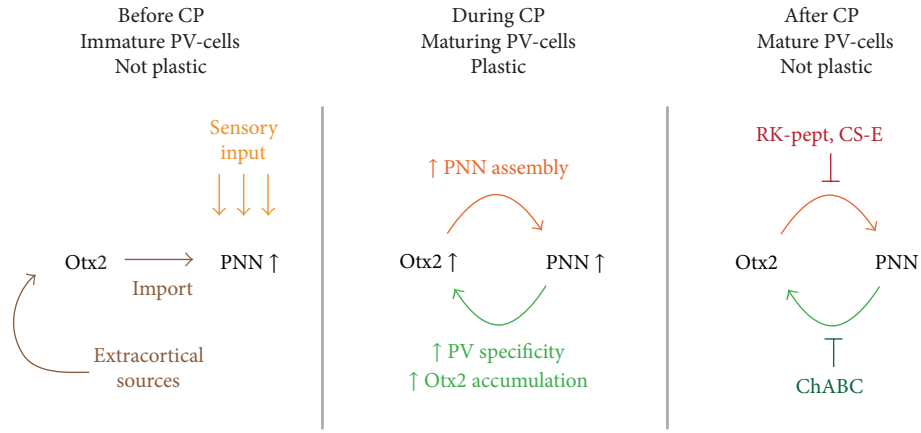

Figure 1: *Otx2-PNN feedback loop for critical period plasticity*. At critical period (CP) onset, sensory activity induces initial formation of the perineuronal nets (PNNs), allowing the internalization of extracortical Otx2 by PV-cells. During CP, the increasing PV-cell Otx2 content enhances PNN assembly. In turn, PNNs ensure the specific accumulation of Otx2 in the PV-cells. In the adult, the constant transfer of Otx2 into the PV-cells, due to the positive feedback loop between the homeoprotein and the PNNs, maintains a mature, consolidated, nonplastic state. Indeed, interfering with Otx2-PNN interaction in the adult (by injecting ChABC to remove PNNs or by infusing the GAG-binding domain of Otx2 (RK-peptide) or a CS-E analogue to block Otx2) reopens a window of plasticity in the visual cortex.

the increased size and the probable structural changes [40]. Otx2 full-length protein binds to a synthetic hexasaccharide analogue of CS-E [41] and six monosaccharide units seem to be the minimum GAG chain length for Otx2 binding. Infusion of this CS-E analogue in the visual cortex of adult mice blocks Otx2 transfer in PV-cells [41], supporting the idea that binding to specifically sulfated GAGs is required for proper transfer of Otx2 into cortical PV-cells. Interestingly, the CS-E subtype is also required for the binding of the semaphorin Sema3A to the PNNs in the visual cortex [42, 43]. In addition, modification of the sulfation pattern of PNNs (using transgenic mice with a low 4S/6S ratio) reduces Otx2 accumulation in the PV-cells of the visual cortex [44]. These mice show extended ocular dominance plasticity in the adult, confirming both the role of Otx2 in the regulation of critical period in the visual cortex and the importance of a specific sulfation pattern for Otx2 binding.

4. A positive Feedback Loop between Otx2 and PNNs

Not only are the complex sugars of the PNNs necessary for Otx2 preferential transfer into PV-cells, but Otx2 is in turn involved in PNN assembly, both during the critical period and in the adult. Indeed, early Otx2 infusion in the visual cortex, before the onset of the critical period for ocular dominance, accelerates PNN expression leading to an early closure of plasticity [32]. In addition, in dark-rearing conditions that delay PV-cell maturation [45, 46], direct infusion of Otx2 in the visual cortex leads to increased amount of PNNs around PV-cells [32]. The opposite effect is observed in *Otx2* conditional knock-out heterozygous mice in which Otx2 protein amounts in the visual cortex are strongly reduced: these mice, which have a delayed ocular dominance critical period, also show a delay in the maturation of the PNNs [32]. It can thus be concluded that Otx2 transfer triggers the maturation of PNNs during postnatal development. In the adult, one of the main sources of cortical Otx2 is the choroid plexus. This structure, present in brain ventricles and responsible for the synthesis of cerebrospinal fluid, is an established site of Otx2 expression throughout life [31, 47]. In the adult mouse, Otx2 is secreted by choroid plexus epithelial cells into the cerebrospinal fluid, and knocking-down *Otx2* specifically in the adult choroid plexus decreases Otx2 cortical content [31]. This decrease in Otx2 is accompanied by a decrease in PV expression and PNN assembly. As already mentioned, this is also the case when Otx2 transfer is blocked at the level of the target cells in the adult cortex, by using the RK-peptide or the synthetic CS-E analogue: both infusions lead to a reduction in the number of PNNs surrounding PV-cells [30, 41]. Otx2 transfer in the adult therefore seems to be required to maintain the PNNs in a mature state.

Both gain- and loss-of-function experiments indicate that Otx2 internalization enhances PNN assembly [30–32, 41]. The ongoing positive feedback of PNNs attracting Otx2, thus triggering their own continued maintenance throughout life, may serve to prevent plasticity in adulthood (Figure 1). Otx2 regulation of plasticity can therefore be explained by a two-threshold model: the critical period is triggered as Otx2 is first captured by PV-cells but then closes as maturing PNNs condense in response to Otx2 accumulation, thus permitting a constant accumulation of Otx2 by PV-cells [31]. However, the mechanisms through which Otx2 regulates the maturation and maintenance of the PNNs are yet unknown. Otx2 could modify the expression of members of the PNNs. For example, the homeoprotein has been shown to regulate the expression of extracellular matrix proteins such as tenascin-C and DSD-1-PG *in vitro* [48]. Otx2 could also regulate the expression of enzymes that modify the extracellular matrix such as the metallopeptidases Adamts, which are expressed by PV interneurons [49]. Otx2 molecular targets for PV-cell regulation are also unidentified. The fact that Otx2 transfer

is necessary and sufficient to open plasticity at P20 and close it 20 days later and that blocking Otx2 is enough to reopen a window of plasticity in the adult cortex suggests a very general action of Otx2. Epigenetic changes have been linked to critical period and adult plasticity [29, 50, 51] and Otx2 could act at the epigenetic level to globally modulate PV-cell maturation. Beyond the understanding of plasticity mechanisms during postnatal development, identifying these plasticity targets of Otx2 could lead to the development of precise tools to reopen windows of plasticity in the adult.

5. Glycans Could Be Involved in the Recognition of Homeoproteins for Unconventional Transfer

GAG moieties vary considerably in size, in the number of disaccharides per core protein, and in the position and degree of modifications, primarily sulfation, allowing huge molecular diversity and structural complexity. Complex sugars are precisely distributed in the postnatal brain, suggesting the existence of a sugar code for specific protein distribution. Therefore, specific sugar epitopes may provide a sugar code for homeoprotein recognition. Sequences homologous to the GAG-binding domain identified in Otx2 are present upstream of the homeodomain of many homeoproteins [52]. GAG-binding sites are often not conserved between proteins of the same family. In the case of chemokines, this allows the specificity and selectivity of GAG-binding across members of the family [40, 53]. The fact that Engrailed, another homeoprotein, does not accumulate specifically in PV-cells when infused in the cortex [30] supports the idea of specific surface binding sites for homeoproteins and of a glycan code for homeoprotein transfer specificity. The identification of precise sugar sequences could lead to the development of novel substances, such as synthetic CS-E analogue [41], to specifically interfere with homeoprotein transfer.

Infusion of homeoproteins in the brain parenchyma requires a coinfusion of polysialic acid to allow their diffusion [30, 32, 54]. Otherwise, the homeoprotein cannot diffuse and is immediately taken up by cells close to the infusion site. In the case of Otx2, the presence of polysialic acid allows the diffusion of the protein until it meets the PNN-enwrapped neurons. This suggests that endogenous traveling Otx2 is associated with low-affinity glycans and, once in the cortex, transfers from the latter low-affinity glycans to high-affinity PNN-associated glycans.

6. Otx2-PNN Interaction Might Coordinate and Synchronize Cerebral Cortex Plasticity

PNNs surround PV-cells not only in the visual cortex but throughout the central nervous system and have been found in the barrel cortex, frontal cortex, amygdala, striatum, substantia nigra, hippocampus, cerebellum, and spinal cord [24, 55–62]. Interestingly, Otx2 is present in PV-cells across all cortical regions, demonstrating that this transcription factor has a noncell autonomous widespread distribution and gains access to PV-cells in most cortical areas that include sensory regions such as the auditory and somatosensory cortices [31]. This makes it tempting to speculate that this factor acts as a global regulator of PV-cell and PNN maturation for cerebral cortex plasticity during development and in the adult [31]. Otx2 transfer could therefore have a wide role in regulating sensory experience during postnatal development. In the visual system, Otx2 transfer is activity-dependent and this raises the question of how activity (e.g., in the visual pathway, the opening of the eyes) operates. The formation of PNNs is also activity-dependent [63] as sensory deprivation by dark-rearing (visual cortex [29]) or whisker trimming (barrel cortex [55]) decreases the number of PNN-bearing neurons. One hypothesis is that as activity-dependent critical periods open following the initial activity of the corresponding peripheral sensory organs, sensory activity regulates an initial PNN assembly allowing for the accumulation of Otx2 en route from the choroid plexus.

Otx2 has been found not only in PV-cells of sensory cortices, but also in structures governing more complex behaviors, such as the amygdala, cingulate, and limbic cortices [31]. PNNs have recently been involved in these regions for the regulation of several types of memory in adulthood. Digestion of PNNs has been shown to increase adult learning capacities in the auditory cortex and perirhinal cortex [64, 65]. Enzymatic removal of PNNs in the prelimbic cortex or in the amygdala of adult rats impaired acquisition and reconsolidation of drug-induced memories [66, 67]. In a mouse model for Alzheimer's disease, digestion of PNNs in the perirhinal cortex enhanced object recognition memory [68]. Considering Otx2 function in maintaining the mature structure of PNNs in the adult, blocking Otx2 transfer could be used to promote cognitive flexibility and enhance memory acquisition in neurodegenerative diseases, for instance. PNNs have also been involved in critical periods for these regions, for instance, for fear extinction in the amygdala. It is of particular interest that, in amygdala, PNNs assemble at the closure of critical periods for fear extinction [57]. Whereas young mice can permanently erase an acquired fear memory by extinction training, adult animals exhibit fear behaviors that are resistant to erasure. In the adult basolateral amygdala, PNN degradation by ChABC reopens a critical period during which fear memories are fully erased by extinction training [57]. Otx2 transfer could therefore also regulate complex functions related to the emotional and anxiety state of the animal. Several reports propose that some psychiatric diseases may find their origin, at least in part, in cortical dysfunctions that occur in a period that precedes the onset of puberty [7, 69–71]. Defective maturation of PV-cells has been reported in cortex of subjects with schizophrenia [72] and has been proposed as one of the causes of psychiatric phenotypes [73–77]. In support of this hypothesis, PNN density is reduced in the amygdala and in the entorhinal and prefrontal cortices of subjects with schizophrenia [78, 79]. Critical periods therefore not only are governing the postnatal development of sensory systems but also have been involved in more complex behaviors, including language [80]. The role of Otx2 in various cortical regions has yet to be confirmed but Otx2 noncell autonomous presence in these areas suggests that this signaling may contribute to

the orchestration of cascading critical periods underlying sensory behaviors and higher cognition.

7. Conclusion

Otx2 homeoprotein accumulation in PV-cells driven by sensory experience triggers a critical period for plasticity. Otx2 transfer regulates the maturation of the PNNs around PV-cells, which eventually closes the critical period. PNNs, in turn, maintain a stable postcritical period state by attracting Otx2 throughout life resulting in a positive feedback loop. PNNs therefore not only are molecular brakes that limit morphological and physiological plasticity, but also can act as "receptors" controlling the concentration of molecular factors that regulate plasticity and modulate PV-cell function, such as Otx2 and Sema3A. A better understanding of the Otx2 and extracellular GAGs interplay requires the identification of the precise glycan sequence that binds to Otx2 in the PNNs and of the mechanisms through which Otx2 regulates PNN assembly, maturation, and/or maintenance. This could allow the development of new GAG-related therapeutic strategies to block Otx2 transfer and reopen windows of plasticity with the hope to cure neurodevelopmental diseases.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Neuron-Glia Interactions in Neural Plasticity: Contributions of Neural Extracellular Matrix and Perineuronal Nets

Egor Dzyubenko,[1,2] Christine Gottschling,[1] and Andreas Faissner[1]

[1]*Department of Cell Morphology and Molecular Neurobiology, Faculty of Biology and Biotechnology, Ruhr-University, Universitätsstraße 150, 44801 Bochum, Germany*
[2]*International Graduate School of Neuroscience, Building FNO 01/114, Ruhr-University, Universitätsstraße 150, 44801 Bochum, Germany*

Correspondence should be addressed to Andreas Faissner; andreas.faissner@ruhr-uni-bochum.de

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Synapses are specialized structures that mediate rapid and efficient signal transmission between neurons and are surrounded by glial cells. Astrocytes develop an intimate association with synapses in the central nervous system (CNS) and contribute to the regulation of ion and neurotransmitter concentrations. Together with neurons, they shape intercellular space to provide a stable milieu for neuronal activity. Extracellular matrix (ECM) components are synthesized by both neurons and astrocytes and play an important role in the formation, maintenance, and function of synapses in the CNS. The components of the ECM have been detected near glial processes, which abut onto the CNS synaptic unit, where they are part of the specialized macromolecular assemblies, termed perineuronal nets (PNNs). PNNs have originally been discovered by Golgi and represent a molecular scaffold deposited in the interface between the astrocyte and subsets of neurons in the vicinity of the synapse. Recent reports strongly suggest that PNNs are tightly involved in the regulation of synaptic plasticity. Moreover, several studies have implicated PNNs and the neural ECM in neuropsychiatric diseases. Here, we highlight current concepts relating to neural ECM and PNNs and describe an *in vitro* approach that allows for the investigation of ECM functions for synaptogenesis.

1. Introduction

The development of the mammalian brain unfolds in a sequence of precisely orchestrated steps. After neurogenesis and gliogenesis neurons have migrated to their final destinations. Thereafter, the process of axon growth and guidance generates a complex network of connections that is crucial for correct central nervous system (CNS) function. Axons terminate in synapses that mediate the transfer and the storage of information. The synapse thus represents the central functional element of the nervous system. It consists of a presynapse, the synaptic cleft, and the postsynaptic membrane [1–3]. This functional unit is formed during development of the nervous system and is subject to malleability in the adult nervous system [3, 4]. There, modifications of synaptic connections on the functional and the structural level are believed to underlie synaptic plasticity that plays a key role in the context of learning and memory [3]. A whole range of cell adhesion molecules are involved in synapse formation and maturation [5]. Synaptic machinery is an intricately organized mechanism where transmembrane proteins work in concert with pre- and postsynaptic factors including cytokines [2, 6], Eph-kinases and ephrin ligands [7], cell adhesion molecules [8–11], neurexins and neuroligins [12], extracellular matrix (ECM) glycoproteins [13, 14], complementary integrin receptors [15], Narp/NP2, wnt7A, and FGF22 [3, 16], and several intracellular scaffolding molecules that anchor postsynaptic receptors [1, 17, 18]. The role of guidance molecules in synapse formation and plasticity was previously reviewed [19], and in this paper we will focus on the role of ECM in neural plasticity.

ECM provides a highly organized environment that mediates a broad spectrum of intercellular interaction in the CNS. In a subpopulation of neurons ECM develops into a specific formation termed perineuronal nets [20, 21]. Perineuronal nets (PNNs) were described by Camillo Golgi as

a honeycomb-like precipitate around a subpopulation of silver-stained neurons [22]. The latter includes fast spiking GABAergic interneurons expressing parvalbumin [23, 24], and sometimes other types of neurons, for example, excitatory pyramidal neurons, can exhibit these macromolecular structures [25, 26]. PNNs localize to the soma and dendrites and delineate synapses on neuronal surfaces, which led to the hypothesis that PNNs contribute to the regulation of neuronal plasticity [22, 27]. Their function is based on the net-like assembly of ECM components that are heterogeneously expressed [28, 29] and interact with the pericellular microenvironment and the surrounding cells. Both neurons and astrocytes contribute to the formation of the tripartite synapse [30]. In the past years, numerous studies have been published examining the structure and function of PNNs in the central nervous system. Within the present review, we will focus on our approach to study the neural ECM in PNNs in the context of astrocyte-neuron interactions and their regulatory function in the establishment of synaptic connections and their maintenance and plasticity.

2. ECM of the CNS

The ECM is composed of glycoproteins and proteoglycans that form an interactive network of macromolecules for which the term matrisome has been proposed [20, 21]. According to this description, the matrisome core comprises about 300 genes whose products structure the extracellular space and function as a scaffold for the binding of various molecular ligands and cells. While it had originally been thought that the ECM of the CNS is confined to the basal lamina of blood vessels and the meninges, a wealth of data has meanwhile shown that it plays crucial roles in the neural stem cell compartment [31, 32], in axon growth and guidance [33], in the visual system [34], and in the lesion response of the CNS [35–37]. The ECM of the CNS consists of glycoproteins including laminins, tenascins, thrombospondins [33, 38], and proteoglycans. The latter ones comprise a core protein and at least one covalently linked unbranched glycosaminoglycan (GAG) chain, which defines the subtypes of heparan sulfate proteoglycans (HSPGs) [39–42] and chondroitin sulfate proteoglycans (CSPGs) [43–45]. In particular CSPGs of the lectican family such as aggrecan [46–54], brevican [55–59], neurocan [46, 55, 60–62], and versican [46, 55, 61, 63, 64] (for a detailed review concerning lecticans see [65]) are abundantly expressed in the developing CNS and enriched in (PNN) structures [66]. These are thought to be involved in processes such as ion-buffering [67], connection to the intracellular cytoskeleton [22], protection against oxidative stress [68], and stabilization of synapses [69] (see below). With the exception of one splice variant of brevican that is anchored to the plasma membrane via GPI [59], all members of the lectican family are secreted into the extracellular space [45].

3. PNNs Composition and Structure

In close proximity to certain types of CNS neurons, the diffuse distribution of ECM changes towards a highly condensed configuration, creating a specific formation termed PNNs [20, 21]. These assemblies are identified as the areas of dense immunocytochemical staining for one of their core molecular components. The most widely used markers to detect PNNs include *Wisteria floribunda* agglutinin (WFA) lectin and antibodies against CSPG core proteins [24, 70, 71]. Although the expression of PNNs components displays some heterogeneity throughout different brain regions [29, 72], several of them can be defined as core components [66, 73]. In particular, the CSPGs of the lectican family [28] are highly enriched in PNNs and share a conserved globular domain at their N-terminus whereby they interact with hyaluronic acid (HA) [49, 55, 65, 74–76], another core component of PNNs (Figure 1). The HA is composed of disaccharides consisting of N-acetylglucosamine and glucuronic acid that forms a linear structured polymer [36, 77] which is not bound to a core protein [36, 78–80]. According to recent reports the membrane based hyaluronic acid synthase (HAS) is at least in part responsible for the attachment of PNNs to the neuronal membrane via binding interactions of PNN constituents such as the lecticans with HA [36, 74, 81]. A further constituent of the PNNs is the CSPG termed DSD-1-PG/phosphacan [55, 66, 82–86]. Phosphacan is a splice variant of the receptor protein tyrosine phosphatase- (RPTP-) β/ζ, a transmembrane receptor linked to several relevant signal transduction pathways [43, 87].

Phosphacan interacts with other ECM constituents, namely, the tenascin glycoproteins, which are also compounds of PNNs. The glycoprotein tenascin-R (Tnr) of the tenascin gene family [48–50, 55, 88–92] is a further prominent component within PNN structures. Tnr has so far exclusively been detected in the CNS where it occurs as a trimeric glycoprotein [90, 92]. Tnr displays binding sites for members of the lectican family, for example, versican [93], brevican [94], and neurocan [95], and has the potential to cross-link ECM components due to its trimeric structure [13].

Link proteins are important for enhancing and maintaining the interactions of CSPGs with HA and involved in the formation of PNNs because thereby they increase the stability of the PNN structure [55, 74] (Figure 1). HAPLN1 (HA and proteoglycan link protein 1)/Crtl1 (cartilage link protein 1), HAPLN2/Bral1 [96], and HAPLN4/Bral2 (brain link proteins 1 and 2) [97] are the most thoroughly studied link proteins relating to PNNs and are known for their interaction with CSPGs and HA [48, 55, 98–101]. Summarizing these indications, it can be stated that the CSPGs of the lectican family, as well as HA, Tnr, and link proteins, determine the scaffold of PNNs in the central nervous system by establishing intensely structured extracellular aggregates [28, 55, 102].

Importantly, a number of regulatory molecules are associated with PNNs. These variable elements can be linked to the main components by either direct interaction with core protein or by binding with GAG chains. One example is tenascin-C (Tnc) that is expressed during the development of the central nervous system [86, 103–108]. Also the glycoprotein semaphorin 3A [76, 109–111] (see below), which plays a crucial role in the process of axon guidance [112, 113], is attached to molecules of PNNs, in particular proteoglycans [114]. Other PNN-associated molecules, in particular matrix

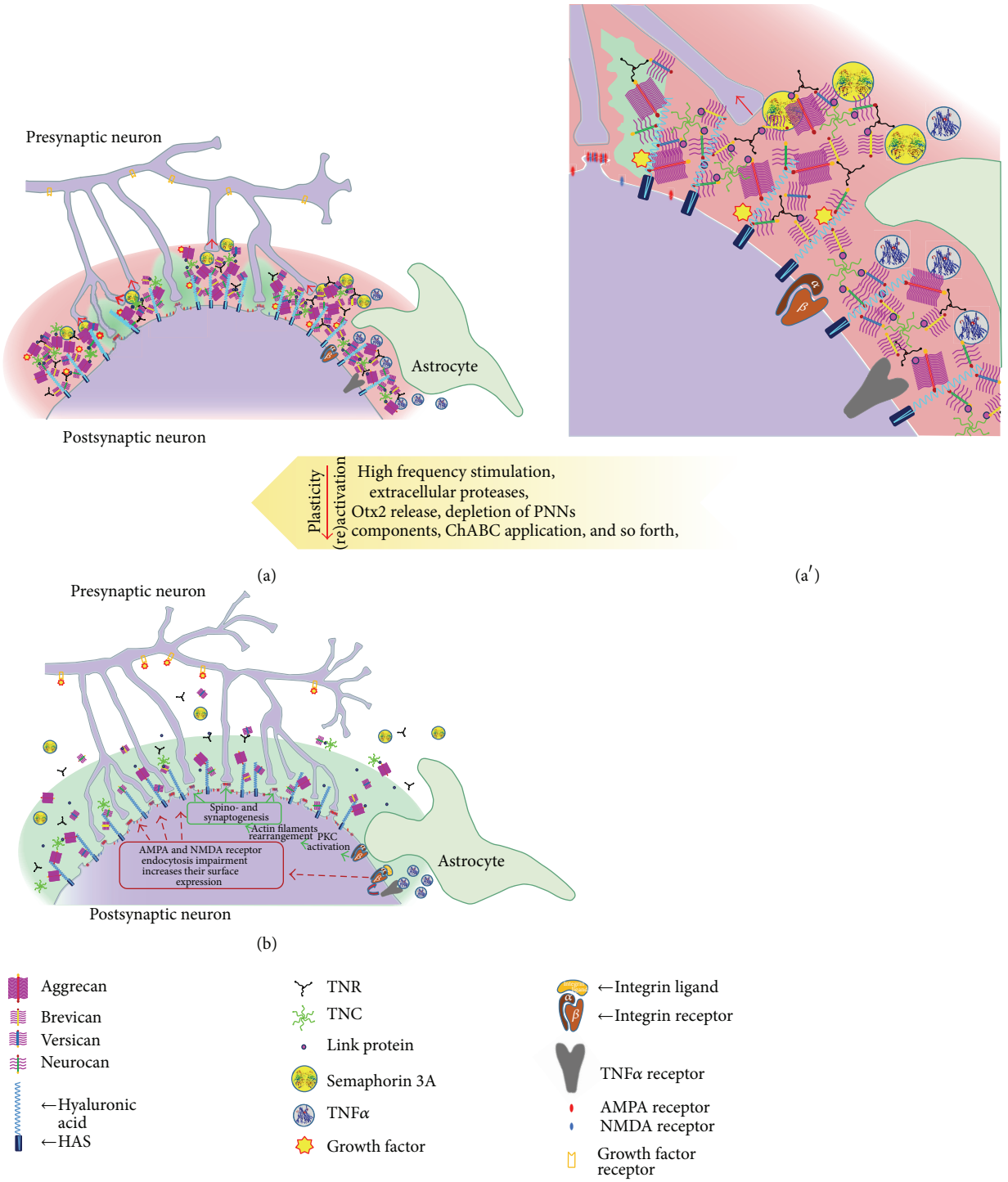

Figure 1: PNNs and neural plasticity. The cartoon depicts the composition of an ECM coat in a PNN on neuronal surfaces, as produced jointly by neurons and astrocytes (a), magnified in (a′). PNNs restrict adult neuronal plasticity, by providing inhibitory environment (depicted in red) restricting astrocyte-induced plasticity and by embedding repulsive guidance molecules. Only several permissive areas are left, indicated in green. Remodelling of PNNs and consequent regain of plasticity (b) can be induced by distinct treatments, as shown by the arrow.

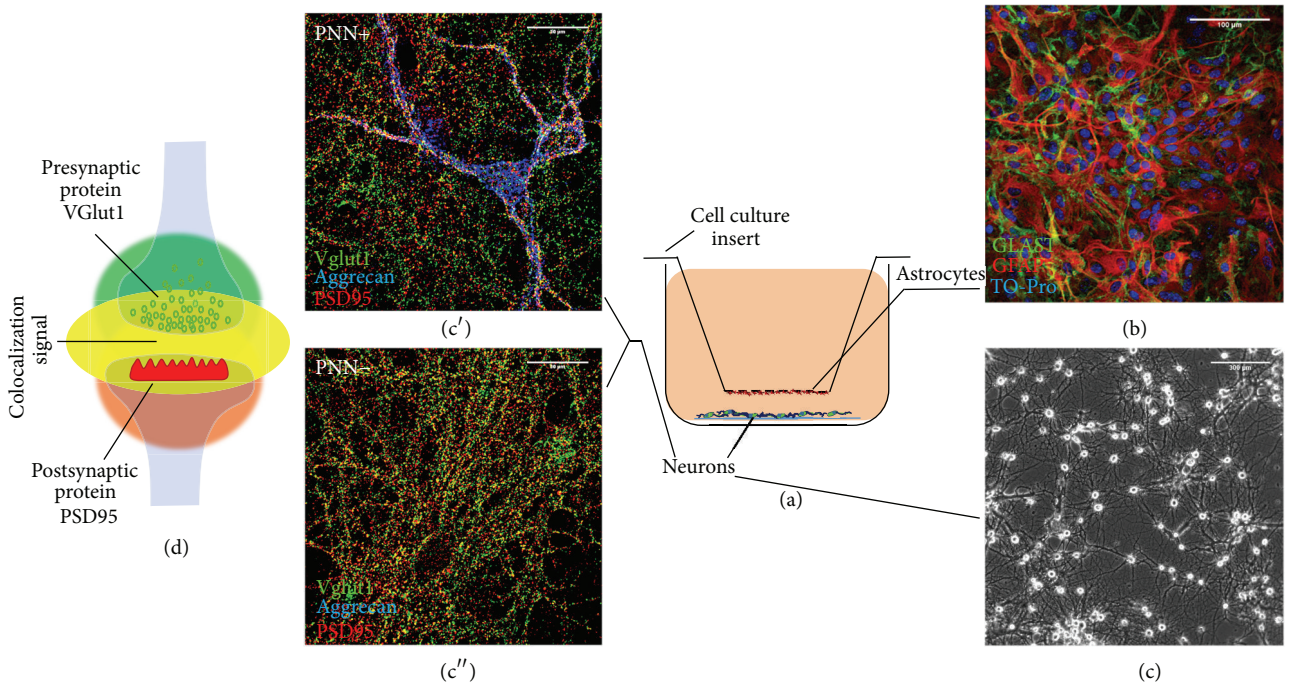

Figure 2: Neuron-astrocyte coculture for the study of synaptogenesis. A schematic view of the neuron-astrocyte indirect coculture system is presented. (a) Primary embryonic day 15 mouse hippocampal neurons are cultivated on coverslips in the presence of primary cortical astrocytes maintained as monolayers in cell culture inserts (b). Thereby, astrocytes and neurons share the same medium in the absence of membrane-mediated contacts. With the use of this system, neurons can be cultivated for up to 4 weeks and form active neuronal networks (c) in completely defined media [132], suggesting a reliable model for synaptogenesis studies. A subgroup of neurons can develop PNNs, as indicated by a specific marker (c′ and c″). Presynaptic and postsynaptic terminals can be visualised using immunocytochemical labelling of presynaptic and postsynaptic proteins. The overlap of pre- and postsynaptic puncta indicates the structural synapses (d). Quantification of synaptic puncta using an analysis software permits the quantitative evaluation of synapse formation *in vitro* under different treatment conditions. For experimental details see [129, 130].

metalloproteases (MMPs) and Otx2, also contribute to neural plasticity and will be discussed further in this review.

4. Astrocyte-ECM-Neuron Interactions in Neuronal Plasticity

The impact of astrocytes on neuronal networks development, their regulation, and plasticity has been a subject of intensive research throughout the last decades [115]. As the new insights were provided, our understanding of glia has switched from an intercellular "glue" to an active component of the CNS [30, 116–118]. We now know that astrocytes not only provide neuronal networks with essential structural and metabolic support, but also modulate neuronal activity and neural plasticity [30, 69, 119–121] and support the formation of neuronal circuits [111, 122]. In addition, astrocyte-neuron crosstalk is crucial for neuroprotection and is involved in neurological diseases progression in several cases [119, 120]. According to the current concept of the tripartite synapse astrocytes are necessary for the establishment and regulation of synapses [69, 117, 122–125]. Furthermore, glial subpopulations can also directly form synapses with neurons [126]. Neurons contact astrocytes in multiple ways including ephrin-based interactions [127] and ECM-mediated integrin signalling [128]. ECM molecules mediate a substantial part of astrocyte-neuron interactions and to consider them as independent entity in a conceptual construct that has been termed the tetrapartite synapse has been proposed [69, 119, 123].

Indirect neuron-astrocyte coculture *in vitro* models provided a powerful tool to study astrocyte-neuronal interactions. Applying this approach, Pyka et al. [129, 130] combined pure cultures of primary embryonic hippocampus neurons with pure cultures of primary astrocytes, using cell culture inserts to avoid direct membrane-mediated cell contacts. Under these conditions neurons survived and developed neuronal networks when sharing the defined culture medium with primary astrocytes (Figure 2). The latter also supported synaptogenesis in the neuronal culture [129]. The use of this coculture system opens the possibility for numerous forms of analysis exclusively for neurons, for example, isolation of mRNA for qRT-PCR, analysis of the transcriptome, expression analysis of distinct proteins via Western Blotting, and immunocytochemistry, while the cells dispose of a long *in vitro* life span. Furthermore, the unique model allows the investigation of the source of ECM within the shared culture medium. Geissler et al. used an analogous model [131, 132] to reveal the impact of several ECM components on the establishment of neuronal networks and the expression of PNNs [131]. In that approach, neurons and astrocytes were obtained from either wild type or quadruple knockout mice,

which lack Tnc, Tnr, brevican, and neurocan [133]. Excitatory synapse formation, postsynaptic currents, and PNNs expression were then analysed in different combinations of wild type and knockout cells. Depletion of the four crucial ECM molecules led to an impairment of PNN formation, decreased frequency of miniature inhibitory and excitatory postsynaptic currents, and disturbed synaptogenesis. Indeed, an initial increase of synapse formation after two weeks was followed by the marked decrease of synapse numbers, an effect that was particularly prominent with PNN-bearing neurons, indicating that the latter may be important for synapse stabilization in the long run [131].

Interestingly, the effects of the quadruple knockout were prominent in knockout neurons and could not be rescued by sharing the secretome of wild type astrocytes [131]. As four genes are depleted in the quadruple knockout mouse, it is difficult to attribute the phenotypic changes to one of the missing ECM constituents. Thus, the knockout of neurocan [133] causes only subtle modifications regarding the late phase in long-term potentiation maintenance, without affecting PNNs or brain development [134]. Moreover, the triple knockout of Tnc, Tnr, and versican generates a similar phenotype as the quadruple knockout [133]. Interestingly, neurocan was almost absent in triple knockout mouse brains. Fibulin-1 and fibulin-2 are upregulated and localized interstitially in the quadruple knockout brain, which may exert a compensatory effect by transiting from a tenascin- to a fibulin-cross-linked ECM [133].

5. PNNs in Neural Plasticity

A number of regulatory functions of the ECM that is collectively synthesized by neurons and glia are attributed to PNNs (Figure 1). Interestingly, these macromolecular assemblies can modulate different types of neuronal plasticity, including circuit remodelling and synaptic plasticity [73, 80, 135]. During CNS development, neuronal plasticity is required for controlled remodelling of neuronal circuits. At this level, the role of PNNs was abundantly demonstrated *in vivo* in the context of ocular dominance plasticity (for review see [73]). It has been noted that the expression of molecules forming the PNNs coincides with the closure of the critical period during the development of the brain [73, 80, 135–138]. Increasing evidence also suggests that PNNs are remodelled in correlation with activity [139, 140]. On the structural level, there is evidence that PNNs restrict neurite growth and the development of synapses [141, 142]. On the synapse level, PNNs compartmentalize the neuronal surface and restrict glutamate receptor mobility [81, 143, 144], thus providing support for synaptic plasticity and stabilizing synapses [37, 142]. Preventing the mobility of AMPA receptors led to a reduction of short-term plasticity in rat primary neurons [80, 143, 145], indicating the possible role of PNNs for memory formation. A number of PNN components were shown to regulate synaptic plasticity in their own right. Neurocan deficiency reduced the stability of late-phase LTP [134], brevican ablation led to significantly impaired LTP [146], and depletion of its binding partner Tnr also caused a reduced LTP [147]. Interestingly, knockout of the related glycoprotein Tnc led to a complete failure in LTD induction, together with impaired LTP development [148]. It was hypothesized that the consequence of the Tnc-knockout was due to the reduced L-type VDCC channel signalling.

5.1. Enzymatic Digestion of PNNs Induces Neural Plasticity. The bacterially derived enzyme chondroitinase ABC (ChABC) has been used in numerous studies for analysing the role of PNNs in neuronal plasticity [138, 149]. This enzyme degrades especially the GAG chains of CSPGs, more precisely chondroitin-6-sulfate, chondroitin-4-sulfate, dermatan sulfate, and HA [150], depending on the pH optimum of the enzyme (pH 8.0: chondroitin sulfate; pH 6.8: HA) [151] without altering the core proteins of the ECM. This degradation results in enhanced neuronal plasticity, for example, a higher expression of synaptic proteins in rat hippocampal neurons [130], restoration of ocular dominance plasticity in the adult cat visual cortex [136, 152], and enhanced regeneration of sensory projections and corticospinal tract axons within the adult rat spinal cord after lesion [37, 153]. The inhibitory effects of CSPGs on synapse formation and plasticity could be caused by the chondroitin sulfate GAG (CS-GAG) chains with varying degree of epimerization and sulfation that might result in functional subdomains along the polymer. These domains could interact with and thereby expose a wide range of protein ligands, including the inhibitory semaphorins (see below) [154, 155]. Alternatively, they can also directly activate specific receptors (for review see [156]) that mediate growth cone stalling or retraction, for example, RPTPσ [157]. In spinal cord lesions, the modulation of RPTPσ promotes recovery after spinal cord injury [158].

5.2. PNNs Restrict Integrin Signalling. Apart from the compartmentalization of the neuronal surface PNNs also control synaptogenesis through integrin signalling. Astrocytes can make contacts with neurons via integrin receptors. Focal integrin activation further leads to global PKC activation, resulting in excitatory synaptogenesis facilitation [21, 128, 159]. Interestingly, CSPGs that are abundantly expressed in PNNs inhibit integrin activation [36, 47]. Moreover, there is an evidence for direct interaction between CS-GAGs and integrins, for example, the interaction of the integrin $\alpha_4\beta_1$ and the melanoma chondroitin sulfate proteoglycan [160, 161]. A number of alternative ways of how CSPGs may reduce integrin activation have been suggested [162]. From this point of view, modulating the CSPG coating provides another ECM-mediated mechanism of astrocyte-dependent control over neuronal synaptogenesis [69, 130].

5.3. PNNs-Associated Molecules and Neural Plasticity. Beyond integrin signalling, PNNs can mediate molecular signals between neurons and astrocytes via a number of regulatory molecules that they capture. A number of growth modulating ligands interact with CS-GAGs, including PTN, FGF, and EGF isoforms [43, 154, 155, 163]. Further ligands include TNFα [164], BDNF, semaphorins [165], and extracellular matrix proteases [164]. Semaphorins are particularly

interesting because members of this gene family, notably semaphorin 3A which is synthesized by both neurons and astrocytes, are growth cone collapse inducing molecules with a strong impact on axon growth and guidance [109, 114, 166], axon regeneration [110], establishment of neuronal polarity [112], and the development of dendritic spines [167]. Semaphorin 3A can synergize with CSPGs to regulate neuronal migration [168] and abounds in PNNs [114, 165, 169]. There, it binds to chondroitin sulfate E GAGs (CSE-GAGs), a disulfated disaccharide epitope (GlcUA-GalNAc(4S,6S)), also named E unit [169–171], and could contribute to the repulsion of synaptic sprouts and inhibition of synaptogenesis [37, 113]. Release of semaphorin 3A by ChABC could reduce the inhibitory properties of PNNs and thus may explain increased synaptogenesis upon ChABC treatment [69, 130].

Along these lines, several of the discovered CSPG-ligands are known to modulate synaptic plasticity and are released from astrocytes. For example, TNFα is involved in astrocyte-mediated synaptic scaling [172]. In response to the absence of presynaptic potential astrocytes release TNFα, which leads to elevated expression of β3-integrins at the postsynaptic site. Integrins further enhance AMPARs surface expression through RAP1 inhibition, which leads to the upscaling of postsynaptic currents [6, 172, 173]. ECM proteases, including MMPs, ADAMTS, and TPA, often show a similar mode of action, although their substrates differ. They exhibit elevated expression after LTP induction and support memory formation and learning. Extracellular proteases are not mainly responsible for ECM degradation but rather regulate neural plasticity through cleaving signalling motifs and active forms of growth factors. The reader is welcome to address a recent review for further information [174]. Remarkably, the metallopeptidases Adamts8, Adamts15, and neprilysin are expressed in fast spiking parvalbumin interneurons that are surrounded by PNNs. It is an intriguing possibility that these proteases may partake in PNN remodelling, in dependence of neuronal activity affecting their release [175].

Another intriguing PNN-associated molecule that regulates neural plasticity is Otx2 [176, 177]. This homeobox-containing protein is involved in transient reopening of the visual plasticity period in amblyopic mice [178]. When an arginine-lysine doublet peptide is infused, Otx2-localization to PV-containing interneurons is disrupted, PNNs expression is decreased, and neuronal plasticity is reopened in adult mice, comparable to the situation after ChABC treatment [176, 177]. These results clearly speak in favour of a control of plasticity by interneuronal Otx2 transfer [179–181].

5.4. PNNs and Ionic Homeostasis. The polyanionic nature of PNNs introduces them as an important element of an neuronal excitability regulatory system [67, 68]. While astrocytes actively remove K^+ and neurotransmitters from the extracellular space, preventing overexcitation [119, 182], PNNs buffer cations in close proximity to neuronal membrane, thus enabling rapid spiking [139, 183, 184]. Together with the fact that PNNs are often found around fast depolarizing Kv3.1b channel expressing interneurons, this observation clearly indicates the potential of this macromolecular buffer for the regulation of inhibition by the interneurons. Moreover, being highly hydrated polyanions some compounds of PNNs are needed to maintain brain extracellular space [67]. In particular, reduction of hyaluronic acid synthesis upon HAS knockout led to severe brain extracellular space reduction, diffusion impairment, and epileptiform activity [185].

To summarize, astrocyte-ECM-neuron interaction provide, to our current knowledge, four main groups of mechanisms to modulate neural plasticity: (i) compartmentalization of neuronal surface to restrict and to stabilize synapse formation; (ii) synaptogenesis restriction through integrin signalling suppression; (iii) mediation of molecular guidance signals for synaptogenesis and synaptic plasticity; (iv) ionic gradients and extracellular space maintenance. All these mechanisms are crucial for CNS development, function, and homeostasis. It is no surprise that malfunctioning of this three-party orchestra can cause multiple neurological diseases, which will be addressed in a later paragraph (see below).

6. Cellular Origins of PNNs Components

The assumed functions of PNNs raise the question about which cellular compartments contribute to their construction. In the realm of the tripartite synapse both astrocytes and neurons could be the source of secreted ECM building blocks of the PNNs (Figure 2). This poses questions relating to their relative roles and the regulatory mechanisms involved in these distinct cellular compartments. To understand how the interplay between glia, neurons, and PNNs affects neuronal plasticity, it is necessary to identify the cellular sources of PNNs components. So far, only few researchers addressed this question [49, 51, 55]. For this reason, we will review the available data about the expression of PNNs components by astrocytes and neurons under physiological conditions (Table 1).

Miyata et al. first showed [51] that cultured neurons can express PNNs in the absence of astrocytes. Using pure cultures of rat cortical neurons, they found the presence of WFA lectin labelled PNNs around parvalbumin-containing interneurons. These PNNs also contained neurocan, phosphacan [85, 87], neuroglycan C, and HA. The expression of PNNs increased as the neurons maturated, matching the timelines of their development *in vivo*. Importantly, under these experimental conditions, WFA labelling surrounded synapsin puncta in immunofluorescence stained preparations. This evidence indicates that the development of structurally integral PNNs does not depend on astrocytes.

Further, Carulli et al. [55] addressed the question of cellular sources of PNN components in rat cerebellum. The authors combined immunohistochemistry with *in situ* hybridization to determine which cell types express mRNAs encoding a variety of proteins contributing to PNNs formation. Briefly, neurocan, aggrecan, and link proteins Crtl1 and Bral2 were expressed by neurons, not glial cells. Versican and phosphacan mRNA localized to NG2 glia and oligodendrocytes but was absent in neurons. Brevican mRNA was found in neurons and astrocytes, while Tnr was expressed by NG2 glia, oligodendrocytes, and neurons. It is interesting to note that link proteins Crtl1 and Bral2, originating from neurons, are crucial for PNNs structural integrity [74, 186].

Table 1: Cellular sources of the major PNNs components.

Name	Neurons		Astrocytes		Oligodendrocytes		NG2 glia		Citations
	In vitro	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	*In vitro*	*In vivo*	
HA	++	++	++	++	–	–	–	–	[49, 55, 74–76]
Aggrecan	++	++	++	–	–	–	–	–	[46–51]
Brevican	–	+[1]	+++	+++	–	–	–	–	[55–57]
Neurocan	+++	+++	+[2]	–	–	–	–	–	[46, 55, 60, 61]
Phosphacan	–	++	–	++	–	++	–	++	[55, 82, 83]
Versican	+[3]	–	+[3]	–	+++	+++	–	++	[46, 55, 63]
Tnr	++	++	+[4]	–	++	++	–	++	[48–50, 55, 88, 89]
Tnc	–	+[5]	+++	+++	–	–	–	–	[103–106]
Crtl1	–	+++	–	–	–	–	–	–	[48, 55, 98]
Bral2	–	+++	–	–	–	–	–	–	[48, 55, 98]
Semaphorin 3A	–	++	++	++	–	–	–	–	[76, 109–111]

Symbol +++ indicates the evidence for strong protein and/or mRNA expression, almost restricted to a certain cell type; symbol ++ indicates moderate expression under physiological conditions; + indicates weak or transient expression in a particular cellular subtype or under certain experimental conditions, indicated by superscript footnotes and described below. Dashes indicate the absence of evidence for cell type specific expression published so far.
[1]Neurons of molecular layer of cerebellar cortex and by large excitatory deep cerebellar nuclei neurons [55].
[2]Astrocytic monolayers in culture [61].
[3]Neurons and astrocytes differentiated from embryonic stem cells [46].
[4]Type 2 but not type 1 astrocytes [89].
[5]Transient expression by neurons of spinal cord and hippocampus during development [103, 105].

In the absence of one of these two proteins, PNNs are attenuated and neuronal plasticity is enhanced [98, 187].

Addressing this issue, Giamanco and Matthews [49] have carefully addressed the question of cellular sources of PNNs components. Applying AraC and KCl in different combinations to mixed cultures of mouse cortical glia and neurons, the authors dissected neuronal and glial contributions to PNNs formation. Briefly, Tnr, neurocan, versican, phosphacan, brevican, Crtl1, Bral2, and HAPLN3 appeared to be expressed in a glia-dependent manner, whereas aggrecan expression was neuron-dependent. Interestingly, HA-synthesis was both neuron- and glia-dependent. However, these data do not rule out that glia-dependent components can be produced by neurons. Although only a limited number of articles focused on the study of cellular origins of PNNs components, accessory information can be drawn from a number of other studies. A thorough search in literature databases targeting the expression of major PNNs compounds in different types of cells of the mammalian CNS under physiological conditions provided further insight. We have further verified whether the cellular origin of a component of our interest was clearly stated. The results of our search are summarized in Table 1.

Summarizing the available reports, both neurons and glial cells can produce the majority of PNNs components, while neurocan and link proteins Crtl1 and Bral2 seem to be neuron-specific, at least *in vivo* and under physiological conditions. It is important to note, however, that the expression profile of PNNs components may change upon activation of glia or under conditions of neuronal hyperactivity. For instance, when glial activation occurs upon brain injury or experimental stress conditions, astrocytes start to express sustainable levels of neurocan [61, 188–190], and phosphacan expression is highly upregulated in reactive astrocytes [82, 189, 191, 192].

7. Neurologic Diseases and PNN Formation

Several neurologic diseases have been identified to exhibit an impaired PNN formation *in vitro* and *in vivo,* including epilepsy and schizophrenia (for detailed review see [145]). As a consequence of the fact that PNN formation and maintenance underlie alterations in these diseases, neuronal plasticity is also affected, which, for example, is the case in epilepsy. Here, the disease is accompanied by epileptic seizures triggered by an altered GABAergic signalling [193] together with an abnormal expression and functionality of GABA receptor seen in primary dentate granule cells in a temporal lobe epilepsy rat model [194–196]. A compromised signalling capacity of GABAergic neurons could also be found in a rat model of Alzheimer's disease, in which rats were treated with amyloid beta [197]. Moreover, animal models regarding the psychiatric disease schizophrenia demonstrate an alteration of the inhibition of GABA together with a loss of parvalbumin-expressing neurons [198–200]. As emphasized previously, PNNs enwrap GABAergic neurons [24, 26, 67].

In addition, several PNN components display a modified expression in animal models mimicking epilepsy and schizophrenia, which is consistent with a change of neuronal plasticity. Different expression patterns of neurocan and phosphacan were observed in the hippocampus in a temporal lobe epileptic rat model compared to healthy control rats [201]. Another study also showed an impairment of neurocan and phosphacan, as well as Tnc, using a different mouse model of epilepsy, in which domoate, a specific glutamate

agonist, is injected next to the hippocampus *in vivo* [202]. Here, immunohistochemical staining revealed a significantly higher expression of neurocan and Tnc seven days after injection, whereas the expression of phosphacan was not increased until 14 days after the injection [202]. Reports in the literature suggest that neurocan expression within hippocampal regions is increased in parallel with a decreased phosphacan expression, followed by the death of pyramidal cells after kainic acid application in rats [203]. PNN formation during schizophrenia is altered in that PNN densities, as revealed by immunohistochemical staining of human postmortem brains with WFA, are highly diminished in the prefrontal cortex of schizophrenic patients [204]. The importance of the existence of a healthy composition of PNNs is shown in another study analysing human postmortem brains of patients suffering from schizophrenia by immunohistochemistry. There, the authors found a higher number of glial cells positive for CSPGs in the amygdala and the entorhinal cortex, but also a decreased density of PNNs in parts of the amygdala and the entorhinal cortex [72]. A continuative study of this group could recently examine a reduction of glial cells positive for aggrecan in the schizophrenic amygdala [205].

These findings contrast the composition of PNNs in the brain of Alzheimer's disease patients. Recently, a study uncovered that PNNs in postmortem brains were unaffected, in particular with regard to the expression of brevican, aggrecan, Tnr, and Crtl1, whereas HA displayed an enhanced expression in amyloid beta plaque areas [206]. In light of these findings, the authors suggested that PNNs might exert a neuroprotective function for the neurons of Alzheimer's disease patients. In future, a substantial effort will be required to unravel the relations between altered PNN formation and neurological diseases in order to gain insight into useful therapeutic strategies.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Research Article

Distribution of N-Acetylgalactosamine-Positive Perineuronal Nets in the Macaque Brain: Anatomy and Implications

Adrienne L. Mueller,[1] Adam Davis,[1] Samantha Sovich,[1] Steven S. Carlson,[2] and Farrel R. Robinson[1]

[1]*Department of Biological Structure, University of Washington, Seattle, WA 98195, USA*
[2]*Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195, USA*

Correspondence should be addressed to Adrienne L. Mueller; alm04@uw.edu

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Perineuronal nets (PNNs) are extracellular molecules that form around neurons near the end of critical periods during development. They surround neuronal cell bodies and proximal dendrites. PNNs inhibit the formation of new connections and may concentrate around rapidly firing inhibitory interneurons. Previous work characterized the important role of perineuronal nets in plasticity in the visual system, amygdala, and spinal cord of rats. In this study, we use immunohistochemistry to survey the distribution of perineuronal nets in representative areas of the primate brain. We also document changes in PNN prevalence in these areas in animals of different ages. We found that PNNs are most prevalent in the cerebellar nuclei, surrounding >90% of the neurons there. They are much less prevalent in cerebral cortex, surrounding less than 10% of neurons in every area that we examined. The incidence of perineuronal nets around parvalbumin-positive neurons (putative fast-spiking interneurons) varies considerably between different areas in the brain. Our survey indicates that the presence of PNNs may not have a simple relationship with neural plasticity and may serve multiple functions in the central nervous system.

1. Introduction

Perineuronal nets (PNNs) are large accumulations of extracellular matrix molecules that form lattice-like structures around neuronal cell bodies and proximal dendrites. They consolidate around neurons near the end of developmental critical periods in V1 [1, 2] and amygdala [3]. They may restrict plasticity through a variety of mechanisms, including stabilizing synapses and inhibiting neuronal sprouting [4].

PNNs are composed of a combination of proteins and proteoglycans, which are secreted by both neurons and glia throughout early postnatal development [5, 6]. Different areas of the central nervous system have different complements of perineuronal net proteins [7]. All PNNs have four elements in common: hyaluronan, tenascin-R, link proteins, and chondroitin sulfate proteoglycans (CSPGs) [8–10]. There are four different CSPGs found in PNNs in the central nervous system: neurocan, versican, brevican, and, most frequently, aggrecan [9, 11]. Hyaluronan forms a molecular scaffold to which CSPGs adhere. These CSPG-hyaluronan connections are stabilized by link proteins. Tenascin-R then forms cross-links between these structures.

Several studies support the idea that PNNs are involved in ending critical periods of synaptic plasticity during development [2, 9, 12–14]. Critical periods in neuronal development are times during which experience can change synaptic connections. A critical period is therefore a time of activity-dependent synaptic plasticity. PNNs finish forming at approximately the same time that critical periods end and synaptic connections mature [1, 15]. PNNs grow in around neurons between postnatal days 7–14 in rat [6] and days 5–90 in rhesus macaques [16]. Artificially extending the critical period by preventing animals from acquiring experience results in a delay in perineuronal net formation [17, 18]. Dissolving PNNs in developed animals can result in at least a partial restoration of the synaptic plasticity evident during critical periods, suggesting that PNN formation is a cause, not just a correlate, of reduced plasticity [2, 3].

PNNs could inhibit synaptic plasticity either by acting as a structural barrier to formation of new processes or synapses or by inhibiting the formation of new synaptic contacts through signaling mechanisms that span the presynaptic or postsynaptic membranes. Several CSPG ligands could mediate inhibitory signals from PNNs, for example, contactin-1 [19], LAR (leukocyte common antigen-related receptor) [20], and PTPσ (protein tyrosine phosphatase σ) [21].

In addition to inhibiting plasticity, PNNs may perform other functions. They could be neuroprotective for highly active neurons that are susceptible to oxidative stress [22, 23]. For example, superoxide dismutase, an enzyme that is important for protecting against oxidative stress, binds to glycosaminoglycan side chains found abundantly on PNN CSPGs [24, 25]. PNNs may also act as cation sinks to balance the milieu around neurons with high fluctuations of ion exchanges [26]. These functions would not necessarily preclude PNN involvement in plasticity as well.

Several papers describe PNNs as primarily surrounding parvalbumin-positive inhibitory interneurons [2, 14, 27–38]. Some studies, however, also show that this correlation is inconsistent across different regions of the brain [30, 36] and even within an area [30, 39–41]. Also one study [42] showed that PNNs surround distinct subpopulations of cholinergic neurons in certain brainstem nuclei. Unfortunately, these studies compare only a limited number of areas and do not measure the proportions of neurons surrounded by PNNs in different areas.

In the cerebellum at least, PNN presence seems to be related to the amount of inhibitory input a cell receives [16]. This result complements recent data suggesting that PNNs, highly negatively charged molecules, ensure that the synapses they surround are inhibitory by influencing the relative internal and external Cl^- levels of the cell [43]. This proposition is very different from previous proposals which place PNNs around inhibitory interneurons and not at the targets of inhibitory interneuron input.

A reasonable start for understanding the functions of PNNs is to see where they are in the brain. To describe where PNNs occur, we surveyed PNN incidence across different regions of the primate CNS to determine where they occur and whether or not the oft-cited colocalization of PNNs and parvalbumin occurs in primates. We show which cell types are surrounded by PNNs and speculate on the possible implications of our results.

We also discuss the comparative distributions of PNNs in different CNS areas and find that PNNs are far more prevalent in the cerebellar nuclei than elsewhere in the brain. We assess the different possibilities for PNN function described above.

2. Materials and Methods

We collected samples of brain from nine adult (Table 1) and three developing rhesus macaques (*Macaca mulatta*), as well as one rat. All animals were sacrificed with barbiturate overdose and perfused through the left ventricle of the heart with 4% paraformaldehyde in 0.1 M phosphate buffer followed by 10%, 20%, and 30% sucrose solutions for cryoprotection.

After perfusion, the brain rested in a 30% phosphate-buffered sucrose solution for several days. We then took pieces from the 25 CNS locations indicated in Figure 1. From these pieces, we cut 25 μm floating sections using a cryostat. Sections were stored in phosphate-buffered saline (pH 7.4). To stain for PNNs, we used *Wisteria floribunda* agglutinin conjugated to fluorescein (WFA-Flscn, 1 : 500; Vector Labs FL-1351). WFA is a lectican that binds to the long sugar side-chain components of CSPGs [37]. Although at least one study suggested that WFA is not a universal marker of PNNs [44], it has recently been shown to be an excellent marker for aggrecan (a core component in the formation of PNNs [45]) and has been routinely used as a general marker for PNNs in the past [8, 34, 36, 46–49]. WFA costains with neurocan, phosphacan, brevican, and an antiserum to nonspecified CSPGs [50]. Also in our hands, WFA and aggrecan (Cat-301 antibody, 1 : 50; Millipore MAB5284) have an extremely high degree of overlap (Figure S1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/6021428). We therefore use WFA as our proxy for PNNs for the purpose of illustrating the broad distribution of PNNs in the macaque central nervous system. We used either NeuN (mouse monoclonal neuronal nuclei N, 1 : 500; Millipore Corp., MAB377) or avidin conjugated with Texas Red (Avidin-TxRd, 1 : 500; Invitrogen, A-820) as a neuronal stain. We used NeuN to label all brain areas except the cerebellar nuclei and avidin to label cerebellar nuclear neurons [51], which are not antigenic for the NeuN antibody [52]. We also stained for a subset of GABAergic inhibitory interneurons with an anti-parvalbumin antibody (mouse monoclonal, 1 : 500, Sigma, P3088). We exposed the sections to the primary antibodies on consecutive days to maximize signal. In the case of NeuN and parvalbumin, sections were additionally exposed to the secondary antibody Alexa Fluor 568 (1 : 1000; Invitrogen, A-21124). A limited number of sections were mounted directly on slides after cryostat sectioning, stored at −80°C, and later stained with the same protocol as the floating sections [53]. These sections were stained with primary antibodies to synaptic vesicle protein 2 (rabbit polyclonal SV2, 1 : 50, Courtesy of Carlson Lab), choline acetyltransferase (ChAT, rabbit polyclonal, 1 : 500; Millipore, AB143), and/or protein tyrosine phosphatase σ (PTPσ, mouse monoclonal, 1 : 100; Millipore, MABN605). In the case of triple-stained sections, we used an Alexa Fluor 647 secondary antibody (1 : 1000; Invitrogen, A-31573). Sections were mounted with Fluoromount media and imaged on a Zeiss Axioskop 2 confocal microscope using LSM 5 Pascal software. The same excitation and acquisition parameters were used for all sections from a single staining session. We occasionally manipulated the gain and offset values of the images following collection for better cell discrimination for counting; however, we did not postprocess any of the images in this paper.

We calculated the fraction of neurons surrounded by PNNs in each area by counting the number of cells with WFA staining (marking PNNs) and dividing it by the number of cells stained with NeuN or avidin (marking neurons). We identified neurons as having PNNs if the staining surrounding the cell was clearly distinguishable from the background, surrounded more than three quarters of the soma, and

Table 1: Percentages of WFA+ neurons by region and animal. The top rows detail each animal's sex and age. The subsequent rows show the percentage of neurons that were surrounded by WFA+ PNNs for each region in each animal that we collected data from.

	Animal								
	A	B	C	D	E	F	G	H	I
Sex	Male	Male	Male	Female	Male	Male	Male	Male	Male
Age	9	8	5	12	5	7	6	21	11
Region									
Primary auditory cortex (A1)	3.91		3.62		3.54		4.56		
Primary motor cortex (M1)			13.12	8.20	8.70	9.14			
Primary somatosensory cortex (S1)	7.16	6.14	6.21	6.66	5.93	11.72			
Primary visual cortex (V1)	4.66	4.81	6.19	6.14	5.36	5.53			
Frontal eye field (FEF)	8.03	2.96	8.88	5.52	6.44	3.49			
Cingulate cortex	2.24		5.18	0.80		5.72			
Mediotemporal cortex (MT)		4.50	5.65	3.26	7.65	4.43			
Orbital gyrus	5.02	2.75	7.72	5.71	5.94	5.73			
Amygdala	5.26	2.88	0.25	0.81	3.86				
External globus pallidus	8.20		18.76		12.84		62.22	12.86	
Internal globus pallidus			14.95		86.49		100.00	67.20	
Hippocampus	1.06		2.92		2.82	0.88			
Thalamus	1.66	0.73	0.00	4.05	0.00	0.00			
Putamen	2.57		0.00		1.22		1.13	0.00	
Inf. colliculus (deep)		6.63	7.41					8.40	10.30
Inf. colliculus (sup.)		5.84	0.67					3.81	5.89
Sup. colliculus (deep)		6.91	2.52					19.91	15.63
Sup. colliculus (sup.)		0.74	6.38					2.60	3.41
Inferior olive (IO)	0.00	0.00	0.00						0.00
Cerebellar nucleus	95.60	91.67	98.08	88.03					
Vestibular nucleus	61.73	62.58	40.00					66.67	40.23
Pontine nuclei	5.47		28.37		43.58			42.25	25.59
Ventral spinal cord	36.60	42.39	43.71	44.62	41.78	55.63			
Dorsal spinal cord	0.19	1.46	1.57	0.51	3.42	1.74			

colocalized with neuronal staining (Figure 2). Four individuals separately counted neurons and PNNs on each image. We averaged these counts and then used these averages to calculate an average percentage of neurons surrounded by PNNs across monkeys. We used a similar procedure to count parvalbumin-positive and WFA-positive neurons but did not calculate percentages from the counts. For this study, we do not use a full stereological approach but instead describe our results in terms of proportions of neurons surrounded by nets because it facilitates comparisons between areas.

We collected tissue from eight adult male rhesus macaques (ages 5, 5, 6, 7, 8, 9, 11, and 21 years) and one adult female (age 12 years), as well as one adult rat (for comparison). The tissue from these animals was primarily assigned to other projects; therefore, we were not able to collect samples from every area in every animal. In the monkeys, we acquired samples from a minimum of four different animals for each area for the PNN/neuron comparisons. Also, in order to examine the expression of PNNs around cerebellar nuclear neurons during development, we examined tissue from one animal each at fetal day 145, postnatal day 5, and postnatal day 90. The value for each area for each animal was calculated based on an average of between 1 and 3 image frames per section and an average of 1–3 sections per area.

3. Results

3.1. Proportions of Neurons Surrounded by PNNs in Different Areas of the Brain. Figure 1 shows the different areas that we examined. Figure 3 shows the percentage of neurons surrounded by PNNs in each of these areas.

3.1.1. Cortex. Figure 4 shows examples of labeling of PNNs and neurons in primary sensory cortices (A1, S1, and V1) and primary motor cortex (M1). In these and all other areas of cerebral cortex, PNNs surround less than 10% of neurons. In all areas of the cortex that we examined, the nets surrounded neurons more frequently in layers three and four than in other layers. Figure 5 demonstrates this pattern in V1. In addition, in M1, the large projection neurons (Betz Cells) in layer five were often surrounded by PNNs.

We also examined cingulate cortex, cortex of the orbital gyrus, area MT, and the frontal eye field (Figure S2). In these areas, approximately 5% of neurons costained with the

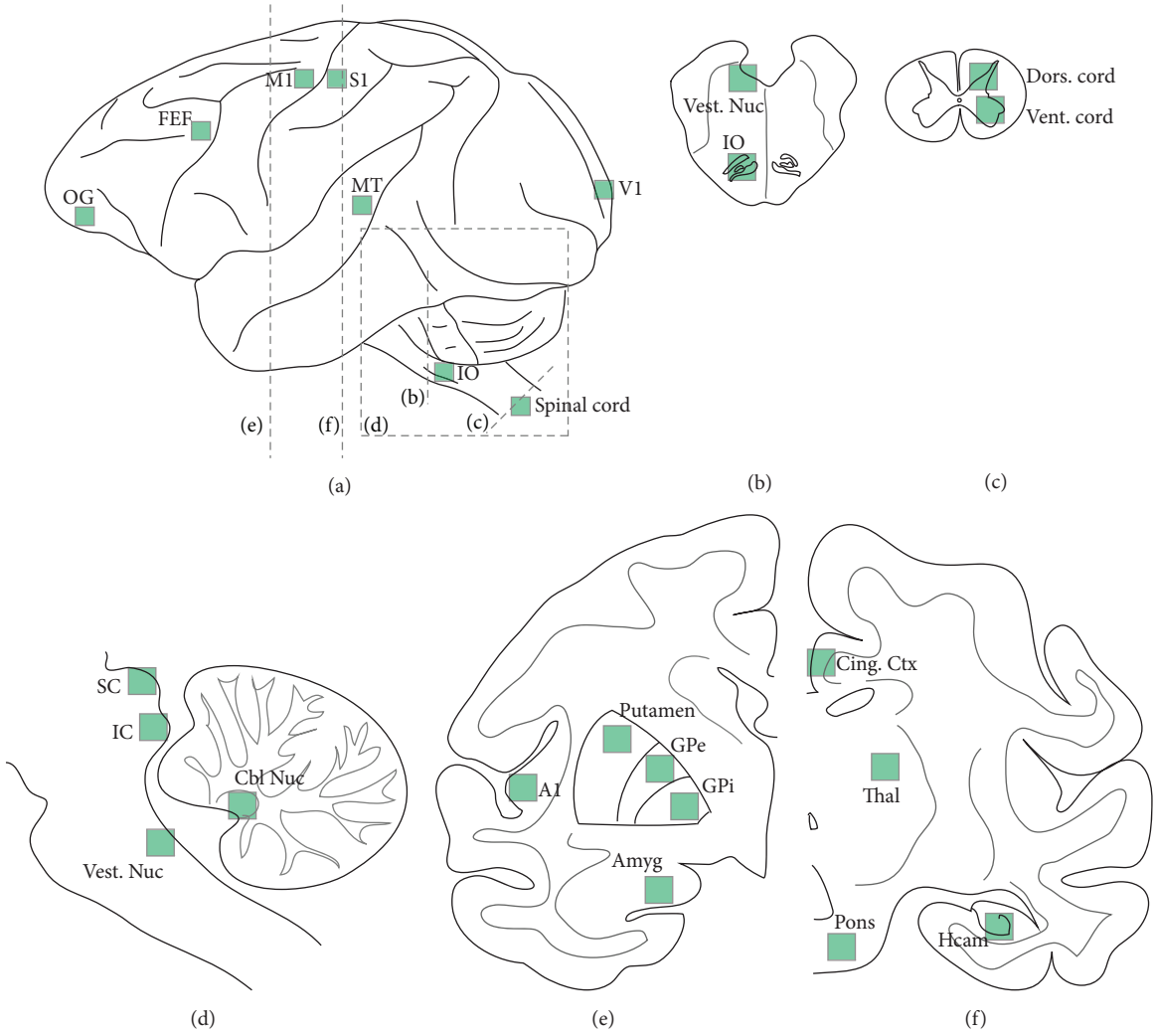

Figure 1: CNS of areas we examined for PNNs. (a) shows the left side of a monkey's whole brain. The green squares show the approximate size and position of our tissue samples. The dashed lines represent planes for the other subfigures. We took samples from the orbital gyrus (OG), the frontal eye field (FEF), primary motor cortex (M1), primary somatosensory cortex (S1), primary visual cortex (V1), and mediotemporal cortex (MT), the inferior olive (IO), and the cervical spinal cord. (b) shows the brainstem locations of our samples from the inferior olive (IO) and the vestibular nuclei. (c) shows the spinal cord where we sampled both the dorsal and ventral horns. (d) shows a sagittal representation of the brainstem and cerebellum where we took samples from the superior colliculus (SC), the inferior colliculus (IC), the cerebellar nuclei (Cbl Nuc), and the vestibular nuclei. (e) shows the left half of the brain in a frontal plane where we took samples from primary auditory cortex (A1), the putamen, the external globus pallidus (GPe), the internal globus pallidus (GPi), and the amygdala (Amyg). (f) shows the right half of the brain in a frontal plane where we took samples from the pontine nuclei, the hippocampus (Hcam), the thalamus (Thal), and cingulate cortex (Cing. Ctx).

perineuronal net marker. Of all the areas of cerebral cortex we examined, PNNs were the most abundant in primary motor cortex.

3.1.2. Subcortical Areas. PNNs surrounded very few cells in the amygdala, hippocampus, and thalamus (Figure S3). The distribution of PNNs in the basal ganglia varied depending on the subregion. There were PNNs around very few neurons in the putamen (1%), more neurons in the external globus pallidus (13%), and about half of the neurons in the internal globus pallidus (51%) (Figure S4). The quality of the labeling differed between external and internal globus pallidus. WFA labeling was strong and sharply defined cells in the internal globus pallidus, but, in the external globus pallidus, the stain was weaker and more diffuse.

3.1.3. Brainstem and Cerebellum. PNNs surround an average of 93% of cells in the cerebellar nuclei, the highest percentage of any area we examined. Figure 6 shows an example of labeling here. The area with the second densest population of PNN-labeled neurons was the vestibular nuclei (55%). The vestibular nuclei are similar to the cerebellar nuclei in that they receive direct input from inhibitory Purkinje cells in the cerebellar cortex. Our estimate of perineuronal

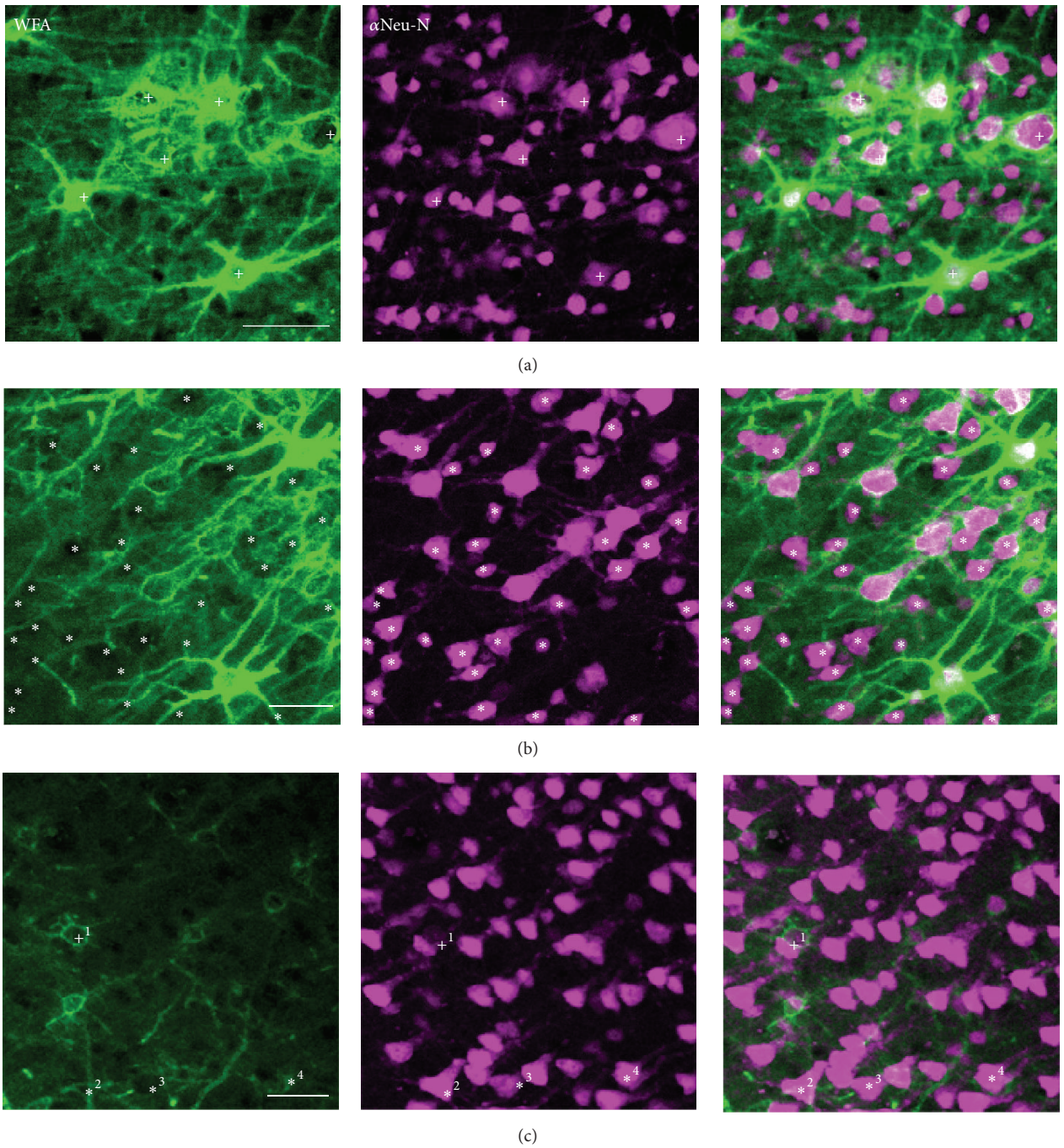

Figure 2: Perineuronal net identification. WFA, NeuN, and combined labeling in primary motor cortex. (a) shows labeling that we identified as PNN-positive, marked with +. (b) shows labeling that we identified as PNN-negative. Cells without PNNs are indicated with ∗. (c) shows some examples of how we classified more ambiguous labeling. 1: a cell with strong WFA and weak NeuN staining, which we classified as WFA+ (+ symbol). 2: labeling which looks like it might surround a cell but does not clearly surround NeuN labeling (∗ symbol). 3: cells that produce absence of background labeling in the WFA channel but are not associated with clear WFA labeling. Even though the background is brighter than the "black" region, we do not identify these cells as WFA+, because they do not have a clear ring-like net (∗ symbol). 4: cell with partial somatic WFA staining, but because the ring around the cell is incomplete, we cannot unambiguously classify the cell as PNN-positive (∗ symbol). Scale bars are 50 μm.

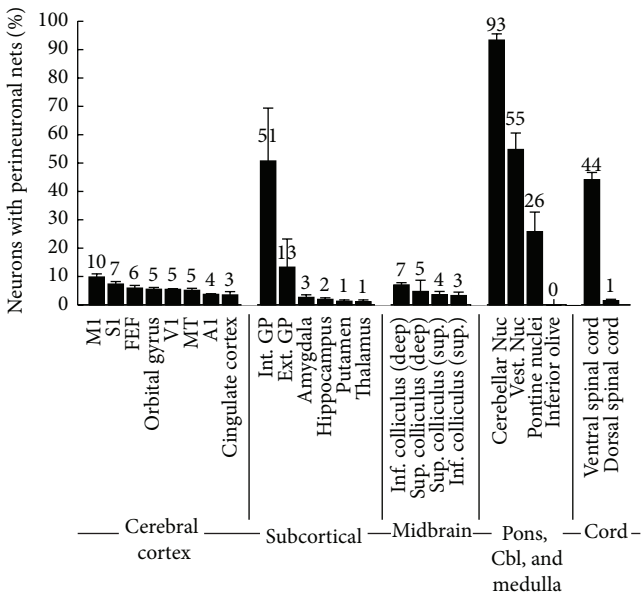

Figure 3: Percentage of neurons surrounded by perineuronal nets in different parts of the brain. Each bar represents the mean percentage of neurons that were surrounded by perineuronal nets in one part of the brain. The values, rounded to the nearest percentage, are shown above each bar. Averages and standard error are calculated from at least 4 monkeys for each area.

net density combines both the medial and lateral vestibular nuclei. Qualitatively, we observed more neurons with PNNs in the lateral vestibular nucleus.

PNNs surround 26% of neurons in the pontine nuclei. In addition to well-defined labeling of a subset of neurons, WFA also diffusely stained the area between cells. Because this diffuse staining made it harder to identify PNN ensheathment, it is possible that our calculation of the proportion of cells surrounded by PNNs is an underestimate. This diffuse labeling was also noticeable in other areas of the brain as well, that is, in the ventral horn of the spinal cord and the internal globus pallidus. Neurons in the inferior olive were completely free from perineuronal net labeling (Figure S5). In both the superior and inferior colliculi, PNNs surrounded a higher fraction of deeper neurons than of superficial neurons (Figure S6).

3.1.4. Spinal Cord. PNNs surround almost 50% of neurons in the ventral horn of the cervical spinal cord but almost none of the neurons in the dorsal horn. Figure 7(a) shows a composite picture of labeling in the dorsal and ventral horns of the spinal cord that illustrates the large difference in the frequency of WFA+ and WFA− cells. Of the WFA+ neurons in the ventral horn, 90% (±3.2%) costained with a marker for primary motoneurons, an anti-choline acetyltransferase (ChAT) antibody. 75.6% (±1.8%) of ChAT-positive cells were surrounded by WFA+ PNNs (Figure 7(b)).

3.2. PNN Presence around Parvalbumin-Positive Inhibitory Interneurons Varies between Areas. We counted the number of parvalbumin-positive cells in a given (0.94 mm^2) area for each of the regions shown in Figure 1 that either did or did not costain for PNNs. As Figure 8 summarizes, we found that (1) not all parvalbumin-positive neurons are surrounded by PNNs; (2) not all neurons with PNNs are parvalbumin-positive; (3) the relative frequencies of these two types of cells (WFA+ and parvalbumin+) differed widely between areas. For example, in primary visual cortex, the populations of PNN-positive and parvalbumin-positive cells were mostly overlapping. However, in the frontal eye field, the two populations were almost completely distinct (Figure S7).

In noncortical parts of the brain, regions in which PNNs are scarce do not exhibit corresponding lack of parvalbumin-positive cells. For example, Purkinje cells in the cerebellar cortex stain strongly for parvalbumin, but only weakly for PNNs (Figure S8). The same is true for neurons in the dorsal horn of the spinal cord (Figure S9). Also, excitatory projection neurons in the cerebellar nucleus, which are densely surrounded by PNNs, are not parvalbumin-positive.

These results show that although in some areas of the brain PNNs surround many parvalbumin-positive inhibitory neurons, this is not a general phenomenon throughout the brain.

3.3. WFA+ Neurons in the Cerebellar Nuclei Do Not Express PTPσ at the Cell Surface. We examined whether PNNs interfaced with PTPσ. Such an interface might allow PNNs to inhibit synaptic connections through intracellular signaling events, a function in which PTPσ is implicated [54, 55]. If this were the case, we would expect to see PTPσ colocalizing with WFA, possibly at positions proximal to synapses, so as to inhibit expansion of synaptic sites. Further, we would expect to find PTPσ expressed on cells presynaptic to the neurons heavily surrounded by PNNs. Figure 9 shows that PNNs in the cerebellar nuclei surround the cell bodies and dendrites of large neurons, leaving gaps for synaptic contacts. We examined these neurons and found that 75.5% of cerebellar nuclear neurons exhibit cytosolic PTPσ. Based on their punctate expression pattern, it is possible that PTPσ is localized to endosomes. 90% of all PTPσ+ neurons were also WFA+. Nonetheless, the expression of PTPσ was primarily cytosolic and did not appear to be linked to the location of synapses (Figure 10).

3.4. PNN Prevalence in Different Age Animals. PNNs appear during early postnatal development but we know little about how the prevalence of neurons surrounded by PNNs changes after that. Here, we examined the prevalence of PNN-surrounded neurons in particular areas of animals of different ages. Our sample size is limited so we cannot perform a statistical analysis but, as Figure 11 shows, it is apparent that for nearly all areas there is no large change in PNN prevalence with age. Though the differences that we saw with age might reflect only between-animal variability, large monotonic changes seem likely to show a real change. One area in which we see such differences is in the deep layers of the superior colliculus. The percentage of neurons in the superior colliculus increases monotonically from age 5 to 20 and is over four times greater at age 20 than at age 5. Also,

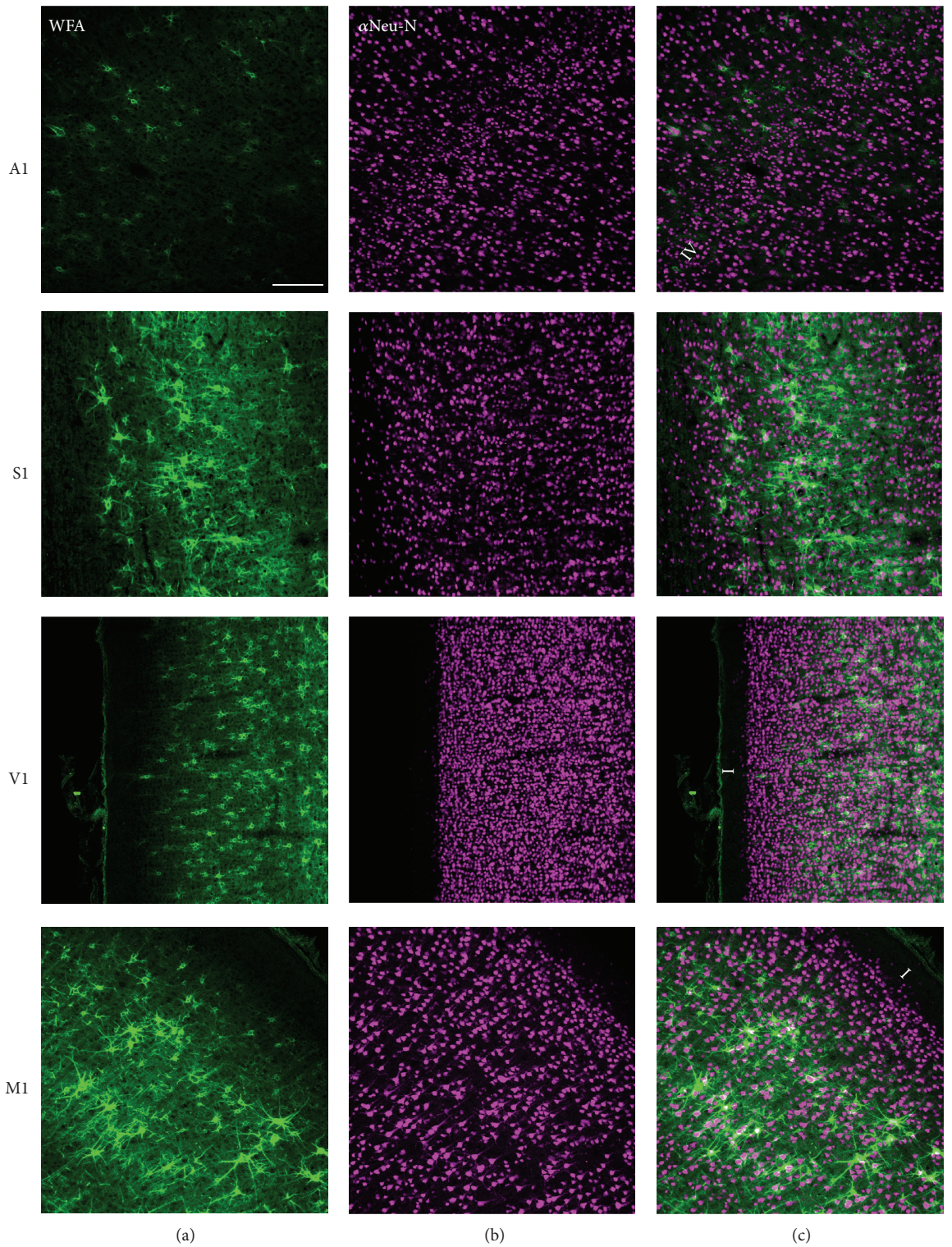

Figure 4: Perineuronal nets in primary sensory and motor cortex. (a) shows perineuronal nets stained with WFA. (b) shows NeuN-labeled neurons. (c) shows merged images of the two. Neurons are predominantly absent from layer I, which is primarily composed of fibers running parallel to the cortical surface. We identify layer I or layer IV, where appropriate, in the rightmost panels. A1: primary auditory cortex, S1: primary somatosensory cortex, V1: primary visual cortex, and M1: primary motor cortex. Scale bar = 200 μm.

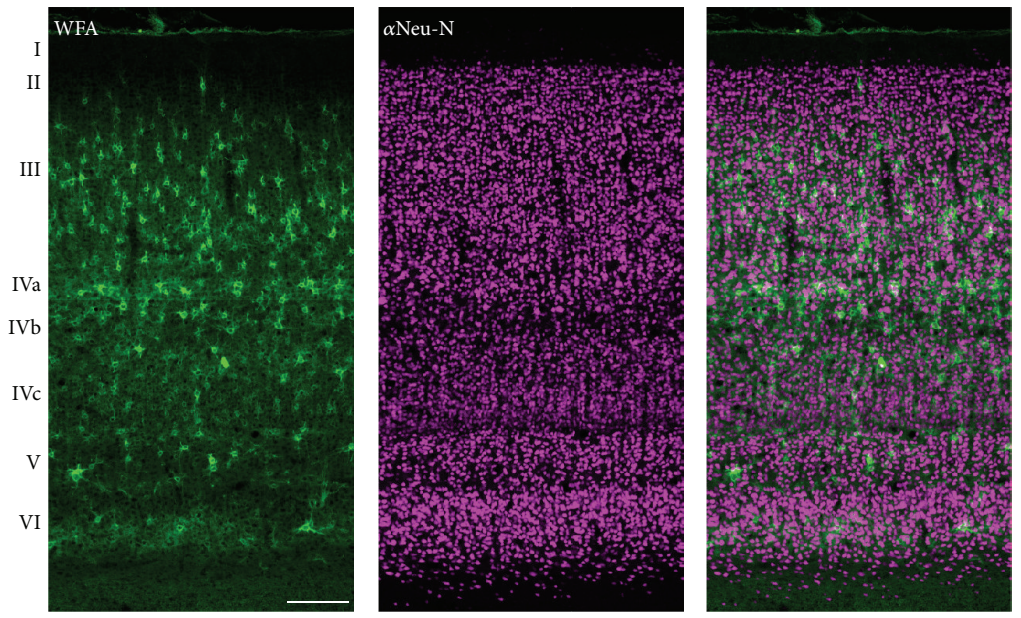

Figure 5: Perineuronal nets in primary visual cortex layers. (a) shows perineuronal nets stained with WFA. (b) shows NeuN-labeled neurons. (c) shows merged images of the two. Scale bar = 200 μm.

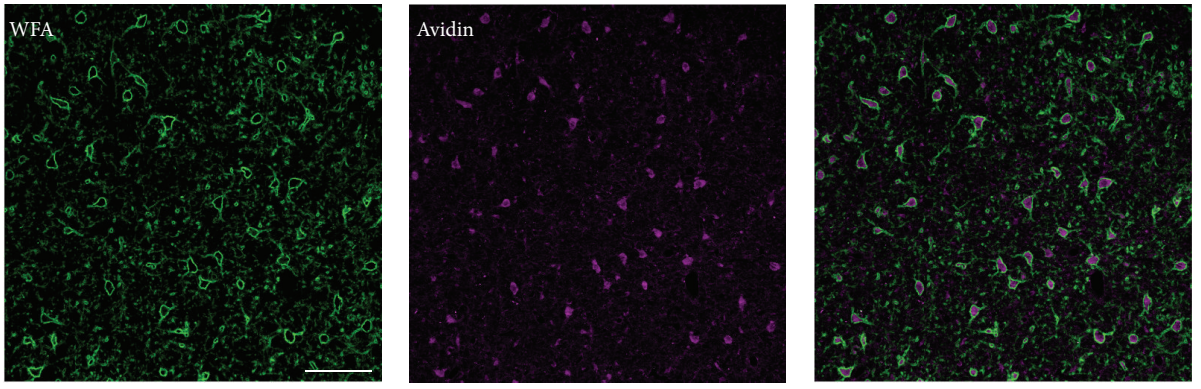

Figure 6: Perineuronal nets in the cerebellar nuclei. (a) shows perineuronal nets stained with WFA. (b) shows avidin-labeled neurons. (c) shows merged images of the two. Scale bar = 200 μm. Note the high proportion of avidin-labeled neurons (pink) surrounded by PNNs (green).

both the cingulate cortex and area MT appear to lose PNNs with age.

We examined the formation of PNNs around neurons in the cerebellar nuclei during development. Figures 12(a)–12(d) show superimposed WFA (PNN) and avidin (neuron) labeling in the cerebellar nuclei at four ages between fetal day 145 and adult. Birth is at about day 164. Figure 12(e) shows that the percentage of neurons surrounded by nets increases rapidly in the 24 days between FD145 and P5 and changes little after that. Our WFA staining shows earlier formation of PNNs than does staining with CAT-301 which found that CAT-301+ nets do not form in monkey cerebellar nuclei until after P5 [16]. We believe that this difference occurs because WFA stains components of PNNs that develop earlier than those stained by CAT-301. When we costained tissue from monkeys at different ages with both CAT-301 and WFA, we found that WFA marked PNNs earlier than did CAT-301.

In contrast, as Figure 12(f) shows, the width of PNNs increases between postnatal day 5 and the adult. Nets are significantly wider in age P90 tissue than P5 ($p < 0.01$), but not significantly different between FD145 and P5 sections ($p = 0.46$). There is also a significant difference ($p = 0.02$) between P90 and adult PNN widths, with the adult being larger. We collected these data from only one animal each. We therefore cannot provide any information about the variability in the developmental timeline between animals.

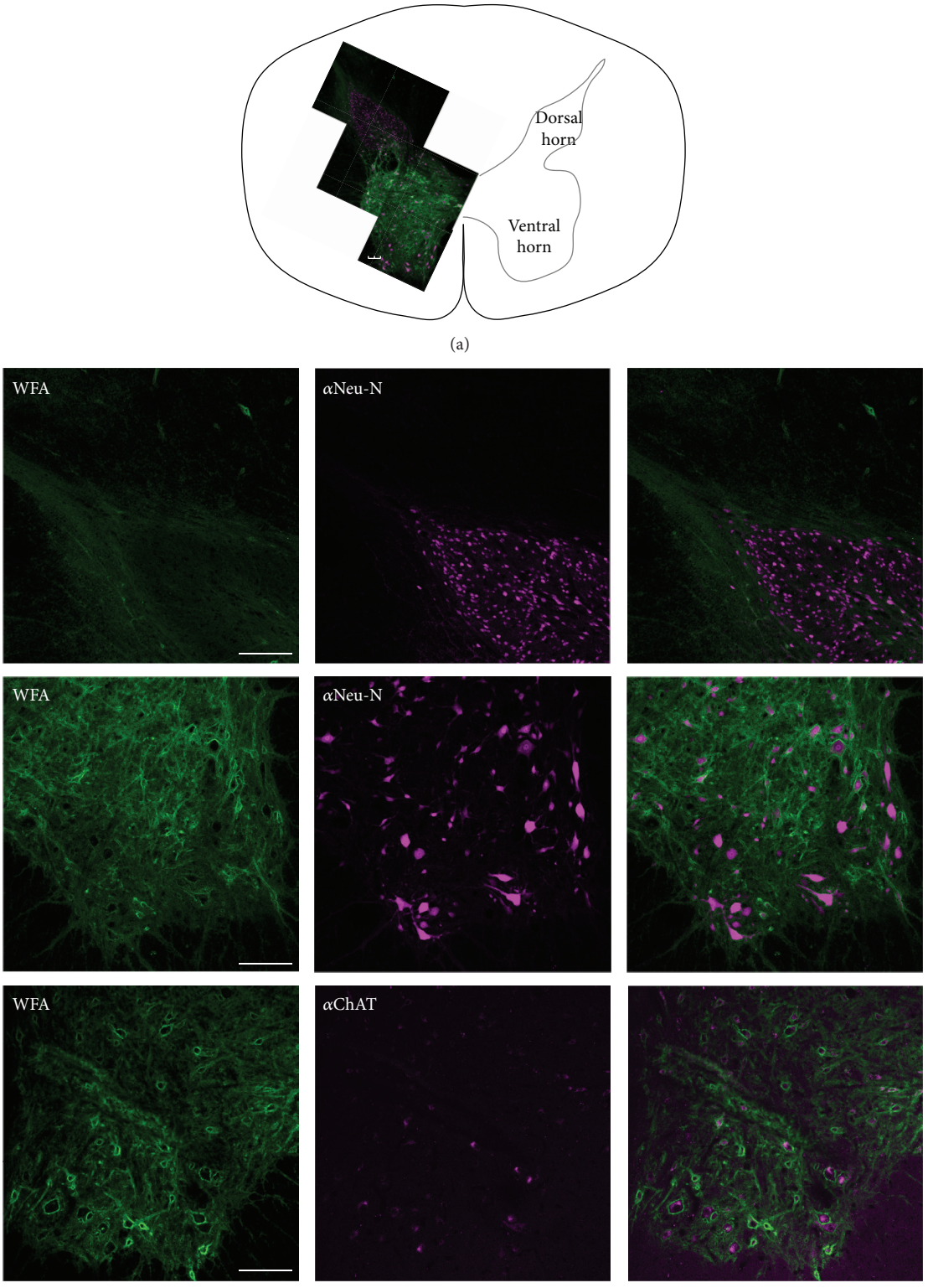

Figure 7: (a) Perineuronal nets in ventral and dorsal horns of the spinal cord. A composite image of perineuronal nets (stained with WFA) and neurons (stained with α-NeuN) is superimposed on the left of a schematic of the cervical spinal cord. (b) Perineuronal nets (WFA+, green) are more prevalent around neurons (NeuN, pink) in the ventral spinal cord (middle panels) than in the dorsal spinal cord (top panels). Cells surrounded by PNNs are often ChAT+, putative primary motoneurons (bottom panels). Scale bars = 200 μm.

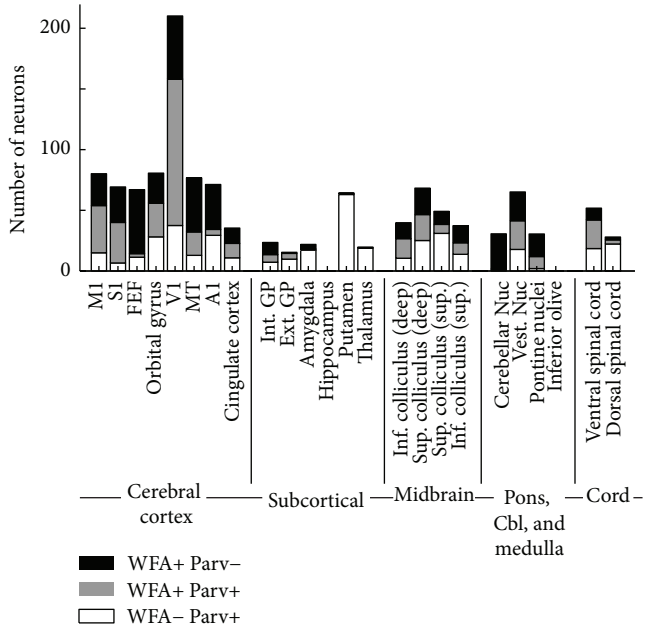

Figure 8: Relative frequencies of perineuronal net- and parvalbumin-positive cells in different areas of the brain of one male animal, age 5. Cells were counted in 0.92 mm^2 areas for each region listed. We tallied cells that stained positive for either PNNs (WFA+ Parv−, black) or parvalbumin alone (WFA− Parv+, white) as well as cells that stained for both markers (WFA+ Parv+, gray) separately. The regions examined were, from left to right, primary motor cortex (M1), primary somatosensory cortex (S1), frontal eye field (FEF), orbital gyrus, primary visual cortex (V1), mediotemporal cortex (MT), primary auditory cortex (A1), cingulate cortex, internal globus pallidus (int. GP), external globus pallidus (ext. GP), amygdala, hippocampus, putamen, thalamus, deep inferior colliculus, deep superior colliculus, superficial superior colliculus, superficial inferior colliculus, cerebellar nuclei (Nuc), vestibular nuclei (Vest. Nuc), pontine nuclei, inferior olive (IO), ventral (V) spinal cord, and dorsal (D) spinal cord.

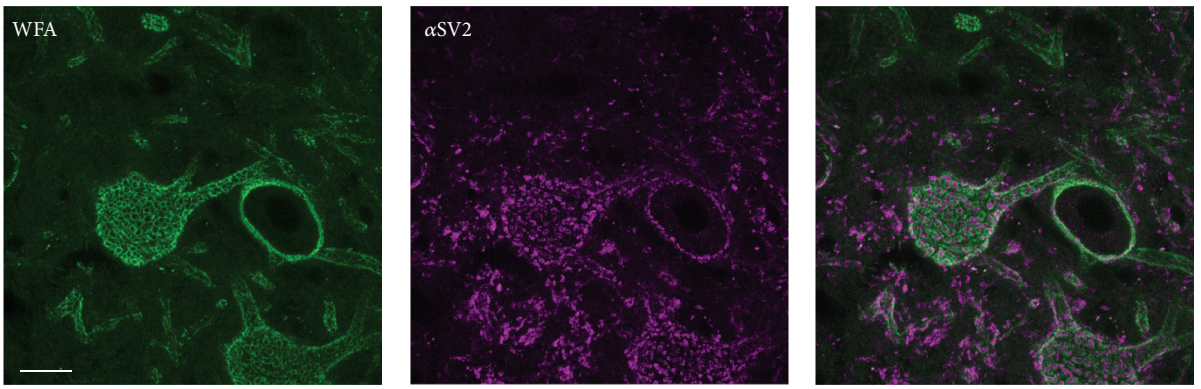

Figure 9: Perineuronal nets surround synapses in cerebellar nuclear neurons. WFA-positive PNNs (green) ensheath the soma and proximal dendrites of neurons in the cerebellar nucleus (pink). They form openings around synapses, labeled with an antibody to synaptic marker SV2 (pink). Z-projection of 10 um confocal stack. Scale bar = 20 μm.

4. Discussion

4.1. Technical Considerations. Although we used an atypical neuronal marker to identify neurons in the cerebellar nuclei (based on McKay et al. [51]), we used this marker exclusively for quantification in this region, an area in which endogenous biotin expression is exceptionally high. Although avidin, which recognizes biotin, is not a standard neuronal stain, costaining with NeuN in regions outside the cerebellum indicates that avidin labels the same number of neurons, or slightly more so than does NeuN. Thus, if anything, our use of this stain may generate a slightly higher estimate of the number of neurons present than does NeuN.

In extremely rare instances, we appeared to find WFA staining that did not encapsulate a neuron. There are several possible explanations. First, a difference in the ideal focal plane during image capture would result in good signal of the extracellular PNNs, but potentially a very weak signal of the spatially displaced neuronal nuclei marker. Second, it is possible for PNNs ensheathing processes to form a circle that

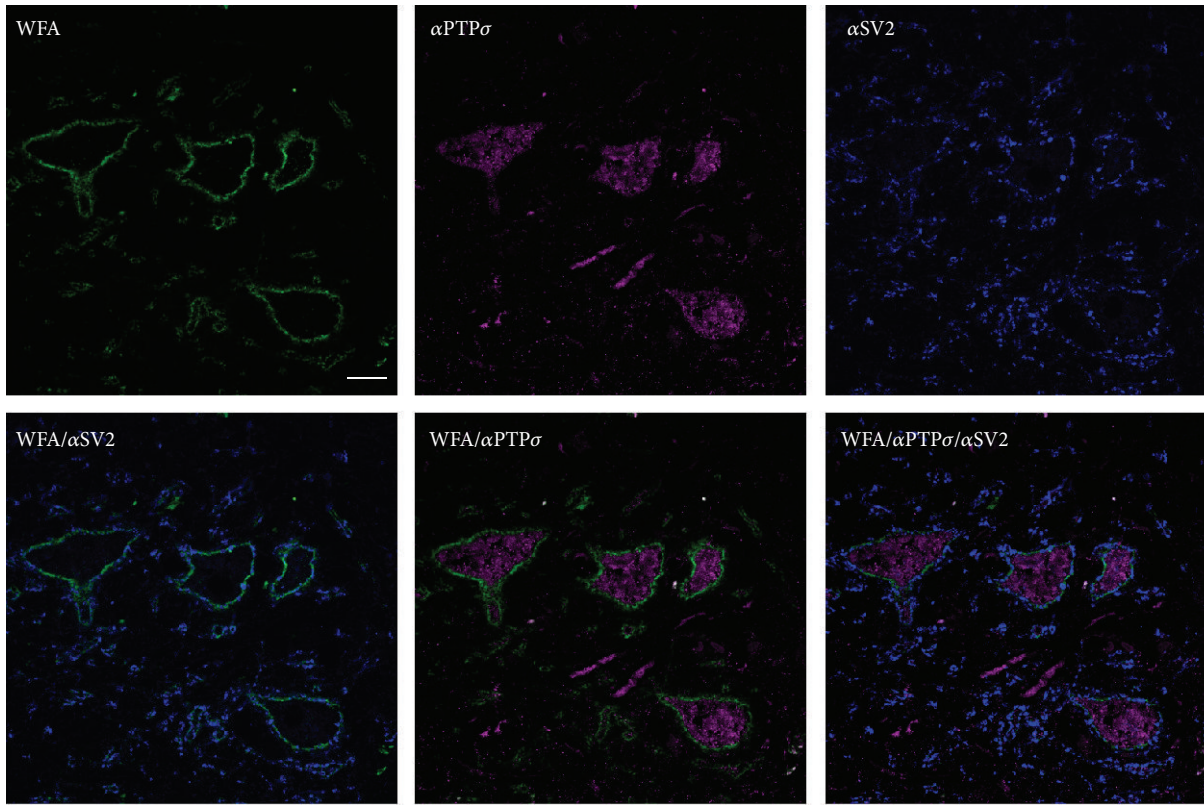

Figure 10: PTPσ does not colocalize with PNNs or synapses. Expression of PNNs (stained with WFA, green), PTPσ (pink), and synapses (stained with anti-SV2 antibody, blue) do not overlap. Scale bar = 40 μm.

looks like a cell but would not express a neuronal marker. Third, although it has never before been established, it is possible that PNNs also surround cells that do not express typical neuronal markers. Regardless of which of these cases is correct, for this study, we counted neither the (absent) cell nor the PNN it putatively ensheathed.

4.2. Perineuronal Nets Are More Prevalent in Motor Structures. Among the areas that we examined, PNNs generally surrounded a larger proportion of neurons in motor areas than in sensory areas. M1 neurons have more nets than S1, A1, or V1. This was especially noticeable in the spinal cord, where we saw a greater-than-40-fold difference in the proportion of neurons surrounded by PNNs across a distance of less than a millimeter. Although our results appear to differ from those of Alpár et al. [56], who found a higher density of PNNs in rat primary visual and somatosensory cortex than elsewhere in the brain, our different methodologies explain this. Alpár et al. calculated density by counting cells with nets per 1 mm^2, whereas we counted the fraction of neurons with nets in a given area. Since there are far more neurons per mm^2 in primary visual cortex than in other areas of the brain [57], this could easily explain our apparently different estimates.

In addition to more PNNs in motor than sensory structures, we found that the highest proportion of neurons surrounded by PNNs was in the cerebellar nuclei, a motor structure. If we assume that the function of PNNs in these structures is to inhibit plasticity, then it is possible that sensory input is more changeable than motor output and that it is beneficial to have fewer nets and more plasticity present in sensory areas. Those areas might require more flexibility in synaptic connectivity than motor areas.

4.3. Implications of Differential Perineuronal Net Expression for Plasticity. PNNs are thought to play a role in restricting plasticity [58–61]. They prevent new nerve fibers and cones from connecting with the postsynaptic cell [62, 63] (reviewed by Rhodes and Fawcett [64]). Further, removal of PNNs allows lateral diffusion of AMPA receptors [65] and allows sprouting in the spinal cord [66, 67]. They mature around synapses during critical periods of development [6, 14]. Supporting this view is the observation that dissolving PNNs with the enzyme ChABC (chondroitinase ABC) causes sprouting and allows restructuring of connections that is similar to what occurs during critical periods in development [2, 3, 7, 68].

One likely candidate for a receptor through which PNNs could mediate inhibition of synaptic plasticity is PTPσ. Shen et al. [21] showed that the transmembrane protein tyrosine phosphatase PTPσ binds CSPGs. Further, cultured neurons without the PTPσ gene exhibited reduced inhibition (quantified by neurite outgrowth) by PNNs. Disruption of the PTPσ gene after spinal cord injury enhanced the ability of axons to penetrate CSPG-rich regions. Also, Liu et al. [34] found that visual deprivation during development resulted in a delay in

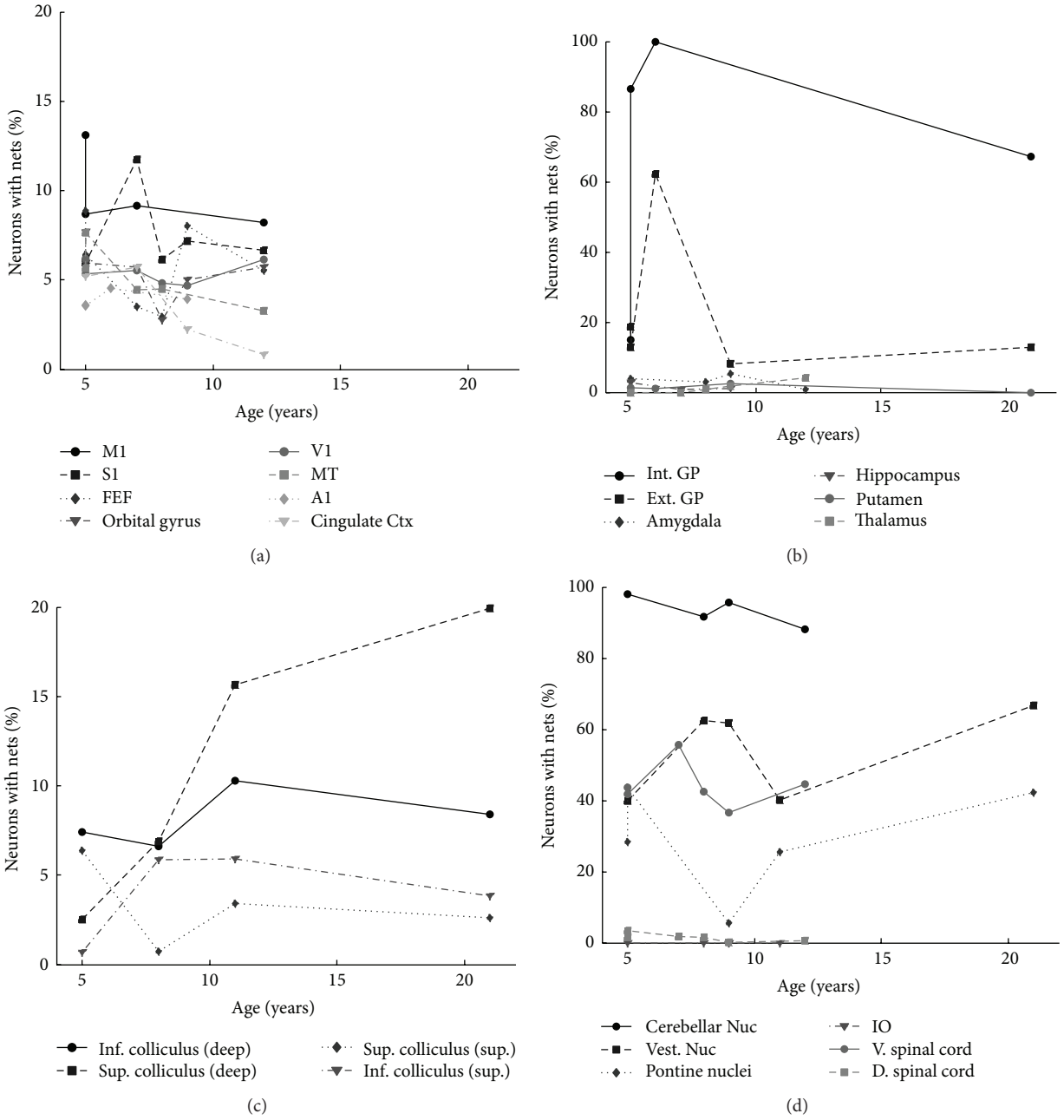

Figure 11: Percentage of neurons with PNNs across animals of different ages. Percentage of neurons surrounded by PNNs in (a) the cerebral cortex, (b) subcortical structures, (c) the colliculi, and (d) the cerebellar nuclei, brainstem, and spinal cord. Each symbol represents a different area in the subplot (see legends).

the end of the visual critical period and PNN maturation, which coincided with stalling of PTPσ expression at critical period levels.

We consider the cerebellum a motor structure because it strongly influences movement via projections to premotor networks in the brainstem and to the origins of the four major descending motor tracts, the corticospinal (via relay in the ventral lateral nucleus of the thalamus), rubrospinal, vestibulospinal, and reticulospinal tracts. The cerebellum is also strongly implicated in motor adaptation. For example, during adaptation of saccade size, neurons in the saccade-related part of the cerebellar nuclei change their output in a way that would cause the observed change in saccade size [69]. This altered output almost certainly affects saccade size and is not just correlated with the change. Inhibitory burst neurons relay these changes to the motoneurons for the lateral rectus muscle in the abducens nucleus [70]. Blocking saccade-related cerebellar output blocks saccade adaptation [71]. Thus, the cerebellar nuclei represent a paradox. They are the structures with the highest percentage of neurons

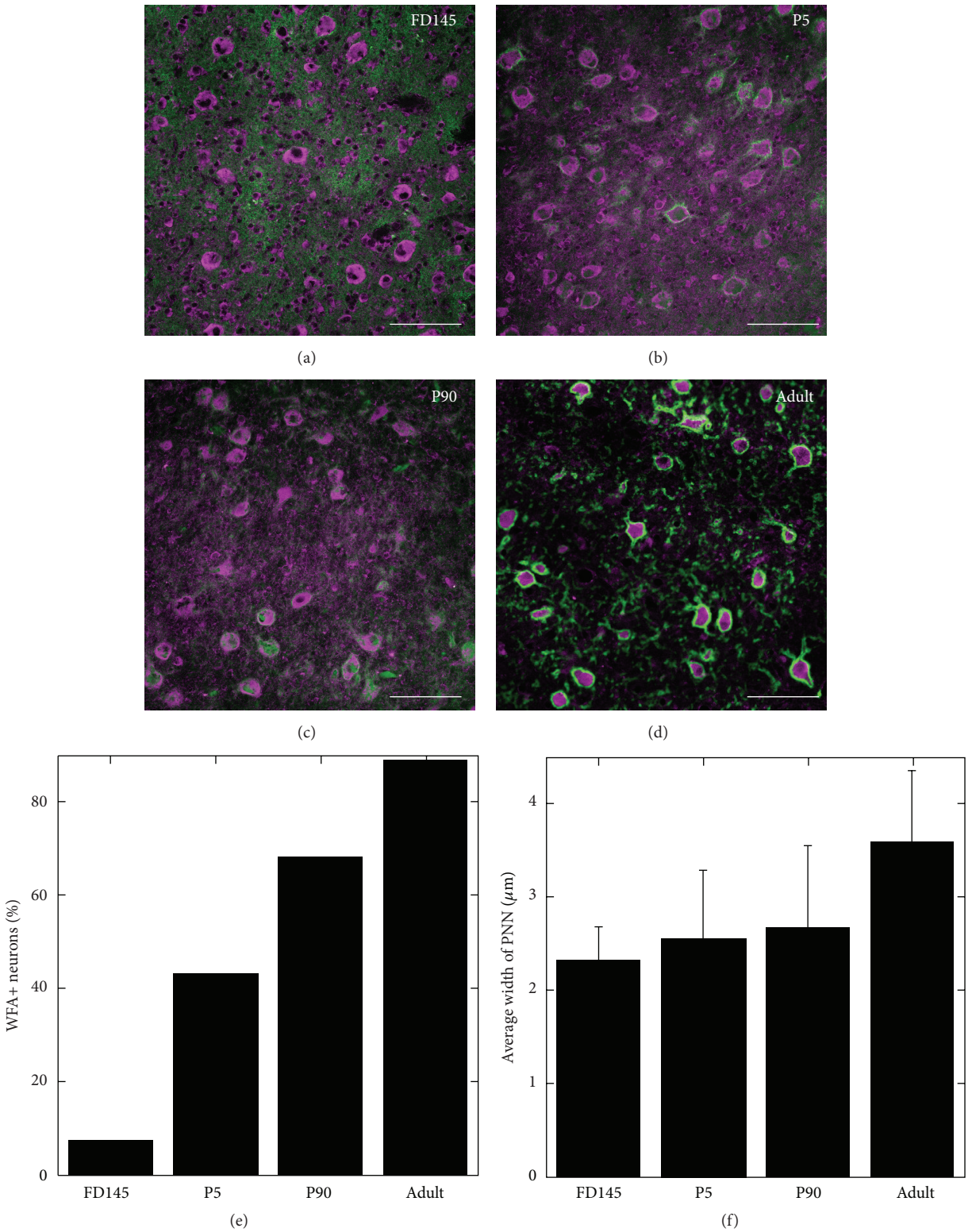

Figure 12: Perineuronal nets in the developing cerebellar nucleus. Perineuronal nets (stained with WFA, green) accrue around neurons in the cerebellar nuclei (stained with avidin, pink) between (a) fetal day 145, (b) postnatal day 5, (c) postnatal day 90, and (d) the adult animal. (e) shows the number of neurons surrounded by nets in (a)–(d). (f) shows the width of PNNs around cells in (a)–(d). Scale bars in (a)–(d) = 100 μm.

surrounded by PNNs but may also participate in motor adaptation that requires plasticity.

Two pieces of evidence suggest that PNNs in the cerebellar nuclei serve a purpose other than inhibiting synaptic plasticity. (1) We recently showed that dissolving PNNs in the cerebellar nuclei had no impact on the strength or persistence of changes in saccade size elicited by long term saccade adaptation [72]. (2) As we show in Figure 10, one of the ligands most likely to mediate inhibition of synaptic plasticity, PTPσ, is not expressed in the cerebellar nuclei in a manner consistent with this function. Therefore, at least in the cerebellar nuclei, PNNs seem to serve a function other than inhibition of synaptic plasticity.

4.4. Implications of Differential Perineuronal Net Expression for Neuroprotection. PNNs may also serve a neuroprotective role. Cabungcal et al. [22] used mice carrying genetic imbalance to demonstrate that PNNs around parvalbumin+ interneurons play a critical role in protecting these neurons from oxidative stress. PNNs limit the effect of genetically impaired antioxidant systems and/or excessive reactive oxygen species in the cell's environment. Parv+ cells without nets are more susceptible to oxidative stress.

Some evidence showed that components of PNNs can interact with iron, which is involved in the generation of reactive hydrogen radicals [23, 73, 74]. Also, PNN CSPGs act as a ligand for extracellular superoxide dismutase (EC-SOD) [24, 25]. EC-SOD catalyzes the dismutation of superoxide radicals which would otherwise damage proteins in the extracellular matrix and plasma membrane.

Another possibility is that PNNs act as a cation sink, surrounding neurons that either sustain or achieve very high firing rates, such as parvalbumin+ interneurons. They could therefore act as a buffering system for the rapid cation exchanges that occur in the extracellular space local to highly active neurons [26, 35]. Consistent with this idea are the many studies that show that PNNs surround fast-spiking parvalbumin-positive interneurons [13–15, 22, 32, 33]. Although our data is consistent with these results for some areas, we also show that for many areas the presence of parvalbumin in a cell does not correlate with PNN presence. Also, we did find that neurons in the inferior olive, small neurons that fire at very low rates (approximately 1-2 Hz), do not have PNNs, while neurons in the cerebellar nuclei, large neurons that fire tonically at rates of 60–100 Hz, did. Purkinje cells in the cerebellar cortex, which have similarly high tonic firing rates, are not surrounded by PNNs at all. This demonstrates there is no simple relationship between the metabolic properties of a cell and its likelihood of being surrounded by PNNs.

4.5. Implications of Differential Perineuronal Net Expression for Ion Homeostasis. Recent data point to a third possible PNN function. The impermeant anions of the cytoplasm and the strongly anionic extracellular PNNs contribute to setting the Cl^- reversal potential in neurons [43]. They therefore also determine the polarity of response to GABAergic input to the cell, which shifts during development. Glykys et al. [43] found a negative correlation between the "intensity" of PNN presence and the internal Cl^- concentration, an expected result from PNNs setting the local extracellular Cl^- concentration. They also found that digesting PNNs increased the internal Cl^- concentration more than threefold. The reduction in the internal Cl^- concentration during development parallels the increase in neuronal cytoplasmic anions during development [75] and experience [76] and increases in PNNs [65]. Further, there is the correlation between the ending of the critical period and the shift in the polarity of GABA [77, 78]. Also consistent with this possibility is the finding by Dityatev et al. [13] that degradation of PNNs resulted in an increase in the excitability of interneurons.

PNNs may surround neurons for which it is important that the Cl^- potential be maintained. This would be particularly important for neurons which receive substantial inhibitory input, which results in fluctuations of Cl^- across the membrane. Thus, PNNs around GABAergic synapses would ensure that these connections remain inhibitory. Our data support such a possibility. In some areas of the brain, the majority of synapses on parvalbumin+ neurons are GABAergic [79], as are the synapses on cerebellar nuclear neurons [16, 28]. However, we also found that neurons in the inferior olive are not surrounded by PNNs. Previous work [80] shows that inferior olive cells receive substantial GABAergic input from neurons in the contralateral cerebellar nuclei. We cannot therefore conclude that there is a simple relationship between the amount of inhibitory input a cell receives and the degree to which it is surrounded by PNNs.

4.6. Comparison of PNN Distributions in Macaques and Other Species. Although this is the first survey of PNN expression across the entire primate brain, several other studies have examined the expression of PNNs in isolated areas and in other species. Unfortunately, many of these reports do not provide cell counts but register only absence or presence of PNNs in a given area.

Many previous studies find PNN expression in the rodent cerebral cortex [81], especially in layers 2–4 [82, 83]. Also, although they do not give specifics regarding the subregions they examined, McGuire et al. [84] found between 3.02 and 14.44% of cells labeled with PNNs in posterior parietal areas. This is similar to our finding that 3–10% of neurons in various regions of the monkey cerebral cortex are surrounded by PNNs. Bertolotto et al. [83] quantified cells/mm^2 for motor, somatosensory, and visual cortex. These numbers in the rodent are very different from those in the monkey and it is not possible to directly compare our results to theirs. Like Bertolotto et al., we found that the overall density (surrounded neurons/mm^2) of PNN+ cells/area was highest in the V1. Depending on which layers of motor and somatosensory cortex we examined, we could find higher densities of PNNs within either motor or somatosensory cortex.

Although we examined only two regions of the nonhuman primate frontal cortex, the FEF and the orbital gyrus, McGuire et al. [84] performed an exhaustive examination of aggrecan expression in the frontal cortex of macaques

using the Cat-301 antibody. They classified aggrecan staining in each cortical area as falling into one of two categories, cellular labeling in layers 3 and 5 or diffuse labeling in layers 2–5. These two categories also differed in the relative numbers of pyramidal to nonpyramidal cell labeling. The FEF, primary motor cortex, SMA, and premotor cortex were all in the first profile while the orbital gyrus, dlPFC, and cingulate cortex were in the second. They found the most intense staining in the PFC frontal cortex in FEF, which was also our finding. McGuire et al. used different counting procedures so it is not possible to directly compare their counts with ours. Still, it appears that they often found higher prevalence of PNNs than we did. This could be due to either their experimental procedure or our having different criteria for what qualifies as a labeled cell. Further, Cruz-Rizzolo et al. [85] performed a detailed qualitative examination of perineuronal net expression in the monkey *Cebus apella* and found less staining in orbitofrontal cortex than putative frontal eye field, as well as highly diverse expression of PNNs throughout the frontal cortex. Also, although Seeger et al. [86] appeared to find similar levels of WFA+ cells throughout the rat cortex, Brückner et al.'s findings [87] in human are again more similar to our own in that far fewer PNNs were found in frontal cortex and association areas than in primary sensory and motor areas.

Vitellaro-Zuccarello et al. [88] and Seeger et al. [86] found that the distribution of PNNs throughout the thalamus was very variable. This does not conflict with our results. We examined only a small subset of the thalamic regions, medial dorsal and ventral posterolateral, but Brückner et al. [89] found WFA staining in the reticular thalamic nuclei in mouse, but less conclusive results using other PNN markers. According to Gáti et al. [90], who examined PNN expression in rat thalamus, PNNs are often absent from traditionally defined relay regions of the thalamus and more prevalent in regions that connect directly to primary cortical regions. Our findings also qualitatively match those found by Seeger et al. [86] (rat), Adams et al. [91] (macaque), and Brückner et al. [92] (human), who examined perineuronal net presence in the primate basal ganglia.

Bertolotto et al. [83], like us, found very little evidence of PNN presence in the hippocampus. However, Brückner et al.'s [89] study in mouse found staining in CA1–3, as did Seeger et al.'s [86] study in rat. This does not conflict with our result because our counts were restricted to the dentate gyrus. Indeed, Lendvai et al. [93] found that, qualitatively, the dentate gyrus has far fewer PNNs than CA1–CA3, the subiculum and entorhinal cortex in human tissue. Ajmo et al. [82] also examined PNN staining in the mouse and rat hippocampus and like us found only scattered neurons labeled with WFA.

Several reports are also in agreement with us regarding the expression of PNNs in the adult cerebellar cortex. Popp et al. [81], Aquino et al. [94], Seeger et al. [86], and Bertolotto et al. [83] all found low or no expression of key PNN proteins around cerebellar Purkinje cells. Like us, Bertolotto et al. found a high density of staining around neurons in the cerebellar nucleus of the mouse. Also, like us, Brückner et al. [89] and Seeger et al. [86] identified strong labeling of PNNs with WFA in many regions of the brainstem including the cerebellar and vestibular nuclei, the colliculi, and the pons.

Galtrey et al. [7] examined the expression of PNN proteins in the rat spinal cord using WFA and NeuN, as we did. They found that PNNs surrounded fewer motoneurons compared to what we found (30% versus 76%) and that a large number of interneurons in the intermediate grey were also surrounded (50%, unquantified in our case) and 20% of neurons in the dorsal horn were surrounded. We found almost no evidence for PNN expression in the dorsal horn, but it is possible that this is a result of our differing demarcations of this zone. Galtrey et al. also noted that there were no PNNs in the cord's dorsalmost laminae. Also, like us, they found diffuse WFA staining around neurons with the tight WFA-labeled ensheathments. This might have made identifying labeled neurons more difficult. Similarly, when Jäger et al. [95] examined PNNs in the human spinal cord, they also found more aggrecan ventrally than dorsally and, as we did, found that not all ChAT+ neurons were also PNN+ (71% in their case, 76% in ours).

To date, very few studies have examined PNN presence in humans. Bertolotto et al. [83] compared their findings in rat to human sample and found very similar results, with the exception that they also identified labeling around some large pyramidal neurons in layer V of the cerebral cortex, as we do in the rhesus macaque, and note that this is something they did not witness in rat tissue.

Our results qualitatively match those of other studies examining PNN presence in different parts of the central nervous system in rodents and humans, with the exception of the inferior olive. Where our results differ, it is likely that this is the result of either interspecies variation or differences in techniques between labs, that is, the use of other markers for PNNs. Overall, this suggests that PNN prevalence is broadly maintained across taxa.

4.7. PNNs around a Small Percentage of Neurons Inhibit Plasticity. Finally, two previous studies restore critical period-like plasticity in V1 [2, 68] and the amygdala [3] of adult rats by dissolving PNNs in these areas. Dissolving PNNs in V1 allowed visual experience to change in the distribution of visual input to V1 and to improve the acuity in a rat's occluded eye [2]. Dissolving them in the amygdala allowed experience to erase fear conditioning in adult rats, conditioning previously thought to be permanent [3]. These striking changes in behavior and brain structure occur after removal of PNNs from brain areas in which, in macaque, only 5% (V1) and 3% (amygdala) of neurons are surrounded by PNNs.

These changes were experimentally induced in rats and it is possible that PNNs surround a much higher percentage of neurons in V1 and amygdala in rat than they do in macaque. We assessed the proportion of PNNs in V1 and amygdala in one rat using the same methods that we used for macaques. We found that in this animal PNNs surrounded 3.5% of V1 neurons and <1% of amygdala neurons.

The fact that WFA+ PNNs surround only a small proportion of neurons in V1 and the amygdala may mean that the large increases in plasticity demonstrated after injecting

chondroitinase result from dissolving the PNNs around only this small proportion of neurons. Alternatively, the injected chondroitinase may dissolve the chondroitin sulfate chains in both the PNNs and the rest of the extracellular matrix within the injection site. It is possible that destruction of this less salient but more broadly distributed matrix, not just the PNNs, increases plasticity. In the absence of more evidence about the function of PNNs, it is just as plausible that the widespread extracellular matrix helps inhibit plasticity as it is that PNNs around only 5% of neurons inhibit plasticity. It will be worthwhile to rule out the former possibility.

5. Conclusion

In summary, the density of PNN-ensheathed neurons is very different in different areas of the central nervous system. No generalization proposed so far accounts well for these differences because we do not yet understand the specialized functions of neurons in these regions, or of PNNs. It appears that in some areas PNN presence around only a small proportion of the neurons is sufficient to block plasticity. However, in other areas, such as the cerebellar nuclei, PNN presence is probably not related to plasticity, but maybe instead to local ion homeostasis.

Because no single explanation can currently account for PNN presence around neurons throughout the central nervous system, it is likely that PNNs serve different functions in different regions of the brain.

Conflict of Interests

The authors declare no competing interests.

Authors' Contribution

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. Study concept and design were the responsibility of Adrienne L. Mueller and Farrel R. Robinson. Acquisition of data was carried out by Adrienne L. Mueller, Farrel R. Robinson, Adam Davis, and Samantha Sovich. Analysis and interpretation of data were done by Adrienne L. Mueller, Farrel R. Robinson, and Steven S. Carlson. Drafting of the paper was done by Adrienne L. Mueller. Farrel R. Robinson and Steven S. Carlson carried out critical revision of the paper for important intellectual content. Farrel R. Robinson and Adrienne L. Mueller obtained funding. Adam Davis, Samantha Sovich, and Steven S. Carlson were responsible for administrative, technical, and material support.

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Review Article

In Sickness and in Health: Perineuronal Nets and Synaptic Plasticity in Psychiatric Disorders

Harry Pantazopoulos[1,2] and Sabina Berretta[1,2,3]

[1] *Translational Neuroscience Laboratory, Mclean Hospital, Belmont, MA 02478, USA*
[2] *Department of Psychiatry, Harvard Medical School, Boston, MA 02215, USA*
[3] *Program in Neuroscience, Harvard Medical School, Boston, MA 02215, USA*

Correspondence should be addressed to Sabina Berretta; s.berretta@mclean.harvard.edu

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Rapidly emerging evidence implicates perineuronal nets (PNNs) and extracellular matrix (ECM) molecules that compose or interact with PNNs, in the pathophysiology of several psychiatric disorders. Studies on schizophrenia, autism spectrum disorders, mood disorders, Alzheimer's disease, and epilepsy point to the involvement of ECM molecules such as chondroitin sulfate proteoglycans, Reelin, and matrix metalloproteases, as well as their cell surface receptors. In many of these disorders, PNN abnormalities have also been reported. In the context of the "quadripartite" synapse concept, that is, the functional unit composed of the pre- and postsynaptic terminals, glial processes, and ECM, and of the role that PNNs and ECM molecules play in regulating synaptic functions and plasticity, these findings resonate with one of the most well-replicated aspects of the pathology of psychiatric disorders, that is, synaptic abnormalities. Here we review the evidence for PNN/ECM-related pathology in these disorders, with particular emphasis on schizophrenia, and discuss the hypothesis that such pathology may significantly contribute to synaptic dysfunction.

1. Introduction

The classic view of psychiatric disorders as "neuronal" disorders has been challenged in recent years by rapidly emerging evidence pointing to the involvement of the extracellular matrix (ECM), glial cells, and their interactions [1–8]. This evidence represents a significant departure from mainstream views and is driving the field toward a growing understanding of these elements as closely interacting components of functional units, such as the "tetrapartite synapse." This latter term, originally proposed by Dityatev et al. [9], aptly describes the functional unit composed of the of pre- and postsynaptic terminals, astroglial processes, and synaptic/perisynaptic ECM complexes [10–14]. Here, we review evidence for the involvement of the ECM in psychiatric disorders and focus on the hypothesis that ECM abnormalities may contribute to a critical pathological component shared by a large subgroup of these disorders, that is, disruption of synaptic functions [15–21]. First, evidence for ECM abnormalities in schizophrenia, the main focus of these authors' studies, is discussed, with particular emphasis on loss of perineuronal nets (PNNs) in several brain regions in this disorder. We then briefly review evidence for a significant involvement of synaptic pathology in this disorder and follow with a discussion on the potential mechanisms linking such pathology to ECM/PNN abnormalities. Finally, evidence for ECM involvement in other psychiatric disorders is reviewed, with reference to molecular families known to play a role in synaptic functions. The specific patterns and causes of ECM abnormalities in each of these disorders are not yet well understood and may be disorder-specific. We postulate that overlapping patterns of ECM/PNN abnormalities may underlie shared synaptic pathology in these disorders.

It should be emphasized here that synaptic regulation is only one of several critical functions performed by the ECM during pre- and postnatal brain development as well as adulthood (for reviews see [22–30]). Thus, in addition to synaptic dysregulation, the consequences of brain ECM abnormalities

may be complex and far-reaching, spanning from disruption of axonal guidance, neuronal differentiation, and migration in early brain development to circuit consolidation and closure of critical periods in postnatal development and finally axonal signal conduction and regulation of the blood/brain barrier in the adult brain [1, 2, 31–38].

2. Schizophrenia

2.1. ECM/PNN Abnormalities in Schizophrenia. Schizophrenia is a chronic, severe, and disabling brain disorder characterized by psychotic symptoms and disruptions of normal emotions and behaviors. Growing evidence points to ECM abnormalities as a component of the core pathophysiology of schizophrenia. Converging results from human genetic and postmortem studies show genetic vulnerabilities for genes encoding several key ECM molecules, including chondroitin sulfate proteoglycans (CSPGs), Reelin, semaphorin 3A, integrins, and remodeling enzymes, as well as dysregulated expression of these molecules in glial cells, and disruption of organized ECM structures such as PNNs (see references below). Animal models indicate that these abnormalities may have far-reaching consequences on neural circuits involved in schizophrenia [39–41]. These findings are briefly reviewed below.

2.1.1. CSPGs. In subjects with schizophrenia, we first reported marked decreases of CSPG-labeled PNNs in the amygdala and entorhinal cortex [42], interconnected brain regions involved in emotion-related learning and associative sensory information processing and in the pathophysiology of schizophrenia [43–47]. In this study, PNNs were detected using the lectin *wisteria floribunda* agglutinin (WFA; Figure 1), which labels PNNs predominantly associated with GABAergic neurons expressing the calcium binding protein parvalbumin (PVB) [48–54]. Consistently, lower numbers of WFA-labeled PNNs were observed in the lateral nucleus of the amygdala and the superficial layers of the entorhinal cortex, where these interneurons are primarily located [42]. A similar distribution pattern of PNN decreases was detected using antibodies against aggrecan, one of the main CSPGs in the brain [55]. In contrast, immunolabeling with antibodies raised against a specific chondroitin sulfate 6 (CS-6; Figure 1) pattern revealed more extensive PNN distribution in the normal human amygdala and decreases in subjects with schizophrenia, including not only the lateral nucleus but also the basal, accessory basal, cortical, and medial amygdala nuclei [55] (Figure 2). PNN reduction in this latter nucleus is of particular interest, as it suggests that GABAergic projection neurons are also affected by PNN abnormalities. Notably, PNN decreases were not accompanied by neuron number reductions [42, 43], pointing to actual loss, or altered neurochemical composition, of PNNs. Lower densities of WFA-labeled PNNs were also detected in layers III and V of the prefrontal cortex of subjects with schizophrenia [56]. Interestingly, the visual cortex did not show similar changes [56], suggesting that while being widespread PNN decreases may spare brain regions that are not heavily involved in the pathology of schizophrenia.

In addition to CSPG-labeled PNN decreases, markedly altered CSPG expression in schizophrenia was also detected in glial cells and "glial clusters" in the amygdala, as well as in olfactory receptor neurons in the olfactory epithelium [42, 55, 57]. In parallel to PNN-related findings, these changes did not appear to depend on altered cell numbers and did not depend on disease-related confounding factors such as exposure to pharmacological treatment, substance abuse, onset and duration of the illness, and so forth, further supporting the idea that altered CSPG expression in schizophrenia may represent a core feature of this disorder [42, 55, 57].

Further support for CSPG involvement in schizophrenia comes from molecular dissection of the neuregulin-ErbB4 pathway, which revealed an association with a genetic polymorphism in PTPRZ1, the gene encoding for receptor phosphotyrosine phosphatase beta/zeta (RPTPbeta) with schizophrenia [58]. RPTPbeta is a transmembrane CSPG shown to play a role in synaptic plasticity and learning [59–63]. Increased mRNA expression of PTPRZ1 has been reported in the amygdala and prefrontal cortex of subjects with schizophrenia [39, 42]. Genetic studies have further identified associations of schizophrenia with the genes encoding for the CSPGs neurocan (NCAN) and neuroglycan-C [64, 65], suggesting that abnormal CSPG expression in schizophrenia may be due, at least in part, to genetic factors.

2.1.2. Reelin. The glycoprotein Reelin is arguably one of the ECM molecules most extensively investigated in schizophrenia and other psychiatric disorders. In subjects with schizophrenia, it has been widely reported to be decreased in a number of cortical areas [66–70]. Several studies have shown that Reelin expression is reduced concurrently with GAD67, one of the main synthetic enzymes for GABA, in cortical GABAergic interneurons, and that these changes may be the consequence of an epigenetic hypermethylation of RELN and GAD67 promoters in these interneurons [70–74]. Notably, Reelin expression was found to be decreased in interstitial white matter neurons, in a study that also confirmed increased density of these neurons in schizophrenia [75, 76]. Together, these studies elegantly link a disruption of Reelin expression in GABAergic neurons to dendritic spine loss and altered neuronal migration in schizophrenia.

2.1.3. Semaphorins. Members of the semaphorin family, and semaphorin 3a in particular, have also been shown to be altered in schizophrenia, potentially in conjunction with Reelin [66, 77]. In particular, increased semaphorin 3A and decreased Reelin expression were detected in the cerebellum of subjects with schizophrenia, while altered expression of multiple members of the semaphorin family was observed in the prefrontal cortex [66]. Genetic polymorphisms of genes encoding for semaphorin 3D and semaphorin receptor plexin A2 have also been associated with schizophrenia ([78, 79], but see also [80]).

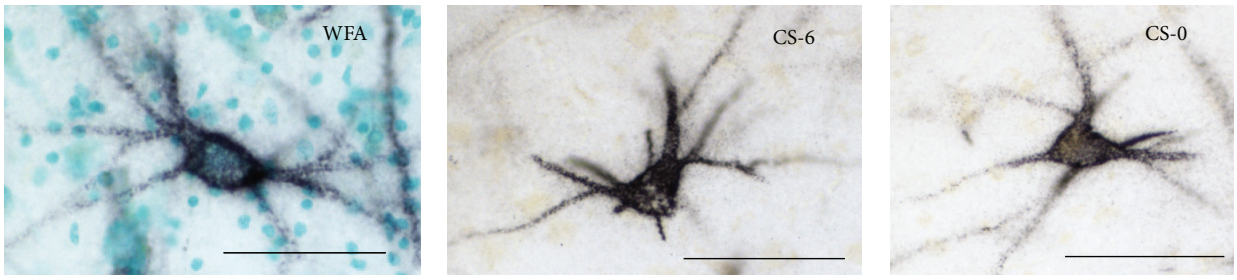

Figure 1: PNNs in the human amygdala. (a) PNN labeled with *wisteria floribunda* agglutinin (WFA) lectin, which binds to N-acetyl-galactosamine on the terminal end of chondroitin sulfate chains. (b) PNN immunolabeled using the mAB antibody 3B3 against native chondroitin sulfate motifs specific for chondroitin-6-sulfate (CS-6). 3B3-immunolabeled PNNs are more numerous and show a much broader distribution in the human amygdala with respect to those labeled with WFA. (c) PNN immunolabeled with mAB antibody 1B5, raised against nonsulfated chondroitin sulfate (CS-0) (3B3 and 1B5 are a generous gift from Dr. Bruce Caterson, University of Cardiff, UK). Scale bar = 50 μm.

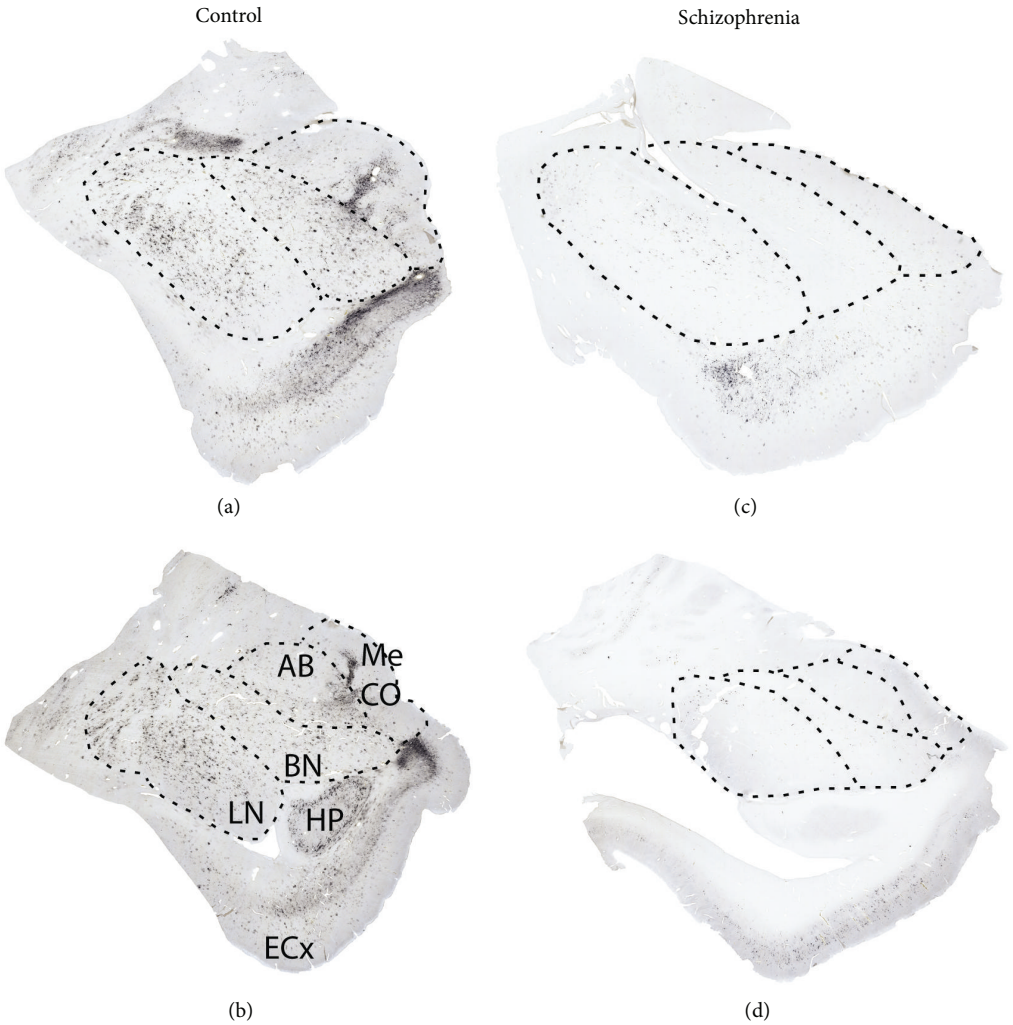

Figure 2: Decreased CS-6 in the amygdala of subjects with schizophrenia. In the amygdala of control subjects, CS-6(3B3)-labeled PNNs and glial cell clusters are distributed in the lateral (LN), basal (BN), accessory basal (AB), and corticomedial (CO-Me) nuclei (a, b). In subjects with schizophrenia, marked decreases of PNNs and glia cell clusters immunolabeled for CS-6(3B3) were observed in the amygdala of (c, d) [55]. ECx: entorhinal cortex. HP: hippocampus.

2.1.4. Integrins. Integrins, a family of heterodimeric cell adhesion molecules (CAMs) consisting of several different α- and β-subunits, interact with ECM molecules to carry out a multitude of developmental and adult brain functions. Support for the idea that integrins may be implicated in the pathology of schizophrenia comes in part from genetic studies pointing to association of this disorder with a number of integrin gene variants, such as SNPs in the ITGA8 and ITGB3 genes [81, 82]. Additional evidence supporting the involvement of integrins in schizophrenia includes increased expression of integrin α(IIb) and β(IIIa) in first episode subjects with schizophrenia and, notably, abnormal cell adhesion in cultures from olfactory mucosa biopsies from patients with this disorder, which was ameliorated by antibodies blocking integrins [83, 84].

2.1.5. Matrix Metalloproteases. Proteolytic ECM remodeling, shown to play a key role in synaptic plasticity, is mediated by matrix metalloproteases (MMPs), "a disintegrin and metalloproteases" (ADAMs), and "a disintegrin and metalloproteases with a thrombospondin motif" (ADAMTS), through their substrates, such as CAMs, CSPGs, and ECM receptors [85–89]. Several of these enzymes have been implicated in the pathophysiology of schizophrenia. Elevated levels of MMP-9, and "tissue inhibitor of metalloproteinases 1" (TIMP-1) which blocks MMP-9 activity, were reported in blood samples from subjects with this disorder [90, 91]. Increased MMP-9 blood serum levels were also identified in treatment resistant patients [92]. A recent gene expression profiling study of the superior temporal gyrus showed altered mRNA expression of MMPs and ADAMTSs in schizophrenia, including MMP-16 [93]. The possibility that genetic vulnerability may contribute to altered expression of matrix metalloproteases in schizophrenia is supported by converging results from several recent genetic association and genome-wide association studies showing that gene variants encoding for a number of these enzymes, including ADAMTSL3, ADAMTS12, ADAMTS16, ADAM22, and MMP-16, may be associated with this disorder [94–98].

2.1.6. Evidence for ECM Abnormalities from Animal Model Studies. Consistent with human studies, animal models provide evidence that abnormalities affecting the ECM, and CSPGs in particular, may contribute to the pathophysiology of schizophrenia. Transgenic mice overexpressing PTPRZ1 show numerous anatomical and behavioral abnormalities also observed in this disorder, including delayed oligodendrocyte maturation, working memory deficits, and altered glutamatergic, dopaminergic, and GABAergic activity [39]. Experimentally induced enzymatic PNN digestion in the mouse hippocampus mimics several functional abnormalities, including increased activity of dopamine neurons in the ventral tegmental area, which is suspected to occur in schizophrenia [40]. Finally, a rodent model for oxidative stress in schizophrenia showed that PNNs protect neurons expressing PVB from oxidative stress, while at the same time they are vulnerable to it [41]. Thus, loss of PNNs may render these neurons more susceptible to the excitotoxic effects of oxidative stress believed to occur in schizophrenia [99–101].

2.2. Synaptic Pathology in Schizophrenia. Solid and growing evidence shows that disruption of synaptic functions represents a core component of the pathology of schizophrenia. Altered synaptic transmission of key CNS neurotransmitters, including glutamate and GABA, altered expression of synaptic molecules, and loss of dendritic spines have been consistently observed in schizophrenia [16, 102–111]. These interlinked components are briefly reviewed below and placed in the context of ECM/PNN abnormalities.

2.2.1. Glutamatergic Synaptic Signaling and GABAergic Inhibitory Neurons. Several neurotransmitter systems have been implicated in the pathophysiology of schizophrenia. For the purpose of this review, we focus on the involvement of glutamatergic transmission and GABAergic inhibitory neurons in schizophrenia and discuss the potential contribution of ECM/PNN pathology to abnormalities affecting these systems. Importantly, abnormalities affecting these neurotransmitters are closely linked to one another. For instance, GABAergic interneurons powerfully regulate intrinsic information processing and glutamatergic efferents (e.g., [112–115]). Conversely, these interneurons are particularly sensitive to glutamate NMDA receptor hypofunction [116, 117].

Abnormalities affecting the GABAergic system have been consistently reported in several brain regions in schizophrenia [115, 118–129]. These include decreases of inhibitory neurons, loss of GABAergic terminals, and expression of glutamatergic receptors on distinct populations of GABAergic interneurons [115, 130, 131]. The regulation of glutamatergic inputs to these neurons, and PVB-positive neurons in particular, has been a specific focus of attention in schizophrenia, as these inputs play a key role in controlling synchronous oscillations at gamma band frequencies, known to be affected in schizophrenia, and are critical to information processing in cortical circuits involved in this disorder [122, 126, 132, 133].

Altered expression of NMDA and, perhaps to a lesser extent, AMPA receptor proteins has been reported in subjects with schizophrenia, pointing to a disruption of synaptic glutamate signaling networks in this disorder [134–142]. In addition, and perhaps more consistently, the expression of a large number of glutamate receptor accessory proteins, including proteins associated with the postsynaptic density, is altered in several brain regions in people with schizophrenia (for review see [141]). Converging evidence from postmortem, genetic, and animal models also points to abnormal expression of the NMDA receptor coagonists, D-serine and glycine, and the endogenous glycine modulatory site antagonist kynurenic acid [143–150]. This well-replicated finding resonates with intriguing evidence for ECM-mediated modulation of the NMDA receptor glycine site [151].

2.3. Potential Contribution of ECM Abnormalities to Synaptic Glutamatergic Transmission on GABAergic Neurons. PNNs tightly surround synaptic contacts on the somata, dendrites, and proximal axon segment of distinct populations

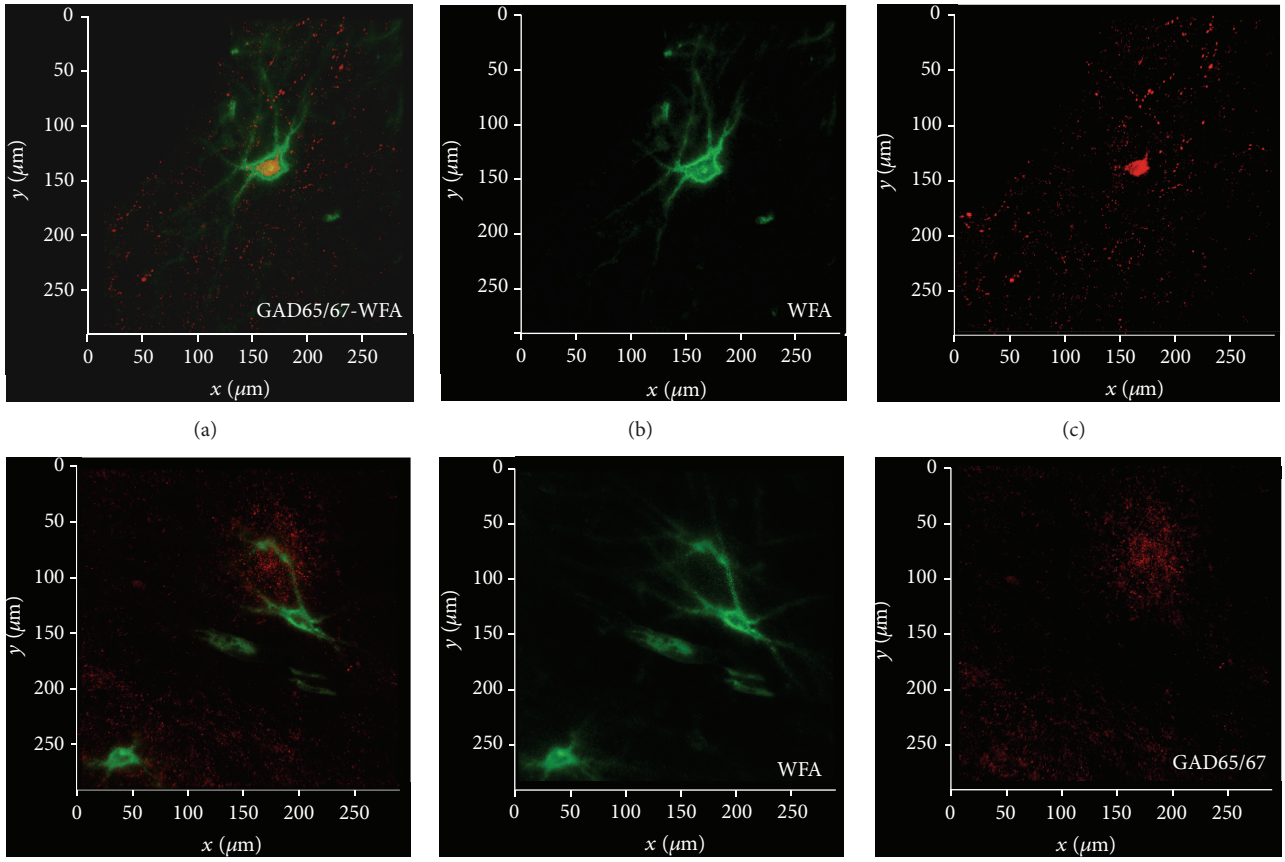

Figure 3: PNNs are associated with heterogeneous neuronal populations. Confocal micrographs depicting WFA-labeled PNNs surrounding GABAergic neurons expressing GAD65/67 in the healthy human amygdala (a–c). Consistently, WFA-labeled PNNs have been shown to be typically associated with neurons expressing parvalbumin. However, we have previously reported that a small subpopulation of these PNNs ensheathes parvalbumin-negative neurons [152]. Preliminary findings shown in (d)–(f) suggest that these neurons do not express GAD65/67 and may therefore correspond to excitatory neurons.

of neurons. These consist of several GABAergic interneuron populations, including, but not limited to, those expressing PVB or somatostatin, and GABAergic projection neurons such as those in the reticular nucleus of the thalamus, the central nucleus of the amygdala, and Purkinje cells in the cerebellum [2, 48, 153–158]. A subset of excitatory cortico-cortical pyramidal cells and spinal cord motor neurons have also been found to bear PNNs [159]. It is likely that the range of neuronal populations associated with PNNs has not yet been fully accounted for, particularly as the molecular and structural heterogeneity of these ECM structures is still not well understood. For instance, in the human amygdala, a small population of WFA-labeled PNNs ensheathes neurons lacking expression of glutamic acid decarboxylase (GAD) and thus likely represented excitatory neurons (unpublished results; Figure 3). In addition, we recently reported that PNNs labeled with an antibody (3B3) targeting a native chondroitin-6-sulfate motif are far more numerous and widely distributed than those labeled with the lectin *wisteria floribunda* agglutinin (WFA) and well represented in amygdalar nuclei virtually devoid of PVB-positive neurons [55]. Therefore, abnormalities affecting PNNs may potentially impact a broad variety of inhibitory, as well as excitatory, neuronal populations. Converging evidence suggests that, in schizophrenia, ECM/PNN components known to regulate glutamatergic and GABAergic inputs on GABAergic neurons are abnormal, potentially contributing to intrinsic information processing and activity outflow.

2.4. Regulation of Glutamatergic and GABA Synapses by PNNs and Perisynaptic ECM Condensations. Growing evidence points to a key role played by PNNs in synaptic regulation, particularly of glutamatergic synapses (for extensive reviews see [11, 12, 38, 160, 161]). In particular, ECM/PNNs affect the diffusion of glutamate receptors laterally within the plasma membrane, as well as receptor clustering within the synapse, thus controlling a fundamental mechanism of synaptic regulation and plasticity [162, 163]. It is thought that this key PNN function may be accomplished by a combination of passive and active mechanisms [160, 162]. Although less is currently known with regard to PNN regulation of GABAergic inputs, a recent study reported that PNN enzymatic

digestion increases the number of inhibitory synapses on PVB-positive interneurons [164]. We briefly describe below some examples according to their potential relevance to schizophrenia. We suggest that, in schizophrenia, concurrent disruption of CSPG expression and molecular families interacting with PNN components may contribute synergistically to glutamatergic synapse dysregulation on neurons associated with PNNs.

2.4.1. PNNs as a Passive Barrier. Highly viscous CSPGs, and other ECM/PNN components, form an effective passive diffusion barrier, controlling the lateral diffusion exchanges of AMPA receptors between the synaptic and extrasynaptic compartments [162]. By restricting the lateral diffusion of AMPA receptors from the extrasynaptic space to the synapse, PNNs allow synaptic desensitization during high frequency firing [160, 162, 165]. Consistent with this function, PNN enzymatic digestion results in increased excitability of interneurons [166]. In pathological states, such as schizophrenia, PNN disruption may result in unregulated lateral membrane diffusion of AMPA receptors, thus impacting excitatory synaptic activation and resembling a more "juvenile" state of synaptic regulation.

2.4.2. Chondroitin Sulfate Proteoglycans. CSPGs are key contributors to the composition of PNNs [167–169] and are also found in other structures described as perisynaptic coats [154, 170–174]. An increasing number of studies show that these molecules are critically involved in the regulation of synaptic plasticity. For example, electrophysiological recordings from in vitro mouse hippocampal slices treated with chABC to remove CSPGs show a twofold decrease in long term potentiation (LTP) but not in short term plasticity [175]. A similar decrease of LTP was also observed in mice lacking a key PNN component, tenascin-R, suggesting that CSPG regulation of long term synaptic plasticity occurs through modulation of PNN composition [175].

Studies focused on specific CSPG molecules and CS-sulfation reveal a complex role in developmental and adult regulation of synaptic plasticity. For example, overexpression of CS-6 sulfation in mice leads to failure to instate an adult form of restricted plasticity, resulting in abnormally persistent synaptic plasticity and reduced PNN formation [176]. In the cortex and hippocampus, expression of the CSPG PTPRZ1 was found to be associated with synaptic remodeling [59–62]. Knockout of PTPRZ1 in mice results in enhanced LTP and deficits in spatial learning exclusively in adults [63]. In contrast, mice overexpressing PTPRZ1 show hippocampal LTP deficits, as well as a number of molecular, anatomical, and behavioral abnormalities reminiscent of those observed in subjects with schizophrenia [39]. Together with the increased expression of PTPRZ1 in subjects with this disorder [39, 42], these findings are consistent with the possibility that increased PTPRZ1 expression may contribute to deficits in synaptic plasticity in schizophrenia. Other CSPGs have also been shown to regulate plasticity. Adult mice deficient for the CSPG brevican display deficits in hippocampal early stage LTP and decreased PNN formation [177], whereas mice deficient for neurocan display decreased late stage LTP but normal PNN formation [178]. Further evidence comes from studies showing that brevican and versican are increased in the hippocampus of rats during memory retrieval in the Morris water maze spatial memory task [179]. Overall, altered expression of CSPGs and CS-sulfation patterns have the potential to contribute to disregulated synaptic plasticity in psychiatric disorders during developmental stages, particularly during critical periods of plasticity, as well as adult regulation of synaptic plasticity.

2.4.3. Reelin. Perhaps one of the most well-replicated findings in the pathophysiology of schizophrenia and, incidentally, autism is a disruption of Reelin expression [66–74, 76]. In the adult brain, Reelin is secreted in the ECM by subpopulations of GABAergic interneurons and takes part in the composition of at least a subpopulation of PNNs [180–182]. Reelin's effects are mediated through its main lipoprotein receptors, apolipoprotein E receptor 2 (ApoER2) and very-low-density lipoprotein receptor (VLDLR) [183, 184], as well as through the CAMs of the integrin family and the Src family kinases [184–188]. Accumulating evidence shows that secreted Reelin powerfully enhances LTP and glutamatergic synaptic transmission by regulating NMDA and AMPA receptors [187, 188]. For instance, Reelin regulates the composition of NMDA receptors, controlling the predominance and/or phosphorylation of the NR2 NMDA receptor subunits, and enhances AMPA responses by increasing the number of AMPA receptors on the postsynaptic membrane [180, 188].

2.4.4. Integrins. Interactions between integrins and ECM/PNN components, including Reelin, thrombospondins, fibrinogen, and others, have been shown to regulate synaptic glutamatergic transmission [160]. Notably, integrin signaling is bidirectional; that is, it can activate intracellular signaling pathways in response to changes in the extracellular environment and impact on cell adhesion in response to intracellular signaling [189]. This may allow integrins to play complex roles in synaptic plasticity, including carrying out structural and functional changes that accompany LTP [189–191]. Integrin-ECM interactions have been shown to regulate AMPA receptor internalization, surface mobility of NMDA receptor subunits, and synaptic dwell time of glycine receptors and their scaffolding molecule gephyrin [151, 192, 193].

2.4.5. Neuronal Activity-Regulated Pentraxin. An intriguing example of interactions between ECM molecules and factors regulating synaptic plasticity is represented by the "neuronal activity-regulated pentraxin" (NARP). NARP is an immediate early gene in which its protein product is secreted in an activity-dependent manner by excitatory terminals onto GABAergic PVB-positive neurons, where it promotes clustering of AMPA receptor subunits [194, 195]. This mechanism regulates homeostatic scaling of excitatory inputs so that increased network activity strengthens the excitatory inputs on PVB-positive interneurons, in turn powerfully inhibiting

excitatory projection neurons [195]. PNNs ensheathing PVB-positive neurons are critical to the maintenance of high levels of NARP at excitatory synapses on these neurons [195]. CSPG enzymatic digestion markedly decreased NARP at excitatory synapses on PVB-positive neurons without decreasing the overall NARP expression [195]. In subjects with schizophrenia, marked Narp mRNA decreases were reported in the prefrontal cortex [196], a brain region where PNN decreases were also detected [56]. Although a causal relationship between these two findings has not been established thus far, it is reasonable to postulate that, in conjunction, NARP and PNN decreases may synergistically impact glutamatergic synapses on PVB-positive neurons in schizophrenia.

2.4.6. Matrix Metalloproteases. Secreted extracellular matrix proteases, such as "tissue plasminogen activator" (tPA) and MMPs, affect excitatory transmission. For instance, tPA has been found to play a role in LTP through several mechanisms, including cleavage of the NR1 subunit of the NMDA receptor, resulting in potentiation of NMDA current, and cleavage of proBDNF resulting in availability of mature BDNF [197–200]. Altered levels of tPA have been reported in subjects with schizophrenia [201–203]. Although it is not currently known whether these abnormalities are linked to comorbidities, such as alcoholism, inflammatory, and autoimmune disorders, or metabolic disorders [201–203], these findings support the intriguing possibility that tPA abnormalities may contribute to a disruption of glutamatergic transmission in schizophrenia. Interestingly, the amygdala, where marked PNN decreases were detected in schizophrenia, is particularly enriched in tPA [42, 55, 204–206]. MMP-9 has also been shown to powerfully regulate synaptic plasticity and LTP in particular, a role mediated by 1-containing integrin receptors [207]. Interestingly, MMP-9 is transiently released in response to enhanced neuronal activity and impacts both synaptic potentiation and dendritic spine enlargement in a dependent manner [207, 208]. As discussed above, the possibility that MMP-9, as well as other MMPs with potentially related functions, may represent genetic vulnerabilities in schizophrenia has been gaining evidence in recent times [98, 208–210].

2.5. Loss of Dendritic Spines in Schizophrenia. Marked reductions of dendritic spines have been consistently reported in schizophrenia, encompassing several cortical areas, including prefrontal and auditory cortical areas and the hippocampus [104, 109–111, 211]. In addition, the expression of postsynaptic density (PSD) proteins, such as PSD95 and Homer-1, and associated glutamate signaling pathway proteins has been shown to be altered in subjects with this disorder, as well as with autism spectrum disorders [107, 108]. In support of the idea that these changes reflect a structural loss of dendritic spines, altered expression of molecules involved in the actin cytoskeleton has been reported in subjects with schizophrenia [102, 103, 105].

2.6. Potential Contribution of ECM Abnormalities to Loss of Dendritic Spines. Dendritic spines contain the membrane-associated postsynaptic density (PSD) and its associated network of neurotransmitter receptors and downstream signaling molecules and are supported by a mesh of filamentous F-actin and scaffolding proteins [212–218]. ECM proteins, their surface receptors, and remodeling ECM enzymes play a critical role in regulating dendritic spine plasticity in adulthood (see review by [197]). CAMs, among which integrins are perhaps the most well studied, link the PSD to the actin cytoskeleton on one side and to the ECM and presynaptic terminal on the other side. Through this arrangement, CAMs mediate ECM and PSD signaling, influencing the dendritic spine actin network and thus the spine shape [219–225]. In turn, the spine size has a direct impact on synaptic strength, as larger spines/PSDs containing more numerous glutamate receptors have stronger effects on neuronal excitation and signal transmission [212, 226–232].

Several ECM molecules found to modulate spine formation, size, and stability through ECM receptors are also implicated in the pathology of schizophrenia (see above). CSPGs have been shown to actively stabilize dendritic spines, while their removal by enzymatic digestion results in increased spine motility [233–236]. Reelin promotes spine remodeling, impacting not only spine size and stability, but also the number of synaptic contacts per spine, effects at least in part mediated by its receptor ApoER2 [237–242]. The potential contribution of decreased Reelin expression to dendritic spine decreases in schizophrenia has long been postulated [243, 244]. Semaphorin 3A, a secreted ECM molecule expressed in PNNs, exerts a powerful effect on synapses, possibly through its plexin and neuropilin receptors [38, 197, 245–248]. Finally, and importantly, ECM proteases including tPA and MMPs have been shown to robustly affect dendritic spine stability [197]. tPA decreases spine stability, and its activation increases spine loss [249, 250]. This effect is particularly interesting as it relates to the impact of chronic stress in the amygdala and hippocampus, where tPA knockout decreases stress-induced spine loss [249, 250]. During development, MMPs play a key role in spine formation and maturation [197, 251, 252]. In mature neurons, MMPs, and their interactions with integrins, are required for spine volume changes induced by LTP and LTD [207, 253].

In summary, ECM molecules and their cell surface receptors mediate a broad range of synaptic regulatory functions impacting glutamatergic and GABAergic synapses, inhibitory neurons, and dendritic spine plasticity on excitatory neurons. The expression of several ECM molecules and their receptors involved in these functions has been shown to be altered in subjects with schizophrenia. Overall, these considerations support the hypothesis that ECM/PNN abnormalities in this disorder may disrupt synaptic functions and plasticity, perhaps leading to a dysregulation of inhibitory circuits and synaptic instability. The impact of these abnormalities is likely to be region specific, given the heterogeneous representation of these molecules in cortical and subcortical regions (e.g., [42, 55, 204–206, 254]).

3. ECM Pathology in Autism Spectrum Disorders

Multiple lines of evidence implicate ECM abnormalities in autism spectrum disorders, a heterogeneous group of neurodevelopmental disorders characterized by persistent deficits in social communication and social interaction and restricted, repetitive patterns of behavior, interests, or activities. Synaptic pathology is a well-established core pathological component of these disorders (e.g., [16]). Genetic studies have identified several ECM and related molecules as potential contributors to the etiology of autism. Analysis of six genome-wide association studies (GWAS) on autism implicates a number of ECM and PNN regulating molecules, including the ECM remodeling enzymes ADAMTS3, ADAMTS5, ADAMTS14, ECM molecules RELN, SEM3A, SEM4D, the hyaluronan surface receptor CD44, and OTX2, a transcription factor involved in PNN formation [255–263].

By far the strongest evidence for ECM involvement in the pathophysiology of autism comes from investigations on Reelin. GWAS, several association studies specifically investigating Reelin involvement in autism, and a meta-analysis report point to Reelin as a vulnerability gene for autism [257, 264–272]. Consistent with these findings, altered expression of Reelin and Reelin signaling pathways has been observed in the frontal, parietal, and cerebellar cortices of subjects with autism [273–275]. Reduced Reelin levels have also been shown in blood samples from subjects with this disorder [273]. Finally, the "reeler" mouse, which carries an autosomal recessive mutation in the Reelin gene, displays neurodevelopmental deficits reminiscent of psychiatric disorders including autism [273].

Emerging evidence suggests a role for heparan sulfate proteoglycans (HSPGs) in the pathophysiology of autism. Decreased HSPG expression was reported in the subventricular zone of subjects with autism [276]. Notably, this decrease was associated with increased neurogenesis in comparison to age-matched controls [276]. These findings are in agreement with an animal model of autism, the BTBR T+tf/J mouse, characterized by abnormal social behavior, communication deficits, and repetitive stereotyped behaviors as well as altered heparan sulfate expression in the subventricular zone, smaller amygdala volume, and other neurodevelopmental deficits reminiscent of those detected in autism [277–280]. Mutant mice lacking heparan sulfate show many features reflective of autism, including impaired social interaction, repetitive behavior, and deficits in ultrasonic vocalization [281]. Taken together, decreased heparan sulfate in autism may contribute to neurodevelopmental abnormalities focused on areas of cell proliferation as well as regions involved in memory and emotional processing.

4. ECM Pathology in Fragile-X Syndrome

Fragile-X is a single gene, inherited intellectual disability with predominant autistic symptoms [282–284]. The role that MMP-9 plays in the pathophysiology of this disorder represents a compelling example of interactions between ECM molecules and synaptic pathology in psychiatric disorders. Fragile-X results from transcriptional silencing of the *Fmr1* gene, which encodes for the mRNA binding protein "Fragile-X mental retardation protein" (FMRP) (for review see [285]). FMRP controls, in an activity-dependent manner, mRNAs encoding for pre- and postsynaptic proteins, scaffolding proteins, neurotransmitter receptors, and signaling molecules [286, 287]. Decreased FMRP expression results in elevated protein synthesis at the synapse, with loss of regulation by neuronal activity, increase in neuronal excitability, and immature, abnormal spine morphology [285, 288–293]. Converging evidence indicates that the interactions between FMRP and the matrix metalloproteinase MMP-9, known to play a role in dendritic spine plasticity in an activity-dependent manner [192, 294], may be critical to these synaptic abnormalities. Recent findings show that FMRP regulates the transport and translation of MMP-9 mRNA within the synapse: decreased FMRP results in increased MMP-9 [295]. Consistently, increased levels of MMP-9 have been reported in Fragile-X syndrome subjects while pharmacologically induced MMP-9 decrease leads to some degree of clinical improvement [296]. Elevated levels of MMP-9 were reported in the amnionic fluid of subjects who went on to develop autism later in life, including a subset of individuals without Fragile-X syndrome [297]. In parallel, Fmr1 knockout mice present with delayed dendritic spine maturation, increased MMP-9, and Fragile-X associated behaviors [295, 298, 299]. Genetic or pharmacological disruption of MMP-9 expression in these mice rescues many of these abnormalities, including dendritic spine maturation and behavioral deficits [299, 300].

5. ECM Pathology in Rett Syndrome

Rett syndrome is a neurodevelopmental disorder characterized by stereotypical hand movements, language regression, decreased rate of brain growth, autonomic dysfunction, and seizures [301]. Deficits in dendritic spines and synaptic plasticity have been consistently reported in subjects with Rett syndrome and in animal models of this disorder [302–306]. Rett syndrome is caused by a de novo genetic mutation in the X-linked methyl-CpG-binding protein 2 gene (MeCP2) [301, 307, 308]. Increased mRNA for RELN was reported in MeCP2 mutant mice [309]. Furthermore, increased PNN labeling with WFA was observed in the motor cortex of subjects with Rett syndrome [310]. Notably, these changes are opposite to those observed in autism and schizophrenia, suggesting that different patterns of pathological deviations of ECM composition may result in synaptic abnormalities, such as those detected in these disorders.

6. ECM Pathology in Mood Disorders

Mood disorders are a category of psychiatric disorders characterized by a persistent altered emotional state; they include bipolar disorder and major depression. Involvement of ECM molecules, and presence of synaptic pathology, in these disorders has been extensively documented [15, 55, 67, 311–315]. For instance, decreased Reelin expression has been

reported in the prefrontal cortex, hippocampus, and cerebellum, as well as in blood, of subjects with bipolar disorder or major depression [311, 313, 316, 317]. Our postmortem studies in bipolar disorder show marked decreased of CS-6(3B3)-immunolabeled PNNs across several nuclei in the amygdala, while more moderate decreases of aggrecan-immunolabeled PNNs were observed in the accessory basal nucleus of the amygdala [55]. Furthermore, similar to schizophrenia and autism, increased levels of MMP-9 have been reported in blood samples from subjects with major depression and young subjects with bipolar disorder in a depressed state [91, 318, 319]. GWAS in bipolar disorder have identified a genetic variant of NCAN, encoding for the CSPG neurocan, as a risk factor for this disorder [314]. Consistently, NCAN gene variants are associated with manic symptoms in human subjects [320], and NCAN knockout in mice results in manic-like behaviors [320].

Intriguingly, some of the most effective treatments for mood disorders impact ECM molecules and PNN composition. For example, chronic treatment with the selective serotonin reuptake inhibitor fluoxetine, effective in treating depression and anxiety, results in decreased numbers of WFA-labeled PNNs in the hippocampus and medial prefrontal cortex of mice, accompanied by increased immature neuronal markers and dendritic spine density on interneurons [321, 322]. Fluoxetine exposure in utero has also been shown to delay the formation of PNNs during adolescence in the amygdala and hippocampus of mice [323]. Lithium, one of the most effective treatments for bipolar disorder, contributes to CSPG digestion [324, 325]. Consistently, numbers of "glial clusters" labeled with a CS-6 specific antibody (CS56), shown to be decreased in the amygdala of subjects with bipolar disorder, showed a positive correlation with lifetime exposure to lithium, raising the possibility that chronic lithium exposure may exert therapeutic effects on CSPG sulfation patterns in bipolar disorder [55].

7. ECM Pathology in Alzheimer's Disease

Alzheimer's disease, an irreversible late life brain disorder that progressively disrupts memory and independent living skills, is associated with dendritic spine loss [326–328]. A key neuropathological feature in Alzheimer's disease is the formation of β-amyloid plaques associated with mutations in the presenilin-1 and presenilin-2 genes [329, 330]. These plaques are generated by cleavage of the amyloid precursor protein (APP) by β-site APP cleaving enzyme-1 (BACE1) [331]. Converging evidence points to a role for HSPGs and CSPGs in the formation of amyloid plaques. Heparan sulfates regulate cleavage of APP by BACE1, and several HSPGs, including syndecan, glypican, and agrin, can be detected within amyloid plaques [332–340]. Notably, syndecan is involved in the formation of dendritic spines [341–344]. Finally, increased expression of HSPGs has been reported in postmortem brain samples from subjects with AD [335–338, 345, 346]. CSPGs, specifically CS-4, CS-6, and nonsulfated CS, have also been reported in β-amyloid plaques [347]. Intriguingly, a splice variant of APP corresponds to the CSPG appican, expressed primarily by astrocytes in the brain [5, 348]. Interestingly, APP cleavage by ADAM10 results in a beneficial form, amyloid-α, and in turn suppresses amyloid-β [349]. Physiologically, ADAM10 and APP are highly concentrated at the PSD site and are involved in the regulation of synaptic plasticity [350], suggesting that disrupted levels of amyloid-α and amyloid-β in Alzheimer's disease may contribute to synaptic deficits. Similarly, MMP-9 has been reported to cleave APP through α-secretase activity, thus promoting the nonamyloidogenic form and functioning as a protective factor from amyloid-β accumulation and subsequent cognitive deficits, accompanied by increased levels of presynaptic proteins [351, 352]. Furthermore, MMP-9 has been shown to degrade extracellular amyloid-β in amyloid plaques, providing further protection against AD pathology [353, 354].

Of relevance to this review, decreased densities of PNNs have been reported in Alzheimer's disease [355]. In particular, decreased WFA-labeled PNNs were observed in the frontal cortex of subjects with Alzheimer's disease, while densities of neurons expressing PVB were not altered [355]. These findings are in agreement with data from our group, showing dramatic decreased numbers and degraded morphology of WFA-labeled PNNs in the entorhinal cortex of Alzheimer's disease patients (Figure 4, unpublished results). Other authors have suggested that aggrecan-containing PNNs may play a protective role against tau pathology in Alzheimer's disease [356].

8. ECM Pathology in Epilepsy

Epilepsy encompasses a spectrum of severe to benign brain disorders characterized by disturbances of the normal pattern of neuronal activity, causing unusual emotions, behaviors, sensations, or sometimes loss of consciousness, convulsions, and muscle spasms. Compelling evidence from animal models supports the involvement of the ECM in seizure disorders. The ECM undergoes extensive remodeling in response to seizures, including increased production of CSPGs by glial cells, and cleavage of CSPGs by MMPs [357–362]. As part of such ECM remodeling, PNNs are decreased, at least in part as a consequence to aggrecan cleavage by MMPs [357, 358]. It has been proposed that ECM remodeling may allow for synaptic reorganization, such as it occurs following seizures [36, 363]. Conversely, intriguing evidence suggests that ECM abnormalities may contribute to susceptibility to seizures. For example, enzymatic PNN digestion lowers the threshold for seizure induction [358]. Similarly, inhibition of MMP activity prevents seizure induction and PNN breakdown in an amygdala kindled seizure model [363]. Kainic acid-induced seizures trigger short term CSPG changes followed by more prolonged ones, resulting in altered neurocan and phosphacan levels in limbic brain regions; this latter phase coincides with increases of spontaneous recurrent seizures [357]. In addition, mice lacking the hyaluronan synthesizing enzyme Has3 present with reduced extracellular space and display increased epileptic activity [364]. Further evidence

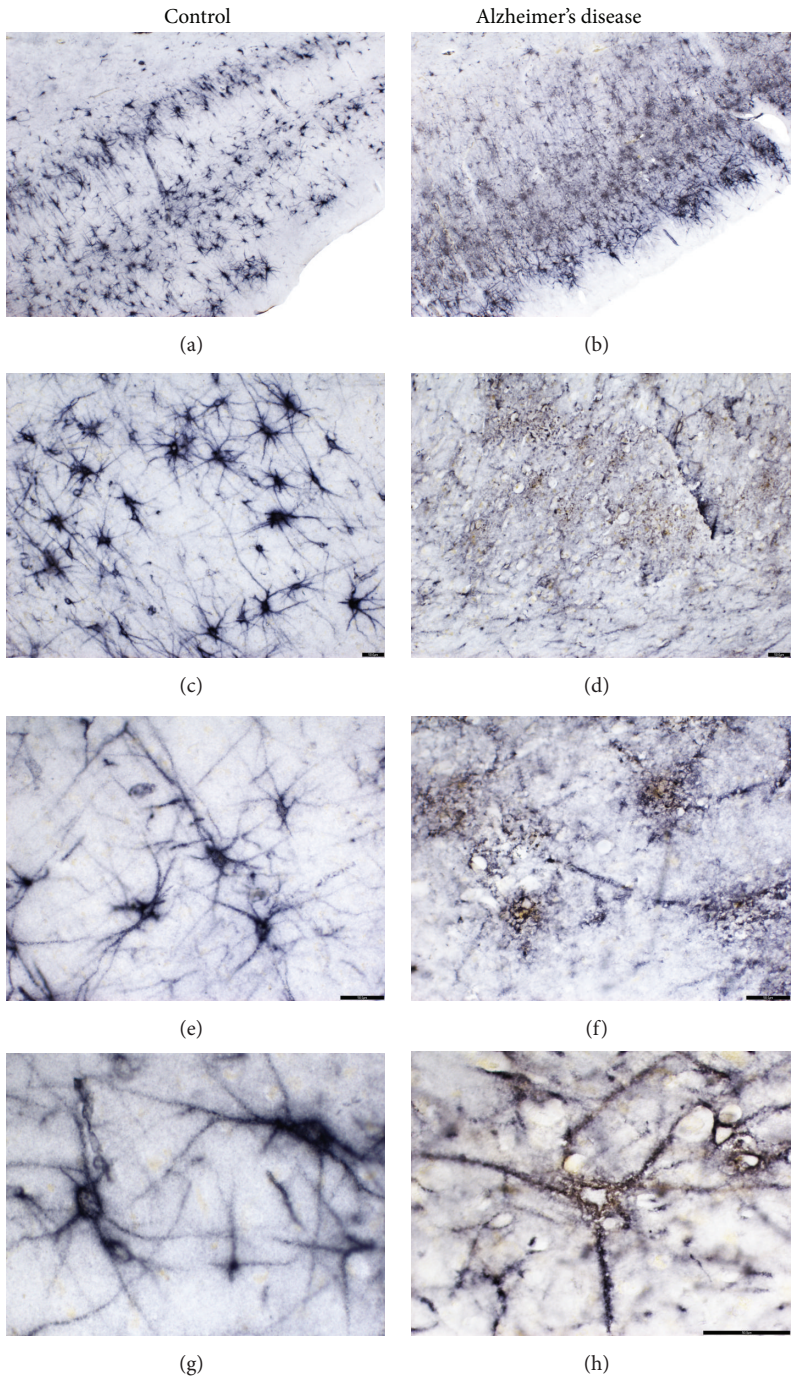

Figure 4: PNN structure is altered in Alzheimer's disease. Examples of WFA-labeled PNNs in the entorhinal cortex of healthy subjects (a, c, e, g) and subjects with Alzheimer's disorder (b, d, f, h). In healthy subjects, WFA-labeled PNNs are distributed across all layers of the ECx, with preferential concentration in layers II-III and layers V-VI (a). In subjects with Alzheimer's disease, WFA labeling appears to be more loosely distributed, in aggregates throughout the ECx, often suggestive of degraded PNNs (b, d, f, h).

that ECM abnormalities contribute to seizure susceptibility comes from studies on MMP-9. Levels of MMP-9 are increased in blood samples from subjects with epilepsy [365–367]. Mice lacking MMP-9 are less susceptible to induction of seizures, whereas rats overexpressing MMP-9 are more susceptible [368]. MMP-9 has been proposed as a potential therapeutic target for this disorder [365].

It is interesting to note that seizures are often comorbid with several of the disorders discussed above, including autism, schizophrenia, Fragile-X, and Rett syndrome, each presenting with ECM abnormalities. Particularly frequent in these disorders are altered levels of MMP-9 and PNN numbers [369–378]. Given the compelling relationship between ECM molecules and seizures, such comorbidity may not be

surprising and, on a speculative level, may point to partially shared mechanisms.

9. Conclusions

The role of PNNs, and more in general ECM, represents an emerging field in the pathophysiology of psychiatric disorders. Evolving in parallel with a growing understanding of the role of the ECM in the regulation of synaptic plasticity, this field is beginning to integrate the concept of the quadripartite synapse in hypotheses on the pathophysiology of synaptic dysregulation in these disorders. Overlapping patterns of ECM abnormalities, in disorders that also share clinical features and synaptic deficits, may underlie common "end-point" mechanisms; that is, anomalies affecting one or more elements of functionally similar molecular families in each of these disorders may lead to convergent effects on synaptic functions and, potentially, clinical domains. Cell and regional specificity may be determined by nonoverlapping pathological aspects in each disorder, as well as by neurodevelopmental determinants specifying the age range at which these anomalies become pathologically relevant.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Reorganization of Synaptic Connections and Perineuronal Nets in the Deep Cerebellar Nuclei of *Purkinje Cell Degeneration* Mutant Mice

M. Blosa,[1] C. Bursch,[1] S. Weigel,[1] M. Holzer,[1] C. Jäger,[1] C. Janke,[2] R. T. Matthews,[3] T. Arendt,[1] and M. Morawski[1]

[1]*Paul Flechsig Institute of Brain Research, University of Leipzig, Liebigstraße 19, 04103 Leipzig, Germany*
[2]*Institut Curie, Centre de Recherche, CNRS UMR3348, Centre Universitaire, 91405 Orsay, France*
[3]*Department of Neuroscience and Physiology, Sunny Upstate Medical University, 750 East Adams Street, Syracuse, NY 13210, USA*

Correspondence should be addressed to M. Morawski; morm@medizin.uni-leipzig.de

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The perineuronal net (PN) is a subtype of extracellular matrix appearing as a net-like structure around distinct neurons throughout the whole CNS. PNs surround the soma, proximal dendrites, and the axonal initial segment embedding synaptic terminals on the neuronal surface. Different functions of the PNs are suggested which include support of synaptic stabilization, inhibition of axonal sprouting, and control of neuronal plasticity. A number of studies provide evidence that removing PNs or PN-components results in renewed neurite growth and synaptogenesis. In a mouse model for Purkinje cell degeneration, we examined the effect of deafferentation on synaptic remodeling and modulation of PNs in the deep cerebellar nuclei. We found reduced GABAergic, enhanced glutamatergic innervations at PN-associated neurons, and altered expression of the PN-components brevican and hapln4. These data refer to a direct interaction between ECM and synapses. The altered brevican expression induced by activated astrocytes could be required for an adequate regeneration by promoting neurite growth and synaptogenesis.

1. Introduction

The function of the nervous system is based on a precise composition and maintenance of a neuronal and synaptic network. The connectivity of the brain is formed during a period of enhanced plasticity in development when appropriate synaptic connections are stabilized in an activity dependent manner. In contrast, once the adult connectivity is established, plasticity of some synaptic contacts is greatly diminished. Functional alterations as they occur in many brain disorders are also accompanied by remodeling of neuronal structures, changes in neuronal activity, and loss of neuronal molecules [1–3]. A number of studies demonstrated that several extrinsic [4–7] and intrinsic [1–3, 8, 9] changes are associated with alterations in synaptic density or shape, dendritic outgrowth, and even extracellular matrix molecules. Especially a specialized form of the extracellular matrix, the perineuronal net, often shows alterations in neurodegenerative diseases [8–11] and acute brain injuries [7, 11–15] and is suggested to prevent regeneration. These perineuronal nets (PNs) enclose the cell bodies and the proximal dendrites of specialized neurons thereby embedding the contacting synaptic boutons [16–18]. PNs are composed of aggregating chondroitin sulphated proteoglycans (CSPGs), hyaluronan, hyaluronan binding link proteins (hapln), and tenascin-R [19–22]. CSPGs of PNs belong to the lectican family including the main members aggrecan, brevican, and neurocan, while aggrecan is prominently detected in PNs [23, 24]. Most of the PN-components are produced by neurons and glial cells, but a few constituents are made by only one of these cell types [25, 26]. PNs are involved in organizing extracellular space, modulating synaptic plasticity, and providing a special extracellular ionic milieu and synaptic stabilization [16, 27–32]. The formation and maintenance of PNs in a number of

systems are activity dependent [31, 33–36]; thus they mainly occur at highly active neurons and altered activity disrupts PN formation [27, 34, 35, 37–42]. To analyze the potential role of PNs in degeneration/regeneration of slow denervation processes and to analyze the declining influence of synaptic input on PNs we use a mouse model for Purkinje cell degeneration (*pcd, pcd-3j/J model*). The pathology is caused by a mutation of the Nna1 gene [43, 44] encoding a protein also known as cytosolic carboxypeptidase 1 (CCP1) [45, 46], which has been demonstrated to be involved in the enzymatic deglutamylation of proteins, and in particular of tubulin [47]. It was therefore suggested that neurodegeneration in the *pcd* mouse is induced by a hyperglutamylation of microtubules in the affected neurons. In a rescue experiment the depletion of the tubulin tyrosine ligase-like protein 1 (TTLL1) [48] could partially prevent degeneration of the Purkinje cells (PCs) [47].

The PCs as part of the cerebellum are involved in motor coordination and posture control; as consequence in the pcd-3j/J model a loss of PCs leads to a moderate ataxia beginning at 3-4 weeks of age [43]. In addition, the degeneration of PCs is accompanied by the loss of cerebellar granule neurons [43, 49], olfactory mitral cells [50], some thalamic neurons [43], and alterations in retinal photoreceptors [50, 51]. Before PCs degenerate, which starts ~P18 and proceeds until ~P45, the PCs and their synaptic contacts show a normal development [52].

The GABAergic PCs receive virtually all input from within the cerebellum and provide the exclusive output of the cerebellar cortex, mainly inhibiting neurons of the deep cerebellar nuclei (DCN). The cells of DCN are a heterogeneous population of inhibitory and excitatory neurons [53–57], but only the large excitatory DCN neurons are surrounded by the condensed specialized extracellular matrix of PNs [25, 58–60]. However, it was repeatedly demonstrated that PN-associated neurons are protected against different neurotoxic insults and degenerative processes while neurons without a PN are not [8, 13, 61, 62].

Here we are investigating the integrity and expression of PNs and their components as well as the synaptic innervation and remodeling of DCN neurons after the degeneration of their main GABAergic input, the PC axons. The PN-associated DCN neurons showed an imbalance of inhibitory and excitatory innervations. We found a reduced GABAergic synaptic input and simultaneously these neurons receive an increased glutamatergic input. Further, the cytochemical analyses showed that the molecular composition of PNs has changed and revealed that brevican and hapln4 are prone to the degeneration processes and may influence the regeneration of the injured tissue.

2. Experimental Procedures

2.1. Animals. Data were collected from 11 pcd3j (C57BL/6-Agtpbp1$^{pcd\text{-}3j}$/J, Stock # 003237) knock out mice and 11 wild type (*wt*) littermates (6 mice of each genotype for immunocytochemistry and 5 mice of each genotype for biochemistry) of both types of sex at the age of 4 months. Animals were genotyped as juveniles by PCR as described on *The Jackson Laboratory's* website (Genotyping protocol database of the Jackson Laboratory). They had free access to food and water and were maintained on a 12/12 h light-dark cycle under conditions of constant temperature (22°C). All animals used in this study were treated in agreement with the German law on the use of laboratory animals. The ethical guidelines of the laboratory animal care and use committee at the University of Leipzig were followed.

2.2. Cytochemistry. The animals were deeply anesthetized with CO_2 and perfused transcardially with 10 mL 0.9% NaCl following 100 mL fixative containing 4% paraformaldehyde and 0.1% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Brains were removed and postfixed in the same fixation solution overnight. The tissue was cryoprotected in 30% sucrose with 0.1% sodium azide, cut in 30 μm thick slices with a cryomicrotome in frontal planes, and collected in phosphate buffer containing 0.1% azide.

Before staining, tissue was pretreated with 60% methanol containing 2% H_2O_2 for 20 min followed by a blocking step with a blocking solution containing 2% BSA, 0.3% milk powder, and 0.5% donkey serum in phosphate buffer for 1 h. All the antibodies (see Table 1) were incubated in blocking solution overnight at 4°C. The visualization was performed by standard fluorescent secondary antibodies (see Table 2). Identification of the investigated brain areas was made by brain atlas of mouse [72].

2.3. Imaging Procedures. Tissue sections were examined with a Zeiss Axiovert 200 M microscope (Zeiss, Jena, Germany) and a Zeiss confocal laser scanning microscope (Zeiss, Jena, Germany; LSM 510 meta). Confocal images of carbocyanine dye 2 (Cy2) fluorescence were obtained with the Argon laser (488 nm) and emission filter BP 505–530. The HeNe 1 laser (543 nm) and the emission filter BP 560–615 were used to detect the carbocyanine dye 3 (Cy3) fluorescence, respectively. Photoshop CS2 (Adobe Systems, Mountain View, CA, USA) was used to process the images with minimal alterations to the contrast and background.

2.4. Quantification. To estimate molecular alterations in *pcd* mice frontal sections were investigated and PN-bearing neurons of *nucleus interpositus* and *nucleus dentatus* of the DCN and the *nucleus vestibularis lateralis* were analyzed. The sections were labeled with anti-human aggrecan antibody (HAG7D4), the most comprehensive marker for PNs and additionally with GAD65/67 or vGlut1 and 2 for double immunofluorescence counting. The tissue was analyzed with a Zeiss Axiovert 200 M microscope equipped with a motorized stage (Märzhäuser, Germany) with MosaiX software and by means of a CCD camera (Zeiss MRC) connected to an Axiovision 4.6 image analysis system (Zeiss, Germany).

Counts were performed using the optical fractionator method [19, 37] on a Zeiss Axioskop 2 plus microscope (Jena, Germany) equipped with a motorized stage (Märzhäuser, Wetzlar, Germany), a Ludl MAC 5000 controller (LEP,

Table 1: Antibodies and markers.

Marker	Detected Component	Source	Dilution		Reference
			IHC	WB	
PN-constituents					
Human antiaggrecan (HAG, clone 7D4)	N-terminal aggrecan, core protein (AGG)	Serotec	1 : 10		[61]
Antiaggrecan (AB1031)	Amino acids 1177–1326 of mouse aggrecan	Millipore	1 : 200		[63]
Antibrevican (B50)	50 kDa cleavage fragment of brevican, brain enriched hyaluronan-binding protein	Dr. R. Matthews	1 : 2000	1 : 2000	[64]
Antibrevican (756)	Mainly 90 kDa cleavage fragment of brevican, brain enriched hyaluronan-binding protein	Dr. R. Matthews	1 : 1000		
Antibrevican (610894)	80 kDa N-terminal fragment and full length up to 145 kDa	BD Bioscience	1 : 1000	1 : 1250	[65]
Biotinylated hyaluronic Acid Binding Protein (bHABP)	Hyaluronan	Cape Cod	1 : 100		[66]
Antihyaluronan and proteoglycan link protein 1 (HAPLN1/Crtl-1)	NS0-derived rhHAPLN1	R&D Systems	1 : 400	1 : 1000	[67]
Antihyaluronan and proteoglycan link protein 4 (HAPLN4)	NS0-derived recombinant human HAPLN4. Gln30-Val402	R&D Systems	1 : 500	1 : 1000	
anti-tenascin-R (clone 619)	Protein backbone of tenascin-R	R&D Systems	1 : 100		[67]
Glial marker					
Glial fibrillary acid protein (GFAP)	a 50 kDa intracytoplasmic filamentous protein in astrocytes	Dako	1 : 1000	1 : 3000	[68]
Transmitter					
Antivesicular glutamate transporter 1 (vGlut1, #135304)	Glutamate transporter 1 in the membrane of synaptic vesicles	Synaptic Systems	1 : 500	1 : 5000	[69]
Antivesicular glutamate transporter 2 (vGlut2, #135404)	Glutamate transporter 2 in the membrane of synaptic vesicles	Synaptic Systems	1 : 500		[69]
GAD65/67	C-terminal region of human GAD 65 and GAD 67	Sigma	1 : 5000	1 : 15000	[61]
Calcium binding Proteins					
Anticalbindin (AB1778)	Recombinant calbindin	Millipore	1 : 1000		[70]
Cytoskeletal proteins					
Anti-β-actin	β-cytoplasmic actin N-terminal peptide	Sigma-Aldrich		1 : 10000	[71]

Table 2: Used secondary antibodies.

Antibody	Marker	Dilution	Source
Streptavidin	Cy3	1 : 250	Dianova
Donkey-anti-mouse	Cy2, Cy3	1 : 1000	Dianova
Donkey-anti-guinea pig	Cy3	1 : 1000	Dianova
Donkey-anti-rabbit	Cy2, Cy3	1 : 1000	Dianova
Donkey-anti-rabbit	HRP	1 : 10.000	DAKO
Donkey-anti-mouse	HRP	1 : 10.000	DAKO
Rabbit-anti-guinea pig	HRP	1 : 10.000	GE Healthcare
Rabbit-anti-goat	HRP	1 : 10.000	GE Healthcare

Hawthorne, NY, USA), and a digital camera CX9000 (MicroBrightField, Williston, VT, USA). Stereo Investigator software 6 (MicroBrightField, Williston, VT, USA) was used to analyze the 30 μm thick sections.

The contours of the DCN were outlined in the Stereo Investigator program using a 10x lens, cell diameter determination and synapse counts were performed using an oil-immersion 63x lens (1.4 numerical aperture).

Somatic boutons were counted from the cell surface of the large glutamatergic PN-bearing projection neurons (≥10 μm diameter) up to a distance of 3 μm in the periphery. For quantification of the boutons in the periphery, the cells were outlined at a distance of twofold diameter of the cell from the cell surface. Peripheral boutons were counted from the end of the somatic zone (≥3 μm) to the outlined area.

2.5. SDS-PAGE and Western Blot Analyses. Mice were deeply anesthetized with CO_2, decapitated, brains rapidly removed, and immediately frozen in liquid nitrogen. On a dry-ice cooled work plate the brains were cut in 2 mm frontal sections and DCN as precise as possible separated and stored in 2 mL tubes at −80°C until further proceeding. The DCN containing tissues of 5 mutant and 5 *wt* mice were homogenized using an Ultra-Turrax tube drive (IKA), in homogenization buffer (20 mM Tris-HCl, 2 mM EDTA, 0.15 M NaCl, 5 mM NaF, 1 mM Na_3VO_4, and 2 mM $MgCl_2$, pH 7.4) containing a protease inhibitor (Complete, Roche, Mannheim, Germany). The homogenate was centrifuged at 10.000 ×g for 14 min at 4°C, followed by determination of the protein concentration in the supernatant by using the BCA Assay. For discontinuous SDS-Page the supernatant containing 35 μg proteins was mixed with 1x SDS sample buffer and denaturized at 70°C for 15 min. The proteins were separated on a 10% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Perkin Elmer, Rodgau, Germany). Blots were blocked with 1% BSA in Tris-buffered saline containing 0.05% tween for 1 h, washed, and incubated with primary antibodies (Table 1) diluted in blocking solution overnight at 4°C. Blots were washed and incubated with HRP-conjugated secondary antibodies (Table 2) for 1 h. HRP activity was detected using ECL Western blotting (Amersham Biosciences) and scanned with DNR Bio-Imaging System and analyzed by using software TINA. The ratios of optical density of the investigated proteins were normalized to β-actin.

2.6. Statistical Analyses. Statistical analysis was performed with SigmaPlot 12.5 (Systat Software, Erkrath, Germany). Values are given as mean ± SEM. For statistical differences between the two genotypes we used *t*-test or Mann-Whitney rank sum test, depending on the distribution of the data.

3. Results

The Purkinje cell degeneration (*pcd*-3j/J) mutant mouse is characterized by the loss of PCs and their axons. The neurons of the DCN and lateral vestibular nucleus (LVN), which are innervated by the cerebellar PCs, are affected as well. In immunohistochemical and biochemical investigations, we observed that the degeneration leads to altered synaptic innervation and ECM conformation in the target areas.

3.1. Calbindin D-28k in pcd Mice. Calbindin *D-28k* is typically used as a marker for the PCs of the cerebellum [20]. The closely spaced somas and axons of the PCs are strongly labeled by antibodies against calbindin. In *pcd* mice this calbindin immunoreaction is significantly reduced. Only very few remaining cells are stained already at one month of age (Figure 1).

3.2. Purkinje Cell Degeneration in the Cerebellum Leads to Reduced GABAergic and an Increase of Glutamatergic Synapses in DCN and LVN. Similar to DCN neurons the neurons of the LVN are highly innervated by the GABAergic Purkinje axons. In addition, they receive excitatory input from mossy fibers and climbing fibers [21]. Previous studies demonstrated that pcd is accompanied by a volume reduction and a decrease in cell number in the DCN and LVN with focusing onto inhibitory neurons [22, 73–75]. Thus, we studied if the degeneration of PCs in the cerebellar cortex modifies the terminating synapses of the afferent fibers at the large excitatory PN-bearing projection neurons in the DCN and LVN. Therefore, we investigated the GABAergic terminals by anti-GAD65/67 antibody labeling and the majority of glutamatergic terminals labeled by a mixture of anti-vGlut1 and 2 antibodies at the PN-ensheathed neurons. The different nuclei (DCN, LVN) show similar distribution of these markers. Hence, the data of the nuclei were pooled.

We could identify a high density of GABAergic terminals at the cell surface (up to 3 μm distance) as well as in the periphery (≥3 μm; for details see Section 2) of each PN-bearing neuron. The number of GABAergic synapses at large PN-ensheathed neurons of *pcd* mice was significantly lower than in the *wt* mice (Figures 2(a) and 3(a); somatic boutons: *wt*: 17.34 ± 0.58 and *pcd*: 10.27 ± 0.41; Mann-Whitney U $p < 0.0001$; peripheral boutons: *wt*: 18.18 ± 0.83; *pcd*: 10.44 ± 0.55; Mann-Whitney U $p < 0.0001$). There were no differences between the densities of somatic versus peripheral GABAergic boutons in both genotypes. Western blot analyses of whole DCN tissue homogenates with anti-GAD65/67 antibody identified the typical molecular weight bands at 65 and 67 kDa, respectively, though the quantification of the GAD band of *wt* and *pcd* showed no

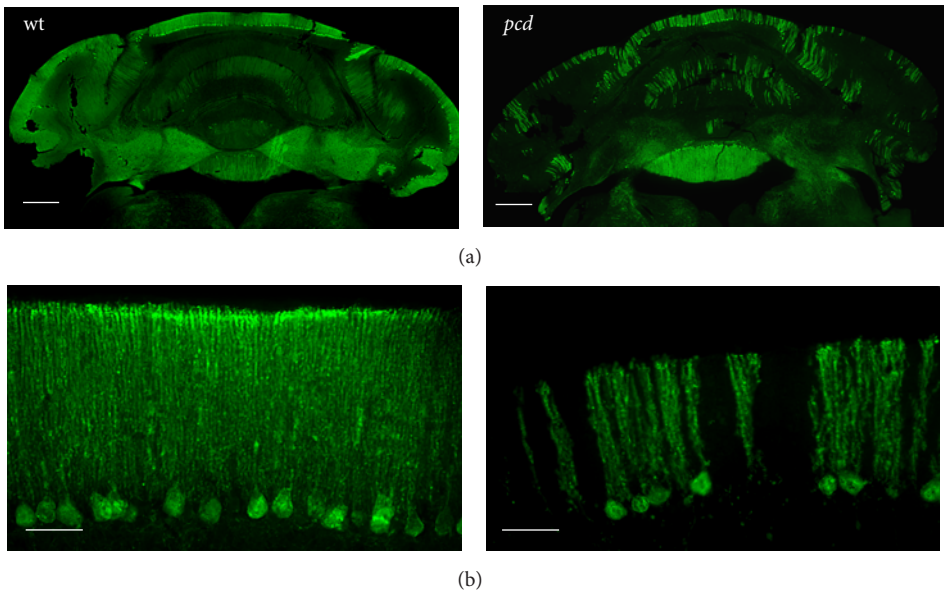

Figure 1: Labelling of calbindin expressing PC neurons in *wt* and *pcd* mice. Purkinje cells and their axons in the cerebellum show a strong immunoreactivity for calbindin. In one month old *wt* anti-calbindin antibodies detect the Purkinje cells and their axon in the cerebellum. The neurons of the cerebellum in one-month-old *pcd* mice reveal less calbindin immunoreactivity. Scale bar: 100 μm (a) and 50 μm (b).

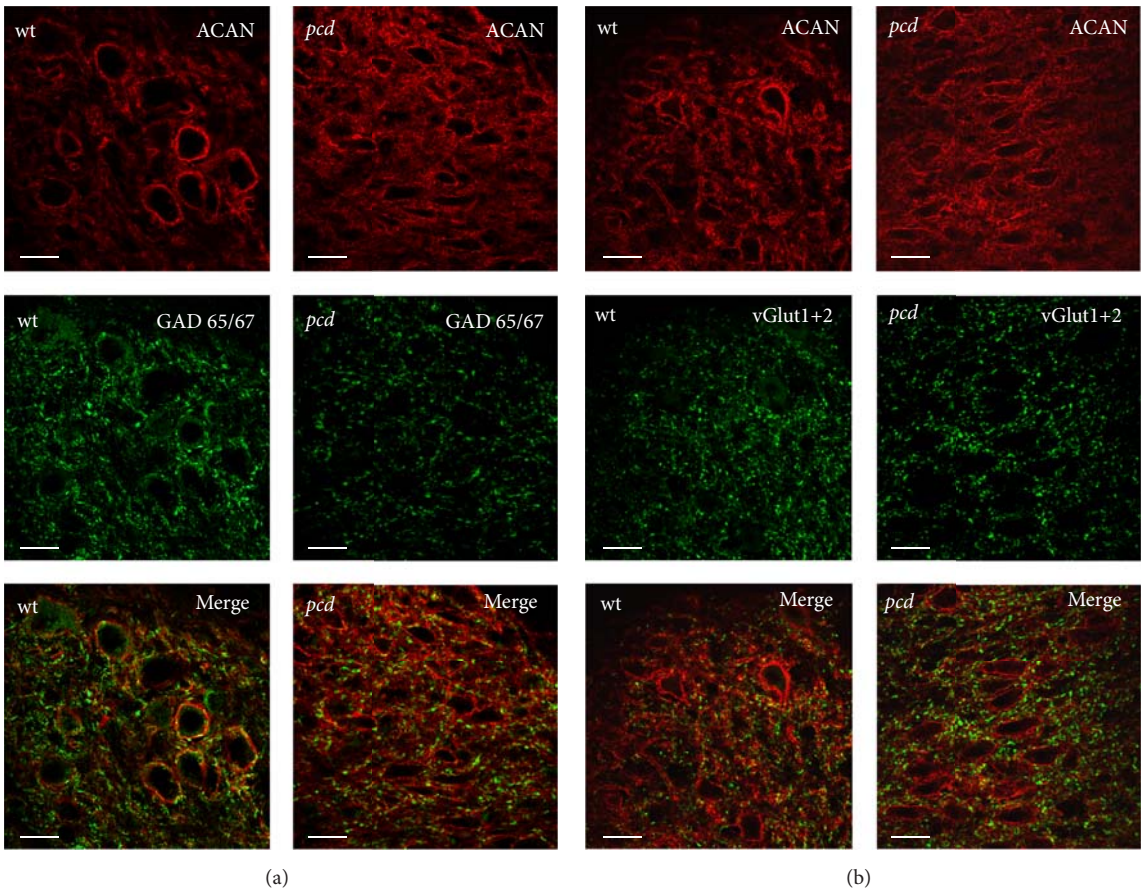

Figure 2: Detection of glutamatergic and GABAergic terminals in DCN. The large DCN neurons are enwrapped by aggrecan-based ECM (red). (a) DCN neurons are innervated by GABAergic boutons, labeled by GAD 65/67. GABAergic terminals seem to be reduced in *pcd*. (b) The glutamatergic boutons at DCN neurons are discovered by moderate vGlut 1 and vGlut 2 staining. The staining in *pcd* appears slightly enhanced. Scale bar: 20 μm.

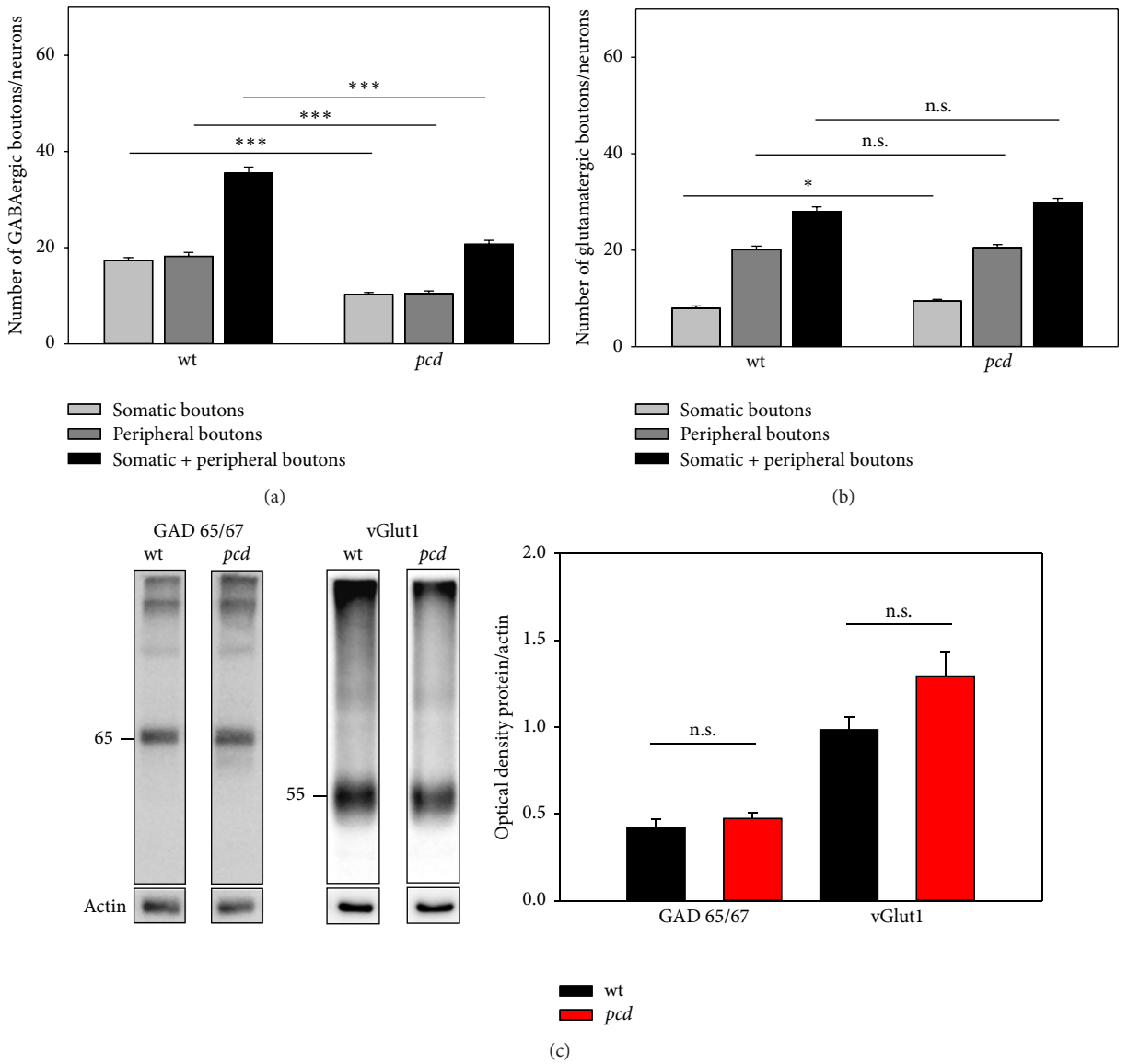

Figure 3: Quantification of GAD65/67 and vGlut in DCN. (a) Quantification shows the distribution of GABAergic terminals in different distances from the PN-bearing neurons. The total number of boutons are reduced in *pcd* compared to *wt*, regardless of the distance. (b) Somatic glutamatergic terminals at DCN neurons appear to be enhanced in *pcd* mice. The peripheral synapses remain unaffected by the insult. (c) Western blot analyses of GAD65/67 and vGlut1 with protein extracts of DCN sections. Typical specific bands are visible in both genotypes. Quantification of these bands reveals slight but no significant differences between *wt* and *pcd* (GAD65/67 $p = 0.419$; vGlut1 $p = 0.087$). Data are given as mean ± SEM.

significant differences (Figure 3(c); *wt*: 0.423 ± 0.05 and *pcd*: 0.473 ± 0.03 t-test $p = 0.419$).

Beside the inhibitory innervation, the DCN neurons receive excitatory glutamatergic input from collaterals from the mossy and climbing fibers [21, 76]. Immunolabeling of the glutamatergic synapses at PN-bearing neurons reveals that the majority of glutamatergic boutons is not directly located at the soma of these neurons, but rather in their periphery, about ≥3 μm away from the cell body. By quantification of somatic and peripheral terminals, we could verify the differences between somatic and peripheral density of the glutamatergic synaptic terminals (Figure 3(b)). The density of somatic glutamatergic synapses in DCN and LVN of *pcd* mice is increased, while the number of peripheral terminals is not altered (Figures 2(b) and 3(b); somatic boutons: *wt*: 7.93 ± 0.51 and *pcd*: 9.43 ± 0.38; Mann-Whitney U $p = 0.010$; peripheral boutons: *wt*: 20.08 ± 0.77; *pcd*: 20.50 ± 0.67; Mann-Whitney U $p = 0.084$). In addition, the quantification of the 55 kDa anti-vGlut1 immunoreactive band in western blot confirmed an increase of vGlut1 in *pcd* mice without reaching significance (Figure 3(c); *wt*: 0.985 ± 0.07 and *pcd*: 1.295 ± 0.14; t-test $p = 0.087$).

3.3. ECM Composition in DCN of pcd Mutant Mice. The axons of the Purkinje cells are the sole output of the cerebellar cortex and innervate the neurons of the DCN. The DCN

mainly contain 2 types of neurons: large excitatory and smaller inhibitory neurons [53, 77]. As previously described, the large neurons of the DCN are ensheathed by very prominent PNs [25, 58]. The DCN neurons in *wt* mice express the major ECM components aggrecan, brevican, neurocan, tenascin-R, hyaluronan, and hapln [25].

3.3.1. Brevican. Recently it was shown that the proteoglycan brevican is enriched at perisynaptic sites and is suggested to be associated with synaptic molecules [18, 78]. Brevican has a metalloproteinase specific cleavage site and can occur as 50 and 80/90 kDa cleavage product and as full length protein of 145 kDa with no chondroitin sulfate (CS) and the CS-bearing variant of over 245 kDa [14, 64]. We investigated the incidence of the cleavage products and the CS-free type of full-length brevican *pcd* mice. For immunocytochemistry, three different antibodies against brevican were used: anti-brevican (BD Bioscience, FL) which detects the full length and the cleavage products, anti-B50 detecting exclusively the 50 kDa cleavage product of brevican and anti-B756, which detects mainly the 90 kDa and the full length isoform. In *wt* DCN immunostaining with all brevican antibodies clearly revealed an immunoreactivity around the large DCN neurons and illustrates the typical brevican-based PN structure surrounding soma and proximal dendrites. In *pcd* mice the DCN neurons show only very weak anti-B50 immunoreaction. Neurons and dendrites are still surrounded by faint immunoreactivity, whereas neurons in nontarget areas of PC axons like the cochlear nucleus (CN) are not affected and show the typical brevican-based PN structure (Figure 4(b)). The FL and B756 antibodies show similar intensities of immunoreactivity in the *pcd* mice. In the DCN not only the neuronal surface is detected, but the whole extracellular space reveals a slight and uniform immunoreactivity. As mentioned above, the neurons of the CN, as an internal reference, show no alterations in the immunoreactivity with FL and B756. The neurons and proximal dendrites still display the typical brevican-based PN structure in *pcd* mice (Figures 4(a) and 4(c)). To further clarify if the altered immunodetection of brevican in *pcd* mice is caused by reduced protein expression we investigated the protein levels of full length and the 50 kDa cleavage product of brevican by western blot analyses. Surprisingly, a significant increased protein level of brevican could be detected in both the full length as well as the 50 kDa cleavage product in the DCN containing tissue of *pcd* mice compared to *wt* mice. The brevican protein amount in the *pcd* mice almost reached a 2-fold increase compared to *wt* mice (Figure 4(d), BCAN: *wt*: 0.599 ± 0.04 and *pcd*: 1.180 ± 0.06; t-test $p < 0.001$).

3.3.2. Link Proteins. Link proteins are known to interact with hyaluronan and CSPGs and stabilize this connection. Hapln1 (Crtl-1) and hapln4 are the two link proteins which are associated with PNs and exclusively expressed by PN-bearing neurons [25, 26, 59]. It is supposed that hapln1 is an important component in PN formation. The upregulation of hapln1 expression correlates with PN development and hapln1 deficient mice showed attenuated PNs [32].

The PN of the large excitatory DCN neurons of *wt* mice is characterized by a strong aggrecan staining and a comparably intensive staining by hapln1. The labeling of both PN-components, aggrecan and hapln1, is for the most part congruent and they seem to be colocalized. In *pcd* mice the detection of aggrecan and hapln1 is less intensive but still clearly present (Figure 5(a)). The immunoreactions of both components appear largely colocalized, although the immunoreactions appear to be redistributed away from the nets into the neuropil and extracellular space, which might be in agreement with immunoblot analyses revealing increased occurrence of hapln1 (Figure 5(c), hapln1: *wt*: 4.164 ± 0.6 and *pcd*: 8.165 ± 0.6; t-test $p = 0.001$).

Hapln4 immunodetection also marks PN-bearing neurons in the DCN of *wt* mice. The large DCN neurons are surrounded by delicate hapln4 staining and the labeling is colocalized with the aggrecan immunoreaction. The DCN neurons of *pcd* mice are immunopositive for aggrecan, but virtually no hapln4 immunoreaction is detectable (Figure 5(b)). In contrast, on western blots the hapln4 protein, in DCN enriched homogenate, is slightly significantly elevated (Figure 5(c), hapln4: *wt*: 0.424±0.04 and *pcd*: 0.637± 0.05; t-test $p = 0.014$).

3.3.3. Hyaluronan and Tenascin-R. Hyaluronan is a very large linear polymer and is supposed to be the backbone of PNs. To visualize hyaluronan in the DCN we used biotinylated hyaluronan binding protein (HABP). Hyaluronan shows a ubiquitous distribution in the DCN of *wt* and *pcd*, with a typical elevated reactivity around PN-bearing neurons, and colocates with aggrecan immunoreaction in PNs (Figure 6(a)). Immunolabeling with tenascin-R reveals similar staining patterns in DCN of both genotypes and show no obvious differences between transgenic and *wt* mice (Figure 6(b)).

3.4. Gliosis in DCN after Purkinje Cell Degeneration. Lesions and injuries are often followed by gliosis and an increased expression of glial proteins and strong formation of glial structures [79], which replace the degenerated tissue and lost cellular structures. The distribution of astrocytes and their expression level were analyzed with anti-GFAP antibodies, an astrocytic marker. The double immunolabeling with anti-aggrecan showed that the *wt* DCN bears only a few GFAP positive astrocytes. In *wt* astrocytes only appear at the edge of the DCN, while the complete DCN of *pcd* mice are marked by a massive glia invasion (Figure 7(a)). These results agreed with the enhanced expression level of GFAP in *pcd* mice and support the assumption that the degeneration of the PCs leads to a strong gliosis in their target area (Figure 7(b), GFAP: *wt*: 0.785 ± 0.03 and *pcd*: 2.086 ± 0.26; Mann-Whitney U $p = 0.008$).

4. Discussion

4.1. Synaptic Input. DCN and LVN neurons are the direct targets of the cerebellar Purkinje cell axons. After their degeneration at an age when nearly all PCs degenerated, the GABAergic terminals in the target regions are significantly

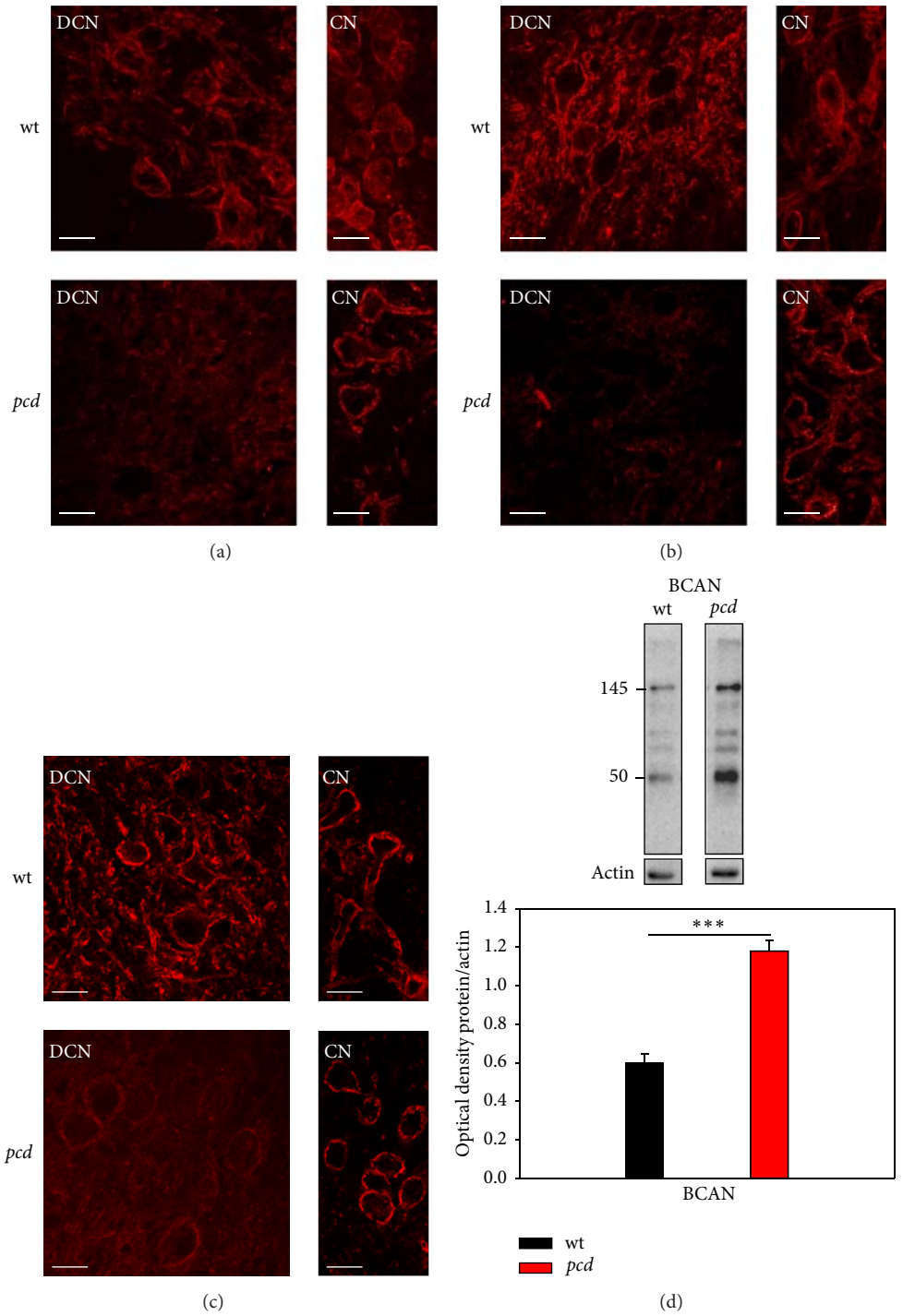

Figure 4: Detection of different brevican fragments. (a) Immunoreaction with pan-specific brevican antibodies (BD Bioscience, FL) clearly surrounds DCN neurons in *wt* but not in *pcd* mice. As internal control nontarget region of PC axons, the cochlear nucleus shows no alterations in immunoreactions with FL. (b) The 50 kDa isoform of brevican seems to be nearly absent around DCN neurons of *pcd* mice, whereas the not affected region (CN) revealed brevican-bearing neurons. (c) The B756 antibodies detect mainly the 80/90 kDa and full length isoforms of brevican. These cleavage products aggregate around the neurons in DCN of *wt* mice and seem to be integrated in PNs. In *pcd*, PNs appear with lower intensity, but with potential higher parenchymatic reaction. PN-detection with all three antibodies in the internal nontarget control region (CN) is unchanged. Scale bar: 20 μm. (d) Biochemical detection of brevican with SDS-PAGE with pan-specific antibodies revealed most known isoforms at 50, 80, 90, and 145 kDa. Quantification of the 50 and 145 kDa brevican isoform showed a significant increased protein expression in *pcd* ($p < 0.001$), respectively, for the different isoforms. Therefore, the diagram is supposed to display the optical density (OD) values of pan-brevican chemiluminescent signal summed values (OD 50 kDa + 145 kDa brevican/actin). Data are given as mean ± SEM.

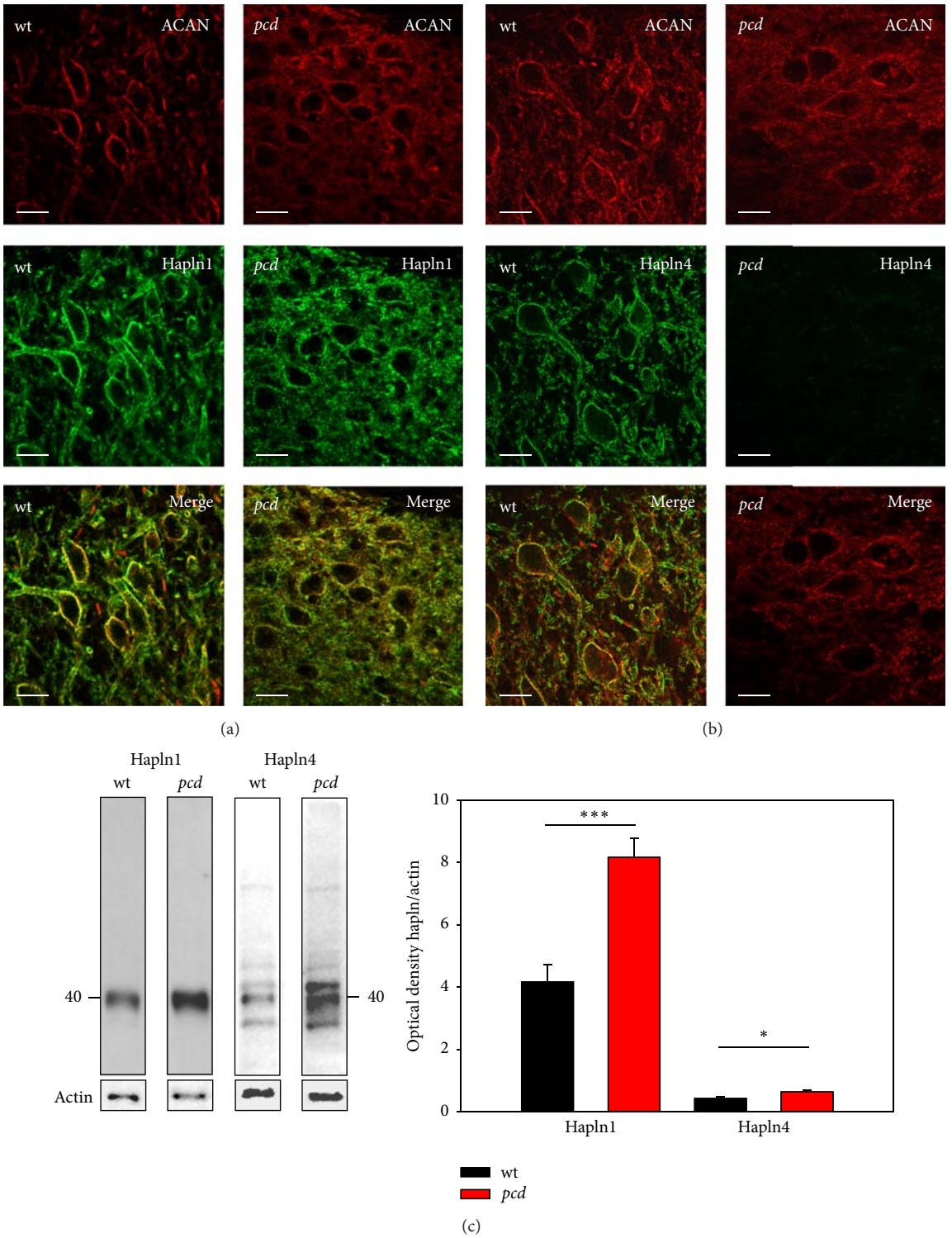

FIGURE 5: Comparison of link protein expression in DCN. DCN neurons are visualized by aggrecan immunoreaction (red). (a) Hapln1 labeling (green) surrounds the DCN neurons in both genotypes, matching the aggrecan immunoreactivity; additionally in *pcd* hapln1 immunoreaction is distributed throughout the whole parenchyma. (b) Hapln4 (green) encloses the DCN neurons in *wt* mice. In contrast, hapln4 in *pcd* exhibits virtually no immunoreaction. Scale bar: 20 μm. (c) Western blot reveals protein bands at approximately 40 kDa for link proteins. Quantification of the link proteins yielded an elevated protein level of both components in *pcd* (hapln1 $p < 0.01$; hapln4 $p < 0.05$). Data are given as mean ± SEM.

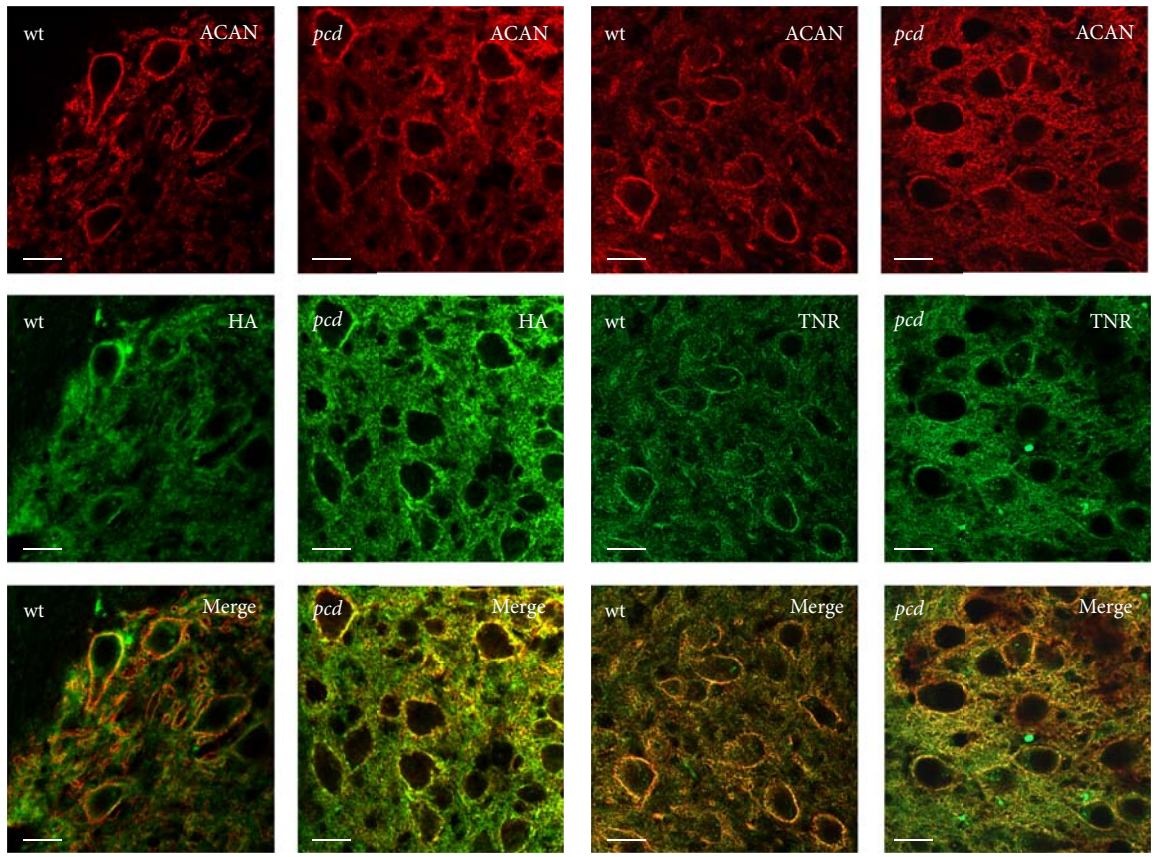

Figure 6: Distribution of hyaluronan and tenascin-R in DCN. The labeling shows important constituents of PNs. The neurons in DCN are surrounded by strong aggrecan immunoreaction (red). (a) Hyaluronan is ubiquitously distributed and concentrated around the neurons. (b) Tenascin-R is equally present in DCN of *wt* and *pcd* mice and mainly encloses the neurons. Scale bar: 20 μm.

reduced [22, 43, 80]. At PN-bearing neurons in DCN and LVN, a subset of large excitatory neurons, the GABAergic terminals are affected as shown by previous investigators [22, 80]. Terminals at the PN-positive neurons are decrease down to 40% independent from terminal localization; somatic terminals as well as peripheral boutons are similarly reduced. In contrast, glutamatergic terminals are increased at PN-bearing neurons after pcd. The degeneration of the PCs and the granule cells in the cerebellar cortex seems to result in a significant reorganization of the synaptic input (reviewed in [75]). Glutamatergic synapses in DCN mainly derive from mossy fibers, which additionally innervate granular cells in cerebellar cortex [81]. Strazielle et al. postulated for another *pcd* model that the loss of Purkinje and granule cells leads to enhanced mossy fiber innervations at DCN neurons [82]. The *pcd-3j/J* model used in this study is also accompanied by an additional decline of cerebellar granule cells. Hence, a similar modification could take place in the DCN of the *pcd* mice and might explain the increased glutamatergic innervations. The excitatory input of the LVN derives mainly from the fastigial nucleus of the DCN [83]; thus the enhanced glutamatergic projection in LVN might be a secondary effect of the lacking inhibition in the DCN neurons. The missing inhibitory innervation and the increased excitatory input might be interpreted as an altered activity of these neurons that could provide an explanation for the ataxic motion. However, in the VN of *pcd* mutants neither the spontaneous activity nor the evoked activation of the neurons are altered ([84, 85] reviewed in [75]). This is in agreement with the observation of axonal sprouting with flat vesicle terminals at the *pcd* DCN neurons [80], which are known to represent inhibitory synapses [86]. The lost GABAergic contacts could be replaced by new non-GABAergic terminals. The massive gain and enhancement of glycinergic boutons observed in DCN and VN could maybe balance the deafferentation of GABAergic axons [74, 80]. Glycine seems to play a predominant role in inhibition and modulates the excitation of DCN neurons; this could temper the symptomatology of the mutants.

4.2. Modifications in PN Formation after pcd. The large neurons in DCN are surrounded by PNs composed of hyaluronan, CSPGs, tenascin-R, and link proteins hapln1 and hapln4 [25, 59]. Most of the PN-components are produced by neurons and several are synthetized by glia as well. Decline of Purkinje cells and consequently deafferentation of DCN

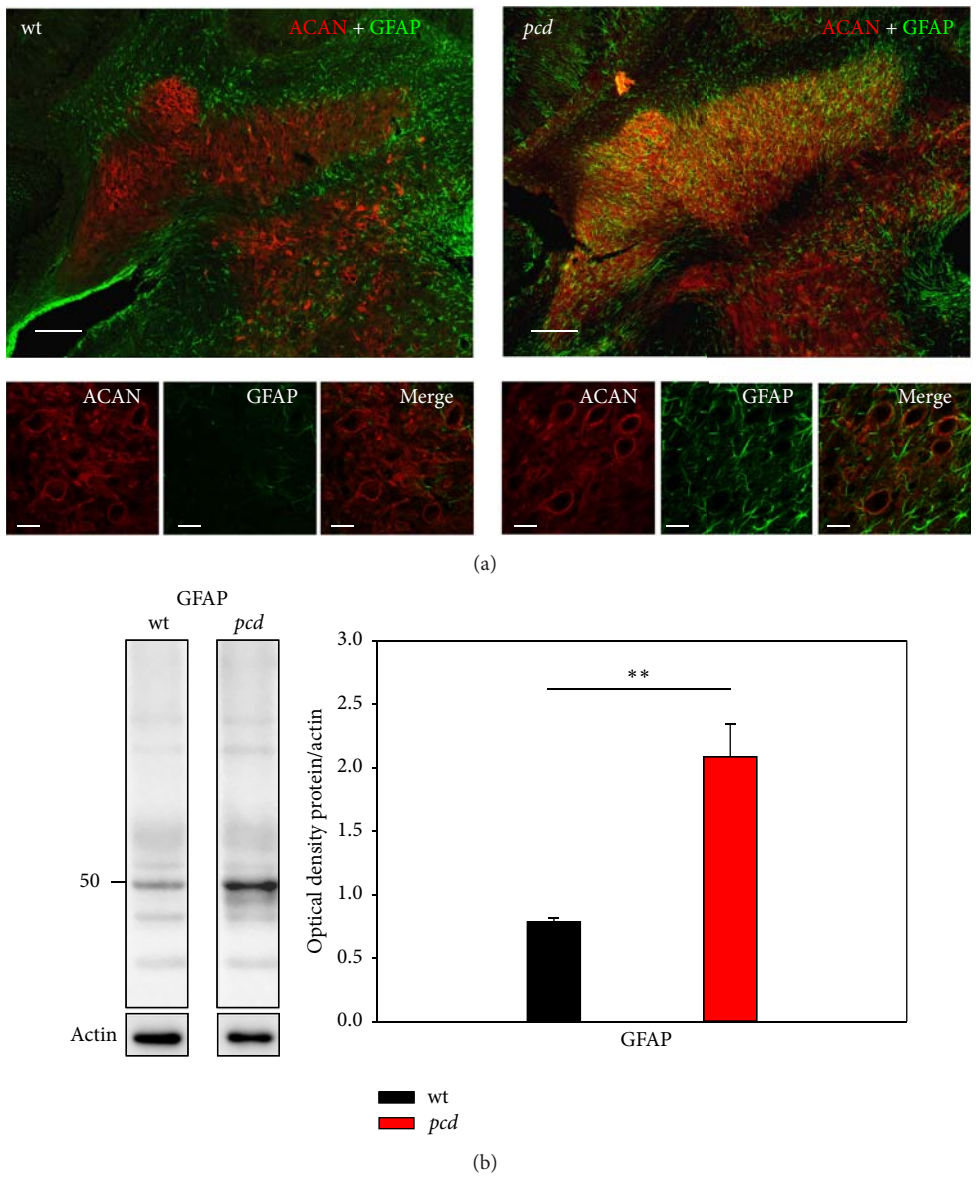

Figure 7: Reactive astrogliosis in the DCN of *pcd* mouse brain. (a) DCN of *wt* mouse brain is characterized by the virtual absence of reactive astrocytes. In *pcd*, the degeneration process is accompanied by a strong astrocytic activation; the DCN seem to be filled with astrocytes. Scale bar: overview 100 μm, detail 20 μm. (b) Western blot analyses confirm the immunocytochemical data. In *pcd* tissue, the GFAP protein level is more than 2-fold increased ($p < 0.01$). Data are given as mean ± SEM.

neurons induce a significant reduction of certain PN-components around the PC-target cells. PNs themselves are still present consisting of the main components hyaluronan, aggrecan, tenascin-R, and hapln1, but brevican and hapln4 are apparently absent. That points to the assumption that brevican and hapln4 are involved in synaptic stabilization and/or maintenance [78, 87]. Brevican is typically enriched at perisynaptic sites and is suggested to accumulate molecules necessary for synaptic formation and preservation [14, 18, 87].

It has been reported that brevican expression is altered after brain injuries [15, 88, 89] and a loss of synapses is associated with a loss of brevican and hapln4 or vice versa [8, 87]. In case of pcd the decay of cells and their axons in cerebellar cortex results in altered expression of brevican and hapln4 in the DCN.

The fact that brevican and hapln4 seem to be no longer an integral part of the PNs in the DCN of the *pcd* mice might confirm the assumption that they might be more sensitive to degeneration than the other PN-components and that both components seem to be strongly dependent on each other for integration into the PNs [90, 91]. In early development, most CSPGs are more soluble and have only a low affinity to bind hyaluronan [92, 93]. Link proteins play an important role in promoting the connection of CSPGs to hyaluronan by inducing conformation changes at the CSPGs allowing a

strong interaction with hyaluronan [59, 94]. Hapln4 in the DCN is supposed to derive mainly from the PC axons [25, 59, 87], so in *pcd* mice the supply of the link protein is interrupted and potentially affects the localization of brevican [59].

4.3. Enhanced Gliosis Determines Protein Properties. CNS injury or degeneration processes are often combined with cell death leading to secretion of molecules triggering an extensive glial response and activation. The activation of different glial cells mostly follows a specific timeline. The first response to acute injuries is the migration of macrophages and microglia, followed by an activation of oligodendrocytes, and finalized with the proliferation of astrocytes [95]. While in the area of insult (cerebellar cortex) in *pcd* mice an activation of microglia and astroglia was observed [96], this study identified that the DCN as a secondary affected area has rare microglia (IBA1 and S100b, data not shown) but extensive astrogliosis. Reactive astrocytes play an important role in regeneration by occupation of the vacated space, uptake of potentially excitotoxic glutamate, stabilization of extracellular fluid and ion balance, and protection from oxidative stress [97]. Beyond these functions, the expression of CSPGs in the injured brain is strongly upregulated due to astrocytes [98]. In regard to these findings several studies focused on brevican, which is expressed by reactive glia in response to brain injuries [14, 92, 93, 97, 99–107]. In the *pcd* mice the SDS-PAGE also reveals an elevation of brevican in DCN after denervation. It is supposed that the cellular source of brevican can switch after injuries and is predominantly produced by astrocytes [14, 108]. Brevican is sensitive to a number of matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinase with a thrombospondin type 1 motif (ADAMTS) creating cleavage fragments of approximately 50 kDa and 80 kDa [64, 109]. In the adult and healthy brain, most MMPs are downregulated. After injuries the expression and enzymatic activity of MMPs and ADAMTS have been shown to be increased [14, 96, 110–118] caused by activated glial cells [118, 119]. MMP9 expression that is enhanced in the cerebellum of *pcd* mice [96] is linked with the growth-associated protein GAP43 and promotes nerve regeneration and axonal sprouting [4, 120, 121]. The increased expression of the 50 kDa cleavage fragment of brevican discovered in this study implies that there might be an increase of protease activity after degeneration in DCN contributing to extracellular matrix proteolysis, which is what loosens the PNs around the DCN neurons and facilitates new sprouting and synaptogenesis [4, 14, 107, 120].

It is not yet clarified if link proteins, which are mainly expressed by neurons, could also switch to glial expression after injury. In *pcd* mice not only the nna1 gene is disrupted, it is reported that the general gene transcription is downregulated [122, 123]. Furthermore inflammatory events, which are known to be associated with degeneration, cause an increased methylation of the genomic region of hapln4 and potentially decrease the neuronal gene expression [93, 124]. The inhibited neuronal gene expression could promote a potential glial expression of hapln4. Sim et al. suggest that a glial hapln4 expression leads to altered protein properties with a rather soluble nature [93]. However, it is supposed that the glial produced brevican is strongly associated with fibronectin which is highly enriched in cerebellum of *pcd* mice [96] and modulates the cell adhesion and motility [93, 104] which may enable the reinnervation of PN-bearing DCN neurons. We speculate that the high amount of brevican could indeed stimulate the expression of the link protein, but hapln4 has no binding partner anymore and cannot be robustly integrated into PNs.

4.4. Technical Consideration. Injuries, diseases, and degeneration-processes often lead to changes of PN-composition. It was reported that remodeling sometimes induces enhanced CSPG expression [88, 108, 125, 126], but it has also been shown that loss of synapses could be associated with reduced CSPG occurrence [4, 8, 87]. Our data display both reduction and enhancement of PN-constituents after degeneration. Deepa et al. [60] showed that detectability is strongly dependent on the solubility of the proteins and the used method. Immunohistochemical methods rather detect proteins in stable complexes and discover soluble fractions less efficiently. In contrast, pretreatment of the tissue for SDS-PAGE with special buffer releases most protein fraction, membrane associated, and soluble fractions [60]. The different techniques could lead to different but not contradictory results.

5. Conclusions

Degeneration of cerebellar Purkinje cells affects large PN-bearing DCN neurons. The following events are an interplay of degeneration and regeneration. Not only the Purkinje cell derived GABAergic terminals decrease, but also the PN-components brevican and hapln4 are virtually absent in the stable structure of the PNs of DCN neurons in *pcd* mice. Simultaneously, the hapln4 and brevican protein expression is increased, probably caused by severe local inflammation processes with migrating astrocytes.

On one hand the attenuated PNs imply that brevican and/or hapln4 are more sensitive to degeneration and might play a vital role in synaptic reorganization and the loss or variation of them might enable new sprouting of synapses. On the other hand, glial produced PN-components reveal altered properties and could influence cell adhesion and motility to facilitate axonal path-finding.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

M. Blosa, C. Bursch, and M. Morawski contributed equally.

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Research Article

Development and Structural Variety of the Chondroitin Sulfate Proteoglycans-Contained Extracellular Matrix in the Mouse Brain

Noriko Horii-Hayashi, Takayo Sasagawa, Wataru Matsunaga, and Mayumi Nishi

Department of Anatomy and Cell Biology, Nara Medical University, 840 Shijo-cho, Kashihara, Nara 634-8521, Japan

Correspondence should be addressed to Mayumi Nishi; nmayumi@naramed-u.ac.jp

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Chondroitin sulfate proteoglycans (CSPGs) are major components of the extracellular matrix (ECM) in the brain. In adult mammals, CSPGs form the specialized ECM structure perineuronal nets (PNNs) that surround somata and dendrites of certain types of neurons. PNNs restrict synaptic plasticity and regulate the closure of critical periods. Although previous studies have examined the starting period of PNN formation, focusing on primary sensory cortices, there are no systematic studies at the whole brain level. Here, we examined the starting period of PNN formation in male mice ranging in age from postnatal day 3 to week 11, mainly focusing on several cortical areas, limbic structures, hypothalamus, and brain stem, using lectin histochemistry with *Wisteria floribunda* agglutinin (WFA). Results showed that early PNN formation was observed in several reticular formations of the brain stem related to the cranial nerves and primary somatosensory cortices. In the limbic system, PNN formation in the hippocampus started earlier than that of the amygdala. Furthermore, in the medial amygdaloid nucleus and some hypothalamic regions, WFA labeling did not show typical PNN-like forms. The present study suggests spatiotemporal differences at the beginning of PNN formation and a structural variety of CSPG-contained ECM in the brain.

1. Introduction

Chondroitin sulfate proteoglycans (CSPGs) are major extracellular matrix (ECM) components in the central nervous system, and many types of CSPGs have been characterized in the brain [1]. Chondroitin sulfates (CS), glycosaminoglycan portions of CSPGs, are known as inhibitory substrates for neurite growth. CSPGs can act as axonal guidance molecules in the developing brain [2–4] and as stabilizing substrates for synapses in the adult brain [5, 6].

CSPGs are composed of a core protein and one or more CS glycosaminoglycans that covalently attach to the serine residues of the core protein. CS is composed of repeating disaccharide units of *N*-acetylgalactosamine and glucuronic acid. The lectin *Wisteria floribunda* agglutinin (WFA) is generally used to detect CS, owing to its ability to bind to the *N*-acetylgalactosamine within carbohydrate structures [7–9]. In the adult brain, WFA is known to label specialized ECM structures called perineuronal nets (PNNs), which are formed by aggregating multiple molecules, including CSPGs, hyaluronan, and glycoproteins. The majority of PNNs surround cell bodies and proximal dendrites of parvalbumin-positive inhibitory neurons in the cerebral cortex and limbic structures [7, 10, 11], whereas a few PNNs are found around pyramidal neurons in both the marsupial and primate cortex [12, 13].

Topographical distribution of CSPG changes during postnatal development, and the shape of PNNs gradually matures a few weeks after birth. Bahia et al. distinguished two successive ECM structures containing CSPGs in the developing barrel field of the primary somatosensory cortex (S1BF) in rats [14]: CSPG shows diffuse and neuropil-associated distribution in the first postnatal week. Next, CSPGs are concentrated around cell bodies with a PNN component

structure at postnatal day 24 (P24). The shape of PNNs continues to develop and reach maturity by postnatal week 9 (9 w). A similar PNN structural development has been observed in the visual cortex [15].

Recent findings suggest the importance of PNNs for regulating synaptic plasticity and closing critical periods [15, 16]. However, the beginning periods of PNN formation in most brain regions other than the cortex have not been fully explored. If the appearance of PNNs implies the end of the critical period, we suspected that PNN appearance periods would be different among brain regions. This is because the maturation speed of each brain region is different depending on its functions. The present study investigated PNN formation in the mouse brain from P3 to 11 w using WFA staining, mainly focusing on the cortex, limbic system, hypothalamus, and brain stem.

2. Materials and Methods

2.1. Animals. All experiments were performed using male C57BL/6N mice. All protocols followed NIH (USA) Guidelines and Guidelines for Proper Conduct of Animal Experiments published by the Science Council of Japan. Animals were purchased from Japan SLC, Inc. (Hamamatsu, Japan). The following number of animals was used at each stage: P3 ($n = 8$), P7 ($n = 8$), P9 ($n = 4$), P14 ($n = 4$), P21 ($n = 4$), 5 w ($n = 4$), and 11 w ($n = 8$). When using mice aged from P3 to P21, pregnant female mice at gestational day 14 were purchased from the same company and their pups were sampled at each stage; day of birth was considered P0. Mice were housed and maintained under standard laboratory conditions: 23°C and 55% humidity in a room with a 12 h light-dark cycle (lights on at 08:00 and off at 20:00), and food and water were available *ad libitum*.

2.2. Fixation and Histochemistry. Fixation and histochemical protocols were conducted as previously described [17]. Briefly, mice were anesthetized with sodium pentobarbitone (100 mg/kg) and transcardially perfused with heparinized 0.01 M phosphate-buffered saline (PBS, pH 7.4), followed by 4% paraformaldehyde in a 0.1 M phosphate buffer (pH = 7.4). Dissected brains were immersed with the same fixative either overnight or for 1 week; the former was applied to animals older than P14 and the latter to those from P3 to P9. Tissue sections were made using a cryostat (Leica, Wetzlar, Germany) at a thickness of 30 μm. Free-floating sections were pretreated with 0.1% H_2O_2 in PBS for 20 min, immersed with PBS containing 0.3% Triton X-100 (PBST) for 15 min, and then treated with the blocking solution of PBST containing 5% normal horse serum for 2 h. When digesting CS, sections were treated with chondroitinase ABC (ChABC; 0.1 U/mL, Sigma, St. Louis, MO, USA) in 50 mM Tris-HCl buffer containing 30 mM sodium acetate (pH = 8.0) for 3 h at 37°C before blocking. The same treatment was performed without using ChABC as experimental counterparts. Sections were incubated with biotinylated WFA (dilution 1 : 1000, Vector Laboratories, Burlingame, CA) overnight at 4°C and developed using a Vectastain ABC Kit (Vector Laboratories). Sections were mounted on glass slides, dehydrated in graded ethanols, and coverslipped with Entellan (Merck, Darmstadt, Germany). Observations were carried out with a BX-43 transillumination microscope equipped with a FX630 CCD camera, and images were captured using 4, 10, 20, and 40x objective lenses, as well as a 100x immersion lens (Olympus). For fluorescent labeling, all procedures up to blocking were the same as above except for skipping H_2O_2 treatment. Sections were incubated with biotinylated WFA (dilution 1 : 500) at 4°C overnight and immersed with a mixture of Alexa 488-conjugated streptavidin (dilution 1 : 1000, Life Technologies) and NeuroTrace 530/615 (dilution 1 : 200, Life Technologies) for 2 h. Sections were mounted on slides and coverslipped with Vectashield containing 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Laboratories). Fluorescent images were captured by a FluoView 1000 confocal microscope in single-plane mode with a 40x objective lens (Olympus).

2.3. Regional Definitions. Brain regions were determined according to the mouse brain atlas [18] and the developing mouse brain atlas [19].

3. Results

The specificity of WFA for CS was confirmed by treating sections with ChABC, a CS digesting enzyme. Our results showed that ChABC treatment almost completely abolished WFA reactivity in all brain regions analyzed (see Figures S1A–G of the Supplementary Material available online at http://dx.doi.org/10.1155/2015/256389). However, in neonatal brain sections, especially at P3 (data not shown) and P7 (Figure S1H), small dot-like WFA reactivity was observed in both ChABC-treated and untreated sections. We considered this dot-like reactivity a false positive and excluded it from the CS-specific reaction.

Diffuse WFA reactivity was observed in the ventral layers of the S1BF at P3 (Figures 1(a) and 1(f)), and this observation was similar to a previous report [14]. At P7, WFA reactivity was observed in cortical IV layer (Figure 1(b)) and slightly condensed around particular cell bodies (Figure 1(g)), which were considered immature PNNs. At both P14 (Figures 1(c) and 1(h)) and P21 (Figures 1(d) and 1(i)), PNN-like labeling was observed around cell bodies without a clear dendritic surrounding. The typical PNN shape that is known to surround both cell bodies and dendrites was observed at 5 w (Figures 1(e) and 1(j)) and was almost equal to that seen at 11 w (data not shown). Serial WFA-labeled sections at P7 revealed that PNN-like labeling was observed in the primary somatosensory cortex (S1) and S1BF, whereas it could not be detected in the prelimbic (PrL), primary motor (M1), secondary motor (M2), cingulate (Cg), primary visual (V1), mediolateral (V2ML), and mediomedial (V2MM) areas of the secondary visual, primary auditory (Au1), and retrosplenial granular (RSG) cortices (Figure 1(k)). These results indicated an intercortical difference in the beginning of PNN formation.

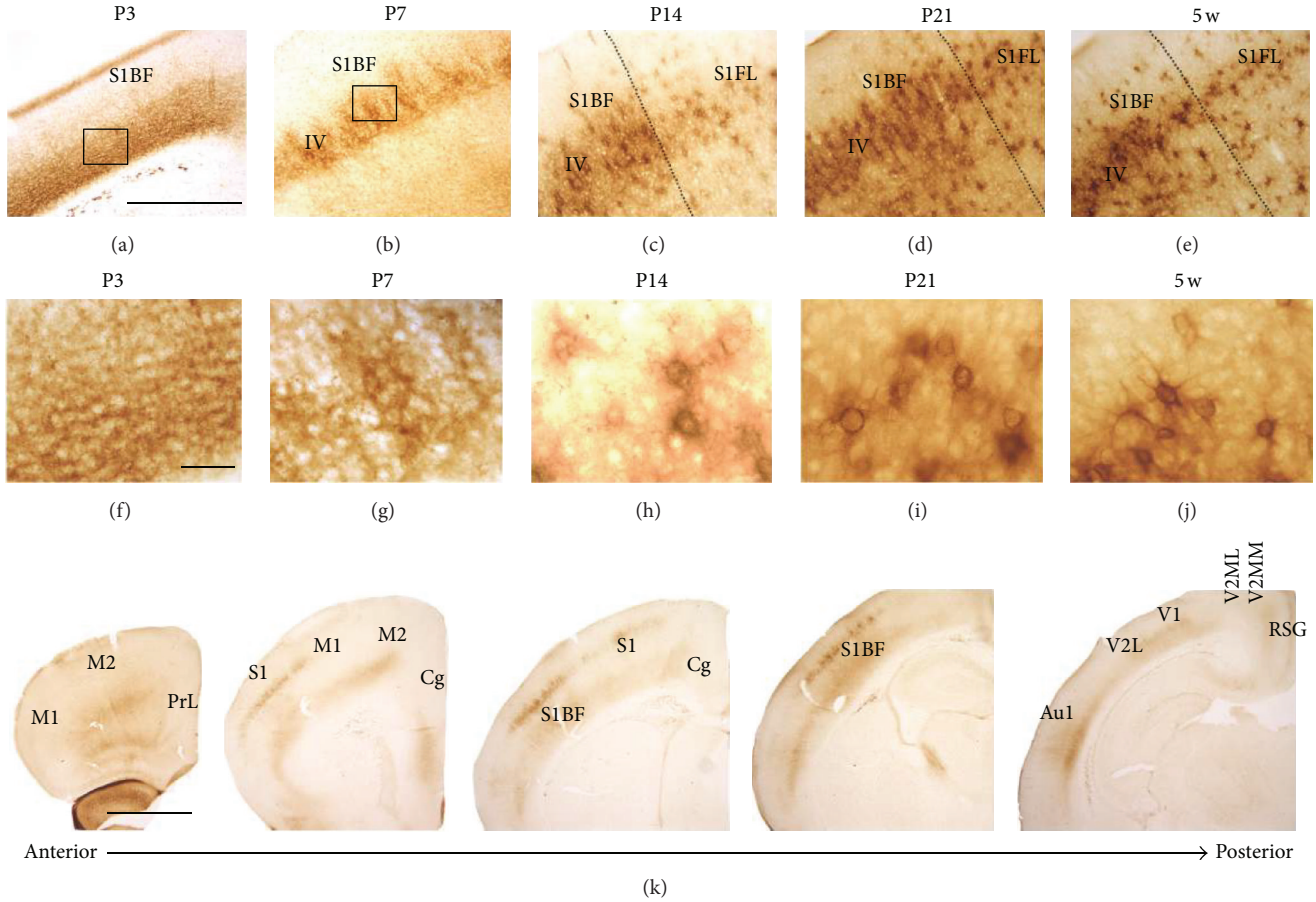

Figure 1: WFA-stained ECM in the developing cortex. (a–j) Low- (a–e) and high- (f–j) power images of WFA staining at P3 (a, f), P7 (b, g), P14 (c, h), P21 (d, i), and 5 w (e, j). Rectangular areas in (a) and (b) indicate the magnified areas shown in (f) and (g), respectively. High-power images (g–j) obtained from cortical IV layer of the S1BF. Dotted lines indicate the border between the S1BF and the S1FL. Accumulation of WFA reactivity around particular cell bodies was observed from P7 onward. WFA reactivity clearly surrounding both cell bodies and dendrites was observed from 5 w onward. (k) WFA-labeled serial coronal sections at P7, indicating that PNN-like WFA reactivity was observed in the S1BF, whereas any other regions indicated did not show PNN-like staining. Au1: primary auditory cortex; Cg: cingulate cortex; M1: primary motor cortex; M2: secondary motor cortex; PrL: prelimbic cortex; RSG: retrosplenial granular cortex; S1: primary sensory cortex; S1BF: barrel field of the primary somatosensory cortex; S1FL: forelimb primary somatosensory cortex; V1: primary visual cortex; V2L: lateral area of the secondary visual cortex; V2ML: mediolateral area of the secondary visual cortex; V2MM: mediomedial area of the secondary visual cortex. Scale bars = 500 (a–e), 50 (f–j), and 1000 (k) μm.

In the hippocampal CA1, PNN-like WFA labeling could not be observed at P3 (Figure 2(a)) and P7 (Figure 2(b)), while it could be detected at P14 (Figure 2(c)). Similarly, clear WFA labeling was first observed in the hippocampal CA2 at P14 (Figure 2(c)). These labels became stronger and clearer at P21 (Figure 2(d)) and 5 w (Figure 2(e)). Higher magnification views confirmed PNN-like labeling in the CA1 at P14 (Figure 2(f)). From P21 onward (Figures 2(g)–2(i)), WFA reactivity clearly surrounded both cell bodies and dendrites in the CA1. WFA reactivity in the CA2 at P14 showed PNN-like labeling (Figure 2(j)), while, from P21 onward, the border of individual PNN structures was difficult to identify (Figures 2(k)–2(m)). Such a distinctive staining pattern in the CA2 is consistent with what has been previously reported [10, 20].

In the amygdala, WFA reactivity was hardly observed by P14 in both the basolateral amygdaloid nucleus (BLA) (Figure 3(a)) and medial amygdaloid nucleus (MA) (Figure 3(e)). At P21, WFA reactivity in both subdivisions became detectable (Figures 3(b) and 3(f)). From 5 w onward, WFA reactivity was clearly observed in the BLA (Figures 3(c) and 3(d)) and MA (Figures 3(g) and 3(h)). High-power images further showed that an immature PNN-like form was observed in the BLA at P21 (Figure 3(i)), which gradually and clearly surrounded both cell bodies and dendrites from 5 w (Figure 3(j)) to 11 w (Figure 3(k)). However, in the MA, no clear WFA reactivity was observed to surround cell bodies and dendrites at P21 (Figure 3(l)) and 5 w (Figure 3(m)). At 11 w, WFA reactivity loosely accumulated around particular cell bodies but not around dendrites (Figure 3(n)).

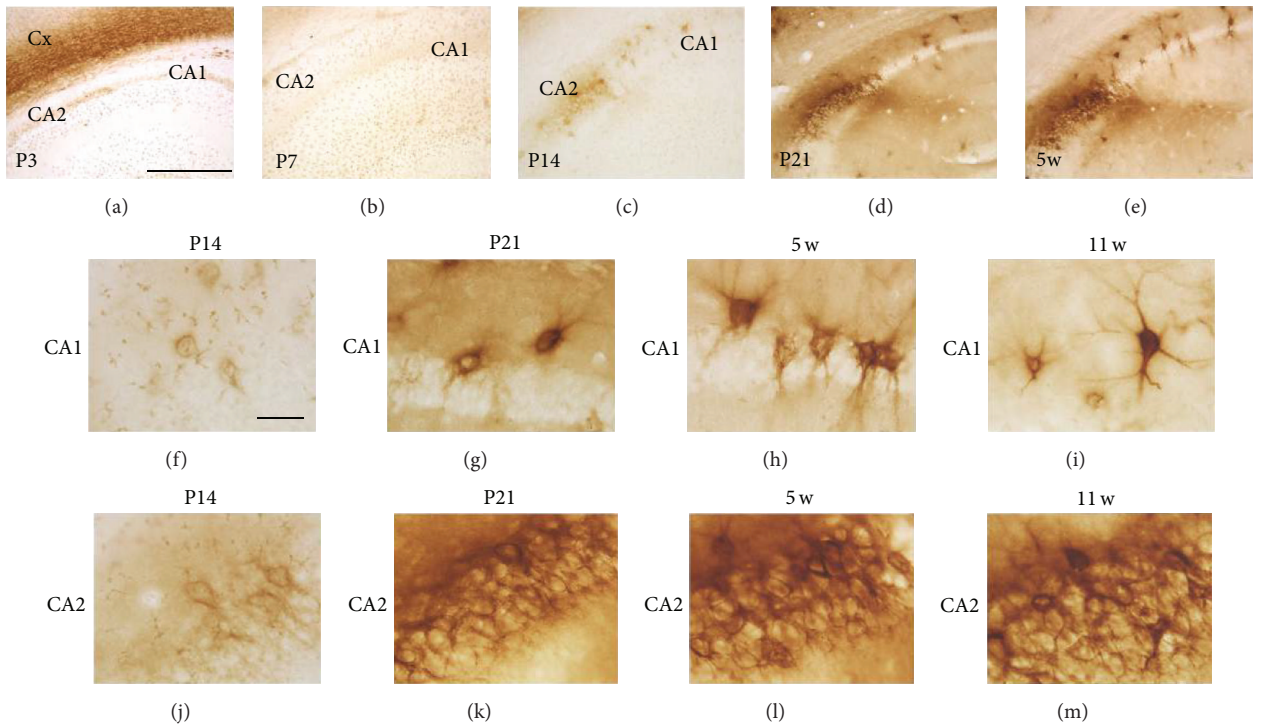

Figure 2: WFA-stained ECM in the developing hippocampus. (a–e) WFA-labeled images at P3 (a), P7 (B), P14 (c), P21 (d), and 5 w (e). PNN-like WFA reactivity was observed from P14 onward. (f–m) Higher magnification images of the CA1 (f–i) and CA2 (j–m) at P14 (f, j), P21 (g, k), 5 w (h, l), and 11 w (i, m). Diffuse PNN-like staining was observed in the CA1 at P14, which gradually surrounded cell bodies and dendrites from P21 onward. WFA reactivity in the CA2 from P21 onward was strong and showed a complex staining manner. Cx: cerebral cortex. Scale bars = 500 (a–e) and 50 (f–m) μm.

In the hypothalamus, WFA labeling patterns and their developmental changes were different within each nucleus or area. In the paraventricular nucleus (PVN), especially in its anterior division, faint PNN-like labeling was observed at P21 (Figure 4(a)), which gradually became clear from 5 w (Figure 4(b)) to 11 w (Figure 4(c)). In the lateral hypothalamus (LH), a comparatively clear PNN-like form was observed at P21 (Figure 4(d)), which became clearer at 5 w (Figure 4(e)) and 11 w (Figure 4(f)). In the ventromedial hypothalamic nucleus (VMH), WFA reactivity was diffusely observed throughout the region at P21 (Figure 4(g)), which became stronger but remained diffuse at 5 w (Figure 4(h)) and 11 w (Figure 4(i)). Contrary to the VMH findings, strong WFA reactivity was observed in the ventral portion of the arcuate nucleus (Arc) neighboring the median eminence (ME) (Figures 4(g)–4(i)). High-power images at 11 w are represented in Figures 4(j)–4(n): PNNs in the PVN surrounded both cell bodies and dendrite-like processes, but they were somewhat diffuse compared with those of the LH (Figure 4(m)) and lateral preoptic area (LPO) (Figure 4(n)). WFA reactivity in the VMH was diffuse and weakly accumulated around particular cell bodies (Figure 4(k)). In the Arc, WFA reactivity densely surrounded cell bodies (Figure 4(l)).

In the gigantocellular nucleus of the pons (Gi), a few PNN structures surrounding both cell bodies and dendrites were observed at P3 (Figures 5(a) and 5(f)) and a substantial number of PNNs could be detected at P7 (Figures 5(b) and 5(g)). From P14 onward, WFA reactivity became stronger and appeared to make plexuses, in which individual PNNs were difficult to identify (Figures 5(c)–5(e) and 5(h)–5(j)).

WFA-labeled ECM structures at 11 w were examined by fluorescent labeling with WFA, Nissl (neuron marker), and DAPI (nuclear marker). PNN-like WFA reactivity that was condensed around both cell bodies and dendrites was observed in many cortical, limbic, and brain stem areas, including the S1BF (Figure 6(a)), hippocampal CA1 (Figure 6(b)) and CA3 (data not shown), BLA (data not shown), lateral septum (data not shown), mesencephalic reticular formation (mRt, Figure 6(c)), red nucleus (RN, Figure 6(d)), vestibular nucleus (Ve, Figure 6(e)), Gi (Figure 6(f)), and spinal trigeminal nucleus (Sp5, Figure 6(g)). In the hypothalamus, the typical PNN structure was observed in the LH (Figure 6(h)) and LPO (data not shown). On the other hand, WFA reactivity in the reticular thalamic nucleus (Rt, Figure 6(i)), MA (Figure 6(j)), VMH (Figure 6(k)), and Arc (Figure 6(l)) was observed to surround cell bodies but not dendrites. WFA reactivity in the MA (Figure 6(j)) and VMH (Figure 6(k)) was particularly more diffuse and showed a more loosely organized accumulation around cell bodies. These results suggested structural variety of CSPG-contained ECM in the adult brain.

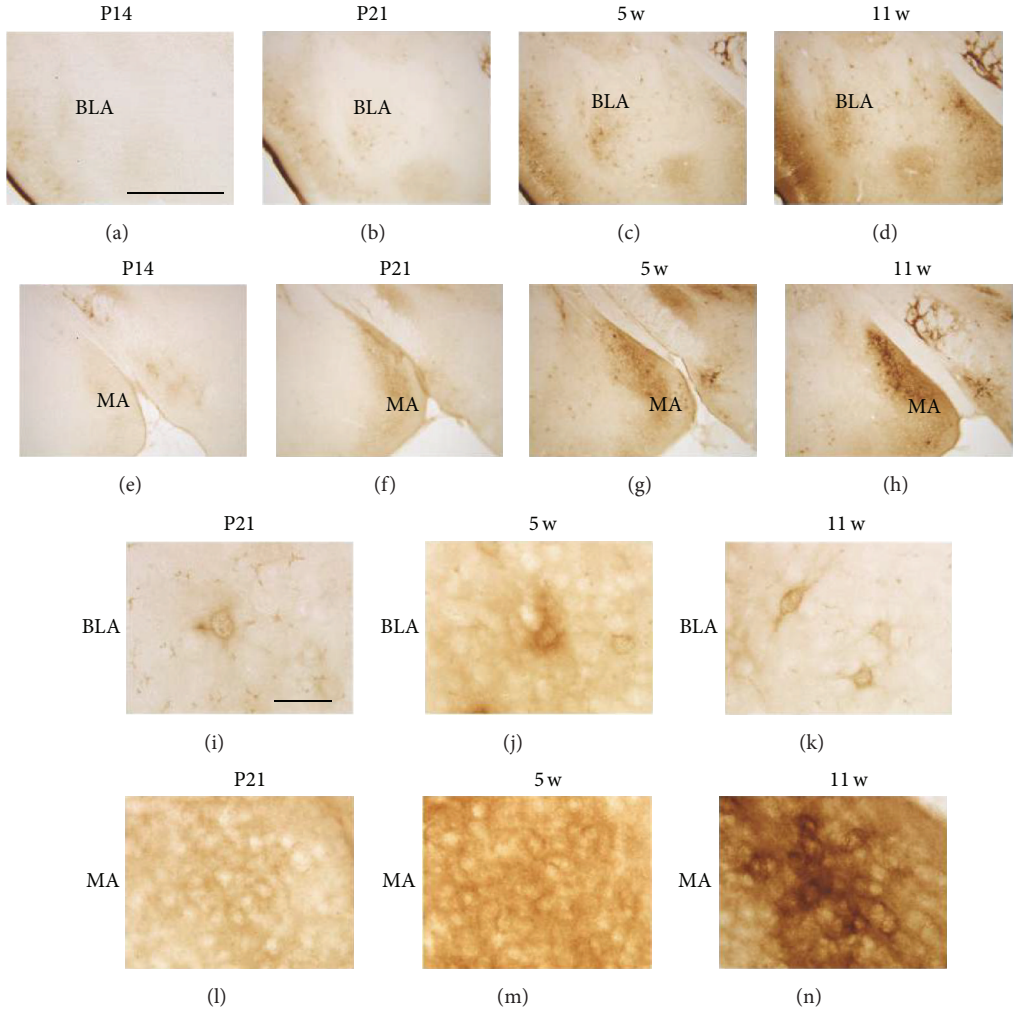

Figure 3: WFA-stained ECM in the developing amygdala. (a–h) WFA-labeled images in the BLA (a–d) and MA (e–h) at P14 (a, e), P21 (b, f), 5 w (c, g), and 11 w (d, h). In both regions, WFA reactivity was observed from P21 onward. (i–n) Higher magnification images of the BLA (i–k) and MA (l–n) at P21 (i, l), 5 w (j, m), and 11 w (k, n). Ambiguous PNN-like staining was observed in the BLA at P21 and 5 w, which surrounded both cell bodies and dendrites at 11 w. Diffuse neuropil-like staining was continuously observed in the MA at all stages shown and a loosely accumulated WFA reactivity around particular cell bodies was observed at 11 w. BLA: basolateral amygdaloid nucleus; MA: medial amygdaloid nucleus. Scale bars = 500 (a–h) and 50 (i–n) μm.

4. Discussion

The present study investigated developmental changes and adult structural variety of CSPG-contained ECM structures. Since the carbohydrate epitope of WFA is thought to be on aggrecan [21], the present results are probably related to the expression and localization of aggrecan. We first hypothesized that if the beginning of PNN formation implies the end of the critical period, the start of PNN formation should be different among brain regions, probably depending on their functions. Consistent with this hypothesis, our results showed a time difference with regard to the beginning of PNN formation. Table 1 indicates the starting period of PNN formation in each brain region, together with known critical periods concerning various biological systems. Since particular regions marked with asterisks did not include typical PNN forms, indicated periods represent when WFA reactivity was first detected. Our results clearly showed that the earliest formation of PNNs occurred in some reticular formation nuclei by P3. Subsequent formation was observed in the primary sensory cortices and other nuclei of the brain stem by P7, and all regions began to form PNNs or express WFA-labeled CSPGs by P21. These findings suggest that the end of the critical period and the speed of brain maturation largely differ depending on regions.

Among the cerebral cortices, including the prefrontal, sensorimotor, and cingulate, immature PNNs were first observed in the S1, S1BF, and piriform cortex (Pir) and successively detected in the primary auditory cortex (Au1) and in the ventral and lateral orbital cortices (VO, LO) (Table 1). At P14, PNN formation began in nearly all regions, except for the V2MM. The critical period for whisker-barrel formation occurs by P7 [22], and formation of the barrel structure requires sensory inputs from whiskers [23],

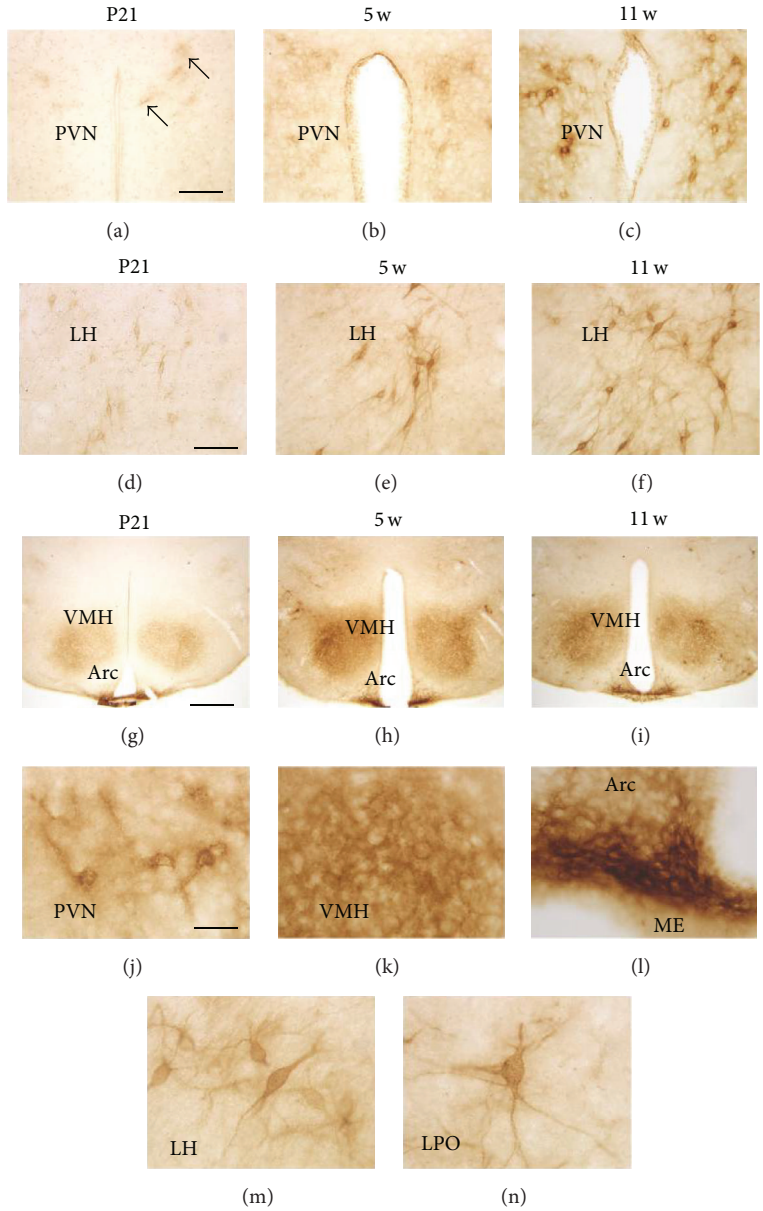

Figure 4: WFA-stained ECM in the developing hypothalamus. (a–i) WFA-labeled images in the PVN (a–c), LH (d–f), and VMH/Arc (g–i) at P21 (a, d, g), 5 w (b, e, h), and 11 w (c, f, i). Ambiguous PNN-like staining (arrows) was observed in the PVN at P21, which became gradually clear over 5 w to 11 w. PNN-like staining was observed at P21 in the LH, which became clearer at 5 w and 11 w. In all stages shown, WFA reactivity in the VMH was diffuse, while that of the Arc was dense, particularly in its ventral portion neighboring the ME. (j–n) Higher magnification images of WFA labeling at 11 w in the PVN (j), VMH (k), Arc (l), LH (M), and LPO (n). PNN structures in the PVN showed a diffuse manner, while those of the LH and LPO clearly surrounded cell bodies and dendrites. In the VMH and Arc, WFA reactivity did not surround dendrites. Arc: arcuate nucleus; LH: lateral hypothalamus; LPO: lateral preoptic area; ME: median eminence; PVN: paraventricular nucleus; VMH: ventromedial hypothalamic nucleus. Scale bars = 100 (a–f), 400 (g–i), and 40 (j–n) μm.

which are relayed to the Sp5 via the trigeminal nerve [24]. Interestingly, PNN formation in the Sp5 was also observed at P7 (Table 1). These results suggest cooperative maturation of the whisker sensory system. In the case of rodents, pups start to move independently by P10 [25]. Additionally, the critical period for the neuromuscular junction is P12 in mice [22]. Thus, our results seem to be consistent with the end of the critical period for the sensorimotor system.

In the hippocampus, PNN formation is observed at P14, except for in the dentate gyrus (Table 1). Furthermore, the notable feature in the hippocampus is dramatic maturation for 1 week from P14 to P21, while PNNs in other regions

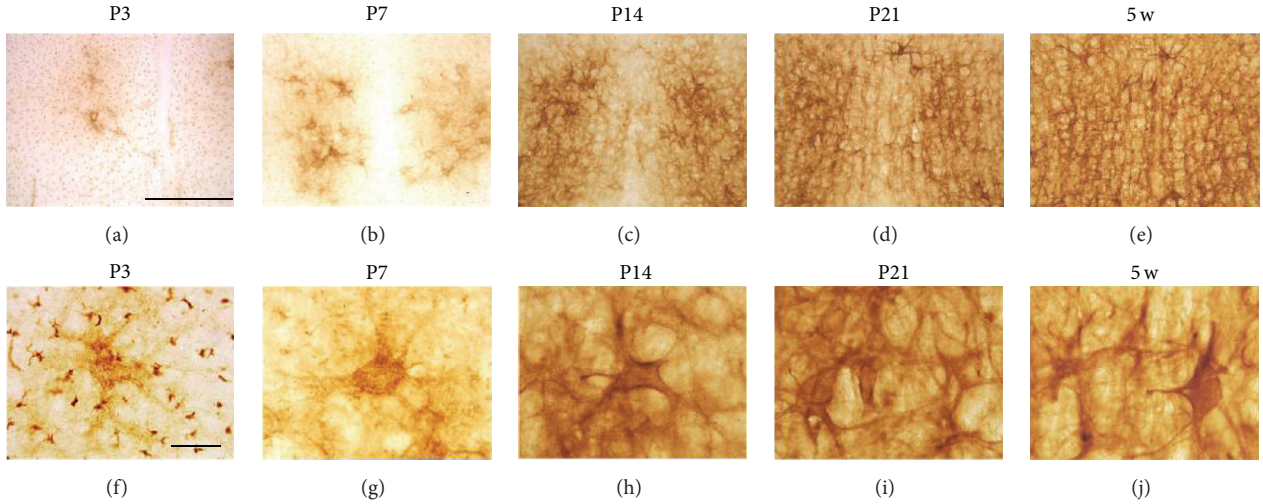

Figure 5: WFA-stained ECM in the Gi of the pons. (a–j) Low- (a–e) and high- (f–j) power images of WFA labeling in the Gi at P3 (a, f), P7 (b, g), P14 (c, h), P21 (d, i), and 5 w (e, j). PNN-like staining was observed at P3 and P7, which became clearer and more complicated from P14 onward. Gi: gigantocellular nucleus. Scale bars = 500 (a–e) and 50 (f–j) μm.

gradually mature, over approximately 2 weeks. Defining the critical period of the hippocampus is a very difficult task. However, the stress-hyporesponsive period (SHRP) may be related to the hippocampal critical period. The SHRP is the critical period for determining the future state of stress responses, including activity of the hypothalamic-pituitary-adrenal (HPA) axis [26, 27]. The hippocampus is a superordinate structure controlling HPA axis activity, which is also known as the hippocampal-HPA axis [28]. The SHRP in rodents approximately matches the first two postnatal weeks. Indeed, our previous study indicated that maternal separation performed during the first two postnatal weeks increases basal plasma corticosterone levels in adulthood, whereas the same intervention during the next week (i.e., from 3 w to 4 w) does not affect adult corticosterone levels [29–31]. Although it remains fully unknown whether PNN appearance in the hippocampus affects the end of the SHRP, this is a challenging issue that requires future study.

In the amygdala, PNN formation was observed by P21 (Table 1), which is mostly consistent with a previous study demonstrating a few obscure PNNs that were observed at P16 and that were found to dramatically increase by P21 [16]. In rodents, persistent fear memory can be observed in animals older than 3 w [32]. Similar to a previous study [16], our results support the functional importance of PNNs in the consolidation and maintenance of fear memory. Interestingly, WFA labeling showed that the MA had a loosely organized ECM structure that was diffuse in the neuropil and avoided surrounding dendrites. A previous study in the cerebellum termed a loosely organized ECM "semiorganized matrix" [33]. This term is thought to be applicable to the ECM structure of the MA. The MA is highly plastic throughout life and known as a sexually dimorphic region. As such, the density of dendritic spines in the MA is largely affected by sex steroids and actively changes during the female estrous cycles [34]. We suspect that the lack of CSPG matrix around MA neuron dendrites was related to the long-lasting maintenance of higher synaptic plasticity throughout adulthood.

Like the MA, many hypothalamic regions do not follow typical PNN structures. WFA labeling patterns observed in the VMH and Arc are also considered to be a semiorganized matrix [33]. Similar to the MA, hypothalamic neurons are ordinarily required to respond to lifelong hormonal and environmental changes for survival and species preservation. Hypothalamic neurons are generally thought to be plastic throughout adulthood [35–40], and the Arc and VMH are no exception because they control appetite and feeding behaviors in response to nutrient availability [35, 38, 41, 42]. Importantly, the structural and functional integrity of PNNs are maintained by complex interactions of multiple ECM molecules. In fact, even the lack of a single ECM molecule can affect both the structure and function of PNNs, as observed in knockout mice that lack genes encoding the hyaluronan proteoglycan link protein (HAPLN) 1 [43], HAPLN4 [44], and tenascin R [45]. Thus, our results remind us that the semiorganized CSPG matrix may not so much restrict synaptic plasticity as highly organized typical PNNs are thought to do [15, 46].

Among the brain regions examined, PNN appearance was earliest in the Gi and oral pontine reticular nucleus (PnO). The Gi is known to innervate the hypoglossal nucleus that controls movement of the tongue muscles [47]. The PnO is involved in the generation and maintenance of rapid eye movement (REM) sleep [48, 49]. These observations suggest that the formation of PNNs is dependent on neuronal activity [50–52], since pups are supposed to move their tongue muscles during suckling, and REM sleep is the most frequent type of sleep among newborns [53]. Furthermore, PNN formation begins by P7 in several regions of the brain stem, the RN, superior colliculus (SC), Sp5, and Ve. Major functions of these regions are as follows: SC, saccadic eye

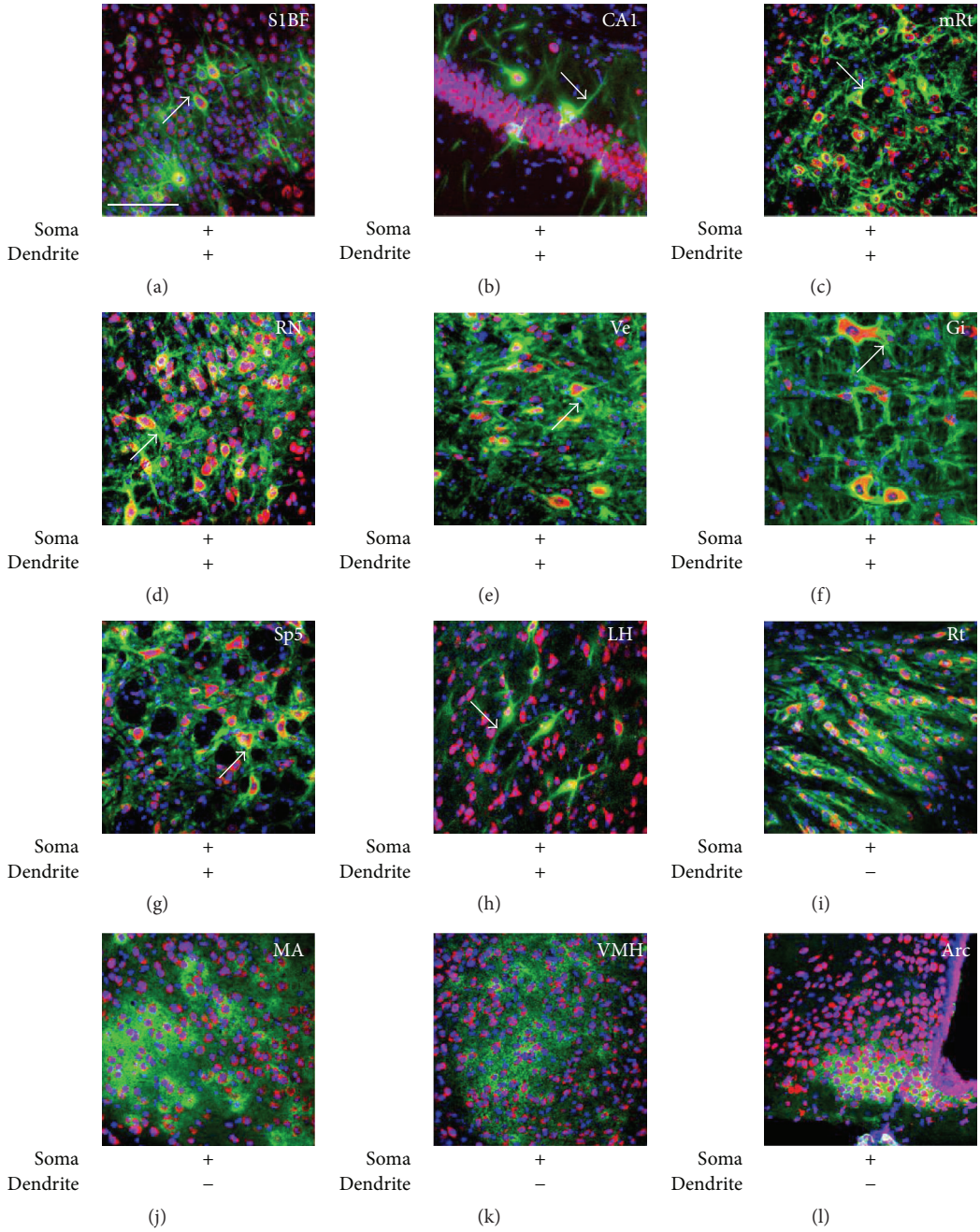

Figure 6: Structural variety of CSPG-contained ECM structures. (a–l) Fluorescent triple labeling of WFA (green), Nissl (red, neuron marker), and DAPI (blue, nucleus marker) at 11 w. Arrows indicate WFA signals around dendrites. The sign of plus (+) or minus (–) below images indicates the presence or absence of WFA reactivity around somata and dendrites. WFA signals in the Cx (a), hippocampal CA1 (b), mRt (c), RN (d), Ve (e), Gi (f), Sp5 (g), and LH (h) were observed around both somata and dendrites, while those of the Rt (i), MA (j), VMH (k), and Arc (l) did not show clear reactivity surrounding dendrites. Arc: arcuate nucleus; Gi: gigantocellular nucleus; LH: lateral hypothalamus; MA: medial amygdaloid nucleus; mRt: mesencephalic reticular formation; RN: red nucleus; Rt: reticular thalamic nucleus; S1BF: barrel field of the primary somatosensory cortex; Sp5: spinal trigeminal nucleus; Ve: vestibular nucleus; VMH: ventromedial hypothalamic nucleus. Scale bar = 100 μm (a–l).

movements, oculosensory reflexes, and eye-head coordination; Sp5, sensory transmission from the face, including the whiskers; and Ve, the maintenance of equilibrium, posture, and the perception of head position. Importantly, the early beginnings of PNN formation in these regions suggest early maturation of nuclei related to the cranial nerves.

5. Conclusions

The present study systematically describes the development and structural variety of the brain ECM. These results strongly support the idea that PNN formation, as well as PNN structural integrity, indicates the degree of brain maturation.

Table 1: The beginning periods of PNN formation in the developing brain. "†" indicates the periods that immature PNNs could be first detected. Asterisks (∗) indicate brain regions not having typical PNN forms and "‡" in these regions indicates the period that WFA signals were first detected. "❡" in the bottom rows shows known critical periods and their references in brackets.

	P3	P7	P9	P14	P21	5 W	11 W
Prefrontal cortex							
Frontal association (FrA)				†			
Orbital, medial (MO)				†			
Orbital, ventral and lateral (VO, LO)			†				
Prelimbic (PrL)				†			
Sensorimotor cortex							
Auditory, primary (Au1)			†				
Motor, primary (M1)				†			
Motor, secondary (M2)				†			
Piriform cortex (Pir)		†					
Somatosensory, primary (S1)		†		†			
Somatosensory, primary, barrel field (S1BF)		†		†			
Somatosensory, secondary (S2)				†			
Visual, primary (V1)				†			
Visual, secondary, and lateral (V2L)				†			
Visual, secondary, and mediolateral (V2ML)				†			
Visual, secondary, and mediomedial (V2MM)					†		
Cingulate gyrus							
Anterior cingulate (Cg)				†			
Posterior cingulate (retrosplenial dysgranular) (RSG)				†			
Basal ganglia							
Caudate putamen (CPu)				†			
Ventral pallidum (VP)					†		
Thalamus							
Habenular nucleus, lateral (LHb)				†			
Reticular thalamic nucleus (Rt)*				‡			
Zona incerta (ZI)				†			
Hypothalamus							
Arcuate nucleus*			‡				
Lateral hypothalamus (LH)				†			
Lateral preoptic area (LPO)					†		
Lateral mammillary nucleus (LM)				†			
Ventromedial hypothalamic nucleus (VMH)*			‡				
Paraventricular nucleus, anterior (aPVN)					†		
Limbic system							
Amygdala, basolateral (BLA)					†		
Amygdala, central (Ce)					†		
Amygdala, medial (MA)*					‡		
Bed nucleus of the stria terminalis (BNST)					†		
Hippocampus, CA1 (CA1)				†			
Hippocampus, CA2 (CA2)*				‡			
Hippocampus, CA3 (CA3)				†			
Hippocampus, dentate gyrus (DG)					†		
Septum, lateral (LS)					†		
Septum, medial (MS)					†		
Brain stem							
Anterior pretectal nucleus (APT)				†			
Gigantocellular reticular nucleus (Gi)	†						
Inferior colliculus (IC)				†			
Interstitial nucleus of Cajal (InC)				†			
Mesencephalic reticular formation (mRt)				†			
Paratrochlear nucleus (Pa4)			†				
Pontine reticular nucleus, oral (PnO)	†						
Precuneiform area (prCnF)				†			
Red nucleus (RN)		†					
Substantia nigra (SN)				†			
Superior colliculus		†					
Spinal trigeminal nucleus (Sp5)		†					
Ventral tegmental area (VTA)					†		
Vestibular nuclei (Ve)		†					

Table 1: Continued.

	P3	P7	P9	P14	P21	5 W	11 W
Critical period							
Whisker-barrel formation [22]		<P7¶					
Neuromuscular junction [22]			<P12¶				
Stress-hyporesponsive period (HPA axis) [26, 27]				<P14¶			
Stress/anxiety [22]					<P21¶		
Erasable fear memory [32]					<P21¶		
Orientation bias [22]						<4 w¶	
Ocular dominance [22]							<5 w¶

The present findings could be useful for determining critical periods among several brain regions.

Abbreviations

Arc: Arcuate nucleus
Au1: Primary auditory cortex
BLA: Basolateral amygdaloid nucleus
Cg: Cingulate cortex
ChABC: Chondroitinase ABC
CS: Chondroitin sulfates
CSPGs: Chondroitin sulfate proteoglycans
ECM: Extracellular matrix
Gi: Gigantocellular reticular nucleus
HAPLN: Hyaluronan proteoglycan link protein
HPA axis: Hypothalamic-pituitary-adrenal axis
InC: Interstitial nucleus of Cajal
LH: Lateral hypothalamus
LO: Lateral orbital cortex
LPO: Lateral preoptic area
M1: Primary motor cortex
M2: Secondary motor cortex
MA: Medial amygdaloid nucleus
ME: Median eminence
mRt: Mesencephalic reticular formation
P: Postnatal day
PBS: Phosphate-buffered saline
PBST: PBS containing 0.3% Triton X-100
Pir: Piriform cortex
PNNs: Perineuronal nets
PnO: Oral pontine reticular nucleus
PrL: Prelimbic cortex
PVN: Paraventricular nucleus
RN: Red nucleus
RSG: Retrosplenial granular cortex
Rt: Reticular thalamic nucleus
S1: Primary somatosensory cortex
S1BF: The barrel field of the primary somatosensory cortex
SC: Superior colliculus
SHRP: Stress-hyporesponsive period
SNR: Reticular part of the substantia nigra
Sp5: Spinal trigeminal nucleus
V1: Primary visual cortex
V2L: Lateral area of the secondary visual cortex
V2M: Medial area of the secondary visual cortex
Ve: Vestibular nucleus
VMH: Ventromedial hypothalamic nucleus
VO: Ventral orbital cortex
w: Postnatal week
WFA: *Wisteria floribunda* agglutinin.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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