NEUROPLASTICITY AND EXTRACELLULAR PROTEOLYSIS

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NEUROPLASTICITY AND EXTRACELLULAR PROTEOLYSIS

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Neuroplasticity refers to the ability of the Central Nervous System (CNS) to alter its structure and function in response to a variety of physiological and pathological processes such as development, cognition, injury or neurological diseases. Since more than four decades, studies on synaptic plasticity in the context of memory and learning attracted a remarkable interest. Soon after first seminal works on synaptic plasticity were published, research in this field was extended by studies on non-synaptic as wells as structural plasticity towards a goal to understand cellular and molecular determinants of cognition. Over the past two decades, yet two additional crucial players in neuroplastic phenomena started to be intensely investigated – glial cells and the extracellular matrix (ECM). Growing awareness that glial cells, especially astrocytes, are important regulators of synaptic functions gave rise to a novel concept of a tri-partite synapse. Also, over the last two decades, a growing body of evidence has accumulated that the extracellular matrix (ECM) in the brain is strongly involved in regulation of neurons, in particular, in synaptic plasticity. Thus, a concept of tetra-partite synapse was put forward by some neuroscientists. The cross-talk between neuron-glia-ECM system involves enzymatic degradation of proteins or peptides and amino acids occurring in each of these brain constituents by means of a variety of proteases. Importantly, it has been realized that proteases such as serine proteases and matrix metalloproteinases, not only accompany "robust" phenomena such as cell division, or development or neurodegenerative conditions but may play a very subtle signaling functions, particularly important in memory acquisition. Indeed, the repertoire of substrates for these enzymes covers a wide variety of proteins known to play important role in the neuroplastic phenomena (e.g. BDNF, TNF-**α**, ephrin systems, various cell adhesion molecules, etc.). In result, the role of metalloproteinases and such serine proteases as tissue plasminogen activator (tPA), neuropsin or neurotrypsin in synaptic plasticity as well as in learning and memory has been particularly well demonstrated. It needs to be emphasized, however, that in spite of a remarkable progress in this field, several basic questions regarding molecular and cellular mechanisms remain unanswered. Potential involvement of so many important players (various proteases and their substrates in neurons, glia and in ECM) points to an enormous potential for plasticity phenomena but makes also studies into underlying mechanisms particularly difficult. In the proposed Research Topic we provide both review of the current state of the art and present some original reports on specific aspects of the role of proteolysis in neuroplasticity phenomena. The present ebook starts with extensive reviews describing involvement of proteolysis not only in synaptic plasticity but also in regulating endogenous excitability and structural changes at the

network, cellular and subcellular levels. Cross-talk between neuroplasticity and proteolysis is also emphasized in the context of development and in relation to various pathologies. Whereas in the first part of the present ebook, the major focus is on metalloproteinases, the successive articles address the role of neuropsin and thrombin. The Research Topic is concluded with a series of articles describing the components of extracellular matrix and adhesion proteins and their elaboration by mechanisms dependent directly or indirectly on proteolysis. We do hope that the present ebook will further stimulate the interest in the fascinating investigations into neuroplasticity-proteolysis cross-talk.

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EDITORIAL
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Editorial: Neuroplasticity and Extracellular Proteolysis

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Keywords: metalloproteinases, proteolysis, neuroplasticity, extracellular matrix, learning and memory

The Editorial on the Research Topic

Neuroplasticity and Extracellular Proteolysis

For long investigations of neuroplasticity and research into the role of extracellular proteolysis have been progressing largely separately but their recent progress was paralleled by a growing awareness that neuronal networks form a functional partnership with glia and extracellular matrix (ECM) and thus a term "tetrapartite synapse" was coined (Dityatev and Rusakov). Extracellular proteolysis emerges now as a key mechanism in mediating interactions between these players and description of this cross-talk opens new avenues in exploring physiological and pathological mechanisms in the CNS. The present research topic aims at presenting some different facets of proteolysis in a broad context of neuroplasticity.

Reinhard et al. provide an overview on MMP-9 at the levels and regulation of enzymatic activity, protein, and mRNA, in the brain development with emphasis on critical periods in the sensory cortices. Important role of the enzyme in neuronal/synaptic plasticity is presented along pointing to gaps in this field. Notably, new results are provided by Kelly et al. that loss of MMP-9 attenuates functional ocular dominance and reduces excitatory synapse density in the visual cortex. Kelly et al. observed no change in the morphology of existing dendritic spines, however, spine dynamics were altered in MMP-9 KO mice. Reinhard et al. further discuss cellular and molecular mechanisms in which MMP-9 plays paramount role to control synapse development and plasticity. The authors review studies on long-term potentiation (LTP) to conclude on essential role of MMP-9 in LTP maintenance, as opposed to its early phase. Interestingly, Gorkiewicz et al. demonstrate that MMP-9 is pivotal for the lasting LTP evoked on the lateral to basal as well as basal to central amygdala connections but not in the cortical to lateral amygdala pathway. Mechanisms of plastic changes may differ between projections raising a possibility of different involvement of metalloproteinases. Wiera and Mozrzymas review involvement of metalloproteinases in mossy fiber synapses onto CA3 pyramidal cells (MF-CA3) projection that show pronounced short-term facilitation, a pre-synaptic and NMDAR-independent LTP and a prominent structural plasticity induced by learning. Reinhard et al. discuss also MMP effects on glutamate receptors trafficking and morphology of the dendritic spines. Excessive MMP-9 provokes dendritic spine thinning and elongation, whereas when MMP-9 activity is counterbalanced by its endogenous inhibitors, the enzyme contributes to the spine head expansion. At the molecular level, MMP-9 function at the spines appears to be mediated by cleavage of cell adhesion molecules (CAM) and integrin signaling. This issue has been reviewed by Conant et al. They discuss the role of a variety of metalloproteinases in the context of LTP and learning and memory with particular emphasis of CAM cleavage which may provide means to generate integrin-binding ligands.

Besides synaptic plasticity, neuronal excitability may undergo plastic changes affecting thus the output of dendritic integration. Wójtowicz et al. discuss role of metalloproteinases in this form of plasticity. Ben Shimon et al. provide an extensive review of the role of thrombin which, through

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direct or indirect activation of Protease-Activated Receptor-1 exerts e variety of effects on classic (LTP) and homeostatic synaptic plasticity and was shown to be involved in epileptogenesis.

The MMP-9 targets are addressed by Kelly et al., who show that critical period for visual cortex development coincides with massive degradation of chondroitin sulfate proteoglycans that is prevented in MMP-9 KO mice. Further, molecule functionally linking MMPs with ECM is CD44, whose molecular interactions, signaling and roles in the nervous system are reviewed by Dzwonek et al. They show great functional diversity of CD44 that is important in a wide range of physiological and pathological phenomena, e.g., axon guidance, cytoplasmic calcium clearance, dendritic arborization, synaptic transmission, epileptogenesis, oligodendrocyte, and astrocyte differentiation, post-traumatic brain repair, and brain tumor development. Bijata et al. add an important piece of information that MMP-9 contributes to dendritic development in hippocampal neurons in vitro and provide evidence that MMP-9 mediated cleavage of beta-dystroglycan is important for dendritic development.

As discussed by Reinhard et al., MMP-9 dysfunctions contribute to major neuropsychiatric pathologies, such as Fraxile X Syndrome (FXS) and other forms of autism spectrum disorders, bipolar disorder, schizophrenia, and epilepsy that may all share neurodevelopmental provenance. Therapeutic approaches aiming at MMP-9 inhibition by non-specific drugs such as doxycycline and minocycline have already been shown to be beneficial also in humans suffering from FXS. The role of MMP-9 in FXS is also discussed by Conant et al., who extend the review of MMP-dependent animal models of psychiatric conditions also to addiction.

GABAergic interneurons (particularly parvalbumin-positive) play a pivotal role in shaping network rhythmicity, which is crucial in cognition. Suzuki et al. examine the correlation between neuropsin and parvalbumin (PV) expression in a model of mice freely moving in a familiar or enriched environment. Whereas, neuropsin knockout resulted in reduction of PV reactivity in mice reared in the familiar environment, in the enriched one, upregulation of PV expression was observed in both groups.

A thorough description of ECM composition is a pre-requisite for exploring the mechanisms whereby proteolysis shapes the neuroplasticity. Malik et al. describe the matricellular proteins of the CCN family which was implicated in regulation of e.g., gene expression, proliferation, differentiation, adhesion, and migration. CCN proteins have been studied mostly beyond CNS, however there is neuronal expression of CCN proteins and their contribution to nervous system development, function, and pathology should be considered.

In aggregate, the papers published in this volume rather open new avenues of research than sum up a scientific field close to completion. Upregulation of extracellular proteases has long been believed to accompany mainly pathological conditions. Extensive research over past two decades has provided strong evidence that these enzymes play pivotal roles in physiological and aberrant synaptic plasticity, as shown in this volume. It is up for the further studies to verify a claim that extracellular proteolysis comes of age, holding an important key to understand brain function, and dysfunction; a key that can be also used to develop diagnostic methods and therapies to combat the neuropsychiatric disorders.

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

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REVIEW
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A delicate balance: role of MMP-9 in brain development and pathophysiology of neurodevelopmental disorders

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The extracellular matrix (ECM) is a critical regulator of neural network development and plasticity. As neuronal circuits develop, the ECM stabilizes synaptic contacts, while its cleavage has both permissive and active roles in the regulation of plasticity. Matrix metalloproteinase 9 (MMP-9) is a member of a large family of zinc-dependent endopeptidases that can cleave ECM and several cell surface receptors allowing for synaptic and circuit level reorganization. It is becoming increasingly clear that the regulated activity of MMP-9 is critical for central nervous system (CNS) development. In particular, MMP-9 has a role in the development of sensory circuits during early postnatal periods, called 'critical periods.' MMP-9 can regulate sensory-mediated, local circuit reorganization through its ability to control synaptogenesis, axonal pathfinding and myelination. Although activity-dependent activation of MMP-9 at specific synapses plays an important role in multiple plasticity mechanisms throughout the CNS, misregulated activation of the enzyme is implicated in a number of neurodegenerative disorders, including traumatic brain injury, multiple sclerosis, and Alzheimer's disease. Growing evidence also suggests a role for MMP-9 in the pathophysiology of neurodevelopmental disorders including Fragile X Syndrome. This review outlines the various actions of MMP-9 during postnatal brain development, critical for future studies exploring novel therapeutic strategies for neurodevelopmental disorders.

Keywords: matrix metalloproteinase-9, critical period, plasticity, neurodevelopment, extracellular matrix

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Introduction

The capacity of the CNS of an animal to adapt to changing internal and external environments must be offset by the capacity to maintain network stability following changes. This stability-plasticity balance is highly regulated and is necessary for adaptation and survival throughout the lifespan of an organism. An important challenge in neuroscience remains to identify the mechanisms

Abbreviations: CNS, central nervous system; CPP, critical period plasticity; CRMP-2, collapsin response mediator protein-2; DG, dentate gyrus; ECM, extracellular matrix; EGL, external granular layer; e-LTP, early-phase long term potentiation; FXS, Fragile X syndrome; *Fmr 1*, Fragile X mental retardation gene 1; FMRP, Fragile X mental retardation protein; ICAM-5, intercellular adhesion molecule-5; IGF-1, insulin growth factor – 1 (IGF-1); IGBP-6, insulin growth factor binding protein – 6; IGL, internal granular layer; L1CAM, L1 cell adhesion molecule; l-LTP, late-phase long term potentiation; MAPK, mitogen-activated protein kinase; MMP-9, matrix metalloproteinase-9; NF155, neurofascin; NLG-1, neuroligin-1; OL, oligodendrocytes; PKC, protein kinase C; PNN, perineuronal net; RGD, arginine-glycine-aspartate; SC, superior colliculus; TIMP-1, tissue inhibitor of metalloproteinase-1.

that regulate the dynamics between stability and plasticity. With some 86–100 billion neurons and an approximately equal or larger number of glial cells, depending upon the brain region (Azevedo et al., 2009), the study of the brain has been likened to the quest for understanding the universe, with multiple cellular and molecular interactions that account for brain plasticity. Yet many key mechanisms underlying synaptic plasticity are found not in these brain cells themselves but in their interactions with molecules in the extracellular space surrounding them (reviewed by Ruoslahti, 1996a,b; Nicholson and Sykova, 1998; Shi and Ethell, 2006; Šišková et al., 2009; Dityatev et al., 2010; Dansie and Ethell, 2011). The ECM can be thought of as a scaffold that surrounds neurons and glia within the extracellular space. It is rich with signaling cues that can guide plasticity while maintaining stable network connections over time, mediating synaptic and trans-synaptic interactions. These components can act as repulsive and/or attractive cues for cellular migration and can mediate cell-ECM or cell–cell interactions. While the role of ECM in the plasticity of neuronal circuits is not yet well understood, cleavage of the ECM through the actions of matrix metalloproteinases (MMP) and other ECM-cleaving enzymes can drive plasticity in response to specific changes in neuronal activity.

Matrix metalloproteinase-9 in particular stands out as an important molecule in CNS development and plasticity. MMP-9 is a Zn^{2+}dependent endopeptidase expressed in both the central- and peripheral nervous systems and acts to cleave components of the ECM as well as cell adhesion molecules, cell surface receptors and other proteases. Research has focused on the role of MMP-9 in the progression of neurologic disorders such as epilepsy, multiple sclerosis, neuroinflammatory and autoimmune disorders. However, converging evidence suggests that MMP-9 plays an important role in both establishing synaptic connections during development and in the restructuring of synaptic networks in the adult brain. As improper maturation of sensory networks during development is implicated in many neurodevelopmental disorders and in cognitive deficits, understanding the mechanisms of MMP-9 mediated synaptic plasticity is essential for the development of therapeutic strategies. Such knowledge will guide future clinical studies on the possible role of MMP-9 in neurodevelopmental disorders. As it is possible to regain a state of plasticity and reverse cognitive deficits by manipulating the onset and closure of 'critical periods' (Bradbury et al., 2002; Pizzorusso et al., 2002, 2006), regulating MMP-9 activity during CPP may aid in further development of precise and targeted treatments for neurodevelopmental disorders.

MMP-9 Activity, Expression and Regulation

Matrix metalloproteinase-9 is a Zn^{2+} dependent endopeptidase that is found in many cell types throughout the body, including neurons and glia, endothelial cells (Genersch et al., 2000), glandular epithelia, supportive connective tissue, and muscle cells (Roomi et al., 2009). Among the 25 known MMPs (reviewed by Ethell and Ethell, 2007), MMP-9, MMP-2 and MMP-3 are widely expressed in the CNS. Furthermore, MMP-9 and MMP-2 share similar substrate specificity and are known as gelatinases. Their substrates include components of the ECM, as well as cell adhesion molecules and cell surface receptors, cytokines, growth factors, and other proteases (reviewed by Ethell and Ethell, 2007). Beside an active site containing Zn^{2+} that is necessary for its enzymatic activity, MMP-9 also contains three fibronectin type II repeats which can directly bind gelatin, laminin and collagens types I and IV (reviewed by Van den Steen et al., 2002).

Matrix metalloproteinase-9 expression is regulated during development, with high levels during early developmental time-points that decrease into adulthood (Vaillant et al., 1999; Oliveira-Silva et al., 2007; Bednarek et al., 2009; Aujla and Huntley, 2014). This pattern is consistent when measuring either the protein levels of MMP-9 with antibodies or the enzymatic activity levels of MMP-9 using gelatin zymography (Oliveira-Silva et al., 2007). Although MMP-9 levels remain low in the adult brain, MMP-9 activity increases in response to synaptic activity (Gawlak et al., 2009; Janusz et al., 2013). MMP-9 expression has been detected in several brain areas, including the hippocampus (Aujla and Huntley, 2014), the brainstem (Oliveira-Silva et al., 2007), the cerebellum (Vaillant et al., 1999), and the neocortex (Bednarek et al., 2009). MMP-9 proteolytic activity co-localizes with excitatory synapses (Gawlak et al., 2009), while it's mRNA is detected within the cell body, along the processes and in synaptoneurosome fractions of neurons (Janusz et al., 2013). MMP-9 is also expressed in astrocytes, microglia and along oligodendrocyte processes (reviewed by Dzwonek et al., 2004; Gawlak et al., 2009).

Importantly, the expression, translation and activity of MMP-9 are tightly regulated. Expression of MMP-9 can be regulated by growth factors, cytokines, oncogenes, metal ions, and hormones through MAPK pathway signaling (reviewed by Van den Steen et al., 2002). MMP-9 translation is suppressed through its binding to FMRP, an mRNA binding protein, implicating MMP-9 in fragile X syndrome (FXS) (Janusz et al., 2013). MMP-9 mRNA is mainly localized in the cell body of hippocampal neurons and is translocated from the cell body to dendritic synapses in response to neuronal activity (Dziembowska et al., 2012) as a part of an FMRP containing granule (Janusz et al., 2013). mGluR activation causes the dissociation of FMRP from the MMP-9 mRNA (Janusz et al., 2013) followed by the association of MMP-9 mRNA with actively translating polyribosomes and *de novo* protein synthesis (Dziembowska et al., 2012). Once the MMP-9 protein is secreted from a cell by a yet to be discovered mechanism, it is in an inactive pro-from, also called a zymogen, where its enzymatic activity is inhibited by a pro-domain that masks the catalytic site through an interaction between Zn^{2+} and a cysteine residue in the pro-domain (reviewed by Ethell and Ethell, 2007). This pro-form cannot cleave its substrates until the pro-domain has been removed from the active site by proteolysis or protein unfolding, a process called a *cysteine switch*. This can be performed by other MMPs, serine proteinases, by nitric oxide and by reactive oxygen species. The action of MMP-9 is further limited by degradation of the enzyme and by

inhibition of its activity via thrombospondins or through tissue inhibitor of metalloproteinase-1 (TIMP-1) – which interestingly is secreted in response to synaptic activity at levels similar to MMP-9 (reviewed by Ethell and Ethell, 2007) and can form a complex with MMP-9 prior to secretion (Roderfeld et al., 2007). Thus, the tight regulation of MMP-9 activity is critical for its function in development and plasticity, and highlights a role for MMP-9 in cell-specific, activity-driven activation and remodeling of the pericellular environment, with spatially and temporally restrained effects.

A large body of research on MMP-9 has focused on its various roles in neurodegenerative disorders (reviewed by Yong, 2005). Nonetheless, it is becoming increasingly clear that MMP-9 has multiple functions in CNS development as well, and may contribute to neurodevelopmental disorders. MMP-9, through cleavage of surface- and cell-adhesion molecules, is implicated in active dendritic spine remodeling and stabilization (reviewed by Bilousova et al., 2009; Benson and Huntley, 2012), affecting the shape and function of dendritic synapses (Michaluk et al., 2011). MMP-9 also plays a role in pre- and postsynaptic receptor dynamics (Michaluk et al., 2009; Peixoto et al., 2012; Ning et al., 2013), in consolidation of long-term potentiation (LTP; Wang et al., 2008), myelination (Oh et al., 1999), and possibly in synaptic pruning (Wilczynski et al., 2008). It is further implicated in axonal elongation (Shubayev and Myers, 2004), pathfinding (Vaillant et al., 2003; Lin et al., 2008; Aujla and Huntley, 2014), regeneration (Ahmed et al., 2005) and degeneration (Costanzo et al., 2006). This review summarizes the various roles of MMP-9 in diverse developmental processes within the CNS, with an emphasis on sensory development, and suggests a role for misregulated MMP-9 in neurodevelopmental disorders.

Brain Development and Critical Period Plasticity

The critical period can be loosely defined as a time when experience-dependent activity can drive alterations in the structure, functional organization and physiological properties of brain regions. It occurs during postnatal development and is marked by increased plasticity and massive synaptic reorganization. The opening of the critical period is driven by the onset of sensory function, when peripheral sensory structures mature and begin to relay external sensory input into central structures. Reorganization takes place among thalamocortical projections, established prior to sensory input (reviewed by Feller and Scanziani, 2005), and among corticocortical connections, which together undergo increased synaptogenesis, axonal arborization, and pruning (Sanes et al., 2005) so that each sensory modality is able to respond optimally to behaviorally relevant stimuli (reviewed by Hensch, 2004). This increased plasticity during the critical period is time-limited, constrained by the development of stabilizing factors such as increased intracortical inhibition and the formation of PNNs, a specialized ECM structure (reviewed by Hensch, 2005). The timing of this plasticity is different depending on species, sensory modality and even on the type of sensory manipulation used within a sensory modality (reviewed by Hensch, 2004). Non-sensory regions likewise have a window of postnatal synaptogenesis, reorganization, pruning and apoptosis (reviewed by Hashimoto and Kano, 2005; reviewed by Ribak et al., 1985; Steward and Falk, 1991; Luo, 2005; Murase and McKay, 2012). Periods of high MMP-9 activity correlate with synaptic reorganization during CPP and developmental windows across CNS regions (see **Table 1**), suggesting a role for MMP-9 in shaping CPP kinetics. Within this review we discuss CPP within rodent models due to the widespread use of these models for genetic manipulation of MMP-9 activity, and note that time-lines provided in this review for CPP windows are subject to variability between experimental procedures, protocols and outcome measures used, and are intended as a basic guideline.

One of the first demonstrations of CPP came from studies by Hubel and Wiesel (1964) in the kitten visual cortex, where they demonstrated that monocular deprivation alters both functional and anatomical organization in the cortex and, to a more limited extent, in the thalamus. Within the rodent visual cortex, the CPP window opens during the third postnatal week and closes into the fifth postnatal week, with adult-like functional properties by P45 (Fagiolini et al., 1994; Gordon and Stryker, 1996), though retinotectal reorganization occurs mostly in the first three postnatal weeks (Serfaty et al., 2005; Oliveira-Silva et al., 2007). Most neurons in the rodent visual cortex (Sawtell et al., 2003) and SC respond to contralateral eye stimulation and this contralateral preference can be manipulated during development by monocular deprivation. After eye closure (monocular deprivation), there is a reorganization of network connections contralateral to the deprived eye within the visual cortex (Spolidoro et al., 2012) and SC (Oliveira-Silva et al., 2007). Neuronal responses to stimulation of the deprived eye undergo depression and weakening of synaptic efficacy within 3 days (Sawtell et al., 2003), along with shrinkage of thalamocortical projections (reviewed by Feller and Scanziani, 2005; Spolidoro et al., 2012). Between 4 and 7 days after monocular deprivation, responses to the non-deprived eye undergo potentiation, with a concomitant expansion in axonal arborization (reviewed by Sawtell et al., 2003; Feller and Scanziani, 2005; Spolidoro et al., 2012). The overall outcome is that more neurons will respond to visual stimulation of the non-deprived ipsilateral eye, termed an ocular dominance shift.

Matrix metalloproteinase-9 has been implicated in various aspects of visual CPP. In the SC, MMP-9 enzymatic activity and protein levels (both the proform and active forms) are high during the period of retinotectal map reorganization before and just after eye opening (from P0 to P14), and decrease during late (P21–P42) postnatal development (Oliveira-Silva et al., 2007). Broad inhibition of MMPs from P0 to P7 alters the topographical organization of retinotectal terminals, while monocular enucleation at P10 increases MMP-9 enzymatic activity in the SC both contralateral and ipsilateral to the enucleated eye (Oliveira-Silva et al., 2007). Within the cortex, MMPs are involved in plasticity following monocular deprivation for either 3 or 7 days in adolescent rats (P21–P45; Spolidoro et al., 2012). Inhibition of MMP activity, using the broad spectrum

TABLE 1 | Matrix metalloproteinase-9 expression in CNS development.

Brain region	Developmental window	Peak of MMP-9 expression	MMP-9 in experience dependent plasticity	Reference
Retinotectal Axons	Retinotectal map formation: P0–P21 (first 3 postnatal weeks) *r.*	Sup. Colliculus: P0–P10 *protein* P0–P14 *activity.*	Monocular enucleation increases MMP-9 enzymatic activity in SC	Oliveira-Silva et al. (2007)
Visual Cortex	Monocular deprivation: P21–P28 *r.*; Postnatal week 3–5 *r.;* P19–P32 *m.*		Monocular deprivation increases MMP-9 mRNA in cortex	Fagiolini et al. (1994), Gordon and Stryker (1996), Spolidoro et al. (2012)
Optic Nerve	Optic nerve myelination: P7–P9 *m.*	Optic Nerve: P7-P11 *activity.* P7 *mRNA*		Oh et al. (1999), Larsen et al. (2006)
Barrel Cortex	Thalamocortical input to Layer 4: P1-P4/5 *m., r.* Intracortical connections: Layer 4-2/3: P10-P14 *r.* Layer 2/3: thru adulthood *r.*		Whisker trimming in adolescent mice increases MMP-9 enzymatic activity; MMP-9 KO mice show reduced potentiation in L4 and L2/3 following trimming	Woolsey and Wann (1976), Kossut et al. (1988), Fox (1992), Maravall (2004), Kaliszewska et al. (2012)
Cerebellum	Granule cell proliferation, Climbing fibers refinement: Postnatal week 1 Granule cell migration; Parallel fiber innervation of Purkinje cells: Postnatal week 2–3 Purkinje cell arborization: Postnatal week 4	Granule precursors, Bergmann glia, Purkinje cells: P10 *protein* EGL, IGL and Purkinje cell layer: P11-P17 *protein*	MMP-9 KO mice at P12 exhibit: Decreased apoptosis of granule precursor cells; Delay in granule cell migration	*r.* Altman (1972a,b), *r*. Vaillant et al. (1999), *r.* Piccolini et al. (2012), *m*. Vaillant et al. (2003)
Hippocampus	Spontaneous activity and apoptosis: P0–P7 *r. m.* Mossy fiber development: first postnatal month *r.* Synapse elaboration: first postnatal month *r.*	Axon tips: P4 *r. protein* DG, CA1, CA3: P4 r. *mRNA* Postsynaptic densities: P8 *r. protein*	MMP-9 mediates cell survival through interactions with laminin MMP-9 KO mice show accelerated synaptic maturation MMP-9 treatment exaggerates immature spine morphology	Ribak et al. (1985), Steward and Falk (1991), Bilousova et al. (2009), Murase and McKay (2012), Aujla and Huntley (2014), Sidhu et al. (2014)
Auditory Cortex	Tonotopic reorganization: P11–P15 *m*. P11–P13 *r.* P9–P20 *m.* P11–P28 *r.*		Cochleotomy leads to increased MMP-9 levels in cochlear nucleus 1 day post-op	Zhang et al. (2002), de Villers-Sidani et al. (2007), Illing et al. (2010), Barkat et al. (2011), Kim et al. (2013)

r., rats; m., mouse; activity, enzymatic activity measure by zymography.

MMP inhibitor GM6001 produces three main effects: (1) reduces the ocular dominance shift after monocular deprivation by selectively preventing the potentiation of open-eye responses without affecting the depression of deprived-eye responses, (2) blocks the deprivation-driven increase in spine density in layer 2/3 excitatory neurons contralateral to the deprived eye, and (3) blocks the potentiation of the deprived-eye responses after eye reopening. Though MMP-9 cannot be directly implicated in cortical ocular dominance plasticity based on these results, the authors noted that the only MMP to show mRNA changes in response to monocular deprivation was MMP-9 (Spolidoro et al., 2012). MMP-2 is, however, expressed in the CNS at higher basal levels than MMP-9, and therefore may account for ocular dominance plasticity without mRNA up-regulation. To our knowledge there is no published work using MMP-9 KO mice to further test the role of MMP-9 in visual CPP, however, MMP-3 KO mice, which have reduced activation of pro-MMP-9, do demonstrate altered visual CPP plasticity including reduced open-eye potentiation, suggesting a possible role for MMP-9 (Aerts et al., 2014).

Matrix metalloproteinase-9 has been more directly implicated in somatosensory barrel cortex plasticity. In rodent barrel cortex each whisker is represented in a one-to-one ratio by a cortical cellular aggregate, termed a 'barrel.' Both the thalamocortical innervation of the barrels as well as the intracortical connections can be modified by removing the whiskers, with less dramatic reorganization after plucking or trimming of the whiskers. There are multiple windows of plasticity between cortical layers and across ages (reviewed by Fox, 2002), and the effect of experimental manipulation on barrel cortex plasticity depends both on the age, length and the type of manipulation used. Early postnatal CPP takes place largely in layer IV barrel cortex from P0 to P4/5 (Woolsey and Wann, 1976; Fox, 1992). It is characterized by receptive field plasticity, a conversion of silent synapses into active synapses, and changes in pre-synaptic thalamocortical arborization and barrel formation (reviewed by Fox, 2002 and Erzurumlu and Gaspar, 2012). Lesions within this window can induce structural reorganization of thalamocortical afferents (reviewed by Fox, 2002; Erzurumlu and Gaspar, 2012). CPP in layer IV to II/III occurs from P10

to P14 (reviewed by Maravall, 2004; Erzurumlu and Gaspar, 2012), whereas horizontal intracortical connections in layers II/III remain plastic through the third postnatal week and, to a lesser extent, throughout adolescence (reviewed by Fox, 2002). Adolescent plasticity is characterized as functional but not structural reorganization, including potentiation of spared input and depression of deprived input. More limited functional plasticity remains into adulthood (reviewed by Kossut et al., 1988; Fox, 2002).

One common manipulation to examine barrel CPP is to trim all but one row of whiskers. This can cause a topographic reorganization of functional sensory maps so that barrel columns in the cortex that have been deprived of peripheral sensory input will respond to deflections of the retained, intact whiskers. Such reorganization is possible in adolescent mice after 1 week of whisker trimming, and even more pronounced after 4 weeks. This manipulation causes an increase in the width of barrel activation when the spared-whiskers are deflected, an effect seen in all cortical layers of a vertical barrel column (Kaliszewska et al., 2012). When the spared-whisker is deflected there is also increased cellular activation in the barrel columns that directly border the spared row, as measured by c-fos density (Kaliszewska et al., 2012). After undergoing this deprivation-induced plasticity for 1 week, MMP-9 enzymatic activity levels are higher in the barrel cortex of mice when compared to the control non-deprived hemisphere, whereas there is no increase in MMP-2 (Kaliszewska et al., 2012). In MMP-9 KO mice, though 4 weeks of deprivation did increase the width of spared-row activation in all cortical layers when compared to the control non-deprived hemisphere, the width of layer IV activation was significantly smaller than in WT mice (Kaliszewska et al., 2012). Cellular activation of the deprived rows was also significantly reduced in layer II/III of MMP-9 KO mice compared to WT mice, but was no different in any other layer (Kaliszewska et al., 2012). The fact that MMP-9 activity did not affect reorganization at all layers indicates a specific action of MMP-9, though the mechanism of the specificity is not known.

Understanding how MMP-9 is affecting plasticity at only certain levels of the circuit is difficult based on these results. The most likely site of functional reorganization in adult barrel cortex after deprivation or denervation may be through the unmasking of previously established silent or 'weak' synapses that project from the intact whisker onto neighboring columns (Simons, 1978; Kossut et al., 1988). It is possible that MMP-9 is necessary for integrating these weaker synapses into the circuit by maturation and stabilization of synaptic contacts (see Synapse Development and Plasticity below). Conversely, increased responses to the intact whisker column may be due to disinhibition from surrounding barrels (Kelly et al., 1999). The decreased potentiation in layer II/III observed in the deprived neighboring barrel columns in MMP-9 KO mice may be an effect of the reduced potentiation in layer IV of the intact barrel, as measurement of response latency suggests that information is transferred within a barrel column first, beginning in layer IV and transferring vertically between layers, before it is transferred outward to neighboring barrels (Armstrong-James et al., 1992; reviewed by Fox, 2002; Feldmeyer, 2012). It is further possible that the increased functional activation in layer IV was reflective of changes in astrocytic activity instead of or in addition to neuronal activity. The method of 2-DG autoradiography used in Kaliszewska et al. (2012) measures metabolic activity including that of astrocytes which are located within the somatosensory barrel cortex and which show an organizational pattern that parallels neuronal organization (Giaume et al., 2009; Logvinov et al., 2011). It is possible that loss of MMP-9 can affect astrocytic function and that as-yet undetermined interactions exist between astrocytes, MMP-9 activity and synaptic plasticity in layer IV barrel cortex. More studies are needed to clarify the mechanisms that underlie changes in plasticity observed here.

To our knowledge, no studies have looked at the role of MMP-9 in auditory system CPP. MMP-9 does not influence the development of spiral ganglion cells, which relay information from cochlear hair cells to the CNS (Sung et al., 2014). On the other hand, after removal of the cochlea and spiral ganglion cells, MMP-9 protein in the cochlear nucleus peaks 1 day post-operation, with an increase in the neuropil and a decrease within cell bodies, suggestive of increased MMP-9 secretion from the cells and indicating that MMP-9 may be involved in degenerative and/or regenerative processes in early auditory pathways (Illing et al., 2010). Indirect evidence suggests MMP-9 may have a role in auditory CPP. In *Fmr1* KO mice, a mouse model of FXS, auditory cortex CPP is deficient (Kim et al., 2013). MMP-9 enzymatic levels are elevated in the brain of *Fmr1* KO mice (Gkogkas et al., 2014; Sidhu et al., 2014) possibly due to a loss of FMRP transcriptional regulation of MMP-9 (Janusz et al., 2013). Genetic deletion of MMP-9 or pharmacological treatment that lowers MMP-9 levels during postnatal development rescues behavioral deficits in *Fmr1* KO mice in adulthood (Dansie et al., 2013; Sidhu et al., 2014). Therefore, it is possible that elevated MMP-9 underlies deficient auditory cortex CPP in the *Fmr1* KO mice, which remains to be tested. This is an important avenue of research because CPP deficits may underlie later auditory processing deficits in FXS patients (Rotschafer and Razak, 2013; reviewed by Rotschafer and Razak, 2014).

In the rodent cerebellum, a peak in MMP-9 expression also correlates with a window of synaptic and cellular reorganization. The cerebellum has a clear laminar distribution of cell types and function, with a deep layer called the granular layer or internal granular layer (IGL), a middle layer called the Purkinje cell layer, and a superficial layer called the molecular layer. During early postnatal development there is yet another layer which covers the surface of the cerebellum, the external granular layer (EGL). Cerebellar postnatal development is marked first by the proliferation of cerebellar granule neuron precursor cells in the EGL during the first postnatal week (Altman, 1972a; reviewed by Luo, 2005) and a refinement of climbing fiber excitatory input onto Purkinje cells (reviewed by Hashimoto and Kano, 2005). During the second postnatal week the EGL thins following the migration of granule precursor cells (Vaillant et al., 1999) and disappears by the end of the third postnatal week. During this same time frame the refinement of climbing fiber connections onto Purkinje cells is followed by the innervation

of parallel fibers (granule cell axons) onto Purkinje cells as the Purkinje cells begin to form secondary and tertiary dendritic branches (Altman, 1972b; reviewed by Hashimoto and Kano, 2005). Finally Purkinje cells undergo an extensive arborization during the fourth postnatal week (reviewed by Luo, 2005). MMP-9 enzymatic activity peaks at P10 and decreases to adult levels by P15 (Vaillant et al., 1999). MMP-9 protein at P10 is detectable in granule precursor cells, Bergmann glial cell bodies and processes, and in Purkinje cell dendrites, a pattern which is similar to MMP-9 mRNA expression (Vaillant et al., 1999). From P11 to P17, both MMP-9 and MMP-3 protein are expressed in the EGL, the IGL, and intensely in the molecular and Purkinje cell layers (Piccolini et al., 2012) where MMP-9 labels the soma of granule and Purkinje cells (Vaillant et al., 2003). At P12 MMP-9 expression in the EGL is concentrated in the premigratory pool of granule precursor cells and is present in migrating granule precursor cells dispersed in the molecular layer (Vaillant et al., 2003). Overlapping expression of MMP-9 and MMP-3 is important because MMP-3 can cleave and activate MMP-9. Indeed, MMP-9 plays an important role in cerebellar development as the application of an MMP-9 blocking antibody and genetic deletion of MMP-9 inhibit axonal outgrowth (parallel fibers) and the migration and apoptosis of granule cell precursors in the developing cerebellum (Vaillant et al., 2003).

In the hippocampus, structural organization is largely established during embryogenesis, though in the germinal zone of the DG active neurogenesis and granule cell migration continue into adulthood (Bayer, 1980). During the first postnatal week, there is spontaneous activity that drives reorganization and neuronal survival (Murase and McKay, 2012), while mossy fibers (Ribak et al., 1985) as well as synapses within the DG and CA1 (Steward and Falk, 1991) undergo elaboration and maturation throughout the first postnatal month. At P4, MMP-9 mRNA levels peak in the DG, CA1 and CA3, and MMP-9 and MMP-2 proteins are shown to co-immunolabel with L1CAM (L1 cell adhesion molecule), a marker of growing axons (Aujla and Huntley, 2014). At a period of peak hippocampal synaptogenesis, MMP-9 and MMP-2 are localized near the postsynaptic densities immunolableled against PSD-95 (Aujla and Huntley, 2014). MMP-9 genetic deletion accelerates the maturation of dendritic spines showing an early increase in the number of mature mushroom-shaped spines at P8, which is normally observed later at P14–P21 (Sidhu et al., 2014). Conversely, an acute treatment of cultured hippocampal neurons with active MMP-9 leads to an increase in immature filopodia-like spines and a decrease in mature mushroom-shaped spines (Bilousova et al., 2009). While global genetic deletion of MMP-9 causes an increase in the number of mature spines during early postnatal hippocampal development and enhanced MMP-9 activity leads to the formation of immature spines in cultured hippocampal neurons, a transient release of MMP-9 at the level of a single synapse was shown to be associated with LTP-induced spine enlargement in adult hippocampus (Wang et al., 2008; see Synapse Development and Plasticity for more details). MMP-9 has been also implicated in spine head maturation during the development of visual cortex (Kelly et al., 2014). It is possible that genetic deletion of MMP-9 can lead to brain area specific compensatory effects of other MMPs (Esparza, 2004; Nagy et al., 2006), as MMP-3 is also expressed in the brain and is implicated in synaptic plasticity (Aerts et al., 2014).

Taken together, these studies suggest MMP-9 activity contributes to multiple aspects of neural network reorganization during early development, including regulation of CPP plasticity. This sensory-dependent, activity driven reorganization primes each region to respond appropriately to external cues. The mechanisms underlying MMP-9-mediated CPP and synaptic reorganization though, are not well understood (see **Table 2**).

Cellular Mechanisms

Synapse Development and Plasticity

The structural changes within the CNS thought to underlie neural plasticity, including learning and memory, can be traced to the

TABLE 2 | Matrix metalloproteinase-9 role in specific plastic events.

		Reference
Synaptic Plasticity		
	Late-phase LTP	Nagy et al. (2006), Okulski et al. (2007), Wang et al. (2008), Wiera et al. (2013)
	NMDA receptor trafficking	Michaluk et al. (2009)
	AMPA receptor trafficking	Szepesi et al. (2014)
	Spine elongation	Bilousova et al. (2009), Michaluk et al. (2009, 2011)
	Spine maturation	Wang et al. (2008), Kelly et al. (2014), Szepesi et al. (2014)
	Integrin-dependent	Wang et al. (2008), Michaluk et al. (2009, 2011)
	Involves ICAM-5	Tian et al. (2007), Ning et al. (2013), Kelly et al. (2014)
Axonal plasticity		
	Pathfinding	Vaillant et al. (2003), Hehr (2005), Aujla and Huntley (2014)
	Regeneration after injury	Ferguson (2000), Shubayev and Myers (2004), Ahmed et al. (2005)
	Degeneration after injury	Costanzo et al. (2006)
	EphB2 mediated growth cone collapse	Lin et al. (2008)
Myelination		
	Oligodendrocyte process extension/formation	Uhm et al. (1998), Oh et al. (1999), Šišková et al. (2009)
	Oligodendrocyte/axon contact	Maier et al. (2006)

level of the individual synapse and spine. Functional synaptic plasticity can be broadly classified into two underlying processes: one being depression, during which the post-synaptic potentials are reduced in amplitude relative to baseline, and the other being potentiation, characterized as an increase in synaptic strength. Additionally these changes in synaptic efficacy can be transient (short-term) or they can be maintained hours to days (long-term), where modification of receptors at synaptic sites through new protein synthesis is thought to underlie long-term changes. The mechanisms of both potentiation and depression can be localized to the presynaptic axon terminal, the postsynaptic dendrite, or both. Synaptic plasticity is a process that can incorporate new or changing input into a system, can form associative networks of connections and can respond to changes in the nervous system environment by scaling connections up or down over time to allow for fine-tuned network modification. As such, synaptic plasticity can be considered to be a fundamental cellular event underlying much of the ability of the brain to adapt and respond to changing environmental demands throughout a lifetime, from CPP sensory reorganization to learning and memory.

Matrix metalloproteinase-9 has been implicated in the maintenance of LTP. LTP can be classified into an early-phase LTP (e-LTP) that is independent of protein synthesis and a late-phase LTP (l-LTP) that requires new protein synthesis. l-LTP in particular may underlie the consolidation of memories by strengthening synapses for extended periods of time. A popular brain structure for the study of synaptic plasticity mechanisms is the hippocampus, due to its well-defined circuit organization and because of its role in learning and memory. Multiple hippocampal pathways have been used to study the role of MMP-9 in synaptic plasticity. In the mossy fiber-CA3 pathway, LTP induction in adolescent rats causes an increase in MMP-9 enzymatic activity, both the proform and the active form of the enzyme, and an increase in MMP-9 protein expression in CA3 neurons (Wiera et al., 2012). Both total genetic deletion of MMP-9 (mice) and a 15-fold overexpression of MMP-9 (rats) impair the maintenance of LTP in the mossy fiber-CA3 pathway in hippocampal adolescent slices (Wiera et al., 2013). In both MMP-9 KO and overexpressing models the initial potentiation after high frequency tetanic stimulation is comparable to wild types, but as early as 7–10 min post-stimulation, both models show a significantly reduced potentiation (Wiera et al., 2013). This indicates that too much or too little MMP-9 can lead to impaired LTP maintenance, including both early- and late-phase LTP. In a different hippocampal pathway, the Schaffer collateral-CA1 pathway, MMP-9 activity is also implicated in LTP. High frequency tetanic stimulation or chemically induced LTP (cLTP) increase the levels of active MMP-9 protein, peaking 30 min post-stimulation, but do not affect the levels of MMP-2 (Nagy et al., 2006). cLTP also increases the proteolytic activity of MMP-9 localized to the neuropil in CA1 stratum radiatum (Nagy et al., 2006). Inhibition of MMP-9 and MMP-2 in slices of both young and adult rats, or inhibition of MMP-9 alone in anesthetized rats *in vivo* (Bozdagi et al., 2007), does not affect initial potentiation and e-LTP but does impair l-LTP, with decreased potentiation by 60 min post-stimulation (Nagy et al., 2006). Total genetic deletion of MMP-9 in this same pathway impairs LTP overall, similar to the mossy fiber-CA3 pathway (Nagy et al., 2006). In the CA1 to prefrontal cortex pathway TIMP-1 overexpression, which reduces MMP activity, prevents l-LTP *in vivo* (Okulski et al., 2007). Similarly direct inhibition of MMP-9 *in vitro* blocks prefrontal l-LTP but not e-LTP (Okulski et al., 2007). The precise mechanisms by which MMP-9 affects synaptic plasticity are not fully understood. In the Schaffer collateral-CA1 pathway, increased MMP-9 levels after tetanic stimulation were dependent on *N*-methyl-D-aspartate (NMDA) receptor activation at postsynaptic sites (Nagy et al., 2006). Conversely in the mossy fiber-CA3 pathway, where LTP is also MMP-9 dependent, LTP occurs at presynaptic sites and is independent of NMDA receptors (Harris and Cotman, 1986; Castillo et al., 1997; Wiera et al., 2013). Moreover the experimental manipulations used to test MMP-9 necessity, by either genetic deletion or chemical inhibition, have differential effects on e-LTP induction, leaving in question the role of MMP-9 in e-LTP.

Matrix metalloproteinase-9 also affects the trafficking of both NMDA and α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate (AMPA) glutamate receptors. The modification and/or insertion of glutamate receptors to the postsynaptic site are thought to fundamentally relate to long-term synaptic plasticity. Bath application of auto activating MMP-9 in cultured hippocampal neurons induces the lateral diffusion of NMDA receptors, but not GluA2-containing AMPA receptors, at synapses (Michaluk et al., 2009). On the other hand, after a chemical LTP (cLTP) induction in cultured hippocampal neurons, MMP activity is necessary for the immobilization and synaptic accumulation of GluA1- and GluA2-containing AMPA receptors (Szepesi et al., 2013, 2014) in dendritic spines. MMP-9 in particular was suggested to be involved in AMPA receptor trafficking because MMP-9 levels, but not MMP-2, were up-regulated following cLTP (Szepesi et al., 2013). The action of MMP-9 on lateral diffusion of NMDA receptors was shown to depend on integrin β1 signaling (Michaluk et al., 2009). Integrins are membrane-bound ECM receptors found on pre-and postsynaptic sites in many types of cells in the CNS. Activation of integrin receptors induces the elongation of dendritic spines in hippocampal culture (Shi and Ethell, 2006). Longer, thinner spines are associated with immature synapses and are highly motile, whereas shorter spines with large spine heads are associated with mature, stable synaptic connections. MMP-9 transgenic overexpression or global bath application of active MMP-9 induces the elongation of dendritic spines, similar to integrin activation, while blocking integrin receptors prevents MMP-9-induced spine alterations (Bilousova et al., 2009; Michaluk et al., 2011). The effects of MMP-9 on integrin receptor signaling are not mediated through direct protein–protein interactions (Michaluk et al., 2009). Rather it is suggested that MMP-9 cleavage of ECM triggers a release of RGD-containing peptides that are potent activators of integrins (Ruoslahti, 1996b). A direct application of RGD-containing peptide to cultured hippocampal neurons was also shown to induce spine elongation and actin

reorganization in dendritic spines in an NMDA-dependent manner (Shi and Ethell, 2006), possibly through integrin-mediated phosphorylation of NMDA receptors (Bernard-Trifilo et al., 2005).

Interestingly, local activation of MMP-9 at the level of individual synapses can induce the maturation of spine heads instead of spine elongation. After cLTP in culture, MMP-9 (and possibly MMP-2) enzymatic activity is localized around a small number of immature spines that preferentially undergo spine head enlargement (as opposed to already mature spines; Szepesi et al., 2014). Additionally, experiments using theta burst stimulation to induce LTP in acute hippocampal slices demonstrated that MMP-9 activity is both necessary and sufficient to induce expansion of spine heads, including small and large spines (Wang et al., 2008). Blocking MMP activity produced a transient increase in head size that was quickly reduced to baseline, analogous to the MMP-9 dependent maintenance of LTP (Nagy et al., 2006; Okulski et al., 2007; Wang et al., 2008). Consistent with other reports, MMP-9-mediated spine head enlargement was also dependent on integrin signaling (Wang et al., 2008). In addition, both MMP-9 activation and cLTP were shown to induce the extension of filopodia-like protrusions from the heads of dendritic spines (Szepesi et al., 2013), which were also linked to a local change in connectivity in response to glutamate release (Richards et al., 2005). Taken together this indicates that MMP-9 can induce spine elongation or maturation depending on the level of MMP-9 activity, its localization and/or substrate. Why MMP-9 would have such differential effects is unclear. If endogenous MMP-9 does preferentially target immature spines to induce their maturation in response to neuronal activity (Szepesi et al., 2014), exogenous application of MMP-9 could disrupt this selectivity, targeting mature synapses instead. It is likewise possible that MMP-9 cleavage of specific synaptic and perisynaptic targets at a specific time and place is necessary for spine head enlargement, but that if the action is not temporally restricted MMP-9 activity prevents these synaptic structures from maturing and stabilizing. Indeed, both genetic deletion and overexpression of MMP-9 impairs the maintenance of LTP in the mossy fiber-CA3 pathway in hippocampal slices (Wiera et al., 2013).

Matrix metalloproteinase-9 up-regulation in response to NMDA activity can also act on ICAM-5 and NLG-1 to regulate dendritic spine morphology. ICAM-5 is a cell adhesion molecule found in excitatory cell bodies and in dendritic shafts and spines. In cultured hippocampal neurons, ICAM-5 is found in association with thin spines and filopodia more so than with mature mushroom-shaped spines (Tian et al., 2007; Ning et al., 2013). ICAM-5 within postsynaptic filopodia binds to presynaptic β1integrin receptors to mediate trans-synaptic signaling and this binding maintains immature spine morphology (Ning et al., 2013). Hippocampal neurons treated with NMDA show an increase in mushroom shaped spines with a concomitant increase in ICAM-5 cleavage, indicating that cleavage of ICAM-5 may induce spine maturation (Tian et al., 2007). Both MMP-9 and MMP-2 can cleave ICAM-5, and active levels of both enzymes are elevated after NMDA stimulation (Tian et al., 2007). Likewise, NMDA induced cleavage of ICAM-5 in dendritic shafts is rescued by inhibition of MMP-2 and MMP-9 (Tian et al., 2007). In the visual cortex during a period of active synaptogenesis, just prior to (P14) and during ocular dominance CPP (P28), there is a developmental shift in ICAM-5 localization from dendritic protrusions at P14 to dendritic shafts by P28 (Kelly et al., 2014). However, this shift does not occur in MMP-9 KO mice (Kelly et al., 2014). Instead there are increased ICAM-5 levels in dendritic protrusions at both P14 and P28 which do not change with age (Kelly et al., 2014). An increase in synaptic contacts between ICAM-5 labeled structures (presumably dendritic structures) and axon terminals is also observed in MMP-9 KO mice (Kelly et al., 2013). Together this may indicate that MMP-9 cleaves ICAM-5 in response to NMDA stimulation to induce spine maturation, while in the absence of MMP-9 ICAM-5 remains in dendritic protrusions and maintains immature synapses through its binding to presynaptic β1 integrin receptors. NLG-1 is a postsynaptic adhesion molecule, specific to excitatory cells, which binds to presynaptic neurexins through trans-synaptic interactions (Peixoto et al., 2012). MMP-9 cleaves NLG-1 both *in vivo* and *in vitro*, and this cleavage induces a rapid destabilization of its presynaptic partner neurexin-1 (Peixoto et al., 2012). This destabilization is paralleled by a reduced frequency of mini excitatory postsynaptic potentials and an altered paired pulse ratio – indicative of reduced presynaptic release probability (Peixoto et al., 2012). MMP-9 cleavage of NLG-1 is dependent on NMDA activation and Ca^{2+}/calmodulin-dependent protein kinase signaling, and occurs locally at the excited spine (Peixoto et al., 2012). Interestingly, NLG-1 cleavage is also regulated by sensory experience associated with CPP in the visual cortex and is increased within 2 h of light exposure after 5 days of light deprivation (dark rearing) in mice during the fourth postnatal week (Peixoto et al., 2012). Together these studies illustrate a role for MMP-9 in the active modification of postsynaptic sites to modulate synaptic efficacy, a process important both during development and throughout adulthood. However, the role of MMP-9 in presynaptic axon reorganization remains less clear.

Axon Regeneration and Plasticity

The basic pattern of axonal tract organization is largely established before birth. During embryonic development axonal processes migrate through the developing nervous system relying on chemical cues as well as cell–cell and matrix–cell interactions in order to find their target locations. They then undergo reorganization in response to sensory stimulation during the critical period, where there is an elaboration of axonal arborizations followed by pruning. The axon arbors are thought to remain relatively stable after CPP closure, but can undergo reorganization following injury or sensory deprivation. MMP-2 and MMP-9 have been implicated in embryonic axon path finding in the *Xenopus* (Hehr, 2005) and MMP-9 was more directly implicated in regeneration of axons after injury in the rat (Shubayev and Myers, 2004; Ahmed et al., 2005). MMP-9 protein is also expressed in growing axons during early postnatal synaptogenesis in the hippocampus (Aujla

and Huntley, 2014) and is necessary for neurite outgrowth in cerebellar granular cells (Vaillant et al., 2003). Still, the role of MMP-9 in CPP axonal reorganization needs further investigation.

During axon outgrowth and regeneration, MMP-9 activity seems to have differential effects depending on the neuronal type. MMP-9 protein is localized to axon growth cones in regenerating nerve fibers 8 days after sciatic nerve injury in rats (Shubayev and Myers, 2004) and its active enzymatic activity can peak as soon as 21 h after sciatic nerve injury in the mouse (Ferguson, 2000), although this early increase may be associated with inflammatory processes at the injury site rather than with regeneration *per se*. MMP-9 in PC12 cell culture can increase the length of sprouting fibers without affecting the total number of sprouting fibers (Shubayev and Myers, 2004). Similarly, MMP-9 and MMP-2 treatment of dorsal root ganglion neurites grown in culture can increase the average length of the neurites (Ferguson, 2000). Conversely, MMP-9 treatment does not affect either the number or the length of neurites in neonatal spiral ganglion neurons cultured from 5 days old rats (Sung et al., 2014). After olfactory nerve injury, increases in MMP-9 protein levels overlap with degeneration of mature olfactory neurons, while MMP-9 decreases during the period of regeneration (Costanzo et al., 2006). In contrast, both MMP-9 and MMP-2 protein and enzymatic levels are increased in regenerating optic nerves compared to non-regenerating nerves, peaking 8 days after injury (Ahmed et al., 2005).

Mechanisms underlying MMP-9 activity in axon growth and repair are also diverse. MMP-9 and MMP-2 can cleave EphB2, a receptor tyrosine kinase that can differentially attract or repulse axonal fibers through interactions with the ephrin-B ligand (reviewed by Sloniowski and Ethell, 2012). MMP-9 cleavage of EphB2 triggers a cell repulsion and subsequent collapse of axon growth cones in cultured hippocampal neurons through Rho GTPase activation (Lin et al., 2008). Additionally, MMP-9 cleaves CRMP-2 (Bajor et al., 2012), a protein that is shown to induce neuronal polarization and the elongation of axons (Yoshimura et al., 2005) in part through its ability to bind with tubulin heterodimers and to thereby promote microtubule formation (Fukata et al., 2002). How MMP-9 activity might affect the function of CRMP-2 is not yet clear, though the putative cleavage site for MMP-9 of CRMP-2 overlaps with its protein binding site (Bajor et al., 2012), indicating that MMP-9 cleavage might interfere with CRMP-2 binding to tubulin, thereby affecting axonal elongation. Furthermore, MMP-9 can create a permissive environment for nerve regrowth after injury by controlling the proliferation of Schwann cells through induction of the Ras/Raf/MEK–ERK pathway to suspend Schwann cell mitosis (Chattopadhyay and Shubayev, 2009). Together these studies indicate numerous roles for MMP-9 in axonal plasticity, including regeneration and degeneration after injury, polarization and growth of neurite fibers, axon repulsion and growth cone collapse, as well as interactions with glial cells. It remains to be seen whether similar mechanisms play a role in postnatal CNS development and CPP.

Myelination

Matrix metalloproteinase-9 is also expressed in OL (Oh et al., 1999) and MMP activity is implicated in the regulation of myelin formation in the CNS (Maier et al., 2006). Myelination is one of the last processes to occur in CNS development, a hallmark of mature, established axonal connections. In the CNS, myelination along axonal processes is performed by OL, a type of glial cell. Each oligodendrocyte can myelinate as many as 40 internodes, a process that occurs in several steps. After OL migrate and proliferate during early postnatal development (reviewed by Pfeiffer et al., 1993) three further steps are involved in the process of myelination: (1) OLs begin a period of extensive outgrowth of processes radially from the cell body toward the axons, (2) once contact with an axon has been made, the OL ensheaths the axon in myelin layers until (3) compaction of the myelin sheath occurs (Uhm et al., 1998). Neuronal signals are key in the maturation of OL, where for example adenosine acts as a neuron-glial signal to inhibit the proliferation of OL progenitor cells and promote differentiation and myelin formation (Stevens et al., 2002). Myelin formation peaks within the first 4 weeks after birth (Foran and Peterson, 1992), generally in a caudal to rostral organization (reviewed by Pfeiffer et al., 1993). Axons with larger diameters are myelinated first, followed by smaller-diameter axons (Matthews and Duncan, 1971; Ye et al., 1995b). However, there are specific periods of myelination within different brain regions, each with a specific onset/peak window as well as different organizational trajectories (Foran and Peterson, 1992).

In the mouse optic nerve, MMP-9 enzymatic activity levels gradually increase from P3 to P11, with elevated transcript levels in the optic nerve at P7 (Larsen et al., 2006) and strong gelatinolytic activity at P9 (Oh et al., 1999), a time period overlapping with the strong expression of myelin basic protein (MBP; Oh et al., 1999). In contrast, in the rodent corpus callosum, MMP-9 protein expression increases from P7 to P28 (Uhm et al., 1998), a time that corresponds to developmental myelination. MMP protein and gelatinolytic activity are localized around the OL cell body, along OL processes and, notably, at the advancing tip of OL processes (Oh et al., 1999; Šišková et al., 2009), implicating MMP-9 in the first step of myelination, OL process outgrowth.

Indeed, in mouse, human and bovine cultures of OLs, (Oh et al., 1999) increased OL process formation following activation of PKC is accompanied by MMP-9 secretion, likely the proform of the enzyme, and inhibition of MMP-9 activity reduces OL process outgrowth (Uhm et al., 1998). In MMP-9 KO mice, though the development of myelin formation in the optic nerve is comparable to WT mice, adult OLs in culture have impaired process outgrowth in response to PKC activation and reduced spontaneous process formation (Oh et al., 1999). In the corpus collosum of MMP-9 KO mice, however, there was a transient reduction in the amount of MBP from P7 to P10 and a decrease in the number of mature OL cells (Larsen et al., 2006). The reduction in the number of mature OL cells indicates a role for MMP-9 not only in process extension but also in OL maturation. Šišková et al. (2009) further

demonstrated that the interaction of MMP-9 with ECM molecule fibronectin affects the distribution of MMP-9 enzymatic activity along OL processes, restricting its activity to OL cell bodies. This change in MMP-9 localization from control conditions is associated with the inhibition of OL differentiation, characterized by fewer primary and secondary processes, implicating MMP-9 enzymatic activity in OL differentiation (Šišková et al., 2009).

Matrix metalloproteinase-9 may also be involved in the second stage of myelination, ensheathment, through indirect interactions with IGF-1. IGF-1 promotes OL proliferation and/or survival, stimulates myelination production and increases myelin thickness, and can be inhibited by IGFBP-6 (Ye et al., 1995a). MMP-9 and MMP-12 can both cleave IGFBP-6, while MMP-9 null mice show increased levels of IGFBP-6 (Larsen et al., 2006) and application of IGF-1 increases the number of mature OLs in MMP-12 null mice (Larsen et al., 2006). These studies suggest a role for MMP-9 and MMP-12 in cleavage of IGFBP-6 that disinhibits IGF-1 and thereby promotes OL maturation and ensheathment. MMP activity is further implicated in mediating contact between OL cells and axons through a cleavage of the OL-specific 155-kDa isoform of NF155, a member of the L1-family of cell adhesion molecules (L1-CAM; Maier et al., 2006). This function is important because the extracellular domain of NF155, localized on the oligodendrocyte, can interact directly with adhesion molecules located on the axonal membrane, such as contactin (Maier et al., 2006). This suggests a role for MMP-mediated cleavage of NF155 in establishing the interactions between myelin and axons. Thus, MMP-9 is involved in multiple steps of the myelination process including OL maturation, process outgrowth and ensheathment of axons through its association with ECM and neuronal receptors to 'guide' the myelination process.

MMP-9 in Pathophysiology of Neurologic Disorders

While the regulation of MMP-9 is important for many aspects of normal CNS development and plasticity, misregulation of MMP-9 levels and activity is increasingly implicated in neurodevelopmental and psychiatric disorders that are associated with aberrant brain development.

Fragile X Syndrome and Other Developmental Disorders

Recently, MMP-9 has been implicated in several psychiatric disorders that are associated with abnormal development including FXS, autism spectrum disorder (ASD), bipolar disorder and schizophrenia. Increased levels of MMP-9 have been demonstrated in human subjects with FXS (Dziembowska et al., 2013; Sidhu et al., 2014), ASD (Abdallah et al., 2012), bipolar disorder (Rybakowski et al., 2013) and treatment-resistant schizophrenia (Yamamori et al., 2013). Still, there is limited literature on the precise function of MMP-9 in these disorders. Whether MMP-9 is responsible for the behavioral deficits associated with these disorders, or is a result of abnormal changes in the brain is still unclear and requires further investigation. However, recent research suggests that enhanced MMP-9 activity is a major factor contributing to the pathophysiology of FXS and epilepsy.

Fragile X syndrome is a trinucleotide repeat disorder that leads to the transcriptional silencing of *Fmr1*. The FMR protein encoded by the gene is involved in translation regulation, and genetic deletion of the *Fmr1* gene in mice mimics FXS-associated deficits, providing a useful model to study FXS. The loss of translational suppression of MMP-9 by FMRP may be driving some of the deficits associated with FXS, such as abnormal dendritic spine development and synaptic plasticity. MMP-9 protein and enzymatic activity levels are increased in the hippocampus of developing (P7), adolescent, and adult *Fmr1 KO* mice (Bilousova et al., 2009; Janusz et al., 2013; Gkogkas et al., 2014; Sidhu et al., 2014). This enhanced MMP-9 activity contributes to abnormal PI3K-Akt-mTOR signaling in the *Fmr1* KO mouse, a pathway implicated in FXS and other ASDs (Sidhu et al., 2014). Moreover, the ability of a tetracycline derivative, minocycline, to inhibit MMP-9 activity is suggested to underlie its beneficial effects in *Fmr1* KO mice. Minocycline treatment lowers MMP-9, but not MMP-2 levels, and restores normal dendritic spine development in young *Fmr1* KO mice (Bilousova et al., 2009), reduces audiogenic seizure severity and rescues abnormal behaviors in both young and adult *Fmr1* KO mice (Rotschafer et al., 2012; Dansie et al., 2013). Genetic deletion of *MMP-9* also repairs abnormal dendritic spine development, normalizes mGluR5-dependent LTD and alleviates abnormal social behaviors and anxiety in *Fmr1* KO mice (Sidhu et al., 2014). In drosophila there are only two MMPs, the secreted form MMP-1 and the membrane-anchored form MMP-2. Inhibition of MMP-1 activity in *dfmr1* null flies, a drosophila model of FXS, rescues some or all of the defects associated with the deletion of *dfmr* (Siller and Broadie, 2011; reviewed by Siller and Broadie, 2012). This was demonstrated using three different approaches: either (1) the overexpression of tissue inhibitor of MMP (TIMP), (2) the generation of double null mutants *dfmr1;mmp1*, or (3) treatment with minocycline (Siller and Broadie, 2011; reviewed by Siller and Broadie, 2012).

In humans with FXS the levels of total MMP-9 enzymatic activity are elevated in the plasma, without changes in MMP-2 levels (Dziembowska et al., 2013). The analysis of postmortem FXS brain tissue samples also indicate elevated levels of MMP-9 protein in both the hippocampus and neocortex (Gkogkas et al., 2014; Sidhu et al., 2014). Minocycline treatment reduces plasma levels of MMP-9 in most but not all subjects, and is associated with improvements in behavior (Paribello et al., 2010; Utari et al., 2010; Dziembowska et al., 2013; Leigh et al., 2013) and a reversal of habituation deficits in auditory event related potentials (Schneider et al., 2013). This suggests that MMP-9 suppression may be a useful therapeutic approach. Together these studies indicate that increased MMP-9 levels in FXS may underlie molecular, cellular and behavioral deficits, many of which are associated with abnormal plasticity, learning and memory in FXS.

MMP-9 in Epilepsy

The role of MMP-9 in epilepsy has been seen in both humans (Suenaga et al., 2008; Konopka et al., 2013) and in animals, and is a useful model of MMP-9 in aberrant plasticity. Epilepsy is a disorder in which seizures become spontaneous and recurrent, with highly synchronized activity. Aberrant neuronal plasticity as a result of seizure activity or brain injury is thought to underlie the progression of epilepsy. In both animal models of epilepsy and human epilepsy subjects (Proper et al., 2000) this aberrant plasticity can include neuronal loss in hippocampal regions, astrogliosis, aberrant pruning of DG spines (Wilczynski et al., 2008), reorganization of interneuron terminals (Pollock et al., 2014), loss and/or disorganized PNN structures (McRae et al., 2012; Pollock et al., 2014), and the sprouting of mossy fibers to create a recurrent network (Wilczynski et al., 2008).

In young and adult human epilepsy patients MMP-9 protein levels are higher compared to controls (Konopka et al., 2013). Though other MMPs are increased as well, including MMP-2, MMP-3 and TIMP-2 in adults, MMP-9 has the most prominent and consistent increase in protein levels across ages (Konopka et al., 2013). This protein expression is localized to neurons, astrocytic processes and synapses, and is more strongly expressed in dysmorphic neurons than healthy neurons (Konopka et al., 2013). One mechanism thought to underlie epilepsy progression may be through a change in the ratio of MMP-9 to TIMP-1, the endogenous inhibitor of MMP-9. Subjects with varying forms of epilepsy have an increase in the MMP-9/TIMP-1 ratio suggesting an increase in the extracellular levels of MMP-9 activity (Suenaga et al., 2008).

In rodent models of epilepsy, a kindling procedure can be used where animals are electrically or chemically stimulated at sub-threshold seizure levels over a period of weeks until spontaneous seizures take place. MMP-9 KO mice require a longer kindling period to develop epilepsy over controls (Wilczynski et al., 2008; Mizoguchi et al., 2011), and experience less severe seizures once fully kindled and an increased survival rate (Wilczynski et al., 2008). Conversely, transgenic MMP-9 overexpressing rats show increased susceptibility to epileptogenesis with kindling (Wilczynski et al., 2008). After epilepsy induction in mice, overall MMP-9 protein levels did not change in the DG; however, there was a marked increase in the number of spines expressing MMP-9 protein and in gelatinolytic activity localized to dendrites (Wilczynski et al., 2008). Interestingly, DG spine density was significantly decreased in WT but not MMP-9 KO mice after kainite-induced seizure indicating MMP-9 may play a destructive role promoting kainate-evoked spine pruning (Wilczynski et al., 2008). The inhibition of MMP-9 in slices after kainate application produces a 90% decrease in the density of mossy fiber sprouting in the DG (Wilczynski et al., 2008). This indicates a functional role for MMP-9 both in synaptic pruning and in mossy fiber sprouting in rodent models of epilepsy.

It is likely that there are multiple targets of MMP-9 which contribute to the progression of epilepsy. It is possible that MMP-9 acts by creating a permissive state through cleavage of ECM molecules, in particular, through cleavage of aggrecan. After seizure induction there is a loss of PNNs around parvalbumin positive interneurons (Pollock et al., 2014), including a specific reduction in aggrecan and an increase in unbound hyaluronan (McRae et al., 2012). Doxycycline hyclate, an antibiotic that inhibits MMP-9, prevents the loss of PNNs after kindling and delays epilepsy onset, possibly through the inhibition of MMP-9 (Pollock et al., 2014). MMP-9 mediated cleavage of ECM may also affect neuronal activity and potentiate NMDAR currents through the activation of integrins (see Synapse Development and Plasticity for details). In addition, MMP-9 cleavage of pro-BDNF into mature BDNF may play a role in the development of epilepsy through mossy fiber sprouting. The BDNF to pro-BDNF ratio increases in kindled WT mice, but not in MMP-9 KO mice, during the progression of epilepsy (Mizoguchi et al., 2011) implicating MMP-9 in pro-BDNF cleavage. Because there is no difference in overall BDNF levels in fully kindled mice, this suggests that MMP-9 cleavage of pro-BDNF may accelerate the onset of epilepsy (Mizoguchi et al., 2011).

Therapeutic Approaches

There are several drugs on the market that can directly or indirectly alter MMP-9 levels and activity, some of which are already in use in humans. For example, diazepam, a drug used to treat epilepsy in humans, is able to suppress the development of epilepsy in kindled mice and reduce MMP-9 levels (Mizoguchi et al., 2011). Likewise both doxycycline (Pollock et al., 2014) and minocycline (Beheshti Nasr et al., 2013; Dansie et al., 2013) treatment can reduce seizure severity or epilepsy onset in mice. Minocycline treatment also rescues behavioral, anatomical and social deficits in young and adult *Fmr1* KO mice (Bilousova et al., 2009; Rotschafer et al., 2012; Dansie et al., 2013). Clinical trials on the effects of minocycline in human subjects with FXS have demonstrated improvement in language, social communication, anxiety and attention (Utari et al., 2010) stereotypy, irritability and hyperactivity (Paribello et al., 2010) as well as on behavioral-improvement scales based on caregiver reports in a randomized double-blind, placebo-controlled trial in children and adolescents with FXS (Dziembowska et al., 2013; Leigh et al., 2013). What's more, electrophysiological measures in the auditory cortex of human subjects with FXS indicate a reduction in auditory hyper-reactivity with minocycline treatment (Schneider et al., 2013).

Considering the widespread action of minocycline in the CNS, the effects of minocycline in FXS may not be limited to the inhibition of MMP-9. Minocycline can inhibit other MMPs and is shown to have anti-inflammatory and anti-apoptotic effects (reviewed by Elewa et al., 2006), can phosphorylate AMPA receptors (Imbesi et al., 2008), and affect MAPK signaling (Nikodemova et al., 2006). Doxycycline similarly has various effects on CNS functions. On the other hand, MMP-9 is itself expressed by many types of tissue throughout the body and, as in the CNS, it can have both detrimental and beneficial effects on biological systems (reviewed by Fingleton, 2008). The potential side effects of non-specific MMP-9 inhibition to treat neurodevelopmental disorders have not been thoroughly investigated, though MMP-9 KO mice have shown detrimental side effects as well as benefits in animal models of cancer (Coussens et al., 2000) and kidney disease (Zeisberg et al., 2006)

and highlight the need for treatments specific to both the CNS and to key points in the progression of a disorder. Nonetheless recent studies discussed herein do demonstrate a relationship between the ability of minocycline to inhibit MMP-9 in FXS and a concomitant improvement on behavioral, morphologic and physiological levels. Side effects of the treatment as reported in these studies are minor and are commonly associated with gastrointestinal discomfort (Paribello et al., 2010; Utari et al., 2010; Dziembowska et al., 2013). Altogether, this body of research points to the need for additional studies to understand the specific role of MMP-9 in the etiology of a disorder, including the time frame during which MMP-9 activity may have the largest deleterious effects, and to develop treatments targeted to MMP-9 in the progression of each disorder to further improve the lives of people with intellectual disabilities and autism.

Conclusion

Untangling the precise functions of MMP-9 is challenging. This is due to the fact that it is expressed by both neurons and glia; that its secretion and activation are regulated by a wide variety of factors; and that it can act on multiple targets, each of which can have differential and even opposing effects. In this review, we summarized the various roles of MMP-9 during early postnatal development within the CNS, with a focus on sensory development.

These developmental events, many of which recruit MMP-9 activity, occur in an organized and interactive fashion through specific cellular targets and in response to sensory experience that drives neuronal activity. MMP-9 recruited to specific extracellular sites can cleave ECM creating room within the extracellular space for cells to migrate and for neuronal processes to grow. Yet beyond being simply permissive, MMP-9, through cleavage and/or activation of cell surface and cell adhesion molecules, is implicated in active dendritic spine remodeling and stabilization; pre- and postsynaptic receptor dynamics; consolidation of LTP; myelination and synaptic pruning. It is further implicated in axonal sprouting, path finding, regeneration and degeneration. MMP-9 is expressed just prior to and during CPP, a peak of reorganization in the sensory cortex, and its activity is down regulated in the adult brain. However, unregulated activity of MMP-9 can have detrimental effects on brain functions and may underlie deficits observed in several neurodevelopmental disorders. It is therefore important to better understand the mechanisms by which MMP-9 mediates early postnatal development and its role in neurodevelopmental disorders.

Several key questions remain unanswered. The role of MMP-9 in auditory CPP, for instance, is yet to be studied. Tonotopic maps in rodent primary auditory cortex do undergo reorganization during a 'sensitive window' after which the network is less plastic. Reorganization of the tonotopic frequency map can take place with as few as 3 days exposure to a single tone, from P11 to P13 (de Villers-Sidani et al., 2007), though time windows used to test auditory CPP vary (Webster, 1983; Zhang et al., 2002; Barkat et al., 2011; Kim et al., 2013). Recent studies have demonstrated an auditory CPP deficit in *Fmr1* KO mice (Kim et al., 2013) that may be related to enhanced levels of MMP-9 activity in the brain. The role of MMP-9 in these auditory deficits remains unclear. Because clarification may lead to new therapeutic strategies to treat auditory hypersensitivity and habituation deficits seen in FXS and ASD, understating this role is important.

Though MMP-9 seems to consistently be associated with early postnatal development across many sensory regions and is downregulated by adolescence and into adulthood, mechanistic studies of MMP-9 have largely focused on l-LTP and synaptic plasticity in cultured hippocampal neurons. Furthermore, it is known that during development many receptors undergo changes in sub-receptor composition, localization and/or distribution within a network, as well as in their physiological properties. Similarly, intracellular signaling molecules, as well as cell surface and cell adhesion molecules and ECM components all have altered expression throughout development. Therefore it is uncertain whether the mechanisms underlying MMP-9 recruitment and activity in adolescent and adult hippocampal neurons are conserved across cortical regions throughout development.

In addition, given the overexpression of MMP-9 in multiple neurodevelopmental disorders, it is important to understand whether MMP-9 plays a regulatory and/or permissive role in critical period development, specifically through its interactions with ECM. The formation of PNN structures in the CNS, a specific sub-type of ECM, has been linked to the closure of visual CPP in rodents (reviewed by Hensch, 2005), associated with developmental song learning in the zebra finch (Balmer et al., 2009), and has been shown to be altered with sensory deprivation in rodent barrel cortex (McRae et al., 2007). MMP-9 can directly or indirectly induce cleavage of multiple components of PNN structures, including laminin, brevican, tenascin-R, and aggrecan (reviewed by Ethell and Ethell, 2007). Therefore elevated levels of MMP-9 during development may induce aberrant cleavage and alterations in PNN structures. It remains to be seen whether PNNs are indeed altered in neurodevelopmental disorders and whether MMP-9 inhibition can recover normal CPP through the stabilization of PNNs.

While MMP-9 activity is important for normal CNS development and for the consolidation of long-term memories from development through adulthood, misregulated expression and/or activity of MMP-9 is associated with many neurological disorders. Therefore, understanding the action of MMP-9 within specific cortical regions, developmental periods and in specific cellular processes is important for providing groundwork for new therapies that target MMP-9 in treatments for neurodevelopmental and other disorders.

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frontiers in Cellular Neuroscience

REVIEW
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Activity dependent CAM cleavage and neurotransmission

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Spatially localized proteolysis represents an elegant means by which neuronal activity dependent changes in synaptic structure, and thus experience dependent learning and memory, can be achieved. *In vitro* and *in vivo* studies suggest that matrix metalloproteinase and adamalysin activity is concentrated at the cell surface, and emerging evidence suggests that increased peri-synaptic expression, release and/or activation of these proteinases occurs with enhanced excitatory neurotransmission. Synaptically expressed cell adhesion molecules (CAMs) could therefore represent important targets for neuronal activity-dependent proteolysis. Several CAM subtypes are expressed at the synapse, and their cleavage can influence the efficacy of synaptic transmission through a variety of non-mutually exclusive mechanisms. In the following review, we discuss mechanisms that regulate neuronal activity-dependent synaptic CAM shedding, including those that may be calcium dependent. We also highlight CAM targets of activity-dependent proteolysis including neuroligin and intercellular adhesion molecule-5 (ICAM-5). We include discussion focused on potential consequences of synaptic CAM shedding, with an emphasis on interactions between soluble CAM cleavage products and specific pre- and post-synaptic receptors.

Keywords: metalloproteases, MMP, adhesion, CAM, glutamate, dendritic spine

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A Brief Overview of the Players: Excitatory Synapses, Metalloproteinases, and Cell Adhesion Molecules

Excitatory Synapses in the Central Nervous System (CNS)

Changes in the number, structure, and/or function excitatory glutamatergic synapses are critical to experience dependent plasticity (Moser et al., 1994; Kopec et al., 2006). In a simplified view, the majority of these synapses are dipartite structures consisting of pre-synaptic axon terminals from which transmitter is released and post-synaptic neurotransmitter receptor-bearing dendritic spines. The latter are small protrusions of varied size and shape that emerge from the dendritic shaft of glutamate-responsive neurons (Alvarez and Sabatini, 2007). Spines with relatively large diameter heads or a mushroom-like morphology tend to be comparatively more stable and to have an increased α-amino-3-hydroxy-5methyl-4-isoxazolepropionic acid (AMPA) receptor (GluA) number (Matsuzaki et al., 2004; Kopec et al., 2006; Bourne and Harris, 2007; Matsuzaki, 2007; Kasai et al., 2010). Importantly, neuronal activity dependent spine head enlargement, with a concomitant increase in synaptic incorporation of GluAs, is thought to underlie lasting enhancement of synaptic transmission or long-term potentiation (LTP). In a more complex view, glutamatergic synapses can be appreciated as multipartite sites in which glial cell processes approximate pre- and post-synaptic contact sites. Glial cells, or their soluble products, may thus modulate the structural and functional dynamics of neurotransmission (Dityatev and Rusakov, 2011).

Metalloproteinases in the CNS

Metzincin proteases are zinc-dependent endopeptidases that include cell-secreted matrix metalloproteinases (MMPs) and transmembrane spanning adamalysins [a disintegrin and metalloproteinase (ADAMs)]. These proteases are increasingly appreciated as important effectors of brain function [reviewed in Rivera et al. (2010), Huntley (2012), Sonderegger and Matsumoto-Miyai (2014)]. Though a variety of MMPs and ADAMs are expressed in man, including more than 23 MMPs identified to date (Page-McCaw et al., 2007), it should be noted that a select subset is likely relevant to physiological and pathological CNS plasticity. Family members with well-described expression in neurons, astrocytes, or microglia include MMP-1, MMP-2, MMP-3, MMP-7, MMP-9, MMP-12, MMP-13, MMP-14, and a disintegrin and metalloproteinase-10 (ADAM-10; Yong et al., 2001; Van Hove et al., 2012a).

The cell regulates overall activity of specific MMP and ADAM family members at several levels including that of gene expression. At the transcriptional level, activator protein 1 (AP-1) and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) increase the expression of varied family members such as MMP-9 (Ganguly et al., 2013; see also Table 1 of Berry et al., 2013 for more on transcription factors and select MMPs). In contrast the transcription factor Yin Yang 1 (YY1) directs histone modification to strongly repress transcription of MMP-9 (Rylski et al., 2008). While less is known about the regulation of ADAM family member expression, we do know that the promoter for ADAM10 contains several transcription factor binding-sites including a retinoic acid-responsive element where retinoic acid receptors and retinoic X receptors can bind and thereby activate gene expression (Prinzen et al., 2005; Tippmann et al., 2009). Retinoic acid receptors are present in synaptoneurosomes and dendrites and contribute to select forms of synaptic plasticity (Aoto et al., 2008; Groth and Tsien, 2008).

Post-translational regulation of MMPs and ADAMs is also important with respect to enzymatic activity. Since MMPs typically act on extracellular substrates, release mechanisms represent a potentially important point of control. In a study that activated fibrosarcoma cells with phorbol myristate acetate (PMA), it was shown that subsequent release of MMP-2 and -9 is soluble NSF attachment protein receptor (SNARE) dependent (Kean et al., 2009). The SNARE protein family is critical for calcium-dependent vesicular fusion and release from neurons (Gerber and Sudhof, 2002; Sudhof, 2013). Since neuronal activity can increase intracellular calcium through mechanisms including activation of voltage gated calcium channels, it is tempting to speculate that calcium-dependent MMP release could be facilitated with the same. Of interest, MMP-2 and MMP-9 containing vesicles are observed in the somatodendritic compartment and found in dendritic spines (Sbai et al., 2008; Wilczynski et al., 2008). Moreover, stimuli that may increase intra-neuronal calcium and can induce LTP, can also evoke local MMP-9 release (Wang et al., 2008).

With respect to transmembrane spanning ADAMs, localization is also regulated. For example, ADAM10 and ADAM17 are mainly associated with the endoplasmic reticulum (ER) and Golgi apparatus, with little protein present at the plasma membrane (Schlondorff et al., 2000; Gutwein et al., 2003). ADAM10 contains an ER retention signal at its C-terminus, suggesting that unidentified proteins are required for the ER exit and transport of this protease to the plasma membrane (Marcello et al., 2010, 2012). The binding of tetraspanins to ADAM10 (Xu et al., 2009; Prox et al., 2012) can promote ADAM10 exit from the ER. Synapse-associated protein-97 (SAP-97), a cargo protein involved in protein trafficking at excitatory synapses, can bind to proline-rich sequences in the cytosolic domain of ADAM10 (Marcello et al., 2012). *N*-methyl-D-aspartate receptor (GluN) activation has been shown to affect phosphorylation of SAP97, as well as the transport of ADAM10 from Golgi outposts to synaptic membranes (Saraceno et al., 2014).

The activation of appropriately localized proteases represents an additional point of control. In the case of MMPs, this is typically achieved following release from the cell through cleavage of the pro-domain by other metalloproteinases or plasmin (Nagase et al., 1990). Cleavage of the pro-domain disrupts a critical Cys-Zn^{2+} interaction that otherwise blocks substrate processing (Van Wart and Birkedal-Hansen, 1990). Non-proteolytic activation of MMPs, however, also occurs. For example, nitration or oxidation may alter tertiary structure to activate pro-forms (Gu et al., 2002). Though less well-studied, MMPs may also be active within the cell (Wang et al., 2002).

Finally, MMP activity can be quenched by processes including low density lipoprotein receptor dependent internalization (Hahn-Dantona et al., 2001), and non-covalent interactions with endogenous tissue inhibitors of metalloproteinases (TIMPs; Gardner and Ghorpade, 2003; Visse and Nagase, 2003; Brew and Nagase, 2010).

In terms of brain plasticity related mechanisms that impact MMP expression, release and/or activity, several stimuli or stressors have been studied (see **Table 1** for a partial summary). These include seizure activity (Zhang et al., 1998; Szklarczyk et al., 2002), cytokines (Khuth et al., 2001; Ogier et al., 2005; Ben-Hur et al., 2006), neurotrophins (Kuzniewska et al., 2013), chronic stress (van der Kooij et al., 2014), spatial learning (Wright et al., 2003; Meighan et al., 2006), head trauma (Phillips and Reeves, 2001; Kim et al., 2005), cocaine (Brown et al., 2008; Smith et al., 2014), methamphetamine (Liu et al., 2008), modafinil (He et al., 2011), ischemia (Planas et al., 2001; Rivera et al., 2002), and viral infection of the central nervous system (CNS; Conant et al., 1999; Johnston et al., 2002; Patrick et al., 2002; Zhang et al., 2003). Upregulation of MMP-9 mRNA and enzymatic activity has also been documented in response to neuronal depolarization by KCl (Von Gertten et al., 2003) and kainate (Szklarczyk et al., 2002; Konopacki et al., 2007; Wilczynski et al., 2008; Rylski et al., 2009). MMP-9 levels are increased with LTP (Nagy et al., 2006), and MMP-9 mRNA can be transported to dendrites to undergo local translation and protein release following glutamate stimulation (Dziembowska et al., 2012). Brain-derived neurotrophin factor (BDNF) also upregulates MMP-9 at the mRNA, protein, and enzymatic activity level in dendrites. This process requires engagement of TrkB receptors with subsequent

TABLE 1 | Stimuli and stressors linked to altered MMP levels in the CNS.

Effector(s)	MP(s)	Regional focus	Model system	Findings and/or biological relevance	Reference
Seizure induction with kainate	MMP-2 and -9	Hippocampus, striatum, diencephalon, midbrain, frontal cortex, and cerebellum	Rat	(1) Increased MMP-2 and -9 activity (2) Increased GFAP immunoreactivity in hippocampus and frontal cortex	Zhang et al. (1998), Szklarczyk et al. (2002)
Hippocampal dependent learning and memory (Morris water maze)	MMP-3 and -9	Hippocampus and prefrontal cortex	Rat	(1) Increased MMP-3 and -9 protein	Wright et al. (2003), Meighan et al. (2006)
Chronic stress	MMP-9	CA1 region of hippocampus	Rat	(1) Increased MMP-9 (2) Reductions in Nectin-3	van der Kooij et al. (2014)
Traumatic brain injury	MMP-3	Hippocampus	Rat	(1) Astrocyte-derived MMP-3 levels and activity increase 7 days after induction of traumatic brain injury	Kim et al. (2005)
Ischemia	MMP-2 and MMP-9	Hippocampus, lateral cortex, and striatum	Rat	(1) MMP-9 levels increase at 4 h post injury (2) MMP-2 levels peak at 4 days post injury, which corresponds with an increase in reactive microglia and macrophage infiltration	Planas et al. (2001), Rivera et al. (2002)
Viral infection	MMP-2 and -9	Cerebral spinal fluid (CSF)	Human (cerebrospinal fluid)	Damage to the blood–brain barrier (BBB) may facilitate the CNS ingress of monocytes that mediate brain injury. Select MMPs, such as MMP-2 and -9, can reduce BBB integrity	Conant et al. (1999)
Psychostimulants (methamphetamine, cocaine, and modafinil)	MMP-9	Hippocampus, medial prefrontal cortex	Rat	Data suggest that MMP-9 expression may be involved in the addiction phenotype and/or remodeling of the nervous system	Brown et al. (2008), Liu et al. (2008), He et al. (2011)

activation of extracellular signal-regulated kinase 1/2 (ERK1/2) and binding of c-Fos to the proximal MMP-9 promoter region (Kuzniewska et al., 2013). In contrast, MMP gene expression can be suppressed by factors including TGF-β, the anesthetic propofol (Zhang et al., 2013), retinoic acid (Osteen et al., 1996; Li et al., 2011; Ye et al., 2011), or sleep deprivation (Taishi et al., 2001).

While a wide variety of studies have examined MMP and ADAM expression in brain or brain-derived cell cultures as a function of specific stimuli, CNS changes have also been examined in a limited number of mutant mouse models. A summary of results from studies using this approach is shown in **Table 2**.

CAMs in the CNS

Cell adhesion molecules represent transmembrane adhesion molecules expressed at cell contact sites including the synapse. These molecules typically belong to one of several superfamilies which include cadherins, neurexins/neuroligins, and Ig-domain containing members [reviewed in Benson and Huntley (2012)]. CAMs can mediate stable cell–cell junctions and select family members also play a role in the initiation of synapse formation. Cell surface levels of these molecules, as well as adhesive contact strength, are modified by processes including clathrin-dependent endocytosis (Kamiguchi and Lemmon, 2000). In addition, varied transmembrane CAMs have important intracellular interactions. For example, while integrin cytoplasmic tails do not possess endogenous kinase activity, they interact with critical effectors of intracellular protein phosphorylation cascades (Clark and Brugge, 1995). Finally, through cis-interactions, transmembrane CAMs may influence the localization of synaptic proteins. As a potential example, *N*-cadherin, GluN1 and L1 are found together in large multiprotein complexes (Husi et al., 2000) suggesting that GluN may be part of a membrane adhesion complex (Sheng and Lee, 2000).

Accumulating evidence demonstrates that disrupted CAM expression can influence experience dependent plasticity. For example, ablation of *N*-cadherin from excitatory forebrain synapses of post-natal mice is associated with an alteration in the composition of glutamatergic synapses, so that levels of the GluA1 subunit and PSD95 are diminished (Nikitczuk et al., 2014). Earlier work by the same group has shown that a conditional *N*-cadherin knockout causes a reduction in the maintenance, but not induction, of LTP (Bozdagi et al., 2010). These studies are of particular relevance in that conditional ablation of *N*-cadherin addresses potential confounds that might be associated with knockout effects on early brain development.

Neuron-specific deletion of dystroglycan, a transmembrane protein that links extracellular matrix and the cytoskeleton, also reduces LTP. Specifically, neuron-specific deletion of this protein is associated with a blunting of high frequency stimulation (HFS) induced LTP at CA3–CA1 synapses (Satz et al., 2010). Dystroglycan is expressed by varied cell types including glia, and glial expression of the molecule is involved in forebrain development (Satz et al., 2010).

A number of studies have also investigated LTP in mice that lack specific Ig-domain CAM family members [comprehensively reviewed in Dityatev et al. (2008)]. Neural cell adhesion molecule

TABLE 2 | Altered neuronal migration and/or plasticity in mutant mouse models.

Animal model	Regional focus	Results	Reference
MMP-3 knock out mouse	Cerebellum	(1) Increased size of the external granular layer (EGL) and enhanced granule progenitor cell proliferation at post-natal days 8–12 (2) Delayed migration of granule cells to the EGL (3) Decreased length and complexity of Purkinje cells at post-natal day 12	Van Hove et al. (2012b)
MMP-3 knock-out mouse	Visual cortex (layer V)	(1) Decreased dendritic length and increased number of apical oblique dendrites in pyramidal neurons	Aerts et al. (2014)
MMP-9 overexpressing rat	Hippocampus	(1) Increased length of dendritic spines in CA1	Michaluk et al. (2011)
MMP-9 knock-out mouse	Cerebellum	(1) Increased granule precursor cell number and decreased apoptosis in the external granular layer at post-natal day 12	Vaillant et al. (2003)
MMP-9 knock-out mouse	Hippocampus (slices)	(1) Impaired magnitude and duration of LTP	Nagy et al. (2006)
MMP-2/-9 double knock-out mouse	Nucleus accumbens	(1) Decreased sensitization and reward behavior following methamphetamine (2 mg/kg) administration	Mizoguchi et al. (2007b)
MMP-2 and MMP-9 single knock out mice (observed in both)	Cerebral cortex	(1) Increased number of cell in cerebral-cortical layers 2–3 (2) Altered ICAM-5 and L1CAM levels as a function of age	Tian et al. (2007)
ADAM-10 knock-out mouse	Hippocampus	(1) Decreased neural progenitor cell number with increased differentiation toward the neuronal lineage (2) Impaired performance on a hippocampal-dependent test of memory	Zhuang et al. (2015)
MMP-9/-12 double knockout	Corpus callosum	(1) Decreased myelination at post-natal day 7 (2) Decreased oligodendroglial cell number at post-natal day 10	Larsen et al. (2006)

(NCAM) is a homophilic binding protein that is expressed on the surface of neurons and glia and has been implicated in neurite outgrowth and synaptic plasticity. Indeed, NCAM-deficient mice show impaired LTP in area CA3 (Cremer et al., 1998), and impaired LTP in NCAM knockouts can be rescued by increasing GluN dependent glutamate transmission (Kochlamazashvili et al., 2012). In related work, Cremer et al. (1998) studied mice with a targeted deletion of a polysialyltransferase that attaches polysialic acid (PSA) to NCAM, and that is expressed predominantly in post-natal life (Eckhardt et al., 2000). These animals were shown to have lower post-natal levels of PSA in the brain as well as impaired LTP in CA1 that is evident by 4 weeks of age. Mice that are deficient in ICAM-5, an additional Ig-domain family member expressed on excitatory neurons of the telencephalon (Oka et al., 1990; Benson et al., 1998), also show changes in glutamatergic synapses. These animals show an increase in the dendritic spine/filopodia ratio at P7, suggesting that full length ICAM-5 may delay spine maturation (Matsuno et al., 2006). Though full length ICAM-5 is gradually excluded from spines during their developmental maturation, it remains in approximately 60 percent of spines in adult hippocampal neurons (Sakurai et al., 1998; Matsuno et al., 2006). An antibody directed against ICAM-5, which would presumably disrupt adhesive interactions important to filopodial maintenance, inhibits LTP in rat hippocampus (Sakurai et al., 1998). In mouse hippocampus, however, LTP is relatively increased in an ICAM-5 null animal (Nakamura et al., 2001). Though confounds include antibody specificity, as well as developmental and compensatory effects in the knockout, results are of interest with respect to ICAM-5 as a potential modulator of glutamatergic function.

Specific neuroligin family members have also been investigated with respect to glutamatergic transmission. These are cell adhesion proteins on the post-synaptic membrane that mediate the formation and maintenance of synapses between neurons. Neuroligins act as ligands for β-Neurexins, which are located on the presynaptic membrane. Of particular interest is a study of an autism-associated point mutation in the neuroligin tail that was evaluated following generation of a knock-in mouse (Etherton et al., 2011). Whole-cell voltage-clamp recordings in hippocampal CA1 pyramidal neurons from the knock-in showed a decrease in mini excitatory post-synaptic current (mEPSC) frequency but not amplitude. Changes in GluA receptor subunit composition or presynaptic release possibility were excluded by additional studies, and it was suggested that the neuroligin-3 cytoplasmic tail modulates recruitment of GluAs to post-synaptic sites of excitatory synapses (Etherton et al., 2011).

In addition to *in vitro* and animal model based studies, human genetic studies are consistent with an important role for CAMs in neuroplasticity. Mutations in contactin-associated protein 2, which may promote neuronal circuit assembly during development (Anderson et al., 2012), predispose to autism. Moreover, polymorphisms in CAMs including cadherin 13 (Johnson et al., 2008; Uhl et al., 2014) are associated with addiction risk.

Synaptic CAMs: Perfectly Poised Substrates for Neuronal Activity Dependent Cleavage

Though a role for CAMs in processes such as LTP could be in whole or large part secondary to the function of full-length molecules, it should also be considered that synaptically localized CAMs represent especially attractive targets for neuronal activity dependent proteolysis. CAM cleavage could disrupt stable interactions with exogenous CAM ligands and/or cause additional effects, including generation of bioactive or dominant negative receptor fragments. Varied CAMs are expressed at synaptic contacts including *N*-cadherin, L1-CAM, ICAM-5,

DSCAM, syndecan 2, syncam 2, and neuroligin (Benson et al., 1998; Peixoto et al., 2012; Sonderegger and Matsumoto-Miyai, 2014), and activity-dependent, membrane-proximal cleavage of these molecules is supported by *in vitro* studies that have demonstrated juxtamembrane shedding for specific family members (Peixoto et al., 2012; Sonderegger and Matsumoto-Miyai, 2014). Data from analysis of cerebrospinal spinal fluid samples also supports shedding of CAMs (Strekalova et al., 2006). In this case, shed CAMs likely access the interstitial space from where they in turn gain access to CSF. In one study, an increase in levels of soluble ICAM-5 ectodomain was detected in the CSF of patients with epilepsy and/or infection (Lindsberg et al., 2002; Tian et al., 2008). In related work, N terminal sequencing of NCAM fragments from the CSF of patients with schizophrenia was performed and a disease-associated increase in levels of ectodomain fragments observed (Vawter et al., 2001).

Regulated cleavage of synaptic CAMs can influence glutamatergic transmission through several non-mutually exclusive mechanisms including reduced synaptic stability, conversion of N-terminal CAM ectodomains into soluble effectors of plasticity, and increased generation of intracellular domains (ICDs) that influence transcription. With respect to ICD generation, it should be noted that ectodomain shedding of CAMs is frequently followed by intramembranous gamma secretase cleavage to generate specific C terminal fragments [reviewed in Jordan and Kreutz (2009)].

Activity Dependent Cleavage of Neuroligin and *N*-Cadherin

Though it has been suggested that ectodomain shedding is highly regulated with only 2% of cell surface proteins released by this process (Hayashida et al., 2010), emerging evidence suggests that neuronal activity dependent CAM shedding represents an important mechanism by which synaptic structure and function are modulated. For example, neuronal activity dependent cleavage of neuroligin-1 is triggered by GluN activation and dependent on MMP or ADAM activity (Peixoto et al., 2012; Suzuki et al., 2012). It occurs in a membrane proximal location and results in destabilization of neuroligin-1's presynaptic partner, neurexin-1β. Destabilization of neurexin is in turn thought to reduce the probability of presynatic neurotransmitter release (Peixoto et al., 2012).

GluN agonists, as well as ADAM and MMP family members that are regulated in a neuronal activity dependent manner, have also been linked to *N*-cadherin shedding (Reiss et al., 2005; Uemura et al., 2007; Williams et al., 2010; Paudel et al., 2013; Porlan et al., 2014). One of many potential sequelae of this event is the associated generation of a C terminal fragment which is quickly processed by gamma secretase to generate a smaller intracellular fragment that destabilizes a protein critical for CREB dependent transcription (Marambaud et al., 2003).

Activity Dependent Cleavage of Ig-domain CAMs

In work related to a potential role of ICAM-5 shedding in developmental plasticity, it has been shown that long term treatment (16 h) of DIV 14 hippocampal neurons with 5 μM NMDA or AMPA stimulated an MMP-dependent increase in supernatant levels of shed ICAM-5. ICAM-5 is expressed on dendritic elements of excitatory/spiny neurons in the telenchephalon (Benson et al., 1998). Since full length ICAM-5 may be a negative regulator of filopodia-to-spine transition (Matsuno et al., 2006), these findings are consistent with the possibility that ICAM-5 shedding contributes to developmental spine maturation.

Studies related to the possibility that ICAM-5 cleavage may occur in a relatively rapid manner to influence activity dependent glutamatergic transmission in the adult CNS have also been performed. NMDA stimulation of cultured hippocampal neurons and high frequency tetanic stimulation of hippocampal slices have both been linked to relatively rapid MMP-dependent ICAM-5 shedding (Conant et al., 2010b). In cultured cells, appreciable release of soluble ICAM-5 into culture supernatants can be detected within 5 min of NMDA exposure (Conant et al., 2010b).

Additional studies have examined neuronal activity dependent cleavage of nectin-1, an Ig-like adhesion molecule expressed at puncta adherentia junctions in the CA3 pyramidal region of adult mouse hippocampus (Lim et al., 2012). Of interest, *in vitro* over-expression of cleavage resistant mutants of nectin 1 is associated with an increase in the density of dendritic spines (Lim et al., 2012). One possibility is that cleavage resistant mutants might lead to an increase in the stability of spines.

Elegant work on a related adhesion molecule, demonstrated enhanced MMP-9 dependent cleavage of nectin-3 in perisynaptic CA1 in the setting of chronic stress (van der Kooij et al., 2014). Intriguingly, inhibition of MMP-9 activity or GluN activation led to a reduction chronic stress related behavioral alterations.

IgLON family members, abundant GPI anchored transmembrane proteins, are also processed in a metalloproteinase dependent manner. The IgLON family is a subgroup of the immunoglobulin superfamily cell adhesion molecules (CAMs) and composed of limbic system-associated protein (LAMP), opioid binding cell adhesion molecule (OBCAM), neurotrimin (NTM) and Kilon. Long term treatment of hippocampal neurons with a broad spectrum MMP inhibitor and subsequent pull down of surface proteins demonstrated that inhibitor-treated neurons show increased levels of specific IgLON family members including NTM (Sanz et al., 2014).

Glutamate and MMP dependent shedding of synaptic cell adhesion molecule 2 (SynCAM-2) has also been described (Bajor et al., 2012), which is of interest given the role of this molecule in synapse organization and function (Biederer et al., 2002; Fogel et al., 2007).

Emerging and Future Studies of Neuronal Activity Dependent CAM Cleavage

Matrix metalloproteinases and ADAMs can also act on a variety of less traditional CAMs including nerve-glia antigen 2 (NG2), β-dystroglycan, and amyloid precursor protein (APP) and netrin-G ligand-3 (Ahmad et al., 2006; Michaluk et al., 2007; Lee et al., 2014; Sakry et al., 2014). There is evidence that these molecules are shed in a neuronal activity-dependent manner, and that they

play a role in developmental and/or adult plasticity. For example, recent work suggests that glutamatergic transmission is altered in NG2 knockout animals (Sakry et al., 2014). Future studies will be necessary to further explore mechanisms by which shedding of these proteins can influence plasticity.

Future studies will also be necessary address issues related to shedding of dimers versus monomers, as well as issues of whether single nucleotide polymorphisms (SNPs) influence shedding. Results from recent work suggest that ADAM dependent shedding of neuregulin-1 requires prior dimerization (Hartmann et al., 2015), and analysis of soluble ICAM-1 in pleural fluid suggests that this molecule may also be shed as a dimer (Melis et al., 2003).

Future studies could additionally explore the question of whether post-translational modifications such as glycosylation can influence the cleavage and/or bioactivity of protease-generated CAM fragments. And finally, unbiased proteomics could be utilized to examine interactions between shed CAMs and other proteins in the background of select physiological and pathological processes.

Metalloproteinases and Synaptic Transmission

Despite their ability to stimulate effects that could both enhance or depress neurotransmission, the majority of studies support a view in which non-pathological neuronal activity stimulates an MMP dependent enhancement of long term memory and its correlates. For example, several groups have demonstrated that MMP inhibitors reduce LTP stimulated by HFS and/or theta burst stimulation (TBS; Nagy et al., 2006; Meighan et al., 2007; Conant et al., 2010b). Inhibition of MMP activity also reduces chemical LTP (cLTP) associated increases in the firing rate and bursting of dissociated cultures of primary hippocampal neurons (Niedringhaus et al., 2012).

Consistent with their effects on hippocampal LTP, varied biochemical and behavioral studies support a role for MMPs in hippocampal dependent learning and/or memory. For example, knockout of MMP-9 impairs contextual fear conditioning (Nagy et al., 2007). Interestingly, in wild-type animals contextual fear conditioning increases hippocampal MMP-9 protein levels as well as MMP-9 dependent cleavage of dystroglycan (Ganguly et al., 2013). These data suggest that MMP-9 plays a role in hippocampal memory association and/or retention. It has also been shown that hippocampal MMP-3 and -9 mRNA levels are increased with Morris water maze (MWM) training, as are levels of active MMP-3 and -9 protein (Meighan et al., 2006). Moreover, treatment with the non-competitive GluN antagonist, MK801, reduces training-associated increases in specific MMP levels, as well as post-training performance assessed by latency to reach platform. Intra-hippocampal or intra-cerebral ventricular injection of a broad-spectrum chemical MMP inhibitor, as compared to artificial CSF control injection, can also reduce time spent in the target quadrant during the MWM probe trial (Meighan et al., 2006). Of interest with respect to anesthetic-modulation of learning and memory, it has been shown that while MWM training can induce a gradual increase in pro- and active-MMP-9, propofol can reduce this increase and also disrupt spatial memory retention 24 h after training (Zhang et al., 2013). In contrast, the wake promoting agent modafinil increases MMP-9 expression in dorsal hippocampal CA3 in a model of REM sleep deprivation (He et al., 2011). In this same model, modafinil increases synapsin 1 expression in an MMP-9 dependent manner. In addiction-related plasticity work, it has been shown that context dependent learning of nicotine induced conditioned place preference (CPP) is associated with an increase in hippocampal MMP-2, -3, and -9 expression, and that exposure to a chemical MMP inhibitor during nicotine induced CPP training can block CPP acquisition (Natarajan et al., 2013). In addition, methamphetamine-induced behavioral sensitization is reduced in mice lacking MMP-2 or MMP-9 (Mizoguchi et al., 2007b).

Matrix metalloproteinase activity can also contribute to enhanced glutamatergic transmission in regions including striatum and amygdala. For example, a chemical MMP inhibitor can disrupt reconsolidation of a fear memory associated with a conditioned stimulus that is independent of contextual cues (Brown et al., 2009). In studies with MMP-9 null mice, Kaczmarek and colleagues have shown that MMP activity in the central amygdala is required for appetitive but not aversive learning (Knapska et al., 2013). In recent work related to cocaine and MMP levels in nucleus accumbens core, an increase in gelatinase activity as detected by *in situ* zymography was detected along neuronal soma and dendrites (Smith et al., 2014). AMPA/NMDA ratios were also increased in medium spiny neurons in cocaine extinguished rats and further increased by cue-induced reinstatement, in an MMP dependent manner. MSNs also showed MMP dependent changes in MSN spine head diameter and/or number in cocaine extinguished and reinstated animals (Smith et al., 2014).

Mechanisms by which MMPs Influence Neurotransmission; A Focus on CAM Cleavage as a Means to Generate Integrin-Binding Ligands

In terms of the mechanisms by which MMPs modulate actin and spine dynamics to enhance glutamatergic transmission, it should be noted that despite their potential to act on varied substrates such as proneurotrophins (Lee et al., 2001), evidence suggests that their ability to enhance LTP is β_1 integrin dependent (Nagy et al., 2006; Meighan et al., 2007). Since changes in the number and size of dendritic spines are thought to underlie LTP, Huntley and colleagues (Wang et al., 2008) also monitored spine size and EPSPs simultaneously in hippocampal neurons with combined 2-photon time-lapse imaging and whole-cell recordings. These investigators observed that persistent spine enlargement and synaptic potentiation required both MMP activity and β_1 integrins (Wang et al., 2008). In related studies, we have previously shown that a β_1 integrin blocking antibody prevents cLTP associated increases in the overall firing rate of hippocampal-derived neurons (Niedringhaus et al., 2012).

Integrin signaling, and signaling through β_1 containing integrins in particular, has been well-associated with changes in dendritic and spine actin dynamics (Huang et al., 2006; Shi and Ethell, 2006). The majority of hippocampal integrin heterodimers contain a β_1 subunit (Pinkstaff et al., 1998; Chan et al., 2006; Huntley, 2012). In addition, β_1 integrins are expressed on dendrites (Mortillo et al., 2012) and implicated in the regulation of post-natal dendritic arbor and synapse density (Warren et al., 2012). Antagonism of β_1 integrin signaling by function blocking antibodies or GRGDS peptide administration is associated with a decay of LTP, and hippocampal infusion of function blocking antibodies to β_1 blocks formation of long-term object location memory (Babayan et al., 2012). Integrin like kinase is also involved in the induction and maintenance of cocaine sensitization, and its silencing prevents sensitization-associated serine-845 phosphorylation of GluA1 (Chen et al., 2010). Though a role for integrin signaling has been implicated in potentiated synaptic activity (Bernard-Trifilo et al., 2005; Nagy et al., 2006; Meighan et al., 2007), the identity of physiologically relevant ligands and important mechanisms by which these ligands are generated has yet to be fully explored.

Recent work is consistent with the possibility that MMP-dependent CAM shedding represents a potential mechanism by which excitatory transmission generates integrin-binding ligands. As opposed to larger ECM components, CAM ectodomains may be relatively soluble. Indeed, previous studies have shown that cleavage of relatively large molecules does not necessarily disrupt three dimensional integrity of the same (Huganir and Racker, 1980). Numerous studies, including those that have demonstrated the integrity of CAM N-terminal fragments in spinal fluid and tissue, support the concept of ectodomain solubility and stability (Lindsberg et al., 2002).

Cell adhesion molecule ectodomains are known to possess integrin-binding motifs and indeed stable CAM/integrin interactions have been described (Conant et al., 2010a; Kelly et al., 2013; Ning et al., 2013). CAM ectodomains can also stimulate integrin dependent signaling. For example, shedding of the L1 adhesion molecule has been shown to stimulate integrin dependent cell migration (Mechtersheimer et al., 2001). In addition, we have observed that soluble ICAM-5 can stimulate a β_1 dependent increase in action potential frequency in cultured hippocampal neurons (Niedringhaus et al., 2012). We also observe co-immunoprecipiation of both full length and shed N terminal ICAM-5 with β_1 in hippocampal lystates from methamphetamine challenged mice, suggesting that the shed ectodomain may interact with β_1 *in vivo* (Conant et al., 2010a). NCAM and NG2 can also interact with integrins, and though not yet tested for effects on integrin-dependent neurotransmission, dorsal hippocampal injection of PSA-NCAM has been shown to partially restore impaired contextual memory in NCAM deficient mice (Senkov et al., 2006). Of interest, mice that overexpress the NCAM ectodomain show memory impairments that are similar to those observed in the knockout (Pillai-Nair et al., 2005). This is consistent with a dominant negative effect as well as the possibility that dysregulated ectodomain shedding is deleterious, and it underlines our need to better explore the bioactivity of CAM ectodomains in both physiological and pathological conditions. A schematic of CAM ectodomain shedding from glia and/or neurons, with subsequent integrin binding, is shown in **Figure 1**.

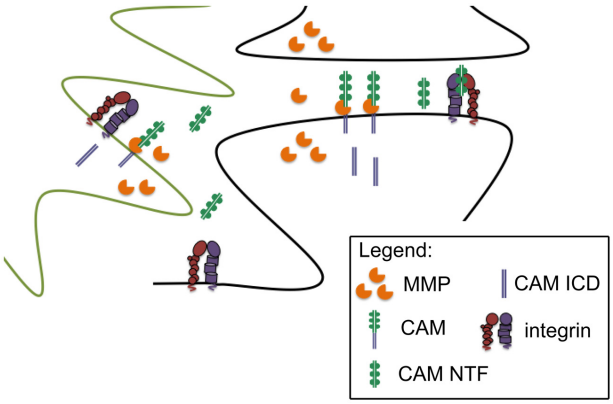

FIGURE 1 | Synaptically localized CAM cleavage. Pre and post synaptic components (black outline) and glial components (green outline) of the synapse are shown. MMPs can be released from neurons and/or glia to cleave peri-synaptic CAMs, thus generating CAM N-terminal fragments with the potential to stimulate intregrin dependent signaling. Though not the focus of this review, cleavage generated CAM intracellular domains (ICDs) may also influence synaptic transmission.

In terms of the functional consequences of MMPs and/or CAM ectodomains at single synapses to in turn affect LTP, several non-mutually exclusive possibilities exist as shown in **Figure 2**. In the first, supported by high resolution imaging (Wang et al., 2008), MMP activity causes a change in actin dynamics with a subsequent widening of spines. This would likely bring an increase in functional GluA receptors to the spine head and increase amplitude of mEPSCs. A second possibility is that MMP generated integrin binding ligands could stimulate the growth of new spines. Integrin signaling has been linked to the same (Shi and Ethell, 2006), and though we did not observe a significant increase in spine number in ICAM-5 ectodomain stimulated DIV 14 rat hippocampal neurons at 1 or 24 h (Lonskaya et al., 2013), it would be premature to rule out the possibility that this measure could be increased at other time points or following exposure to additional CAM ectodomains. The potential for integrin binding ligands to cause an unsilencing of post-synaptic components that were previously silent due to deficient synaptic levels of GluA receptors should be considered as a third possibility. Integrin signaling can activate protein kinases that would in turn phosphorylate specific GluA subunits to enhance their synaptic entry (Lim et al., 2008; Chen et al., 2010). Consistent with this possibility, in previous work we have observed both an ICAM-5 ectodomain stimulated increase in the phosphorylation and membrane localization of GluA1, and an increase in the frequency of mEPSCs (Lonskaya et al., 2013). A fourth possibility is that MMP-dependent signaling stimulates in increase in spine head protrusions to affect glutamatergic

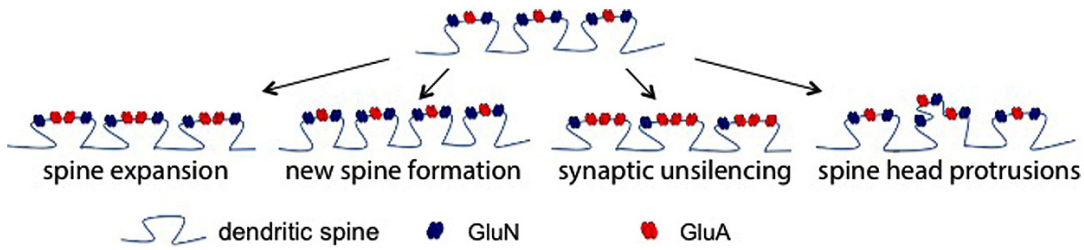

FIGURE 2 | Post-synaptic mechanisms by which MMP activity could enhance glutamatergic transmission. There are several non-mutually exclusive possibilities by which MMP activity could influence excitatory neurotransmission. Shown (top) is a dendritic segment with representative spines. As the schematic suggests with arrows to show potential changes, existing spine expansion, new spine formation, synaptic unsilencing, and formation of spine head protrusions represent post synaptic changes that might contribute to MMP and/or integrin dependent changes in the post synaptic element.

transmission. While LTP is generally thought to represent an experience dependent increase in dendritic number and/or size (Kopec et al., 2006), increased complexity of spines might also occur. It has been shown that MMP-9 can stimulate an increase in spine head protrusions (Szepesi et al., 2013). This finding is of significance in that these protrusions may be PSD-95 and GluA positive (Richards et al., 2005; Szepesi et al., 2013), and they may be functionally active in terms of mediating glutamateric neurotransmission (Richards et al., 2005).

Future studies related to CAM-integrin interactions will be necessary to examine a variety of additional questions including that of which integrin binding ligands are generated with learning and memory *in vivo*. Further study of whether ectodomain shedding plays and important role in select MMP dependent endpoints including changes in spine size or number (Shi and Ethell, 2006; Wang et al., 2008), the development of spine head protrusions (Szepesi et al., 2013), and developmental changes in neurite outgrowth and dendritic arbor (Van Hove et al., 2012a) may also be warranted. MMPs have also been linked to changes in neuronal excitability (Wojtowicz and Mozrzymas, 2014), and since integrin signaling can also influence ion channel function (Wildering et al., 2002), this could represent a parallel topic for future exploration.

An additional avenue for exploration includes the question of whether the downstream effects of CAM shedding can synergize with events that follow from MMP-dependent processing of additional synaptic substrates. As an example, we consider protease activated receptor-1 (PAR-1). A select subset of MMPs target PAR-1, a G protein coupled receptor that is activated by cleavage in N-terminal domain and consequent exposition of a tethered peptide ligand (Vergnolle et al., 2001; Soh et al., 2010). The receptor is expressed on select neuronal subpopulations and has been detected in synaptoneurosomes (Han et al., 2011; Maggio et al., 2013a,b). While activation of neuronal PARs has the potential to enhance GluN subunit phosphorylation and GluN function (Gingrich et al., 2000), whether PAR activation enhances neuronal integrin signaling is unknown. In non-neural cells, however, it has been shown that PAR-1 activation can enhance integrin affinity for ligands (Shattil et al., 2010).

CAM Shedding: From Physiology to Pathology

While regulated physiological release of MMPs can contribute to adaptive plasticity, it is important to note that dysregulated release has the potential to disrupt the same (Wojtowicz and Mozrzymas, 2014). Consistent with this, MMP inhibitors have been shown to ameliorate neuronal injury in a number of disease models. In many of these studies, however, reduced MMP-dependent damage to blood brain barrier integrity is likely to account for much of the observed neuroprotection (Asahi et al., 2001). There is, however, an increasing appreciation synaptic proteolysis in particular as significant a contributor to neurological disease. To follow, we will briefly discuss two disorders in which altered synaptic proteolysis may be critical to disease expression. The first is fragile X syndrome (FXS) and the second is psychostimulant addiction.

Fragile X Syndrome

Fragile X syndrome is a leading genetic cause of intellectual disability and autism (Santoro et al., 2012). At present, no cure is available. Symptoms include developmental delay and increased susceptibility to seizures, while pathological findings include relatively dense and immature dendritic spines (Galvez and Greenough, 2005; McKinney et al., 2005; Pan et al., 2010; Santoro et al., 2012). Expansion of the trinucleotide CGG repeat in excess of 200 repeats located in the 5′ untranslated region of the X chromosome-linked *FMR1* gene cause FXS (Verkerk et al., 1991; Sutcliffe et al., 1992). This leads to transcriptional silencing and a consequent lack of functional protein product, fragile X mental retardation protein (FMRP). FMRP regulates expression of a subset of dendritically localized mRNAs, and thus levels of dendritically localized proteins may be altered in FXS (Darnell et al., 2012; Santoro et al., 2012). FMRP generally acts to inhibit the translation of target genes, but in select cases it may have actions that lead instead to enhanced translation (Darnell et al., 2012; Santoro et al., 2012). For example, FMRP is thought to enhance translation of superoxide dismutase (Bechara et al., 2009), and thus antioxidant enzyme activity might be reduced in FXS. This has implications for glial activation, which has been observed in association with

the disease (Rossignol and Frye, 2012). Moreover, neuronal and/or glial expression of MMPs may be elevated in response to increased glial activation and/or oxidant stress (Gu et al., 2002).

Published studies have linked MMP activity to FXS. Levels of MMP-9 are increased in affected humans and in a murine model (Sidhu et al., 2014). Increased dendritic translation of MMP-9 has also been observed (Janusz et al., 2013). Recent work suggests that expression of additional MMPs may be increased as well. For example, transcripts for MMP-2, -3, -7, -9, and -24 are increased in heavy polysomes from *Fmr1* mutant mice (Gkogkas et al., 2014). In terms of functional consequences, dendritic spine abnormalities in a mouse model of FXS can be reduced by minocycline, an inhibitor of MMP activity that can access the brain (Bilousova et al., 2009). Spine abnormalities are also reduced in the background of MMP-9 deficiency (Sidhu et al., 2014). Moreover, exogenous MMP administration to cultured neurons has been associated with relevant changes in spine morphology (Bilousova et al., 2006). Interestingly, MMP knockout can also reduce neuronal circuit defects in a drosophila model of disease (Siller and Broadie, 2011).

The mechanism(s) by which excess MMP activity stimulates the FXS phenotype have yet to be determined. Several possibilities have been suggested, including increased signaling by MMP-dependent activation of pro-neurotrophins and/or generation of integrin-binding laminin fragments (Sidhu et al., 2014). It is tempting to speculate, however, that excess generation of integrin-binding CAM fragments might play a role. Future studies to address this question may therefore be warranted.

Addiction

Matrix metalloproteinase-dependent changes in synaptic structure and function are also thought to contribute to the maladaptive learning and memory associated with addiction to stimulants including methamphetamine. Methamphetamine is a widely abused illicit drug that has high addictive potential. A variety of studies suggest that methamphetamine is linked to metabolic changes in the brain as well as to synaptic injury (Pu et al., 1996; Volkow et al., 2001; Chang et al., 2009). Evidence for increased MMP expression, release, and/or activation in the setting of methamphetamine exposure comes from several groups. This stimulant can increase release of MMP-1 from cultured neural cells (Conant et al., 2004). This observation is consistent with results from rodent studies in which methamphetamine stimulates increased binding of AP-1, a transcription factor critical to the expression of MMPs including MMP-1 (Akiyama et al., 1996). Additional studies have demonstrated that 5 days of exposure to the drug (2 mg/kg/day) is associated with increased MMP-2 and -9 protein in the frontal cortex and nucleus accumbens of rats (Mizoguchi et al., 2007a). Moreover, an acute high dose of MA (40 mg/kg) is followed by increased mRNA expression of MMP-9 in murine CNS (Liu et al., 2008). Similarly, cocaine, which is similar to methamphetamine in its potential to increase catecholamine levels, has been shown to increase MMP-9 activity in the medial prefrontal cortex at 1, 3, and 24 h post-administration (Brown et al., 2008).

Methamphetamine has the potential to increase MMP expression through several non-mutually exclusive mechanisms including increased catecholamine dependent signaling, activation of glutamate receptor signaling, and increased oxidant stress. For example, methamphetamine associated increases dopamine can act on D1 type dopamine receptors to enhance substrate proteolysis (Iwakura et al., 2011). Since both D1 and D2 type are linked to $\beta\gamma$ subunits that can activate PKC and release of intracellular calcium, activation of either receptor type might stimulate calcium dependent MMP release and/or PKC dependent activation of a transmembrane MMP. Another possibility is that MA increases levels of glutamate, as has been shown by Yamamoto and colleagues (Mark et al., 2004), and that glutamate signaling can in turn stimulate increased MMP expression and/or activity. Relatively high concentrations of MA also stimulate an increase in signaling by reactive oxygen intermediates (Lee et al., 2002), which can enhance both the expression and the activation of select MMPs (Gu et al., 2002).

Increased MMP activity may also contribute to synaptic and behavioral changes observed with stimulant exposure. It has been shown that methamphetamine-induced behavioral sensitization is reduced in mice lacking MMP-2 or MMP-9 (Mizoguchi et al., 2007b). Protease activity has also been shown to contribute to cocaine associated CPP (Brown et al., 2007). In recent work focused on structural and function changes at the levels of the synapse in the setting of cocaine exposure paradigms, an increase in the AMPA/NMDA current ratio was increased in extinguished rats, and further increased 15 min following cue-induced reinstatement (Smith et al., 2014). An increase in the AMPA/NMDA ratio after extinction was restored to control by a selective MMP-2 inhibitor, while the altered reinstatement ratio was restored by either and MMP-2 or -9 inhibitor (Smith et al., 2014). Complementary measures of spine density and spine size supported a view in which MMP-2 could increase the density and head size of spines in extinguished animals and that MMP-9 activity contributed to an increase in spine size with reinstatement.

Overall, studies related to the role of MMPs in addiction are exciting and should stimulate further work to address underlying mechanistic components.

Summary

Matrix metalloproteinases were named for their ability to cleave extracellular matrix proteins such as laminin and collagen. While matrix remodeling effects may be essential during development and wound healing, proteolysis of cell surface receptors including CAMs could represent a critical means by which MMPs can fine tune synaptic structure and function in a more stable or relatively mature CNS. This possibility is supported by work showing that neuronal activity stimulates proteolysis of synaptically localized CAMs, and that CAM shedding can influence varied measures of synaptic transmission. Future studies will be necessary to examine CAM cleavage as affected by variables including cell type, brain region, stimulus type/duration, and developmental stage. Future studies will also be necessary to determine which

CAM cleavage products are most likely to influence MMP dependent plasticity *in vivo*, and to determine the extent to which CAM shedding combines with additional MMP-stimulated events to influence experience dependent plasticity.

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REVIEW
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Diverse impact of acute and long-term extracellular proteolytic activity on plasticity of neuronal excitability

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Learning and memory require alteration in number and strength of existing synaptic connections. Extracellular proteolysis within the synapses has been shown to play a pivotal role in synaptic plasticity by determining synapse structure, function, and number. Although synaptic plasticity of excitatory synapses is generally acknowledged to play a crucial role in formation of memory traces, some components of neural plasticity are reflected by nonsynaptic changes. Since information in neural networks is ultimately conveyed with action potentials, scaling of neuronal excitability could significantly enhance or dampen the outcome of dendritic integration, boost neuronal information storage capacity and ultimately learning. However, the underlying mechanism is poorly understood. With this regard, several lines of evidence and our most recent study support a view that activity of extracellular proteases might affect information processing in neuronal networks by affecting targets beyond synapses. Here, we review the most recent studies addressing the impact of extracellular proteolysis on plasticity of neuronal excitability and discuss how enzymatic activity may alter input-output/transfer function of neurons, supporting cognitive processes. Interestingly, extracellular proteolysis may alter intrinsic neuronal excitability and excitation/inhibition balance both rapidly (time of minutes to hours) and in long-term window. Moreover, it appears that by cleavage of extracellular matrix (ECM) constituents, proteases may modulate function of ion channels or alter inhibitory drive and hence facilitate active participation of dendrites and axon initial segments (AISs) in adjusting neuronal input/output function. Altogether, a picture emerges whereby both rapid and long-term extracellular proteolysis may influence some aspects of information processing in neurons, such as initiation of action potential, spike frequency adaptation, properties of action potential and dendritic backpropagation.

Keywords: extracellular proteases, intrinsic excitability, E-S potentiation, plasticity, LTP, hippocampus

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Introduction

Learning and memory require alteration in the number and strength of existing synaptic connections. Functional *in vitro* and *in vivo* studies confirmed that memory traces may be encoded by use-dependent modification of synapses. A hallmark of such synaptic plasticity is the long-term potentiation (LTP) or depression (LTD) which can be evoked by patterned

stimulation of afferent fibers at high and low frequency, respectively (Malenka and Bear, 2004). In experimental conditions, LTP and LTD were most extensively studied in hippocampal formation in excitatory, and more recently, in inhibitory connections and exact molecular mechanisms vary depending on the synapses and circuits in which they operate (Malenka and Bear, 2004; Nicoll and Schmitz, 2005; Kullmann and Lamsa, 2007). The last decade brought convincing evidence that indeed memory formation uses the same "repertoire" of functional and molecular synaptic modifications as those previously found to accompany long-term plasticity phenomena (Moser et al., 1998; Sacchetti et al., 2001; Whitlock et al., 2006; Nabavi et al., 2014). In particular, synaptic potentiation following behavioral training and that evoked by high-frequency electrical stimulation in the hippocampus occlude each other (Whitlock et al., 2006) strongly indicating common mechanisms.

In most studies aiming at addressing mechanisms of synaptic plasticity, patterned stimulation was commonly used to evoke LTP or LTD. However, no patterned exogenous stimulation can reproduce a complex pattern of endogenous activity of neuronal networks occurring *in vivo*. In addition, changes solely in synaptic strength of synaptic connections such as LTP alone were found to be insufficient to explain memory formation (Zamanillo et al., 1999; Shimshek et al., 2006). For instance, in some reports hippocampal spatial learning occurred in the absence of synaptic LTP (Jeffery, 1997; Silva et al., 1998; D'hooge and De Deyn, 2001). In fact neurons could significantly enhance the information storage capacity by scaling dendritic and somatic excitability (Poirazi and Mel, 2001; Häusser and Mel, 2003; Polsky et al., 2004) and learning (for review, see Zhang and Linden, 2003). Thus, memory storage may involve multiple levels and could be supported by long-term modifications of neuronal input-output properties by far more complex mechanisms than synaptic plasticity alone. Indeed, in the neuronal network in which, besides synaptic strength, additionally neuron firing rate, firing threshold or gain can be modulated, one would expect substantially larger memory storage and information processing capabilities than in the case of alterations in excitatory synaptic function alone. In their early seminal papers, Bliss and colleagues (Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973) noticed that in rabbit dentate gyrus population spike amplitude following tetanically evoked synaptic LTP increased far beyond the probability predicted by increased synaptic input [a phenomenon referred to as excitatory post synaptic potential (EPSP)-to-Spike potentiation, E–S potentiation, Bliss and Gardner-Medwin, 1973; Bliss and Lomo, 1973]. Later, studies at the level of single neurons confirmed existence of E-S potentiation and E-S depression (reviewed in Campanac and Debanne, 2008). Interestingly, although E-S potentiation is often observed following stimulation patterns inducing synaptic LTP, changes solely in neuronal excitability in the absence of synaptic gain were reported indicating that synaptic plasticity and intrinsic neuronal excitability are not tightly coupled or at least do not scale linearly (e.g., Ohno et al., 2006). Consequently, taken the importance of action potential generation in transmission of information in neuronal networks, intrinsic neuronal plasticity has been proposed to reflect one of the cellular correlate of behavioral learning and neuronal homeostasis (reviewed in Zhang and Linden, 2003).

In the recent years, rapid, activity-regulated extracellular proteolysis has been implied as an indispensable factor supporting neuronal plasticity learning and memory. In particular, proteolytic activity within and in the vicinity of the synapses has been shown to play a pivotal role in determining synapse structure, function, and number (Huntley, 2012; Sonderegger and Matsumoto-Miyai, 2014). In particular, extracellular proteases determine structural modification of synapses through various pathways, including proteolysis of the extracellular matrix (ECM) proteins, cell adhesion molecules, and neurotrophic factors. Such proteolysis induces changes in the properties of substrate proteins or releases functional domains (ligands) of the substrate proteins, which activate a signal transduction cascades. Several excellent reviews hold information about mechanism whereby extracellular proteolysis could support synaptic plasticity (Ethell and Ethell, 2007; Agrawal et al., 2008; Huntley, 2012; Tamura et al., 2013; Sonderegger and Matsumoto-Miyai, 2014; Ben Shimon et al., 2015), and therefore we will not extensively review this topic here. Importantly, considering the wide range of proteases and their substrates it seems unlikely that they support solely synaptic plasticity. In addition, although the phenomenon of long-term synaptic potentiation and E-S potentiation was discovered at the same time (Bliss and Lomo, 1973), the latter received less attention and molecular mechanisms remain poorly understood. Most recently, we studied for the first time the impact of matrix metalloproteases (MMPs) activity on E-S potentiation in hippocampal CA3 region (Wójtowicz and Mozrzymas, 2014). We found that inhibition of MMPs had detrimental effect on E-S potentiation induced by various LTP-induction protocols. Most strikingly, the reduction in spike potentiation following inhibition of MMPs was more pronounced than it would be expected from impaired synaptic plasticity alone, suggesting that synaptic and nonsynaptic components are regulated by MMPs separately (Wójtowicz and Mozrzymas, 2014). It is important to bear in mind that synaptic and nonsynaptic mechanisms are tightly functionally coupled and deciphering the information processing requires a thorough consideration of these two elements. The fact that recent findings indicate that both of them can be potently regulated by MMPs, places these enzymes at a very strategic position in regulating signal transduction in the neuronal networks. Therefore, here we review the impact of extracellular proteolysis on neuronal plasticity with particular emphasis on nonsynaptic targets and discuss how these processes ultimately alter input-output/transfer function of neurons, supporting cognitive processes. Due to its pivotal role in learning and memory, we will focus on the hippocampal formation but examples of results obtained from studies on other structures will be also presented.

Neuronal Excitability Following Certain Forms of Learning and Memory *in Vivo*

Following induction of synaptic plasticity in acute brain slices, a shift in neuronal excitability is often observed (for a

review, see Daoudal and Debanne, 2003). In most frequently studied CA1 and CA3 hippocampal regions, significant potentiation of population spike amplitude, beyond that predicted solely by gain in synaptic input, occurs following tetanic stimulation of afferent fibers or by pairing synaptic inputs with antidromic action potentials (Andersen et al., 1980; Abraham et al., 1987; Chavez-Noriega et al., 1990; Jester et al., 1995; Wójtowicz and Mozrzymas, 2014). However, the question arises to what extent E-S potentiation occurs *in vivo* following learning?

Past decades of research brought evidence that certain paradigms of learning are associated with E-S potentiation. Initially, persistence of long-term plasticity of intrinsic properties of cell membranes has been demonstrated in invertebrate models (for review, see Mozzachiodi and Byrne, 2010). Later studies showed the same rule exists in vertebrates (reviewed in Zhang and Linden, 2003). With this regard, eyeblink conditioning has been one of the most widely used model systems to study mechanisms of learning and memory. In this paradigm, auditory or visual stimulus is paired with an aversive, eyeblink-eliciting unconditioned stimulus (e.g., a puff of air to the cornea, mild shock or whisker deflection). When applied repeatedly, association is formed such that a learned blink occurs and precedes the unconditioned stimulus. Such learning paradigm involves both cerebellum and hippocampus and the learning-induced potentiation of hippocampal synapses shares similarities with classically obtained hippocampal LTP with respect to components of molecular machinery involved in plastic changes and changes in synapse morphology (reviewed in Christian and Thompson, 2003). Some authors proposed that this paradigm is an example of declarative explicit learning task (Clark and Squire, 1998). With regard to E-S potentiation, it has been shown that trace eyeblink conditioning in rabbits, rats and mice leads to long-term upregulation of neuronal firing in the CA1 region of the hippocampus. A hallmark of increased neuronal excitability is that it occurs as early as 1 h after behavioral training, peaks 24 h after training, and decays over a period of about 1 week (Disterhoft et al., 1986, reviewed in Christian and Thompson, 2003). Interestingly, changes in synaptic input were not typically observed indicating a pivotal role of E-S coupling in this form of plasticity. While studying classical conditioning in a model of trace fear conditioning, enhanced intrinsic excitability of CA1 pyramidal neurons was described (Kaczorowski and Disterhoft, 2009; Mckay et al., 2009).

More recently, environmental enrichment was used to study naturally occurring changes in synaptic efficacy in the hippocampus that underlie experience-induced modulation of learning and memory in rodents. Environmental manipulations, in particular enriched environment, caused an increase in population spike amplitude in the dentate gyrus *in vivo*, while the effects on synaptic transmission were either absent or highly variable, depending on the exposure pattern to the new environment (Irvine et al., 2006). It has been shown that following learning in that paradigm, neurons exhibited decreased spiking threshold and fired significantly more action potentials while no changes were observed on the level of EPSPs (Malik and Chattarji, 2011). Thus, enriched environment may enhance the synaptic plasticity in CA1 neurons but also it may strongly affect the neuronal spiking (Malik and Chattarji, 2011). In agreement with above data, learning in Morris water maze was used to assess the function of neurons from dorsal hippocampus. In particular, dorsal but not ventral hippocampal CA1 neurons exhibited enhanced excitability in animals which learned the water maze task as compared with those from neurons of control rats (Oh et al., 2003). Finally, in a most recent study, periods of neuronal rhythmic firing in rat barrel cortex pyramidal neurons were shown to trigger long-lasting changes in membrane excitability *in vivo* in the absence of altered synaptic input, membrane resting potential or membrane resistance (Mahon and Charpier, 2012). Altogether a picture emerges that both synaptic and nonsynaptic forms of plasticity are substrates for long term memory and work synergistically (Giese et al., 2001; Debanne et al., 2003; Zhang and Linden, 2003). Importantly, changes in neuronal excitability are learning-specific since they are observed exclusively in animals that learned, but not in pseudoconditioned controls or animals that failed to learn (Moyer et al., 1996; Oh et al., 2003; Song et al., 2012). Consistent with a role for intrinsic plasticity in memory consolidation, learning-specific changes in intrinsic neuronal excitability can also serve a metaplasticity function. Thus, the period of enhanced neuronal excitability would match the period during which animals display enhanced learning. Indeed, in one study, olfactory learning resulted in transient enhancement of hippocampal intrinsic excitability and this resulted in facilitation of acquisition of the hippocampus-dependent Morris water maze task (Zelcer et al., 2006). Thus, intrinsic plasticity would support the learning-induced facilitation of learning.

Studies on E-S potentiation phenomenon in acute brain slices were limited to 2–3 h therefore it is important to ask how long E-S potentiation can last? Scarce *in vivo* data indicate that changes in intrinsic excitability accompanying certain paradigms of learning last beyond several hours. In particular, learning-associated upregulation in neuronal intrinsic excitability was observed up to 7 days following learning but not later (reviewed in Sehgal et al., 2013). Therefore, it has been suggested that because of its limited time span it unlikely to encode the memory itself but rather it facilitates successful memory formation (Sehgal et al., 2013). In the next section, we will discuss the mechanisms that were proposed to underlie E-S potentiation.

The Major Factors Contributing to Potentiation of Neuronal Excitability

As mentioned above, both synaptic and nonsynaptic forms of plasticity often work synergistically. Therefore, the major difficulty in studying the mechanism of altered neuronal activity, firing rate, firing threshold and gain is to separate intrinsic plasticity effects from those induced by increased excitatory synaptic drive. Plasticity of neuronal firing rate, firing threshold or gain within hippocampal circuits requires

several factors that can be roughly divided in two groups. The first group is related to enhanced local or global intrinsic membrane excitability, mediated by changes in the expression level or biophysical properties of ion channels affecting dendritic integration, spike generation, signal propagation in dendrites and the axon, and regulation of plasticity thresholds. The second one has synaptic origin and involves decreased somatic inhibition to excitation ratio resulting in local and effective modulation of neuronal output.

Plasticity of Intrinsic Membrane Excitability

A decade ago, in a seminal paper, Frick and colleagues reported that long-term synaptic potentiation was accompanied by an enhanced local excitability of pyramidal neuron dendrites (Frick et al., 2004). It is now clear that dendrites are not just passive integrators of excitatory synaptic input but actively participate in information processing (reviewed in Johnston and Narayanan, 2008; Sjöström et al., 2008). In particular dendrites can convey fast regenerative action potential-like events mediated by voltage-activated Na^+ channels (back propagating action potentials, bAPs) or slower events mediated by voltage-activated Ca^{2+} channels called Ca^{2+} dendritic spikes. The basis for these phenomena is the nonuniform and site specific distribution of several voltage gated channels, including Na^+ channels, A-type K^+ potassium channels and T-, R- or L-type Ca^{2+} channels across pyramidal neuron extremities. In particular, depending on channel type, phosphorylation state or inactivation kinetics in axon, soma or dendrites, these voltage gated conductances support different functions (reviewed in Waters et al., 2005; Johnston and Narayanan, 2008, see also "Long-Term Effects of Extracellular Proteolysis on Neuronal Excitability" Section below). Functionally, bAPs may act as retrograde signals to dendritic tree indicating the level of neuronal output and similarly to dendritic spikes enhance Ca^{2+} entry through N-methyl-D-aspartate receptors (NMDARs) following removal of Mg^{2+} block in these receptors and activation of Ca^{2+} voltage gated channels (Stuart and Sakmann, 1994; reviewed in Stuart et al., 1997; Waters et al., 2005). Importantly, change in the number, distribution or activity of various ion channels located throughout the neuron may result in altered intrinsic membrane excitability and neuronal input/output function.

Several mechanisms were suggested to operate concomitantly in rapid neuronal activity-dependent modulation of ion channel properties in dendrites. Importantly, mechanisms involved in synaptic plasticity and plasticity of intrinsic membrane excitability share several effectors. First, similar to synaptic plasticity, raise in Ca^{2+} concentration following activation of NMDARs is necessary for induction of E-S potentiation in hippocampus (Jester et al., 1995; Lu et al., 2000; Daoudal et al., 2002; Wójtowicz and Mozrzymas, 2014). Local Ca^{2+} microdomains may support modulation of properties of ion channels regulating neuronal excitability through mechanisms involving calmodulin, calmodulin-dependent kinase II (CaMKII), phosphatase calcineurin and other intracellular effectors (reviewed in Sjöström et al., 2008). Thus, Ca^{2+} from NMDARs would support E-S potentiation by kinase activation and in parallel LTD of GABAergic transmission (e.g., calcineurin activation, Lu et al., 2000). While protein kinase A and C (PKA, PKC) were shown to be crucial for CA1 region neuronal excitability (Hoffman and Johnston, 1998; Yuan et al., 2002), the role of CaMKII was proposed to be negligible (Ohno et al., 2006). A second mechanism involves Ca^{2+}-dependent gene expression involving cAMP response element binding protein (CREB) and NF-AT transcription factors. Another mechanism underlying intrinsic plasticity is the regulation of production, trafficking and insertion of ion channels, which results in an altered density of ion channel proteins in the membrane (reviewed in Beck and Yaari, 2008). Finally, activation of receptors of certain neurotransmitters (e.g., acetylcholine, dopamine, serotonin) and activation of metabotropic glutamate and GABA receptors (mGluRs and $GABA_BRs$) was shown to rapidly affect ion channel function through PKC and PKA kinases activity. Channels that were shown to be rapidly modulated through above mentioned mechanisms include e.g., L-type voltage gated Ca^{2+} channels (VGCCs), large-conductance Ca^{2+}-activated BK-type K^+ channels, hyperpolarization activated cyclic nucleotide-gated (HCN) cation-selective channels mediating H-current and many others (reviewed in Sjöström et al., 2008). Thus, following enhanced excitatory synaptic activity, rapid change in channel function may occur with respect to time constants of activation and inactivation, single channel conductance and surface expression and degradation (reviewed in Johnston and Narayanan, 2008; Sjöström et al., 2008). For instance, downregulation of H-current mediated by HCN1/2 channels results in enhanced EPSP summation (reviewed in Beck and Yaari, 2008). In addition, downregulation of A-type K^+ channels mediating A-current by hyperphosphorylation may result in enhanced bAPs and altered firing (Bernard et al., 2004). In opposite, upregulation of T-type Ca^{2+} channels results in enhanced dendritic Ca^{2+} spikes and altered neuronal firing (Beck and Yaari, 2008).

Besides key aspects of neuronal firing, such as action potential threshold and firing mode, it seems equally important to know how neurons fire repetitively in response to prolonged depolarization. During repetitive firing Ca^{2+}-sensitive K^+ currents are activated which leads to hyperpolarization that lasts tens to hundreds of milliseconds. Afterhyperpolarization (AHP) current affects somatic and dendritic membrane potentials such that by reducing EPSP amplitude in apical dendrites, AHP controls the threshold for neuronal spiking and LTP in CA1 hippocampal region (reviewed in Sehgal et al., 2013). Most importantly, the extent of such AHP determines the rate of repetitive neuronal firing and is downregulated by some learning tasks involving hippocampal formation in intact animals (Disterhoft et al., 1986; Coulter et al., 1989; de Jonge et al., 1990). Moreover, there is a strong and inverse relationship between AHP and magnitude of LTP in acute brain slices: the larger the AHP the smaller the LTP (Sah and Bekkers, 1996; Cohen et al., 1999; Kramar et al., 2004). In addition, age-related learning deficits are reversed with pharmacological manipulations that reduce the normally enlarged post-burst AHP of the CA1

pyramidal neurons observed in aged animals (Disterhoft and Oh, 2006). Most recent work indicates that long-term changes in intrinsic excitability following learning are not only limited to pyramidal neurons, but also hold for interneurons. Learning in the hippocampus-dependent trace eyeblink conditioning task was reported to reduce AHP in somatostatin-positive population of interneurons and enhance inhibition onto CA1 pyramidal neurons (Mckay et al., 2013). Altogether, function of ion channels outside synapses that play an important role in neuronal membrane excitability is regulated following enhanced neuronal activity patterns that lead to neuronal plasticity and formation of memory traces.

Balance in Somatic Inhibition-Excitation Ratio is Shaping Neuronal Output

The strategic locus of inhibitory drive to principal hippocampal neurons remain soma (Freund and Katona, 2007). By reducing inhibitory tone or responsiveness to inhibitory inputs, neuronal response to integrated excitatory input would increase. Thus, the interplay between excitatory and inhibitory weights remains crucial for basal neuronal input-output function and control of synchrony of principal cell populations (Isaacson and Scanziani, 2011). With regard to neuronal plasticity, changes in the balance between excitation and inhibition (E/I) could directly regulate the plastic potential of neuronal networks. For instance, during cortical development, increasing efficacy of GABAergic inhibition results in progressive reduction in plastic potential of neuronal networks and more extensive hard wiring of existing circuits (Hensch, 2005). Most importantly, invasive or noninvasive (e.g., pharmacological) interventions resulting in decreased strength of certain inhibitory inputs were recently successfully used to enhance experience dependent neuronal plasticity in adult nervous system (reviewed in Bavelier et al., 2010).

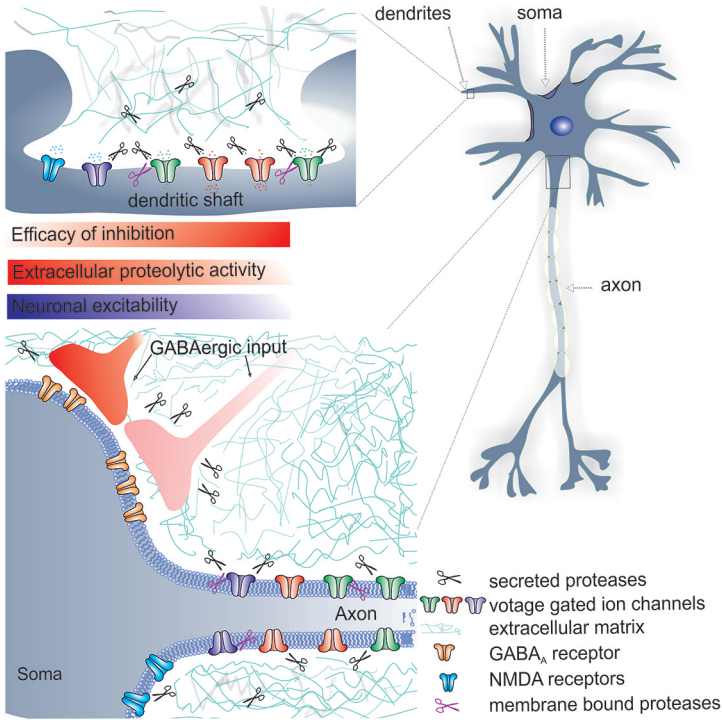
Changes in inhibition were proposed to be particularly important for neuronal plasticity following sensory experience. It has been show that in the adult primary auditory cortex, the dynamics of synaptic receptive field plasticity is associated with a reduction of synaptic inhibition which was followed by a large increase in excitation (Froemke et al., 2007). Therefore, in addition to modulation of intrinsic membrane properties, additional important mechanism proposed to underlie E-S potentiation has synaptic origin and involves modulation in somatic inhibition. This view is supported by several studies. For instance, E-S potentiation was completely or partially abolished in the presence of blockers of GABAergic transmission in the hippocampus (Chavez-Noriega et al., 1989; Tomasulo and Ramirez, 1993; Daoudal et al., 2002). Moreover, direct recordings from CA1 pyramidal cells following tetanization of Sch-CA1 projection showed increased excitation/inhibition ratio (Abraham et al., 1987). One proposed candidate for effector mediating suppression of GABAergic inhibition was Ca^{2+}-sensitive phosphatase calcineurin, since blockade of this phosphatase prevented induction of E-S potentiation without interfering with synaptic LTP (Lu et al., 2000). Most recently, brief repetitive stimulation of Schaffer collaterals was found to enhance intrinsic neuronal excitability in parvalbumin-positive subpopulation of GABAergic interneurons (PV+ basket cells; Campanac et al., 2013). It was proposed, that following enhanced neuronal activity that results in synaptic plasticity of excitatory synapses, activity of pyramidal neurons could be effectively controlled and balanced by increase in inhibitory input from PV+ basket cells (Campanac et al., 2013). It should be remembered however, that excitatory synaptic transmission onto certain interneurons types also undergoes LTP, keeping the balance between synaptic excitation and inhibition onto principal neurons intact (Lamsa et al., 2005). Moreover, some reports questioned the role of GABAergic inhibition in shaping E-S potentiation (Hess and Gustafsson, 1990; Jester et al., 1995).

The Role of Extracellular Proteolysis in Shaping Neuronal Excitability

Acute Actions of Extracellular Proteolysis on Neuronal Spiking

Low levels of several proteases are normally present in hippocampus and include for instance trypsin (Koshikawa et al., 1998), neuropsin (Tomimatsu et al., 2002), neurotrypsin (motopsin, Mitsui et al., 2007), tissue plasminogen activator (tPA; Pawlak and Strickland, 2002), thrombin (Rohatgi et al., 2004) and MMPs (e.g., MMP-2–9, Szklarczyk et al., 2002; Wiera et al., 2012). Activity of these serine proteases and metalloproteases was shown to be crucial for synaptic plasticity and memory (reviewed in Tomimatsu et al., 2002; Shiosaka, 2004; Sonderegger and Matsumoto-Miyai, 2014). Following enhanced neuronal activity such as that occurring during formation of memory traces, proteases may be rapidly released and activated in extracellular space and support synaptic plasticity. For instance, tPA is rapidly secreted following membrane depolarization and Ca^{2+} entry (Gualandris et al., 1996; Parmer et al., 1997; Baranes et al., 1998), while activity of protease neuropsin was shown to rapidly increase as early as 5 min following neuronal activity and activation of NMDARs (Matsumoto-Miyai et al., 2003). Similarly, neurotrypsin may be exocytosed as early as 30 s–2 min post stimulation of neurons with KCl and then removed within several minutes (Frischknecht et al., 2008). In addition, matrix metalloprotease 9 (MMP-9) was shown to follow vesicular release (Sbai et al., 2008) and its activity was detected as early as 5 min following stimulation of cultured neurons with glutamate or blockers of inhibitory transmission (Michaluk et al., 2007). More recently, rapid release and enhanced activity of MMP-9 in the synapse was shown to occur as early as 5–10 min following stimulation (Dziembowska et al., 2012; Stawarski et al., 2014). Thus, taken that use-dependent plasticity of intrinsic excitability has been reported in numerous regions of the brain, including hippocampus, cerebellum and neocortex (see for reviews Daoudal and Debanne, 2003; Zhang and Linden, 2003) the question arises to what extent proteolytic activity in extracellular space could support these processes? In the following sections, we discuss literature related to proteolysis-mediated and activity-dependent changes in neuronal excitability and E-S potentiation (see also **Figure 1**).

With regard to rapid actions, several studies indicate that thrombin, trypsin and their protease activated receptors (PAR-1



Protease	Downstream protease target implicated in E-S coupling	Putative region of action	Functional outcome and reported time of action	Reference
Thrombin	Protease activated receptor 1 (PAR-1)	Dendrites, soma	-membrane depolarization (10-20s), enchanced NMDAR-mediated synaptic EPSPs, increased spiking (3 min)	Han et al., 2011
		Soma	- reduce GABAergic currents (1 minute), - decreased or increased inhibition (in CA3 and CA1 region respectively) enchanced firing and population spike (10 min)	Hashimotodani et al., 2011 Maggio et al., 2012
		???	- enchanced neuronal excitability (EEG level (2-4 min) - lower threshold for generating seizures - promotes persistent sodium current by lowering threshold for activation of sodium channels (10 minutes)	Lee et al., 1997 Maggio et al., 2008 Isaeva et al., 2012
Trypsin	Protease activated receptor 2 (PAR-2)	Dendrites	- promotes depression of spiking (antiepileptogenic role) - affects BK channel current (promotes hyperexcitability)	Gan et al. 2011 Moss et al., 2015
		Dendrites, soma	- regulates N-type voltage-gated Ca2+ channels	Kim et al., 2014
		???	- reduction in afterdischarges (EEG), 10 min	Lohman et al., 2008
Neuropsin	N.D.	Dendrites	-larger potentiation of population spike than EPSPs (15-30 min.)	Tamura et al., 2006
Plasmin	N.D.	Soma	- reduce GABAergic currents (1 minute)	Mizutani et al., 1997
		???	- equal potentiation of EPSPs and population spikes (10-15 min)	Mizutani et al., 1996
Tissue plasminogen activator (tPA)	N.D.	Soma (?)	- tPA KO mice: increased feed forward inhibition, E-S depression, lack of E-S shift following LTP induction and reduced paired-pulse spike facilitation	Frey et al., 1996
Matrix metallo-proteases	N.D.	Dendrites	- support E-S potentiation (5 min) - prevent NMDARs potentiation (5 min)	Wojtowicz and Mozrzymas, 2014
	NMDARs	Dendrites	- MMP-7 cleave GluN1 subunit of NMDARs and suppress Ca2+ current	Szklarczyk et al., 2008
		Dendrites	- MMP-9 affects kinetics of NMDAR current - MMP-9 regulates NMDAR lateral mobility	Gorkiewicz et al., 2009 Michaluk et al., 2009
		Dendrites	- MMP-3 cleaves NMDARs GluN1 subunit	Pauly et al., 2008
ß-secretase 1 (BACE-1)	ß2 subunit of voltage gated sodium channels	Soma, dendrites, axon	- cleaves beta2 subunit of sodium channel (2h), cultured neurons of BACE1 overepressing or BACE1 knockout mice exhibited altered density of sodium channels, sodium current amplitude, firing threshold	Kim et al., 2007 Hu et al., 2010
	KCNQ channels	Soma, axon	- regulates repolarizing M-current and spike frequency adaptation (48h)	Hessler et al., 2015
Chondroitinase ABC	ECM, glycosaminoglycans of PNNs, Kv3.1b channels	Soma	- increased spiking of PV+ GABAergic interneurons in vitro (48h treatment of cultures)	Dityatev et al., 2007

FIGURE 1 | Summary of the known role of several proteases in rapid (requiring minutes to hours) changes in EPSP-to-spike (E-S) potentiation and/or neuronal spiking. A cartoon indicates the putative regions of CA1 pyramidal neuron where extracellular proteolysis may affect intrinsic neuronal excitability and E-S potentiation via (a) cleavage of extracellular matrix (ECM) constituents and modulation of ion channels; (b) modulation of inhibitory inputs efficacy (see text for details). The list of known targets of extracellular proteolysis and putative regions of action is listed in the table. N.D.- no data available.

and PAR-2) may play a crucial role in shaping neuronal excitability. Activation of PARs is initiated by site-specific proteolytic cleavage in the N-terminal extracellular region, which uncovers a tethered ligand activating Gα q/11, Gα i/o, or Gα12/13-proteins and results in activation of multiple intracellular signaling pathways depending on the activating ligand (reviewed in Ben Shimon et al., 2015, this Special Issue). Interestingly, thrombin and it's receptor PAR-1 were shown to mediate pathway specific effects on neuronal excitability. For instance, during EEG recording *in vivo*, thrombin was shown to rapidly enhance neuronal excitability as early as 2–4 min following application (Lee et al., 1997). In another study, exogenous thrombin application rapidly (within 10 min) decreased inhibitory currents and increased CA3 pyramidal neurons spontaneous action potentials discharges, while in CA1, it produced enhanced inhibitory input (Maggio et al., 2013). Such site specific acute actions of thrombin were ascribed to enhanced expression of PAR-1 in these regions (Maggio et al., 2013). Interestingly, thrombin application or PAR-1 activation produced rapid increase in population spike that was more pronounced than accompanying EPSP enhancement, an effect that saturated the ability of the tissue to undergo tetanus-induced LTP (Maggio et al., 2008). In addition, thrombin lowered the threshold for generating epileptic seizures in CA3 region of the hippocampus (Maggio et al., 2008). Also in dentate gyrus neurons, PAR-1 activation resulted in rapid membrane depolarization within 10–20 s and increased spiking of dentate granule cells (Han et al., 2011). Thus, the role of PAR-1 receptors seems to be of particular importance in rapid modulation of neuronal excitability of hippocampal neurons (see also **Figure 1**). It should be stressed, that PAR1 activation in astrocytes can trigger release of glutamate into the extracellular space and activate neuronal NMDA receptors, further supporting membrane depolarization (Lee et al., 2007). Thus, by releasing glutamate and D-serine, a single astrocyte can affect, in a synchronous manner, neuronal plasticity in many thousands of nearby excitatory synapses by activating NMDARs (Henneberger et al., 2010). Moreover, activation of PAR-1 by thrombin mediates induction of MMP-9 expression (Choi et al., 2008), a metalloprotease crucial for neuronal plasticity, learning and memory (Huntley, 2012). Altogether, it seems that PAR1-mediated signaling may be even more complex and interaction between proteases, PARs and neuronal excitability will require more detailed studies.

Another member of the family of receptor for proteases activated by trypsin, namely PAR-2, was previously implicated in rapid protease-dependent alteration of neuronal excitability and epileptic activity *in vivo* (Lohman et al., 2008). Notably, acute activation of PAR-2 resulted in significant membrane depolarization and depression of neuronal spiking in cultured

hippocampal neurons (Gan et al., 2011). In another study, rapid PAR-2 activation via SLIGRL peptide resulted in reduced trypsin release and decreased afterdischarges within electroencephalograms (EEG) following electrically evoked kindling in amygdala as early as 10 min following peptide application (Lohman et al., 2008). Interestingly, activation of PAR-2 with trypsin reduced N-type voltage-gated Ca^{2+} current and PAR-2 agonists reduced action potential firing frequency in rat peripheral sympathetic nerve neurons (Kim et al., 2014). In conclusion, these studies indicate that PAR-2 activation reduces neuronal excitability and therefore has an anti-epileptogenic role. However, most recently, PARs were also implicated in regulation of neuronal excitability through modulation of ion channels function and opposite conclusions were drawn. In bronchopulmonary sensory neurons, activation of PAR-2 for 2 min with activating peptide reduced large conductance Ca^{2+}-activated potassium channel (BK) current, which resulted in increased excitability (Moss et al., 2015). Thus, it is plausible that activation of PAR-2 in various neurons results in different functional outcomes and therefore it remains to be further established *in vivo* whether PAR-2 has an anti-seizure and antiepileptogenic role (see also **Figure 1**).

Not only thrombin and trypsin were shown to rapidly modulate neuronal spiking. Another protease neuropsin, a trypsin-like serine protease strongly expressed within hippocampus (Chen et al., 1995) was shown to be crucial for hippocampal dependent learning and synaptic LTP (Tamura et al., 2006). In support of this view, exogenous application of recombinant neuropsin lasting several minutes potentiated EPSPs and to even a larger extent also population spike for whole duration of recording (3 h, Tamura et al., 2006) further documenting that extracellular proteases may trigger fairly rapid and persistent E-S potentiation. Unfortunately, while the mechanism of synaptic plasticity was investigated, the mechanism of E-S potentiation in this system remains to be established. However, in another study from that group, neuropsin knockout mice exhibited significantly reduced inhibitory inputs from parvalbumin interneurons among many other abnormalities (Hirata et al., 2001), suggesting that excitation/inhibition balance could be under control of neuropsin (see also section below).

The above mentioned findings suggest that proteases may differentially affect synaptic and spike components. In our most recent study, we addressed the role of MMP-3 and MMP-2/9 activity in supporting E-S potentiation in the CA3 region of the hippocampus (Wójtowicz and Mozrzymas, 2014). We found that acute MMP-3 inhibition with specific inhibitor NNGH impaired EPSP and spike potentiation as early as 5 min following LTP induction with tetanic stimulation or pairing of mossy fiber synapses activity with CA3-CA3 synapses. In contrast, the effect of MMP-2/9 inhibition was visible not earlier than 1.5 h post LTP induction (Wójtowicz and Mozrzymas, 2014). Thus, MMP-3 and MMP-2/9 may differentially shape expression of E-S potentiation in the CA3 region of the hippocampus. Most strikingly, the reduction in spike potentiation following MMP-3 inhibition was more pronounced than would be expected from impaired synaptic plasticity alone, suggesting that synaptic and nonsynaptic components are regulated separately. Of note, MMP-3 activity regulates availability of several proteins known to support long-term plasticity, such as NMDARs, brain-derived neurotrophic factor (BDNF), cell adhesion molecules and other MMPs including MMP-9 (reviewed in Van Hove et al., 2012). Thus, properly balanced MMP-3 activity may be permissive for expression of E-S coupling. The exact mechanism underlying such MMP-3 impact on E-S potentiation remains elusive. However, we may suggest at least two possible explanations. First, we found that LTP of NMDARs function is lacking upon application of MMP inhibitors (Wójtowicz and Mozrzymas, 2014). Therefore, taken the great importance of Ca^{2+} flux mediated by NMDARs for E-S potentiation (see above), MMP-activity could support E-S potentiation by supporting NMDARs function. Second, since MMP-3 cleaves immunoglobulin-like cell adhesion molecule 5 (ICAM-5, Conant et al., 2010) and recently, a soluble N-terminal fragment of ICAM-5 was shown to rapidly (10–15 min) induce neuronal spiking in dose and time-dependent manner (Niedringhaus et al., 2012) we can speculate that MMP-3 could cleave ICAM-5 and support E-S potentiation by providing N-terminal fragment of this immunoglobulin like protein.

Finally, when considering acute action of proteases on neuronal E-S coupling, it should be born in mind that in most cases, data were obtained in the *in vitro* models, where proteases were applied in excess to the entire preparation and timing of such administration could not be very precise. To give an example, it was reported that neuropsin at concentrations below 1.8 mU/ml potentiated EPSPs and population spike amplitude to similar extent (Tamura et al., 2006). However, doubling this concentration resulted in a significant depression of synaptic transmission (Tamura et al., 2006). In another study, protease plasmin applied for 6 h at 100 nM impaired the maintenance of synaptic LTP (Nakagami et al., 2000). However, in another study plasmin applied at the same concentration (100 nM) for 10 min was shown to equally potentiate EPSPs and population spikes following patterns of stimuli inducing short-term potentiation (Mizutani et al., 1996) which typically promote neuronal plasticity, but not E-S potentiation. Thus, it needs to be emphasized that in physiological conditions, the enzyme concentration, site of action and timing are likely to be of crucial importance.

Proteolytic Regulation of Ion Channels and Membrane Excitability

Intracellular proteolytic processing and regulation of voltage-gated calcium channels via the carboxy-terminal domain is well documented (for a review, see Catterall, 2011). However, studies relating directly rapid extracellular proteolysis with function of voltage- or ligand gated channels with respect to neuronal spiking are sparse. Recent studies suggest that a complex interaction occurs between certain extracellular proteases and function of voltage-gated channels. In particular, a number of voltage-gated ion channels contribute to spike afterdepolarization (ADP). ADP is mediated largely by persistent sodium current (I_{NaP}), which is opposed by outward current mediated by KCNQ/M potassium

channels (I_M) and has a crucial impact on neuron ability to generate multiple spikes. Notably, in young (postnatal day 6–15) rats, exogenously applied thrombin was shown to increase neuronal excitability in just several minutes in an NMDAR-independent manner. In addition, exposure of CA3 pyramidal neurons to exogenous thrombin for 10 min was shown to result in less negative membrane potential (depolarization by 2 mV) and it produced a hyperpolarizing shift of voltage dependence of tetrodotoxin-sensitive persistent voltage-gated sodium channel activation which resulted in more pronounced I_{NaP} current at negative potentials (Isaeva et al., 2012). Thus, in addition to action on PAR-1, thrombin may promote neuronal excitability via regulation of certain ion channels.

From the point of view of neuronal plasticity, ligand-gated ion channels providing Ca^{2+} are indispensable. NMDA-receptors are known to play a pivotal role in neuronal plasticity, learning and acquisition of spatial reference memory (Malenka and Bear, 2004; Nakazawa et al., 2004). Therefore possible interaction of extracellular proteolysis with NMDARs function has been addressed in several studies. Importantly, as stated above, Ca^{2+} flux mediated by NMDARs remains necessary for expression of E-S potentiation (Wójtowicz and Mozrzymas, 2014).

It appears that some proteases released following enhanced neuronal activity could potentially regulate NMDAR-currents. For example, matrix metalloprotease (MMP-7, but not MMP-2/9) was shown to cleave GluN1 subunit of NMDARs and suppress Ca^{2+} current (Szklarczyk et al., 2008) while MMP-9 proteolysis was implicated in lateral mobility of these receptors (Michaluk et al., 2009). In addition, another matrix metalloprotease (MMP-3) was shown to cleave NMDARs at glycine binding site, but the functional consequences of this cleavage remain to be determined (Pauly et al., 2008). Neuropsin knockout mice exhibited significantly smaller NMDAR-mediated currents in principal neurons of the basal amygdala (Attwood et al., 2011). However in the hippocampus, direct application of neuropsin did not modulate NMDAR-mediated current (Komai et al., 2000). Another protease tPA was reported to cleave the GluN1 subunit of the NMDA receptor, but in the case of this protease such cleavage enhanced NMDAR function (Nicole et al., 2001). However, the proteolytic degradation of NMDARs was questioned in other *in vitro* studies were high doses of tPA were used (Liu et al., 2004; Pawlak et al., 2005). In particular, nonproteolytic effect of tPA on NR2B-containing NMDA receptors was reported (Pawlak et al., 2005). Therefore, it remains to be established *in vivo* to what extent neuropsin or tPA could rapidly modulate NMDAR function following enhanced synaptic activity.

In a study from our laboratory, the impact of MMP-9 on NMDAR currents was tested in cultured neurons. We showed that recombinant MMP-9 increased NMDAR desensitization and shortened the decay time constant of evoked NMDAR-mediated current in cultured neurons *in vitro* (Gorkiewicz et al., 2009). Altogether, the prevalent view from these studies is that excess of proteases activity decrease NMDAR-mediated currents. This contrasts with the general view that proteolytic activity supports NMDAR-dependent synaptic LTP. Second, with regard to MMP, in our study, we found that LTP of NMDARs function is lacking upon application of MMP inhibitors (Wójtowicz and Mozrzymas, 2014). Thus, MMP-activity is necessary to support rather than suppress NMDARs function. Therefore, it is not clear to what extent proteases could directly affect NMDAR function *in vivo*.

We have recently showed that the permanent deprivation but also excess of protease MMP-9 activity negatively affect LTP expression in MF–CA3 and Sch-CA1 hippocampal projections (Wiera et al., 2013, 2015). Thus, in the hippocampus, epileptiform activity involves enhanced excitation among pyramidal cells and excessive NMDA receptor activation (Miles and Wong, 1987; Meier et al., 1992) which is associated with extensive synaptogenesis and recurrent collaterals and reduced synaptic inhibition (Dingledine et al., 1986; Merlin and Wong, 1993). Several lines of evidence indicate that extracellular proteolysis mediated by trypsin, plasmin or thrombin and several other enzymes could be involved in these processes (Yamada and Bilkey, 1993; Mizutani et al., 1996; Lee et al., 1997). Indeed, overexpression of serine protease inhibitor 1 (Nexin-1, PN-1) resulted in enhanced GABAergic and glutamatergic transmission, increased polyspiking and enhanced NMDAR function resulting in enhanced LTP (Lüthi et al., 1997). Thus, while synaptic transmission seems to be differentially regulated by PN-1 activity, PN-1 manipulation always resulted in enhanced neuronal excitability. Moreover, mutant mice lacking tPA, or plasminogen, are resistant to seizure induction and neuronal cell death after kainic acid administration (Tsirka et al., 1995, 1997). Thus, modulation of NMDARs function most likely represents a fine-tuning related to specific time windows and loci of the proteolytic action which is dependent on the neuronal network activity. Indeed, considering a key role of NMDARs in secretion of several proteases (Michaluk et al., 2007), one can expect some feedback mechanisms in which, following enhanced synaptic activity and activation of NMDARs, released proteases regulate NMDARs function and thereby Ca^{2+} entry affecting synaptic plasticity which, in turn, affects further release of proteases.

Finally, some evidence document more general role of proteolysis in regulation of ion channels beyond hippocampus. For instance, an α-secretase ADAM10 was shown to cleave ectodomain of β2 subunit of voltage-gated sodium channel in Chinese hamster ovary cells (Kim et al., 2005). However, to what extent ADAM10 protease could affect function of this channel remains to be elucidated. Moreover, as mentioned in above section, rapid modulation of N-type voltage-gated Ca^{2+} channels and BK channel function occurs following PAR-2 activation in sensory or peripheral nerves *in vitro* (Kim et al., 2014; Moss et al., 2015). Interestingly, MMPs 2/9 were shown to directly alter the gating properties and function of retinal cyclic nucleotide-gated channel in concentration-dependent manner by proteolysis of extracellular domain of the receptor (Meighan et al., 2012, 2013). It is also worth mentioning, that in nonneuronal cells, epithelial sodium channel (ENaC) was shown to be regulated by serine proteases (Rossier and Stutts, 2009), cysteine protease cathepsin-S (Haerteis et al., 2012) or metalloprotease meprin β (Garcia-Caballero et al., 2011).

Thus, it is important to realize that besides modulation of key receptors involved in synaptic plasticity, rapid extracellular

proteolysis may also affect the function of voltage-gated ion channels. However, since some of above mentioned studies were carried out *in vitro*, future *in vivo* studies are necessary to verify this interesting possibility. Since voltage gated sodium channels have recently been implicated not only in regulating membrane excitability, but in addition to that in adhesion, migration, path finding and transcription (Brackenbury and Isom, 2008), interaction between extracellular proteases and voltage-gated sodium channels most likely goes beyond regulation of neuronal excitability.

Long-Term Effects of Extracellular Proteolysis on Neuronal Excitability

While above mentioned studies addressed acute actions of extracellular proteolysis, several studies in which knockout or overexpression of protease genes have further indicated, that prolonged deficit or excess of extracellular proteolysis may have a crucial impact on neuronal excitability and E-S potentiation. For instance, in neuropsin mutant mice, increased susceptibility for hyperexcitability (polyspiking) was reported in response to repetitive afferent stimulation with no deficits in hippocampal synaptic LTP (Davies et al., 2001). This suggests that neuropsin is a protease supporting both synaptic plasticity and plasticity of neuronal excitability. In the amygdala, PAR-1 was proposed to promote contrasting neuronal responses depending on the emotional status of an animal by a dynamic shift between distinct G protein-coupling partners. In particular, in PAR-1 knockout mice, basal amygdala pyramidal neurons exhibited enhanced firing rate 48 h following fear conditioning when compared to wild type controls (Bourgognon et al., 2012). Similar results were obtained in naïve mice that were bilaterally infused with SCH79797, a PAR-1 function inhibitor before above mentioned conditioning paradigm (Bourgognon et al., 2012). Altogether, PAR-1 may be involved in regulation of neuronal excitability in amygdala in experience-dependent manner.

Complementary conclusions were drawn by manipulating PN-1. This endogenous serine proteases inhibitor is provided by glia and neurons and affects activity of thrombin, tPA, plasmin, trypsin and several other serine proteases in ECM (Lüthi et al., 1997). In agreement with studies discussed above, animals lacking and overexpressing PN-1 exhibited severe changes in EPSP-spike coupling in the hippocampus. Notably, mice knocked out in PN-1 gene exhibited increased susceptibility to kainic acid-induced seizures, characteristic polyspiking, while at the same time they exhibited decreased NMDA/AMPA ratio and no changes in basal EPSCs or IPSCs (Lüthi et al., 1997). Altogether, these data indicate that the proper balance between serine proteases and metalloproteases and their endogenous inhibitors (serpin inhibitors, tissue inhibitors of metalloproteases TIMPs) in long-term window are strongly involved in control of neuronal excitability.

With regard to extracellular proteolysis, most recent studies indicate that regulation of neuronal intrinsic excitability might rely on increased proteolytic cleavage of certain ion channel subunits. First, membrane bound protease BACE1 (beta-site APP cleaving enzyme 1, memapsin 2, Asp 1) expressed in hippocampal dentate gyrus, hilus and stratum lucidum (Laird et al., 2005) was shown to be crucial for function of sodium and potassium channels. Nav1.1 and 1.3 channels are mainly located at somatodendritic regions, while Nav1.2 and 1.6 are distributed to axons (Lai and Jan, 2006). In rat primary cortical cultures, prolonged (2 h) exposure to BACE1 resulted in cleavage of β2 subunit of sodium voltage-gated ion channels (Nav1.1). Moreover, overexpression of BACE1 reduced sodium-current *in vitro* indicating that BACE1 activity regulates cell-surface sodium channel function (Kim et al., 2007). Consequently, neurons from BACE1 knockouts exhibited overexpression of voltage gated sodium channels (Nav1.1, 1.2, 1.6), had decreased firing threshold, larger population spikes and developed spontaneous epileptic seizures (Hu et al., 2010), but exhibited unaltered LTP, LTD or NMDARs function (Laird et al., 2005). Interestingly, in a most recent study, BACE1 was shown to regulate neuronal excitability through an nonenzymatic interaction with KCNQ channels mediating I_M current. Notably, BACE1$^{-/-}$ hyperexcitability was explained by loss of repolarizing I_M-current (Hessler et al., 2015). Potassium channels mediating I_M-current do not participate appreciably in action potential repolarization due to their slow kinetics. However, this current is crucial during AHP of medium duration (Gu et al., 2005; Tzingounis and Nicoll, 2008) and shapes spike frequency adaptation. In addition, I_M-current is also present in axons and presynaptic terminals, where it modulates firing patterns and transmitter release (Martire et al., 2004; Vervaeke et al., 2006; Sun and Kapur, 2012; Battefeld et al., 2014). Thus, BACE1 seems to play an important role in neuronal excitability and is essential for cognitive, emotional, and synaptic functions (Laird et al., 2005) and BACE1 inhibitors may normalize membrane excitability in Alzheimer's disease patients with elevated BACE1 activity (Kim et al., 2007).

Impact of Extracellular Proteolysis on Inhibitory Transmission and Excitation/Inhibition Balance

As mentioned in previous chapters, change in the balance of excitation/inhibition is considered to be a powerful mechanism gating information flow in neuronal networks. Therefore altered GABAergic inhibition is expected to significantly affect the outcome of dendritic integration and ultimately the information flow. Moreover, inhibition of $GABA_A$Rs facilitates the induction of synaptic plasticity (Stelzer et al., 1994). With this regard, exogenous thrombin application rapidly (less than 1 min) and robustly (80%) decreased inhibitory currents in cultured neurons (Hashimotodani et al., 2011). Consequently, acute application of thrombin was shown to lower the threshold for generating epileptic seizures and to change spontaneous activity of CA3 hippocampal pyramidal cells through activation of PAR-1 (Maggio et al., 2008). Indeed, in acute brain slices, thrombin rapidly (within 10 min) decreased inhibitory currents and increased CA3 pyramidal neurons spontaneous action potentials discharges (however in CA1, it produced rather enhanced inhibitory input, Maggio et al., 2013). Exogenously applied plasmin was also shown to quickly (1 min) reduce GABAergic currents in CA1 pyramidal neurons (Mizutani et al., 1997).

In several transgenic models, GABAergic transmission was modulated following long-term manipulation of proteolytic activity in extracellular milieu. For instance neuropsin KO

mice exhibited increased propensity to hyperexcitability (polyspiking) in response to repetitive afferent stimulation. These animals were additionally more prone to seizure activity on kainic acid administration and heightened immediate early gene (c-fos) expression throughout the brain (Davies et al., 2001). Importantly, neuropsin mutant mice displayed normal hippocampal LTP and exhibited no deficits in spatial navigation tasks (Davies et al., 2001) implying that neuropsin may regulate neuronal spiking to larger extent than synaptic plasticity (see also below). Given that most neuropsin is stored in the extracellular space as a nonactive proform and that neuropsin activity occurs rapidly following neuronal activity (5 min, Matsumoto-Miyai et al., 2003) it is expected that local neuropsin-mediated proteolysis could significantly change the E-S coupling. Indeed, loss of neuropsin was implicated in decreased efficiency of somatic inhibitory input, reduced synchronization of pyramidal cells and ultimately impaired long-term plasticity of glutamatergic transmission (Tamura et al., 2012).

Mice overexpressing the protease tPA showed an enhanced LTP and exogenous application of tPA enhanced L-LTP in rat hippocampal slices (Baranes et al., 1998; Madani et al., 1999). Thus, tPA proteolysis remains important for synaptic LTP. In a seminal paper, Frey and coworkers investigated the role of protease tPA in E-S plasticity (Frey et al., 1996). In acute brain slices from tPA knockout mice they described increased feed forward inhibition and E-S depression, lack of E-S shift following LTP induction and reduced paired pulse spike facilitation. By using $GABA_A$R-antagonist bicuculline, they found much more pronounced population spike increase without any impact on EPSPs. Thus, tPA differentially supports EPSP and spike plasticity and most likely GABAergic system is involved in these processes.

We also studied the role of GABAergic system in mediating effects of MMPs activity on E-S potentiation in CA3 hippocampal region (Wójtowicz and Mozrzymas, 2014). We found that by blocking $GABA_A$Rs with low, nonsaturating doses of picrotoxin, population spike in CA3 region of the hippocampus increased four-fold in the absence of any change in synaptic drive (Wójtowicz and Mozrzymas, 2014; Supplementary Figure 1) confirming a crucial role of somatic inhibitory input in braking neuronal firing. However, we still observed effects of MMP inhibition on E-S potentiation in the presence of $GABA_A$Rs blocker, indicating that at least in our system, effects associated with MMPs inhibition are not primarily related to GABAergic input.

In the last decade, the regulation of GABAergic transmission via endocannabinoid-mediated retrograde signaling were intensely investigated (Heifets and Castillo, 2009; Kano et al., 2009). Following postsynaptic membrane depolarization, release of endocannabinoids can retrogradely activate presynaptic receptors (CB1) and suppress GABA release in inhibitory synapses, a phenomenon called depolarization induced suppression of inhibition (DSI; Wilson and Nicoll, 2001; Wilson et al., 2001). It has been recently shown *in vivo* that CA1 hippocampal place cell firing, following injection of waking patterns of CA1 place cells discharge recorded during a spatial task resulted in endocannabinoid-mediated decrease of GABAergic transmission through CB1Rs (Dubruc et al., 2013). This resulted in enhanced firing probability and better spike precision compared to situation of no DSI (Dubruc et al., 2013). It has been postulated that such improved spike-time precision could play a role in both spike-timing coordination and network oscillations in the hippocampus (Dubruc et al., 2013). It is expected that modulation of endocannabinoid signaling may have a profound impact on neuronal input-output function and eventually information processing. With this regard, recently, activation of neuronal protease activated receptor (PAR-1) has been shown to affect this system. Notably, in cultured hippocampal neuron *in vitro*, activation of neuronal PAR-1 via thrombin or specific activating peptide was shown to trigger retrograde signaling mediated by endocannabinoid 2-AG and presynaptic CB1 receptors, ultimately resulting in massive suppression of inhibitory synapses (Hashimotodani et al., 2011). Taken that activation of astrocytic PAR-1 results in potentiation of excitatory transmission (Lee et al., 2007; Mannaioni et al., 2008) it is expected that acute activation of PAR-1 (e.g., by thrombin) may result in enhanced EPSP-spike coupling via suppression of somatic inhibition and enhanced synaptic excitation.

In conclusion, it appears that both acute and long-term manipulations in proteases activity may affect effectiveness of inhibitory drive and neuronal excitability.

Proteolytic Degradation of Extracellular Matrix and Neuronal Excitability

Among many substrates, proteases released to extracellular space cleave ECM components, yielding cryptic peptides and providing space for structural rearrangements of neuronal compartments. The molecular mechanism underlying interaction of ECM components and neuronal excitability remains elusive. It appears however, that proteolytic processing of some components of ECM or adhesion molecules following enhanced neuronal activity may affect neuronal excitability by affecting function of certain ionotropic channels.

Recently, hyaluronic acid was shown to directly gate L-type voltage gated channels in hippocampal CA1 neurons and subsequently regulate postsynaptic Ca^{2+} entry, synaptic plasticity and learning and memory (Kochlamazashvili et al., 2010). While these effects were observed in excitatory synapses, it is interesting to consider more broad regulation of neuronal function by ECM rigidity. Integrity of specialized ECM areas surrounding neuronal cell bodies, perineuronal nets (PNNs) were shown to be crucial for hippocampal-dependent learning and memory (Gogolla et al., 2009; Kochlamazashvili et al., 2010; reviewed in Wang and Fawcett, 2012). PNNs were shown to envelop Kv3.1b expressing neurons and it has been suggested that these structures likely support the high firing frequencies of fast-spiking interneurons (Hartig et al., 1999). Indeed, *in vitro* enzymatic treatment of PNNs with chondroitinase ABC which resulted in degradation of glycosaminoglycans (e.g., chondroitin sulfate and hyaluronan) was shown to result in increased excitability and spike firing in parvalbumin positive interneurons *in vitro* but not in pyramidal cells (Dityatev et al., 2007).

In another *in vivo* study, exogenous application of chondroitinase in the ventral hippocampus resulted in a selective increase in dopaminergic neurons and pyramidal cells firing rate, as determined a week later (Shah and Lodge, 2013). Long term treatment (9 days long) of neuronal cultures with hyaluronidase transformed the normal network firing burst into oscillations and epileptiform-like activity (Vedunova et al., 2013). However, whether this increased neuronal firing was induced by enhanced synaptic drive or changes in intrinsic neuronal excitability was not investigated.

On the other hand, ectodomains of some cell adhesion molecules are cleaved by several secreted (e.g., MMPs) or membrane bound (e.g., ADAM10) proteases and released into extracellular space following neuronal activity (e.g., nectin-1, L1 and NCAM, ICAM-5; Thelen et al., 2002; Hinkle et al., 2006; Lim et al., 2012; Niedringhaus et al., 2012) and may additionally regulate neuronal firing. In particular, MMP-3 was shown to cleave ICAM-5 following enhanced neuronal activity and LTP (Conant et al., 2010). Soluble N-terminal fragment of this immunoglobulin like protein was shown to induce neuronal spiking in dose and time-dependent manner through integrin β1 signaling (Niedringhaus et al., 2012). Thus, a cascade involving secreted proteases and the products of rapid proteolysis containing RGD motif could rapidly affect neuronal function through integrin signaling.

The importance of ECM rigidity in neuronal excitability is additionally evident in several knockout models. For instance, tenascin-R knockout mice exhibited hyperexcitability in the CA1 region but were not susceptible to pilocarpine induced seizures (Brenneke et al., 2004). Tenascin-R knockout mice exhibited decreased somatic GABAergic inhibition due to altered $GABA_B$-mediated transmission, as well as increased basal excitatory synaptic transmission and impaired NMDAR-mediated LTP (Saghatelyan et al., 2001; Brenneke et al., 2004; Bukalo et al., 2007). Of note, tenascins can bind to neuronal sodium channels and play an important role in regulation of sodium channel density at axon initial segments (AISs) and nodes of Ranvier (Srinivasan et al., 1998). This further emphasizes that components of ECM and PNNs may not solely build a scaffold for neurons, but play an important yet subtle role in regulation of neuronal excitability and E-S coupling.

Finally, proteins secreted in latent form and activated by proteolysis in the extracellular environment may add another level of complexity to the input-output functions of neurons. One prominent example involves BDNF. This protein is secreted in neuron activity-dependent manner (Hartmann et al., 2001; Gärtner and Staiger, 2002) and it's availability in extracellular space is regulated by proteolytic processing involving e.g., tPA-mediated activation of plasmin (Seidah et al., 1996) and MMP-9 (Mizoguchi et al., 2011). BDNF is transported anterogradely and retrogradely and can activate TrkB receptors both pre- and postsynaptically. Importantly, the association of BDNF with TrkB affects ion channels including voltage gated sodium (e.g., Nav1.9), potassium (e.g., Kv1.3, Kir3), calcium channels as well as NMDARs and AMPARs within a range of seconds to minutes through intracellular signaling cascades (Cunha et al., 2010). While the impact of BDNF and its receptor TrkB on synaptic plasticity has been extensively studied (for a review, see Cunha et al., 2010) it's role in neuronal excitability has received less attention. In one study BDNF was shown to acutely potentiate population spike more than would result from EPSP amplification alone (Messaoudi et al., 2002). Acute BDNF application resulted in significantly enhanced E-S coupling indicating that neuronal excitability is not linearly affected by EPSP boost (Messaoudi et al., 2002). Consequently, BDNF is localized and upregulated in areas implicated in epileptogenesis (reviewed in Binder et al., 2001). Altogether, indirectly, by regulating availability of BDNF and other secreted proteins, proteolytic activity in ECM may serve a potentially important role in shaping E-S potentiation, but verification of this possibility requires further studies.

Regulation of Neuronal Output by Axon Initial Segment

The AIS is a specialized unmyelinated part of the axon involved in the initiation of action potentials. This region is enriched in several types of voltage gated sodium (Na_V), potassium K^+ (K_V1), Ca^{2+} channels and specific molecular complexes such as adhesion proteins (CAMs), ECM and cytoskeleton adaptors (Clark et al., 2009). However, similar to dendrites, AIS does not seem to simply passively generate and convey action potentials. In a recent study using fluorescence recovery after photobleaching (FRAP), recovery of $K_V2.1$ channels cluster within AIS occurred rapidly with a time constant of approximately 11 s (Sarmiere et al., 2008). This suggests mobility of ion channels is not restricted by AIS. In addition, functional studies showed that variable position of AIS within the axon produces significant differences in neuronal excitability (Kress et al., 2010). Notably, $Na_V1.6$ channels distributed more proximally and exhibited lower overall density in dentate granule cells than CA3 pyramidal cells and that resulted in higher voltage threshold of dentate granule neurons (Kress et al., 2010). In addition, recent studies have showned a novel level of regulation of neuronal excitability in response to excitatory and inhibitory inputs which relies on changes in length and molecular composition of AIS (Grubb and Burrone, 2010; Kuba et al., 2010). Assembly of the AIS requires interactions between scaffolding molecules and voltage-gated sodium channels, but the molecular mechanisms that stabilize the AIS remain unclear. Members of chondroitin sulfate proteoglycans have been found to surround AIS such as aggrecan, brevican (John et al., 2006), neurocan, versican, tenascin-R (Bruckner et al., 2006). Consequently, recent studies have highlighted the role of the ECM degrading enzymes including matrix metalloproteinases, serine proteases (thrombin and the urokinase plasminogen activator system) and cysteine proteases in AIS stabilization. For instance, tenascin-C and -R were implicated in regulation of action potential initiation due to impact of this ECM component on density of sodium channels (Srinivasan et al., 1998). Tenascin-C additionally plays an important role in modulating L-type voltage gated calcium channels (Evers et al., 2002; Kochlamazashvili et al., 2010)

while tenascin-R integrity may be essential for proper function of perisomatic GABAergic inhibition. Notably, tenascin-R knockout mice showed reduction of such GABAergic inhibition provided to CA1 neurons by basket cells (Bukalo et al., 2007).

In mature nervous system, AIS is surrounded by a dense ECM of unique composition, including the chondroitin sulfate proteoglycan brevican which shows preferred expression within AIS (John et al., 2006). Brevican is degraded by matrix metalloproteinases and aggrecanase-1 (ADAMTS4; Nakamura et al., 2000) and it's interaction with CAMs such as neurofascin isoform NF-186 is essential for molecular assembly of the AIS (Hedstrom et al., 2007; Zonta et al., 2011). Similarly, since L1cam protein interacts with ankyrins which couple voltage-gated sodium channels to the spectrin-based membrane skeleton (Srinivasan et al., 1988), cleavage of L1cam by neuropsin following enhanced neuronal activity was proposed to result in dynamic rearrangement of clustered membrane proteins (Shiosaka, 2004) such as sodium channels. In summary, it seems plausible that rapid proteolytic processing of some constituents of ECM and cell adhesion molecules may affect stability of AIS. However, it is currently not clear what is the time scale of this process and functional studies addressing neuronal firing following modulation of AIS stability following proteolysis are lacking.

Discussion

Plasticity of local or global neuronal excitability can have profound consequences for neuronal input-output function and paradoxically also for synaptic plasticity for at least two reasons. First, changes in intrinsic properties of a part of a dendrite will directly affect local summation of EPSPs (reviewed in Beck and Yaari, 2008). Second, the amplitude of bAPs in dendrites is an important factor for LTP induction in hippocampal neurons and local synaptic LTP is associated with enhanced local membrane excitability which affects other synapses in the vicinity of the activated synapses (reviewed in Johnston et al., 2003). Therefore by affecting local dendritic Ca^{2+} spikes bAPs may additionally boost synaptic plasticity in respective parts of the dendrite (reviewed in Frick and Johnston, 2005). Finally, the Ca^{2+} influx that accompanies the bAPs or synaptic activity during the LTP induction protocols may also regulate the expression and function of dendritic ion channels. Ultimately, global changes in neuronal intrinsic excitability are expected to be critically important in entraining other neurons within hippocampal networks into a synchronized population discharge (Traub and Wong, 1982). However, the underlying mechanism is not fully understood.

We are just beginning to unravel the importance of intrinsic changes in physiology of the central nervous system accompanying neural plasticity, learning and memory. Importantly, changes in intrinsic neuronal properties are increasingly being recognized as an important, pathophysiologically relevant aspect of psychiatric and neurological disorders. In a recent review, intrinsic plasticity of nucleus accumbens medium spiny neurons excitability has been proposed to be an important player in development of addiction (Kourrich et al., 2015). Thus, drugs such as cocaine may affect several voltage gated conductances and the outcome of such modulation may also contribute to the shaping of the addiction phenotype (Kourrich et al., 2015). In addition, recent advances indicate that some disorders of the central nervous system, such as chronic pain and epilepsy, are associated with significant alterations of intrinsic properties of neurons (reviewed in Beck and Yaari, 2008). In particular, increased intrinsic dendritic excitability has been found to underlie disorders in mouse models of chronic epilepsy and Alzheimer disease (reviewed in Beck and Yaari, 2008). Cognitive decline in Alzheimer disease was also previously proposed to be a result of destabilization of Ca^{2+} homeostasis and modulation of intrinsic excitability (Disterhoft and Oh, 2006; Santos et al., 2010; Kaczorowski et al., 2011).

In this review, we highlighted several mechanisms by which proteases released into extracellular space following enhanced neuronal activity may rapidly participate in shaping neuronal input-output function in various neuronal compartments, not limited to synaptic loci (**Figure 1**). Studies discussed above and our most recent study addressing the role of MMPs in E-S potentiation (Wójtowicz and Mozrzymas, 2014) emphasize that extracellular proteolysis may be capable of fine-tuning information flow and storage by affecting targets beyond synapses. Thus, in our view, this opens an interesting field for future research, addressing the role of rapid and long-term extracellular proteolysis on important aspects of information processing in neurons, such as propagation of synaptic input to soma, initiation of action potential, spike frequency adaptation, properties of action potential and dendritic backpropagation. In particular, we are lacking functional studies addressing activity-dependent proteolytic modulation of dendritic or somatic channel function.

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REVIEW
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Extracellular proteolysis in structural and functional plasticity of mossy fiber synapses in hippocampus

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Brain is continuously altered in response to experience and environmental changes. One of the underlying mechanisms is synaptic plasticity, which is manifested by modification of synapse structure and function. It is becoming clear that regulated extracellular proteolysis plays a pivotal role in the structural and functional remodeling of synapses during brain development, learning and memory formation. Clearly, plasticity mechanisms may substantially differ between projections. Mossy fiber synapses onto CA3 pyramidal cells display several unique functional features, including pronounced short-term facilitation, a presynaptically expressed long-term potentiation (LTP) that is independent of NMDAR activation, and NMDA-dependent metaplasticity. Moreover, structural plasticity at mossy fiber synapses ranges from the reorganization of projection topology after hippocampus-dependent learning, through intrinsically different dynamic properties of synaptic boutons to pre- and postsynaptic structural changes accompanying LTP induction. Although concomitant functional and structural plasticity in this pathway strongly suggests a role of extracellular proteolysis, its impact only starts to be investigated in this projection. In the present report, we review the role of extracellular proteolysis in various aspects of synaptic plasticity in hippocampal mossy fiber synapses. A growing body of evidence demonstrates that among perisynaptic proteases, tissue plasminogen activator (tPA)/plasmin system, β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) and metalloproteinases play a crucial role in shaping plastic changes in this projection. We discuss recent advances and emerging hypotheses on the roles of proteases in mechanisms underlying mossy fiber target specific synaptic plasticity and memory formation.

Keywords: MMP, tPA, mossy fiber, synaptic plasticity, LTP, CA3, ADAM, BACE1

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INTRODUCTION: EXTRACELLULAR PROTEOLYSIS IN NEURONAL PLASTICITY

Activity-dependent plasticity of synaptic transmission plays a crucial role in memory encoding and in the development of the nervous system. Functional plasticity is typically accompanied by structural alterations of synapses and therefore, their morphological changes are considered a manifestation of memory trace formation (Caroni et al., 2012). Moreover, most manipulations that block structural plasticity also impair long-term plasticity and memory. Over the last decades, accumulating evidence has revealed that both neuronal function and morphology can be potently

regulated by extracellular matrix (ECM) which itself shows substantial plasticity (Mukhina et al., 2012; Levy et al., 2014). Proteolytic cleavage of ECM proteins provides thus potent mechanisms to modulate the network activity and to refine neuronal circuits (Lee et al., 2008; Wlodarczyk et al., 2011). Indeed, spatially and temporally limited proteolysis of perisynaptic ECM components and membrane adhesion proteins is crucial for memory related reorganization of synaptic compartments and adjustment of signaling pathways occurring upon induction of plastic changes (Sonderegger and Matsumoto-Miyai, 2014).

The emerging pivotal role of extracellular proteolysis in neuroplasticity phenomena and cognitive tasks has provided a strong drive towards investigations into the underlying subcellular, structural and molecular mechanisms. A variety of proteases and their substrates have been found in the extracellular space in different brain regions such as cerebellum, hippocampus, cortex and amygdala. Moreover, for some proteases and respective substrates, clear correlations between their activity and experience-induced plasticity or cognitive processes have been observed (Bajor and Kaczmarek, 2013; Sonderegger and Matsumoto-Miyai, 2014). Importantly, the use of pharmacological tools as well as of transgenic animals clearly indicated that plasticity phenomena, in various neuronal pathways, may critically depend on the activity of proteases (Hirata et al., 2001; Nagy et al., 2006; Li et al., 2010b; Almonte et al., 2013). However, the major difficulty in deciphering the role of any molecular player in the plasticity phenomena is that underlying molecular signaling typically shows a high degree of pathway to pathway variability even within the same area. For instance, as it will be discussed below in detail, classic CA3-CA1 and mossy fiber–CA3 hippocampal projections show profoundly different long-term potentiation (LTP) mechanisms but in both cases LTP maintenance strongly depends on metalloproteinases (Meighan et al., 2007; Wiera et al., 2013). This points to, on one hand, a widespread presence and universal role of proteases in the neuronal plasticity but, on the other, it should be expected that the mechanisms of their involvement in shaping the plasticity are diversified among different pathways. Moreover, a closer look at the local neuronal circuits revealed that even the synapses originating from the same presynaptic axon may deeply differ in plasticity mechanisms depending on e.g., identity of postsynaptic neurons (Toth et al., 2000; McBain, 2008). The multiplicity of proteases, complexity of neuronal pathways and diversity of synaptic connections pose the major challenge in studies into the roles of proteases in the neuroplasticity phenomena and for this reason the underlying mechanisms remain poorly understood. It seems thus that the optimal strategy to study the role of proteases in the neuronal plasticity is to focus on a specific pathway and to try to correlate its physiological properties with the impact of up- and down-stream molecular players. Mossy fiber-CA3 projection in the hippocampus has been implicated in important cognitive functions including novelty detection, pattern completion and partially in pattern separation (Yassa and Stark, 2011; Kesner, 2013) and is known to be characterized by unique structural and functional plasticity (Evstratova and Tóth, 2014). In particular, extensive activity-dependent structural alterations at the level of projection topography and microstructure of different MF synapses make this pathway particularly interesting in the context of the extracellular proteolysis. Herein, we aim at providing a concise overview on the roles of extracellular proteases in functional and structural plasticity at the hippocampal mossy fiber synapses. To this end, we first present the most important features of this pathway, including its morphology and function with a particular emphasis on functional and structural plasticity, and, on this background, we review the role of the extracellular proteolysis in this projection. In particular, we discuss the impact of metalloproteinases (MMP) from metzincin family, tissue plasminogen activator (tPA), plasmin and β-site amyloid precursor protein-cleaving enzyme 1 (BACE1) and describe some recognized and putative substrates of plasticity-related extracellular proteases, which are abundantly expressed in mossy fiber terminal field. Although this review will be focused on the MF-CA3 pathway, for the sake of comparison, results obtained on the role of proteases in other, so far more extensively studied pathways (such as e.g., CA3-CA1) will be also mentioned.

THE MOSSY FIBER PROJECTION IN HIPPOCMAPUS

The mossy fiber input to CA3 region is a part of the classical trisynaptic hippocampal circuit: from the entorhinal cortex (EC) to the dentate gyrus (DG) from which granule cells project their axons (mossy fibers) to CA3 which sends Schaffer collaterals to CA1 which, in turn, sends the projection to EC through the subiculum. Dentate granule cells receive a strong polymodal input, which conveys densely coded information from the EC and participate in its conversion into relatively sparse and highly specific code transferred into the CA3 region where it undergoes associations (Witter, 2007; Kesner, 2013; Evstratova and Tóth, 2014). To perform the function of an efficient high-pass filter and, at the same time to encode a faithful information, mossy fibers developed unique anatomical and functional features. The term "mossy fibers" was coined by Ramon y Cajal who observed numerous appendages (boutons) on MF axons, which gave these fibers appearance of being covered by moss (Ramon y Cajal, 1911). Unmyelinated mossy fibers travel through the hilus where they strongly branch out and enter hippocampal CA3 region where, as a dense axonal bundle, extend parallelly to the pyramidal cell layer in stratum lucidum (Witter and Amaral, 2004; **Figure 1**).

The most peculiar feature of MFs is that each MF axon forms three types of glutamatergic presynaptic terminals with characteristic morphology and dissimilar functions (**Figure 1**). Giant mossy fiber boutons (MFBs, 3–10 μm in diameter), the hallmark of MF projection, form synaptic contacts on hilar mossy cells (7–12 per one axon) and on proximal dendrites of the CA3 pyramidal cells (MF-PC projection, 11–15 boutons per one axon; Acsády et al., 1998). Moreover, mossy fibers form also elongated filopodia protruding from MFBs

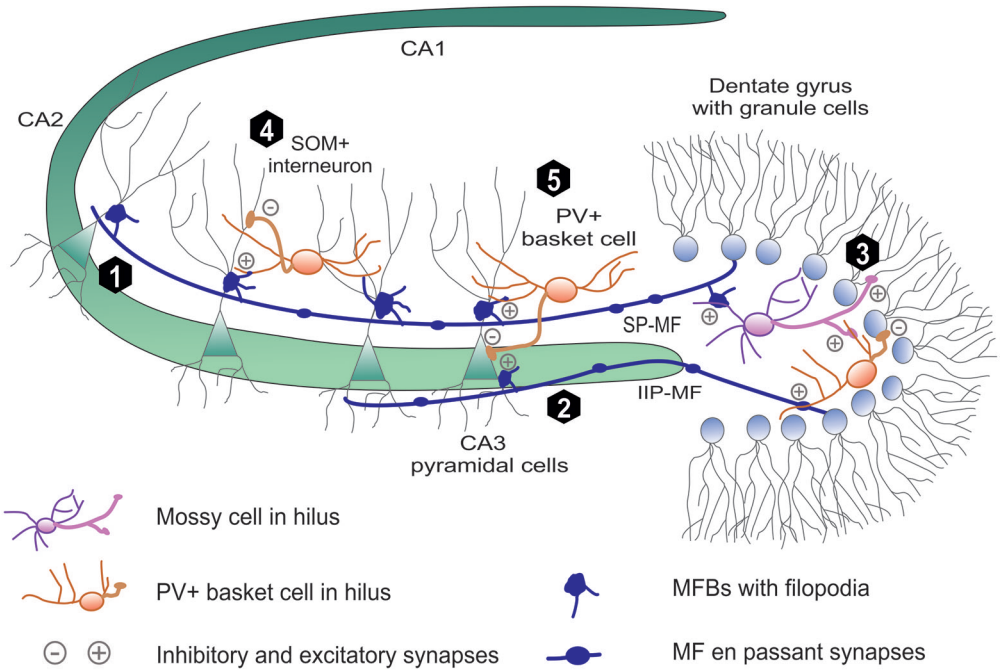

FIGURE 1 | Mossy fiber projection in hippocmapus. The mossy fiber projection consists of axons of the dentate gyrus (DG) granule cells. Mossy fiber axons project through (1) suprapyramidal bundle (SP-MF) in stratum lucidum as well as (2) intra- and infrapyramidal projection (IIP-MF) located mainly in stratum oriens. In CA3 and hilus MFs form three types of synapses: on pyramidal cells via mossy fiber butones and on local internurons via filopodia and en passant synapses. In hillus (3) MF axons synapse on excitatory mossy cells, which backpropagate excitation to granule cells. Additionally mossy fibers directly or via mossy cells excite local hilar parvalbumin-positive internurons (basket cells) that provide feedback inhibiton to granule cells mainly in distinct lamella across the septohippocampal axis (not shown). Mossy fibers in CA3 excite local interneurons that are responsible for feedforward inhibition of CA3 pyramidal cells. These local interneuurons (e.g., PV-positive basket cells) synapse on (5) PC soma or (4) PC denditic tree (e.g., somatostatin-positive interneurons).

and en-passant terminals both innervating local inhibitory interneurons (MF-INT, approximately 150 in hilus and 50 in CA3 region). MF passing through the hilus excite hilar mossy cells that, in turn, provide granule cells with a direct feedback excitation (Jinde et al., 2013). Additionally, mossy fibers directly or via mossy cells excite local hilar GABA-ergic interneurons (e.g., PV+ basket cells) that ensure strong feedback inhibition of granule cells (Jinde et al., 2012). In the CA3 area, mossy fibers run in two main bundles (**Figure 1**), the main suprapyramidal projection in stratum lucidum and the intra- and infrapyramidal projection (IIP) that runs first within the proximal extent of stratum pyramidale and startum oriens, to cross over to stratum lucidum in the CA3a region (Schwegler and Crusio, 1995). As it will be described in more detail in next sections, IIP axons in CA3 show high degree of plasticity which correlates with various forms of hippocampus-dependent learning. CA3 pyramidal neurons make synaptic contacts with MFBs via specialized complex of clustered spines called thorny excrescences (Gonzales et al., 2001). In rat brain, on average, each of 300,000 CA3 pyramidal cells is contacted by ∼50 different MFBs originating from distinct mossy fiber axons (there are ca. 10^6 granule cells in the rat DG), which indicates a convergence of neuronal information (Henze et al., 2000).

Large MF boutons in stratum lucidum are characterized by several unique morphological properties which underlie their functioning, including the ability to undergo the plastic changes. Adult mossy fibers, together with glutamate, release numerous co-transmitters like adenosine (Kukley et al., 2005), Zn^{2+} ions (Pan et al., 2011) and neuropeptides (Jaffe and Gutiérrez, 2007). The single giant MFB contains a large number of synaptic vesicles (up to 25000) with 1400–5700 vesicles as a readily releasable pool (Hallermann et al., 2003; Rollenhagen et al., 2007). Typical MFB contains from 18 to 45 separate but closely spaced active zones (Rollenhagen and Lübke, 2010; Wilke et al., 2013). Due to a small distance between individual release sites within a single MFB, the impacts of glutamate released from neighboring active zones may sum up and a crosstalk between calcium signals may take place presynaptically. Thus, even if single MF-PC active zone is characterized by a low release probability (0.01–0.05; Jonas et al., 1993), the unitary excitatory postsynaptic potentials may show a high amplitude (∼10 mV) due to the numerous active zones in single MFB (Bischofberger et al., 2006; Evstratova and Tóth, 2014). Finally, close distance of the MFBs to the soma of the CA3 pyramidal cells assures minimal electrotonic dissipation of this input. Collectively, anatomical and functional studies demonstrate that MF-CA3 projection mediates a powerful monosynaptic excitatory input onto CA3 pyramidal neurons through MFBs and a disynaptic feedforward inhibition through MF-INT synapses (**Figure 1**). Importantly, the latter one fine-tunes recruitment of small neuronal memory-related ensembles in the CA3 field during learning (Ruediger et al., 2011).

FUNCTIONAL PLASTICITY HIPPOCAMPAL MOSSY FIBER SYNAPSES

One of the most characteristic features of the MF-PC synapses in CA3 region is strong short-term plasticity (STP). Increase in the frequency of action potentials leads to up 10-fold increase in the postsynaptic EPSP amplitude. It has been reported that intra-bouton accumulation of residual calcium during stimulation within a short time window transiently increase release probability in MF-PC synapses and drives frequency facilitation (Regehr et al., 1994; Evstratova and Tóth, 2014). As a result, even a single MF, when excited with a high frequency, is capable to trigger an action potential in CA3 pyramidal cells *in vivo* (Henze et al., 2002). In contrast, high-frequency stimulation at the CA3 MF-INT synapse leads either to relatively small frequency facilitation or even to a short-term depression (Toth et al., 2000). Since a single MF axon in CA3 forms more than ten times more synapses onto interneurons than on PC, during the low-frequency transmission, potent feedforward inhibition blocks further signal relay to CA3. Conversely, strong frequency facilitation at MF-PC synapses counterbalances powerful feedforward inhibition and efficiently activates the CA3 pyramidal cells in case of MF high frequency bursts of activity (Urban et al., 2001; Lawrence and McBain, 2003). Therefore, due to such a specific balance between strong frequency facilitation in MF-PC synapses and feedforward inhibition, MF-CA3 projection is often referred to as a conditional detonator (Urban et al., 2001). Recently, strong evidence was reported, that loose coupling of Ca^{2+} channels to Ca^{2+} sensors in MFBs together with endogenous calcium buffers with limited capacity underlie conditional detonator function of MF-PC synapses (Vyleta and Jonas, 2014). These properties enable MF-CA3 pathway to constantly change its input-output relationship as a function of granule cell spiking frequency.

The overall mean firing rate of granule cells is low, although, during hippocampus-dependent learning they may discharge high-frequency spike packages (Mistry et al., 2011) and it was found that high-frequency stimulation of MF projection induces LTP at MF-PC synapses both *in vivo* and *in vitro* (Zalutsky and Nicoll, 1990; Gundlfinger et al., 2010). It is generally accepted that the induction of LTP at MF-PC synapses is independent of NMDA receptors and expressed presynaptically as increased probability of neurotransmitter release (Tong et al., 1996; Reid et al., 2004; Nicoll and Schmitz, 2005) although NMDAR-dependent, postsynaptically expressed LTP in this pathway was also reported (Kwon and Castillo, 2008; Rebola et al., 2008). The mechanism of LTP induction at MFB requires local increase in Ca^{2+} concentration, activation of calcium/calmodulin-sensitive adenyl cyclase, protein kinase A (PKA) and protein kinase C (PKC) and subsequent phosphorylation of proteins associated with the machinery of neurotransmitter release. Four proteins were found to be essential for MF-PC LTP: small GTPase Rab3A and synaptotagmin-12, both located on synaptic vesicles and RIM1α with Munc13–1 both present in the active zone (Castillo et al., 1997, 2002; Yang and Calakos, 2011; Kaeser-Woo et al., 2013). It appears that cAMP-dependent phosphorylation of synaptotagmin-12 and interaction between RIM1α, Munc13-1 and Rab3A is required for MF-PC LTP (Kaeser et al., 2008). Additionally, presynaptic activation of the ERK and PKC signaling pathways plays a role in the activity-dependent modulation of MF synaptic vesicle mobilization and neurotransmitter release (Son et al., 1996; Vara et al., 2009). Interestingly, MF–mossy cell synapses exhibit both long- and STP that are similar to those described at MF-PC synapses (Lysetskiy et al., 2005).

Although the major expression mechanism of LTP in MF-PC synapses is presynaptic, some reports suggest involvement of the postsynaptic compartment. In this context, attention is drawn to the following factors: (1) feedback retrograde signaling from postsynaptic adhesion receptor EphB2 to presynaptic ephrin-B (Contractor et al., 2002; Armstrong et al., 2006); (2) tuning of presynaptic calcium influx and plasticity by arachidonic acid released in activity-dependent manner from postsynaptic cell membrane, which modulates voltage-gated potassium channels in MFB leading to axon potential broadening (Geiger and Jonas, 2000; Carta et al., 2014); and (3) zinc ions released as a co-neurotransmitter with glutamate from MFB which activate postsynaptic TrkB receptor in a Src kinase-dependent manner (Huang et al., 2008). Additionally, synaptic zinc also inhibits expression of postsynaptic LTP in MF-PC synapses (Pan et al., 2011).

While MF-PC synapses express NMDAR-independent LTP and LTD, ultrastructural studies have shown that NMDARs are nonetheless present at these synapses (Berg et al., 2013) and can mediate substantial postsynaptic current (Jonas et al., 1993; Kwon and Castillo, 2008). Interestingly, co-activation of synaptic NMDAR, mGluR5 and adenosine A_{2A} receptors, together with a rise in postsynaptic calcium, Src and PKC activity lead to selective enhancement of NMDAR-mediated transmission in MF-PC projection (Kwon and Castillo, 2008; Rebola et al., 2008). Moreover, this LTP of NMDARs currents acts as a metaplastic switch allowing MF-PC synapses to express classical postsynaptic NMDAR-dependent LTP of AMPA-ergic transmission (Rebola et al., 2011). Additionally, recently, it was shown that NMDAR-mediated transmission in MF-PC plays a role in heterosynaptic metaplasticity that affects plasticity in upstream CA3-CA3 synapses (Hunt et al., 2013).

I addition to the fact that mossy fibers form different synapses on various cell types, it has been demonstrated that the direction of long-term synaptic plasticity and the loci of its expression also depend on the type of postsynaptic neurons. Contrary to MF-PC, at MF-INT synapses, high-frequency stimulation leads to two types of long-term depression (Maccaferri et al., 1998). Synapses expressing AMPA receptors impermeable to Ca^{2+} ions exhibit postsynaptic and NMDAR-dependent LTD (Lei and McBain, 2002, 2004). In contrast, MF-INT synapses containing Ca^{2+} permeable AMPA receptors show presynaptic LTD in the form of reduced neurotransmitter release probability (Lei and McBain, 2004). Induction of this type of LTD requires activation of postsynaptic AMPA and presynaptic mGluR7 receptors, generation of retrograde messenger, that, in turn, causes a reduction of Ca^{2+}

influx into the presynaptic filopodium (Pelkey et al., 2005, 2008).

Homeostatic plasticity (in contrast to associative, input specific and Hebbian long-term plasticity) involves cell-wide adjustments in synaptic strength. Recently, Lee et al. (2013) have shown that hippocampal MF-CA3 projection in adult animals reveals a substantial homeostatic plasticity. Indeed, MF-PC synapses undergo selective, independent and bidirectional homeostatic alterations in response to chronic changes in network activity. Homeostatic scaling in MF-PC synapses can act as a superior gain control mechanism, that modules overall network activity, leaving information encoded in remaining hippocampal synapses intact (Chater and Goda, 2013; Lee et al., 2013).

STRUCTURAL PLASTICITY OF HIPPOCAMPAL MOSSY FIBER PROJECTION AND SYNAPSES

Over the last decade, compelling evidence has indicated that hippocampal mossy fiber projection both in juvenile and adult rodents shows substantial structural plasticity at the broad range of anatomical scales—from modification of a gross axonal MF topography through changes in synapse number to remodeling of MFB ultrastructure. Using Timm's staining method, it has been demonstrated that learning can induce a remodeling of the mossy fiber circuitry. For example, learning in Morris water maze resulted in increased spatial expansions mossy fiber axons in the CA3 stratum oriens (Ramirez-Amaya et al., 1999; Holahan et al., 2006; McGonigal et al., 2012). Extensive training in this paradigm led to increase length of infrapyramidal bundle of mossy fibers which persisted for at least 30 days (Ramirez-Amaya et al., 1999). Additionally, in rats, the extent of mossy fiber IIP projection (**Figure 1**) correlates with acquisition of spatial memory in water maze or radial maze (Lipp et al., 1988; Schwegler and Crusio, 1995; Pleskacheva et al., 2000). Consistent with these data, induction of LTP in DG *in vivo* enlarged IIF-MF projection (Adams et al., 1997). Interestingly, MF synapses on the basal dendrites in CA3 pyramidal cell are located even closer to the soma and axon-initial segment than MFBs in the stratum lucidum (Gonzales et al., 2001). This places IIP-MF projection in a strategic position to influence action potential generation in CA3 pyramidal cells (Carnevale et al., 1997).

It is particularly evident that structural plasticity of the mossy fibers is expressed also at the synaptic level (**Figure 2**). For example, long-term stress can lead to a reduction in the density of mossy fiber synapses and to a deterioration in spatial learning performance (Sandi et al., 2003). Importantly, these impairments can be reversed through extended training for spatial tasks (Sandi et al., 2003; Scharfman and MacLusky, 2014). Conversely, enriched environment increases the number of synapses in the stratum lucidum (Tashiro et al., 2003). Furthermore, the individual MFBs differ from each other in degree of mobility and dynamics of plasticity-related structural changes. Different MFBs on the same axon show various extent of intrinsic structural plasticity, usually having one hyperplastic MFB (De Paola et al., 2003). High-frequency stimulation transforms a fraction of stable mossy fiber terminals into highly motile ones in a process involving AMPA receptors, BDNF signaling, activation of PKA and *de novo* protein synthesis (De Paola et al., 2003). In addition to diverse intrinsic motility, there is also evidence for age-related changes in MFB structural plasticity. While most hippocampal mossy fiber terminals shrink and their structural plasticity decreases with age, usually one MFB per axon gradually increases in size, suggesting the existence of "plastic hot spots" in single MF (Galimberti et al., 2006). Additionally, acquisition of a large amount of spatial information by mice housed in an enriched environment leads to increase in MFB complexity to such extent that some of them extend process with a new satellite MFB that synapse on the same or a different pyramidal cell (Galimberti et al., 2006; Gogolla et al., 2009b; **Figure 2C**). Moreover, the fraction of MFBs containing satellites continuously increases with mice age. It has been also reported that in animals housed in enriched environment an expansion of MFBs along apical dendrite of pyramidal cells takes place in a process that is dependent on MF-PC LTP (Gogolla et al., 2009b). Age and experience-related MFBs growth and its maintenance depends on the MF spiking activity, GABA-ergic transmission and glutamate release from MF synapses but is independent on NMDA receptors (Galimberti et al., 2006).

Recently, it was shown that also filopodia extending from MFB (contacting local interneurons) are characterized by high intrinsic mobility and prominent structural plasticity (**Figure 2D**). Learning was shown to be correlated with upregulation of feedforward inhibition connectivity in stratum lucidum, resulting in roughly doubling the excitatory synapses on PV-positive inhibitory interneurons (Ruediger et al., 2011). This process was implicated in increased precision of memory encoding in the hippocampus (Ruediger et al., 2012).

LTP induction in the MF-PC projection alters also the ultrastructure of pre- and postsynaptic compartment. For example, chemical LTP (induced using PKA activator—forskolin) causes an increase in the presynaptic MFB membrane surface and in the number of active zones on a single MFB (**Figure 2B**). MF-PC LTP was also shown to affect postsynaptic structure by increasing the number of protrusions from thorny excrescences, penetrating into the MFB (Zhao et al., 2012). These changes were found to be dependent on actin polymerization and cofilin phosphorylation (Frotscher et al., 2014).

Taken together, above described findings from distinct species and using a variety of experimental approaches clearly indicate that the local reorganization of neuronal MF circuits, synaptogenesis and modification of pre- and postsynaptic structures of MF-CA3 synapses accompany hippocampus-dependent learning and memory formation (Ruediger et al., 2012). However, the mechanisms underlying this extraordinary richness of plastic changes only start to be revealed. It needs to be reiterated that a spectacular structural plasticity of the MF-CA3 projection and synaptic ultrastructure strongly suggests involvement of extracellular proteolysis and this issue will be discussed in detail below.

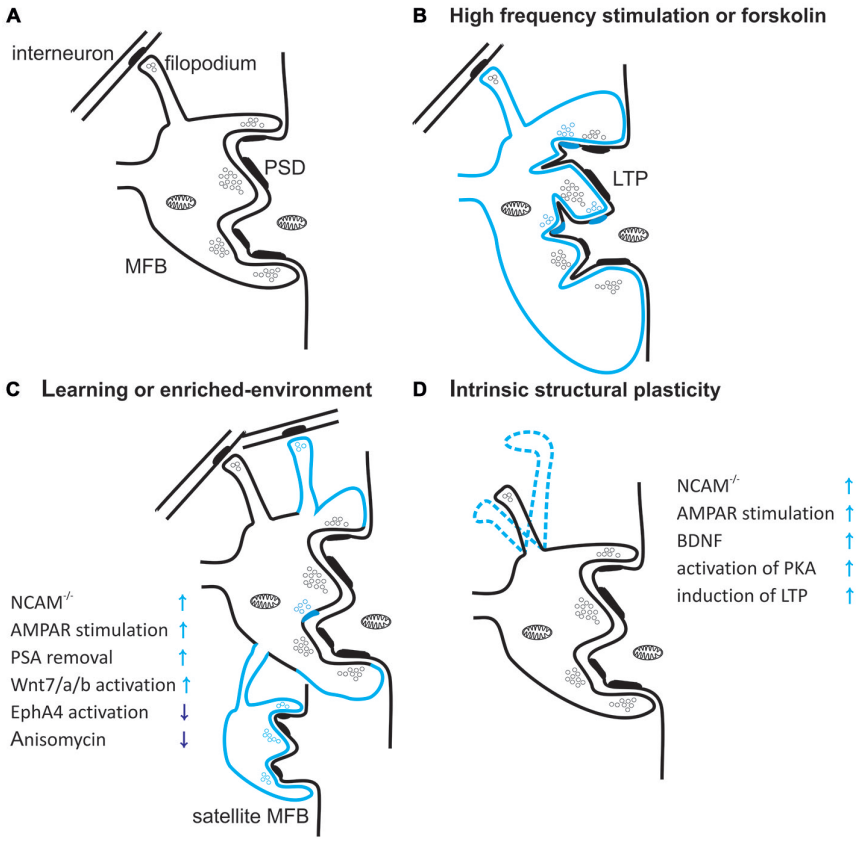

FIGURE 2 | Structural plasticity of mossy fiber presynaptic terminals. (A) Schematic diagram showing mossy fiber bouton (MFB) contacting thorny excrescence of CA3 pyramidal cell. Thick postsynaptic lines reflect postsynaptic densities. Note that filopodial extension of mossy terminal is specialized to innervate GABAergic interneurons. **(B)** Induction of long-term potentiation (LTP) in MF-PC synapses with high-frequency stimulation or forskolin leads to increase in MFB volume and in the number of release sites per MFB. Blue color reflects structural plastic changes. Additionally, LTP promotes development of small protrusions extending from the large complex spines into the presynaptic bouton. **(C)** Spatial learning or enriched-environment promotes an expansion of mossy fibers terminals in the stratum lucidum. Furthermore, housing mice in an enriched environment leads to an increased local complexity of MFBs with frequent appearance of satellite boutons with functional release sites. Additionally hippocampus-dependent learning increases the number of filopodia protruding from MFBs and contacting feedforward projecting PV+ interneurons. **(D)** MFB filopodia are characterized by a dynamic intrinsic structural plasticity in the adult hippocampus *ex vivo*. Filopodia undergo spatial rearrangement in shape in mature hippocampal slice cultures. Plasticity processes that increase MF transmission affect also filopodia motility.

THE ROLE OF EXTRACELLULAR PROTEOLYSIS IN STRUCTURAL AND FUNCTIONAL PLASTICITY IN MOSSY FIBER—CA3 PATHWAY

In general, extracellular proteolysis may produce a variety of molecular factors involved in synaptic plasticity by means of several processes including: (1) cleavage of structural ECM constituents and membrane adhesion proteins; (2) processing and direct activation of receptors, ligands and enzymes; (3) release of ectodomains and exposure of cryptic epitopes with signaling properties; and (4) inactivation or degradation of signaling proteins or enzymes. Regulated proteolysis in the extracellular space causes changes in perisynaptic proteome and local loosening of ECM structure which may offer a permissive environment for structural plasticity (Tsien, 2013). In addition, proteolytic activation of receptors, release of its ligands or exposure of the protein-protein interaction epitopes can activate intracellular signal transduction pathways that modulate the function of synaptic receptors and expression of the plasticity-related genes in the nucleus (Levy et al., 2014). Thus proteolytic activity appears to be an ideal candidate for linking structural and functional forms of plasticity. Indeed, secretion of proteases typically depends on neuronal activity and proteolysis mediated by active enzymes affects the extracellular milieu which, in turn, triggers modulatory processes participating in the plasticity phenomena. Interestingly, in all tested hippocampal synapses, there is at least one extracellular protease that regulates their physiology.

All mammalian proteases, depending on the catalytic mechanism of peptide bond hydrolysis, are classified into five distinct classes: aspartic, threonine, cysteine, serine and metallo-proteases (listed from the smallest to the largest class in human genome; Puente et al., 2003). Here we focus on membrane-bound

or soluble extracellular proteases present in mossy fiber synaptic and perisynaptic space that affect neuronal plasticity.

Metzincins

Among extracellular proteases the most abundantly expressed in the brain are members of the metzincin family of metalloproteinases (Rivera et al., 2010). The name reflects the presence of the conserved methionine residue and catalytic zinc ion in the active site of the enzyme. Metzincin family consists of matrix metalloproteinases (MMPs), a disintegrin and metalloproteinases (ADAMs) and a disintegrin and metalloproteinases with thrombospondin motif (ADAMTSs). Over the last years, growing evidence has revealed that at least three metzincins: MMP-9, ADAM-10 and ADAM-17 regulate synaptic plasticity, learning and memory.

The MMP family includes more than 20 different genes. Depending on homology and substrate specificity, MMPs may be grouped into collagenases (MMP-1, -8, -13), stromelysins (MMP-3, -10), matrilysins (MMP-7 and only-human MMP-26), gelatinases (MMP-2, -9), transmembrane (MMP-14, -15, -16, -23, -24) or GPI-anchored (MMP-17, -25). MMPs have two major characteristics. The first is the presence of conserved cysteine residue in N-terminal propeptide sequence that maintains the enzyme in the form of an inactive zymogen (pro-MMP). The second is the HEXGHXXGXXH sequence in catalytic domain that binds zinc ion (Sekton, 2010). Activation of most MMP proteases depends on destabilization of interaction between Cys residue in prodomain with catalytic zinc. This cysteine switch occurs due to proteolytic cleavage of the prodomain that may take place extracellularly (most of MMPs) or in the lumen of endoplasmic reticulum (Furin-activated: MMP-11, -21, -28 and all transmembrane MMPs). In physiological conditions MMPs may also undergo activation through chemical modification of aforementioned cysteine in the prodomain by S-nitrosylation (e.g., by synaptically released NO) or S-glutathionylation (Vandooren et al., 2013).

MMP-9

Over the last decade, compelling evidence has documented a particularly strong involvement of MMP-9 in neuronal physiology, learning and memory (Dziembowska and Wlodarczyk, 2012; Huntley, 2012; Stawarski et al., 2014). Indeed, MMP-9 regulates functions related to CNS development and neurogenesis (Vaillant et al., 2003; Wojcik-Stanaszek et al., 2011b), dendritic spine morphology (Michaluk et al., 2011; Sidhu et al., 2014), synaptic plasticity (Nagy et al., 2006; Wiera et al., 2013; Wojtowicz and Mozrzymas, 2014), neuronal excitability (Wójtowicz et al., 2015) and learning (Kaliszewska et al., 2012; Peixoto et al., 2012; Knapska et al., 2013; Smith et al., 2014).

Recent work showed, that presynaptic NMDA-independent LTP in the MF-CA3 projection requires MMP activity, as in the presence of a broad-spectrum MMP inhibitor (FN-439) LTP is strongly impaired (Wojtowicz and Mozrzymas, 2010). However, this inhibitor was effective only during the first 45 min after LTP induction, indicating that MMPs are critical for LTP only within a limited time window. Similar results were obtained in the CA3-CA1 pathway, where LTP is induced and expressed postsynaptically (Meighan et al., 2007). These results point to a universal role of metalloproteinases in synaptic plasticity as MMPs involvement in LTP was observed in synapses in which plasticity profoundly differs in the loci of induction/expression and NMDAR-dependence. The identity of MMP protease engaged in MF-CA3 LTP has been examined in our laboratory and it was found that in slices from MMP-9 knockout mice, LTP is impaired (Wiera et al., 2013) to a similar extent as in the presence of a broad-spectrum MMP inhibitor or a specific inhibitor of gelatinases (Wojtowicz and Mozrzymas, 2014). Interestingly, both knockdown and overexpression of MMP-9 strongly attenuated LTP maintenance showing that a fine-tuned level of MMP-9 activity is necessary for efficient LTP maintenance in the MF-CA3 pathway (Wiera et al., 2013). Importantly, treatment with exogenous active MMP-9 resulted in a stable presynaptic potentiation of MF-CA3 transmission. Additionally, in MMP-9 knockout mice, exogenous MMP-9 restored the LTP maintenance comparable to that observed in controls (Wiera et al., 2013). Thus fine-tuned MMP-9 activity appears to play a permissive role in consolidation of MF-CA3 LTP. Furthermore, LTP induction in the MF-CA3 projection is associated with increased MMP-9 (but not MMP-2) expression and activity in CA3 stratum lucidum and stratum radiatum (Wiera et al., 2012). This finding appears consistent with observation that MMP-9 mRNA is transported along microtubules to dendritic spines (Sbai et al., 2008; Dziembowska et al., 2012). Janusz et al. (2013) have shown in neuronal cultures that dendritic MMP-9 mRNA is inactive due to an association with FMRP protein and is directed to translation after synaptic activity-dependent polyadenylation (Janusz et al., 2013). MMP-9 is secreted outside the cell, as an inactive proenzyme and is subsequently activated (Murphy and Nagase, 2011; **Figure 3**). Thus the question arises, how MMP-9 is activated in the extracellular space of the stratum lucidum? A good candidate for this purpose could be synaptically released nitric oxide (Gu et al., 2002; O'Sullivan et al., 2014). However, block or knockdown of nitric oxide synthase does not affect the magnitude of LTP in MF-CA3 projection (Doreulee et al., 2001) arguing against this possibility. Several other mechanisms await verification. In particular, tPA/plasmin system is highly expressed in the mossy fibers and may activate MMP-9 (Tai et al., 2010; Hwang et al., 2011). Similar to MMP-9, its endogenous inhibitor TIMP-1 is also upregulated in stratum lucidum by neuronal activity (Newton et al., 2003). This raises the possibility that extracellular MMP-9 activation and its subsequent inhibition by TIMP-1 defines a specific time window for the proteolytic activity that allows for long-term plastic changes in MF synapses. These functional and pharmacological studies were paralleled by behavioral investigations which indicated that novel-object recognition, a task dependent on MF-CA3 synaptic transmission and plasticity, (Kesner, 2007; Bednarek and Caroni, 2011; Hagena and Manahan-Vaughan, 2011) is impaired in MMP-9 knockout mice (Mizoguchi et al., 2010).

MMP-9 was also found to play an important role in the structural aspect of the synaptic plasticity. Indeed, several

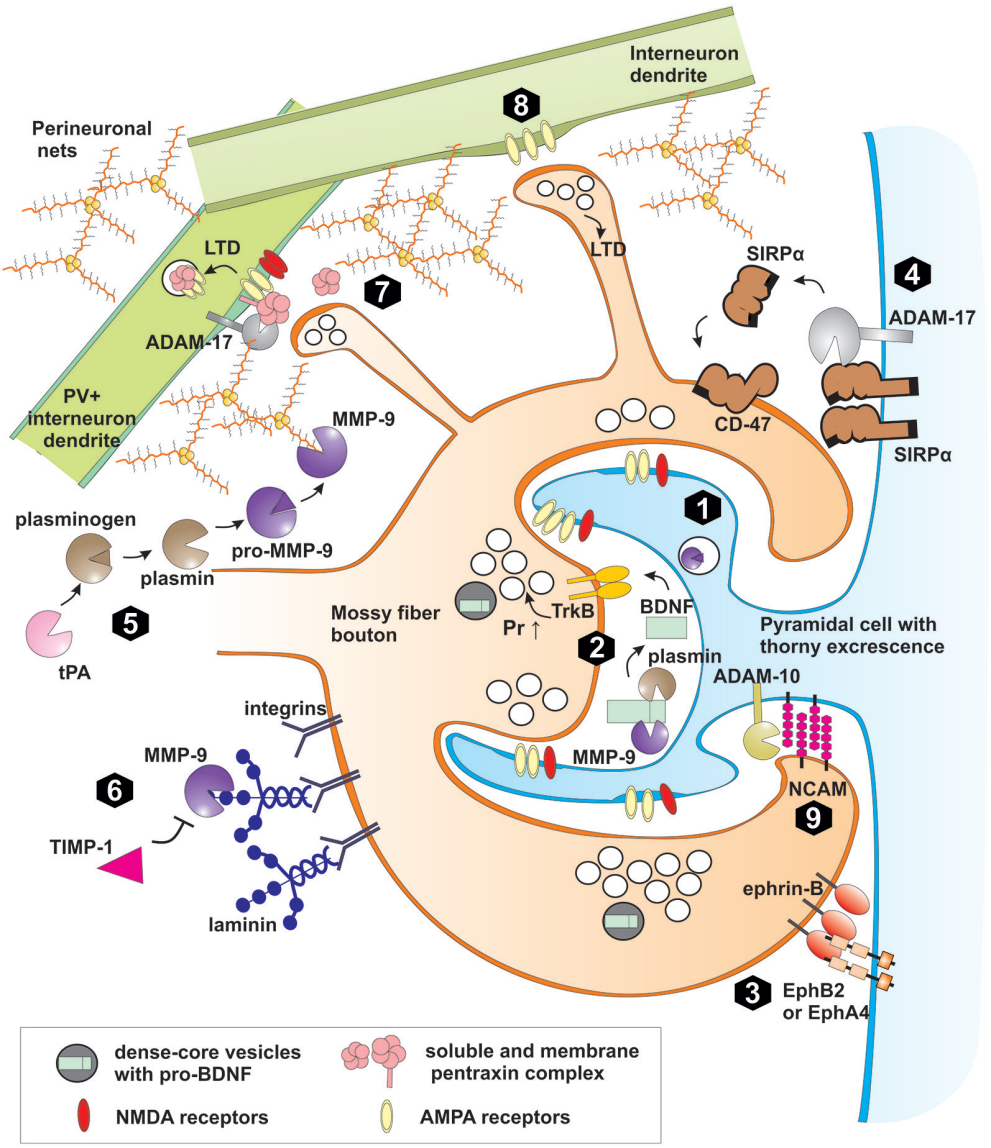

FIGURE 3 | Extracellular proteolysis in plasticity of mossy fiber synapses. (1) High frequency stimulation leads to synthesis, release of matrix metalloproteinase (MMP)-9 and tissue plasminogen activator (tPA) to extracellular space and LTP induction in MF-CA3 synapse through increased probability of neurotransmitter release (Pr ↑). (2) MMP-9 or plasmin both activated by tPA cleaves pro-BDNF to mature form. BDNF activates TrkB receptor in the pre and postsynaptic membranes. TrkB activation affects the machinery of neurotransmitter release. (3) Trans-synaptic Eph receptor-ephrin B signaling is crucial for induction of hippocampal mossy fiber LTP. MMPs as well as neuropsin may cleave postsynaptic EphB receptors or presynaptic ephrin ligands. (4) Activity-dependent cleavage by MMPs and ADAM-17 of postsynaptic Signal regulatory protein α (SIRPα) followed by shedding of the ectodomain. The released extracellular domain of SIRPα binds to presynaptic CD47 receptor and promotes the maturation of the presynaptic terminals and LTP. (5) Activation of pro-MMP-9 in stratum lucidum requires proteolytic processing of the propeptide domain. Plasmin activated by tPA is a possible proteolytic activator of MMP-9. After activation MMP-9 and other MMPs (e.g., MMP-3) may cleave proteins of perineuronal nets (PNNs) that enwrap PV+ interneurons and restrict synaptic plasticity. (6) MMP-9 regulates mossy fiber synaptic physiology through integrin receptors by cleavage of extracellular matrix (ECM) components (e.g., laminin), release of latent integrin ligands and activation of integrin-dependent signaling pathways. Binding of endogenous inhibitor TIMP-1, secreted in neuronal activity-dependent manner terminates the activity of MMP-9 in the extracellular space. (7) Pentraxin complex composed of membrane pentraxin receptor Npr and two soluble proteins NPTX1 and NPTX2 binds and clusters synaptic AMPA receptors in excitatory synapses on interneurons. During induction of LTD, ADAM-17 cleaves Npr releasing pentraxin complex leading to AMPARs internalization. (8) In a subset of MF-INT synapses containing calcium permeable AMPA receptors and lacking NMDA receptors, presynaptic LTD can be developed in response to MF high frequency stimulation. (9) Synaptic ADAM-10 cleaves NCAM adhesion protein affecting the morphology of MFB.

studies demonstrate that administration of exogenous protease or MMP-9 overexpression can regulate dendritic spine shape and stability. For example, Wang et al. (2008b) have shown that acute MMP-9 application gives rise to a spine enlargement and morphological modification towards a mushroom-like phenotype in the CA1 hippocampal neurons. On the other hand, prolonged treatment of cultured hippocampal neurons with active MMP-9 tended to induce a filopodia-like phenotype

of dendritic spines (Michaluk et al., 2011) which is accompanied by increased membrane mobility of NMDAR, synaptic clustering of AMPA receptors (Michaluk et al., 2009; Szepesi et al., 2014) and increased decay constant of mEPSCs (Wiera et al., 2015). These findings suggested that prolonged activity of MMP-9 gave rise to a functional and morphological "juvenilization" of dendritic spines and synaptic currents. Additionally, it was also shown that exogenous active MMP-9 induces growth of thin spine head protrusions (Szepesi et al., 2013) already described in MF-PC synapses (Zhao et al., 2012). However, so far, little is known about involvement of MMP-9 in MF structural changes after LTP and during the acquisition of hippocampus-dependent memory. Temporal lobe epileptogenesis is often accompanied by an aberrant sprouting of hippocampal mossy fibers and the formation of abnormal MF synapses with granule cells. It has been shown that blocking of the MMP activity with broad-spectrum inhibitor decreases abnormal growth of mossy fibers during pentylenetetrazole-induced kindling (Yeghiazaryan et al., 2014). Moreover, MMP-9 protein and activity were detected in DG molecular layer where sprouted MF synapses are formed and in CA3 region of epileptic hippocampus (Wilczynski et al., 2008; Takács et al., 2010; Konopka et al., 2013). Interestingly, development of seizures in kindled mice depends on the conversion of pro-BDNF to mature BDNF by MMP-9 (Mizoguchi et al., 2011). Taken together, these studies indicate that the MMP-9 activity is essential to support the synaptic modifications associated with MF-CA3 LTP and structural plasticity in hippocampus.

Proteomics experiments using mass spectrometry have shown that the number of putative MMP-9 substrates may reach up to several hundred proteins. Among them there are extracellular, cytoplasmic and nuclear proteins (Overall and Blobel, 2007; Xu et al., 2008; Butler and Overall, 2009; Cauwe et al., 2009; Cauwe and Opdenakker, 2010; Zamilpa et al., 2010). Whether a putative MMP-9 substrate is really cleaved *in vivo* depends on several factors including: (1) expression level of both protease and substrate; (2) access of the proteases to the substrate (e.g., to proteins present in the cell nucleus or in the vicinity of the cell membrane); (3) binding of a protease to the components of ECM, which defines sites of protease activity; (4) duration of time window between MMP activation and inhibition by binding of TIMP inhibitors; (5) interaction between the MMP-9 hemopexin, hinge domain or fibronectin repeats with substrate that increase the cleavage specificity. Given these limitations, only a small part of the putative MMP-9 substrates is actually proteolyzed *in vivo* giving rise to physiologically important effects. While largely untested, there are many putative MMP-9 substrates present in stratum lucidum and hilus, which after cleavage may shape structural and functional plasticity of mossy fiber synapses.

One of aforementioned MMP-9 neuronal substrate is pro-BDNF, which is secreted from mossy fiber terminals and postsynaptic cell during burst activity (Danzer and McNamara, 2004; Li et al., 2010a; Dieni et al., 2012). MMP-9 potentially cleaves pro-BDNF to produce mature BDNF that binds to and activates TrkB receptor. Thus MMP-9 may regulate the availability of active BDNF in the perisynaptic space (Ethell and Ethell, 2007; Mizoguchi et al., 2011). Moreover, putative activation of pro-BDNF released from mossy fibers by dendritic MMP-9 may act as a coincidence detector of pre-post activity during the induction of MF-PC LTP (**Figure 3**). NMDA receptor is one of the few known synaptic coincidence detectors of pre-post activity as it is activated by presynaptically released glutamate and postsynaptic depolarization. However, LTP in the MF-PC synapses is independent on NMDAR and is routinely induced experimentally in the presence of APV. Therefore, in the MF-PC synapses, induction of LTP may require a different coincidence detector and activation of pro-BDNF by MMP-9 is a good candidate for such a mechanism.

MMP-9 activity affects both postsynaptic NMDAR-dependent LTP in CA3-CA1 pathway and NMDAR-independent, presynaptic LTP in MF-PC synapses, raising the question how MMP-9 can regulate such different types of long-term plasticity. Early data suggested that BDNF/TrkB signaling is activated both in pre- and postsynaptic compartment (reviewed in Edelmann et al., 2014). In hippocampal CA3-CA1 synapses, BDNF augments postsynaptic transmission through activation of MAPK pathway, modulation of GluN2B-conatinnig NMDAR and induction of calcium inward currents (Minichiello, 2009). In contrast, mossy fibers express TrkB receptor that is activated by synaptic BDNF (Helgager et al., 2014). Moreover, activation of TrkB signaling enhances glutamate release targeting presynaptic proteins engaged in MF-PC LTP such as: synapsins, Rab3a and Rim1α (Jovanovic et al., 2000; Thakker-Varia et al., 2001; Zakharenko et al., 2003; Yano et al., 2006; Simsek-Duran and Lonart, 2008). These results together indicate that MMP-dependent processing of pro-BDNF and TrkB activation may be necessary for functional presynaptic plasticity and structural alteration of MF-CA3 synapses, although the latter possibility awaits thorough investigations.

Integrin adhesion receptors, a major family of ECM receptors in the brain, are involved in numerous molecular mechanisms related to synaptic plasticity, learning and memory (McGeachie et al., 2011). In particular, integrin signaling modulates morphology and intrinsic motility of dendritic spines (Levy et al., 2014). Moreover, MMP-9 involvement in structural and functional plasticity is strongly dependent on integrin signaling. Indeed, integrin β1 blocking antibody abolishes the effect of exogenous MMP-9 on NMDAR membrane diffusion (Michaluk et al., 2009), dendritic spine morphology in neuronal cultures (Wang et al., 2008b) and blocks synaptic potentiation after administration of MMP- 9 in CA3-CA1 pathway (Nagy et al., 2006). Interestingly, mossy fibers show immunoreactivity for α2-, β4- and β5-integrins (Wu and Reddy, 2012) while perisynaptic astrocyte processes in stratum lucidum contain β1-integrin (Schuster et al., 2001). Although MMP-9 cleaves directly β4-integrin *in vitro* (Pal-Ghosh et al., 2011), the present evidence indicates rather an indirect interaction. β4-subunit containing integrins present in mossy fibers are considered as laminin receptors (Srichai and Zent, 2010). MMP-9 can locally cleave perisynaptic ECM proteins (such as laminin or fibronectin), that, in turn, might reveal their RGD sequence and activate integrin signaling that could locally support activity-dependent and intrinsic structural plasticity (Levy et al., 2014). As already

mentioned, some MFBs are hyperplastic and develop additional satellite boutons (Galimberti et al., 2010). Maintenance of these local hot spots of structural plasticity in single axon may require MMP-9 activity and integrin signaling (**Figure 3**). In hippocampal CA3-CA1 and cortical synapses, MMP-9 cleaves the membrane adhesion protein ICAM-5 that is most abundant on dendritic filopodia (Tian et al., 2007; Lonskaya et al., 2013). In neuronal cultures postsynaptic ICAM-5 interacts with presynaptic β1 integrin and blocks structural transition from filopodium to mature spine (Ning et al., 2013; Gahmberg et al., 2014). ICAM-5 cleavage promotes spine maturation and release of soluble extracellular domain of ICAM-5 increases synaptic AMPA receptor expression and cofilin phosphorylation (Lonskaya et al., 2013). Although β1-integrin is not expressed in mossy fiber synapses, the role of ICAM-5 proteolysis and activation of perisynaptic integrins in stratum lucidum await clarification.

Recently, it was shown that MMP-dependent cleavage of signal regulatory protein α (SIRPα) leads to release of extracellular SIRPα domain that binds to presynaptic CD47 receptor and promotes maturation of presynaptic terminals in cell cultures. Moreover, synaptic transmission and LTP in CA1 region is significantly impaired in SIRPα null mice (Toth et al., 2013). Interestingly, both SIRPα and CD47 are most abundant in the stratum lucidum (Matozaki et al., 2009). This raises the possibility that MF activity leads to MMP-9 secretion, shedding of SIRPα ectodomain that, in turn, regulate presynaptic MF-PC plasticity (**Figure 3**). It will be interesting to further elucidate the molecular determinants of MMP-dependent SIRPα proteolysis in MF synapses, and to find out how CD47 activation regulates presynaptic functions.

In addition to substrates described above, MMP-9 cleaves structural proteins that form the backbone of the excitatory synapses such as neuroligin-1 (Peixoto et al., 2012), EphB receptors (Lin et al., 2008), netrin-3 (van der Kooij et al., 2014), SynCAM-2 (Bajor et al., 2012) and β-dystroglycan that is present mainly at inhibitory synapses (Lévi et al., 2002; Michaluk et al., 2007; Ganguly et al., 2013). MMP-9 processes also short-lived proteins responsible mainly for synaptic signaling, including interleukins (Amantea et al., 2007), β-amyloid peptide (Ridnour et al., 2012) and insulin-like growth factor-binding proteins (Nishijima et al., 2010). Thus, MMP-9 proteolysis may be involved in mechanisms underlying various types of memory, characterized by a different time span. Indeed, the short-term synaptic changes are likely to be related to cleavage of proteins with fast turnover, whereas truncation of proteins with long half-lives would produce long-lasting changes which are important for LTP maintenance.

In the last decade, thanks to proteomic techniques, it was realized that the repertoire of metzincin substrates is much wider than expected and, surprisingly, a part of putative or confirmed MMP substrates reside inside the cell. Moreover, the active forms of MMP-2, -3, -9, -12 have been found in the cytosol, mitochondria, and nucleus of neurons and glia (Cauwe and Opdenakker, 2010; Mannello and Medda, 2012). Intraneuronal activity of MMPs has been reported in pathological processes such as Parkinson's disease (Choi et al., 2011), cerebral hypoxia (Yang et al., 2010; Wojcik-Stanaszek et al., 2011b) or epilepsy (Konopka et al., 2013). Interestingly, induction of LTP in MF-CA3 pathway in slices, using high-frequency stimulation of mossy fibers, increases *in situ* MMP-9 activity in the cytoplasm of CA3 pyramidal neurons (Wiera et al., 2012). Additionally, increase in MMP-9 protein was observed in the cytoplasm and nuclei of CA3 pyramidal cells after the induction of MF LTP (Wiera et al., 2012). In the same study, nuclear MMP-2 was also reported in neurons, although its expression and activity has not changed after LTP. It seems thus that unraveling substrates of intraneuronal MMPs and mechanisms of their regulation emerges as a novel challenge in studies on MMP-9 role in neuronal physiology.

ADAM-10 and ADAM17/Tace

Membrane-anchored proteases, belonging to the ADAM subfamily of metzincins, are able to cleave transmembrane proteins close to the surface of cell membrane in a process referred to as ectodomain shedding. Most of ADAMs are built with (from C-terminus): a cytoplasmic domain, a type I transmembrane sequence, an EGF-like domain, a cysteine-rich region, a disintegrin domain and a metalloproteinase domain (in some ADAMs inactive). Two most studied neuronal ADAMs are ADAM-10 and ADAM-17 also known as TNF-α converting enzyme TACE (Weber and Saftig, 2012). Studies on the roles of ADAM-10 and ADAM-17 in learning-related processes are hampered by the fact that ADAM-10 knockout mice are embryonic lethal and ADAM-17-deficient mice die perinatally or show increased mortality in adulthood depending on genetic background (Rivera et al., 2010). ADAM-10 and ADAM-17 are known due to their α-secretase activity responsible for APP cleavage and generation of extracellular, soluble, non-amyloidogenic sAPPα domain that is endowed with signaling functions (Saftig and Reiss, 2011). Conditional neuronal ADAM10 knockout mice show impairments in hippocampus-dependent learning, almost abolished LTP and atypical prevalence of stubby spine morphology in CA1 region (Prox et al., 2013). Additionally, constitutive overexpression of ADAM-10 negatively affects learning and memory in mice (Schmitt et al., 2006). These findings are reminiscent of the observation that LTP maintenance in MF-CA3 projection requires fine-tuned proteolytic activity mediated by MMP-9 (Wiera et al., 2013). Increased synaptic activity, through deacetylase SIRT-1 induces ADAM-10 transcription and subsequent translation (Donmez et al., 2010; Gao et al., 2010). ADAM-10 as an integral membrane protein is present in postsynaptic densities of excitatory synapses where it is trafficked due to interaction with synaptic scaffold protein SAP97 (Musardo et al., 2014). In response to synaptic stimulation ADAM-10 cleaves neuronal N-cadherin in primary hippocampal neurons (Malinverno et al., 2010). Interestingly, N-cadherin regulates mossy fiber fasciculation (Bekirov et al., 2008) and have an important role in modulating synaptogenesis, spine formation, and synaptic plasticity in CA3-CA1 projection (Arikkath and Reichardt, 2008). ADAM-10 has also been reported to cleave ephrin-A2 that forms trans-synaptic complex with EphA receptors in cell cultures (Hattori et al., 2000).

Galimberti et al. (2010) have found that after disruption of EphA4 signaling in mossy fiber circuits, single MFB establishes significantly more satellite boutons, suggesting that ADAM-10-dependent proteolysis of synaptic ephrin-A may be involved in regulation of the number of MFBs in hilus and stratum lucidum.

ADAM-10 and ADAM-17 both shed many classical cadherin adhesion proteins (Weber and Saftig, 2012). One of them, cadherin-9 is expressed selectively in mossy fibers and CA3 pyramidal cells and knockout or interference with cadherin-9 adhesion severely disrupts morphology of MFBs and filopodia (Williams et al., 2011). Additionally, both ADAM-10 and ADAM-17 cleave synaptic adhesion protein NCAM in neuronal cultures (Hinkle et al., 2006). NCAM, expressed in stratum lucidum, is responsible for MF growth and lamination, but is dispensable for MF-CA3 LTP recorded in slices (Cremer et al., 1997, 1998; Bukalo et al., 2004). Nevertheless, NCAM knockout mice have more filopodia emanating from MFBs and, additionally, they are hyperdynamic and excessively branched (De Paola et al., 2003). Interestingly, polysialylated form of NCAM (PSA-NCAM) is present in stratum lucidum and affects structural plasticity of MFB (Seki and Arai, 1999; Galimberti et al., 2010). Thus, activity-dependent proteolysis of NCAM may constitute an important factor in shaping the structural plasticity of the mossy fibers projection (**Figure 3**).

Recent studies in cell cultures and hippocampal CA3-CA1 pathway in slices point compellingly to a role for ADAM-10 in LTD (Marcello et al., 2013). Induction of LTP in this projection decreases whereas LTD stimulates ADAM-10 synaptic localization and activity (reviewed in Musardo et al., 2014). In addition, activation of mGluR1/5 receptors in neuronal cultures induces MMP and ADAM-17-dependent cleavage of synaptic pentraxin receptor Npr, release of its extracellular domain, internalization of AMPA receptors and expression of LTD (Cho et al., 2008). As discussed above, the hallmark of MF-CA3 pathway is the opposite impact of high frequency stimulation on synaptic plasticity in MF-PC and MF-INT synapses in stratum lucidum (**Figure 3**). At the network level, induction of LTD in mossy fiber excitatory synapses onto PV^+ interneurons (MF-PV^+ INT) causes decreased recruitment of feedforward perisomatic inhibition, which affects time window for synaptic integration in CA3 pyramidal cells (McBain, 2008). Recently, Pelkey et al. (2015) have shown that neuronal pentraxins control strengthening of synapses on PV+ interneurons. There are three neuronal pentraxins: two secreted to extracellular space (NPTX1 and NPTX2 also called Narp) and one membrane neuronal pentraxin receptor (Npr). All three pentraxins form heteropentamers that are soluble or tethered to the cell membrane by Npr (Kirkpatrick et al., 2000). Neuronal pentraxins are broadly expressed in the hippocampus, cerebral cortex and cerebellum (Pribiag and Stellwagen, 2014). Moreover, NPTX1 is highly expressed by CA3 PC and, to a lesser extent, by DG granule cells (Schlimgen et al., 1995; Dodds et al., 1997). Activity-regulated protein NPTX2 (Narp) is abundant in mossy fiber terminal field (Tsui et al., 1996; Xu et al., 2003), similar to Npr (Cho et al., 2008). NPTX2 knockout mice are hypersensitive to kindling-induced seizures (Chang et al., 2010) in an analogos way as transgenic mice overexpressing MMP-9 (Wilczynski et al., 2008) and in agreement with attenuation of seizures by inhibitor blocking ADAM-17 activity (Meli et al., 2004). Additionally, both NPTX2 and Npr are enriched at excitatory synapses on PV+ interneurons where they cluster GluA4-containing AMPA receptors, increasing thereby synaptic currents (Chang et al., 2010; Gu et al., 2013; Pelkey et al., 2015). Furthermore, Npr and NPTX2 double deficient mice show impaired feedforward inhibition and increased duration of critical period plasticity in hippocampus (Pelkey et al., 2015). Thus, ADAM-17-dependent pentraxin cleavage is likely to be involved in target cell-specific synaptic plasticity in MF-INT synapses. Future studies are needed to elucidate the relationship between extracellular proteolysis, activity dependent synaptogenesis in stratum lucidum and induction of LTD in MF-INT synapses. Moreover, MMP activity and synapse-specific cleavage of Npr in MF-INT synapses may determine the local short-lasting opening of a "critical period" plasticity of excitatory transmission between principal cells in adult hippocampus.

Hippocampal neurons and glial cells express a plethora of other extracellular metalloproteinases that may also modify synaptic functions and several lines of evidence support the notion that, besides MMP-9, also other metzincins participate in certain types of synaptic plasticity. Indeed, MMP-7 affects dendritic spine morphology and NMDAR currents in primary neuronal cultures (Bilousova et al., 2006; Szklarczyk et al., 2008) and ADAMTS-4 cleaves ECM proteoglycans enhancing neuroplasticity (Lemarchant et al., 2014). Moreover, learning is associated with elevated expression of MMP-3 in rodents (Olson et al., 2008; Wright and Harding, 2009) and MMP-12 is upregulated after bicuculine-induced chemical LTP (Pinato et al., 2009). This suggests that additional metalloproteinases may regulate synaptic transmission and synaptic morphology in the mossy fiber pathway, but further studies are needed to understand their roles at MF synapses in the hippocampus.

Serine Proteases: tPA/Plasmin System

Tissue plasminogen activator (tPA) is a serine protease that converts plasminogen to plasmin. In neurons *tpa* is an immediate-early gene and the increase in synaptic activity induces its expression (Qian et al., 1993). tPA together with plasmin modulate long-term plasticity in hippocampus, cortex and amygdala as well as learning and memory (reviewed in Samson and Medcalf, 2006; Almonte and Sweatt, 2011). tPA and plasmin are abundant in mossy fiber terminal field (Salles and Strickland, 2002; Taniguchi et al., 2011) and both are stored in dense-core vesicles and released in an activity-dependent manner from axons or dendrites (Lochner et al., 2006). Interestingly, tPA appears to play a similar role in the mossy fibers to that discussed above for MMP-9. Indeed, akin to MMP-9, blocking the activity of tPA/plasmin system or tPA knockout impairs induction of MF-CA3 LTP while exogenous active tPA enhances LTP and potentiates basal MF synaptic transmission (Huang et al., 1996; Baranes et al., 1998). Moreover, tPA knockout mice exhibit impaired object recognition memory, which is critically dependent on

MF-CA3 projection and on MMP-9 activity (Benchenane et al., 2007; Kesner, 2007; Mizoguchi et al., 2010; Bednarek and Caroni, 2011). Furthermore, blocking tPA or MMP-9 activity decreases aberrant mossy fiber sprouting in epileptic hippocampus (Wu et al., 2000; Yeghiazaryan et al., 2014). These similarities between MMP-9 and tPA involvement in MF plasticity suggest their potential crosstalk. Indeed, direct or indirect activation of pro-MMP-9 by tPA/plasmin is the most plausible scenario (Chakraborti et al., 2003), but this hypothesis requires verification.

Both plasmin and MMP-9 can cleave and activate pro-BDNF to mature BDNF during the maintenance phase of CA3-CA1 LTP and epileptogenesis (Pang et al., 2004; Nagappan et al., 2009; Mizoguchi et al., 2011). Moreover, BDNF, in a feedback-manner activates TrkB and promotes transcription of *tpa* and *mmp-9* genes in neuronal culture (Kuzniewska et al., 2013; Bennett and Lagopoulos, 2014). Besides, activation of pro-BDNF and pro-MMP-9, tPA/plasmin system cleaves also GluN1, GluN2A and GluN2B subunits of NMDA receptor *in vitro*, changing its kinetic and pharmacological properties (Benchenane et al., 2007; Yuan et al., 2009; Ng et al., 2012). This raises a possibility that tPA/plasmin may be involved in NMDA-dependent metaplasticity described in MF-PC synapses (Hunt et al., 2013).

Serine Proteases: Neuropsin

In MF-PC synapses, the postsynaptic EphB2 interaction with presynaptic ehrin-B2 builds high affinity adhesion complex, which mediates MF-PC trans-synaptic signaling (Contractor et al., 2002). EphB2 and ephrin-B2 bind to each other with a high affinity which is it unlikely to spontaneously dissociate. To "reset" the synapse to the state prior the formation of ephrin-Eph receptor complex, cell internalizes the whole complex together with a part of neighboring membrane (Zimmer et al., 2003) or may release proteases that cleave ephrin or its receptor. In the MF-CA3 and CA1-CA3 projections, interference with the EphB-ephrinB complex, using recombinant soluble ligands or genetic knockouts causes impairment of LTP and dendritic spine structural plasticity (Contractor et al., 2002; Grunwald et al., 2004; Armstrong et al., 2006; Kayser et al., 2008). It was shown in the amygdala that stress-related learning and induction of LTP is accompanied by a neuropsin-dependent cleavage of EphB2 (Attwood et al., 2011). The activity of serine protease neuropsin has a pivotal role in establishing early phase of LTP in CA3-CA1 pathway, synaptic tagging and hippocampus-dependent learning (reviewed in Shiosaka and Ishikawa, 2011; Tamura et al., 2013). Additionally, neuropsin cleaves neuregulin-1, releasing part of its extracellular domain which activates a receptor tyrosine kinase—ErbB4 in synapses onto PV-positive hippocampal interneurons (Tamura et al., 2012). In turn, ErbB4 activation strengthens GABAergic feedforward synaptic transmission (Lu et al., 2014). Further studies, including physiological, morphological and behavioral approaches are necessary to clarify whether neuropsin-dependent cleavage of EphB-ephrin complex and neuregulin-1 regulates synaptic plasticity of MF-CA3 pathway, feedforward inhibition in CA3 and experience-driven structural plasticity of mossy fiber synapses.

Aspartate Protease: Beta-Secretase-1

BACE1 has been identified as a major neuronal β-secretase responsible for formation of β-amyloid peptide, which is thought to be responsible for the amyloid plaques formation, which is the hallmark of the Alzheimer's disease. Importantly, BACE1 protein is abundantly expressed in the mossy fiber projection in stratum lucidum (Laird et al., 2005) and undergoes retrograde transport from dendrites to axons (Buggia-Prévot et al., 2013). In MF-PC synapses, LTP is absent in BACE1 knockout mice (Wang et al., 2008a, 2014) and this impairment may be rescued by pharmacologically induced increase in the presynaptic calcium influx (Wang et al., 2010). Augmentation of short-term plasticity (STP) in MF-PC synapses in BACE1 deficient mice suggests BACE1-dependent tuning of presynaptic function (Laird et al., 2005; Wang et al., 2008a). Moreover, regulation of release probability by BACE1 is synapse-dependent, because knockout of BACE1 protein increases STP in MF-PC, but not in MF-INT synapses (Wang et al., 2014).

Hitt et al. (2012) have indicated yet another aspect of BACE1-dependent hippocampal plasticity. BACE1-deficient mice exhibit shortened and disorganized intra- and infrapyramidal (IIP) mossy fiber projection. Moreover, during the developmental growth of IIP-MF projection, BACE1 may cleave an adhesion protein—a close homolog of L1 (CHL1) which is abundant in mossy fiber terminal filed (Hitt et al., 2012; Kuhn et al., 2012). Additionally, CHL1-deficient mice show impairments in MF-dependent novel object recognition memory (Pratte and Jamon, 2009). It remains unclear, however, whether BACE1-dependent CHL1 cleavage is involved in the experience-driven outgrowth of IIP-MF axons, MF-CA3 LTP and learning. Because CHL1 protein is expressed also by PV+ interneurons in the hippocampus, it is also possible that the BACE1 activity may regulate the connectivity of feedforward inhibition in CA3 (Nikonenko et al., 2006; Wang et al., 2014).

FEAUTURE DIRECTIONS: EXTRACELLULAR PROTEOLYSIS AND TARGET CELL-DEPENDENT PLASTICITY

A unique feature of hippocampal mossy fibers at early postnatal development is the release of GABA (Safiulina et al., 2006). The immature hippocampus is characterized by a network-driven giant depolarizing potentials, which are generated by a synergistic action of glutamate and GABA, both depolarizing at early developmental stages (Ben-Ari et al., 2007). Pairing single postsynaptic spikes with unitary MF $GABA_AR$-mediated postsynaptic potentials consistently upregulates or downregulates synaptic strength according to the temporal order of stimulation. Positive pairing induces LTP which is L-type calcium channel and BDNF-dependent (Sivakumaran et al., 2009; Caiati et al., 2013). In mature hippocampus high-frequency stimulation was shown to increase the dynamics of structural changes in MF terminals (De Paola et al., 2003). The

impact of juvenile mossy fiber GABA-ergic plasticity on the postnatal developmental growth of MF axons and their activity-dependent morphological changes remain to be determined. In particular, the role of neuronal activity-induced proteolysis in the extracellular space in the mossy fiber GABA-ergic plasticity (Safiulina et al., 2010) or mossy fiber developmental growth (Holahan et al., 2007; Galimberti et al., 2010) awaits elucidation. Most interestingly, in this context, the highest expression of MMP-9 and tPA in hippocampus and cortex occurs in early postnatal development (Zheng et al., 2008; Aujla and Huntley, 2014).

As already mentioned, plasticity in the MF-PC and MF-INT synapses shows an interesting property of target-cell specificity (McBain, 2008). The mechanism of this differential plasticity is not fully understood but synapse-specific interactions between ECM elements and extracellular proteases are likely candidates. Interestingly, recently, for the first time, it has been also shown that MMP-dependent degradation of ECM proteins promotes reorganization of inhibitory innervation in hippocampus during increased neuronal activity (Pollock et al., 2014). A very characteristic ECM structures in the brain are so called perineuronal nets (PNNs) which consist of aggregates of proteins and glycosaminoglycans surrounding soma, proximal dendrites and axon initial segment, mainly of PV+ interneurons (Wang and Fawcett, 2012). PNNs appear at later stages of interneuron maturation and affect their electrophysiological properties (Miyata et al., 2005; Van den Oever et al., 2010; Liu et al., 2013; Slaker et al., 2015). Administration of chondroitinase, that enzymatically digests glycosaminoglycans and disrupts PNN structure, reopens critical period plasticity in adult animals (Pizzorusso et al., 2002) as well as increases spine motility and structural plasticity in the visual cortex (de Vivo et al., 2013). It is worth emphasizing, that chondroitinase is not an endogenous protein and other neuronal enzymes like proteases and sulfotransferases are responsible for PNN remodeling (Matsui et al., 1998; Matthews et al., 2000; Miyata et al., 2005; Valenzuela et al., 2014; Rankin-Gee et al., 2015). It has been already shown that MMPs cleave almost all proteoglycans present in PNNs, e.g., aggrecan, neurocan, brevican, and phosphacan (Van Hove et al., 2012). Interestingly, expression and activity of most plasticity-related extracellular proteases peak during critical period (Zheng et al., 2008; Aujla and Huntley, 2014). Moreover, tPA activity permits synapse remodeling during ocular dominance plasticity similarly as does PNN digestion (Mataga et al., 2002; Oray et al., 2004; Gogolla et al., 2009a). Roger Tsien has recently proposed an intriguing hypothesis that very long-term memories may be stored as patterns of holes excised in the PNNs by extracellular proteases released and activated upon increased neuronal activity (Tsien, 2013). In this scenario, molecular determinants of proteolytic activity exerted upon PNNs, which embed MF-INT and inhibitory synapses, are expected to play a central role in this mechanism of memory storage. Multiplicity of important physiological mechanisms which are critically regulated by PNN remodeling underscores the importance of ECM proteolysis in modulation of neuronal network functions, especially in a long time scale. It is expected that future studies will shed more light on how crosstalk between PNNs, mossy fiber synapses and extracellular proteolysis contributes to the formation of hippocampal memory engrams.

Modification of perisynaptic environment by ECM proteolysis might give rise to yet another mechanism of synaptic signals modulation. It is known that the time course of the synaptic agonist transient is a key determinant of the synaptic currents kinetics (Mozrzymas, 2004; Barberis et al., 2011) and geometry of the synaptic cleft as well as of its nearest environment plays a crucial role in shaping the time course of synaptic agonist (e.g., Barbour et al., 1994; Cathala et al., 2005). Considering a particularly complex structure of synapses in the MF-CA3 projection this mechanism of synaptic signals regulation can be of particular importance but whether or not it is the case still awaits to be investigated.

There is compelling evidence that memory allocation to specific neurons and synapses in neuronal circuits is not random, and that specific processes, such as plastic changes in neuronal excitability and synaptic tagging, determine the exact sites where memories are stored (Rogerson et al., 2014). Precise temporal regulation of transcription and translation of new proteins are necessary for synapse strengthening and maintenance of plastic changes, in spite of the continuous protein turnover. It remains unexplained how one translation machinery in a single neuron, may be responsible for strengthening of specific individual synapses among thousands of synapses present in the dendritic tree. A growing body of evidence suggests that process of synaptic tagging and capture may explain the synapse specificity of LTP (Reymann and Frey, 2007). A strong stimulation of synapse (evoking spikes in postsynaptic neuron) induces translation of plasticity-related proteins (PRPs) which are essential for long-term maintenance of LTP and memory. At the same time, the stimulation modifies the synapses by creating hypothetical "tags" which ensure that some of the PRPs generated in the cell body will be captured only at the stimulated (activated) synapses. Because proteolysis, in contrast to other posttranslational protein modifications (such as e.g., phosphorylation), is irreversible, it is a good candidate for a synaptic tag. This possibility seems to be supported by the observation that, in most cases, inhibitors or genetic knockout of plasticity-related proteases impair LTP maintenance. The initial evidence in support of this hypothesis came from elegant *in vitro* electrophysiological studies showing neuropsin-dependent synaptic tagging and cross-tagging in the CA1 stratum oriens (Ishikawa et al., 2011). However, the idea that synaptic tagging could be related to activity-dependent extracellular proteolysis only started to be investigated. Strong mossy fiber input to CA3 may prime synaptic plasticity in upstream CA3-CA3 synapses in a process similar to a cross-tagging (Kobayashi and Poo, 2004). Since burst activity in single MF axon can generate action potentials in postsynaptic cell (Henze et al., 2002) and plays an instructive role in associative synaptic plasticity at CA3 recurrent excitatory synapses (Wojtowicz and Mozrzymas, 2014), mossy fiber LTP may drive expression of plasticity-related proteases in postsynaptic CA3 PC which is expected to affect, in turn, synaptic plasticity of the associational-commissural CA3 network.

GENERAL CONCLUSIONS

Activity-driven structural and functional refinement of neuronal connections is thought to be a key element in mechanisms of information storage and memory formation. In this context, activity and experience-dependent extracellular proteolysis is attracting increasing attention as soluble or membrane proteases emerge as key players in controlling neuronal plasticity. It should be emphasized that the interplay between proteases and ECM proteins affects both structural and functional changes in synapses, thereby mechanistically connecting these aspects of plasticity. Indeed, in the mossy fiber pathway ECM constituents and proteases regulate long-term plasticity, experience-driven structural pre- and postsynaptic changes, synapse maturation and reversal of this process, synaptogenesis and even neurogenesis in DG (Wojcik-Stanaszek et al., 2011a). Tight regulation of MMP-9, tPA, BACE-1 and ADAMs expression is also important because their elevated activity can contribute to mossy fiber sprouting and epileptogenesis. Thus, unraveling functions of synaptic proteases and their synaptic degradomes may be of key interest far beyond of the scope of neuronal plasticity and may shed new light on investigations of clinical relevance. But still little is known about extracellular protease expression and function in hippocampal interneurons as well as about precise localization of active proteases in synaptic and perisynaptic space. Although we know much about putative, tested mainly *in vitro* substrates of particular ECM proteases, synaptic degradomes are not fully described and await systematic studies. Such a knowledge will help to understand molecular determinants involved in brain development, plasticity and memory encoding. In conclusion, extracellular perisynaptic space it is not passive or merely supportive, but it is acting in concert with ECM macromolecules and extracellular proteases potently shaping the structure and function of synapses and neuronal networks.

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Matrix metalloproteinase 9 (MMP-9) is indispensable for long term potentiation in the central and basal but not in the lateral nucleus of the amygdala

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It has been shown that matrix metalloproteinase 9 (MMP-9) is required for synaptic plasticity, learning and memory. In particular, MMP-9 involvement in long-term potentiation (LTP, the model of synaptic plasticity) in the hippocampus and prefrontal cortex has previously been demonstrated. Recent data suggest the role of MMP-9 in amygdala-dependent learning and memory. Nothing is known, however, about its physiological correlates in the specific pathways in the amygdala. In the present study we show that LTP in the basal and central but not lateral amygdala (LA) is affected by MMP-9 knock-out. The MMP-9 dependency of LTP was confirmed in brain slices treated with a specific MMP-9 inhibitor. The results suggest that MMP-9 plays different roles in synaptic plasticity in different nuclei of the amygdala.

Keywords: LTP, MMP-9, amygdala, synaptic plasticity, learning

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Introduction

Matrix metalloproteinase-9 (MMP-9) is a member of a matrix metalloproteinase family of zinc-dependent extracellular and membrane bound endopeptidases that cleave components of the extracellular matrix. Its activity is tightly controlled by an endogenous inhibitor, tissue inhibitor of matrix metalloproteinases 1 (TIMP-1, Dziembowska and Wlodarczyk, 2012). MMP-9 has been shown to be involved in synaptic plasticity, as well as in learning and memory involving the hippocampal formation. In particular, it has been observed that MMP-9 knock-out mice display a deficit in late phase of long term potentiation (L-LTP), but not in its early phase (E-LTP) in the hippocampal CA3 to CA1 pathway (Nagy et al., 2006). Similarly, chemical inhibition of MMP-9 causes destabilization of LTP in the mossy fibers-CA3 pathway (Wojtowicz and Mozrzymas, 2010). Moreover, deficits in spatial learning and contextual fear conditioning were observed in MMP-9 knock-out mice (Nagy et al., 2006). It has also been demonstrated that overexpression of TIMP-1 *in vivo*, as well as specific chemical inhibition of MMP-9 in acute slice preparation block late phase of LTP in the subiculum to medial prefrontal cortex pathway (Okulski et al., 2007). On the other hand, very little is known about the role of MMP-9 in neuronal plasticity in other brain structures, including the amygdala.

Nagy et al. (2006), as well as Brown et al. (2009) reported that interfering with MMP-9 activity did not affect amygdala-dependent fear conditioning to an acoustic cue. Recently, we have shown that appetitively, but not aversively motivated discrimination learning depends on MMP-9 activity within the central amygdala (Knapska et al., 2013).

Little is also known about the molecular heterogeneity corresponding to the function of the amygdalar nuclei. It has, however, been well established that this brain structure is complex, with over a dozen subdivisions distinguished by anatomical, as well as functional features (see: Sah et al., 2003; Knapska et al., 2007). Such functional heterogeneity suggests that different molecular mechanisms may underlie processing of information in different nuclei of the amygdala. For instance, studies on expression of gene activity markers in response to different kinds of behavioral training have revealed patterns of activation specific to the learning task (see Knapska et al., 2007).

To address the question about molecular heterogeneity of the amygdalar nuclei at the level of synaptic plasticity we investigated the role of MMP-9 in LTP measured at three different amygdalar pathways: from the external capsule (EC) to the lateral amygdala (LA), from the LA to the basal amygdala (BA) and from the BA to the medial section of the central amygdala (CeAm). We used coronal brain slices from mice lacking *mmp-9* gene (MMP-9 KO). The slices were subjected to a tetanic stimulation protocol that produces both early (E-) LTP and, a subsequently emerging, protein synthesis-dependent late (L-) LTP. Additionally, we induced LTP in the pathways from the EC to LA and from the BA to CeAm in rat brain slices treated with S24994, a chemical inhibitor of MMP-9.

Materials and Methods

MMP-9 homozygous knock-out mice on a C57BL/6 background were obtained from Dr. Z. Werb (University of California, San Francisco). These mice were bred with C57BL/6NtacF wild-type mice for at least two generations and then maintained and bred continuously with each other as heterozygotes for >10 generations. The MMP-9 KO and MMP-9 WT mice used in this study were always littermates. The experiments were performed on male 2- to 4 month-old mice. For experiments with MMP-9 inhibitor 2- to 3-month-old male Wistar rats were used. All of the animals were group-housed and maintained on a 12 h/12 h light/dark cycle with water and food provided *ad libitum*. The animals were treated in accordance with the ethical standards of European (directive no. 86/609/EEC) and Polish regulations resulting from this directive. All of the experimental procedures were approved by the Local Ethics Committee. Animals were anesthetized with isoflurane and decapitated. The brains were quickly removed and placed in cold artificial cerebrospinal fluid (aCSF; 117 mM NaCl, 4.7 mM KCl, 2.5 mM $NaHCO_3$, 1.2 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$ and 1 mM glucose), bubbled with carbogen (95% O_2 and 5% CO_2). Both hemispheres were cut into 400 μm coronal slices with a vibratome. The slices were then transferred to a recording interface chamber and perfused with carbogenated aCSF at 33°C for at least 1 h before the LTP experiments started. Field excitatory postsynaptic potentials (fEPSP) were recorded using glass electrodes (1–3 MΩ resistance). Electrodes positions are shown in **Figure 1**. Test pulses at 0.033 Hz, 0.1 ms, were delivered by a bipolar metal electrode (FHC). The intensity of a test stimulus was adjusted to obtain fEPSP with amplitude that amounted to a half of the maximal response. After at least 15 min of stable baseline recording, a theta burst protocol (TBS) was used to evoke LTP. Three trains of stimuli were applied every 5 min. One train was composed of five sequences of pulses separated by 1 s. Each sequence consisted of five bursts of stimuli at 5 Hz. The bursts consisted of eight pulses at 100 Hz. After the end of the theta burst protocol, test pulses were subsequently applied for at least 90 min. Recordings were amplified and digitized, and amplitudes were analyzed online and off-line (CED, Cambridge, UK). The same protocol was used in the experiments with S24994 (Hanessian et al., 2001; Jourquin et al., 2003), a specific MMP-9 inhibitor. After 15 min of baseline recordings S24994 was delivered (100 nM), and, 15 min later, TBS protocol was used to induce LTP. S24994 was present in ACSF throughout all remaining recording time. ANOVA with repeated measures was used for statistical analysis of responses averaged in 5 min intervals; $p < 0.05$ was considered significant.

Results

In the first experiment we examined whether MMP-9 is important for LTP in the LA. We induced LTP by stimulation of the EC, in brain slices from MMP-9 KO and MMP-9 WT mice (**Figure 1A**). We observed no statistically significant difference between LTP recorded in slices from MMP-9 KO and MMP-9 WT mice (155.1 ± 15.7% of baseline vs. 161.6 ± 18% of baseline).

Next we investigated LTP in the pathway from the LA to BA. TBS stimulation protocol evoked LTP that was stable within first 70 min in slices from both MMP-9 KO (137.2 ± 7.2% of baseline) and MMP-9 WT controls (147 ± 7.4% of baseline). However, thereafter LTP in MMP-9 KO declined to the baseline level, whereas it remained stable in MMP-9 WT mice (repeated measures ANOVA: $F_{(1,12)} = 5.95$, $p = 0.04$ for comparison between two groups for the last 50 min of recording, **Figure 1B**).

Finally, we investigated impact of MMP-9 knock out on LTP in the CeAm. LTP evoked in slices from MMP-9 KO was stable only within first 30 min (129.7 ± 5.9% of baseline), then it decayed to the baseline level within 15 min. LTP in MMP-9 WT group was stable throughout entire recording period (141 ± 81% of baseline; repeated measures ANOVA: $F_{(1,12)} = 14.73$, $p = 0.004$ for the last 60 min of recording, **Figure 1C**).

We also investigated basal synaptic transmission in MMP-9 KO and WT mice in all three pathways. There were no differences in I-O relationship between MMP-9 KO and WT mice neither in the EC–LA nor in the LA to the BA and BA to the CeAm amygdala pathways (data not shown).

To confirm MMP-9 dependency of LTP, we investigated the brain slices treated with S24994, a specific MMP-9 inhibitor.

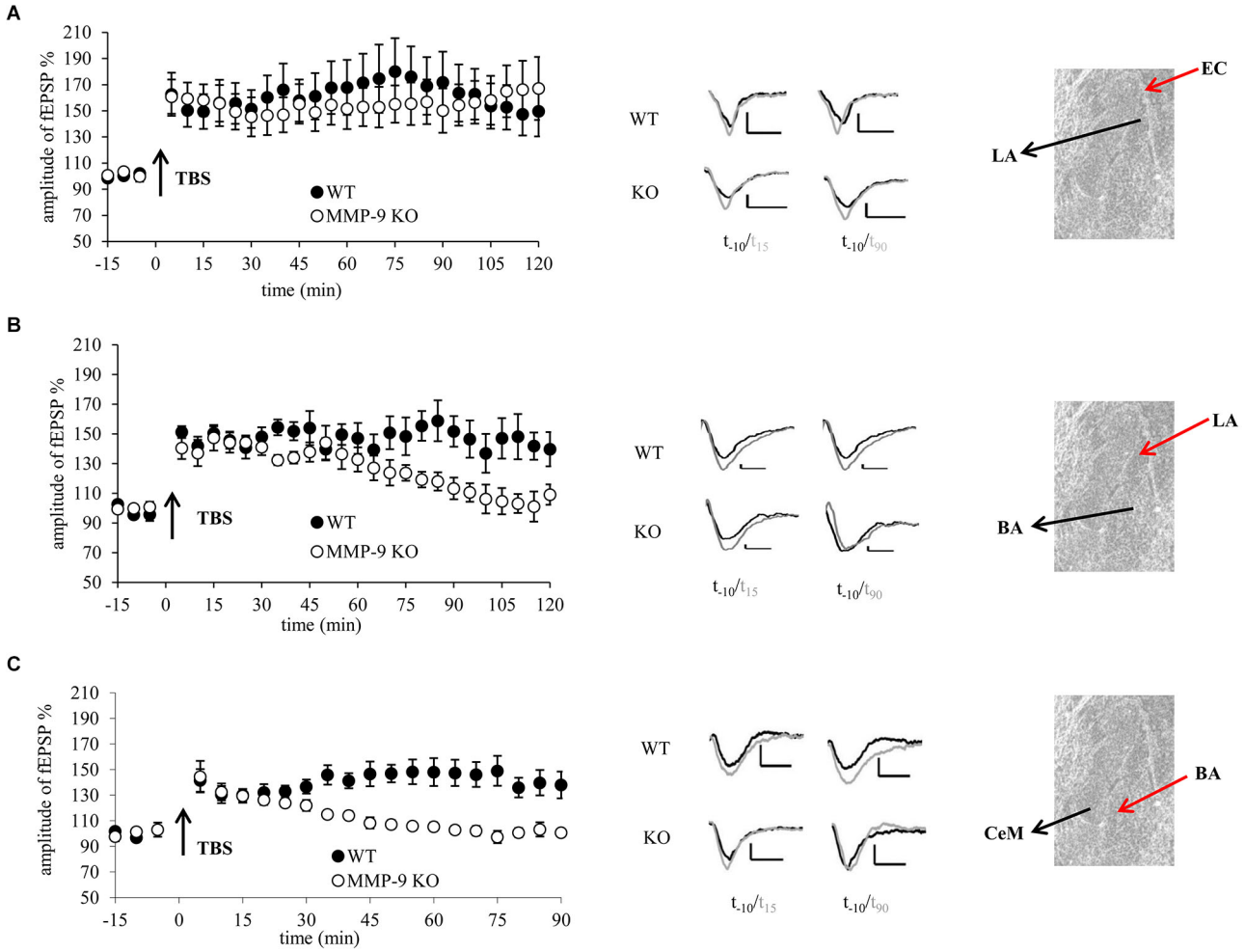

FIGURE 1 | Genetic inhibition of MMP-9 results in destabilization of LTP in the central and basal but not in the lateral amygdala. **(A)** fEPSP in the EC–LA amygdala pathway was similar in slices from mice lacking functional MMP-9 gene (MMP-9 KO, open circles *n* = 6) and control animals (WT, filled circles, *n* = 5). **(B)** fEPSP evoked in the LA-BA pathway in slices from MMP-9 KO mice (open circles, *n* = 7) within first 70 min had the same magnitude as LTP in slices from control animals (WT, filled circles, *n* = 7); however afterwards it went down to the baseline level. **(C)** fEPSP induced in the BA-CeAm pathway in slices from MMP-9 KO mice (open circles, *n* = 7) had the same amplitude as LTP evoked in control slices (filled circles, *n* = 7) within first 30 min after induction. Then, LTP in MMP-9 KO slices gradually decreased to the baseline level. Left panels show graphs with time course of maximal EPSP amplitudes normalized to baseline. Black arrows mark the time of application of TBS stimulation. Error bars represent SEM. Middle panels show exemplary traces of fEPSP recorded 10 min before (black) and 15 and 90 min after (gray) induction of LTP. Scale bars = 0.2 mV and 5 ms. Right panels present photographs of mouse amygdala (Nissl staining) with positions of stimulating (red arrow) and recording (black arrow) electrodes.

We studied two amygdalar pathways: from the EC to LA and from the BA to CeA. In the LA, LTP in slices treated with S24994 did not significantly differ from LTP obtained in control, untreated slices (128 ± 8.1% of baseline vs. 137.3 ± 10.5% of baseline, **Figure 2A**). In the CeAm, LTP induced in the presence of MMP-9 inhibitor was very similar to LTP recorded in the control slices within first 30 min (133.4 ± 21% of baseline and 123.4 ± 4.7% of baseline, respectively). However, LTP under MMP-9 inhibition returned to the baseline level within 50 min, whereas, in control slices, LTP remained elevated for at least 90 min (repeated measures ANOVA : $F_{(1,7)}$ = 6.824, p = 0.039 for the last 60 min of recording, **Figure 2B**).

Results Summary

In summary, in the present study we show that LTP in the lateral to basal (LA-BA) and basal to the medial division of the central amygdala (BA-CeAm) pathways in MMP-9 KO mice are disrupted in its late phase, whereas LTP in the cortico-LA pathway remains intact. The results obtained in MMP-9 knock-outs were confirmed in slices treated with specific MMP-9 inhibitor.

Discussion

LTP in the LA has been proposed as a putative cellular mechanism for fear learning (LeDoux, 2000; Maren, 2005;

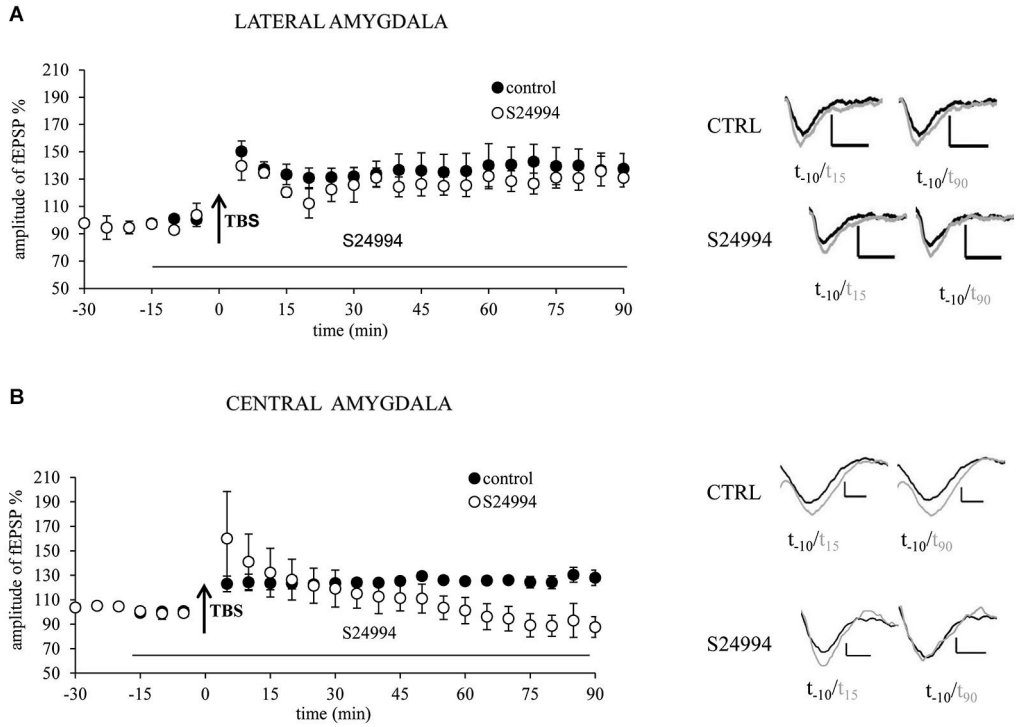

FIGURE 2 | Chemical inhibition of MMP-9 with highly specific inhibitor S24994 results in destabilization of LTP in the central but not in the lateral amygdala. **(A)** MMP-9 inhibitor S24994 (100 nM) administrated 15 min before LTP induction did not affect fEPSP in the EC–LA amygdala pathway (open circles, *n* = 4) in comparison with control, untreated slices (filled circles, *n* = 5). **(B)** MMP-9 inhibitor S24994 (100 nM) administrated 15 min before LTP induction destabilized fEPSP in the BA-CeAm pathway 30 min after its induction (open circles, *n* = 4), whereas fEPSP evoked in control slices was unaffected (filled circles, *n* = 5). Black arrows mark the time of application of TBS stimulation. Error bars represent SEM. Beneath the graphs there are exemplary traces of fEPSP 10 min before (black) and 15 and 90 min after (gray) induction of LTP. Scale bars = 0.2 mV and 5 ms.

Whitlock et al., 2006). Specifically, the cortico-LA pathway carrying information from the auditory cortex has been implicated in encoding of acoustically cued fear memory (LeDoux, 1995; Schroeder and Shinnick-Gallagher, 2005). Our electrophysiological data presented here, showing that genetic and chemical inhibition of MMP-9 does not affect long-term plasticity in the LA, are consistent with the observation that mice lacking MMP-9 have no deficits in cued fear conditioning (Nagy et al., 2006). On the other hand, little is known about LTP in the CeA, which is downstream from the sites of associativity in the LA and BA and is most often considered as a primary output nucleus for information processing in the amygdala (Davis, 1986; LeDoux, 2000). Here, we show that MMP-9 is important for stabilization but not for induction of LTP in the BA-CeAm pathway. This result is consistent with our behavioral data on MMP-9 KO mice and mice with blocked MMP-9 activity in the CeA, in which we showed impairments in appetitively but not in aversively motivated discrimination learning (Knapska et al., 2013). Moreover, we observed impaired formation of late phase of LTP in the LA-BA pathway. It has been shown that the BA is involved in learning of different behaviors such as fear conditioning and extinction, and appetitively and aversively motivated instrumental learning (Everitt et al., 2003; Maren, 2005, 2011). The role of MMP-9 in the BA in learning and memory needs further studies.

Although MMP-9 is present and active in all amygdalar nuclei, including the LA, BA and CeAm (Knapska et al., 2013), the present results suggest different role of MMP-9 in various parts of the amygdala. The possible explanation of this phenomenon may be based on the fact that LTP at the BLA-CeAm synapses studied herein has been reported to be independent of GABA inhibition (Fu and Shinnick-Gallagher, 2005) whereas LTP at the EC-LA pathway strictly depends on GABA-ergic modulation (Ehrlich et al., 2009). To date, MMP-9 was found to be present in a subset of dendritic spines bearing asymmetric (i.e., glutamatergic) synapses and was not detected on the synapses that are symmetric and expressing GABAa receptors (Wilczynski et al., 2008; Gawlak et al., 2009). We have also demonstrated a similar synaptic localization of MMP-9 activity in the CeA (Knapska et al., 2013). This may suggest that MMP-9 plays a role in plasticity of glutamatergic rather than inhibitory synapses. In line with this reasoning, MMP-9 was shown to be able to modify kinetics of NMDA receptors (Gorkiewicz et al., 2010), which are located post-synaptically and crucial for post-synaptically induced form of LTP (Lynch, 2004). Finally, it is known that neurons in the BLA send axons that form glutamatergic

synapses onto neurons within the CeAm (Pape and Pare, 2010).

Discussion Summary

In summary, we show that LTP in the basal and central but not in the LA is affected by MMP-9 deficiency. These results suggest functional and molecular diversity between the amygdalar nuclei. There have been some evidence supporting this idea (Savonenko et al., 1999; Knapska et al., 2006, 2007), but in the present study we have shown for the first time that one protein plays different roles in synaptic plasticity in different nuclei of the amygdala.

Acknowledgments

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frontiers in Cellular Neuroscience

ORIGINAL RESEARCH
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Proteolytic regulation of synaptic plasticity in the mouse primary visual cortex: analysis of matrix metalloproteinase 9 deficient mice

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The extracellular matrix (ECM) is known to play important roles in regulating neuronal recovery from injury. The ECM can also impact physiological synaptic plasticity, although this process is less well understood. To understand the impact of the ECM on synaptic function and remodeling *in vivo*, we examined ECM composition and proteolysis in a well-established model of experience-dependent plasticity in the visual cortex. We describe a rapid change in ECM protein composition during Ocular Dominance Plasticity (ODP) in adolescent mice, and a loss of ECM remodeling in mice that lack the extracellular protease, matrix metalloproteinase-9 (MMP9). Loss of MMP9 also attenuated functional ODP following monocular deprivation (MD) and reduced excitatory synapse density and spine density in sensory cortex. While we observed no change in the morphology of existing dendritic spines, spine dynamics were altered, and MMP9 knock-out (KO) mice showed increased turnover of dendritic spines over a period of 2 days. We also analyzed the effects of MMP9 loss on microglia, as these cells are involved in extracellular remodeling and have been recently shown to be important for synaptic plasticity. MMP9 KO mice exhibited very limited changes in microglial morphology. Ultrastructural analysis, however, showed that the extracellular space surrounding microglia was increased, with concomitant increases in microglial inclusions, suggesting possible changes in microglial function in the absence of MMP9. Taken together, our results show that MMP9 contributes to ECM degradation, synaptic dynamics and sensory-evoked plasticity in the mouse visual cortex.

Keywords: dendrite, spine, plasticity, ocular dominance, primary sensory cortex (S1), primary visual cortex (V1), matrix metalloproteinase 9 (MMP9)

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Introduction

Distinct areas of the brain show increased cortical plasticity during defined critical periods of development (Hensch, 2005a,b; Morishita and Hensch, 2008; Erzurumlu, 2010; Maffei et al., 2010; Levelt and Hübener, 2012; Hubener and Bonhoeffer, 2014). This localized increased plasticity requires the coordination of multiple signaling events, including synaptic scaling (Desai et al., 2002), synaptic stabilization and circuit reorganization (Hensch, 2005b). While many mechanisms contribute to the opening and closing of critical periods of plasticity, the restructuring of the extracellular matrix (ECM)

may be an important regulator of plasticity. As development proceeds, cells in the brain become gradually encased in an accumulation of structural proteins that form a non-permissive environment for reorganization. The ECM is composed of a lattice of structural proteins, including laminin, tenascins, thrombospondin, and lectins that form a mesh-like network around maturing cells called a perineuronal net (PNN). While the PNN generates stability and can promote cell maturity, it also generates a barrier which can prevent further interactions between neurons and advancing axons, act as a scaffold for the binding of molecules which may then inhibit synaptic formation, and restrict receptor mobility at the synapses, influencing receptor exchange. Thus ECM maturation can limit activity-dependent plasticity at the end of the critical period and into adulthood (Berardi et al., 2004).

The ECM is differentially regulated during development due to the secretion of proteolytic molecules that promote plasticity (Ethell and Ethell, 2007). Matrix metalloproteinases (MMPs) have recently emerged as key players involved in long term memory and the underlying synaptic changes. These proteins constitute a large family of zinc-dependent endopeptidases which can cleave and remodel the ECM and are involved in many physiological and pathological processes (McCawley and Matrisian, 2001; Sternlicht and Werb, 2001). Of the 25 known MMPs, MMP2 and MMP9 are the most prevalent in the brain (Yong, 2005), where they can regulate substrates with roles in synaptogenesis, synaptic plasticity and long-term potentiation—including other proteases, growth factors, cell adhesion molecules, cytokines and neurotransmitter receptors (reviewed in Ethell and Ethell, 2007). MMPs are locally synthesized, secreted from both neurons and microglia, and their activity is regulated by other proteases such as tissue plasminogen activator protein (tPA) and plasmin as well as tissue inhibitors of metalloproteinases (TIMPs). Both tPA and MMP9 have been implicated in the induction of plasticity (Mataga et al., 2002, 2004; Szklarczyk et al., 2002; Nagy et al., 2006, 2007), and may have critical roles at the level of the dendritic spine (Mataga et al., 2004; Oray et al., 2004; Tian et al., 2007; Conant et al., 2010) where local proteolytic activity triggers dendritic remodeling.

The rodent visual system serves as an ideal model to study mechanisms of synaptic plasticity and circuit remodeling (Hensch, 2005a; Tropea et al., 2009). Within the binocular visual cortex, cells exhibit *ocular dominance (OD)*, whereby they respond preferentially to input coming from one eye over the other. Following monocular deprivation (MD), responsiveness shifts from the closed eye to the open eye, and this is called *Ocular Dominance Plasticity* (ODP; Gordon and Stryker, 1996). There are two stages of ODP: first a weakening of deprived eye inputs and reorganization of intracortical connections in the superficial layers (Trachtenberg et al., 2000; Trachtenberg and Stryker, 2001), followed by strengthening of non-deprived eye inputs and anatomical reorganization of thalamocortical afferents (Shatz and Stryker, 1978; Antonini and Stryker, 1993; Antonini et al., 1999; Frenkel and Bear, 2004). While there is evidence that local upregulation of proteases (Mataga et al., 2002, 2004; Oray et al., 2004) and their downstream effects on spine motility and turnover (Mataga et al., 2004; Oray et al., 2004) play an important role in ODP, it is unknown whether protease-mediated ECM remodeling accompanies ODP.

Here, we investigated changes in the ECM during ODP and the contribution of MMP9 to this process. We show that specific components of the ECM are rapidly remodeled and that MMP9 regulates both functional plasticity and ECM remodeling. We also show that loss of MMP9 alters the development of cortical excitatory synapses and induces modifications in microglia. Our results provide evidence for the importance of MMPs in proteolytic regulation of synaptic plasticity *in vivo*.

Materials and Methods

Animals

Animals were treated in strict accordance with the University of Rochester Committee on Animal Resources and the 2011 NIH Guide for the care and use of laboratory animals. Mice were group housed with food and water available ad libitum under a fixed 12 h light/dark cycle. The following mouse lines were used in this study: C57Bl/6 (Charles River, Wilmington, MA), green fluorescent protein (GFP)-M (Feng et al., 2000), MMP9 knock-out (KO) (The Jackson Laboratory; B6.FVB(Cg).Mmp9tm1Tvu/J—C57Bl/6 background), and MMP9 KO/GFP-M (generated by crossing GFP-M and MMP9 KO mice, in house).

Extracellular Matrix Immunohistochemistry

For brain harvesting, mice were anesthetized with sodium pentobarbital (150 mg/kg; i.p.) at P30-P35 and perfused through the aortic arch with ice-cold phosphate-buffered saline (0.1 M PBS, 0.9% NaCl in 50 mM phosphate buffer [pH 7.4]) followed by 4% paraformaldehyde (PFA; in 0.1 M PBS, pH 7.4). Brains were post-fixed in 4% PFA for 2 h and transferred to an increasing gradient of sucrose (10, 20, 30% in ultra-pure water) at 4°C. Brains were sectioned coronally at a 50 μm thickness on a freezing, sliding microtome (Microm; Global Medical Instrumentation, Ramsey, MN).

Fixed brain sections containing visual cortex were immersed in 0.1% sodium borohydride (in 0.1 M PBS) for 30 min at room temperature (RT), washed in 0.1 M PBS, and processed freely floating. For *hyaluronic acid* (HA) detection, sections (ND = 5, 2dMD = 5, 4dMD = 5, 7dMD = 5) were blocked in a solution containing 2% bovine serum albuminin (BSA) and 0.1 M phosphate buffered saline (0.9% NaCl in 50 mM phosphate buffer [pH 7.4]) for 1 h. Sections were then incubated for 72 h in a solution containing biotinylated hyularonic acid binding protein (HABP, 1:200, Seikagaku Biobusiness Corp, Amsbio, UK) in 2% BSA in 0.1 M PBA at 4°C in a humidified chamber. Specific activity was detected using an ABC reagent (1:100; Vector Laboratories Inc, Burlington CA) and visualized with 3, 3-diaminobenzidine (0.5 mg/ml) and hydrogen peroxide (0.03%) in buffer solution (DAB peroxidase kit; Vector Laboratories). For *heparan sulfate proteoglycan* (HSPG) detection, sections (ND = 5, 2dMD = 5, 4dMD = 5, 7dMD = 5) were first processed with a 2% hydrogen peroxide/

70% methanol antigen retrieval step. Sections were digested with 5 mU/ml of heparitinase (from flavobacterium heparinum, Seikagaku Corporation, Tokyo, Japan, Cat100703) diluted in a buffer containing 100 mM sodium chloride and 1 mM calcium chloride for 3 h at 37°C. Sections were blocked in a solution containing 2% BSA in 0.1 M PBS followed by a primary incubation in anti-Δ-heparan sulfate monoclonal antibody (3G10) (1:100, Seikagaku Corp) in a humidified chamber overnight at 4°C. Sections were placed in a secondary incubation containing anti-mouse biotinylated IgG (1:200, Vector Laboratories), 2% BSA in 0.1 M PBS. Specific activity was detected using an ABC reagent (1:100; Vector Laboratories Inc, Burlington CA) and visualized with 3, 3-diaminobenzidine (0.5 mg/ml) and hydrogen peroxide (0.03%) in buffer solution (DAB peroxidase kit; Vector Laboratories). To determine chondroitin sulfate proteoglycan (CSPG) composition, sections were processed for *wisteria floribunda agglutinin* (WFA) which recognizes N-acetylgalactosamine, a sugar that is found in the glycosaminoglycan chains of CSPGs (Murakami et al., 1999). Sections (C57Bl/6: ND = 5, 2dMD = 4, 4dMD = 4, 7dMD = 4; MMP9 KO ND = 5, 2dMD = 5, 4dMD = 5, 7dMD = 5) were processed free-floating by first blocking sequentially in a streptavidin and biotin solution (Biotin/Streptavidin Block Kit, Vector Labs, SP-2002, per kit instructions). Sections were further blocked in a solution containing 3% BSA, 20 mM Lysine, and 0.2% Triton-X in 0.1 M PBS. Sections were incubated in a serum solution containing 1% BSA in 0.1 M PBS and biotinylated-WFA (1:200; Vector Laboratories, B1355) at 4°C in a humidified chamber for 24–48 h. Specific activity was detected using an ABC reagent (1:100; Vector Laboratories Inc, Burlington CA) and visualized with 3, 3-diaminobenzidine (0.5 mg/ml) and hydrogen peroxide (0.03%) in buffer solution (DAB peroxidase kit; Vector Laboratories). Processed tissue sections were mounted out of a solution containing 1% Gelatin/99% ethanol in 0.1 M PB onto clean slides. Once dried, slides were dehydrated in an ascending concentration of ethanol into xylene. Slides were coverslipped using DPX mounting media Electron Microscopy Sciences (EMS).

Brightfield microscopy images were taken on a BX51 Olympus scope at X 10 magnification (UPlanFL N; X 10/0.30;Olympus, Tokyo, Japan) and 40X magnification (UPlanFL N; X 40/0.50; Olympus) mounted with a Spot Pursuit RT color digital camera (Diagnostic Instruments, Sterling Heights, MI, USA). Following image acquisition, images were analyzed using Image J software.[1]

Laminar Determination

For laminar determination, alternating sections were counterstained with a 0.5% Cresyl violet (CV) Acetate stain (1% CV in dH_2O in an acetate buffer [9:1 ratio of acidic component (0.6% Glacial acetic acid in dH_2O) to basic component (1.36% sodium acetate in dH_2O)]. ECM analysis was performed in individual layers (2/3, 4, and 6). Determination of layer was performed based on cellular size and density by an experienced observer using neighboring sections stained with CV. The distinct cytological architecture allowed high magnified image collection at the vertical center of each layer. Layer 2/3 (the external pyramidal layer) contains predominantly small and medium sized pyramidal neurons. Layer 4 (the internal granular layer) contains different types of smaller stellate and pyramidal neurons, providing an obvious cytological transition from L2/3 and a definitive border with L5. L5 (the internal pyramidal layer), contains large pyramidal neurons while L6 (the polymorphic or multiform layer) contains few large pyramidal neurons and many small spindle-like pyramidal and multiform neurons. (Mountcastle, 1997) For examples see **Figures 1A,F**, **2A**, **4A**.

Extracellular Matrix Analysis

All image collection and analysis was done blind to genotype and manipulation. Samples were blinded before imaging and uncoded following the completion of analysis.

HA and HSPG Analysis

Images were imported into Image J, background subtracted and overall image intensity measurements (8-bit, 0–255) were

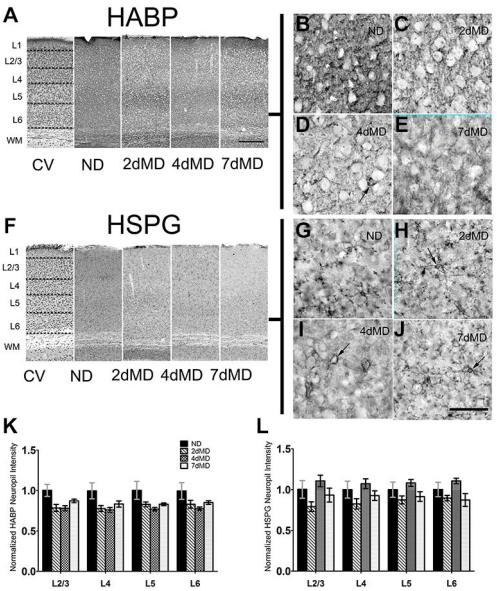

FIGURE 1 | Hyaluronic acid (HA) and heparin sulfate proteoglycans (HSPGs) are differentially regulated in rodent sensory cortex. **(A)** Representative examples of low-mag (4X) images showing laminar demarcation in cresyl violet (CV) alongside HABP immuno-reactive tissue. **(B–E)** hyularonic acid binding protein (HABP) immunohistochemistry in all conditions shows immunoreactivity in neuropil and occasional dense cellular labeling (arrow). **(F)** Representative examples of low-mag (4X) images showing laminar demarcation in CV alongside HSPG immuno-reactive tissue. **(G–J)** Anti-HSPG immunoreactivity displays dense staining around cell bodies (arrows) and in neuropil in all conditions. **(K,L)** HABP labeling intensity in cortical neuropil was altered by deprivation (Two-way ANOVA; main effect of deprivation, $p < 0.05$; Bonferroni *post hoc* analysis, $p > 0.05$ for all comparisons). **(J)** HSPG labeling intensity was unchanged when compared to ND controls suggesting differential regulation of HSPGs following monocular deprivation (MD). Values were normalized to their respective ND controls for presentation purposes only. All values reported are the mean ± standard error of the mean (SEM). Scale bars **(A,F)** = 250 μm; **(B–E,G–J)** = 200 μm.

[1]Freeware: http://rsb.info.nih.gov/ij/

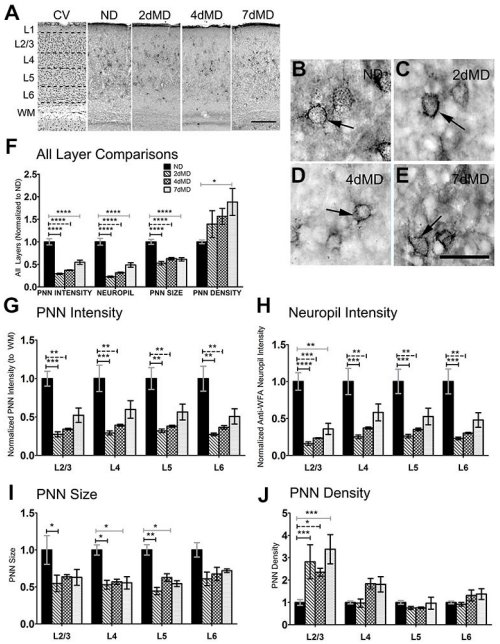

FIGURE 2 | Chondroitin sulfate proteoglycans (CSPGs) are degraded following MD. (A) Representative examples of low-mag (4X) images showing laminar demarcation in CV alongside CSPG (anti-wisteria floribunda agglutinin (WFA)) immuno-reactive tissue. **(B–E)** Anti-WFA immunoreactivity labels CSPG deposits in perineuronal nets (PNNs) (arrows) in all experimental conditions (arrows). **(F)** Quantitative analysis of several labeling parameters across all layers in experimental conditions. Values were normalized to ND controls for presentation purposes only. Significant differences following MD in PNN intensity, Neuropil intensity and PNN size were observed. Changes in PNN density were restricted to 7dMD. **(G–J)** Quantitative analysis of individual labeling parameters across layer. Degradation of CSPG immunoreactivity was most prominent in overall PNN intensity **(G)** and surrounding neuropil **(F)** following 2dMD and 4dMD, where deposition parameters often returned to baseline by 7dMD. PNN size **(I)** was significant reduced in L2/3-5 following 2dMD. This size reduction was also evident in L4 and L5 after 7dMD. A laminar specific increase in PNN density **(J)** was noted only in L2/3. Scale bar **(A)** = 250 μm. **(B–E)** = 50 μm. Statistics = **(F)** = One-way ANOVA within each measurement parameter with Bonferroni multiple comparisons *post hoc* analysis; **(G–J)** = Two-way ANOVA with Bonferroni multiple *post hoc* comparisons. All values reported are the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.001$, $^{***}p < 0.0001$, $^{****}p < 0.00001$.

taken from the primary binocular visual cortex contralateral to the deprived eye. Background was determined in each image based on the average of multiple areas inside selected cell bodies that appeared unstained or very lightly stained. For each section (five sections per animal), values were normalized to the background subtracted values obtained in the white matter (WM) below layer 6 where staining was present, albeit less intense than in the cortex, and where immunoreactivity was expected to be insensitive to MD to control for staining variance across animals. The values were further normalized to the average ND value for display purposes only.

CSPG Analysis

Images were imported into Image J and background subtraction was performed on each image (n = 5 per animal). PNNs were identified by an experienced observer. All PNNs were included irrespective of staining intensity as long as they were determined to be in focus in the image. PNNs were manually traced using the tracing tool in ImageJ. Staining intensity of the PNN was collected as well as PNN size. These structures were then removed from the image, a layer mask applied and the neuropil intensity was determined. To control for variability in staining PNN and neuropil intensity values were normalized to the intensity observed in the WM as for HABP and HSPG analysis. PNN density was determined as the number of PNNs observed divided by the area analyzed. For all parameters values were normalized to the average ND value in each condition for display purposes only.

Intrinsic Signal Optical Imaging

To induce ODP, mice (C57Bl/6: ND = 10, 4dMD = 9, 7dMD = 9; MMP9 KO: ND = 5, 4dMD = 6, 7dMD = 7) were monocularly deprived for 4 and 7 days at the height of the critical period for cortical plasticity. On P28 ± 2, lid margins were resected and lids sutured under isoflurane anesthesia (2–3%). After 4 and 7 days of MD, animals were anesthetized with isoflurane (2–3%) along with chlorprothixene (2 mg/kg) and the sutures were removed for imaging. The skull over visual cortex was cleared, covered with agarose (1%) and a coverslip and illuminated with 700 nm light. Anesthetic level was maintained with isoflurane (0.75%) during imaging. IOS was performed using a DALSA 2 M30 CCD camera (Kalatsky and Stryker, 2003). An image of the vascular pattern was obtained through the skull by illumination with a green filter (550 nm). Intrinsic signal images were then captured using a red filter (700 nm). Visual stimuli consisting of white horizontal square-wave bars on a neutral background moving downward (270°) and upward (90°) for 6 min per run, were presented to each eye separately. The amplitude of the fast fourier transform component in the binocular visual cortex was analyzed offline using Matlab to determine OD (Kalatsky and Stryker, 2003; Tropea et al., 2010). OD was compared between MMP9 deficient mice and C57Bl/6 controls. An ocular dominance index (ODI) was calculated as (contralateral-ipsilateral)/(contralateral + ipsilateral) based on the average pixel intensities of the images obtained during visual stimulation of each eye (Cang et al., 2005). Positive ODI values indicate a contralateral bias; negative values indicate an ipsilateral bias.

Dendritic Spine Analysis

To examine the effects of MMP9 depletion on dendritic spine density and morphology, MMP9 KO mice were crossed with GFP-M mice (Feng et al., 2000). MMP9KO/GFP (n = 6; 657 spines total) mice were compared to GFP-M controls (n = 6; 927 spines total). Animals were perfused between P32 and P35. Brains were sectioned on a freezing sliding microtome to a 50 μm thickness. Sections were mounted out of a 0.1 M PBS solution and coverslipped with Prolong Gold (Invitrogen) anti-fade media. Confocal microscopy image acquisition and spine analysis was performed as described previously (Bogart et al., 2011). Briefly, layers 2/3 within the primary somatosensory cortex (S1) were identified for imaging on a Zeiss LSM 510 confocal microscope

(Care Zeiss, Thornwood NY). The distributions of imaged areas within S1 were similar between experimental conditions. GFP-labeled brain sections were excited at 488 nm and imaged through an HFT 514/633 dichroic and 530–600 nm band pass filter. Excitation power and settings for pinhole and detector gain were optimized to minimize photobleaching and utilize the full dynamic range of fluorophore emission intensity. High resolution (512 × 512 pixels) confocal image stacks of layer 5 apical dendritic branches located in layer 2/3 were collected using a 100 × oil-immersion lens (NA 1.46), at a digital zoom factor 2 (pixel size 0.082 μm), and a *z*-step of 0.5 μm. Additional *z*-stacks were collected using lower power objectives to document the position of acquired images within the dendritic arbor stacks. Dendritic segments of the primary apical dendrite in layer 2/3 were located between 70 and 150 μm from the pial surface and were selected based on the quality of GFP expression and resulting signal-to-noise ratio, so that spines could be identified and measured as accurately as possible.

Following image acquisition, *z*-stacks were exported to TIF format using Zeiss's Axiovision software (release 4.6). Image analysis was then done using Image J. To quantify spine density, spines were identified by manually stepping through the *z*-stack, and marked on the projected image. Only spines located in the plane of their parent dendrite branch were marked and counted. Spines falling out of plane and those projecting from the parent dendritic branch in the *z*-dimension were systematically excluded from our counts even if they were visually identifiable as spines. For the purposes of this study we define spines as all visible dendritic protrusions and filopodia are included in the analysis. After all spines on a segment were marked, segment length was measured using the segmented line tool. 3D segment length was accounted for by measuring the absolute difference in depth between the two ends of the segment and using the Pythagorean Theorem. Spine density was then computed as the number of spines per micron of dendrite. Since spine density varies with dendritic diameter (Irwin et al., 2002), we ensured that analyzed dendrites were well matched between the two genotypes. Dendritic diameters were not significantly different between the two groups (CTL ND = 1.628 μm ± 0.148, MMP9 KO = 2.040 μm ± 0.190, Student's *t*-test, $p = 0.118$). We also analyzed the dimensions of dendritic spines. Spine length was measured on maximum intensity projections using a segmented line tool to draw a line from the most distal point of the spine head to the base of the spine neck where it connects to the parent dendritic branch. Measurements of spine head and neck width were made based on fluorescence measurements. The fluorescence profile of a line placed along the center of the head and neck was determined and fit to Gaussian using custom-written algorithms in MATLAB (The MathWorks, Inc., Natick, MA). The full-width half-max was taken as a measure of spine head width. This method may overestimate the size of small spines that fall under the limit of the resolution of our confocal microscope. The amplitude of the Gaussian fit to the spine neck fluorescence profile was normalized to the amplitude of the fit to the spine head profile as a relative measure of spine neck width. Background fluorescence was subtracted before fitting on a dendrite-by-dendrite basis. Great care was taken to avoid saturation in images, and saturated points were removed from the fluorescence profiles. Spines with more than two saturation points were removed from the analysis as it was determined that accurate fits were obtained if fewer than three points were omitted. This affected less than 2% of the population of spines.

Two-photon Imaging and Dendritic Spine Turnover Analysis

For two-photon imaging, mice (GFP-M = 5, 1, 432 spines total; MMP9 KO ND = 8, 927 spines total) were anesthetized with a fentanyl cocktail (fentanyl; 0.05 mg/kg of body weight; midazolam; 5 mg/kg; medetomidine; 0.5 mg/kg; i.p.); the skull was exposed, cleaned and glued to a thin metal plate. S1 was identified according to stereological coordinates. The skull above the imaged area was thinned with a dental drill. During surgery and imaging, the animal's temperature was kept constant at 37°C with a heating pad and anesthesia was maintained with periodic administration of fentanyl. Imaging and data analysis were carried out as previously described (Majewska et al., 2006). A custom-made two-photon scanning microscope (Majewska et al., 2000) was employed, using a wavelength of 920 nm and a 20 × 0.95 NA objective lens (Olympus, Melville, NY) at 8.5× digital zoom. A map of the blood vessels was taken as a reference point. After image acquisition the animal's scalp was sutured and the animal was allowed to recover before being placed back in its home cage. Four days later, the animal was re-anesthetized and the skull re-exposed. The blood vessels map and dendritic architecture were used to identify the same imaging regions. Dendritic protrusions were identified as persistent if they were located within 0.5 μm laterally on the subsequent imaging session. Elimination and formation rates refer to the numbers of lost spines and new spines, respectively, observed on the second imaging time point divided by the total number of spines present in the first imaging session.

Immunoperoxidase Reactivity for Electron Microscopy

Sections (C57Bl/6 ND = 3, MMP9 KO ND = 3) were immersed in 0.1% borohydride (in 0.1 M PBS) for 30 min at RT, washed in 0.1 M PBS, and processed freely floating following a pre-embedding immunoperoxidase protocol previously described (Riad et al., 2000; Tremblay et al., 2010b). Briefly, sections were rinsed in 0.1 M PBS, followed by a 2 h pre-incubation at RT in a blocking solution containing 5% normal goat serum and 0.5% fish gelatin. Sections were then incubated for 48 h at RT in rabbit anti-Iba-1 (1:1,000 in blocking solution; Wako Pure Chemical Industries) and rinsed thoroughly in 0.1 M PBS (pH 7.4). Sections were incubated for 2 h at RT in goat anti-rabbit IgG conjugated to biotin (Jackson Immunoresearch) and with streptavidin-horseradish peroxidase (Jackson Immunoresearch) for 1 h at RT in blocking solution. Immunoreactivity was visualized with diaminobenzidine (0.5 mg/ml) and hydrogen peroxide (0.03%) in buffer solution (DAB Peroxidase Substrate Kit; Vector Laboratories). Sections were then post-fixed flat in 1% osmium tetroxide and dehydrated in ascending concentrations of ethanol. They were treated with propylene oxide, impregnated

in Durcupan EMS overnight at RT, mounted between ACLAR embedding films EMS, and cured at 55°C for 48 h. Areas of the primary visual cortex (V1, Layer 2), at the level approximating the transverse planes A + 0.16 to A + 0.72 (Franklin and Paxinos, 2008), were excised in a trapezoid shape from the embedding films in a selected orientation to accurately determine the pial surface during ultrathin sectioning and re-embedded at the tip of resin blocks. Ultrathin sections (60–80 nm; evidenced by the sections silver sheen) were cut with an ultramicrotome (Reichert Ultracut E) and collected on bare square-mesh grids.

Electron Microscopy Imaging and Data Analysis

Eighty pictures were randomly taken at 40,000× in layer 2 of V1 (approximately 10 microns from the pial surface) in each animal at the tissue-resin border corresponding to a total surface of ∼1,000 μm^2 of neuropil per animal (as in Tremblay et al., 2007, 2009, 2010a; Bouvier et al., 2008, 2010; Kelly et al., 2010; Mortillo et al., 2012, among others). Images were captured on a Hitachi 7650 Transmission Electron Microscope using a Gatan 11 megapixel Erlangshen digital camera and Digitalmicrograph software. TIFF images were exported into Adobe Photoshop (CS5.5) and adjusted for brightness and contrast in preparation for analysis. Cellular profiles were identified using a series of criteria previously defined in single-ultrathin sections (Peters et al., 1991; Tremblay et al., 2009, 2010a; Lu et al., 2011). Asymmetrical synapse density and the length of the postsynaptic density (PSD) were analyzed using Image J software (500 μm^2/animal/genotype; n = 3 CTL ND, n = 3 MMP9 KO ND). Only asymmetrical synapses that displayed visible neurotransmitter vesicles in the presynaptic terminal and an abutting postsynaptic membrane containing an electron dense PSD were included in the study. Using the line tool in Image J software, a straight line was drawn from edge to edge of the PSD to determine PSD length. Neighboring structures to Iba-1 immunoreactive elements were classified into the following categories: *dendrite (shaft)*, *dendritic spines, putative filopodia*, *axon terminal*, and glial elements (including *microglia* and *astrocytes)*. We conservatively classified all the subcellular profiles that were difficult to identify as "unknown". See Kelly et al. (2014) for electron microscopy element classification details. To control for the fact that large processes have the ability to interact with more microglial processes and contain more inclusions, we normalized our observations to the size of the microglia processes analyzed.

Microglial Morphology Analysis

To examine the effects of MMP9 depletion on microglial morphology, C57Bl/6 (CTL, n = 6 mice) and MMP9 KO (n = 6 mice) brain slices were processed with anti-Iba-1, a microglial marker. For brain harvesting, mice were anesthetized with sodium pentobarbital (150 mg/kg; i.p.) at P32 and perfused through the aortic arch with ice-cold phosphate-buffered saline (0.1 M PBS, 0.9% NaCl in 50 mM phosphate buffer [pH 7.4]) followed by 4% paraformaldehyde (PFA; in 0.1 M PBS, pH 7.4). Brains were post-fixed in 4% PFA for 2 h and transferred to an increasing gradient of sucrose (10, 20, 30% in ultra-pure water) at 4°C. Brains were sectioned coronally at a 50 μm thickness on a freezing, sliding microtome (Microm; Global Medical Instrumentation, Ramsey, MN). Fixed brain sections containing visual cortex were immersed in 0.1% sodium borohydride (in 0.1 M PBS) for 30 min at RT, washed in 0.1 M PBS, and processed freely floating. Sections were blocked in a solution containing 0.5% BSA, 5% normal serum and 0.3% Triton-x for 2 h. Sections were then incubated for 24–48 h in rabbit anti- Iba-1 [ionized calcium binding adaptor molecule-1; microglia- marker; 1:2500; Wako; (Imai et al., 1996)]. Sections were washed in 0.1 M PBS and incubated for 2 h in a solution containing anti-rabbit Alexa 594 (Invitrogen). Sections were mounted out of a 0.1 M PBS solution and coverslipped with Prolong Gold (Invitrogen) antifade media. Z-stack images from layers 2/3 within binocular visual cortex were collected on a Zeiss LSM 510 confocal microscope (Care Zeiss, Thornwood NY, USA) and images were imported into Image J for analysis. The process area, soma area, process/soma length (LA, longest axis of the process/soma area) and process/soma width (SA, perpendicular to the process length) were measured using Image J. The circularity index was based on the following equation: 1−(LA−SA/LA+SA).

Statistical Analysis

Statistical analysis was performed using Prism VI statistical analysis software (Graphpad Software, Inc; La Jolla, CA). All values reported are the mean ± standard error of the mean (SEM). For all analyses, significance was based on $\alpha = 0.05$. When comparing between groups, significance was determined using two-tailed unpaired Student t-tests and Bonferroni multiple comparison *post hoc* analysis. Initial multi-group comparisons were performed using one-way and two-way ANOVAs. On two occasions, an outlying number was omitted following the use of the Grubbs Outlier Test.[2]

Results

Degradation of the ECM promotes neurite outgrowth, axon regeneration and functional recovery (Bradbury et al., 2002; Pizzorusso et al., 2002), and may therefore play important roles during experience-dependent plasticity. To determine if ECM composition contributes to plastic changes in the rodent visual cortex, we profiled the degree of degradation of several components of the ECM following MD in the binocular visual cortex. MD was performed within the visual critical period beginning on postnatal day (P)28 (Gordon and Stryker, 1996, for animals numbers throughout the results please See "Materials and Methods" Section). We decided to focus on three important components of the brain ECM: HA, which comprises the fundamental base of the ECM lattice (Yamaguchi, 2000), heparin sulfate proteoglycans (HSPGs) and CSPGs which are strongly associated with PNNs (Deepa et al., 2006). We hypothesized that MD would stimulate ECM degradation to facilitate plasticity. As plasticity is elicited differentially across cortical layers following MD (Trachtenberg and Stryker, 2001; Oray et al., 2004), we

[2]http://graphpad.com/quickcalcs/Grubbs1.cfm

performed quantitative analysis of the overall immunoreactivity across cortex and in specific layers to determine possible layer-specific effects of ECM degradation over time following deprivation (**Figures 1A,F**). Immunohistochemical analysis of HA (using HA binding protein; HABP) displayed a prominent deposition in the neuropil (**Figures 1B–E**) with occasional densities around cell bodies, similar to previous reports (as shown in **Figure 1D**; Costa et al., 2007). While levels of HABP immunoreactivity were decreased in all layers at all time points following MD (two-way ANOVA, effect of deprivation $p < 0.0001$), no statistically significant effects were observed in individual layers (Bonferroni *post hoc* tests; $p > 0.05$). This suggests that although deprivation elicits degradation of HA, the effect is subtle. (**Figure 1K**).

Similarly, HSPG immunoreactivity revealed both pronounced deposition around cell bodies, forming concentrated rings around several cells that extended into proximal neuronal processes (**Figures 1G–J**), and in the neuropil, resulting in diffuse patterning throughout all layers in all conditions. HSPG deposition was not significantly affected following MD (**Figure 1L**) suggesting differential regulation of HSPG as compared with HA during MD.

WFA immunoreactivity, indicative of CSPG distribution, was prevalent in PNN structures that thoroughly encased a subset of neuronal cell bodies (**Figures 2A–E**). This dense perineuronal accumulation was found in all deprivation conditions, where it extended into the surrounding neuropil. Given the fundamental role of CSPG composition in plasticity regulation (Bandtlow and Zimmermann, 2000), and also the more complex nature of its immunoreactivity, we investigated several CSPG immunoreactivity parameters, including PNN staining intensity, PNN size, PNN density (number of PNNs/unit area) and overall neuropil immunoreactivity. Beginning as early as 2dMD, we noted a significant reduction in PNN intensity, PNN size, and neuropil composition ($p < 0.05$–0.001, two-way ANOVA, Bonferroni multiple comparisons *post hoc* analysis) when compared to ND controls (**Figure 2F**). When analyzed independently, all cortical layers showed significant changes in PNN intensity and neuropil composition at both 2dMD and 4dMD (**Figures 2G,H**; two-way ANOVA, Bonferroni multiple comparisons *post hoc* analysis) with a recovery by 7dMD. Interestingly, a reduction in PNN size was found primarily after 2dMD in superficial layers which recovered by 7dMD in most layers (**Figure 2I**). Finally, changes to PNN density were restricted to superficial layers in all MD conditions (**Figure 2J**). These findings suggest that ECM degradation occurs rapidly following MD during the time when deprived eye inputs weaken, and recovers when plasticity is maximal at 7 days of deprivation (Frenkel and Bear, 2004).

ECM degradation is regulated by proteases (such as MMPs) secreted by both neurons and glia (Webster and Crowe, 2006; Dziembowska and Wlodarczyk, 2012; Konnecke and Bechmann, 2013), and this process is thought to promote synaptic plasticity (Mataga et al., 2002, 2004; Pizzorusso et al., 2002; Oray et al., 2004). MMP9 in particular is highly expressed in the brain, and is rapidly upregulated during plasticity (Szklarczyk et al., 2002). In the somatosensory cortex, MMP9 activity is upregulated following whisker deprivation and affects experience-dependent barrel remodeling (Kaliszewska et al., 2012), suggesting that a similar mechanism could be used in the visual cortex. We first examined whether CSPG composition in the primary visual cortex was altered by loss of MMP9 (**Figure 3**). Analysis of PNN intensity, neuropil intensity, PNN size and PNN density (**Figures 3A–D**, two-way ANOVA with Bonferroni multiple comparisons *post hoc* analysis) showed a similar deposition and distribution of CSPGs in the visual cortex of WT and MMP9 KO mice. While lower levels of PNN and neuropil staining, PNN size and density were observed in MMP9 KO mice (two-way ANOVA, effect of genotype $p < 0.05$), few of these changes reached statistical significance and were generally restricted to layer 5 (Bonferroni *post hoc* analysis; $p < 0.05$). The magnitude of the changes was also small compared to the degradation observed following MD (**Figure 2**), suggesting that developmental loss of MMP9 has a limited effect on CSPG regulation. To determine if MD-induced ECM degradation was mediated by MMP9, we monocularly deprived MMP9 KO mice for 2, 4, and 7 days and assayed CSPG degradation using anti-WFA immunoreactivity. CSPG deposition in MMP9 KO mice was observed in dense accumulations around cell bodies, as well as dense labeling in the neuropil in all deprivation conditions (**Figures 4A–E**) as in WT animals. In contrast to the ECM degradation we observed in control animals following MD (**Figure 2**), CSPG composition remained intact in MMP9 KO mice (**Figures 4G–J**), suggesting that MMP9 contributes to ECM remodeling during ODP.

Next we wanted to determine whether MMP9-mediated ECM degradation contributes to functional OD shifts. Therefore we performed intrinsic signal optical imaging (iOS) on control (C57Bl/6; CTL) and MMP9 KO mice following 4 days of monocular deprivation (4dMD) and quantified binocularity by calculating an ODI. An ODI above 0 represent a contralateral

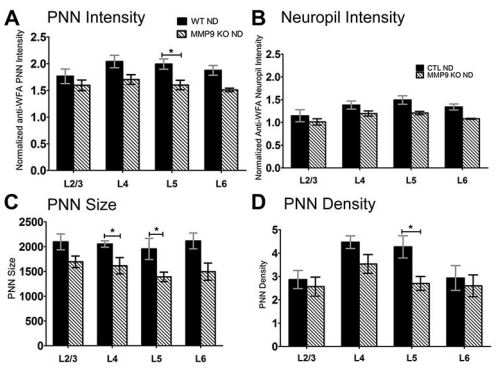

FIGURE 3 | CSPGs composition comparisons between WT and matrix metalloproteinase 9 (MMP9) knock-out (KO) mice. (A–D) Quantitative analysis of PNN intensity, Neuropil intensity, PNN size, and PNN density between WT and MMP9 KO ND mice showing few differences between the ND conditions. Significant decreases were noted in MMP9 KO mice in L5 of PNN intensity, PNN size and PNN density suggesting a laminar effect of MMP9 depletion. Statistics = Two-way ANOVA with Bonferroni multiple comparisons *post hoc* analysis. All values reported are the mean ± SEM. $^*p < 0.05$.

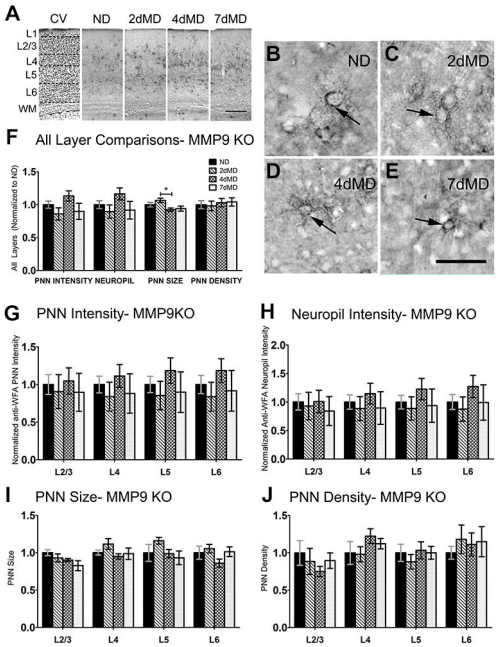

FIGURE 4 | CSPG degradation is absent in MMP9 KO mice. **(A)** Representative examples of low-mag (4X) images showing laminar demarcation in CV alongside CSPG (anti-WFA) immuno-reactive tissue in MMP9 KO mice. **(B–E)** Anti-WFA immunoreactivity labels CSPG deposits in PNNs (arrows) in all experimental conditions in MMP9 KO mice. **(F)** Quantitative analysis of several labeling parameters in all layers across experimental conditions. Values were normalized to ND controls for presentation purposes only. Little degradation is apparent following MD. **(G–J)** Quantitative analysis of individual labeling parameters across layer. No significant degradation of CSPG immunoreactivity was evident suggesting that the lack of MMP9 attenuated the effects of MD on extracellular matrix (ECM) degradation. Scale bar **(A)** = 250 μm; **(B–E)** = 50 μm. Statistics = Two-Way ANOVA with Bonferroni multiple comparison *post hoc* analysis. All values reported are the mean ± SEM. $^*p < 0.05$.

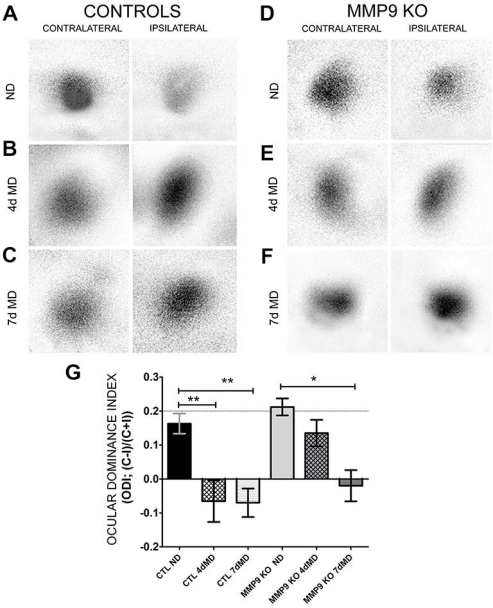

FIGURE 5 | Ocular dominance plasticity (ODP) is attenuated in MMP9 KO mice. (A–F) Representative examples of visually-evoked amplitude maps measured using intrinsic signal optical imaging (iOS) in control (C57Bl/6) and MMP9 KO mice. **(G)** Quantitative analysis of ODP comparing changes in the ocular dominance index (ODI). Values above 0 denote an ODI with a contralateral bias, while values below value suggest an ODI with an ipsilateral bias. CTL ND (black bar) have a characteristic and expected contralateral bias that significantly shifts to an ipsilateral bias after both 4dMD (CTL 4dMD, white hatched bar) and 7dMD (CTL 7dMD, white dotted bars). MMP9 KO ND (gray bar) displayed a contralateral bias, similar to CTL ND. Following 4dMD (gray hatched bar), MMP9 KO mice failed to demonstrate an ODP shift. ODP was apparent in MMP9 KO 7dMD (gray dotted bar). Statistics = Two-Way ANOVA with bonferroni multiple comparisons. All values reported are the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.001$.

bias, while values below 0 represent an ipsilateral bias (Cang et al., 2005). CTL mice showed the expected contralateral bias in the absence of MD (**Figures 5A,G**, black bar, 0.163 ± 0.03). Following MD, responsiveness shifted from a contralateral to ipsilateral bias (**Figures 5B,G**, white hatched bar (−0.06 ± 0.06), white dotted bar (−0.07 ± 0.04). Non-deprived MMP9 KO mice also showed a strong contralateral response, with an ODI comparable to CTL ND mice (**Figures 5D,G**, gray bar, 0.212 ± 0.02). After 4 days of MD, however, ODIs failed to shift towards the ipsilateral eye (**Figures 5E,G**, gray hatched bar, 0.135 ± 0.04). To determine whether plasticity was reduced or slowed by the absence of MMP9, we repeated the experiment after 7 days of MD. By 7dMD, MMP9 KO mice displayed a significant ipsilateral shift similar to that observed in CTL 7dMD mice (**Figures 5C,F,G**, gray dotted bar, −0.02 ± 0.04, $p < 0.05$, two-way ANOVA, Bonferroni multiple comparisons) suggesting that MMP9 deficiency delays the induction or manifestation of ODP.

The effects of MMP9 KO on plasticity may be explained by the lack of ECM remodeling following deprivation or by other changes that may occur in the brain due to developmental loss of MMP9 that can leave the cortex in a less plastic state. Our finding that CSPG deposition is altered to a small extent in MMP9 KO mice, suggests that developmental changes in the ECM are unlikely to limit plasticity. However, MMPs can have profound effects on the development of neural circuits (Aerts et al., 2015), as well as the function of other cell types in the CNS (Hansen et al., 2013). Therefore we decided to further characterize the MMP9 KO mice to determine what other changes could contribute to the blunted experience-dependent plasticity we observed. We decided to focus specifically on dendritic spine development and microglial phenotypes, both of which have been shown to be critical for visually-driven plasticity (Oray et al., 2004; Tremblay et al., 2010a).

MMP9 and the ECM can regulate the structure of dendritic spines, the postsynaptic sites of excitatory synapses, (Szklarczyk et al., 2002; Ethell and Ethell, 2007), and dynamic changes in dendritic spine structure accompany ODP (Oray et al., 2004). To ascertain whether changes in ECM composition in MMP9 KO mice may differentially regulate cortical excitatory synapses and thus affect plasticity, we used electron microscopy to quantify asymmetric synapse density (**Figures 6A–C**) and PSD size

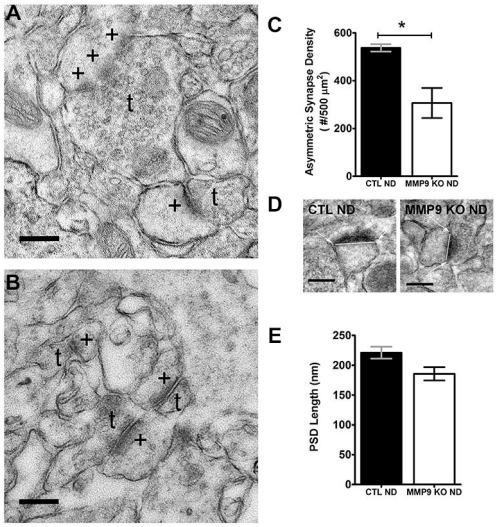

FIGURE 6 | Synapse Density but not postsynaptic density (PSD) size is affected in MMP9 KO mice. Representative examples of images obtained from CTL ND **(A)** and MMP9 KO ND **(B)** visual cortex following pre-embedding electron microscopy ultra-structural preparation for synaptic profiling. Images show the appearance of asymmetric (excitatory; glutamatergic) synapses (denoted by +) abutting presynaptic terminals containing neurotransmitter vesicles (denoted by *t*). **(C)** Quantitative analysis of the total number of asymmetrical synapses in CTL ND (black bar) vs. MMP9 KO ND (white bar) showing a significant decrease in MMP9 KO synapses. **(D)** Examples of PSD length measurements performed using Image J software in CTL ND (first panel) and MMP9 KO ND (second panel). **(E)** Quantitative analysis of PSD lengths in CTL ND (black bar) and MMP9 KO ND (white bar). No significant difference was observed. Scale bars = 200 nm **(A,B)**; 250 nm **(D)**. Statistics = Students *t*-test. All values reported are the mean ± SEM. $^*p < 0.05$.

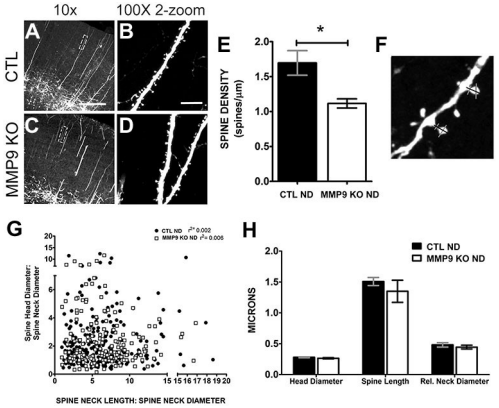

FIGURE 7 | Dendritic spine density and morphology are differentially affected in MMP9 KO mice. Representative confocal images of CTL **(A,B)** and MMP9 KO **(C,D)** cortical dendrites of layer 5 pyramidal neurons. **(E)** Quantitative analysis of spine density showed significantly more spines in CTL ND (black bar, 1.697 ± 0.176) compared to MMP9 KO mice (white bar; 1.116 ± 0.065; Student's *t*-test with Mann Whitney test, $^*p < 0.05$). **(F)** Example of spine measurements. Spine neck length (black bar) and spine head and neck width (gray bars) measurements were made using Image J software. **(G)** Correlation analysis depicting no differences between head and neck comparisons across genotypes (Linear regression analysis; CTL ND $r^2 = 0.002$, MMP9 ND $r^2 = 0.006$). **(H)** Head diameter, spine length, and relative neck diameter were comparable between the two genotypes (Two-way ANOVA with bonferroni multiple comparisons). Scale bar = 200 μm. All values reported are the mean ± SEM.

(**Figure 6D**) in layer 2 of visual cortex. We found that MMP9 KO mice had significantly fewer asymmetric synapses in the same cortical area when compared to CTL mice (**Figure 6C**). This change in synaptic density was not accompanied by changes in PSD size (**Figure 6E**). These data suggest that deficits in excitatory synapse development may contribute to the plasticity phenotype observed in MMP9 KO mice, and that synaptic density rather than synaptic strength may be affected.

To further explore the differences in synaptic behavior caused by loss of MMP9, we crossed MMP9 KO mice with GFP-M transgenic mice (Feng et al., 2000) in which GFP is expressed in L5 pyramidal neurons (**Figures 7A–D**). Because GFP expression is low in visual cortex during adolescence we assayed dendritic spine morphology in S1, a brain area in which dendritic spine development mirrors that in primary visual cortex (Elston and Fujita, 2014). Confocal microscopy revealed GFP-labeled dendritic branches in both GFP-M (CTL ND, **Figure 7A**) and MMP9 KO-GFP (MMP9 KO, **Figure 7C**) adolescent (P28) S1, with clearly discernable dendritic spines on the apical dendritic branches (in L2/3) in both genotypes (**Figures 7B,D**). As the formation and elimination of synapses is believed to be one of the mechanisms underlying adaptive remodeling of neural circuits (Trachtenberg et al., 2002; Majewska et al., 2006) and previous reports implicate a role for MMP9 on dendritic spine development (Szklarczyk et al., 2002; Tian et al., 2007; Stawarski et al., 2014), we wanted to know how the density, structure and dynamics of dendritic spines were affected by MMP9 loss. We first wanted to determine whether dendritic spines in S1 were regulated similarly to those in V1. Therefore, we first compared changes in synaptic density between GFP-M and MMP9 KO-GFP mice in S1 to see if initial development of networks was compromised. We found a significant reduction in spine density in MMP9 KO mice (**Figure 7E**, $p < 0.05$), consistent with the decrease in asymmetric synapse density observed in V1 (**Figure 6C**). This suggests that MMP9 may have similar roles in synaptic development across sensory cortical areas. Since spine density changes are often associated with alterations in spine morphology (Wallace and Bear, 2004), we measured the dimensions of each spine, including spine head and relative neck diameter (**Figure 7F**, gray bars) and dendritic spine length (**Figure 7F**, black bar measuring from dendritic shaft to tip of protrusion). Changes in spine shape can predict spine developmental profiles; where thinner spine heads and longer necks suggest an immature phenotype while a wider head and short neck might suggest maturity (reviewed in Sala and Segal, 2014). Interestingly, while we noted a significant reduction in dendritic spine density in MMP9 KO mice, we found no differences in the distribution of spine "types", as spine head and neck comparisons did not differ across genotypes and these comparisons correspond to relative distributions of mushroom, thin and stubby spines (**Figure 7G**). Similarly,

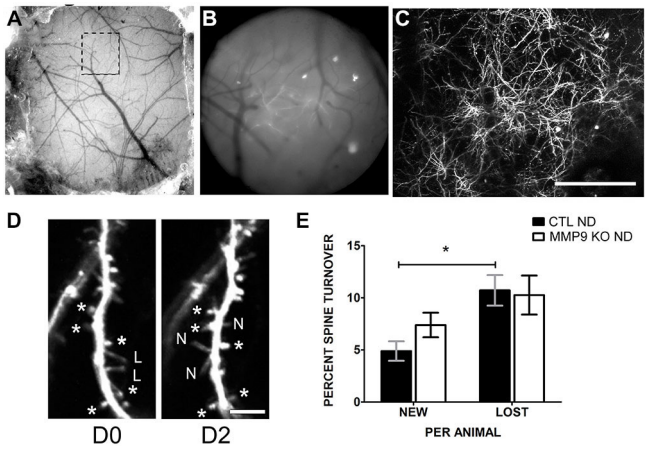

FIGURE 8 | Dendritic spine turnover is altered in MMP9 KO mice. **(A)** Example of thin skull preparation viewed through dissecting scope showing clear demarcation of brain vasculature. **(B)** Magnified epifluorescent image of the region of interest (ROI, hatched box in **(A)**) visualized with a 20 × 0.95 NA objective lens. Vasculature landmarks aided in relocating ROI in chronic imaging. **(C)** Two-photon image of the same region (as **A** and **B**) taken at 1× digital zoom which served as a reference map of magnified vessels and dendritic branches. **(D)** 8x digital zoom of dendritic branches and spines on D0 (first panel) and identical branches at D2 (second panel). Asterisks (*d**) denote reference spines between images. L = lost spines, N = new spines. **(E)** Quantitative analysis of percent dendritic spine turnover in CTL ND (black bars) and MMP9 KO ND (white bars) mice. CTL ND mice showed significantly more lost spines than gained spines after 2 days (Two-Way ANOVA, $^*p < 0.05$). Spine loss and gain were not significantly different in MMP9 KO ND mice. Scale bar = 200 μm **(A–C)**, 5 μm **(D)**. All values reported are the mean ± SEM.

spine morphology was unchanged when compared to GFP control mice (**Figure 7H**) suggesting no clear changes in the morphological classes of spines present in GFP CTL and MMP9 KO GFP mice, supporting the data obtained in visual cortex showing no difference in PSD size between the two genotypes.

Alterations in spine density may be the result of aberrant spine formation or elimination. To determine if spine dynamics are affected by MMP9 deficiency, we investigated dendritic spine turnover in GFP-M CTL mice and MMP9 KO GFP *in vivo* using chronic two-photon (2P) imaging. The skull over S1 was thinned to reveal the cortical vasculature (**Figures 8A–C**) which was used as a reference for chronic imaging. Dendritic spines from the apical tufts of L5 pyramidal neurons were imaged on D0 (first day imaging, P28) and the same spines were reimaged 2 days later (D2) (**Figure 8D**). Under basal conditions (CTL ND), CTL GFP-M mice displayed a significantly greater rate of spine loss compared to spine gain (**Figure 8E**, black bars, $p < 0.05$), as has previously been described during adolescence (Zuo et al., 2005). This effect was not seen in mice lacking MMP9, where loss and gain rates were well matched (**Figure 8E**, white bars). These results suggest that spine dynamics are altered in MMP9 KO mice.

Microglia secrete proteases (Webster and Crowe, 2006; Konnecke and Bechmann, 2013), including MMP9, that contribute to localized proteolytic activity during physiological and pathological events. Furthermore, microglial interactions with synapses have recently been shown to promote healthy brain homeostasis, including the regulation of cell death, synapse elimination, neurogenesis, and neuronal surveillance (Paolicelli et al., 2011; Tremblay et al., 2011; Wake et al., 2013). Thus MMP9 depletion may alter microglia morphology and function and influence dendritic spine pruning (Szklarczyk et al., 2002). To determine the effects of MMP9 deficiency on microglia, we first investigated changes in microglial morphology as these can indicate changes in microglial function. Whereas pathological microglial activation is characterized by the thickening, polarization and retraction of branches, as well as an increase in soma size, a resting/ quiescent state is characterized by a ramified, circular arbor and small soma (Hanisch and Kettenmann, 2007; Kettenmann et al., 2011). CTL and MMP9 KO tissue from the binocular visual cortex was processed for the anti-microglial marker, Iba-1, visualized with immunofluorescence and analyzed using confocal microscopy. Confocal *z*-stack images allowed us to fully assess the extent of microglial process arbor (**Figure 9A**) and soma (**Figure 9D**). While we noted no significant difference in the area occupied by the microglial process arbor in CTL and MMP9 KO microglia (**Figure 9B**), we found a small but significant decrease in the process circularity index (**Figure 9C**, $p < 0.05$, Student's *t*-test) signifying arbor elongation in MMP9 KO microglia. MMP9 KO microglia also had a small but significant increase in soma size (**Figure 9E**, $p < 0.05$, Student's *t*-test) but not soma circularity (**Figure 9F**). These results suggest that MMP9 deficiency has a limited effect on microglial morphology but the morphological changes observed suggest a more activated state.

To further investigate the potential activation of microglia, we preformed pre-embedding immuno-peroxidase electron microscopy on samples taken from the primary visual cortex at P28 in both control C57BL/6 and experimental MMP9 KO mice. Sections were processed for Iba-1 immunoreactivity, resulting in clear demarcation of microglial processes, internal content and neighboring elements in both control (CTL ND, **Figure 10A**) and MMP9 KO mice (MMP9 KO ND, **Figure 10B**). We observed no difference in microglial process size in MMP9 KO mice, but found significantly larger pockets of extracellular space surrounding microglia (**Figure 10C**). Furthermore, we found significantly more inclusions (intracellular vacuoles often containing engulfed cellular content) in the MMP9 KO (**Figure 10D**). To determine whether this increase in inclusions was due to changes in microglial behavior towards synapses (Paolicelli et al., 2011), we quantified microglial contacts with stereotypic excitatory synapses. We measured the degree of microglial contact with excitatory pre- and postsynaptic terminals (characterized by synaptic vesicle accumulation in the presynaptic terminal abutting a postsynaptic terminal containing a visible PSD) often surrounding the synaptic cleft. We found no differences in microglial contacts with the synaptic cleft (**Figure 10E**) suggesting that increased phagocytosis in MMP9 KO microglia may not be specific to synapses. We also found a similar profile of structures that contacted microglial processes in CTL or MMP9 KO microglia (aside from a significant increase in the number of pockets of extracellular space surrounding MMP9

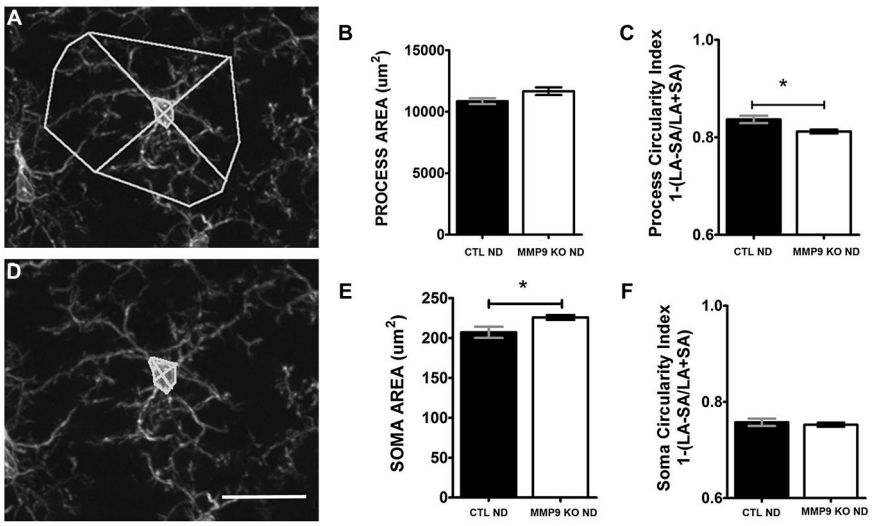

FIGURE 9 | MMP9 KO mice show changes in microglial morphology. (A) The area occupied by microglial processes was measured using Image J software. **(B)** Quantitative analysis of microglial process area was not significantly different between CTL ND (black bars, 0.837 ± 0.008) and MMP9 KO ND (white bars, 0.812 ± 0.004; unpaired student's *t*-test). **(C)** Process circularity index [1−(LA−SA)/(LA+SA)] was significantly decreased in MMP9 KO ND mice (unpaired student's *t*-test, $^*p < 0.05$) **(D)** The area of microglial soma was measured using Image J software. **(E)** Quantitative analysis of the soma area showed significantly larger microglial soma in MMP9 KO ND (unpaired student's *t*-test, $^*p < 0.05$) with no significant changes in overall soma circularity **(F)** Scale bar = 20 μm. All values reported are the mean ± SEM.

KO microglia), further suggesting a lack of microglial targeting to specific elements in MMP9 KO mice as compared to CTL (**Figures 10F,G**).

Discussion

In this study, we analyzed the role of MMP9, one of the primary MMPs in the brain, in experience-dependent plasticity. We show that several components of the ECM are differentially regulated following MD in the rodent primary visual cortex, and that the degradation of CSPGs after MD is not present in MMP9 KO mice. ODP was also attenuated in MMP9 KO mice following MD showing that MMP9 is an important player in experience-dependent plasticity. Inspection of excitatory synapses and dendritic spines revealed significantly lower asymmetric synapse density in layer 2, and lower spine density on the apical tufts of layer 5 pyramidal neurons in MMP9 KO mice with no change in spine morphology. MMP9 KO mice also displayed defects in dendritic spine turnover. The effect of MMP9 deficiency on microglial morphology was small although increases in microglial inclusions in MMP9 KO mice suggest mild redirection of microglial function towards an activated phenotype. Collectively, our findings suggest an instrumental role for MMP9 in activity-dependent plasticity in the rodent sensory cortex.

The Extracellular Matrix Plays a Fundamental Role in the Regulation of Activity-Dependent Plasticity

Synaptic plasticity in the brain involves a complex series of interactions that enable the reorganization of connections as a response to sensory experience. In the rodent visual cortex, deprivation (via lid suture) triggers circuit reorganization that enables strengthening of intact inputs and weakening of deprived circuits (Gordon and Stryker, 1996; Frenkel and Bear, 2004). Plasticity occurs at the level of dendritic spines, the small protrusions originating from dendritic shafts, which contain synaptic machinery and are the primary sites of interneuronal communication (Holtmaat and Svoboda, 2009). Plasticity can be implemented through changes in spine number, spine shape, and signaling strength (Sala and Segal, 2014). Recent studies have shown that spine structure can be regulated by the ECM (reviewed in Ethell and Ethell, 2007; Levy et al., 2014; Stawarski et al., 2014) and that MMPs play a fundamental role in regulating ECM degradation and signaling to enable plasticity.

The ECM in the neuropil and in the PNN, a specialized extracellular structure formed around certain neurons, can form a barrier around maturing neurons, and limit circuit reorganization by (1) preventing interaction with neurons and advancing axons; (2) providing a scaffold for inhibitors; and (3) limiting receptor mobility at the synapse (Wang and Fawcett, 2012). Degradation of the ECM can reactivate plasticity in adults (Pizzorusso et al., 2002) suggesting that ECM composition is an important factor in activity-dependent plasticity. HA comprises a fundamental base of the ECM lattice in the neuropil and in the PNN and the tightness of this lattice can be altered by HA content and the proteins that bind HA (Yamaguchi, 2000). HSPGs are present in the neuropil and in PNNs and are important for binding various molecules that could affect synaptic plasticity. CSPGs (lecticans) versican, brevican, neurocan, CAT-301 antigen (aggrecan), phosphacan and link proteins (Hockfield and McKay, 1983; Celio and Blümcke,

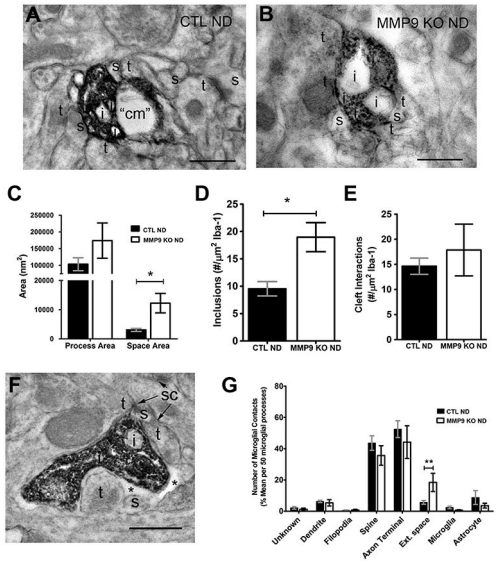

FIGURE 10 | Ultrastructural analysis of microglia suggest mild activation in the absence of MMP9. (A,B) Iba-1 immunoperoxidase EM in non-deprived control mice (CTL ND) and non-deprived MMP9 KO mice (MMP9 KO ND). **(C)** Quantitative analysis of microglial process area showed no significant differences, while surrounding extracellular space was significantly increased in MMP9 KO ND compared to CTL ND (One-Way ANOVA, $*p < 0.05$). **(D)** Average number of inclusions per area of Iba-1 labeling in 50 microglia per animals demonstrating a significant increase in inclusions in MMP9 KO ND mice (unpaired student's *t*-test, $*p < 0.05$). **(E)** Average number of synaptic contacts per area of Iba-1 labeling in 50 microglia per animals showing no significant differences between CTL ND and MMP9 KO ND mice. **(F)** Iba-1 immunoperoxidase EM showing close apposition of a microglial process to excitatory synaptic sites (sc). **(G)** Quantification of neighboring contacts to Iba-1 immunoreactive microglial processes. We found no significant differences between microglial contacts which showed similar interactions with spines and terminals in both CTL and MMP9 KO mice (One-Way ANOVA, $**p < 0.001$). All values reported are the mean ± SEM. *t, axon terminal; s, dendritic spine; i, inclusion; cm, cellular membrane; sc, synaptic contact; *, extracellular space*. Scale bar = 500 nm.

1994; Celio et al., 1998) are strongly present in PNNs where they are involved in multiple processes, including regulation of neuronal plasticity (Pizzorusso et al., 2002; Spolidoro et al., 2012), and neuroprotection (Brückner et al., 1999; Morawski et al., 2004). The composition of the lecticans within the PNN is a fundamental determinant of ECM structure and functionality (Yamaguchi, 2000). Therefore, understanding how these different ECM components are regulated within PNNs and in the neuropil surrounding synapses that are undergoing remodeling after MD is important to untangling the function of the ECM during ODP.

In the visual cortex, HA and HSPGs showed primarily neuropil staining with occasional staining around cell bodies, while CSPGs were present in the neuropil and densely within PNNs. In the neuropil, we observed a trend towards degradation of HA throughout the cortical layers and the time scale of deprivation with no changes in HSPG content (**Figure 1**), but significant degradation of neuropil CSPGs in all layers beginning at 2dMD (**Figure 2**). These findings suggest that the ECM is remodeled relatively early in ODP when responses from the deprived eye are weakened (Frenkel and Bear, 2004), and when synaptic rearrangement and local upregulation of proteases occurs (Mataga et al., 2002, 2004; Oray et al., 2004). Degradation of HA, in the absence of HSPG remodeling, may be sufficient to affect the overall "tightness" of the ECM (Yamaguchi, 2000) where the amount of HA, the structural makeup of its domains, and affinity for binding other lecticans all contribute to the fluidity of the structure. This in turn may lead to delayed remodeling of associated CSPGs which could impact the strengthening of non-deprived eye responses. Interestingly, we also noted a significant decrease in CSPG immunoreactivity within PNNs (**Figure 2**), suggesting that neuropil and PNN ECM content is regulated in a similar manner. While PNN size was relatively unaffected by MD, PNN density increased in layer 2/3 possibly to compensate the degradation of CSPGs throughout cortex, Interestingly, MMP9 loss only mildly affected the distribution of CSPGs in the absence of deprivation suggesting that MMP9 does not play a large role in the development of ECM structure or that its loss is compensated in these mice through other mechanisms.

MD-induced degradation of CSPGs was abolished in MMP9 KO mice, suggesting that CSPG content is regulated by MMP9. It is likely that this regulation is coupled with the effects of tPA activity that has been shown to be upregulated following MD (Muller and Griesinger, 1998; Mataga et al., 2002, 2004), especially given that exogenous application of tPA can mimic the structural effects of ODP (Oray et al., 2004). Furthermore, the conversion of the pro-MMP9 zymogen (inactive) to active MMP9 lies downstream of tPA/plasmin activation (Ramos-DeSimone et al., 1999) suggesting that MD induces a pathway in which pro-MMP9 is cleaved and active MMP9 activates the tPA/plasmin axis to implement changes in the ECM.

Regulation of Plasticity by Matrix Metalloproteinases 9 (MMP9)

MMPs belong to a family of zinc-dependent endopeptidases that are crucial effectors in the development and remodeling of various tissues (Sternlicht and Werb, 2001). MMP9 is the best-characterized MMP family member involved in activity-dependent structural and functional changes at CNS synapses. Increased neuronal activity enhances MMP9 expression in an NMDA-dependent manner (Sternlicht and Werb, 2001; Szklarczyk et al., 2002; Nagy et al., 2006) and MMP9 has been shown to regulate late phase LTP *in vivo* (Nagy et al., 2006), learning and memory (Bozdagi et al., 2007), experience-dependent plasticity (Kaliszewska et al., 2012), and dendritic spine morphology (Tian et al., 2007; Michaluk et al., 2011). Indeed, the synaptic localization of MMP9 implies a fundamental role in neuronal plasticity.

In the visual cortex, ODP was attenuated in MMP9 KO mice following 4dMD but not 7dMD (**Figure 5**) suggesting an effect of MMP9 during specific phases of ODP. Following closure of one eye, ODP occurs in a biphasic manner, in which first there is a decrease in deprived eye responses, followed by increased potentiation in the ipsilateral non-deprived eye (Frenkel and Bear, 2004). Our results suggest that MMP9 affects initial phases of ODP, consistent with results seen in tPA KO

mice (Mataga et al., 2002), and in ECM changes described in this study which occur at 2dMD. Alternatively, MMP9 KO may delay the effects of MD, possibly due to an inhibitory ECM milieu, and require longer deprivations to elicit plasticity. In contrast, previous reports suggest that MMP9 inhibition (infusion of the global MMP inhibitor, GM6001) only affects the potentiation of the open-eye responses due to the attenuating effects of MMP9 on ODP following 7dMD only (Spolidoro et al., 2012). An important difference between the two studies is the approach to abrogating MMP activity. Our model is less invasive and allows for complete loss of MMP9 activity without off-target effects, whereas GM6001 is a non-specific MMP inhibitor. Our genetic model, however, potentially allows for developmental compensation for MMP9 loss. In addition, the use of a mouse model (this study) vs. rat model (Spolidoro et al., 2012) may introduce differences in plasticity mechanisms (Kadish and Van Groen, 2003). While more work will be needed to reconcile these different results, collectively, it is clear that MMP9 is necessary for specific forms of experience dependent plasticity that may vary depending on the experimental design.

While our data suggests that MMP9 plays important role in the remodeling of the ECM and that this remodeling may be a critical step in ODP, MMP9 loss during development likely alters other pathways that could impact plasticity. To explore this possibility we further characterized the MMP9 KO cortex for other changes which could lead to an altered potential for ODP. MMP9 is important in late-phase LTP (Nagy et al., 2006; Wang et al., 2008); a process often accompanied by changes in dendritic spine density and morphology (Yuste and Bonhoeffer, 2001). In our study, MMP9 KO mice also exhibited reduced excitatory synapse density in V1 and reduced spine densities on the apical dendrites of S1 layer 5 neurons, without changes in PSD size or spine morphology, respectively (**Figure 7**). MMP9-mediated cleavage of ECM/CAM (cell adhesion molecules) results in products that can act on integrin receptors to transduce the signals provoking actin cytoskeleton modification (reviewed in Wlodarczyk et al., 2011, but also Nyman-Huttunen et al., 2006), suggesting that a reduction in spine density in MMP9 KO mice maybe due to a defect in signaling that regulates spine formation and elimination during early periods of development. MMP9 KO mice also do not show increased elimination of dendritic spines as compared to spine formation (**Figure 8E**), which is a hallmark of normal adolescent cortex. This may suggest that a compensatory mechanism is activated to counteract the low density of spines by normalizing spine formation and elimination.

Despite decreased overall excitatory synapse density in MMP9 KO mice, PSD size and spine morphology (**Figures 6E**, **7G–H**) was unaffected indicating that while the initial mechanisms of synapse formation may be disrupted, synapses that are formed continue to mature normally. This was unexpected to us as MMP9 has been reported to modulate dendritic spine morphology (Tian et al., 2007; Wang et al., 2008; Bilousova et al., 2009; Michaluk et al., 2011; Dziembowska et al., 2013). In transgenic rats overexpressing an autoactivating mutant of MMP9, dendritic spines appeared thinner and longer (Michaluk et al., 2011). Similarly, in the fragile X mouse model, there was an increase in the ratio of filopodia to mature spines; reversed by the MMP9 inhibitor, minocycline (Bilousova et al., 2009). MMP9 overexpressing mice also showed a preponderance of immature spine phenotypes (Gkogkas et al., 2014), while MMP9 KO mice have previously been described to have longer and larger spines (Sidhu et al., 2014), although this effect was observed only earlier in development. The regulation of ICAM-5, a telencephalin-associated intracellular adhesion molecule, by MMP9 (and other MMPs) provides a compelling link between MMP9 and spine morphology in which cleavage of ICAM-5 by MMPs is able to regulate spine maturation (Tian et al., 2007; Conant et al., 2010). In these studies, MMP9 appeared to have a pleiotropic effect, resulting in both elongation of filopodia as well as maturation of spine heads. Interestingly, the soluble (cleaved) portion of ICAM-5 (sICAM-5) can also bind to β1 integrin receptors on nascent spines, resulting in the phosphorylation of cofilin and triggering spine growth (Conant et al., 2011; Ning et al., 2013). Indeed, MMP9-dependent synaptic effects through β1 integrin receptors signaling suggest that MMP9 cleaves ECM proteins with exposed integrin activating epitopes (cryptic RGD motifs), resulting in the surface diffusion of NMDA receptors (Michaluk et al., 2009). Taken together with the results of our study, these data show that MMP9 has multiple effectors that contribute to regulating dendritic spine morphology in a complex manner, however effects on synapse density and plasticity can be seen in the absence of changes in spine morphology. Additionally, our results suggest that altered development of visual cortical circuitry in the absence of MMP9, along with the inability to remodel the ECM in response to deprivation, may be responsible for blunted ODP in MMP9 KO mice.

Matrix Metalloproteinases and Microglia

Microglia are the resident immune cells of the brain and are instrumental in pathological responses. Classically thought to play a pivotal role during onset, maintenance, relapse and progression of inflammatory conditions (Kettenmann et al., 2011; Konnecke and Bechmann, 2013), microglia have recently been shown to contribute to mechanisms that regulate neurodevelopmental processes, including synaptic interactions and remodeling (Wake et al., 2009, 2013; Paolicelli et al., 2011; Tremblay and Majewska, 2011; Tremblay, 2011). Microglia secrete MMP9 (Webster and Crowe, 2006; Konnecke and Bechmann, 2013) as well as other proteases and protease inhibitors, although the role of MMP9 can vary depending on the condition studied. In injury models, inflammation triggers increases in MMP9 and the proinflammatory cytokine, TNF-α, at injury sites (Hansen et al., 2013). Indeed, the upregulation of MMPs in the CNS have several potentially detrimental roles, including the promotion of neuroinflammation, disruption of the blood brain barrier, demyelination, and damage to axons and neurons (reviewed in Konnecke and Bechmann, 2013). Secretion of MMP9 from microglia can be beneficial as well, contributing to ECM degradation around synaptic sites and promoting synapse reorganization and functional recovery following traumatic brain injury (Chan et al., 2014). Although MMP9 inhibition has been reported to attenuate microglia activation (Hansen et al., 2013), here we show that in MMP9

KO mice, microglial morphology is largely unaltered with some evidence of changes which suggest a change in microglial phenotype towards a more activated state (decrease in process circularity (**Figure 9C**) and an increased number of inclusions (**Figure 10D**)). This may be a direct result of MMP9 loss or the result of compensatory upregulation of other proteases (Sekine-Aizawa et al., 2001; Esparza et al., 2004; Greenlee et al., 2007). Thus changes in microglia are subtle in MMP9 KO mice, although these changes might also contribute to altered plasticity in these mice.

Conclusion

MMPs belong to a large family of endopeptidases shown to be instrumental regulators in both physiological and pathological events throughout the PNS and CNS (Sternlicht and Werb, 2001). Their biochemical complexity and the large variety of substrates accounts for the richness of their functions (Ethell and Ethell, 2007). In this study we show that MMP9 may contribute to early stages of ODP. MMP9 affects many neuronal and glial processes and its effects on ODP may result from a combination of ECM degradation, remodeling of excitatory synapses, and changes in microglia function. Given evidence that proteases act in a complimentary and often redundant manner (Sekine-Aizawa et al., 2001), future studies should investigate the interaction between MMP9 and other closely associated molecules such as tPA and MMP2 during plasticity.

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Role of neuropsin in parvalbumin immunoreactivity changes in hippocampal basket terminals of mice reared in various environments

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In vitro approaches have suggested that neuropsin (or kallikrein 8/KLK8), which controls gamma-aminobutyric acid (GABA) neurotransmission through neuregulin-1 (NRG-1) and its receptor (ErbB4), is involved in neural plasticity (Tamura et al., 2012, 2013). In the present study, we examined whether parvalbumin (PV)-positive neuronal networks, the majority of which are ErbB4-positive GABAergic interneurons, are controlled by neuropsin in tranquil and stimulated voluntarily behaving mice. Parvalbumin-immunoreactive fibers surrounding hippocampal pyramidal and granular neurons in mice reared in their home cage were decreased in neuropsin-deficient mice, suggesting that neuropsin controls PV immunoreactivity. One- or two-week exposures of wild mice to novel environments, in which they could behave freely and run voluntarily in a wheel resulted in a marked upregulation of both neuropsin mRNA and protein in the hippocampus. To elucidate the functional relevance of the increase in neuropsin during exposure to a rich environment, the intensities of PV-immunoreactive fibers were compared between neuropsin-deficient and wild-type (WT) mice under environmental stimuli. When mice were transferred into novel cages (large cages with toys), the intensity of PV-immunoreactive fibers increased in WT mice and neuropsin-deficient mice. Therefore, behavioral stimuli control a neuropsin-independent form of PV immunoreactivity. However, the neuropsin-dependent part of the change in PV-immunoreactive fibers may occur in the stimulated hippocampus because increased levels of neuropsin continued during these enriched conditions.

Keywords: KLK8, neuregulin1, ErbB4, hippocampus, interneuron, GABA, synaptic plasticity

INTRODUCTION

The secretory serine protease neuropsin is considered to be involved in activity-dependent neural plasticity such as the early phase (E-) of long-term potentiation (LTP) and kindling epileptogenesis (Chen et al., 1995; Okabe et al., 1996; Tamura et al., 2006). The identification of neuropsin's substrates (neuregulin-1 (NRG-1), fibronectin, vitronectin, and L1cam) have aided in elucidating the understanding of the function of neuropsin (Shimizu et al., 1998; Matsumoto-Miyai et al., 2003; Tamura et al., 2012). Hippocampus of the neuropsin-deficient mice exhibited reduction in a mature mushroom-type synapses and increase in a small immature synaptic terminals labeled by L1cam antibody and the changes were reversed by the intraventricular application of recombinant neuropsin (Nakamura et al., 2006). In addition, deficient mice exhibit a disappearance of E-LTP (Komai et al., 2000; Tamura et al., 2006), and the synaptic association between two independent inputs of the Schaffer-collateral pathway, which is the basis of hypothetical synaptic tagging and which was reversed by a bath application of recombinant neuropsin (Ishikawa et al., 2008, 2011). These results suggest that neuropsin is involved in synaptic modulation during the early stages of neural plasticity by cleaving (or modifying) the extracellular domain of target proteins.

Our recent study has suggested that neuropsin induces the neural activity-dependent release of the NRG-1 ligand, regulates interaction of an NRG-1 with ErbB4 into parvalbumin (PV)-positive neurons, and as a result, strengthens gamma-aminobutyric acid (GABA) neurotransmission (Tamura et al., 2012). It is well known that ErbB4 in the PV-positive GABAergic interneurons triggered phosphorylation by cleaving NRG-1 in an LTP-dependent manner (Longart et al., 2007; Vullhorst et al., 2009; Buonanno, 2010; Shamir et al., 2012; Tamura et al., 2012). Thus, neuropsin may play a role only in the early period (less than a few hours) of inhibitory signaling and regulate hippocampal pyramidal neurons through GABAergic neurotransmission.

The GABAergic system in the hippocampus is involved in synchronization and gamma oscillation (Klausberger et al., 2005; Sohal et al., 2009; Volman et al., 2011; Hou et al., 2014) and possibly in cognitive functions in humans and animals. Several studies have shown a functional relevance of these molecules in human cognitive function; NRG-1, ErbB4, and neuropsin are all risk factors for schizophrenia/bipolar disorder (Britsch et al., 1998; Stefansson et al., 2003; Corfas et al., 2004; Izumi et al., 2008; Mei and Xiong, 2008). In addition, animal studies have supported this because both ErbB4- and neuropsin-deficient mice exhibit impaired working memory and memory acquisition (Tamura et al., 2006; Wen et al., 2010).

Multiple lines of evidence have suggested that neuropsin is involved in synaptic plasticity. However, the role played by neuropsin in the hippocampus of voluntarily behaving animals remains unknown. In the present study, we examined: (1) whether an enriched environment (EE) stimulated the induction of neuropsin and PV immunoreactivity in the hippocampus; and (2) whether neuropsin regulated PV immunoreactivity in mice reared in familiar and EEs.

RESULTS

To analyze the effects of neuropsin on hippocampal neurons in voluntarily behaving mice, we first examined neuropsin mRNA and protein levels in animals reared in familiar and EEs. Prior to the experiment, a single mouse was reared in a familiar cage (10- × 20- × 14-cm^3 home cage; **Home**) for acclimation (**Figures 1Ai–iv**). Furthermore, two control and two types of enriched conditions were set up (**Figures 1Ai–iv**), and the mRNA levels of the hippocampi of the animals reared in each of the environments were analyzed by real-time polymerase chain reaction (RT-PCR). The rearing conditions were as follows (**Figure 1A**): (i) mice were kept in the home cage during all of the experimental periods; (ii) mice were transferred into a large cage (28 × 33 × 16 cm^3; **Con**); (iii) mice were exposed to the enriched condition of a running wheel in the large cage (**Run**); and (iv) mice were exposed to another enriched condition of a running wheel and a plastic tunnel for hiding in the large cage (**EE**). With both behavioral stimuli, the running distance was calculated by counts of rotation/day × circumference of the wheel (Section Materials and Methods). Mice exposed to both enriched conditions ran longer daily and reached approximately 9 km/day after 1 week (**Figure 1B**).

NEUROPSIN mRNA AND PROTEIN LEVELS WERE INCREASED IN THE HIPPOCAMPUS BY ENVIRONMENTAL STIMULI IN THE VOLUNTARILY BEHAVING MICE

After mice were transferred into an EE (**Figure 1Aiv**) or large control cage (Con; **Figure 1Aii**), the time course of the expression of neuropsin mRNA was determined by quantitative PCR during 1-, 2- or 3-week rearing. A significant increase was observed after 1 and 2 weeks, and it returned to basal levels after 3 weeks (**Figure 1C**). Furthermore, we compared the levels of expression of neuropsin mRNA in the hippocampi of mice reared in the two enriched conditions, Run and EE, after 1 week. In both cases, the levels of neuropsin mRNA were significantly upregulated (**Figure 1D**). However, when the wheel was locked with a stopper till it stopped rotating (Lock), the levels of expression were the same as the control level (Con) (**Figure 1D**). The changes in neuropsin were further quantified by an enzyme-linked immunosorbent assay (ELISA) for neuropsin protein. Two weeks of rearing of the mice in EE resulted in a significant increase in neuropsin immunoreactivity (**Figure 1E**). These results suggested that environmental stimuli contribute to an upregulation of neuropsin expression.

NO SIGNIFICANT CHANGES WERE FOUND IN THE TOTAL CELL NUMBER OF PV-IMMUNOREACTIVE INTERNEURONS IN THE NEUROPSIN-KNOCKOUT (NPKO) MICE REARED IN THE FAMILIAR CAGE

Because neuropsin interacts with PV-immunoreactive neurons through ErbB4 signaling, as shown by Tamura et al. (2012), the hippocampal PV-immunoreactive neurons were examined in the neuropsin-deficient mice. In our earlier study, no remarkable changes in PV-immunoreactive cell number were found in the pyramidal cell layer of the CA1 subfield (Hirata et al., 2001). To confirm the results and extend the findings to other hippocampal subfields, we performed thorough quantitative analyses in each layer of the dentate gyrus (sectioned by broken blue lines of **Figure 2A**), CA1/2 (sectioned by broken green lines of **Figure 2A**), and CA3 (sectioned by broken red lines of **Figure 2A**). In agreement with our previous study, negligible changes in PV-immunoreactive cell numbers were observed in the granular cell layer of the dentate gyrus (**Figure 2B**), and the total numbers of PV cell bodies in each subfield were not changed, even in the NPKO mice (**Figure 2C**). In addition, no morphological changes in PV-immunoreactive cells, such as cell size or dendritic arborization, were observed in both genotypes (data not shown; Hirata et al., 2001). No significant changes in the number of GAD67-immunoreactive cell bodies were observed in the previous and present study (data not shown; Hirata et al., 2001). Therefore, PV-positive inhibitory interneurons were considered to be maintained normally even in the neuropsin-deficient mice.

MARKED DECREASES IN THE INTENSITY OF PV-IMMUNOREACTIVE FIBERS IN THE DENTATE GYRUS AND THE CA1 AND CA3 SUBFIELDS WERE OBSERVED IN NPKO MICE REARED IN A FAMILIAR CAGE

In the hippocampal slices, paired-pulse inhibition experiments result in a reduction in GABAergic release from ErbB4-containing PV-positive neurons in the CA1 subfield of deficient mice (Tamura et al., 2012). Such physiological deficits in the GABAergic nerve terminals of neuropsin-deficient mice suggest that neuropsin controls granular and pyramidal neurons through basket and/or axo-axonic fiber networks. Therefore, we examined PV-immunoreactive nerve terminals in the granular layer of the dentate gyrus and the stratum pyramidale of the CA1-3 subfields (**Figures 3A,C,E**) and compared them to those in neuropsin-deficient mice (**Figures 3B,D,F**). The intensities of the PV-immunoreactive fibers were significantly decreased, particularly in the granular cell layer of the dentate gyrus (**Figure 4A**) and the stratum pyramidale of the CA3 subfield

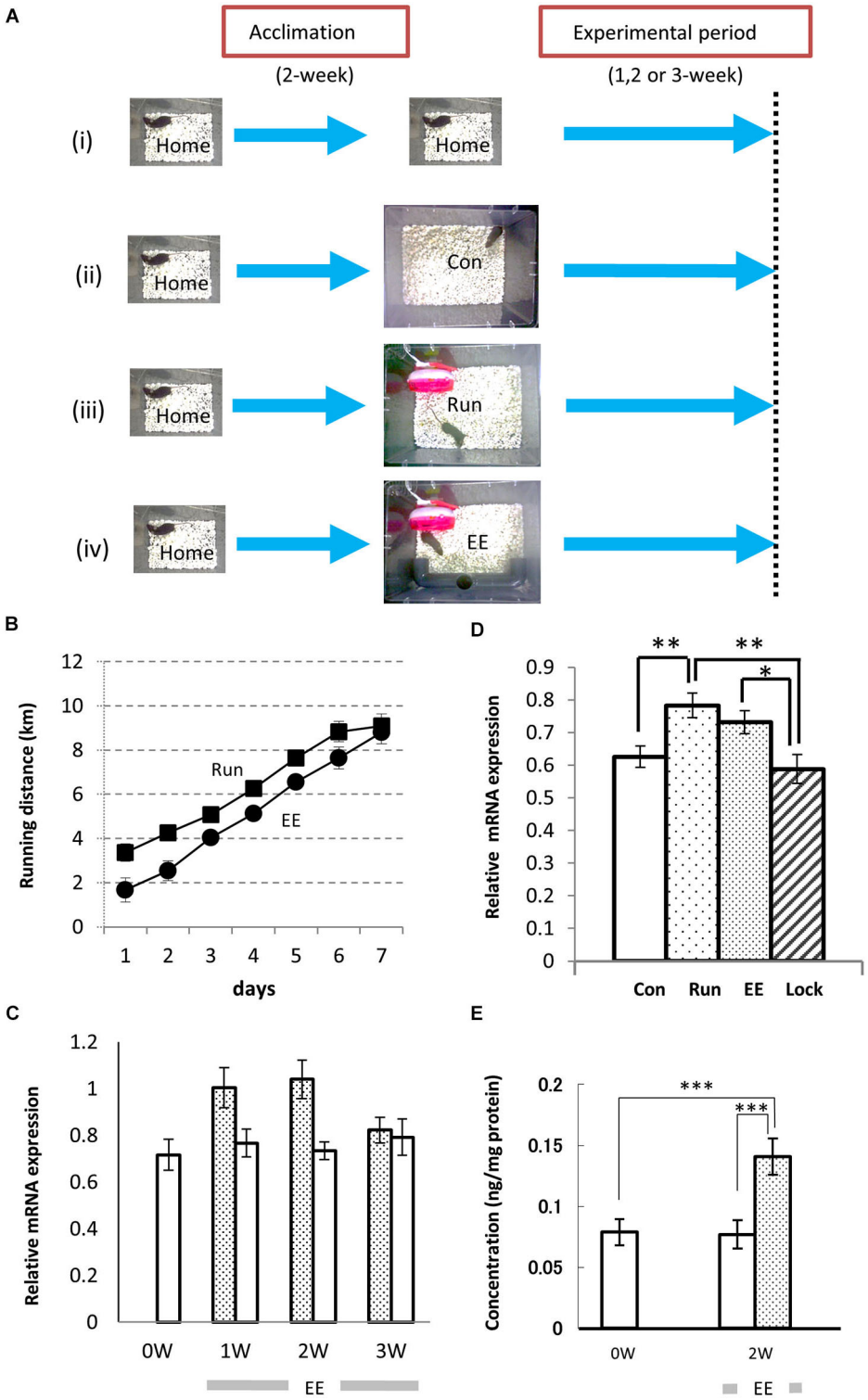

FIGURE 1 | Environmental stimuli upregulate the levels of expression of neuropsin mRNA and protein in the hippocampus. **(A)** The enrichment protocol was applied to voluntarily behaving mice. After a 2-week acclimation in the home cage, the mice were maintained in the same home cage (i) or transferred to a large cage (28 cm × 33 cm × 16 cm; Con, (ii), a large cage containing only a plastic running wheel (Run; iii), a large cage with a running wheel and a plastic opaque tube (EE; iv), or a large cage with a nonrevolving wheel, which was fixed with glue, and a plastic tunnel (Lock). **(B)** The running distances (counts of rotation/day × circumference of wheel) were increased daily both in the Run (Filled squares) and EE (Filled circles) rich environments. **(C)** The time course of the change in neuropsin mRNA during longer

(*Continued*)

FIGURE 1 | Continued
experimental periods (1–3 weeks). One week (1 W) or 2 weeks (2 W) of exposure to the environmental enrichment (EE) resulted in an increase in neuropsin mRNA. After 3 weeks (3 W), the increase in the levels of expression of neuropsin mRNA in the hippocampus of EE mice returned to the Con level. Statistical significance was determined by a one-way analysis of variance (ANOVA) between the control (Con: open bars) and EE (hatched bars) groups (n = 7). *p < 0.05. **(D)** Significant increases in the levels of neuropsin mRNA expression in the hippocampus were seen in both the EE and Run groups during the 1-week experimental period. Note that no increase in mRNA was found when the running wheel was locked (Lock; n = 7). The error bars indicate standard error of the mean (SEM). The levels of statistical significances were *p < 0.05 or **p < 0.01. **(E)** The enzyme-linked immunosorbent assay represents a change in neuropsin protein levels during the experimental period. A significant increase in the immunoreactivity of neuropsin was found in the EE group. ***p < 0.005. The experimental period was 2 weeks (2 W).

(**Figure 4C**) and less significantly decreased in the stratum pyramidale of the CA1 subfield (**Figure 4B**). The intensities of PV terminals, which were measured in randomly selected boxed areas, were significantly reduced in the dentate gyrus and CA3 subfield (**Figures 4A,C**). Higher-magnification photographs that were obtained under a Nomarski interference condenser showed that thick PV-immunoreactive axons surrounding the cell somas in the granular and pyramidal layers (**Figures 3G,I,K**, thick open arrows) were present in the wild-type (WT) mice, and these axons were mostly reduced in the deficient hippocampus (**Figures 3H,J,L**). However, weak punctate immunoreactivity remained in the surrounding granular and pyramidal neurons, even in the deficient hippocampus (arrowheads; **Figures 3H,J,L**).

Furthermore, we examined whether the decreases in the densities of PV-immunoreactive fibers were caused by any axonal loss of inhibitory fibers because of the neuropsin deficiency. We examined the vesicular GABA transporter (VGAT; a transporter that mediates the accumulation of GABA into inhibitory synaptic vesicles) with immunostaining in WT and neuropsin-deficient mice. No apparent changes were observed in the three areas that were examined in the subfields of the hippocampus (**Figures 3M–R**, **4D–F**). The results of the PV and VGAT immunoreactivities suggested that GABAergic terminal boutons maintained the inhibitory vesicles, whereas the PV immunoreactivity was reduced in the boutons in the deficient hippocampus.

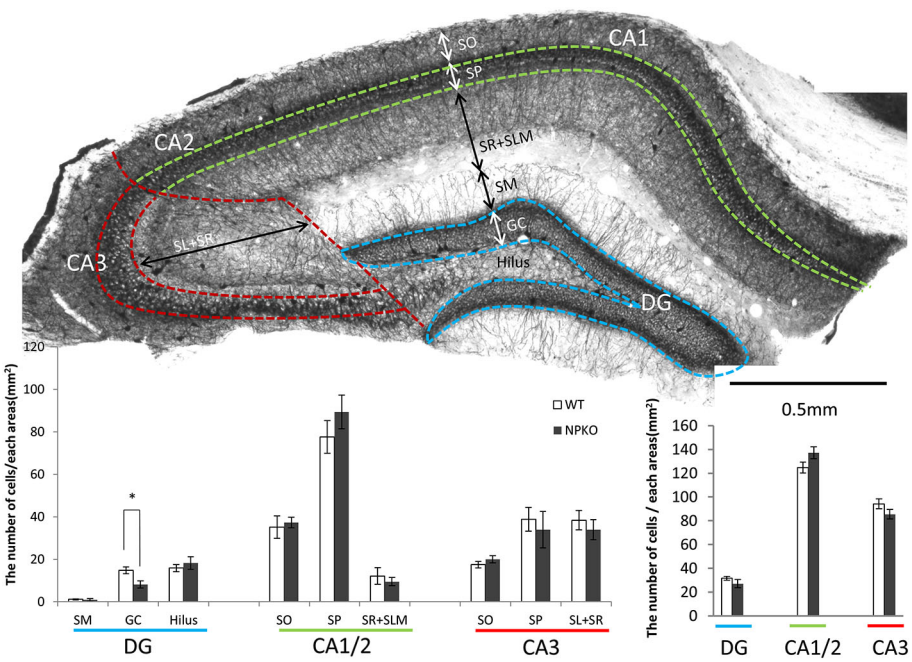

FIGURE 2 | The quantitative analysis of parvalbumin (PV)-immunoreactive neurons between wild-type (WT) and neuropsin-deficient (NPKO) mice. **(A)** Parvalbumin-immunoreactive neurons and fibers in a coronal section of mouse hippocampus. A 3,3′-diaminobenzidine reaction revealed thick positive nerve terminals surrounding the CA1-3 pyramidal neurons and granular neurons. The blue, green, and red broken lines on a whole section represent the boundaries dividing the sublayers and subfields of the hippocampus. Scale bar: 0.5 mm. **(B)** Number of PV-immunoreactive neurons in each sublayer in the dentate gyrus (DG; blue line), CA1 and two subfields (CA1/2; green line), and CA3 subfield (CA3; red line) of the hippocampus (n = 4; the average from 10 slices from each mouse were calculated). Open bar: WT, Closed bar; NPKO mice. **(C)** Total number of PV-immunoreactive neurons in each area. There was no difference in the total positive cell number between WT (Open bar) and NPKO (Closed bar) mice. Abbreviations: CA1, CA1 subfield; CA2, CA2 subfield; CA3, CA3 subfield; DG, dentate gyrus; GC, granule cell layer; SL, stratum lucidum; SM, stratum moleculare; SO, stratum oriens; SP, stratum pyramidale; SR, stratum radiatum. The asterisks indicate significance: *p < 0.05. The error bars indicate SEM.

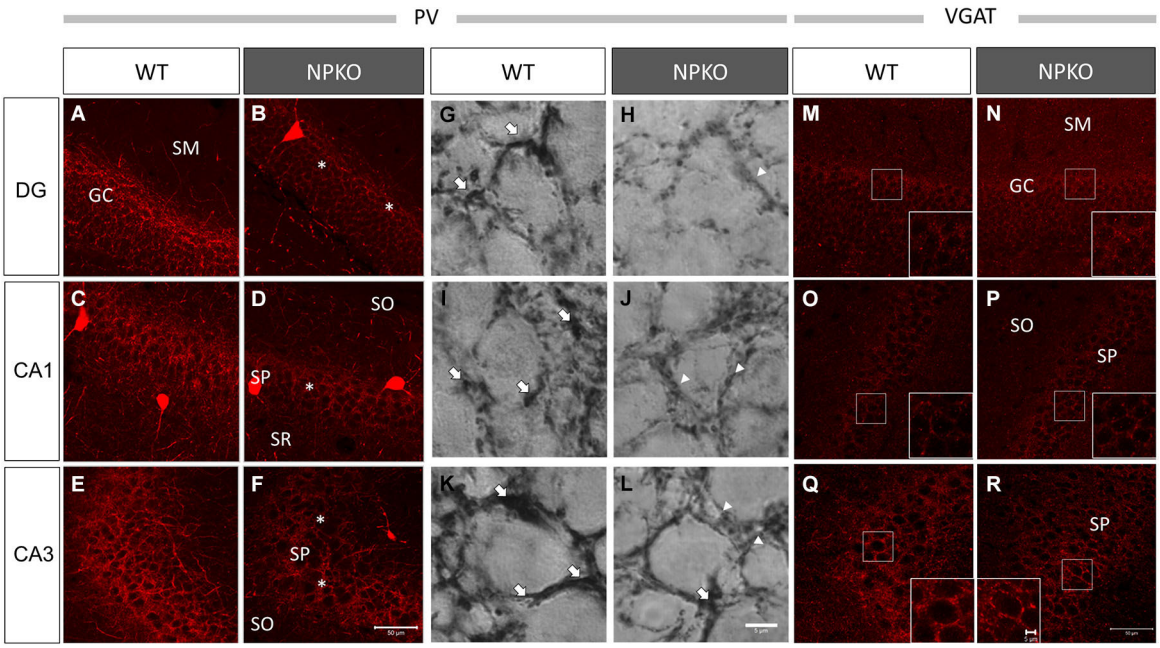

FIGURE 3 | Marked downregulation of PV immunoreactivity in the NPKO mice compared to the WT mice. Confocal microscopic images **(A–F)** and Nomarski differential interference images **(G–L)** are presented. Dense PV-immunoreactive fiber networks were localized in the GC **(A)** and SP **(C,E)** of CA1 and CA3 subfields. The decrease in PV immunoreactivity was significant in the GC **(B)** and SP of the CA3 subfield **(F)**, and the decrease was less significant in the SP of the CA1 sublayer **(D)**. The Nomarski images show that the PV-immunoreactive fibers surrounding the granular and pyramidal cells were thick fiber bundles (Open arrows in **G**,**I**,**K**), whereas, in the NPKO mice, only weakly immunoreactive fibers remain (Open arrowheads in **H**,**J**,**L**). The scale bars indicate 50 μm **(A–F)** and 6 μm **(G–L)**. Vesicular GABA transporter (VGAT) immunoreactivity was not changed between the WT and NPKO mice. Both of the immunofluorescent images of the WT **(M,O,Q)** and NPKO **(N,P,R)** mice show similar staining patterns in DG, CA1, and CA3. Because VGAT concentrates in GABAergic nerve endings, the inhibitory synaptic apparatuses may be intact in the NPKO mice. The insets show higher magnification of the boxed areas of the photographs. The scale bars indicate 50 μm and 5 μm in the insets.The abbreviations listed in **Figures 3–6** are the same as those in **Figure 2**.

A MARKED INCREASE IN THE INTENSITY OF PV-IMMUNOREACTIVE FIBERS WAS OBSERVED IN MICE RUNNING VOLUNTARILY

We examined PV immunoreactivity in the voluntarily running mice because neuropsin mRNA and protein levels were upregulated by transferring mice into rich environments. Parvalbumin immunoreactivity was upregulated in the granular layer of the dentate gyrus and stratum pyramidale in the CA3 subfield of the mice allowed to voluntarily run (Run) (**Figures 1Aiii, 5A–I**).

TRANSFER TO A RICH ENVIRONMENT INCREASED PV IMMUNOREACTIVITY EVEN IN THE NPKO MICE

Because voluntary running itself induced an increase in PV immunoreactivity in the hippocampus (**Figure 5**), we further analyzed whether the increase was because of the effects of neuropsin on PV neurons. In NPKO mice, PV immunoreactivity was slightly increased only after transferring the mice into a novel large cage (**Figure 1Aii**; Con; WT vs. NPKO; **Figures 6A–F, J–L**). Two weeks of rearing in the large cage with the running wheel (**Figure 1Aiii**; Run) resulted in a marked increase in PV immunoreactivity, even in the knockout mice (WT vs. NPKO; **Figures 6A–C, G–L**). These results showed that at least some of the plastic changes in PV immunoreactivity by the environmental stimuli were because of a neuropsin-independent mechanism.

DISCUSSION

Increasing levels of PV in the hippocampal basket cells indicated a state of plasticity. Donato et al. (2013) reported that low PV content parallels enhanced memory and structural synaptic plasticity (plastic state; state for high capacity of acquisition), while high PV content parallels a low plasticity (consolidated state; state for fixation of memories). When PV cells are activated and promoted into a high PV state, these may impede hippocampal plasticity (Donato et al., 2013; Hensch, 2014). Optogenetic technology has revealed that the specific driving of PV interneurons induces enhanced gamma oscillation, which contributes to cognitive functions, such as memory formation and sensory processing (Sohal et al., 2009). In contrast, reduced PV parallels with the decreases in gamma oscillation in computational models (Volman et al., 2011), which was similar to what is observed in diseases involving cortical dysfunction, such as schizophrenia (Lewis et al., 2012). In addition, researchers have shown that physical exercise induces plastic changes in inhibitory neurons in the hippocampus. Arida et al. have reported that both 10 or 45 days of voluntary running (acute or chronic exercise) increases PV expression in cell bodies (Arida et al., 2007; Gomes da Silva et al., 2010). Thus, PV levels may correlate with the plastic state in the inhibitory system, even in the voluntarily acting mice and in subsequent cognitive behavior of the animals. In the present study, we focused on the

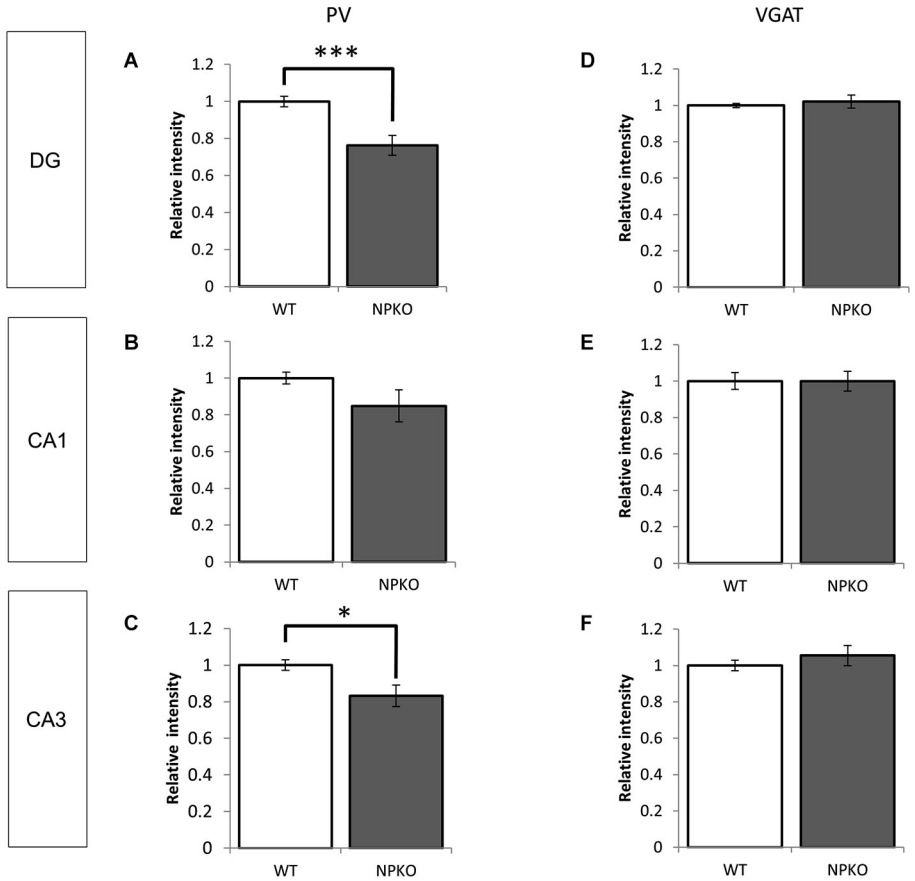

FIGURE 4 | Densitometry analyses of PV and VGAT immunoreactivities in the hippocampus of WT and NPKO mice. The relative fluorescent intensities of PV- **(A–C)** and VGAT-immunoreactive fibers **(D–F)** were measured and compared between WT and NPKO mice. A significant downregulation of the PV-immunoreactive fibers was observed in the DG **(A)** and CA3 **(C)** of NPKO mice. $*p < 0.05$, $***p < 0.005$. A moderate but nonsignificant reduction in the PV-immunoreactive intensity was also observed in the CA1/2 subfields (**B**; $n = 3$). Six to 11 images (200 × 200 pixels) of similar areas in the WT and NPKO mice were combined. No change in the relative intensity of VGAT immunoreactivity was observed in all observed regions ($n = 5$). Five to 14 images of similar areas in the WT and NPKO mice were combined. The error bars indicate SEM. The relative intensities of the PV and VGAT immunoreactivity were analyzed by ImageJ software.

involvement of neuropsin, a plasticity-related serine protease on PV-immunoreactive nerve terminals in the pyramidal and granular layers, when a mouse exercised (in a rich environment) and transient upregulation of the neuropsin was observed during 1- or 2-week exposure of wild mice to voluntary exercise.

Previous studies on animals in restricted conditions have demonstrated that neuropsin is involved in the physiological regulation of hippocampal and amygdaloid plasticity in exploring behavior (Tamura et al., 2006), the induction of hippocampal E-LTP *in vivo* (Tamura et al., 2006), and anxiety (Horii et al., 2008; Attwood et al., 2011). Moreover, *in vitro* studies have demonstrated that the processing of NRG-1 by neuropsin allows an interaction of NRG-1's functional domain with ErbB4, which consequently regulates $GABA_A$ receptor-dependent transmission and increases the potentiation of E-LTP (Tamura et al., 2012). The physiological impairments that resulted from the neuropsin deficiency are reversible by the addition of neuropsin protein, neuropsin-treated recombinant NRG-1 (cleaved NRG-1), or the ErbB4-activating domain of the NRG-1 peptide *in vivo* (Tamura et al., 2006, 2012; Ishikawa et al., 2008). Thus, neuropsin may be a key cleavage enzyme for NRG-1 for inducing NRG-1-ErbB4 signaling. This system controls hippocampal plasticity through a GABAergic inhibitory network in a neural activity-dependent manner. In the present study, we observed in voluntarily behaving mice that the intensity of PV immunoreactivity in the tranquil mice was significantly decreased in the neuropsin-deficient mouse, suggesting that neuropsin regulates PV immunoreactivity in mice under the relaxed condition. Therefore, the major function of neuropsin on the inhibitory neurons may strengthen the transient GABAergic transmission via ErbB4 NRG-1 receptor after continual occurrence of plasticity in the nonstressful condition.

Although we found an upregulation of neuropsin mRNA and protein in the hippocampus with an increase in hippocampal PV-immunoreactive terminals after environmental change, we did not find any detrimental effects on PV immunoreactivity in the neuropsin-deficient mouse hippocampus. Instead, we found an increase in PV immunoreactivity when mice were

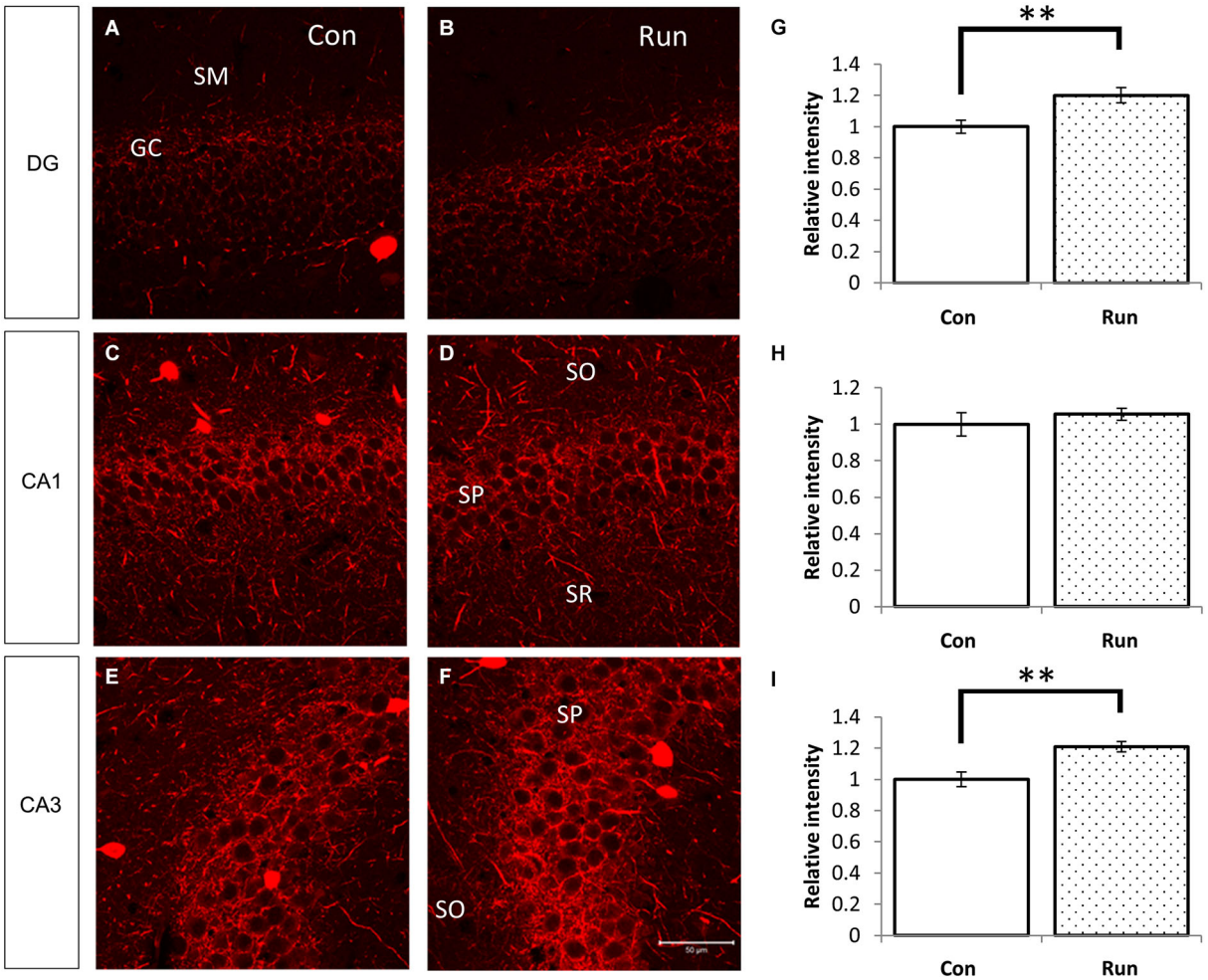

FIGURE 5 | Voluntary running upregulates PV-immunoreactive fibers in the hippocampus. Parvalbumin-immunoreactive fibers were increased in mice that were reared in the cage with a running wheel (Run) compared to the control (Con) **(A–F)**. The hippocampi of voluntarily running mice showed higher fluorescent intensities of PV immunoreactivity in the granular layer of the DG and the pyramidal layer of CA3 **(A,B,E,F)**, and the difference in CA1 was not as remarkable **(C,D)**. Densitometry analyses of the relative intensities of the photographs of PV immunoreactivity **(G–I)**. Significant increases in the PV immunoreactivity in the DG **(G)** and CA3 **(I)** of the Run group were observed, but no statistical differences were observed between the Run and Con groups in the pyramidal layer of CA1 **(H)**. Student's *t*-test: **, $p < 0.01$. The error bars show SEM [$n = 4$ (Con) and 3 (Run)]. Nine 200 × 200 pixel images of similar areas from each mouse were combined.

placed in novel environments. When mice were transferred to the unfamiliar cage, the apparent inverse effects in the neuropsin-deficient mice may be attributable to alternative signaling activating PV cells other than neuropsin. A similar phenomenon was observed in L-LTP induced by strong tetanic stimulus into the Shaffer-collateral pathway (Ishikawa et al., 2008, 2011). This case represents the existence of a neuropsin-independent form of long-term plasticity. However, we do not have direct evidence that neuropsin acts on PV immunoreactivity in the dynamic plasticity state because neuropsin deficiency did not impair PV immunoreactivity during environmental changes. However, a continual increase in neuropsin mRNA and protein in the enriched cage may demonstrate that neuropsin-dependent plasticity is hidden by the stronger and long-lasting neuropsin-independent control for PV.

In summary, in the nonstressful condition, neuropsin was involved in the control of PV networks in the hippocampus, whereas, in EEs, even with changing to a different cage, mice received strong psychological inputs. Consequently, the PV network was regulated by a neuropsin-independent system and presumably by a neuropsin-dependent system.

MATERIALS AND METHODS

ANIMALS

A total of 130 C57BL/6J mice and 50 neuropsin-deficient mice (6–10 weeks of age) were used in this study. The neuropsin-deficient mice were generated as previously described (Hirata et al., 2001). The mice were backcrossed with the C57BL/6J strain 12 times. The mice were maintained under a 12-h light/dark cycle and given food and water *ad libitum* according

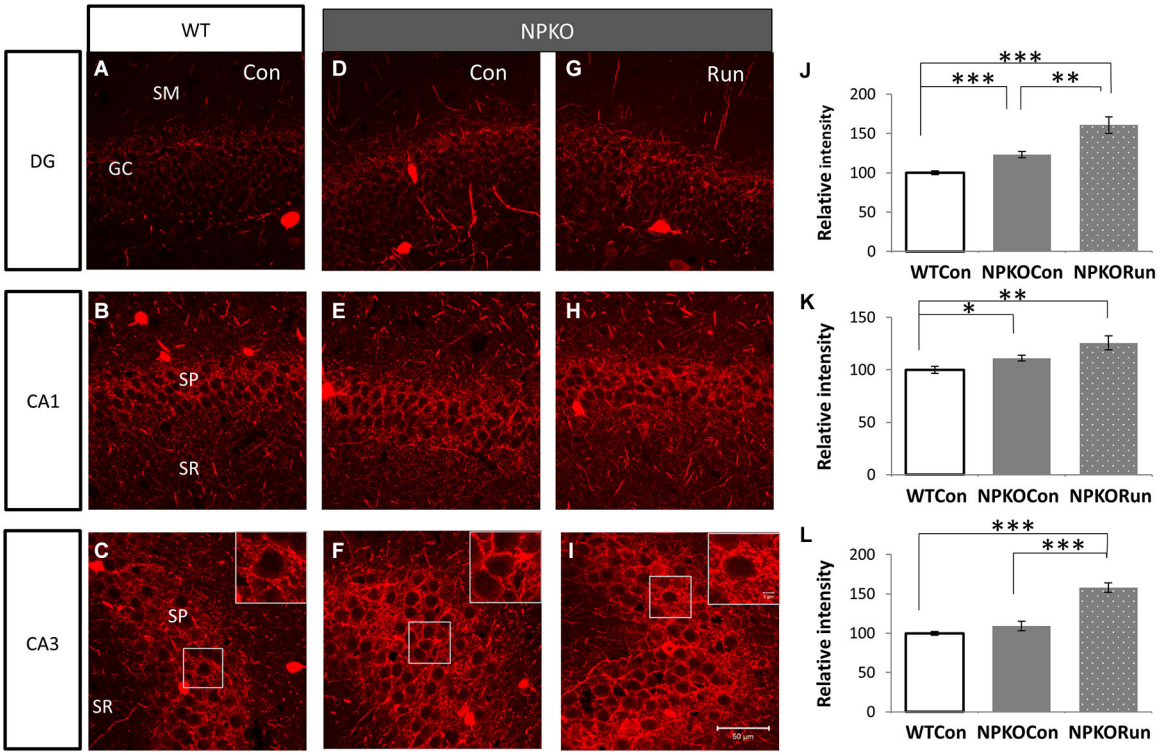

FIGURE 6 | The neuropsin-dependent form for the induction of PV immunoreactivity during voluntary running cannot be confirmed in the NPKO mice. The induction of PV immunoreactivity in the mice reared in the cage with a running wheel (Run) was compared between the knockout (NPKO; **D–F**) and WT-type hippocampus (**A–C**; same photographs with those of **Figures 5A,C,E**). After transfer from the home cage to a large experimental cage (Con: **Figure 1Aii**), a slight increase in PV immunoreactivity was observed **(D–F)**. In the Run group, a further increase in PV immunoreactivity, even in the knockout mice, was observed in DG, CA1, and CA3 **(G–I)**. Densitometric analysis of PV immunoreactivities in the hippocampus of WT and NPKO mice **(J–L)**. Parvalbumin immunoreactivity was not impaired but rather was increased in the NPKO mice when they were transferred from the home cage into a large experimental cage (without toys). The hippocampi of mice that could voluntarily run showed even higher fluorescent intensities of PV immunoreactivity in the granular layer of the DG and the pyramidal layer of CA1 and 3 **(J–L)**. $*p < 0.05$, $**p < 0.01$, $***p < 0.005$.

to the guidelines of the Nara Institute of Science and Technology. The study was approved by the institutional animal care and user's committee.

ENVIRONMENTAL STIMULATION

Mice were individually housed until the behavioral experiments. After 2 weeks of acclimation, the mice were transferred into each environmental setting (**Figures 1Aiii,iv**). The objects in the cages were maintained in the same position. For the environmental stimulation conditions, a plastic running wheel (Sanko Shokai Co., Ltd., Osaka, Japan) for free, voluntary running (Run) or a running wheel and a plastic opaque tube for a more complex, EE were placed in a large cage (28 cm × 33 cm × 16 cm). For the controls, no objects (Con) or a nonrevolving wheel, which was fixed with glue and a plastic tunnel (Lock), were placed in the large-sized cage. Moreover, some mice were kept in home cages (Home). Running distance was calculated by counting the rotation numbers × the circumference of the plastic wheels (11 cm diameter). Rotation number was checked at 10 A.M. daily, and mice that ran less than 10,000 rotations per day (less than 3.5 km) were excluded from the groups; both 8/19 and 8/18 mice were excluded from Run and EE groups, respectively.

TISSUE PREPARATION AND IMMUNOHISTOCHEMISTRY

Mice were deeply anesthetized with an intraperitoneal injection of 10% urethane (15 mg/kg) and perfused with 50 mL of 0.1 M phosphate-buffered saline (PBS; pH 7.4), which was followed by 100 mL of PBS containing 2% paraformaldehyde through the ascending aorta. The brains were removed and postfixed overnight in the same fixative at 4°C. The brains were then consecutively rinsed for 1 h in cold (4°C) 70%, 80%, 90%, and 95% ethanol and then placed in 100% ethanol overnight. Furthermore, the brains were rinsed through 50% and 75% mixtures of ethanol and polyester wax (VWR International LLC, Radnor, PA, USA) at 42°C and placed in 100% polyester wax two times for 1 h each. The brains were embedded in 100% polyester wax. Sections (6-μm-thin and 50-μm-thick) were cut with a microtome (Microm HM400, Microm International GmbH, Walldorf, Germany) for immunofluorescent observations and differential interference observations, respectively.

The sections were dewaxed three times by immersion in 100% ethanol and were then immersed in PBS. All immunostaining was performed with the floating method. The sections were blocked for 2 h in 5% bovine serum albumin in 0.3% Triton X-100, incubated overnight at 4°C with a monoclonal or polyclonal

antiparvalbumin antibody (monoclonal, 1:3.000; Sigma-Aldrich Japan K.K., Tokyo, Japan; polyclonal, 1:1500, Abcam plc, Cambridge, UK) that was diluted with 1% bovine serum albumin in 0.3% Triton X-100 in PBS. After washing with PBS, the sections were incubated overnight at 4°C with Alexa-labeled secondary antibodies (1:500). For light microscopic and differential interference observations of the immunoreactivity, a Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA, USA) was used and visualized by a diaminobenzidine reaction.

A polyclonal antibody for VGAT was produced by immunization as described elsewhere (Takayama and Inoue, 2010). The sections were incubated overnight with the VGAT antibody (1:400), and the same protocol as that described above was used.

RNA EXTRACTION AND REAL-TIME RT-PCR

The mice hippocampi were dissected, and total RNA was extracted with TRIzol reagent (Life Technologies, Grand Island, NY, USA). The relative levels of mRNA expression were measured by LightCycler480 (Roche Diagnostics Japan, Tokyo, Japan). Cp values were determined by the second derivative maximum method. The *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene was used as an internal control. The primer sequences for the *neuropsin* gene were (forward) 5′-CCCACTGCAAAAAACAGAAG-3′ and (reverse) 5′-TGTCAGCTCCATTGCTGCT-3′. The primer sequences for *GAPDH* were (forward) 5′-CGGGAAGCCCATCACCATC-3′ and (reverse) 5′-GAGGGGCCATCCACAGTCTT-3′. The primer concentrations were 0.2 μM for both genes. Samples were preheated at 95°C for 10 min. The PCR conditions were 95°C for 10 s, annealing temperature of 60°C for 10 s, and extension temperature of 72°C for 14 s. The sample concentration was calculated by the average of the triple measurements.

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) FOR NEUROPSIN

The dissected hippocampi were homogenized with lysis buffer [1 mM ethylenediaminetetraacetic acid, 0.5% (w/v) Triton X-100 in PBS], and the supernatants that were collected after centrifugation (15,000 rpm, Tomy Seiko Co., Ltd., Tokyo, Japan) were used as samples. An antineuropsin monoclonal antibody (clone F12; Medical & Biological Laboratories Co., Ltd., Nagoya, Japan) was adsorbed in 96-well ELISA plate (Sumiron Co., Ltd., Tokyo, Japan). After blocking with 5% skim milk in PBS, sample aliquots were incubated at 4°C overnight. An antineuropsin rabbit polyclonal antibody, peroxidase-labeled antirabbit immunoglobulin, and substrate for peroxidase 3,3′,5,5′-tetramethylbenzidine were used for detection and were measured with a microplate reader at 450 nm absorbance (Model 3350; Bio-Rad Laboratories, Inc., Tokyo, Japan).

OBSERVATION, QUANTITATIVE ANALYSIS, AND FLUORESCENT DENSITOMETRY ANALYSIS OF THE IMMUNOSTAINING

Observations were performed with a LSM 710 Zeiss Axio Observer ZI confocal laser-scanning microscope (Carl Zeiss AG, Jena, Germany) that was equipped with a differential interference prism and fluorescent light source.

The number of PV-immunoreactive neurons was determined in 40 immunostained coronal sections (50-μm thickness) from four WT and four neuropsin-deficient mice. Sections that were reacted with diaminobenzidine as a chromogen were observed and photographed under a Zeiss Axioplan 2 microscope. The numbers of positive cells were counted in each area of the photographs that was encircled by the boundaries shown in **Figure 2A**.

For densitometry analyses, hippocampal sections from all groups were immunostained with the same reaction media of primary antibodies and Alexa-labeled secondary antibodies together. Photographs were taken under the same strength of Laser power and exposure time of Zeiss confocal microscope, and intensities of immunopositive nerve terminals were measured and compared from the photographs of WT and NPKO mice (NPKO; 3–5 mice) with ImageJ software. The 200 × 200-pixel boxes which do not contain positive cell bodies were randomly selected on photographs. Background (intensities measured at the nuclei of the pyramidal cells) was subtracted from the 200 × 200-pixel images. Five to 14 coronal hippocampal sections from each mouse were used for the analysis.

STATISTICAL ANALYSIS

Statistically significant differences were determined by Student's *t*-tests, or one-way analysis of variance (ANOVA) with a Dunnet's *post hoc* test. All of the data are presented as means ± standard errors of the mean. The number of mice is indicated by *n*, and *p* values less than 0.05 were considered statistically significant.

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REVIEW
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Thrombin regulation of synaptic transmission and plasticity: implications for health and disease

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Thrombin, a serine protease involved in the blood coagulation cascade has been shown to affect neural function following blood-brain barrier breakdown. However, several lines of evidence exist that thrombin is also expressed in the brain under physiological conditions, suggesting an involvement of thrombin in the regulation of normal brain functions. Here, we review ours' as well as others' recent work on the role of thrombin in synaptic transmission and plasticity through direct or indirect activation of Protease-Activated Receptor-1 (PAR1). These studies propose a novel role of thrombin in synaptic plasticity, both in physiology as well as in neurological diseases associated with increased brain thrombin/PAR1 levels.

Keywords: thrombin, proteases activated receptor 1 (PAR1), clotting factors, long term potentiation, synaptic plasticity, hippocampus

Thrombin in the Blood and in the Brain

Coagulation is a fundamental biological process, by which cellular as well as protein components in the blood form a clot to stop bleeding from injured vessels (Kalz et al., 2014). It consists of a cascade of molecular events leading to the activation of thrombin, which catalyzes the conversion of fibrinogen to fibrin, the building blocks of the hemostatic plug (**Figure 1**; Siller-Matula et al., 2011; Lippi et al., 2012). Thrombin is a serine protease which is activated by the enzymatic cleavage of two sites on prothrombin by activated Factor X (FXa; Furie and Furie, 2005). The activity of FXa is enhanced by binding to activated Factor V (FVa, which forms the prothrombinase complex with FXa; **Figure 1**; Furie and Furie, 2005).

Prothrombin is produced in the liver and is post-translationally modified in a vitamin K-dependent reaction that converts 10 glutamic acids on prothrombin to gamma-carboxyglutamic acid (Gla; Huang et al., 2003). In the presence of Ca^{2+}, the Gla residues promote the binding of prothrombin and other coagulation factors to exposed phospholipid bilayers, which accelerates but also restricts clotting procedure in a dose-dependent manner to injured sites (Huang et al., 2003). Deficiency of vitamin K or administration of the anticoagulant *warfarin*, an inhibitor of the vitamin k epoxide reductase, inhibits the production of Gla residues and slows the activation of coagulation (Huang et al., 2003). As part of its activity in the coagulation cascade, thrombin also promotes platelet-activation and aggregation (**Figure 1**; Borissoff et al., 2011).

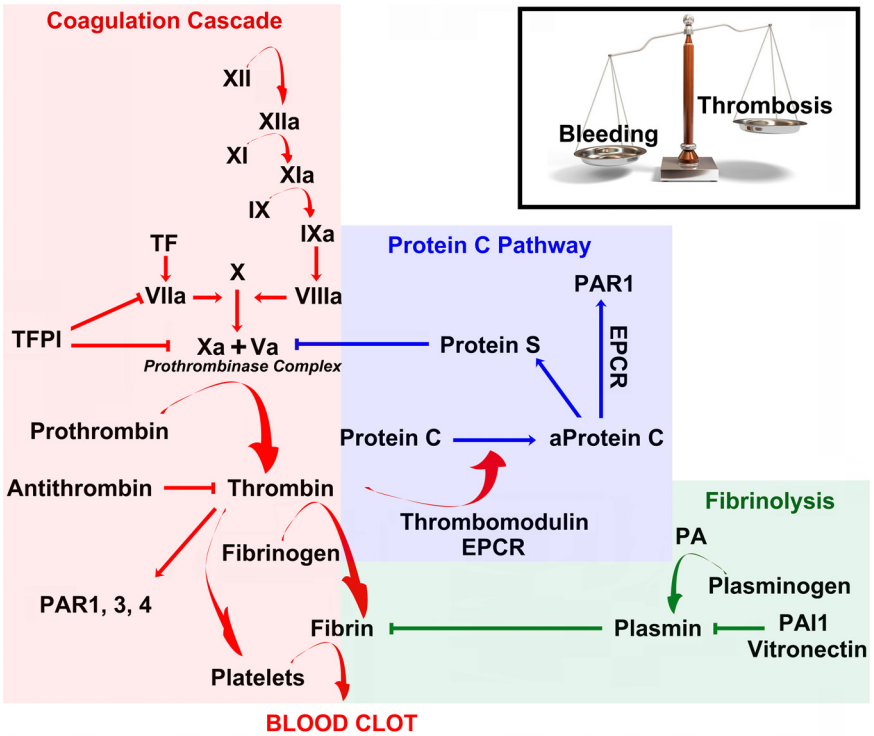

FIGURE 1 | Coagulation, anticoagulation and fibrinolysis maintain a delicate physiological balance between bleeding and thrombosis. Schematic diagram of the coagulation cascade (red), Protein C pathway (blue) and fibrinolysis (green). See main text for more detailed explanation. Abbreviations: TFPI, Tissue Factor Pathway Inhibitor; PAR, Proteases activated receptors; EPCR, Endothelial Protein C Receptor; PA, Plasminogen Activator; PAI, Plasminogen Activator Inhibitor.

Conversely, thrombin initiates a feedback mechanism which leads to its own inhibition. Once bound to thrombomodulin, an integral membrane protein expressed by endothelial cells, it increases its affinity to and activates Protein C. Activated Protein C (aPC) in turn leads to the inactivation of the prothrombinase complex (= FXa + FVa) at sites with an intact endothelium (**Figure 1**; Mosnier et al., 2014). In addition, freely circulating thrombin is blocked by antithrombin-III (**Figure 1**), a serine protease inhibitor, which is enhanced in its activity by the anticoagulant *heparin* (Jo et al., 2014). Taken together, blood thrombin activity is tightly regulated by a set of positive- and negative-feedback mechanisms, which promote clotting at injured sites and prevent coagulation at healthy sites. Considering its key role in the coagulation cascade it is not surprising that novel oral anticoagulants (NOACs) have been developed that act as direct (i.e., dabigatran) or indirect thrombin inhibitors (i.e., rivaroxaban and apixaban, via FXa inhibition) (Levy et al., 2014).

Beyond its role in the dynamic process of blood clot formation, thrombin has pronounced pro-inflammatory effects (Esmon, 2014). Acting via specific cell membrane receptors, the Protease-Activated Receptors (PARs), which are abundantly expressed in all arterial vessel wall constituents, thrombin has the potential to exert pro-atherogenic actions, such as leukocyte migration, cellular proliferation, regulation of vascular permeability and tone, platelet-activation, and edema formation (Coughlin, 2000, 2001; Sambrano et al., 2001; Chen and Dorling, 2009; Schuepbach et al., 2009; Spiel et al., 2011). PARs belong to a unique family of G protein-coupled receptors (Luo et al., 2007). Their activation is initiated by an irreversible, site-specific proteolytic cleavage in the N-terminal extracellular region, which uncovers a tethered ligand activating $G\alpha_{q/11}$, $G\alpha_{i/o}$, or $G\alpha_{12/13}$ -proteins (Coughlin, 2000; Macfarlane et al., 2001; Traynelis and Trejo, 2007). Activation of PARs can recruit multiple intracellular signaling pathways depending on the activating ligand (Russo et al., 2009). This agonist-biased signal transduction and the resulting diversity of intracellular signaling pathways appear to be crucial for the multiple actions of PARs (Russo et al., 2009; Bourgognon et al., 2013).

Interestingly, PARs are also expressed in the brain and while PAR2 represents a class of trypsin/tryptase-activated receptors, PAR1, PAR3, and PAR4 are most effectively activated by thrombin (Gingrich and Traynelis, 2000). In the brain, PAR1 has been detected in both neurons and astrocytes, with the latter demonstrating stronger immunohistochemical signal in human brain tissue (Junge et al., 2004). High levels of PAR1 are detected in the hippocampus, cerebral cortex, and striatum of humans (Junge et al., 2004). While the precise molecular pathways activated by neural PAR1 are yet under investigation, in the brain PAR1-activation has been shown to modulate synaptic

transmission and plasticity through the enhancement of N-methyl-D-aspartate receptor (NMDAR) currents (Gingrich et al., 2000; Lee et al., 2007; Maggio et al., 2008; Becker et al., 2014; Vance et al., 2015). In addition, PAR1-deficient animals have been reported to have alterations in hippocampus-dependent learning and memory processes (Almonte et al., 2007, 2013), indicating that PAR1 plays a critical role in memory formation and synaptic plasticity under physiological conditions.

A variety of neurological conditions have been associated with changes in the expression of PAR1 in the brain. In Parkinson's disease, a significant increase in the number of astrocytes expressing PAR1 has been reported in the substantia nigra pars compacta (Ishida et al., 2006). In addition, upregulation of PAR1 in astrocytes has been observed in HIV encephalitis (Boven et al., 2003), implicating this receptor in neuroinflammatory responses. This idea is supported by the evidence of elevated levels of thrombin in an experimental model of multiple sclerosis (Beilin et al., 2005) and other inflammatory brain diseases (Chapman, 2006). Stimulation of PAR1 by thrombin causes proliferation of glia and produces reactive gliosis, infiltration of inflammatory cells, and angiogenesis (Striggow et al., 2001). Finally, expression of PAR1 is increased in experimental models of Alzheimer's disease (Pompili et al., 2004) and brain ischemia (Striggow et al., 2001).

Both thrombin and its inactive precursor prothrombin have been also detected in the brain (Dihanich et al., 1991; Xi et al., 2003). Prothrombin mRNA shows the highest expression in the cerebral cortex and a moderate expression in the hippocampus and cerebellum (**Figure 2A**; Dihanich et al., 1991). In the hippocampus pronounced immunohistochemical labeling of thrombin is observed in pyramidal cell layers, while lower (clustered) thrombin signal is seen in the fiber-layers (**Figure 2B**). Although the precise cellular source of thrombin in the brain and the molecular mechanisms responsible for its formation and release warrant further investigation, experimental evidence has been provided that neural prothrombin expression and thrombin activity are highly regulated under physiological and pathological conditions (Xi et al., 2003; Stein et al., 2015). Hence, the molecular machinery of thrombin/PAR1 signaling is detected not only in the vascular system but also present in brain tissue, where it seems to act as a modulator of neural plasticity.

Concentration-Dependent Effects of Thrombin on Synaptic Plasticity

Long term potentiation (LTP) or depression (LTD) of synaptic strength, homeostatic plasticity and metaplasticity are considered to play important roles for the ability of the brain to effectively learn and adapt to novel challenges (Malenka, 2003; Turrigiano, 2012; Hulme et al., 2013). Consistent with the crucial role of synaptic plasticity in brain function (and similar to the blood coagulation cascade), plasticity is a highly regulated process composed of multiple feed-forward and feed-back mechanisms. This situation tunes neural networks in a way that promotes stability, but at the same time allows for rapid and site-specific responses; including changes in the threshold, direction and

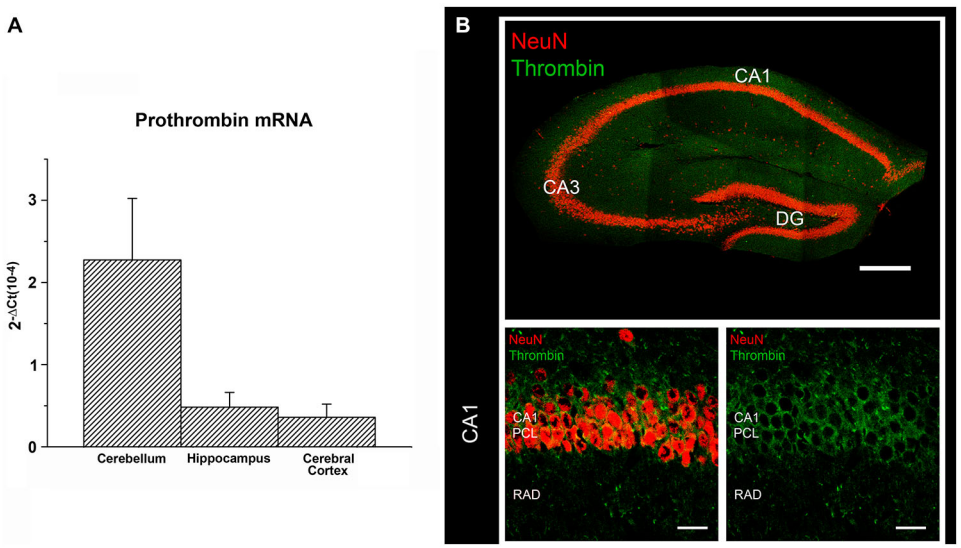

FIGURE 2 | Prothrombin and Thrombin are expressed in the brain. (A) Prothrombin mRNA measured by (RT)qPCR in cerebellum, hippocampus and cerebral cortex of C57BL/6J mice (primers: 5′ CCGAAAGGGCAACCTAGAGC, 5′ GGCCCAGAACACGTCTGTG). The results were normalized to HPRT gene expression within the same cDNA sample and calculated using the ΔC_T method with values being expressed as $2^{-\Delta Ct}$. **(B)** Immunostaining for thrombin (Santa Cruz Biotech, goat polyclonal IgG, cleaved thrombin HC (m361), sc-23335; dilution 1:100 in PBS-based solution) and NeuN (Millipore, mouse monoclonal, MAB377, dilution 1:1000 in PBS-based solution) in the dorsal hippocampus of adult C57BL/6J-mice. Thrombin labeling is predominantly detected in the pyramidal cell layer (PCL) of CA1. The fiber-layers (e.g., stratum radiatum, rad) display weaker but more clustered immunohistochemical signal for thrombin. Scale bar at low magnification = 300 μm; scale bar at higher magnification = 20 μm.

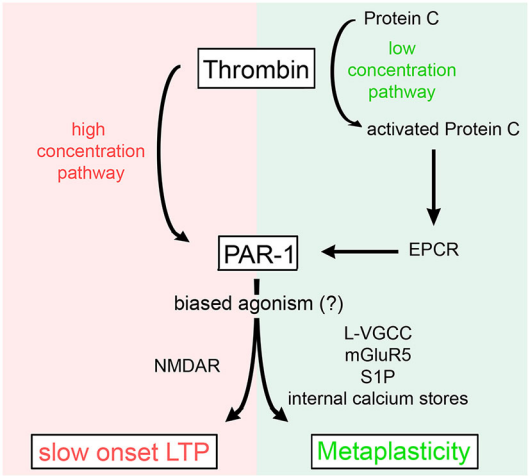

FIGURE 3 | Concentration-dependent effects of thrombin on synaptic plasticity. High levels of thrombin cause a slow onset NMDAR-dependent LTP by direct activation of PAR1. Low concentrations of thrombin activate Protein C which binds to EPCR and activates PAR1 to induce metaplasticity, i.e., a reduction in the LTP threshold [by recruitment of L-type voltage gated calcium channels (L-VGCC), metabotropic glutamate receptors 5 (mGluR5), sphingosine-1-phosphate (S1P), and internal calcium stores]. For further details refer to the main text and Maggio et al. (2013c).

duration of synaptic events. Conceptually this *stable dynamic state* of synaptic plasticity is comparable to the blood coagulation cascade, which is kept in a delicate balance between bleeding and site-specific thrombosis. Therefore, it has been hypothesized that similar signaling pathways are employed in blood coagulation and synaptic plasticity.

Indeed, robust experimental evidence exists that thrombin and other coagulation factors are involved in the regulation of LTP in the hippocampus (Gingrich et al., 2000; Almonte et al., 2007; Maggio et al., 2008, 2013b,c; Mannaioni et al., 2008; Hamill et al., 2009). Furthermore, it has been shown that PAR1-signaling mediates thrombin-induced synaptic plasticity, which requires the activation of NMDARs (Lee et al., 2007; Han et al., 2011; Oh et al., 2012; Park et al., 2013).

In a recent attempt to shed more light on the physiological vs. pathological functions of thrombin and PAR1 in the brain, we studied the effects of different concentrations of thrombin and PAR1 activating peptide (PAR1-AP) on hippocampal LTP. This set of experiments disclosed that thrombin regulates the threshold for synaptic plasticity in a concentration-dependent manner (**Figure 3**; Maggio et al., 2013c). Specifically, $[Thrombin]_{high}$ induced an NMDAR-dependent slow onset LTP, which occluded the ability of neurons to express further LTP (Maggio et al., 2008, 2013c). Conversely, $[Thrombin]_{low}$ promoted L-type voltage gated calcium channel (L-VGCC), metabotropic glutamate receptor 5 (mGluR-5), and intracellular Ca^{2+} store-dependent LTP, which required aPC and the activation of the Endothelial Protein C Receptor (EPCR, detected on astrocytes and neurons) (Maggio et al., 2013c). In a follow-up study, we further clarified the role of aPC and EPCR in regulating $[Thrombin]_{low}$-mediated synaptic plasticity (Maggio et al., 2014). We demonstrated that aPC induces metaplasticity and thereby enhances the ability to induce LTP (Maggio et al., 2014). This effect of aPC is mediated by EPCR-dependent PAR1-activation that triggers the production of Sphingosine-1-Phosphate (S1P). In turn, the activation of S1P-receptor 1 and intracellular Ca^{2+} stores, ultimately lead to a reduction in the LTP threshold (Maggio et al., 2014). Hence, high concentrations of thrombin saturate the ability of neurons to express further LTP via direct PAR1-activation (Maggio et al., 2008), while at low concentration thrombin mediates metaplasticity by enhancing LTP via aPC-EPCR mediated PAR1-signaling (**Figure 3**; Maggio et al., 2013c).

This finding is of considerable interest in clinical settings of cerebrovascular events: upon blood-brain barrier (BBB) opening, exposure to blood-derived thrombin and aPC could interfere with endogenous, i.e., neural thrombin/aPC mediated synaptic plasticity. On the one hand high thrombin concentrations may affect the ability of the brain to use LTP-like plastic processes for acquisition of new "memories", due to the $[Thrombin]_{high}$-mediated saturation of LTP. On the other hand aPC/PAR1 mediated synaptic plasticity (Maggio et al., 2013c, 2014), could facilitate LTP through other mechanisms and may counteract the $[Thrombin]_{high}$ effect (Maggio et al., 2008). The precise role and interplay of thrombin vs. aPC mediated synaptic plasticity under physiological and pathological conditions and the biological consequences for the course of diseases is currently under investigation.

PAR1-Activation Affects Homeostatic Synaptic Plasticity

To learn more about the role of thrombin/PAR1-signaling under pathological conditions and to study its role in other forms of synaptic plasticity, we used the entorhinal denervation *in vitro* model (Del Turco and Deller, 2007) and assessed the role of PAR1 in *denervation-induced homeostatic synaptic plasticity* (Vlachos et al., 2012). This model takes advantage of the highly organized entorhino-hippocampal projection, which originates in the entorhinal cortex, forms the perforant pathway, and terminates in the hippocampus. Lesioning this fiber tract results in the partial denervation of neurons in the hippocampus, without directly damaging the dendritic tree of target neurons (Müller et al., 2010). In turn, neurons respond to the loss of entorhinal input with a slow adaptive, i.e., compensatory increase in excitatory synaptic strength (Vlachos et al., 2012, 2013a,b), which is considered to play an important role in stabilizing the activity of neuronal networks under physiological and pathological conditions (Marder and Goaillard, 2006; Maffei and Fontanini, 2009; Turrigiano, 2012; Vitureira et al., 2012). While the presence of PAR1 was not required for homeostatic plasticity of excitatory synapses to occur, our study revealed that PAR1-activation occludes the ability of partially denervated neurons to increase their excitatory synaptic strength in a compensatory manner (Becker et al., 2014). Thus our results provided first experimental evidence that PAR1 activity affects neural homeostasis, which could be of relevance for

neurological diseases associated with increased brain thrombin concentrations, neuronal cell loss and denervation of connected brain regions (see also Maggio and Vlachos, 2014).

The Role of Thrombin/PAR1-Signaling in Ischemic LTP

Finally, in a recent study we demonstrated that thrombin/PAR1-signaling has a fundamental role in oxygen glucose deprivation (OGD)-mediated alterations in synaptic plasticity (Stein et al., 2015). A brief (i.e., a few minutes) deprivation of oxygen and glucose, as seen for example in the early stage of acute ischemia or transient ischemic attack, potentiates synaptic transmission at hippocampal CA3-CA1 synapses in an NMDAR-dependent manner and induces ischemic LTP (iLTP) without causing neuronal cell death (Crépel et al., 1993; Hsu and Huang, 1997; Calabresi et al., 2003). It has been proposed that iLTP could have a major impact on the functional reorganization of neuronal networks during the early phase of ischemic stroke (Calabresi et al., 2003). In this context, we were able to demonstrate that OGD increases thrombin activity in neural tissue, i.e., in acute hippocampal slices, and triggers the induction of iLTP. Moreover, we showed that the induction of iLTP impairs the ability of neurons to express further LTP in a thrombin/PAR1-dependent fashion. Indeed, iLTP was blocked when thrombin activity or PAR1 signaling were inhibited and the ability of neurons to express LTP was rescued (Stein et al., 2015). We concluded from this series of experiments that iLTP resembles a $[\text{Thrombin}]_{\text{high}}$-mediated LTP and hypothesized that counteracting thrombin signaling in the brain during the acute phase of stroke may improve synaptic plasticity (Stein et al., 2015).

Together, these studies provided robust experimental evidence that PAR1-signaling (which is activated, e.g., by thrombin or aPC) is involved in the regulation of distinct forms of synaptic plasticity under physiological and pathological conditions.

A Role for Thrombin/PAR1 in Epileptogenesis

Although serine proteases are expressed at very low concentration in neural tissue (Luo et al., 2007), their levels increase significantly in the brain following BBB breakdown. In this condition, a large, non-selective increase in the permeability of brain capillaries and tight junctions takes place, such that high molecular weight proteins (Ballabh et al., 2004) and other blood components gain access to the brain. This particular event occurs in several neurological conditions (Ballabh et al., 2004; Tomkins et al., 2008), including hemorrhagic/ischemic stroke (Hjort et al., 2008; Bang et al., 2009) and traumatic brain injury (Barzó et al., 1997; Tomkins et al., 2008; Itzekson et al., 2014). Even though there is a paucity of information concerning the amount of thrombin crossing the BBB, it has been reported that thrombin levels increase more than 200 fold (from 100 pM to 25 nM) in the cerebrospinal fluid of patients with subarachnoid hemorrhage (Suzuki et al., 1992). In addition, if bleeding occurs directly within the brain tissue, active thrombin and other proteases freely diffuse into the brain parenchyma until blood coagulation closes off the injured vessels (Suzuki et al., 1992). A direct consequence of the contact of thrombin with the brain tissue is the onset of seizures (Lee et al., 1997). In 1997, Lee et al. reported that intracerebral injections of thrombin results in focal motor seizures. Interestingly, the injection of thrombin together with its inhibitor alpha-(2-naphthylsulfonyl-glycyl)-4-amidinophenylalaninepiperidide (alpha-NAPAP) prevented both clinical and electrographic seizures (Lee et al., 1997). Similarly, mice engineered to lack Nexin-1, an endogenous thrombin inhibitor, have an increased susceptibility for kainic acid-induced seizures (Luthi et al., 1997).

Following this line of evidence we were able to demonstrate that thrombin-induced seizures are mediated by activation of PAR1 (Maggio et al., 2008). In hippocampal slices, thrombin at a concentration of 5 nM (1 U/ml) increased spontaneous firing of CA3 pyramidal cells (Maggio et al., 2008). In order to examine whether thrombin facilitates the onset of epileptic discharges in conditions mimicking BBB breakdown in the slice (Chen and Swanson, 2003; Beart and O'Shea, 2007), we exposed neurons to thrombin in presence of elevated $[K^+]_o$ or glutamate. In normal slices, addition of 4 mM $[K^+]_o$ did not produce any noticeable spontaneous seizures, which were clearly seen when $[K^+]_o$ were increased to 15 mM. Similarly, 500 μM but not 100 μM glutamate produced spontaneous seizure-like activity in the slice. Remarkably, thrombin facilitated the response to the lower concentration of $[K^+]_o$ (4 mM) and glutamate (100 μM) to produce seizure-like activity. This facilitatory action of thrombin on the production of seizure-like activity was mediated by PAR1, since it was mimicked by PAR1-AP and blocked by the PAR1-inhibitor SCH79797. However, it did not depend on NMDARs as it was not affected by the selective NMDAR-inhibitors ifenprodil or by 2R-amino-5-phosphonovalericacid (APV), indicating that $[\text{Thrombin}]_{\text{high}}$-mediated LTP does not explain the acute effects of thrombin on seizure-induction (Maggio et al., 2008). While thrombin induced epileptogenesis seems to be NMDAR-independent, it is possible that thrombin could also indirectly modulate synaptic transmission, e.g., through thrombin-mediated proteolysis and remodeling of the extracellular matrix (e.g., Dityatev, 2010).

More recently, Isaeva et al. (2012) exposed hippocampal slices prepared from young postnatal rats (P6 to P15) to large concentrations of thrombin (10 U/ml), to find that thrombin depolarizes membrane potential of neurons and produces a hyperpolarizing shift of tetrodotoxin-sensitive *INap* through a PAR1-mediated mechanism (Isaeva et al., 2012). In addition, we have reported that thrombin affects synaptic transmission in hippocampal CA3 neurons by enhancing both frequency and amplitude of mEPSCs, while reducing frequency and amplitude of mIPSCs (Maggio et al., 2013b). Together, these studies showed a pro-epileptic effect of thrombin, which induces membrane and synaptic changes leading to seizures via PAR1-activation. However, whether the thrombin-induced increase in neuronal activity leads to the development of epilepsy at a later stage remains unclear. Nevertheless, it has been demonstrated that high concentrations of thrombin induce apoptosis (Xi et al., 2003; Luo et al., 2007), which may lead to maladaptive circuit

reorganization and to the development of epilepsy. Additional work is required to test this intriguing possibility and to assess whether thrombin could play a crucial role in epileptogenesis.

Open Questions and Conclusions

While robust experimental evidence exists that thrombin affects brain functioning through the concentration-dependent regulation of distinct forms of synaptic plasticity, it remains unclear how thrombin exerts such diverse effects by acting on the same receptor, i.e., via PAR1. Conceptually, several possibilities, or a combination of different mechanisms, need to be considered. In endothelial cells for example the recruitment of distinct PAR1-mediated downstream signaling pathways has been reported: while thrombin mediated PAR1-activation recruits $G_q/G_{12/13}$, aPC seems to act via PAR1-mediated G_i (Riewald and Ruf, 2005). This observation is consistent with a study showing that PAR1 interacts with various G proteins (Russo et al., 2009; McCoy et al., 2012). Also, precise location of PAR1 in neuronal or astrocytic membrane compartments and the current state of neuronal networks seem to influence selectivity of PAR1 to distinct ligands and the recruitment of diverse intracellular signaling pathways by PAR1 (Russo et al., 2009; Bourgognon et al., 2013). Such agonist-biased signal transduction may explain the involvement of PAR1 in mediating and influencing various forms of plasticity, depending on the actual thrombin concentration (**Figure 3**). However, whether distinct subtypes of PAR1 exist, which recruit specific downstream signals, and even show different affinities for thrombin and aPC needs to be tested. The role of simultaneous or selective activation of PAR1 in distinct cell types and/or cellular compartments remains unclear in this context as well. While some evidence for astrocytic PAR1 signaling has been provided (e.g., Vance et al., 2015), the contribution of PAR1 signaling in pre- vs. postsynaptic compartments, in distinct types of interneurons, microglia cells, oligodendrocytes or endothelial cells warrants further investigation. Thrombin may also exert its function through other receptors and proteolysis of specific components in the extracellular matrix (e.g., fibrinogen) under physiological and pathological conditions (e.g., epileptogenesis, see Dityatev, 2010). Indeed, evidence exists that fibrinogen plays an important role in microglia recruitment (Davalos et al., 2012). Hence, it is interesting to speculate that thrombin could modulate neuroinflammatory responses by acting on PAR1 and fibrinogen (Tripathy et al., 2013). Whether thrombin-induced, fibrin-mediated pathways also act on synaptic transmission and plasticity, and the role of PAR1 in this context has not been addressed so far.

Considering the suggested role of thrombin as a major factor for seizures, the cellular source of thrombin in the brain and the molecular machinery responsible for its formation and release needs to be further clarified. Nevertheless, it is interesting to speculate that new oral anticoagulants, which target thrombin could have an unexpected antiepileptic effect (Maggio et al., 2013a), and may restore the ability of neurons to express plasticity under conditions of increased brain thrombin concentrations and PAR1-activity, as seen for example upon BBB breakdown. Its central role in neural plasticity classifies PAR1 as an interesting therapeutic target in thrombin associated pathologies. We are confident that future studies will provide new important insights on the role of thrombin/aPC/PAR1-mediated neural plasticity and excitability and will shed more light on concentration-, ligand-, source-, target-, and state-dependent recruitment of PAR1 signaling cascades. These information may contribute to the development of new strategies to restore brain function under pathological conditions.

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Dystroglycan controls dendritic morphogenesis of hippocampal neurons *in vitro*

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Dendritic outgrowth and arborization are important for establishing neural circuit formation. To date, little information exists about the involvement of the extracellular matrix (ECM) and its cellular receptors in these processes. In our studies, we focus on the role of dystroglycan (DG), a cell adhesion molecule that links ECM components to the actin cytoskeleton, in dendritic development and branching. Using a lentiviral vector to deliver short-hairpin RNA (shRNA) that specifically silences DG in cultured hippocampal neurons, we found that DG knockdown exerted an inhibitory effect on dendritic tree growth and arborization. The structural changes were associated with activation of the guanosine triphosphatase Cdc42. The overexpression of DG promoted dendritic length and branching. Furthermore, exposure of the cultures to autoactivating matrix metalloproteinase-9 (aaMMP-9), a β-DG-cleaving protease, decreased the complexity of dendritic arbors. This effect was abolished in neurons that overexpressed a β-DG mutant that was defective in MMP-9-mediated cleavage. Altogether, our results indicate that DG controls dendritic arborization *in vitro* in MMP-9-dependent manner.

Keywords: dystroglycan, dendritic arborization, extracellular matrix, MMP-9, Cdc42

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Introduction

Dendritic structure and arborization have a profound impact on the processing of neuronal information (Gulledge et al., 2005; Stuart et al., 2007). The molecular mechanisms that regulate the formation of dendritic trees are precisely controlled. Aberrations in these mechanisms are the basis of several neurodevelopmental disorders, including mental retardation and autism (Kulkarni and Firestein, 2012). Appropriate dendritic branching is driven by numerous extracellular signals, including neurotrophic factors, cell adhesion molecules, and neuronal activity, which lead to changes in cytoskeletal organization (Jan and Jan, 2010; Arikkath, 2012). However, our current understanding of how signal transduction from the extracellular matrix (ECM) controls neuronal morphogenesis is still incomplete.

Dystroglycan (DG) is a cell adhesion receptor composed of α and β subunits that form a link between the ECM and intracellular actin cytoskeleton. DG was identified as a glycan component of the dystrophin glycoprotein complex (DGC) in skeletal muscles (Ervasti and Campbell, 1991), but it is also expressed in the nervous system, epithelia, and endothelia (Durbeej et al., 1998). Extracellular α-DG binds to numerous laminin G domain ligands, including laminins, agrin, and perlecan (Gee et al., 1994; Talts et al., 1999), and presynaptic neurexins in the brain (Sugita et al., 2001). The transmembrane β-DG anchors α-DG to the cell membrane via the N-terminal domain and interacts with the cytoskeletal proteins dystrophin and utrophin via the

C-terminal cytoplasmic domain (Ervasti and Campbell, 1991; Barresi and Campbell, 2006). Both subunits undergo posttranslational modifications, such as proteolysis, glycosylation, and phosphorylation (Moore and Winder, 2012). Abnormalities in these processes underlie the pathogenesis of several complex disorders, including dystroglycanopathies and cancer (Singh et al., 2004; Moore and Hewitt, 2009).

Distinct functions of glial and neuronal DG in the brain have been reported (Satz et al., 2010). These studies indicate a crucial role for extracellular α-DG interactions, as the cerebral cortex developed normally in transgenic mice that lacked the DG intracellular domain. β-DG is also known to interact directly with F-actin and thereby may influence actin cytoskeleton remodeling, accompanied by morphological changes (Chen et al., 2003). Moreover, β-DG has been identified as a matrix metalloproteinase-9 (MMP-9) substrate that is digested in response to enhanced neuronal activity (Michaluk et al., 2007). The involvement of MMP-9 in neuronal plasticity and the modulation of dendritic spine morphology has been reported (Michaluk et al., 2011; Dziembowska and Wlodarczyk, 2012; Stawarski et al., 2014). However, the contribution of MMP-9 and its substrates to dendritic morphogenesis is still unclear. Notably, blockade of the proteolytic activity of MMP-9, similarly as DG deletion in neurons, impairs hippocampal long-term potentiation (LTP; Nagy et al., 2006; Satz et al., 2010).

Although β-DG has been shown to be necessary for the formation of filopodia and microvilli-like structures in numerous cell types (Spence et al., 2004a; Batchelor et al., 2007), detailed knowledge of the mechanisms of action of DG in neuronal development is still lacking.

In the present study, we evaluated the effects of the knockdown or overexpression of DG on dendritic arborization in hippocampal neurons *in vitro*. Our results showed that DG deletion simplified dendritic arbor morphology and decreased the total length of dendrites. Conversely, the enhanced neuronal expression of DG resulted in a significant increase in dendritic length and branching. Moreover, we found that treatment of the cultures with autoactivating MMP-9 (aaMMP-9) decreased the complexity of dendritic arbors, and this effect was abolished in neurons that were transfected with a plasmid carrying a β-DG with a mutation in the MMP-9 cleavage site. Furthermore, the DG knockdown-induced simplification of dendritic arbor morphology was dependent on Cdc42 guanosine triphosphatase (GTPase) activity.

Materials and Methods

Animals

All of the animal procedures were performed in accordance with the guidelines of the First Local Ethical Committee on Animal Research in Warsaw (permission no. 554/2013), based on the Polish Act on Animal Welfare and other national laws that are in full compliance with EU directives on animal experimentation.

short-hairpin RNA (shRNA) Constructs and Lentivirus Production

Two short-hairpin RNAs (shRNAs) for DG [SH1 DG (GCUCAUUGCUGGAAUCAUUGC; described previously by Jones et al., 2005) and SH2 DG (UGUCGGCACCUCCAAUUU)] were introduced to a pSilencer (with the U6 promoter) plasmid (Ambion) as double-stranded oligonucleotides. The shRNAs were then subcloned into the pTRIP-PL-WPRE vector together with the SynGFP sequence, which enables the production of lentiviruses that carry shRNA with the simultaneous expression of green fluorescent protein (GFP) driven by the synapsin I promoter. As a control, we used a pTRIP-PL-WPRE vector without shRNA. The lentiviruses were produced in the Laboratory of Cell Engineering, Nencki Institute of Experimental Biology.

Constructs for Dystroglycan Overexpression

The following expression plasmids were used: DG (α + β) without any tag (OE DG), DG (α + β) fused with GFP (OE DG-GFP), β-DG fused with GFP (OE β-DG-GFP), and β-DG fused with GFP with a mutation in the MMP-9 cleavage site (OE β-DG-MUT-GFP). The neurons were transfected with the aforementioned vectors on the third day *in vitro* (DIV). A red fluorescent protein (RFP)-encoding vector was used to visualize the morphology of transfected cells. All of the overexpressed proteins were under the control of the synapsin I promoter.

The DAG-1 coding sequence was amplified via RT-PCR from total rat RNA using the following DG primers: forward (GCATGTCTGTGGACAACTGGCTACTG) and reverse (CGCGTCGACTTAAGGGGGTACATATGGAGG). The cDNA of the full-length rat DAG1 gene, which encodes DG, was cloned into pDrive vector (pDrive-DG). To generate OE DG, DAG-1 cDNA was subcloned into a vector with the synapsin I promoter (pSyn-GFP). The pSyn-GFP plasmid was cleaved with HpaI and SalI enzymes, and the pDrive-DG plasmid was cleaved with SalI and SnaBI enzymes. OE DG was used to generate OE DG-GFP. We amplified GFP cDNA using the following GFP primers that contained overhangs that introduced NdeI and AseI restriction enzyme sites: forward (CTGATCCATATGTACCCCCTATGGTGAGCAAGGGCGAG) and reverse (GGCCGGATTAATTACTTGTACAGCTCGTCCA). The OE DG plasmid was cleaved with NdeI and AseI enzymes. We checked the obtained constructs for the proper orientation of GFP.

OE DG-GFP was used to generate OE β-DG-GFP. We deleted the cDNA region that encodes α-DG, leaving signaling sequences and regions that encode β-DG using the following primers: forward [TCTATTGTGGTCGAGTGGACCAACA (DG-F-DEL)] and reverse [GGCTTGAGCCACAGCCACAGA (DG-R-DEL)]. The mutation in the cleavage site (OE β-DG-MUT-GFP) was generated by inserting two missense point mutations using the following primers: forward [GCACAGTTCATCCCGTGGCACCACCC (DG-MUT-F)] and reverse [TGCTCGGCAACTGCCAGAGCCCGCCACAG (DG-MUT-R)]. The missense mutation was generated by changing His715 and Leu716 to alanines. The enzymes that

were used for cloning were purchased from either New England Biolabs or Thermo Scientific.

Hippocampal Neuronal Culture and Transfection

Dissociated hippocampal cultures were prepared from postnatal day 0 (P0) Wistar rats as described previously (Michaluk et al., 2011). Cells were plated at a density of 120,000 cells per 18-mm-diameter coverslips (Assistant, Germany) that were coated with 1 mg/ml poly-D-lysine (Sigma) and 2.5 μg/ml laminin (Roche). The cells were kept at 37°C in 5% CO_2 in a humidified incubator for 1 week. The cells were transfected with Effectene (Qiagen) or Lipofectamine 2000 Reagent (Invitrogen) according to the manufacturer's protocol on the indicated DIV with the given plasmids. The experiments described below were performed on 8 DIV.

Cortical Neuronal Culture and Nucleofection

Dissociated cortical cultures were prepared from P0 Wistar rats as described previously (Michaluk et al., 2007). Following isolation, 4×10^6 cortical neurons per nucleofection were centrifuged for 5 min at 800 rcf. The pellet was gently resuspended in 100 μl of nucleofection solution and mixed with 3 μg of the given plasmids. The cell suspension was transferred to a nucleofector cuvette and nucleofected using the O−03 program (Amaxa, Lonza). Immediately after nucleofection, 1 ml of pre−warmed complete growth medium was added to the cuvette. Cells were transferred to six-well plates that contained 1 ml of medium. One hour later, the medium was replaced with 2 ml of prewarmed fresh complete growth medium. The cells were kept at 37°C in 5% CO_2 in a humidified incubator for 1 week. The experiments were performed on 8 DIV.

For the Cdc42 activation assay, the cortical cell suspension was plated on six-well plates that were covered with 1 mg/ml poly-D-lysine (Sigma) and 2.5 μg/ml laminin (Roche) at a density of 1×10^6 cells per well.

Lentiviral Treatment of Neuronal Cultures

Primary hippocampal and cortical neurons were infected on 2 DIV with the described lentiviruses. For infection, 1,000 viral particles per cell were used. To determine the silencing effect of the shRNA constructs, the specified amounts of the prepared lentiviral vectors were added to the culture medium according to previously tested ratios.

Cdc42 Activation Assay

On day 5 *in vitro*, 4×10^6 cortical neurons were homogenized in lysis buffer [25 mM HEPES (pH 7.5), 150 mM NaCl, 1% Non-idet P-40, 10 mM MgCl2, 1 mM ethylenediaminetetraacetic acid (EDTA), and 2% glycerol] and centrifuged at 14,000 × *g* for 10 min. The cell extracts were incubated with GST-PAK-PBD (Cell BioLabs) fusion protein that had been conjugated with glutathione beads at 4°C overnight and washed three times with lysis buffer. GST-PAK-PBD-bound Cdc42 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently immunoblotted with Cdc42-specific antibody.

HEK 293 Culture and Transfection

Human embryonic kidney 293 (HEK 293) cells were grown in Dulbecco's modified Eagle medium (DMEM; Gibco) that contained 10% fetal bovine serum (FBS; Invitrogen) and 100 U/ml penicillin/streptomycin (Gibco) at 37°C in 5% CO_2. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Treatment with Recombinant MMP-9

The expression of the auto-activating mutant of MMP-9 G100L (aaMMP-9) was evaluated as described previously (Michaluk et al., 2007). Primary hippocampal cultures were treated with 100 ng/ml of aaMMP-9 every day beginning on 3 DIV.

To evaluate Cdc42 activity, primary cortical cultures were treated with 400 ng/ml of aaMMP-9 on day 5 *in vitro* for 10 min. To assess resistance to MMP-9 proteolytic activity, β-DG-MUT-GFP-expressing HEK 293 cells were exposed to aaMMP-9 at a concentration of 400 ng/ml for 3 h.

Inhibition of Cdc42 Activity

To block Cdc42 activity, the cultures were treated 3 DIV with ZCL 278 (50 μM; Tocris), previously described as a selective Cdc42 inhibitor (Friesland et al., 2013).

Western Blot

The cells were lysed in RIPA buffer (Sigma) that contained 150 mM NaCl, 1.0% IGEPAL CA-630, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 8.0. Protein lysates were then separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore). The membranes were then blocked with 10% non-fat milk in Tris-buffered saline with 0.1% Tween 20 (TBST). After blocking, the membranes were incubated overnight at 4°C with primary antibodies: anti-β-DG (1:500; ab49515, Abcam), horseradish peroxidase (HRP)-GFP (1:1000; LS-C50850, LifeSpan BioSciences), mouse anti-α-DG (1:1500; 05-593; Millipore), and anti-β-actin (1:10000; A5441, Sigma) diluted in 5% non-fat milk in TBST or anti-GFP (1:1000; MAB3580, Chemicon) and anti-Cdc42 (1:500; 11A11, Cell Signaling) diluted in 5% bovine serum albumin in TBST. The blots were washed three times with TBST followed by 1 h incubation with peroxidase-conjugated secondary antibody, diluted 1:5000 in TBST that contained 5% non-fat milk. After washing, bands were detected using the ECL PRIME chemiluminescence detection system (GE Healthcare).

Immunocytochemistry

For immunocytochemical studies cultured cells were fixed 8 DIV with 4% paraformaldehyde (PFA) for 10 min at room temperature and permeabilized with 0.1% Triton X-100 in phosphate-buffered saline (PBST) for 10 min. After blocking non-specific binding sites with 10% normal donkey serum (NDS) diluted in PBST for 1 h at room temperature, the cells were incubated with primary antibodies in PBST that contained 5% NDS at 4°C overnight. The following primary antibodies were used: mouse anti-α-DG (1:100; 05-593, Millipore), mouse anti-β-DG (1:50; ab49515, Abcam), rabbit anti-β-DG (1:100; sc-28535, Santa Cruz Biotechnology), and mouse anti-MAP-2 (1:500;

M4403, Sigma). After washing with PBS, secondary antibodies conjugated with Alexa Fluor 647 or Alexa Fluor 555 (Molecular Probes, Invitrogen) were applied for 1 h at room temperature. After washing with PBS, the coverslips were mounted in anti-quenching medium (Fluoromount G, Southern Biotechnology Associates, Biozol, Eching, Germany) and subjected to imaging analysis.

The specificity of used antibodies was previously confirmed by other studies (e.g., Michaluk et al., 2007; Pribiag et al., 2014). Indeed, in our hands these antibodies have successfully verified the efficiency of DG knockdown or DG overexpression.

Image Acquisition and Analysis

To study dendritic morphology, images of neurons were acquired using a Leica TCS SP5 or SP8 confocal microscope with a PL Apo 40×/1.25 NA oil immersion objective. Morphometric analyses were performed using ImageJ with NeuronJ software (Meijering et al., 2004) and the Sholl plugin (Perycz et al., 2011). The axons were excluded during marking tracings for Sholl analysis.

Statistical Analysis

The data are expressed as mean and SEM. Pair-wise datasets were tested for significant differences using Student's *t*-test.

Results

Knockdown of Dystroglycan Simplifies Dendritic Arbor Morphology

First we determined the time course of the expression of DG in primary hippocampal cultures. We detected low level of DG on 2 DIV, which was gradually increased up to 7 DIV (**Figure 1A**). To define the possible role of DG in dendritic tree arborization, we decreased its expression using lentiviral shRNA vectors. To verify DG knockdown efficiency, the cells were infected 2 DIV with shRNA1 (LV SH1 DG), shRNA2 (LV SH2 DG), or an empty lentivirus (LV GFP). After 6 days, immunofluorescence staining and Western blot were performed (for detailed kinetic, see Supplementary Figure S1). As shown in **Figure 1B**, neurons that were infected with LV SH1 DG exhibited a reduction of endogenous β-DG expression compared with LV GFP-infected cells. Immunoblotting also revealed efficient β-DG down regulation caused by LV SH1 DG and LV SH2 DG, whereas high levels of β-DG expression were detected in cultures that were infected with LV GFP and control non-infected cultures (**Figure 1C**).

To evaluate the effect of DG silencing on dendritic morphogenesis, 4 days after infection the cultures were additionally transfected with an RFP-encoding vector, which allowed the observation of neuronal morphology. Images for the morphometric analysis were acquired 8 DIV. We selected this time point because intensive dendritogenesis in primary hippocampal neurons occurs during the first week *in vitro*. Representative images of neurons in all of the groups are presented in **Figure 1D**. Infection with LV SH1 DG and LV SH2 DG clearly decreased the complexity of dendritic arbor morphology compared with controls (non-infected cells or cells infected with LV GFP). To precisely evaluate the pattern of dendritic branching, we performed a Sholl analysis (Sholl, 1953), which measures the number of dendrites that cross circles at various radial distances from the cell soma. The results showed that DG silencing induced the shrinkage of dendritic arbors compared with controls, reflected by a downward shift of the Sholl plot (**Figure 1E**). At most of the measured distances (30–140 μm from the cell body), the number of crossings was significantly reduced in DG-deficient neurons ($p < 0.001$). Moreover, the shRNA-mediated knockdown of DG considerably decreased the total dendritic length (**Figure 1F**; non-infected cells: $l = 1417 \pm 38$ μm, $n_{cell} = 109$; LV GFP: $l = 1367 \pm 38$ μm, $n_{cell} = 85$; LV SH1 DG: $l = 759 \pm 32$ μm, $n_{cell} = 87$; LV SH2 DG: $l = 713 \pm 32$ μm, $n_{cell} = 51$; $p < 0.001$). These results may indicate that DG is an important regulator of dendritogenesis in developing primary hippocampal neurons.

Overexpression of Dystroglycan Promotes Dendritic Growth and Branching

To further confirm the role of DG in dendritic morphogenesis, we developed three plasmid vectors to overexpress DG in primary neurons. The first plasmid encoded DG that was fused with GFP at the C-terminus (OE DG-GFP). However, to exclude the possibility that the observed effect was attributable to disturbances in the C-terminal domain of DG, we also produced a DNA construct without GFP (OE DG). Additionally, to study the function of β-DG alone (without the α subunit) in neuronal morphogenesis, we created a vector that overexpressed β-DG (with a signaling sequence) fused with GFP (OE β-DG-GFP).

To verify the effectiveness of the aforementioned constructs, we first evaluated the expression of α- and β-DG by immunofluorescence and Western blot. In neurons that were transfected with the OE DG, OE DG-GFP, or OE β-DG-GFP vector, we observed enhanced β-DG immunostaining compared with neighboring cells (**Figure 2A**). At the same time we noted increased immunoreactivity of α-DG only in neurons transfected with the OE DG or OE DG-GFP plasmid (**Figure 2A**). For the Western blot analysis, we used nucleofection technology (Amaxa, Lonza) to obtain high-efficiency transfection. The immunoblotting of protein lysates with anti-β-DG and anti-GFP antibodies revealed a band of approximately 70 kDa, which corresponds to β-DG fused with GFP (**Figure 2B**). The level of overexpression was lower than the endogenous levels of β-DG, however by using synapsin promoter we obtained increased expression of β-DG exclusively in neurons, while endogenously it is also expressed in glial cells present in hippocampal cultures. In addition, we observed increased level of α-DG in HEK 293 cells transfected with OE DG-GFP vector (see Supplementary Figure S2). These results confirmed that we were able to obtain DG overexpression in transfected cells, and this DG overexpression was properly ascribed to the α and β subunits.

To assess the effect of DG overexpression on dendritic arbor morphology, the cultures were transfected with the OE DG, OE DG-GFP, or OE β-DG-GFP vector and co-transfected with an RFP-coding vector 3 DIV. Images for the Sholl analysis

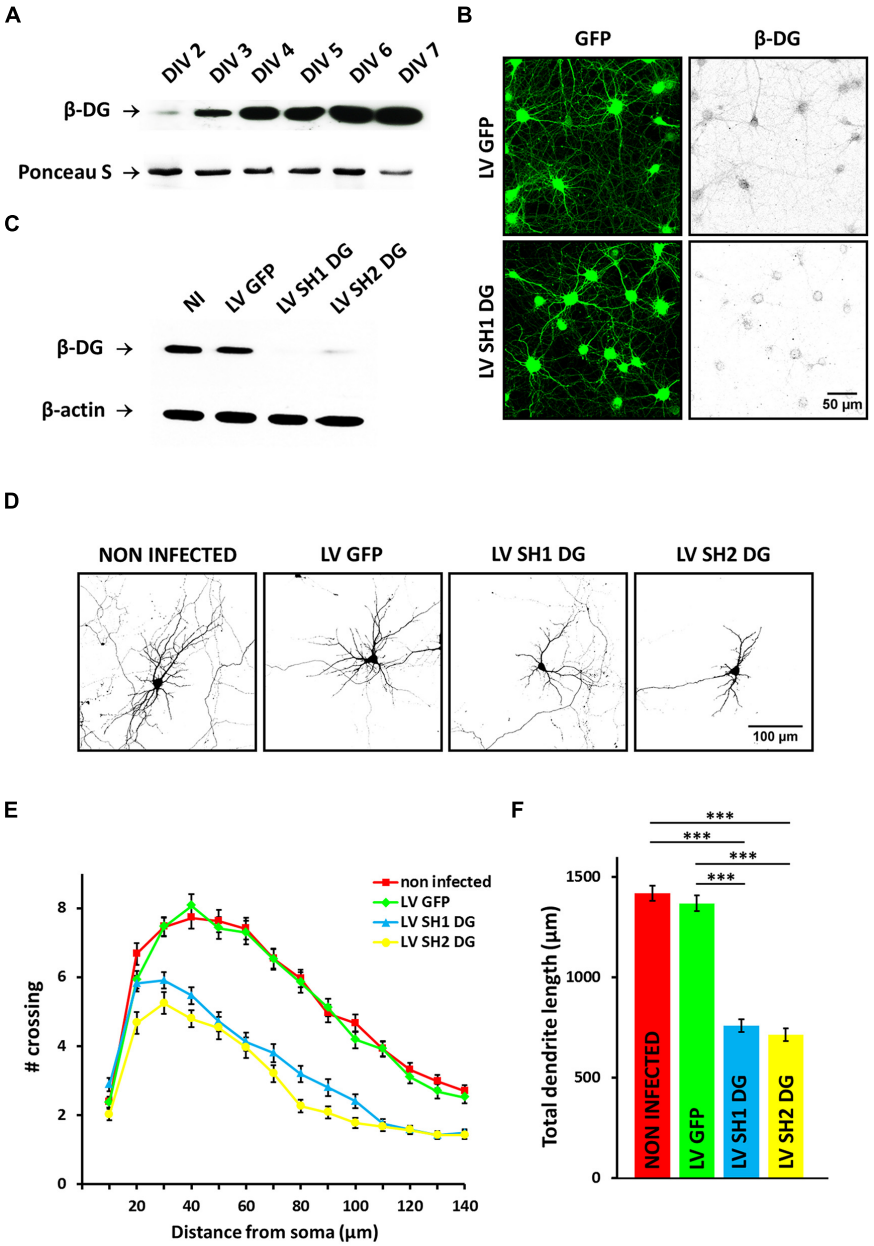

FIGURE 1 | Knockdown of dystroglycan (DG) impairs dendritic tree development. (A) Time-course of the DG expression in primary hippocampal cultures – representative western blot analysis is shown. **(B)** Confocal images of hippocampal neurons infected with either empty lentivirus [LV green fluorescent protein (GFP)] or a lentivirus that carries short-hairpin RNA (shRNA) for DG (LV SH1 DG) and immunostained with anti-β-DG antibody. **(C)** Western blot analysis of β-DG expression in protein lysates from non-infected neurons (NI) and neurons infected with LV GFP, LV SH1 DG, or LV SH2 DG. β-actin served as a loading control. **(D)** Representative images of neurons used for the morphometric analysis. The cells were transfected with an RFP-coding vector to visualize the exact morphology. **(E)** Sholl analysis of hippocampal neurons treated as indicated (NI, $n_{cell} = 109$; LV GFP, $n_{cell} = 85$; LV SH1 DG, $n_{cell} = 87$; LV SH2 DG, $n_{cell} = 51$). At distances of 30–140 µm from the cell body, the differences between DG-deficient neurons (LV SH1 DG and LV SH2 DG) and the control groups (NI and LV GFP) were statistically significant ($p < 0.001$). **(F)** Total dendritic length of neurons treated as in **(C)**. Three independent experiments were performed. The data are expressed as mean ± SEM. $^{***}p < 0.001$ (Student's *t*-test).

were acquired 8 DIV. As shown in **Figure 2C**, neurons that overexpressed DG exhibited enhanced dendritic arborization, regardless of the presence of the GFP tag in the construct. This fact indicates that the presence of GFP likely did not interfere with the C-terminal-mediated function of DG.

The morphometric analysis revealed an upward shift of the Sholl plot in neurons that were transfected with the OE DG, OE DG-GFP, or OE β-DG-GFP vector compared with non-transfected cells (**Figure 2D**), indicating an increase in the complexity of dendritic trees. At most of the measured

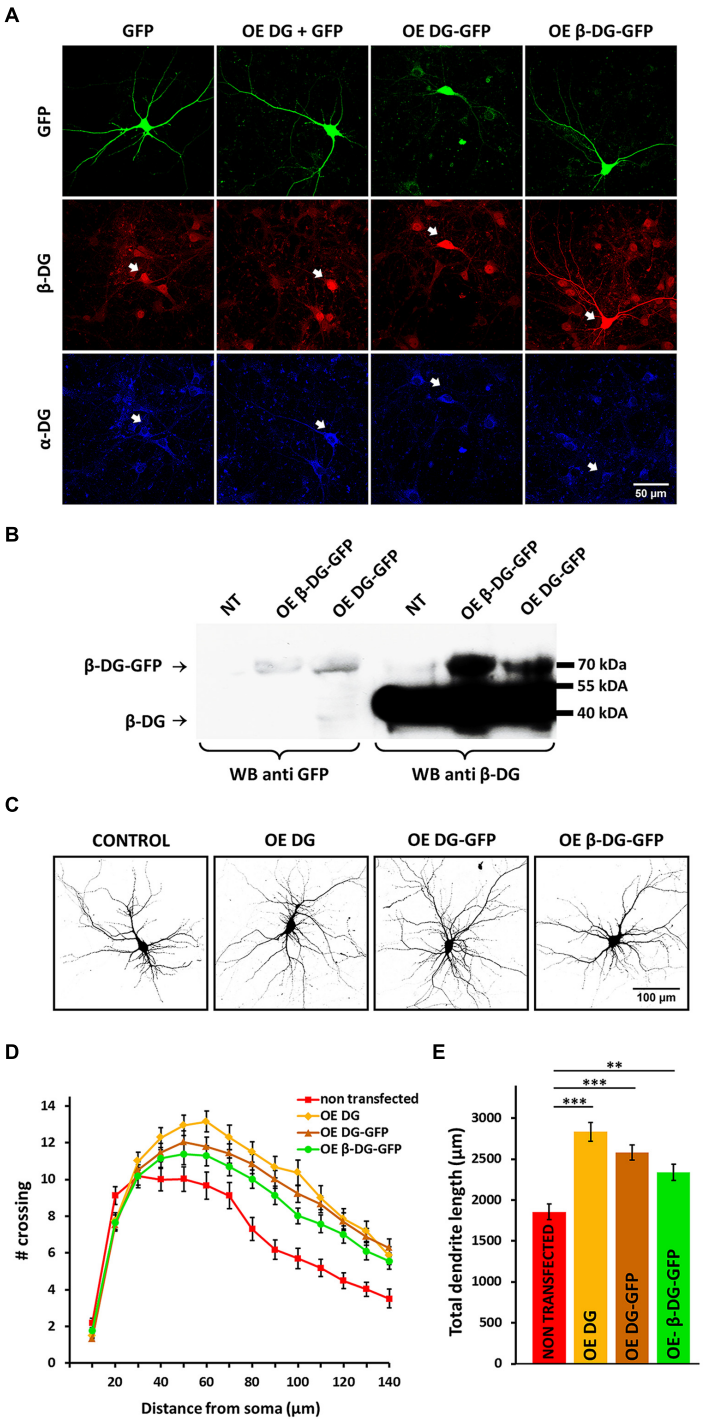

FIGURE 2 | Overexpression of DG stimulates dendritic outgrowth and arborization. (A) The upper panel shows confocal images of hippocampal neurons transfected with a GFP-coding vector (GFP) or vectors used for DG overexpression: OE DG (co-transfected with GFP), OE DG-GFP, and OE β-DG-GFP (green). The lower panels show α-DG (blue) and β-DG (red) immunofluorescence staining of cells in the same fields. The arrowheads indicate transfected cells. **(B)** Immunoblot analysis of GFP and β-DG expression in cortical neurons after nucleofection with either OE DG-GFP or OE β-DG-GFP plasmid. Non-transfected cells (NT) served as a control. **(C)** Representative images of neurons used for the morphometric analysis. The cells were co-transfected with an RFP-coding vector to visualize the exact morphology. **(D)** Sholl analysis of hippocampal neurons treated as indicated (NT, n_{cell} = 36; OE DG, n_{cell} = 40; OE DG-GFP, n_{cell} = 57; OE β-DG-GFP, n_{cell} = 56). At distances of 80–120 µm from the cell body, the differences between DG-overexpressed neurons (OE DG, OE DG-GFP, and OE β-DG-GFP) and the control group (NT) were statistically significant ($p < 0.001$). **(E)** Total dendritic length for neurons treated as in **(C)**. Three independent experiments were performed. The data are expressed as mean ± SEM. $^{**}p < 0.01$, $^{***}p < 0.001$ (Student's *t*-test).

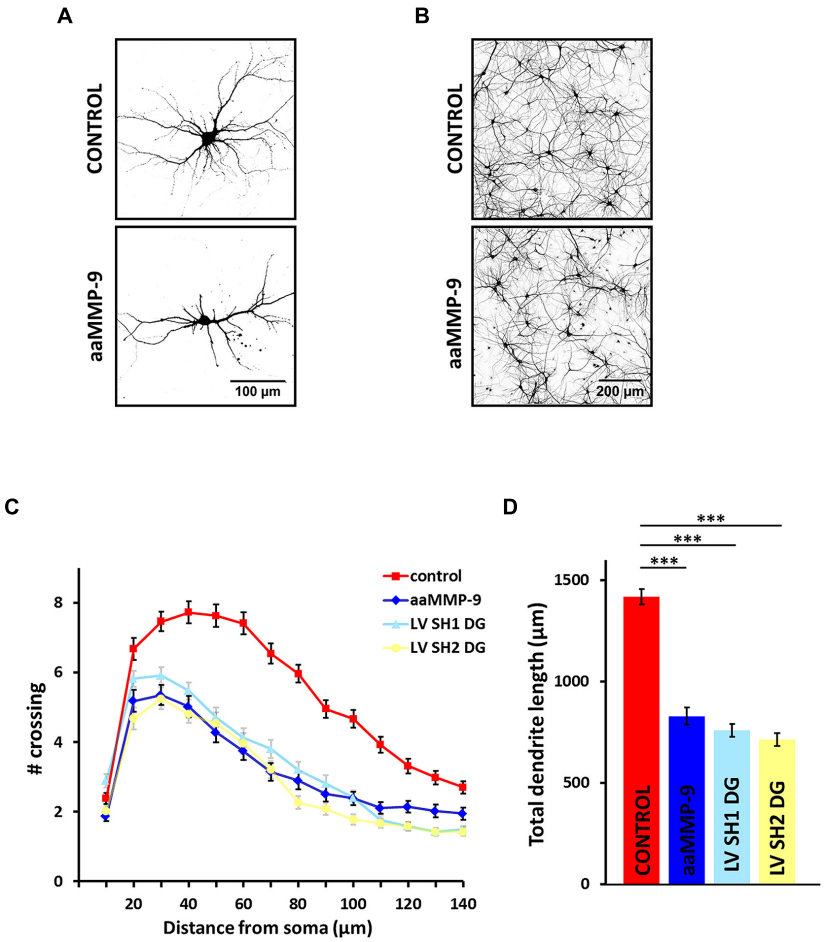

FIGURE 3 | Matrix metalloproteinase-9 (MMP-9) activity inhibits dendritic growth and arborization. (A) Representative images of hippocampal neurons from control culture and cultures treated with autoactivating MMP-9 (aaMMP-9). The cells were transfected with an RFP-coding vector to visualize the exact morphology. **(B)** Representative images of hippocampal cultures treated as in **(A)** following immunofluorescence staining with anti-MAP2 antibody. **(C)** Sholl analysis of hippocampal neurons exposed to aaMMP-9 ($n_{cell} = 55$) vs. control ($n_{cell} = 36$) and neurons infected with either LV SH1 DG ($n_{cell} = 87$) or LV SH2 DG ($n_{cell} = 51$). At distances of 20–130 µm from the cell body, the differences between aaMMP-9-treated neurons and the control group were statistically significant ($p < 0.001$). At distances of 10–90 µm from the cell body, no statistically significant differences were found between aaMMP-9-treated neurons and neurons infected with LV SH1 DG or LV SH2 DG. **(D)** Total dendritic length for neurons treated as in **(C)**. All of the experiments were performed in triplicate. The data are expressed as mean ± SEM. ***$p < 0.001$ (Student's *t*-test).

distances (80–120 µm from the cell body), the number of crossings significantly increased in neurons that overexpressed DG or β-DG ($p < 0.001$). The overexpression of DG also affected dendritic outgrowth (**Figure 2E**). The total dendritic length increased in neurons that were transfected with OE vectors compared with non-transfected cells (OE DG: l = 2835 ± 114 µm, $n_{cell} = 40$, $p < 0.001$; OE DG-GFP: l = 2582 ± 92 µm, $n_{cell} = 57$, $p < 0.001$; OE β-DG-GFP: l = 2338 ± 97 µm, $n_{cell} = 56$, $p < 0.002$; non-transfected cells: l = 1856 ± 92 µm, $n_{cell} = 36$).

The enhancing effect on dendritic trees was observed only in cells that were transfected 3 DIV. When transfection was performed 5 DIV, these morphological changes were not evident (data not shown). This observation indicates that DG is particularly necessary during the early stages of dendritic development. Interestingly, the overexpression of β-DG alone (without α-DG) fused with GFP evoked a very similar effect on dendritic arbors as overexpressed full-length DG. These data suggest an important role for β-DG in neuronal development.

MMP-9 Inhibits Dendritic Growth and Branching

β-dystroglycan has been previously shown to be proteolytically processed by MMP-9 in activated neurons (Michaluk et al., 2007). Thus, we decided to examine the effect of exogenous MMP-9 on dendritic arborization. We treated primary hippocampal cultures with recombinant aaMMP-9 once per day beginning 3 DIV. As shown in **Figure 3A**, 5-day exposure to aaMMP-9 reduced dendritic tree arborization compared with control untreated neurons. Similarly, immunostaining against microtubule-associated protein 2 (MAP2) showed decreased complexity of the neural network in aaMMP-9-treated cultures (**Figure 3B**).

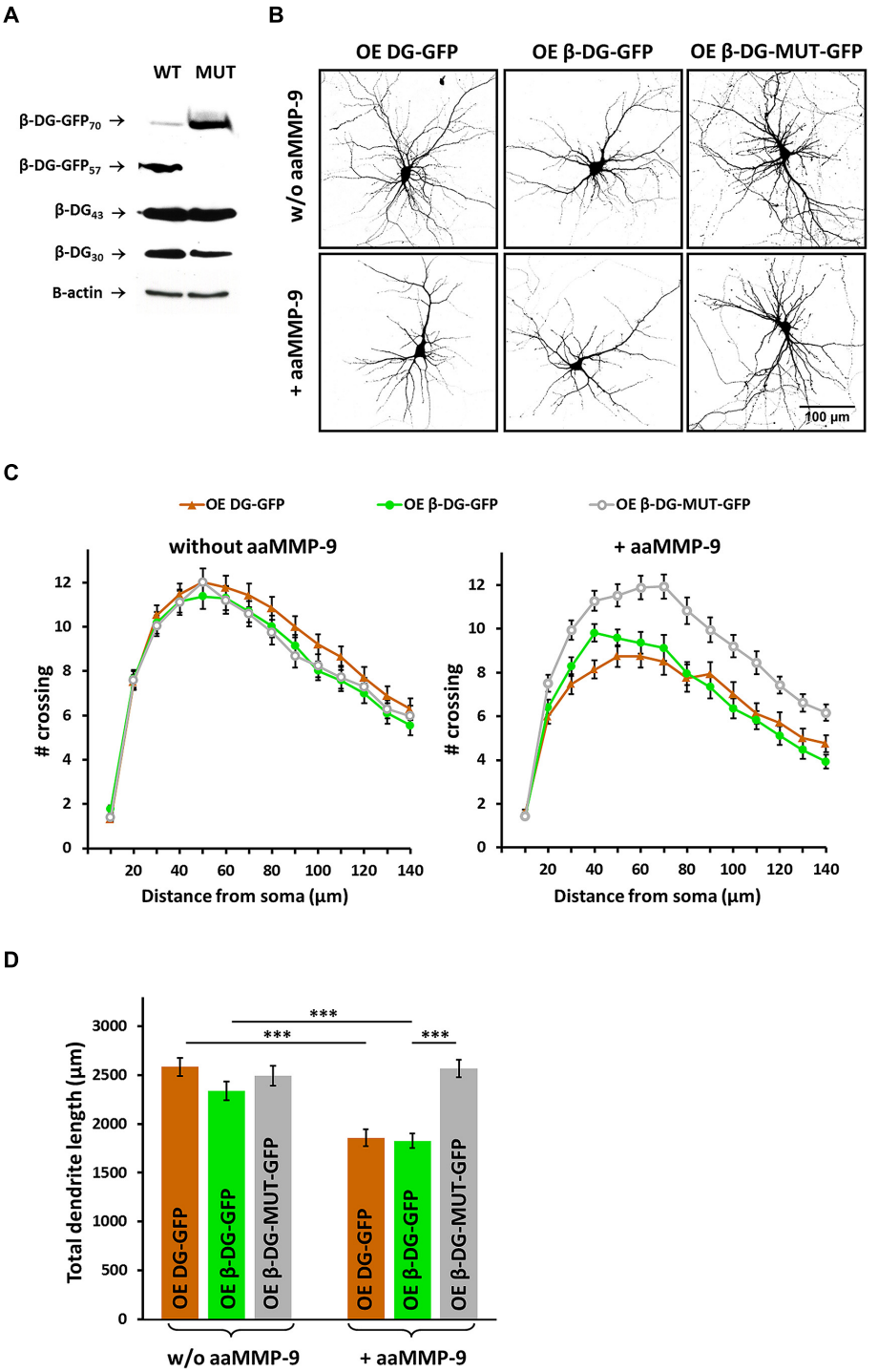

FIGURE 4 | Matrix metalloproteinase-9 dependent cleavage of β-DG is important for dendritic development. (A) Immunoblot analysis of β-DG and GFP expression in protein lysates from HEK 293 cells transfected with either OE β-DG-GFP (WT) or OE β-DG-MUT-GFP (MUT) plasmids and then treated with aaMMP-9. β-actin served as a loading control. **(B)** Representative images of neurons used for the morphometric analysis. The cells were co-transfected with an RFP-coding vector to visualize the exact morphology. **(C)** Sholl analysis of hippocampal neurons treated as indicated ($n_{cell} = 50 \pm 4$). In control cultures (without aaMMP-9), no statistically significant differences were found between DG-overexpressed neurons (transfected with either OE DG-GFP or OE β-DG-GFP) and neurons transfected with the OE β-DG-MUT-GFP vector. In cultures treated with aaMMP-9, at distances of 30–140 µm from the cell body, the differences between DG-overexpressed neurons (OE DG-GFP and OE β-DG-GFP) and neurons that overexpressed mutated β-DG (OE β-DG-MUT-GFP) were statistically significant ($p < 0.02$). **(D)** Total dendritic length for neurons treated as indicated. All of the experiments were performed in triplicate. The data are expressed as mean ± SEM. ***$p < 0.001$ (Student's *t*-test).

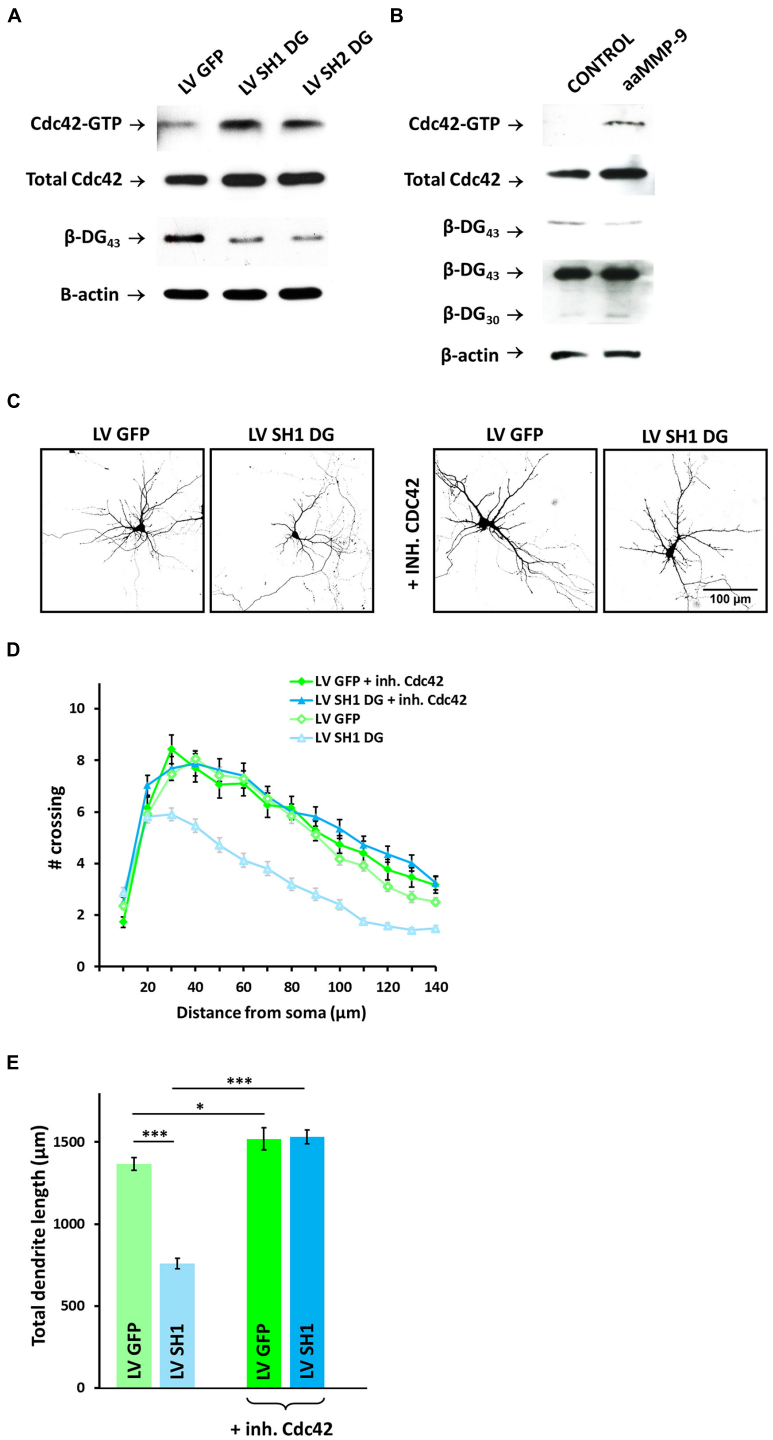

FIGURE 5 | Cdc42 activity contributes to dendritic atrophy caused by DG knockdown or MMP-9 treatment. (A) Western blot analysis of active Cdc42 (Cdc42-GTP) and total Cdc42 in cortical neurons infected with LV GFP, LV SH1 DG, or LV SH2 DG. Silencing efficiency was checked by immunoblotting with anti-β-DG antibody. β-actin was used as a loading control. **(B)** Western blot analysis of active Cdc42 (Cdc42-GTP) and total Cdc42 in control and aaMMP-9-treated cortical neurons. Immunoblotting with anti-β-DG served as a control for the proteolytic activity of MMP-9. β-actin was used as the loading control. **(C)** Representative images of neurons used for the morphometric analysis. The cells were transfected with an RFP-coding vector to visualize the exact morphology. **(D)** Sholl analysis of hippocampal neurons treated as indicated (LV GFP, $n_{cell} = 85$; LV SH1 DG, $n_{cell} = 87$; LV GFP + Cdc42 inhibitor, $n_{cell} = 28$; LV SH1 DG + Cdc42 inhibitor, $n_{cell} = 51$). At distances of 20–140 µm from the cell body, the differences between DG-deficient neurons that were treated with the Cdc42 inhibitor and DG-deficient neurons that were not treated with the Cdc42 inhibitor were statistically significant ($p < 0.001$). **(E)** Total dendritic length for neurons treated as indicated. Three independent experiments were performed. The data are expressed as mean ± SEM. $^{*}p < 0.05$, $^{***}p < 0.001$ (Student's *t*-test).

The morphometric analysis revealed that enhanced MMP-9 activity evoked the shrinkage of dendritic arbors compared with untreated controls, reflected by a downward shift of the plot (**Figure 3C**). At most of the measured distances (20–130 μm from the cell body) the number of crossings was significantly reduced in aaMMP-9-treated neurons ($p < 0.001$). MMP-9 activity also decreased the total dendritic length (**Figure 3C**; untreated cells: $l = 1417 \pm 38$ μm, $n_{cell} = 109$; aaMMP-9: $l = 829 \pm 43$ μm, $n_{cell} = 55$; $p < 0.001$). Interestingly, the morphology of aaMMP-9-treated neurons was very similar to the morphology of DG-deficient neurons. At distances of 10–90 μm from the cell body, no statistically significant differences were found between aaMMP-9-treated neurons and neurons that were infected with LV SH1 DG or LV SH2 DG. These groups were also very comparable with regard to total dendritic length, which was approximately 50% less than in controls (**Figure 3D**). These results may indicate that MMP-9 is also an important regulator of dendritogenesis and suggest that DG and MMP-9 cooperate in regulating the morphology of dendritic arbors.

MMP-9-Mediated Cleavage of β-DG is Involved in Neuritogenesis

We found that the silencing of DG and enhanced activity of MMP-9 exert similar effects on dendritic morphology. To test whether this similarity arises from the fact that MMP-9 inactivates DG by proteolytic processing, we modified the protease cleavage site in the OE β-DG-GFP vector using site-directed mutagenesis. The OE β-DG-MUT-GFP vector was introduced into HEK 293 cells, and its resistance to cleavage by MMP-9 was determined. We performed Western blot using an anti-GFP antibody to verify the cleavage of overexpressed β-DG and anti-β-DG to verify the cleavage of endogenous β-DG. As shown in **Figure 4A**, the mutation prevented the cleavage of β-DG by MMP-9 compared with wild type β-DG.

We then attempted to determine whether chronic MMP-9 activity can prevent the enhancement of dendritogenesis that is caused by DG overexpression. Neurons were transfected with OE DG-GFP, OE β-DG-GFP, or OE β-DG-MUT-GFP and treated once per day with aaMMP-9 beginning 3 DIV. As shown in **Figure 4B**, aaMMP-9 treatment disrupted dendritogenesis in neurons that overexpressed DG or β-DG but did not influence the morphology of neurons that were transfected with OE β-DG-MUT-GFP.

The results of the Sholl analysis showed that treatment with aaMMP-9 induced the shrinkage of dendritic arbors in neurons that were transfected with either OE DG-GFP or OE β-DG-GFP, whereas we did not observe any differences in neurons that were transfected with OE β-DG-MUT-GFP after aaMMP-9 stimulation (**Figure 4C**). At most of the measured distances (20–140 μm from the cell body), the number of crossings in OE DG-GFP- or OE β-DG-GFP-transfected neurons significantly reduced upon exposure to aaMMP-9 ($p < 0.02$ and 0.04, respectively). The number of crossings in OE β-DG-MUT-GFP-transfected neurons did not significantly change after stimulation with aaMMP-9.

We also evaluated the effect of aaMMP-9 treatment on total dendritic length. In neurons that overexpressed DG-GFP or β-DG-GFP, daily stimulation with aaMMP decreased this parameter (from $l = 2582 \pm 92$ μm, $n_{cell} = 57$ for OE DG-GFP to $l = 1856 \pm 86$ μm, $n_{cell} = 38$ for OE DG-GFP + aaMMP-9, $p < 0.001$; from $l = 2338 \pm 97$ μm, $n_{cell} = 56$ for OE β-DG-GFP to $l = 1825 \pm 75$ μm, $n_{cell} = 50$ for OE β-DG-GFP + aaMMP-9, $p < 0.001$; **Figure 4D**). In contrast, the total dendritic length was unchanged in OE β-DG-MUT-GFP-transfected neurons upon aaMMP-9 stimulation (from $l = 2494 \pm 102$ μm, $n_{cell} = 46$ for OE β-DG-MUT-GFP to $l = 2566 \pm 90$ μm, $n_{cell} = 47$ for OE β-DG-MUT-GFP + aaMMP-9, $p = 0.39$; **Figure 4D**).

We also compared the effect of aaMMP-9 on OE β-DG-GFP-transfected neurons with neurons that expressed OE β-DG-MUT-GFP. We observed a significantly lower number of crossings (50–140 μm from the cell body, $p < 0.002$) and a decrease in total dendritic length ($p < 0.001$) in cells that were transfected with wild type β-DG-GFP. These results indicate that the MMP-9-mediated cleavage of β-DG is important for morphological changes that accompany neuritogenesis.

Morphological Effects of DG Knockdown and MMP-9 Treatment are Rescued by the Inhibition of Cdc42 Activity

Cdc42 GTPase is well known to play an important role in the growth and branching of axons and dendrites. DG has been shown to cooperate with Cdc42 to induce the formation of filopodia structures. Therefore, we examined whether DG knockdown influences the activity of Cdc42 in cultured cortical neurons using a pull-down assay with GST-PAK-PBD. We observed an increase in the level of GTP-bound Cdc42 (i.e., active Cdc42) in SH1 DG- and SH2 DG-infected neurons compared with control untreated cells (**Figure 5A**). The enhanced activation of Cdc42 GTPase was associated with a reduction of the complexity of dendritic trees since the similar Cdc42 activation was also observed in cultures that were exposed to aaMMP-9 (**Figure 5B**).

We next determined whether Cdc42 inhibition rescues dendritogenesis in DG-deficient neurons. LV SH1 DG-infected hippocampal neurons were treated with 50 μM of the selective Cdc42 inhibitor ZCL 278 (Friesland et al., 2013). As shown in **Figure 5C**, the disruption of dendritogenesis was rescued by blocking Cdc42 activity. Moreover, we did not observe any significant differences in dendritic arborization in LV GFP-infected neurons evoked by treatment with the Cdc42 inhibitor.

The Sholl analysis revealed similar upregulation of dendritic tree complexity in LV GFP-infected neurons, regardless of ZCL 278 treatment, and LV SH1 DG-infected cells that were exposed to ZCL 278 compared with DG-deficient neurons that were only infected with LV SH1 DG. The number of crossings was significantly higher at most of the measured distances (20–140 μm from the cell body; $p < 0.001$; **Figure 5D**).

Similarly, a high degree of rescue was observed with regard to total dendritic length (from $l = 759 \pm 32$ μm, $n_{cell} = 87$ for SH1

DG to l = 1531 ± 40 μm, n_{cell} = 51 for SH1 DG + ZCL 278, $p < 0.001$; **Figure 5E**). This parameter was only slightly increased upon Cdc42 inhibition in LV GFP-infected cells.

These results suggest that DG plays a crucial role in the process of neuritogenesis through the inhibition of Cdc42 activity. Furthermore, the enhancement of proteolytic MMP-9 activity may cause DG inactivation through cleavage, leading to the disinhibition of Cdc42 and impairments in dendritic arbor development.

Discussion

Dystroglycan is a major ECM receptor that is expressed in various tissues, including the central nervous system (Durbeej et al., 1998; Zaccaria et al., 2001). Deletion of the gene that encodes DG (*Dag1*) causes early embryonic lethality in mice, indicating its important role during mammalian development (Williamson et al., 1997). Previous studies demonstrated that DG expressed on glial cells is responsible for basement membrane formation, whereas neuronal DG facilitates hippocampal LTP, one of several phenomena that underlie synaptic plasticity (Satz et al., 2010). The number and pattern of synapses that are received by each neuron are well known to be determined by dendritic outgrowth and arborization (Koleske, 2013). However, unknown is whether DG affects dendritic development, which is crucial in the formation of functional neural networks.

The present study provides evidence that DG plays an important role in early stages of neuritogenesis in cultured hippocampal neurons. We detected a high level of DG expression at 1 week in cultures. These results contradict previous research published by Lévi et al. (2002) showing a very weak expression of DG during maturation of hippocampal neurons *in vitro*. These discrepancies may be due to differences in the models used in both studies. We found that the lentiviral-mediated shRNA knockdown of DG reduced dendritic growth and branching. In contrast, the overexpression of either full-length DG or β-DG alone had the opposite effect (i.e., the promotion of dendritic tree development). Furthermore, our results indicated that the observed disturbances in dendritic arborization might be caused by the MMP-9-mediated proteolysis of DG and are associated with Cdc42 GTPase activation.

Neuron–ECM interactions are considered essential for proper dendritic development and function (Myers et al., 2011; Skupien et al., 2014; Thalhammer and Cingolani, 2014). DG is a high-affinity receptor for several ECM components, including laminin (Ervasti and Campbell, 1991, 1993). The importance of the DG-laminin interaction is supported by the fact that mice with astrocyte-specific DG deletion exhibit discontinuities in pial surface basal lamina (Moore et al., 2002). Since in the present study we used cells grown on laminin, it would be interesting to check whether the simplification of dendritic arbors evoked by DG silencing is attributable to the weakening of laminin binding.

We further found that the overexpression of OE DG, OE DG-GFP, or OE β-DG-GFP promoted hippocampal dendritic development. Interestingly, the overexpression of β-DG caused the same effect on dendritic arborization as overexpression of full-length DG that contained the α and β subunits. This may indicate the important role of downstream signaling mediated by β-DG. DG has been reported to be a scaffold protein that interacts with components of the ERK-MAP kinase cascade (Spence et al., 2004b; Moore and Winder, 2010). Moreover, the cytoplasmic tail of β-DG has been shown to interact with utrophin, an actin-binding protein, and directly with the actin cytoskeleton (Chen et al., 2003).

We observed a pronounced effect of DG on dendritic maturation. We tested whether the MMP-9-mediated cleavage of DG is functionally involved in this process. We found that daily treatment of hippocampal cultures with aaMMP-9 for 5 days inhibited dendritic growth and branching. Interestingly, this effect coincided with the effect induced by infection with LV SH DG. Given the results that indicated that MMP-9 interferes with proper dendritic arbor formation, we next considered how β-DG mutation in the MMP-9 cleavage site might affect dendritogenesis. We found that the overexpression of β-DG-MUT-GFP abolished the growth-inhibiting effect of MMP-9 on dendritic trees. Matrix metalloproteinases were once believed to regulate neurite extension by simply degrading the surrounding ECM to create a passage for process outgrowth (Ethell and Ethell, 2007; Sarig-Nadir and Seliktar, 2010). However, more recent *in vitro* and *in vivo* studies suggest that proteolytic cleavage is directed toward specific ligands and receptors to regulate neuronal development and regeneration (Fujioka et al., 2012). In the present study, we found that MMP-9 may act as a regulator of dendritic arbor maturation, and the digestion of DG is important for this activity.

Actin reorganization that accompanies morphological changes is generally known to be regulated by Rho-family small GTPases, such as Rho, Rac, and Cdc42. Their activity is strictly controlled by multiple guanine nucleotide exchange factors (GEFs), guanine nucleotide dissociation inhibitors (GDIs), and GTPase-activating proteins (GAPs). DG was previously shown to bind the cytoskeletal adapter protein ezrin and form a complex with a Rho GEF that is responsible for activating Cdc42 and thus causing filopodia formation in fibroblasts (Batchelor et al., 2007). In the present study, we examined the expression of active Cdc42 in LV SH DG-infected neurons and neurons treated with aaMMP-9. In both cases, we observed an increase in Cdc42 activity. This result was surprising because in most studies published to date, Cdc42 inhibition resulted in significant simplification of dendritic trees in many types of neuron (Govek et al., 2005; Negishi and Katoh, 2005). However, the functional outcome of the activity of GTPases must be considered within the context of additional considerations, such as the temporal control of activation and cell type. Notably, the signaling pathways that employ Rho GTPases are highly regulated and can produce different cellular responses under different cellular conditions. For example, both constitutively active Cdc42 and Rac1 that is expressed in *Drosophila* giant fiber neurons inhibit neurite outgrowth (Allen et al., 2000). Moreover, our data are consistent with the observation that the suppression of Cdc42 signaling during mouse cortical development is required

to promote the branching of dendritic trees (Rosário et al., 2012). In the same paper, the authors showed that mutations in Cdc42 GAP (NOMA-GAP) resulted in hyperactive Cdc42 and simplified dendritic branches. The complex role of Cdc42 in dendritogenesis has recently been reviewed (Simó and Cooper, 2012). One unresolved issue involves the DG-dependent intracellular signaling pathways that regulate Cdc42 and cytoskeletal rearrangement.

Altogether, we established that DG is important for dendritic outgrowth and arborization in primary hippocampal neurons. Further studies are required to elucidate the signaling mechanisms of DG action. However, our findings indicate the involvement of MMP-9-mediated proteolytic cleavage of the β-DG extracellular domain and intracellular activation of Cdc42 GTPase. These results may provide insights into the role of interactions between cell adhesion receptors and the ECM in neuritogenesis and synaptic plasticity and open the way to *in vivo* studies that may help to understand the molecular bases of several neurological disorders, including mental retardation associated with dystroglycanopathies.

Author Contributions

Conceived the study: MB, IF. Experimental design: MB, IF. Performed the experiments: MB, IF. Analyzed the data: MB, IF. Contributed reagents/materials/analysis tools: MB, JW, IF. Wrote the paper: MB, JW, IF.

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Supplementary Material

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fncel.2015.00199/abstract

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REVIEW
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CD44: molecular interactions, signaling and functions in the nervous system

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CD44 is the major surface hyaluronan (HA) receptor implicated in intercellular and cell-matrix adhesion, cell migration and signaling. It is a transmembrane, highly glycosylated protein with several isoforms resulting from alternative gene splicing. The CD44 molecule consists of several domains serving different functions: the N-terminal extracellular domain, the stem region, the transmembrane domain and the C-terminal tail. In the nervous system, CD44 expression occurs in both glial and neuronal cells. The role of CD44 in the physiology and pathology of the nervous system is not entirely understood, however, there exists evidence suggesting it might be involved in the axon guidance, cytoplasmic Ca^{2+} clearance, dendritic arborization, synaptic transmission, epileptogenesis, oligodendrocyte and astrocyte differentiation, post-traumatic brain repair and brain tumour development.

Keywords: CD44, adhesion molecule, hyaluronan receptor, extracellular matrix receptor

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Introduction

CD44 is a transmembrane glycoprotein mediating cell responses to the extracellular microenvironment. Also known as the hermes antigen, Pgp1, MDU3 and Inlu-related p80 glycoprotein, it was first described as a surface molecule present in lymphocytes, thymocytes and granulocytes (Dalchau et al., 1980), and later recognized as a novel human erythrocyte cell surface antigen, lymphocyte homing receptor, and leukocyte surface glycoprotein (Stefanová et al., 1989). Today, CD44 is being described as an adhesion molecule expressed in various cell types, and an important participant in a number of signaling pathways. The present review summarizes the current knowledge on CD44 expression and function in the nervous system.

Structure

CD44 molecule consists of several functional domains. The N-terminal extracellular domain contains motifs serving as docking sites for various ligands—primarily hyaluronan (HA), but also extracellular matrix (ECM) glycoproteins and proteoglycans, growth factors, cytokines and matrix metalloproteinases (**Figure 1**; Yu and Stamenkovic, 1999; Ponta et al., 2003). The aminoterminal domain of CD44 is separated from the plasma membrane by a short stem structure with proteolytic cleavage sites for such metalloproteinases as disintegrin, metalloproteinase domain-containing proteins 10 and 17 (ADAM-10 and ADAM-17) or membrane type 1-matrix metalloproteinase (MT1–MMP; Okamoto et al., 1999; Nagano and Saya, 2004). The cleavage of CD44 triggers the release of cells bound to HA—a process important

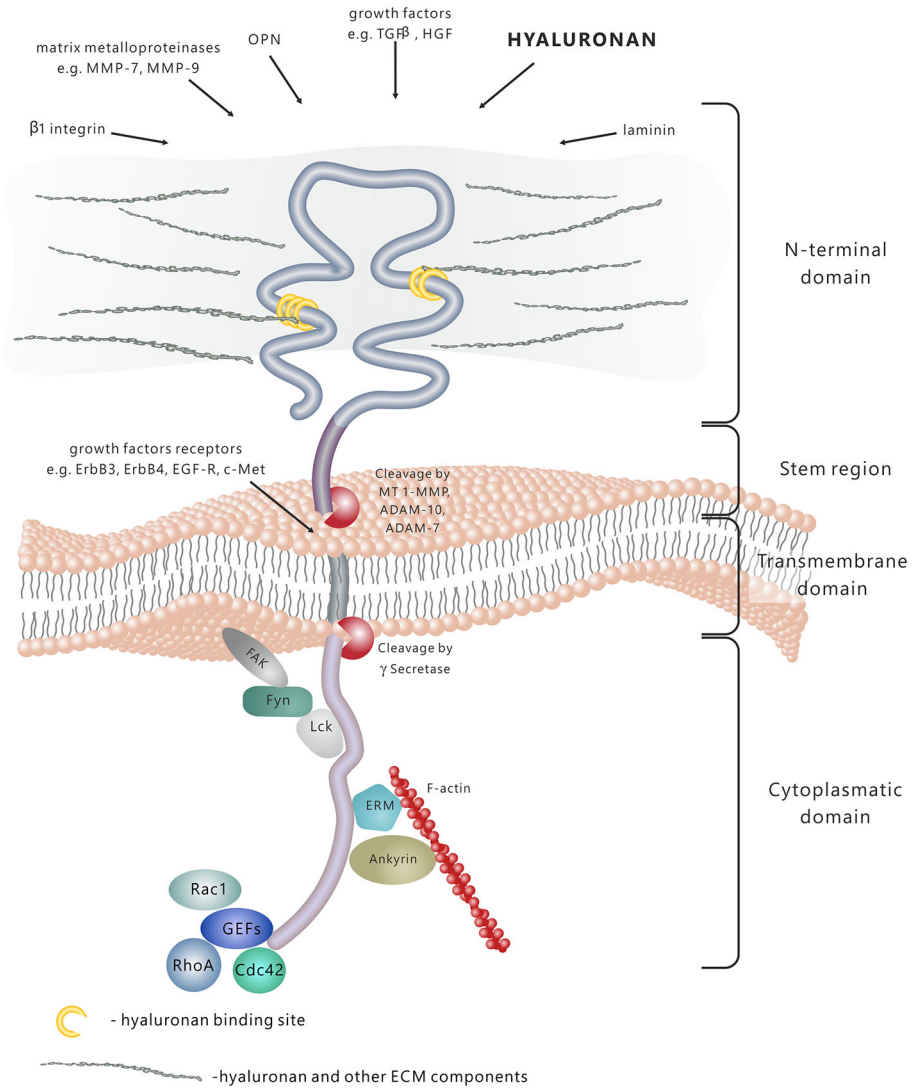

FIGURE 1 | CD44 protein structure and signaling. CD44 is a transmembrane molecule composed of several domains. The N-terminal extracellular domain can bind various ligands, including hyaluronan (HA), extracellular matrix (ECM) glycoproteins and proteoglycans, growth factors, cytokines and matrix metalloproteinases. In result of the proteolytic cleavage within the stem region, the extracellular domain is released into the extracellular space. The transmembrane domain anchors and stabilises the molecule in the plasma membrane. The cytoplasmic domain is responsible for signal transduction through binding to different molecules, including cytoskeleton components, kinases and activators of small Rho GTPases (GEFs-guanine nucleotide exchange factors).

in the regulation of cell migration. Notably, the stem region is the variable portion of the protein due to the alternative splicing and insertion of exons 6–15. The C-terminal cytoplasmic tail region plays a crucial role in the CD44 involvement in intracellular signal transduction. It has been shown that it binds to cytoskeletal elements such as ankyrin, the ERM proteins: ezrin, radixin, and moesin, and moesin-ezrin-radixin-like protein (MERLIN; Martin et al., 2003), it also interacts with signaling molecules, namely members of Src family kinases (SFKs) Src, Lck, Fyn and Lyn (Ponta et al., 2003), and activators of small Rho GTPases (Bourguignon et al., 2005). In addition, the cytoplasmic tail of CD44 may be cleaved off by γ-secretase and translocated to the cell nucleus, where it acts as a transcriptional regulator (Nagano and Saya, 2004).

The human gene for CD44 consists of 20 exons and is located in the 11p13 locus (Thorne et al., 2004). The most common form of CD44, also called standard CD44 (CD44s), is encoded by exons 1–5, 16–18 and 20. There also exists a large number of CD44 variants (CD44v), generated by extensive alternative splicing and containing the "standard" exons with a combination of exons 6–15 (Ponta et al., 2003). The predominant CD44 isoform expressed in the nervous system is the standard form (Sretavan et al., 1994; Jones et al., 2000; Bouvier-Labit et al., 2002), although the presence of CD44 splice variants has been

demonstrated in normal human brain tissue (Kaaijk et al., 1997), as well as in certain primary tumours of the brain and peripheral nerve (Kaaijk et al., 1995; Sherman et al., 1997; Resnick et al., 1999).

Expression and Activation in the Nervous System

Originally, CD44 expression was reported to take place predominantly in the white matter of the human brain (McKenzie et al., 1982; Bignami and Dahl, 1986; Cruz et al., 1986). Likewise, further studies have shown that the source of CD44 protein was glial cells, in particular astrocytes (Girgrah et al., 1991; Vogel et al., 1992). *In vitro* studies with the use of primary cell cultures from human foetal and adult brain have evidenced the expression of CD44 in astrocytes and oligodendrocytes (Moretto et al., 1993; Bouvier-Labit et al., 2002). The subsequent analysis of developing brain has identified CD44 as a marker for astrocyte-restricted precursor cells (ARPs) committed to give rise to astrocytes *in vitro* and *in vivo*, in both rodent and human tissue (Liu et al., 2004). In mouse cerebellum, CD44 expression was observed not only in astrocyte precursor cells, but also in neural stem cells and oligodendrocyte precursor cells (OPCs) at early postnatal stages (Naruse et al., 2013). CD44 expression in glial cells has been further demonstrated by studies examining the role of this molecule in the Schwann cells in developing nerves (Sherman et al., 2000) and at the neuromuscular junction of the adult rat skeletal muscle (Gorlewicz et al., 2009). The immunostaining of surgical specimens of temporal cortex and hippocampus for CD44 revealed astrocyte diversity in the human brain in terms of CD44 expression (Sosunov et al., 2014). High expression of CD44 has been observed in astrocytes with long, unbranched processes and "fibrous"-like astrocytes, whereas the "protoplasmic" astrocytes, which display a "bushy" morphology, exhibit no CD44 expression. Likewise, during development, CD44 expression was limited specifically to Bergmann glia and fibrous astrocytes among three types of astrocytes in cerebellum (Naruse et al., 2013). Interestingly, the expression of CD44 in astrocytes was shut off during postnatal development of the cerebellum.

Although the number of reports point to glial expression of CD44 in the nervous system, and describe adult neurons generally as CD44-negative cells (Vogel et al., 1992; Akiyama et al., 1993; Jones et al., 2000), the neuronal expression has also been observed. Expression of different splice variants of CD44, namely CD44v4, CD44v5 and CD44v10, in neurons (axonal membranes and cytoplasm) of the cerebral cortex, putamen, thalamus, hippocampus, cerebellum and spinal cord, in addition to the expression of CD44s in the white matter astrocytes, has been found in human brain sections (Kaaijk et al., 1997). It has also been shown that the embryonic optic chiasm neurons expressed the standard isoform of CD44 protein (Sretavan et al., 1994), and that strong upregulation of CD44 took place in axotomised facial motoneurons, but not in the gray matter astrocytes (Jones et al., 1997). In yet another study, the cellular location of CD44 in the brain tissue in seven different brain lesion models has been investigated (Jones et al., 2000). The results demonstrated an inducible expression of CD44 in cholinergic neurons of the forebrain, brainstem and spinal cord post distant axotomy, as opposed to its upregulation in astrocytes and other non-neuronal cell types following a more severe trauma involving local disruption of the blood-brain barrier. The antigen has been found in the cell bodies, dendrites and axons of neurons. CD44 immunoreactivity has also been detected in the dentate gyrus inner molecular layer (IML) of the mouse hippocampus after pilocarpine-induced status epilepticus (SE), where it coincided with early mossy fiber sprouting (MFS; Borges et al., 2004). Recently, CD44 mRNA has been detected in the neurons of striatum, extended amygdala and certain hypothalamic, cortical and hippocampal regions of non-stimulated brain (Glezer et al., 2009), as well as in the central respiratory control system (ventral respiratory group—VRG) within the brain stem (Matzke et al., 2007). CD44 expression was also detected in developing cerebellar Purkinje and granule neurons, but was limited to granule neurons in the adult cerebellum (Naruse et al., 2013). The expression of CD44 protein in pyramidal neurons of rat hippocampus and cortex is also developmentally regulated (Skupien et al., 2014). It increases postnatally, peaks around day P10 and then decreases. Notably, electron microscopy analysis revealed the dendritic localization of CD44 immunostaining within the pyramidal neurons of the developing hippocampus. There can be several causes for the apparent discrepancies among the studies describing expression of CD44 in neurons and glia. First, the CD44 expression appears to strongly depend on the developmental stage, especially in neurons. In fact, the studies claiming neurons to be CD44-negative were done mainly in the adult brain (Akiyama et al., 1993; Jones et al., 2000). The discrepancies may result also form differences in sensitivity of detection methods, and the differences in CD44 expression levels in glia (higher expression) and neurons (lower expression). In addition, since CD44 is a subject to extracellular and intramembrane proteolysis, the antibodies against its N-terminus may fail to detect the molecule in cells in which the rate of proteolytic processing is high. Such differences in the CD44 proteolysis, however, remain to be investigated.

Biological Function in the Nervous System

The main function ascribed to CD44 is acting as a receptor for HA—the key component of ECM in the brain playing a crucial role in many physiological and pathological processes, such as the neuronal development, synaptic plasticity (e.g., learning and memory), epileptogenesis, response to injury, neurodegeneration and brain tumour invasion (Kochlamazashvili et al., 2010; Wlodarczyk et al., 2011). The neuronal function of hyaluronic acid receptors, particularly CD44, in the nervous system remains to be elucidated, although there exists evidence that, among other processes, CD44 might be involved in the axon guidance during neuronal development (**Figure 2**). *In vitro* studies indicated that CD44 had an inhibitory effect on embryonic retinal axon growth (Sretavan et al., 1994), while experiments

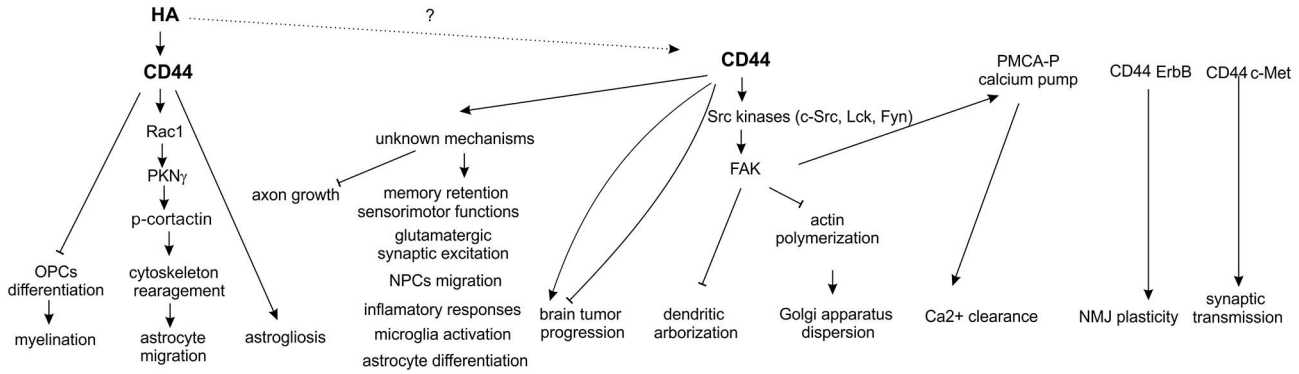

FIGURE 2 | Functions of CD44 in the nervous system.

using specific anti-CD44 blocking antibodies demonstrated that the functional CD44 molecule in chiasmatic neurons was essential for axon crossing and axon divergence at the mouse optic chiasm (Lin and Chan, 2003). Furthermore, it appears that CD44 participates in the extension of axons from retinal ganglion cells growing on a laminin substrate (Ries et al., 2007).

Recently, a novel signaling pathway has been described in pyramidal neurons of the hippocampus and cerebral cortex, involving CD44 and Src tyrosine kinase-induced cascade, regulating Golgi apparatus morphology and dendritic tree arborization (Skupien et al., 2014). It was shown that the loss of CD44 resulted in an increase in the complexity of dendritic arbors in hippocampal neurons cultured *in vitro*, and in cortical neurons electroporated *in vivo*. Moreover, the knockdown of CD44 resulted in structural alteration of the Golgi apparatus, an organelle that is essential for mediating dendritic polarity, growth, and maintenance. Additionally, CD44 interacted with Src kinase in the brain, and the activation of both c-Src and its main substrate, focal adhesion kinase (FAK), was decreased upon CD44 knockdown.

The similar signaling pathway, involving CD44 and plasma membrane Ca^{2+} ATPase (PMCA), has been described in sensory neurons (Ghosh et al., 2011). The proposed molecular mechanism envisages PMCA as a point of cross-talk enabling the interaction of ECM and CD44, which regulates the Ca^{2+} signaling through the activation of SFKs (Src family kinases: Lck, Fyn) and FAK cascade. Since Ca^{2+} clearance from the neuronal cytoplasm regulates a number of Ca^{2+}-dependent processes in neurons, including excitability, plasticity and neurotransmitter release, it appears that CD44 might be involved in all the above processes. Moreover, hyaluronic acid has been implicated in hippocampal synaptic plasticity by modulating postsynaptic L-type voltage-dependent Ca^{2+} channels (L-VDCCs; Kochlamazashvili et al., 2010). On the other hand, it has been demonstrated that activity of neuronal L-VDCCs is regulated by phosphorylation of its α_{1c} subunit by Src kinase (Bence-Hanulec et al., 2000; Gui et al., 2006). It would be interesting to investigate whether regulation of Src activity by CD44 can be involved in L-VDCCs-dependent synaptic plasticity.

In the nervous system and other tissues, CD44 can act as a co-receptor for receptor tyrosine kinases (RTKs) and serve as a platform for signaling molecule assembling. For instance, in the peripheral nervous system, CD44 enhances the neuregulin signaling by mediating the ErbB receptor heterodimerization in Schwann cells of the developing peripheral nerves (Sherman et al., 2000). In the adult rat nerve-muscle synapse, CD44 present in terminal Schwann cells has been shown to bind to ErbB3 receptors and to be involved in the neuromuscular junction plasticity (Gorlewicz et al., 2009). Moreover, the CD44v6 isoform can act as a co-receptor for c-Met *in vitro* (Orian-Rousseau et al., 2002). The CD44-c-Met interaction during embryogenesis has been further demonstrated by *in vivo* studies (Matzke et al., 2007) showing that c-Met is haploinsufficient in the $CD44^{-/-}$ background. The $CD44^{-/-}$ c-$Met^{+/-}$ mice die at birth due to a breathing defect caused by impaired synaptic transmission in the respiratory rhythm generating network, and alterations in the phrenic nerve, while c-Met $^{-/-}CD44^{+/+}$, and $CD44^{-/-}$ c-$Met^{+/+}$ mice develop normally and do not exhibit phenotypic abnormalities. The above results suggest that CD44 and c-Met are involved in synaptogenesis and axon myelination in the central and peripheral nervous systems.

It has been shown that CD44-deficient mice were born at the Mendelian ratio without any obvious developmental or neurological defects (Schmits et al., 1997). However, recently, Raber et al, applied a battery of behavioral and cognitive tests to determine if CD44-null mice display cognitive or other neurological disturbances (Raber et al., 2014). The results support an important role for CD44 in locomotor and sensorimotor functions, and in spatial memory retention, but do not specify whether these effects are due to the lack of CD44 in neurons or in glia. Given that CD44 is expressed in neural stem cells implicated in spatial memory (Oishi and Ito-Dufros, 2006; Deng et al., 2009; Naruse et al., 2013) the observed deficits can indicate that CD44 depletion in mice influences adult neurogenesis which in turn affects memory.

Interestingly, Matzke et al. (2007) have shown that the glutamatergic synaptic excitation in CD44 knockout mice was strongly reduced, while no change was detected in the

glycinergic and GABAergic synaptic inhibition or in the overall synaptic activity of pre-Bötzinger complex neurons within the brain stem (Matzke et al., 2007). The above observations seem to indicate a potential role of CD44 in synaptic transmission, yet this requires more in-depth research. The absence of overt neurological defects in CD44 knockout mice might be explained by efficient compensation by another protein, all the more so since HA-mediated motility receptor (RHAMM) and intercellular adhesion molecule-1 (ICAM-1) have previously been shown to take over the CD44 role in the CD44 knockout mice (Nedvetzki et al., 2004; Olaku et al., 2011).

Although, the expression of CD44 in astrocytes has been described, the function of the interaction of HA and its receptor in these cells is poorly understood. It has been shown that interaction of hyaluronic acid with CD44 induce Rac 1-dependent PKNγ (protein kinase N-γ) activity which, in turn up-regulates the phosphorylation of the cytoskeletal protein, cortactin. This HA/CD44 interaction with Rac1-PKNγ leads to cytoskeleton activation and enhanced astrocyte migration (Bourguignon et al., 2007). Similarly, in neural precursor cells, overexpression of CD44 protein significantly enhanced the *in vitro* and *in vivo* trans-endothelial migration of these cells (Deboux et al., 2013). Moreover, it was shown that CD44 overexpression in glial precursor cells inhibits differentiation towards oligodendrocytes and increases the differentiation into astrocytes (Liu et al., 2004). Accordingly, a high molecular weight form of HA was also shown to inhibit maturation of OPCs into myelin-forming cells (Back et al., 2005).

Association with the Nervous System Pathologies

The limited data from descriptive studies, indicating a widespread upregulation of CD44 expression in neurons and non-neuronal cells post brain injury, suggest an important role of this cell surface glycoprotein in the neuronal, glial and leukocyte response to trauma and in the nervous system repair (Jones et al., 2000; Shin et al., 2005). However, the molecular mechanisms underlying the above processes remain to be elucidated.

Few studies conducted to investigate the CD44 role in epileptogenesis have provided inconsistent results. It has been observed that CD44 was strongly upregulated in the dentate IML 3 days post pilocarpine-induced SE in mice, then it declined over the next 4 weeks (Borges et al., 2004). CD44 appeared to be one of the earliest proteins upregulated in the IML, which coincided with early MFS. On the other hand, repeated kainate injections did not induce any changes in the CD44 expression in the IML, which also correlated with MFS absence in the mice hippocampus. In view of the above, it has been hypothesized that CD44 was involved in the response to axon terminal degeneration and/or neuronal reorganization preceding MFS. Contrary to the above observations, studies on CD44 expression in an *in vitro* model of MFS have shown that high CD44 expression in the molecular layer coincided with minimal MFS, and that reduced CD44 expression/function following the kainic acid (KA) treatment or use of blocking antibodies was associated with increased MFS (Bausch, 2006). The time course of KA-induced decrease in CD44 expression corresponded with the temporal progression of KA-induced MFS in hippocampal slice cultures, suggesting that reduced CD44 expression might contribute to MFS. HA has been also implicated in epileptogenesis. Studies with the use of microelectrode array recording and Ca^{2+} imaging in hippocampal neurons cultured *in vitro* revealed that enzymatic removal of HA by hyaluronidase induced epileptiform activity in neuronal network (Vedunova et al., 2013). Additionally, mice deficient in HA synthase (HAS) genes, especially Has 3 knock-outs, exhibit epileptic phenotype along with a pronounced reduction in the level of tissue HA (Arranz et al., 2014).

Recently, it was shown that the silencing of CD44 expression during the early development of neuronal cells exerted a significant protective effect on young neurons that were exposed to subtoxic conditions, by preventing dendritic shortening induced by glutamate exposure (Skupien et al., 2014). Therefore, CD44 might be a novel therapeutic target in neurological disorders in which alterations in dendritic tree arborization have been observed.

One of a few studies with the use of CD44 knockout mice has provided evidence for the potential role of CD44 in the response to ischaemia in brain tissue (Wang et al., 2002). These findings indicated that CD44 deficiency in mice protected their brain from cerebral ischemia injury, and it has been suggested that this effect might be associated with selective reduction in inflammatory cytokines, for instance interleukin-1b. These results are further supported by studies showing that CD44 expression in activated microglia can be involved in the pathogenesis of neuroinflamatory diseases. Up-regulation of CD44 expression in microglia/macrophages were observed after transient or permanent forebrain ischemia in rats (Wang et al., 2001; Kang et al., 2008), in experimental cryolesions, a model for rat brain injury (Shin et al., 2005) and in mouse model of amyotrophic lateral sclerosis (ALS; Matsumoto et al., 2012).

Increased expression of CD44 and HA accumulation has also been observed in the brain white matter of patients with multiple sclerosis (MS), as compared with the normal brain tissue (Girgrah et al., 1991; Back et al., 2005). Consistently, elevated levels of both CD44 and HA has been detected in areas where there was loss of myelin, in the acute and chronic lesions in mice with experimental autoimmune encephalomyelitis (EAE), a murine model of MS (Back et al., 2005). Transgenic mice that overexpressed CD44 under the control of a myelin-specific promoter had widespread CNS dysmyelination and progressive demyelination that occurred in the absence of an inflammatory response (Tuohy et al., 2004). These findings provide strong evidence that CD44 proteins expressed by oligodendrocytes and Schwann cells, play a role in promoting demyelination. Moreover, HA staining intensity correlated with the levels of CD44 in CD44-overexpressing mice, indicating that HA accumulates in demyelinating CNS lesions as a result, at least in part, of elevated CD44 expression by glial cells (Back et al., 2005). HA inhibits remyelination and OPCs maturation after

chemical demyelination of mouse white matter (Back et al., 2005) but, *in vitro* studies showed that blocked maturation of oligodendrocytes depends on HA interaction with Toll-like receptor 2 (TLR2) rather than with CD44 expressed by OPCs (Sloane et al., 2010). CD44 expression was strongly induced by activated astrocytes surrounding the demyelinated lesions suggesting that CD44 may play a role in facilitating inflammatory responses during reactive gliosis (Girgrah et al., 1991; Haegel et al., 1993). In MS the interactions between astrocytes and lymphocytes occurs during the entry of activated lymphocytes into the CNS, and in the sites of lesions. It was shown that CD44 is involved in direct contacts between T-cells and astrocytes in EAE mice (Haegel et al., 1993). Recently, an increased EAE disease severity and inflammation was demonstrated in CD44-KO mice (Flynn et al., 2013). CD44-deficient mice with EAE had more pro-inflammatory T-cell profile and increased permeability of the brain-blood barrier (BBB) than WT controls. The data suggest that CD44 acts as a negative regulator of inflammation with roles in T-cell differentiation, adhesion and trans-endothelial migration, and BBB permeability.

Protoplasmic astrocytes in patients with Alexander disease, a primary disorder of astrocytes, caused by heterozygous mutations in GFAP (glial fibrillary acidic protein), convert to reactive cells that lost their bushy-like morphology and become multinucleated and hypertrophic (Sosunov et al., 2013). This phenotypic conversion is accompanied by acquiring of CD44 by normally CD44-negative protoplasmic astrocytes of gray matter suggesting that CD44 can play a role in this process e.g., by regulation of changes in astrocyte shape. White matter of patients with vanishing white matter disease, that is associated with maturation defect of astrocytes and oligodendrocytes, is enriched in CD44-expressing astrocyte precursor cells and accumulates HA (Bugiani et al., 2013). Accumulation of HA synthesized by CD44-positive reactive astrocytes was also observed during chronic human neonatal white matter injury (Buser et al., 2012; Back and Rosenberg, 2014).

Chronically elevated CD44 expression and HA accumulation occur in non-human primate CNS with normal aging as a result of age-related astrogliosis and are linked to the aberrant accumulation of OPCs (Cargill et al., 2012). CD44 expression is also highly and persistently upregulated by astrocytes in brains of patients with Alzheimer's disease (AD; Akiyama et al., 1993).

Several studies have demonstrated the correlation between high CD44 expression and poor prognosis in patients with brain tumour. CD44s and CD44v are frequently expressed in primary brain tumours and seem to be essential for the invasive growth of various CNS-derived tumour cell types *in vitro* and *in vivo* (Kuppner et al., 1992; Nagasaka et al., 1995; Sherman et al., 1997; Breyer et al., 2000; Monaghan et al., 2000; Pusch et al., 2010). The precise mechanism of CD44 action in the development and invasiveness of nervous system tumours has not been elucidated to date. In glioblastoma multiforme (GBM), the most aggressive brain tumour, CD44 inhibits the activation of the mammalian equivalent of Hippo signaling pathway and plays a key role in regulating the stress and apoptotic responses of human GBM cells (Xu et al., 2010). In addition, it has been found that a subset of human GBM cases showed high expression of CD44 in brain tumour stem-like cells (BTSC), and that the growth of these tumours might depend on CD44v6/AKT signaling pathway (Jijiwa et al., 2011). Su and colleagues have demonstrated that CD44 overexpression was induced by the Src kinase activity in malignant peripheral nerve sheath tumour (MPNST) cells, and that it contributed to tumour invasiveness (Su et al., 2003). By contrast, in neuroblastomas, CD44 expression is often low in advanced tumours (Combaret et al., 1997; Gross et al., 1997, 2000; Kramer et al., 1997). Shtivelman and Bishop (1991) have shown that several upstream *cis*-acting elements contribute to the downregulation of CD44 gene expression in neuroblastoma cells (Shtivelman and Bishop, 1991).

Overall, it appears that both the overexpression and lack of expression of CD44 might be involved in the development and invasiveness of various brain tumour types. The results of the performed studies are consistent with the hypothesis put forward by Herrlich and colleagues, stating that CD44 might play a dual role in cancer pathogenesis, acting as either an oncogene or a tumour-suppressing factor (Herrlich et al., 2000). By binding to growth factors and presenting them to their receptors, CD44 might be involved in the activation of tumour-promoting signaling pathways. On the other hand, in certain conditions, binding of HA might lead to the recruitment of tumour suppressor proteins (e.g., merlin) into the cytoplasmic tail of CD44 and result in cell growth arrest. Clearly, the posed hypothesis is elegant in its attempt to explain the dual role of CD44 in tumorigenesis. Nevertheless, its confirmation requires further research, for instance the identification of individual signaling cascades involved in the above-suggested mechanisms—particularly in the brain tissue.

In conclusion, there is growing evidence that the previously underappreciated CD44 molecule might be a key signal transducer in neurons, where it can act as a c-Met co-receptor to regulate synaptic transmission or through the activation of Src-dependent signaling pathways to influence dendritic arborization and calcium ions clearance. Additionally, CD44 can also modulate many other processes important for neuronal functions summarized in **Figure 2**, i.e., memory retention and axonal growth, but the exact cellular mechanisms of CD44 action in these phenomena are not discovered yet. One can expect that, at least some of the neuronal functions exerted by CD44, depend on HA binding but still the experimental evidences consistent with this notion are missing. In glial cells, unlike in neurons, binding of HA to CD44 receptor was shown to play an important roles in both physiological (myelination and oligodendrocyte maturation) and pathological (astrogliosis, demyelination) processes. However, except of the well-defined signaling cascade that include CD44/HA-dependent activation of small RhoGTPase Rac1 and PKNγ and subsequent cytoskeletal rearrangement in migrating astrocytes, CD44-related cellular mechanisms in glial cells (astrogliosis, microglia activation, oligodendrocyte and astrocyte maturation, see **Figure 2**) still remain to be elucidated. Furthermore, explanation of precise molecular mechanisms of CD44 action might potentially form basis for novel therapeutic interventions in a number of CNS disorders.

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Matricellular proteins of the Cyr61/CTGF/NOV (CCN) family and the nervous system

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Matricellular proteins are secreted proteins that exist at the border of cells and the extracellular matrix (ECM). However, instead of playing a role in structural integrity of the ECM, these proteins, that act as modulators of various surface receptors, have a regulatory function and instruct a multitude of cellular responses. Among matricellular proteins are members of the Cyr61/CTGF/NOV (CCN) protein family. These proteins exert their activity by binding directly to integrins and heparan sulfate proteoglycans and activating multiple intracellular signaling pathways. CCN proteins also influence the activity of growth factors and cytokines and integrate their activity with integrin signaling. At the cellular level, CCN proteins regulate gene expression and cell survival, proliferation, differentiation, senescence, adhesion, and migration. To date, CCN proteins have been extensively studied in the context of osteo- and chondrogenesis, angiogenesis, and carcinogenesis, but the expression of these proteins is also observed in a variety of tissues. The role of CCN proteins in the nervous system has not been systematically studied or described. Thus, the major aim of this review is to introduce the CCN protein family to the neuroscience community. We first discuss the structure, interactions, and cellular functions of CCN proteins and then provide a detailed review of the available data on the neuronal expression and contribution of CCN proteins to nervous system development, function, and pathology.

Keywords: nervous system, matricellular proteins, extracellular matrix, signal transduction, CCN

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Introduction

Matricellular proteins are secreted proteins that exist at the border of cells and the extracellular matrix (ECM). However, instead of playing a role in structural integrity of the ECM, these proteins, that act as modulators of various surface receptors, have a regulatory function and instruct a multitude of cellular responses. Thrombospondin-1 (TSP-1), SPARC, and Tenascin-C (TN-C) are considered canonical matricellular proteins, however numerous new ones have been described. Among them are members of the Cyr61/CTGF/NOV (CCN) protein family. Over the last two decades neurobiologist have started to appreciate role of ECM in the nervous system development, physiology and pathology. Accordingly, important contributions of canonical matricellular proteins were described. In contrast, the CCN proteins, although expressed in the nervous system, received relatively little attention in this context. Instead major research efforts focused on their importance for cardiovascular system and skeleton development as well as their links with cancer. Yet, over last few years evidence has accumulated that at least some of

CCN family members play important role in the nervous system, especially during development. Thus, the major aim of this review is to introduce the CCN protein family to the neuroscience community. We first briefly introduce matricellular proteins in general. Next, we provide basic facts about structure, interactions, and cellular functions of CCN proteins that are needed as a background to understand the involvement of these proteins in the nervous system. The following parts of this article contain a detailed review of the available data on the neuronal expression and contribution of CCN proteins to nervous system development, function, and pathology.

Matricellular Proteins

The idea of matricellular proteins arose almost 20 years ago with the identification of pericellular matrix proteins, which were shown to not be directly involved in structuring the ECM but rather regulate cell function (Bornstein, 1995; Bornstein and Sage, 2002; Murphy-Ullrich and Sage, 2014). TSP-1, SPARC, and TN-C became prototypic matricellular proteins, and their properties served as selection criteria for the subsequent identification of other matricellular proteins (Bornstein and Sage, 2002; Murphy-Ullrich and Sage, 2014). Specifically, matricellular proteins should: (i) be highly expressed during development or upon injury; (ii) modulate cell-matrix interactions; (iii) bind to cell surface receptors, the ECM, growth factors, cytokines, or proteases; and (iv) induce de-adhesion or support states of "intermediate adhesion" (i.e., a condition characterized by the reorganization of F-actin stress fibers to dismantle focal adhesions). Consequently, the group of matricellular proteins expanded and now includes additional TSPs (2–5), tenascins (R, W, X, Y), osteopontin, and members of the CCN family (discussed in more detail below; Murphy-Ullrich and Sage, 2014). The role of matricellular proteins has been mostly studied in wound healing, cancer, and the production of connective tissue. Some of these proteins, however, have also been studied in the nervous system. The classic matricellular proteins TSP-1 and SPARC were shown to accelerate synaptogenesis when released by astrocytes (Christopherson et al., 2005; Jones et al., 2011). Osteopontin expression increases after ischemic or mechanical brain injury and likely contributes to regenerative processes (Yan et al., 2009; van Velthoven et al., 2011; Plantman, 2012). The use of TN-C knockout mice revealed that TN-C is important for neuromuscular junction formation and plasticity (Cifuentes-Diaz et al., 1998), hippocampus and cortex structure and electrical properties (Irintchev et al., 2005; Gurevicius et al., 2009), hippocampal and cerebellar plasticity (Evers et al., 2002; Andjus et al., 2005), olfactory detection (de Chevigny et al., 2006) and learning and memory (Strekalova et al., 2002). TN-C also positively regulates neurite outgrowth (Rigato et al., 2002; Michele and Faissner, 2009). Similarly, TN-R was shown to regulate multiple processes in the nervous system, including neurogenesis (Xu et al., 2014), neuronal migration (Saghatelyan et al., 2004), axon navigation and neurite growth (Becker et al., 2003, 2004; Zacharias and Rauch, 2006), the formation of perineuronal nets (Brückner et al., 2000; Morawski et al., 2014), and neuron excitability and plasticity (Saghatelyan et al., 2001; Nikonenko et al., 2003; Gurevicius et al., 2004). In contrast, the CCN protein family received relatively little attention from neurobiologists. Nonetheless, CCN matricellular proteins are expressed in the nervous system and some examples exist to confirm their importance for the proper development and function of the nervous system.

CCN Family

The CCN gene family (Cyr61/CTGF/NOV) consists of six members: *CCN-1* (*Cyr61*), *CCN-2* (*CTGF*), *CCN-3* (*NOV*), *CCN-4* (*WISP1*), *CCN-5* (*WISP2*), and *CCN-6* (*WISP3*). The family name was derived from early nomenclature for the first three identified proteins. These names often reflect the history of the discovery of particular CCN proteins or an initial view of their functions but may not necessarily reflect the current state of knowledge. For example, *CCN1* (*Cyr61)* was identified as 61st gene among genes, the expression of which was induced by trophic factors, e.g., epidermal growth factor (EGF) in fibroblasts (Lau and Nathans, 1987). Because of its mitogenic activity in connective tissue, *CCN2* was named connective tissue growth factor (*CTGF*; Bradham et al., 1991). *CCN3* was cloned as a gene that is overexpressed in nephroblastoma because of proviral insertion sites, and it was consequently named after this observation, i.e., Nephroblastoma overexpressed (*NOV*; Joliot et al., 1992). *CCN4* and *CCN5*, initially called expressed in low-metastatic type 1 cells (*ELM-1*; Hashimoto et al., 1998) and card-only protein 1 (*rCOP-1*; Zhang et al., 1998), respectively, were later identified as genes that were upregulated in response to Wnt-1 and consequently renamed WNT-inducible signaling pathway protein 1 (*WISP1*) and *WISP2* (Pennica et al., 1998). Finally, *CCN6* was identified as a *WISP1* homolog and named *WISP3*. This diversity of names within the CCN gene family (**Table 1**) became a major source of confusion and difficulty in attempts to follow research progress in the CCN field. Therefore, after the first international workshop on the CCN Family of genes, CCN researchers proposed a unification of the nomenclature for CCN family members (Brigstock et al., 2003); see also http://ccnsociety.com/ccn_nomenclature/index.html), which will be used hereinafter in the present review (**Table 1**). CCN family genes encode relatively small proteins (∼40 kDa) that share ∼40–60% similarity in their primary structure. Their most prominent common features are their functional domain composition and conservation of 38 cysteins through the peptide (**Figure 1**). Consequently, CCN proteins have several similarities in their molecular mechanisms of action and types of regulated cellular processes (e.g., cell adhesion, migration, proliferation, survival, and differentiation). However, the activity of particular CCN members may vary, depending on the cellular/tissue context (see below). Several extensive reviews have been published on the molecular biology of CCN proteins and their function in physiology and pathology (Leask and Abraham, 2006; Holbourn et al., 2008; Chen and Lau, 2009; Katsube et al., 2009; Jun and Lau, 2011; Perbal, 2013).

TABLE 1 | Alternative names and receptors of CCN proteins.

CCN1
Alternative names: Cyr61[1], CTGF-2, IGFB10, IGFB-rP4
Receptors: $\alpha_6\beta1$[2], $\alpha_V\beta_3$, $\alpha_M\beta_2$, $\alpha_2\beta_1$, $\alpha_V\beta_5$, $\alpha_D\beta_2$, $\alpha_{2b}\beta_3$, HSPG
CCN2
Alternative names: CTFG, IGFBP8, IGFBP-rP2, HBGF0.8, ecogenin, FISP12
Receptors: $\alpha_V\beta_3$, $\alpha_6\beta_1$, $\alpha_5\beta_1$, $\alpha_M\beta_2$, TrkA, $p75^{NTR}$, HPSG, LRP-1, LRP-6
CCN3
Alternative names: NOV, NOVH, IGFBP9, IGFBP-rP3
Receptors: $\alpha_V\beta_3$, $\alpha_6\beta_1$, $\alpha_5\beta_1$, $\alpha_v\beta_5$, Notch
CCN4
Alternative names: Wisp-1, Elm-1
Receptors: $\alpha_v\beta_5$ (Hou et al., 2013; Stephens et al., 2015); $\alpha_V\beta_3$ or β_1 (Stephens et al., 2015), $\alpha_5\beta_1$ (Liu et al., 2013)
CCN5
Alternative names: Wisp-2, CTGF-L, CTGF-3, HICP, Cop-1
Receptors: β_1 integrin (Ohkawa et al., 2011), $\alpha_v\beta_3$ (Myers et al., 2014), $\alpha_6\beta_1$ (Haque et al., 2015)
CCN6
Alternative names: Wisp-3
Receptors: β1 integrin (Batmunkh et al., 2011), $\alpha\upsilon\beta_5$ (Schütze et al., 2007)

Abbreviations: [1]*Cyr61 cystein rich 61, CTGF—connective tissue growth factor, IGFB IGFB-rb—IGFB-related protein, HBGF—heparin-binding growth factor, FISP12 fibroblast-inducible secreted protein, NOV—nephroblastoma overexpressed, Wisp—Wnt-inducible secreted protein, Elm-1 expressed in low metastatic cells, HICP—heparin-induced CCN-like protein, Cop-1 card only protein 1.* [2]*if not stated otherwise please refer to Jun and Lau (2011) for more detailed information.*

Structure, Molecular Interactions, and Basic Modes of Action of CCN Proteins

CCN proteins are structurally very similar. With the exception of CCN5, CCNs contain four functional domains that are preceded by an export signaling peptide at their N-terminus, namely insulin-like growth factor binding protein (IGFBP), a von Willebrand factor type C repeat (VWC), thrombospondin type-1 repeat (TSP-1 or TSR), and a cystein knot-containing domain (CT; **Figure 1**). The last of these is missing in CCN5. IGFBP and VWC constitute the N-terminal half of CCN proteins, which is separated from the C-terminal half that contains TSP-1 and CT by a "hinge" region. Additional forms of CCNs, which have different activities from full-length proteins, can be produced by proteolysis (Brigstock et al., 1997; Steffen et al., 1998) or alternative splicing (Perbal, 2009). Each of the CCN protein domains is responsible for interactions with different sets of molecules (**Figure 1**). Consequently, CCN proteins bridge a variety of cell surface receptors, signaling molecules, and the ECM. Thus, modular composition, which is unique for the CCN family, explains the functional distinctiveness of CCNs from other proteins that contain one of the domains but raises the issue of what makes them functionally different from each other. Three-dimensional structure modeling revealed subtle changes in electrostatic surfaces that might be responsible for the differential interactions of particular CCN family members (Holbourn et al., 2008). However, most experimental evidence points to other sources of diversity, namely differential, often non-overlapping CCN expression patterns and different sets of receptors and/or proteins that interact with CCNs and are expressed by target cells.

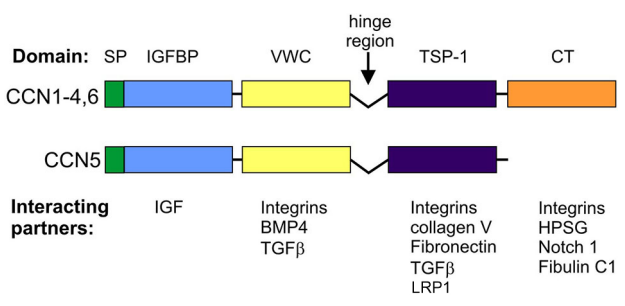

FIGURE 1 | Domain structure and canonical interactions of CCN family members.

CCN proteins have two major modes of action: (i) interactions with cell surface receptors; and (ii) interactions with receptor ligands. To date, the best studied are the cellular effects of CCN protein interactions with cell adhesion receptors [i.e., integrins and heparan sulfate proteoglycans (HSPGs); **Table 1**]. At the molecular level, such binding results in the cellular context-specific activation of signaling cascades, including extracellular signal-regulated kinases (ERKs), phosphoinositide 3-kinase (PI3K), and small GTPases of the Rho family (Leask and Abraham, 2006; Chen and Lau, 2009; Jun and Lau, 2011). This in turn leads to myriad cellular responses, of which changes in gene expression and cytoskeleton dynamics have been the most extensively studied. Some non-canonical outcomes (e.g., sustained reactive oxygen species production and cell senescence in response to CCN1-induced Rac1 activation) have also been described (Chen et al., 2007). One of the characteristic futures of CCN members is an ability to interact with various integrins (**Table 1**). Specific CCN proteins can bind different integrins, triggering dissimilar cellular responses. For example, in fibroblasts, CCN1 interactions with $\alpha_6\beta_1$ stimulate cell adhesion, whereas its binding to $\alpha_v\beta_3$ results in the induction of DNA synthesis. Moreover, the binding sites for different β-integrins do not overlap; therefore, simultaneous interactions with two integrins and a combined outcome are possible. However, cellular responses even to the same CCN protein-integrin couple may vary depending on the cell type (see below).

Integrins and HSPGs are currently the best described CCN receptors, but they are not the only ones. For example, the adhesion of rat hepatic stellate cells to a CCN2-coated surface requires binding to low-density lipoprotein receptor-related protein 1 (LRP-1) and HSPG (Gao and Brigstock, 2003). Binding to LRP-6 is likely responsible for the inhibitory effects of CCN2 on the canonical Wnt pathway in developing

Xenopus laevis embryos. CCN2 binds tyrosine kinase receptor-A (TrkA) and p75 neurotrophin receptor ($p75^{NTR}$; Wahab et al., 2005), whereas Notch is a receptor for CCN3 (Sakamoto et al., 2002; Minamizato et al., 2007). In addition to receptor binding, CCNs interact with receptor ligands and modulate their binding affinity or bioavailability. In the former case, the most thoroughly described interactions include those between CCNs and bone morphogenetic proteins (BMPs), which has an inhibitory effect, and between CCNs and transforming growth factor β (TGFβ), which has an activational effect (Abreu et al., 2002). CCN2 and CCN1 bind vascular endothelial growth factor (VEGF) and basic fibroblast growth factor (bFGF), respectively, and modify their bioavailability (Chen and Lau, 2009).

General Functions of CCN Proteins

Detailed descriptions of the functions of CCN proteins have been provided in several reviews (Leask and Abraham, 2006; Holbourn et al., 2008; Chen and Lau, 2009; Katsube et al., 2009; Jun and Lau, 2011; Perbal, 2013). The diversity of CCNs' modes of action results in a variety of cellular responses to CCN proteins. CCNs have been shown to regulate often converse cellular processes (e.g., cell proliferation/differentiation, cell death/survival, and cell adhesion/migration; for review see Jun and Lau, 2011; **Table 2**). Unsurprising is that different CCNs regulate opposite cellular processes. For example, CCN1, CCN2, and CCN3 are considered positive regulators of adhesion, whereas CCN5 and CCN6 are considered negative regulators (for review see Jun and Lau, 2011). However, opposing or very diverse cellular responses to the same CCN protein have also been reported. For example, CCN1 binds $\alpha_6\beta_1$, $\alpha V\beta_3$, $\alpha_2 B\beta_3$, and $\alpha M\beta_3$ integrins and positively regulates the adhesion of fibroblasts and smooth muscle cells, activated endothelial cells, platelets, and monocytes and macrophages, respectively (for review see Jun and Lau, 2011). However, the binding of CCN1 to $\alpha V\beta_5$ and $\alpha_6\beta_1$ induces the migration of fibroblasts and smooth muscle cell chemotaxis, respectively (for review see Jun and Lau, 2011). As mentioned above, the binding of CCN1 to $\alpha_v\beta_3$ integrin in fibroblasts does not affect adhesion/migration but rather regulates DNA synthesis. This example shows that CCN1 can have pleiotropic activities, depending on the cell type, functional/developmental cell status, and type of accessible receptors (e.g., integrins). The same is true for other CCN proteins (for review see Jun and Lau, 2011; **Table 2**).

In addition to the intrinsic properties of a cell, the surrounding environment/tissue can also modulate responses to a particular CCN. Therefore, a deeper understanding of the physiological roles of CCNs and their links to human diseases became possible only with *in vivo* models. These *in vivo* studies point to a primary role for CCN family members in angiogenesis and cardiovascular and skeletal development. For example, CCN1, CCN2, and CCN3 stimulate *in vivo* blood vessel growth, whereas *CCN1* knockout ($CCN1^{-/-}$) embryos die from placental vascular deficiency (Mo et al., 2002). The surviving embryos exhibit atrioventricular septal defects. $CCN1^{+/-}$ mice are viable, but 20% of the adult mice have ostium primum atrial septal defects (Mo and Lau, 2006). Also *CCN2*-deficient mice have defects in vasculature remodeling (Hall-Glenn et al., 2012). CCN2, CCN3, CCN4, and CCN6 are mostly known for their importance in bone formation and/or remodeling. *CCN2* knockout mice exhibit skeletal deformations, reduced ossification, and the expansion of mineralizing cartilage zones. In *CCN3* knockout mice, Matsushita et al. (2013) observed accelerated bone regeneration. A study of transgenic mice that overexpress CCN4 in preosteoblasts reported that CCN4 stimulates osteogenesis (Ono et al., 2011). Finally, mutations in *CCN6* were repeatedly found in progressive pseudorheumatoid dysplasia, an autosomal recessive disorder (Hurvitz et al., 1999). Surprisingly, however, *CCN6* knockout mice have no obvious bone phenotype (Kutz et al., 2005; Hann et al., 2013), whereas some defects that may be relevant to human disease were observed in zebrafish morphants (Nakamura et al., 2007). In addition to the major research areas described above, CCNs have been extensively studied in the context of proper wound healing (Jun and Lau, 2011). Mounting evidence also indicates links between CCNs and tumorigenesis (Jun and Lau, 2011). In contrast, knowledge concerning the role of CCNs in the nervous system is relatively lagging. Part of the problem is that full, nonconditional knockout results in embryonic (*CCN1*, *CCN5*) or perinatal (*CCN2*) death. Often such early lethality precludes studies on several tissue/organ functions, including in the nervous system. In cases of CCN knockout animals that survive embryonic development, no apparent

TABLE 2 | Cellular functions of CCN proteins.

CCN1
Cell adhesion[1] (+)[2], cell migration (+), DNA synthesis/proliferation (+), survival (+), apoptosis (+), differentiation (+), senescence (+)
CCN2
Cell adhesion (+), cell migration (+), survival (+), apoptosis (+)
CCN3
Cell adhesion (+), cell migration (+/−), DNA synthesis/proliferation (−/+), survival (+), apoptosis (+)
CCN4
Cell adhesion (+), (Liu et al., 2013; Stephens et al., 2015), migration (+) (Liu et al., 2013), proliferation (+) (Liu et al., 2013), survival (+) (Hou et al., 2013; Schlegelmilch et al., 2014)
CCN5
Cell migration (−), DNA synthesis/proliferation (−) (Haque et al., 2015), survival (+) (Ohkawa et al., 2011)
CCN6
Proliferation (+) (Batmunkh et al., 2011; Fang et al., 2014), migration (+) (Fong et al., 2012; Fang et al., 2014), survival (Huang et al., 2010)

[1]*if not stated otherwise please refer to Jun and Lau (2011) for more detailed information.* [2]*+ positive effect, −negative effect, −/+ either positive or negative effect depending on cellular context.*

nervous system problems were reported, but usually deeper analyses in this regard have not been performed because different authors have different major interests (e.g., bone development). Another reason for the low recognition of CCNs' roles in the nervous system stems from scattering of relevant information. To date, a comprehensive summary of the current state of knowledge concerning CCN expression patterns, the involvement of CCNs in neuronal development and physiology, and their links to nervous system pathology has been lacking.

Expression and Functions of CCN Family Members in the Nervous System

Major research on CCN proteins has not focused on the nervous system, but for some CCNs, the central nervous system (CNS) is one of the major sites of expression. This observation supports the hypothesis of a possible role for the CNN protein family in different aspects of CNS physiology and pathology. In fact, the role of some CNN family members in the CNS has already been demonstrated, and the number of reports concerning this issue is constantly growing.

CCN Expression in the Mammalian Central Nervous System

CCN proteins are expressed in non-neuronal tissues mainly during highly dynamic processes that involve tissue remodeling. CCN expression is thus observed during embryonic development and under pathological conditions (e.g., inflammation, injury, and cancer). Similarly, in the CNS, the expression of CCNs can be induced under such conditions. For example, both CCN1 and CCN2 were elevated in gliomas and glioblastomas (Pan et al., 2002; Xie et al., 2004a,b). However, CCN proteins are also expressed in the properly developing and adult CNS. Although knowledge of the pattern of CCN expression during embryogenesis and postnatal development and in the adult CNS is incomplete and restricted mainly to CCN1, CCN2, and CCN3, the number of studies that are investigating this issue is growing. Additional information concerning CCN expression patterns in the rodent and human brain can be extracted from large databases, including the collection of Allen Brain Atlases (ABA), e.g., Allen Mouse Brain Atlas (Lein et al., 2007)[1] and Allen Developing Mouse Brain Atlas[2] for *in situ* hybridization data, BrainStars* (BS*) for microarray data (Kasukawa et al., 2011)[3], and the Human Protein Atlas (HPA) for immunohistochemistry data (Uhlén et al., 2015).[4] The major advantages of these databases, which resulted from large-scale projects, are standardized procedures, reagents, and biological material. In this review, we use this knowledge to discuss previously published data and identify gaps in the literature.

[1]http://www.brain-map.org
[2]http://developingmouse.brain-map.org
[3]http://www.brainstars.org
[4]http://www.proteinatlas.org

CCN1 Expression in the Developing and Adult Central Nervous System

CCN1 mRNA has been detected in different areas of the developing and adult human and rat brain and in rat hippocampal and cortical neurons cultured *in vitro* (Albrecht et al., 2000; Malik et al., 2013). Interestingly, *CCN1* mRNA levels in the rat hippocampus were higher during embryonic development and dropped postnatally, suggesting that this protein may play an important role during embryonic brain development (Malik et al., 2013). A similar developmental trend in *CCN1* expression was observed in cultured hippocampal neurons (Malik et al., 2013). An analysis of ABA data showed fluctuations of *CCN1* mRNA amount in embryonic brain but confirmed higher *CCN1* mRNA levels in the mouse embryonic day 18 (E18) brain compared with early postnatal weeks. The ABA suggests partial expression recovery in the cortex from postnatal day 28 (P28) onward (**Figure 2**). In the adult brain (P56) the ABA provides evidence for *CCN1* mRNA in deeper cortical layers (**Figure 3**). BrainStars* provides evidence of *CCN1* mRNA in the adult mouse CNS, with the highest levels in the retina. In the adult human brain, *CCN1* mRNA was detected throughout the CNS, with the strongest expression in the spinal cord, frontal, temporal, and occipital cortices, hippocampus, and caudate nucleus (Albrecht et al., 2000). This observation appears to be confirmed by the HPA, in which mild CCN1 protein levels were present in the cortex and hippocampus, but the highest levels were detected in the cerebellum (**Figure 4**). An analysis of the cell specificity of immunohistochemical signal showed that CCN1 protein can be detected mostly in neurons, whereas its expression in glia is lower or undetectable (**Table 3**).

Our data from *in vitro* hippocampal neuron cultures showed that CCN1 expression can be modulated by various stimuli [e.g., brain-derived neurotrophic factor (BDNF), insulin, and bicuculline], both in developing and "mature" neurons. The effects of bicuculline suggest a role for enhanced excitatory transmission in the regulation of *CCN1* mRNA levels. Similar results were obtained in organotypic hippocampal slices (Iacono et al., 2013). *In vivo*, *CCN1* mRNA level increases with decreases in excitatory transmission that are caused by *N*-methyl-D-aspartate receptor inhibition (Ito et al., 2007). Nevertheless, the mechanism behind this phenomenon might be indirect and involve increases in the activation of other neurotransmitter systems. Elevations of *CCN1* mRNA were also observed upon methamphetamine administration (Ito et al., 2007), administration of the dopamine receptor agonist clozapine (Sakuma et al., 2015), and the activation of muscarinic acetylcholine receptors (Chung and Ahn, 1998; Albrecht et al., 2000).

CCN2 Expression in the Developing and Adult Central Nervous System

Similar to *CCN1*, *CCN2* is also expressed in different parts of the CNS and rat primary neuron cultures *in vitro* (Albrecht et al., 2000), and its levels are regulated developmentally. *CCN2* mRNA is present in neural tissue in developing mouse embryos already at embryonic day 9.5 (Ivkovic et al., 2003). Williams

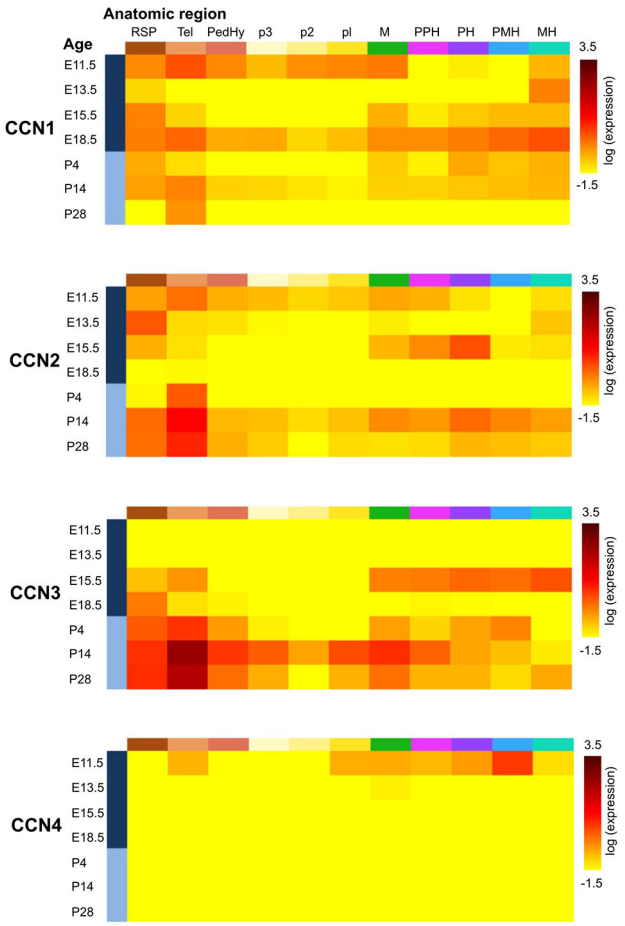

FIGURE 2 | Expression of CCN1–4 genes in the developing mouse brain according to the Allen Developing Mouse Brain Atlas [© 2013 Allen Institute for Brain Science. Allen Developing Mouse Brain Atlas (Internet)]. Gene expression summary for selected brain structures were downloaded from http://developingmouse.brain-map.org/ and are expressed as a heatmap of expression energy (for more details please refer to http://help.brain-map.org/display/devmouse/In+Situ+Hybridization+(ISH)+Data#InSituHybridization(ISH)Data-CorrelationSearch). Heatmaps source: CCN1: http://developingmouse.brain-map.org/gene/show/15780; CCN2: http://developingmouse.brain-map.org/gene/show/13996; CCN3: http://developingmouse.brain-map.org/gene/show/17900; CCN4: http://developingmouse.brain-map.org/gene/show/22159. RSP—rostral secondary prosencephalon; Tel—telencephalic vesicle; PedHy—peduncular (caudal) hypothalamus; p3 prosomere 3; p2 prosomere 2; p1 prosomere 1; M—midbrain; PPH—prepontine hindbrain; PH—pontine hindbrain; PMH—pontomedullary hindbrain; MH—medullary hindbrain (medulla).

et al. (2007) reported presence of *CCN2* mRNA in olfactory bulb at embryonic days 14, 17.5 and at postnatal day 3. In the adult brain presence of both *CCN2* mRNA and CCN2 protein was reported in the hippocampus (Hertel et al., 2000) and olfactory bulb (Williams et al., 2007). The ABA shows moderate levels of *CCN2* mRNA between E11.5-E15.5 and a complete lack of *CCN2* expression just before birth. However, *CCN2* mRNA levels gradually increase from P4 until adulthood (**Figure 2**). Such dynamic regulation of *CCN2* expression in early postnatal development was studied in detail in the rodent olfactory bulb. *CCN2* mRNA in the glomerular and mitral cell layers could be detected around P5. In the glomerular layer, *CCN2* mRNA level remained stable throughout postnatal development and in the adult brain, whereas it gradually decreased in the mitral cell layer and was barely detectable already on P12 (Khodosevich et al., 2013). In the adult rat brain, *CCN2* mRNA showed a highly selective distribution pattern in the forebrain, with strong labeling that was restricted to specific regions of the olfactory bulb, endopiriform nucleus, and supracallosal layer in the cerebral cortex. Particularly strong staining was observed in large neurons of layer VI in the cerebral cortex (Heuer et al., 2003). In the adult mouse brain, *CCN2* mRNA could be detected in layer VI of the cortex and mitral cell and glomerular layers of the olfactory bulb (Khodosevich et al., 2013). These observations are consistent with the ABA (**Figure 3**) and BS* data. Presence of *CCN2* mRNA was also shown throughout the adult human CNS, including different brain regions and the spinal cord. In fact, the *CCN2* expression pattern is similar to *CCN1*, with the exception that tissue levels of *CCN2* transcripts were lower in the hippocampus, caudate nucleus, and corpus callosum (Albrecht et al., 2000). In the healthy human brain, CCN2 protein was predominantly detected in neurons and partially in subtypes of glial cells (Schwab et al., 2000, 2001; **Table 3**). The HPA also suggests the presence of CCN2 protein in glial cells, especially in cortical areas (**Table 3**). Supporting further glial expression of *CCN2*, its expression has been detected in rat CNS astrocytes and tanycytes (Kondo et al., 1999). In the normal human spinal cord, the low expression of *CCN2* was observed in cells with astroglial morphology, particularly in white matter (Spliet et al., 2003). In the senile human brain, exclusively *CCN2* neuronal expression was found throughout different brain areas, particularly in entorhinal layer pre-alpha neurons and cortical pyramidal cells, both in somatodendritic and axonal compartments (Ueberham et al., 2003). Expression of *CCN2* also appears to be regulated by neuronal activity. Khodosevich et al. (2013) demonstrated that CCN2 protein level is dynamically regulated in response to changes in olfactory input.

CCN3 Expression in the Developing and Adult Central Nervous System

CCN3 is also expressed in the developing and adult CNS. In fact, the CNS appears to be the major site of *CCN3* expression during development compared with other tissues. This was demonstrated in chick embryos (Joliot et al., 1992) and human embryos in the first trimester of embryogenesis, in which *CCN3* mRNA and CCN3 were most abundantly present in motor neurons and the floor plate of the spinal cord (Kocialkowski et al., 2001). *CCN3* expression undergoes dynamic changes during development, suggesting that CCN3 protein might play a role in maintaining or establishing specific brain functions. The abundance of *CCN3* mRNA increases throughout the postnatal period during rat brain development. Increases in amount of *CCN3* mRNA and CCN3 protein were detected in the developing rat brain after birth, with a pronounced peak between P15 and P150 (Su et al.,

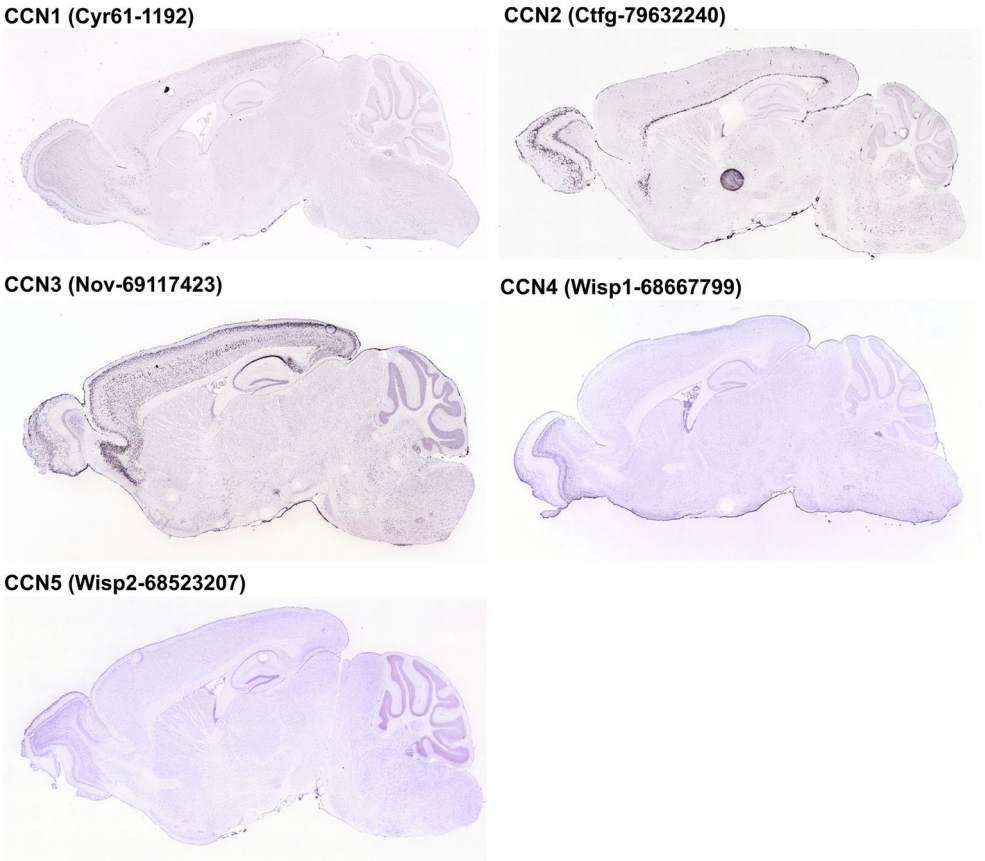

FIGURE 3 | Expression of CCN1–5 genes in the adult mouse brain according to the Allen Brain Atlas [© 2014 Allen Institute for Brain Science. Allen Mouse Brain Atlas (Internet)]. Images of brain sections were downloaded from http://mouse.brain-map.org/. Experiment name is given in parentheses.

2001). A similar increase seems to occur in the developing murine brain according to the ABA (**Figure 2**) through a majority of brain areas and the highest levels of *CCN3* mRNA are present in olfactory bulb, cortex and CA1 field of hippocampus (**Figure 3**). Intriguingly, the ABA also reports relatively low levels of *CCN3* mRNA in the cerebellum. But *CCN3* mRNA and CCN3 protein were detected in the rat cerebellum during the postnatal period, with the most prominent increase from P7 to P14 (Le Dréau et al., 2010b). In the human brain during early developmental stages, *CCN3* mRNA was mainly observed in somatomotor neurons in the lower CNS. At later stages, however, *CCN3* was expressed in the higher CNS (Su et al., 1998). High levels of *CCN3* mRNA were reported in the cortex, hippocampus, amygdala, and spinal cord (Albrecht et al., 2000). Surprisingly, the HPA reveals no CCN3 protein expression in the analyzed areas (**Figure 4**).

CCN3 expression is particularly observed in neurons. For example, presence of *CCN3* mRNA was shown in rat neuronal cultures *in vitro* (Albrecht et al., 2000) while CCN3 protein was detected in rat cerebellar Purkinje cells (Su et al., 2001; Le Dréau et al., 2010b), and neurons of dorsal root ganglia and the dorsal horn of the rat spinal cord (Kular et al., 2012). However, CCN3 immunoreactivity was also observed in astrocytes in the rat cerebral cortex, corpus callosum, and hippocampus (Le Dréau et al., 2010a). *CCN3* mRNA and CCN3 protein are also expressed in specific structures in the developing chicken eye, including the lens, ciliary body, optic nerve, pecten, and retina (Laurent et al., 2012).

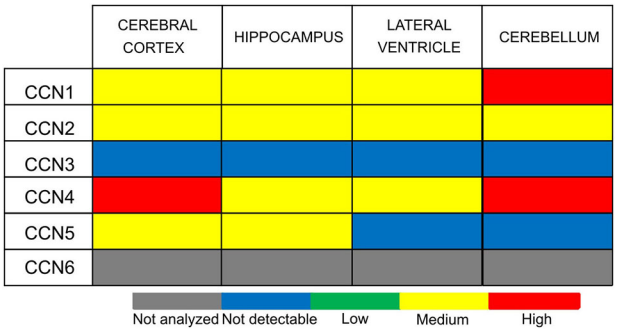

FIGURE 4 | CCN protein abundance in the selected human brain regions according to the Human Protein Atlas (HPA).

TABLE 3 | Cellular specificity of CCN protein expression in selected regions of the CNS according to the Human Protein Atlas.

	Cerebral cortex	Hippocampus	Lateral ventricle	Cerebellum
CCN1	endothelial cells—medium glial cells—low neuronal cells—medium neuropil—medium	glial cells—not detected neuronal cells—medium	glial cells—low neuronal cells—medium	cells in granular layer—medium cells in molecular layer—low Purkinje cells—high
CCN2	endothelial cells—medium glial cells—medium neuronal cells—medium neuropil—medium	glial cells—low neuronal cells—medium	glial cells—low neuronal cells—medium	cells in granular layer—medium cells in molecular layer—medium Purkinje cells—medium
CCN3	endothelial cells—not detected glial cells—not detected neuronal cells—not detected neuropil—not detected	glial cells—not detected neuronal cells—not detected	glial cells—not detected neuronal cells—not detected	cells in granular layer—not detected cells in molecular layer—not detected Purkinje cells—not detected
CCN4	endothelial cells—high glial cells—medium neuronal cells—medium neuropil—medium	glial cells—medium neuronal cells—medium	glial cells—medium neuronal cells—medium	cells in granular layer—medium cells in molecular layer—medium Purkinje cells—high
CCN5	endothelial cells—not detected glial cells—not detected neuronal cells—medium neuropil—not detected	glial cells—not detected neuronal cells—medium	glial cells—not detected neuronal cells—not detected	cells in granular layer—not detected cells in molecular layer—not detected Purkinje cells—not detected
CCN6	not analyzed	not analyzed	not analyzed	not analyzed

CCN4, CCN5, and CCN6 Expression in the Central Nervous System

In contrast to CCN1–3, very little is known about CCN4, CCN5, and CCN6 expression patterns. The data that are available are also somewhat contradictory. A study of *CCN4*, *CCN5*, and *CCN6* mRNA levels in human embryonic and adult tissues showed no expression in the brain (Pennica et al., 1998), but other studies do not fully support this finding. Indeed, during rat embryonic development, *CCN4* mRNA presence was reported exclusively in osteoblasts and osteoblastic progenitor cells of the perichondral mesenchyme (French et al., 2004). The ABA also does not reveal the presence of *CCN4* mRNA during development after E11.5 (**Figure 2**). But, both the ABA and BS* suggest that *CCN4* mRNA is present in the adult murine brain, although at relatively low levels, with the highest expression in the olfactory bulb (ABA, BS*) and retina (BS*; **Figure 3**). Also, the HPA shows presence of CCN4 protein in the adult human cerebral cortex and cerebellum (**Figure 4**), with the highest expression levels in cortical endothelial cells and Purkinje neurons (**Table 3**). Additionally, CCN4 protein was shown to be expressed in rat primary neurons during oxygen-glucose deprivation (Wang et al., 2012). *CCN5* mRNA, according to the ABA, is not present in P56 murine brain (**Figure 3**). But the BS* reports low to moderate *CCN5* mRNA levels throughout the adult mouse CNS, with the highest expression in the retina. Moreover, Ohkawa et al. (2011) detected some *CCN5* mRNA in the cerebellum and spinal cord of 15 week old mice. In contrast, CCN5 immunostaining was reported in fetal mouse brain (E12–16) but not human brain (4 months; Jones et al., 2007). Cytoplasmic and nuclear CCN5 immunostaining was also reported in cortical neurons in the adult rat (Gray et al., 2007). The presence of CCN5 protein in the adult cortex is supported by the HPA (**Figure 4**), which additionally provides evidence of its hippocampal expression. Virtually nothing is known for CCN6. No data are reported concerning its expression in either the ABA or HPA. Only a BS* microarray analysis detected almost equal amounts of CCN6 transcript in all 51 analyzed brain regions.

Role of CCN Proteins in the Central Nervous System

CCNs can be detected in the CNS. Importantly, CCN levels dynamically change during development. This observation supports their possible role in CNS development and physiological functions. Here, we will discuss the available data that indicate the involvement of CCN proteins, mainly CCN1, CCN2, and CCN3, in CNS development and physiology.

CCN1

In 1998, CCN1 was suggested to play an important role in neuronal differentiation, in which it was induced by bFGF during the differentiation of immortalized hippocampal progenitor (H19–7) cells (Chung and Ahn, 1998). Indeed, we recently confirmed that CCN1 is required for proper development of the dendritic tree in rat hippocampal neurons *in vitro* and acts downstream of Ras, ERK, and PI3K. Moreover, CCN1 overexpression promoted dendritic branching, and this effect depended on β1-integrin (Malik et al., 2013).

CCN1 is secreted by retinal Muller glial (RMG) cells that are cultured *in vitro* in response to glial cell line-derived neurotrophic factor (GDNF) treatment, which is known to have pro-survival effects in the retina. Thus, the neuroprotective and pro-survival activity of CCN1 was studied in a mouse model of retinitis pigmentosa (Kucharska et al., 2014). In organotypic retinal cultures that were derived from these mice, CCN1 treatment increased the survival rates of photoreceptors. The authors showed that CCN1 activates

the mitogen-activated protein kinase (MAPK)/Erk and Janus-associated kinase (JAK)/Stat pathways but not PI3K/Akt pathway in retinal explants from retinitis pigmentosa mice. Interestingly, in primary porcine cultures, CCN1 did not stimulate photoreceptors themselves but rather only RMG cells and retinal pigment epithelium (RPE) cells, suggesting that the protective effect on photoreceptors occurs indirectly. In disagreement with the results that were obtained in the retinitis pigmentosa model, CCN1 treatment in pure porcine RMG cultures stimulated not only the MAPK/Erk and JAK/Stat pathways but also the PI3K/Akt pathway, whereas it led to activation of the PI3K/Akt and MAPK/Erk pathways in RPE cultures.

The stimulation of muscarinic acetylcholine receptors (mAChRs) induced *CCN1* mRNA expression in primary neurons and the rat brain, in which *CCN1* mRNA was detected in cortical layers V and VI and thalamic nuclei. mAChRs modulate neuronal functions, including long-term potentiation and synaptic plasticity in neuronal circuits that are involved in learning and memory formation. The authors suggested a role for CCN1 in the cholinergic regulation of synaptic plasticity (Albrecht et al., 2000).

CCN2

Consistent with its expression pattern, CCN2 was shown to play an important role in the rodent olfactory bulb, where it acts as a proapoptotic factor that eliminates newborn neurons in an activity-dependent and locally restricted manner. CCN2 acts via glial-derived transforming growth factor β2 (TGF-β2) to promote the apoptosis of newly generated periglomerular interneurons in the glomerular layer (Khodosevich et al., 2013). CCN2 levels, which are dynamically regulated in response to changes in olfactory input, adjust the survival of postnatally born neurons in an odorant-specific fashion. Importantly, CCN2 levels decrease in the mouse olfactory bulb if the sensory input is suppressed (Khodosevich et al., 2013). In contrast, olfactory activity enhances *CCN2* expression specifically in odor-activated glomeruli in the olfactory bulb. This phenomenon has important behavioral consequences, and *CCN2* knockdown mice perform better in odorant detection and olfactory discrimination than controls.

As mentioned above, CCN2 binds TrkA and p75NTR, receptors that transduce neurotrophin signals (Wahab et al., 2005). The authors showed that CCN2 stimulates TrkA and induces its autophosphorylation. The nerve growth factor (NGF)-induced activation of TrkA is widely known to control neuronal cell survival and axonal growth (Kuruvilla et al., 2000; Atwal et al., 2003). Nonetheless, the physiological importance of CCN2's influence on neurotrophin signaling in brain development, physiology, and disease remains to be evaluated.

CCN3

A role for CCN3 in brain development has also been reported. Specific patterns and dynamic changes in *CCN3* expression in the rat cerebellum (Le Dréau et al., 2010b) likely reflect its function in postnatal cerebellum development. Supporting this possibility, an *in vitro* study showed that CCN3 reduces cerebellar granule neuron precursor (GNP) proliferation that is induced by Sonic Hedgehog (SHH). SHH maintains GNPs in a proliferative state and delays their differentiation, resulting in a decrease in the proportion of postmitotic neurons. By counteracting SHH-driven effects, CCN3 reduces the proliferation of GNPs and consequently promotes their differentiation. This anti-proliferative action of CCN3 requires glycogen synthase kinase 3β (GSK3-β) activity. Moreover, CCN3 stimulates the migration and chemotaxis of cerebellar GNPs *in vitro* (Le Dréau et al., 2010b). The role of CCN3 in the retina has also been studied. *CCN3* is expressed in the chick retina during development in distinct cell types and is regulated by the Notch and BMP signaling pathways (Laurent et al., 2012). However, the ectopic expression of CCN3 had no effect on retinal development. These authors did not describe the effects of CCN3 depletion, and the possible role of CCN3 in the developing retina remains unclear (Laurent et al., 2012).

CCN5

A role for CCN5 in neuronal development has also been suggested. CCN5 was shown to enhance neurite outgrowth in Neuro2a cells. The underlying mechanism remains elusive, although the authors suggested the involvement of the CCN5-triggered, β1-integrin-dependent activation of Akt (Ohkawa et al., 2011). Thus, far no data are available concerning neuronal functions of CCN4 and CCN6. Most likely almost complete lack of data concerning role of CCN4, 5 and 6 in the nervous system reflects their low abundance in the nervous system and relatively poor repertoire of tools to reliably study their expression. Alternatively, those proteins may attract less attention when identified in large "omics" screens because of limited knowledge concerning their cellular functions.

CNN Proteins and Nervous System Dysfunction

As in non-neuronal tissues, CCN proteins play a role in responses to injury in the CNS. This role has been particularly attributed to CCN2. CCN2 appears to be involved in gliosis and glia scar formation in response to different types of brain injury, such as trauma, cerebral infarction, and excitotoxic brain damage. After kainic acid-induced lesions of the CA3 area of the hippocampus (i.e., a model of excitotoxic brain damage), CCN2 protein was detected in the CA1, CA3, and dentate gyrus, mainly in neurons that mostly died on subsequent days. At later stages when repair processes are active, CCN2 was found extracellularly and in GFAP-positive astrocytes (Hertel et al., 2000).

CCN2 upregulation has also been observed in reactive gliosis adjacent to the site of mechanical injury and in brain tissue in stroke patients and following traumatic brain injury (Schwab et al., 2000, 2001). A role in neuroinflammation has also been suggested for CCN3. CCN3 protein was shown to be expressed in astrocytes and regulate astrocyte chemokine synthesis *in vitro* and *in vivo* (Le Dréau et al., 2010a). CCN3 was suggested to attenuate inflammatory pain (Kular et al., 2012). CCN3 protein expression decreased in dorsal root ganglia and the dorsal horn of the spinal cord in a rat model of inflammatory pain. Interestingly,

intrathecal administration of CCN3 siRNA during early stages of an inflammatory pain model resulted in a significant increase in mechanical allodynia. In contrast, CCN3 treatment significantly attenuated mechanical allodynia in rats but had no effect on basal pain perception in control animals. As demonstrated both *in vitro* and *in vivo*, CCN3 influences matrix metalloproteinase (MMP) expression, which might contribute to the possible mechanism of CCN3's involvement in inflammatory pain (Kular et al., 2012).

The role of several CCN proteins has been suggested in neurodegenerative disease, based largely on the observation that they are expressed in patients' brains in both neurons and astrocytes. Notably, however, a causal role for CCN proteins in neurodegeneration has not yet been proven, and increased levels of CCNs might result from inflammation that accompanies neurodegeneration. CCN2 appears to be involved in the progression and persistence of astrogliosis in neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS) and Alzheimer's disease (AD). In AD patients' brains, CCN2 protein was detected in the entorhinal cortex, hippocampus, and temporal cortex, and the signal was associated with neurofibrillary tangles and neurons that are associated with amyloid plaques (Ueberham et al., 2003). Moreover, CCN2 was detected in perivascular astrocytes and astrocytes that were associated with plaques (Ueberham et al., 2003). Another study showed that *CCN2* expression in the AD brain is correlated with the progression of clinical dementia in AD and amyloid plaques but not neurofibrillary tangle pathology. The authors suggested that CCN2 may play a role in the pathogenesis of AD by promoting amyloid β peptide levels. This hypothesis was supported by findings from a Tg2576 AD model in mice that were exposed to a diabetogenic diet. These mice developed insulin resistance, accompanied by elevations in CCN2 levels in the brain and the promotion of AD-type amyloid plaque burden (Zhao et al., 2005). The authors also found that CCN2 activated the MAPK and PI3K/Akt pathways in human H4-APP_{751} neuronal cells and suggested that this may lead to an increase in amyloid β peptide levels and contribute to AD pathology (Zhao et al., 2005).

CCN2 expression was also linked to ALS. In normal human spinal cord, the low expression of CCN2 protein is observed in cells with astroglial morphology, particularly in white matter. CCN2 protein levels are increased in ALS cases. It can be detected in vimentin-positive reactive astrocytes and remaining motor neurons in the ventral horn in long-term-surviving patients (Spliet et al., 2003).

CCN4 has been linked to neurodegeneration protection. Treatment with CCN4 blocked primary neuronal injury and apoptosis during oxygen-glucose deprivation, and this effect depended on PI3K-Akt signaling (Wang et al., 2012).

Conclusions and Perspectives

CCN proteins have not been studied vigorously or systematically in the nervous system, but their known functions in other tissues and organs support their potential involvement in the proper development and physiology of the nervous system. Such a role is also supported by a few discoveries of CCNs' involvement in neuroprecursors proliferation, neuronal survival, and differentiation. Examples of nervous system pathologies that are linked to changes in CCN gene expression support the importance of CCNs in the nervous system. Yet, based on these few examples it would be premature to build a comprehensive model of CCN protein-dependent regulation of the nervous system development and physiology. As in case of other systems, it is very likely that the final outcome of CCN activities may depend on cell type and repertoire of receptors expressed by a given cell at a particular developmental/differentiation stage. What is more, similar to studies on skeletal development, studies in the context of the whole organism are key to understanding CCNs' modulatory functions. Therefore, further progress in our understanding of the functions of CCNs in the nervous system will require the development of animal models using spatially and developmentally regulated knockouts of CCN family genes. Studies on the role of CCNs in the nervous system can accelerate in the future only with such models.

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