Novel Roles of Metalloproteinases in Neurite Outgrowth and Synapse Formation

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ABSTRACT

The establishment of the brain circuitry relies on the precise targeting and connectivity of neuronal projections. Neurons respond to a vast repertoire of extracellular cues by temporally and spatially regulating the presence of cell surface cognate receptors. Cleavage of cell surface proteins, a mechanism mediated by members of the metalloproteinase subfamily, alters the neuronal sensitivity to repressive or permissive cues present in the extracellular milieu. Metalloproteinases can desensitize neurons to ligands in the environment by rapidly remodeling the repertoire of surface proteins, altering intracellular signaling, or releasing active fragments in the extracellular space. **This thesis establishes critical roles for metalloproteinases in spatial and temporal regulation of neurite outgrowth and synapse formation**. In Chapter 2, we identified MT3-MMP as a membrane-bound metalloproteinase prominently expressed in the mouse cerebral cortex. MT3-MMP loss of function decreases the density and components of excitatory synapses, suggesting a potential role in the development of excitatory connections. Through genetic mutations and shedding experiments from aged cortical neurons, we identify the Nogo-66 receptor (NgR1) as a downstream MT3-MMP proteolytic effector. Consistently, treatment with Ecto-NgR1 fragments or a cleavage enhanced NgR1 construct potentiates excitatory synapse formation. In Chapter 3, we describe a role of metalloproteinases in cortical outgrowth. Through a proteomic analysis, we identify members of the IgLON cell adhesion superfamily as novel metalloproteinase substrates. IgLON surface expression correlates with the sensitivity of aged cortical neurons to metalloproteinase inhibitors. Furthermore, immobilized IgLONs promote growth and desensitize neurons to metalloproteinase inhibitors, implicating IgLON proteolysis in the specificity of subdomain axon targeting. In Chapter 4, we evaluate a metalloproteinase-dependent mechanism in the growth of DRG neurons. IgLON family members are also present and processed from the surface of embryonic DRGs. Introducing IgLON family members or exposing DRG neurons to immobilized IgLON substrates represses neurite outgrowth, implicating IgLONs as endogenous repressors of neurite outgrowth. Together, these studies demonstrate new roles for metalloproteinases in excitatory synapse formation and neurite outgrowth. Remodeling the surface proteome

by the action of metzincin metalloproteinases might provide new avenues to potentiate neurite outgrowth and synaptic connectivity after injury or neurological disorders.

RÉSUMÉ

L'établissement des circuits cérébraux repose sur la croissance contrôlée et la connectivité des projections nerveuses. Les neurones répondent à une panoplie de signaux extracellulaires en régulant de manière temporale ou spatiale des récepteurs membranaires de même affinité. Le clivage des protéines de surface, un mécanisme induit par les membres de la sous-famille des métalloprotéinases, altère la sensibilité des neurones aux signaux répressifs ou permissifs présents dans le milieu extracellulaire. En effet, les métalloprotéinases peuvent désensibiliser les neurones aux ligands de leur environnement en remodelant rapidement le répertoire des protéines de surface, en altérant la signalisation intracellulaire ou en relâchant des fragments actifs dans l'espace extracellulaire. **Cette thèse met en évidence des rôles critiques des métalloprotéinases dans la régulation spatiale et temporale de la croissance des neurites et la formation synaptique.** Dans le chapitre 2, nous avons déterminé que la MT3-MMP, une métalloprotéinase liée à la membrane, est exprimée de manière prédominante dans le cortex cérébral de souris. La perte de fonction des MT3-MMP engendre une diminution de la densité des récepteurs de synapses excitatrices ainsi que leurs composantes, suggérant un rôle potentiel dans le développement de connexions excitatrices. En induisant des mutations génétiques et des expériences de clivage dans des neurones corticaux âgés, nous avons identifié le récepteur Nogo-66 (NgR1) comme un effecteur protéolytique de la MT3-MMP. Par suite, des traitements avec des fragments Ecto-NgR1 ou avec des constructions de NgR1 augmentés par le clivage potentialisent la formation de synapses excitatrices. Dans le chapitre 3, nous décrivons le rôle des métalloprotéinases dans la croissance corticale. Utilisant l'analyse protéomique nous avons identifié des membres de la superfamille des molécules d'adhésion cellulaire IgLON comme nouveaux substrats des métalloprotéinases. L'expression des IgLON de surface corrèle avec la sensibilité des neurones corticaux âgés mis en présence d'inhibiteurs de métalloprotéinases. De plus, nous avons déterminé que des IgLONs immobilisés incitent la croissance et la désensibilisation neuronale aux inhibiteurs des métalloprotéinases, liant la protéolyse des IgLON dans la spécificité du sous-domaine de ciblage axonal. Au chapitre 4, nous évaluons un mécanisme dépendant des métalloprotéinases dans la

croissance neuronale des ganglions de la racine dorsale (DRGs). Les membres de la famille des IgLON sont aussi présents et traités de la surface des DRGs embryonnaires. L'introduction des membres de la famille des IgLON ou l'exposition des neurones DRGs à des substrats immobilisés de IgLON répriment la croissance des neurites, suggérant que les IgLON sont des répresseurs de la croissance des nerfs. Mis ensemble, ces études démontrent de nouveaux rôles pour les métalloprotéinases dans la formation des synapses excitatrices et la croissance des neurites. Le remodelage du protéome de surface par l'action de la metalloprotéinases metzincin pourrait mener à de nouvelles avenues pour potentialiser la croissance des neurites et la connectivité synaptique après lésions ou problèmes neurologiques.

LIST OF ABBREVIATIONS

ADAM _____a disintegrin and adamalysin metalloproteinase
ADAMTS _____ADAM with thrombospondin sequence
AP-1_____activator protein 1
APMA_____4-amino phenylmercuric acetate
ArgBP1_____arg protein tyrosine kinase binding protein-1
ATP_____adenosine triphosphate
BIII_____beta-III tubulin
BACE-1_____beta-site APP-cleaving enzyme 1
BB-94_____batimastat
BSA_____bovine serum albumin
CE-NgR1_____cleavage enhanced Nogo-66 receptor
CHL-1_____cell adhesion molecule L1-like
CHO_____chinese Hamster Ovary
CM_____conditioned media
CNS_____central nervous system
CRE_____cis-regulatory elements
CSB_____cell surface biotinylation
CSPG_____chondroitin sulphate proteoglycans
CT-NgR1_____carboxy-terminal Nogo-66 receptor
DCC_____deleted in colorectal carcinoma
DIV_____days in vitro
DMSO _____dimethyl sulfoxide
DMEM_____dulbecco's medium
DRG_____dorsal root ganglion cell
ECM_____extracellular matrix
EGF_____epidermal growth factor
EGFP_____enhanced green fluorescent protein
ERK_____extracellular-signal regulated kinase
FGFR2_____fibroblast growth factor receptor 2
FL-NgR1_____full-length Nogo-66 receptor
GAG_____glycosaminoglycan
GAPDH_____glyceraldehyde 3-phosphate dehydrogenase
GEF_____guanine exchange factor
GFP_____green fluorescent protein
GI242530X___selective ADAM-10 inhibitor
GM-I_____inactive analalogue for GM6001
GM6001_____ilomastat
GPI_____glycosphosphatidylinositol
GPCR_____G-protein coupled receptor
HRP_____horse-radish peroxidase
IC-3_____borad metalloproteinase inhibitor
IL-1β_____ interleukin-1Beta
KUZ_____kuzbanian

LGI-1_____leucine-rich glioma-inactivated protein 1
LINGO-1____leucine rich repeat and immunoglobulin-like domain containing protein 1
LRR_____leucine-rich repeat
LSAMP_____limbic system-associated membrane protein
MAI_____myelin-associated inhibitor
MAG_____myelin-associated glycoprotein
MAPK_____mitogen activated protein kinases
MBP_____myelin-basic protein
MMP_____matrix metalloproteinase
MP_____metalloproteinase
MT-MMP___membrane-type metalloproteinase
N-Cad_____N-Cadherin
NCAM_____neuronal cell-adhesion molecule
NEGR1_____neuronal growth regulator-1
NF-κB_____nuclear factor κB
NgR1_____nogo-66 receptor 1
NgR2_____nogo-66 receptor 2
NgR3_____nogo-66 receptor 3
NLG-1_____neuroligin-1
NTM_____neurotrimin
OBCAM____opioid-binding cell adhesion molecule
OMgp_____oligodendrocyte-myelin glycoprotein
$P75^{NTR}$_____neurotrophin receptor p75
PI-PLC_____phospholipase-C
PirB_____paired-immunoglobulin-like receptor B
PKC_____protein kinase C
PLL_____poly-l-lysine
PLP_____myelin proteolipid protein
PNGase F___peptide-N-glycosidase F
PNS_____peripheral nervous system
PSD95_____postsynaptic density protein 95
PTP-sigma___protein tyrosine phosphatase sigma
PTRK_____receptor protein tyrosine kinase
RECK_____reversion inducing cysteine rich protein with Kazal motifs
RhoA_____ras homolog gene family member A
RGC_____retinal ganglion cell
Robo_____roundabout
ROCK_____rho-associated protein kinase
ROS_____reactive oxygen species
RT-PCR_____reverse transcription polymerase chain reaction
SH3_____SRC homology 3 domain
SH3PX1_____sortin nexin
SLITRK_____SLIT and NTRK-like protein
SOS_____son of sevenless
SPARC_____secreted protein acidic acid and cysteine rich
SYN-1_____synapsin-1

SynCAM_____synaptic cell-adhesion molecule
SynPhy_____synaptophysin
SRC_____proto-oncogene tyrosine protein kinase
STAT_____signal transducer and activator of transcription
TIMP_____tissue inhibitor of metalloproteinase
TPA_____12-O-tetradecanoylphorbol-13-acetate
TUNEL_____terminal deoxynucleotidyl transferase dUTP nick-end labeling
TGF-β_____transforming growth factor beta-1
TNF-α_____tumor necrosis factor-alpha
VGLUT1_____vesicular glutamate transporter-1
VEGF_____vascular endothelial growth factor

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INTRODUCTION

During development, the nervous system must establish a series of high fidelity connections to mediate appropriate communication. Neurons respond to numerous ligands and adhesion molecules in the environment through engagement of cell surface receptors that signal intracellularly to control the speed and direction of neuronal growth and the formation of stable synaptic connections. Neurons grow in a series of discrete steps through their environment necessitating spatiotemporal strategies to dynamically modify how neuronal projections respond to extracellular cues (1). In particular, neurons control their response to the environment by regulating the complement of proteins present at the cell surface through regulated transcription, local translation, membrane trafficking and proteolysis, the focus of this thesis. Proteolytic cleavage is a post-translational modification orchestrated by proteases that results in the release, fragmentation, clipping or degradation of proteins. The process can occur intracellularly, in the extracellular matrix (ECM), or at the cell surface (2). Cleavage of cell surface proteins can result in degradation of receptor molecules, a process termed ectodomain shedding, which can desensitize a neuron to ligands in the environment, generate biologically active fragments in the extracellular space and also initiate regulated intracellular proteolysis (RIP) and subsequent intracellular signaling.

Proteolytic cleavage was initially discovered in 1962, when experiments conducted by Gross and Lapierre led to the observation that explant cultures from tadpole tails have the ability to degrade collagen substrates (3). The interesting collagenolytic activity reported during amphibian metamorphosis was the cornerstone for the characterization of a large family of clinically relevant endopeptidases, later known as metalloproteinases. Metalloproteinases are members of a large family of metzincin proteinases that include astacins, serralysins, pappalysins, matrix metalloproteinases (matrixins, MMPs) and adamalysins (ADAMs, A Disintegrin and Metalloproteinase) (4-6). They are ubiquitously expressed in the nervous system and include intracellular, cell surface and secreted family members. The matrix metalloproteinase subfamily is further categorized into four different groups dependent on structure and substrate affinities, including: collagenases, gelatinases, stromelysins, matrilysins and membrane-type matrix metalloproteinase (MT-MMPs) (Figure 1).

The first study implicating proteolytic cleavage in axonal guidance decisions was performed in *Caenorhabditis Elegans* where mutations in a metalloproteinase ortholog prevented stereotypical cell migration (7). The importance of metalloproteinases in nervous system development has been subsequently confirmed in multiple species. Metalloproteinase mutations in the fruit fly *Drosophila Melanogaster* or in mice and application of broad metalloproteinase inhibitors in the visual system of the frog *Xenopus Laevis,* all result in altered central nervous system (CNS) development (8-10). Recently, aberrant metalloproteinase activity have been reported in different neurological disorders including Multiple Sclerosis, Alzheimer's disease, stroke, malignant glioma and amyotrophic lateral sclerosis (11-18), Metalloproteinases thus occupy important roles in many normal and pathological processes in the nervous system.

Under basal conditions, metalloproteinases target growth factors, receptors and adhesion molecules to generate biologically active/inactive peptides capable of modulating migration, cell adhesion and growth. Soluble shed fragments activate or arrest signaling pathways in an autocrine or paracrine fashion. For example, the shedding of the ectodomain of the L1 cell adhesion molecule promotes adhesion and cell migration through the interaction of cleaved L1 proteins with the cell surface αvβ5 integrin receptor (19-21). Proteolytic cleavage can also facilitate outgrowth in the hostile inhibitory environment of the injured CNS. Cleavage of inhibitory ligands including Nogo and Chondroitin sulphate proteoglycans has been reported to promote neurite outgrowth (22-25). Shedding of receptors including Nogo-66 receptor 1 (NgR1), Neuropilin-1, Ephrin (Eph) and Roundabout (Robo) can also desensitize neurons to the inhibitory extracellular milieu (26-29). Furthermore, RIP of $p75^{NTR}$, a co-receptor for NgR1, could initiate intracellular proteolysis, nuclear translocation and gene expression by activation of y-secretases or the signal peptide peptidase-like (SSPL) proteases, resulting in activation of different signaling pathways that regulate cell death, axon growth, growth cone collapse, degeneration, or cell survival (30-36).

The following thesis dissertation will focus on the ability of matrix metalloproteinases and ADAM family members to modulate neurite outgrowth and synapse formation

through the processing of extracellular proteins present at the cell surface.

1.1 Matrix metalloproteinases and Adamalysins

To date, 24 MMPs and 21 ADAM family members have been identified in humans with distinct yet overlapping substrate specificities. At the structural level, most metalloproteinases consist of four distinct domains: an N-terminal pro-peptide structure, a catalytic subunit, a hinge region that connects the carboxy- and amino-terminal domains and a C-terminal hemopexin subunit (37). The metalloproteinase crystal structure reveals that the pro-peptide domain is in close proximity to the catalytic site (38). A conserved zinc-binding methionine (Met) group in the catalytic subunit, critical for metalloproteinase activity, confers the metallo-prefix to their name and membership to the metzincin family of proteases (4). The catalytic site has a zinc-binding motif that contains three histidines (HEXXHXXGXXH). The interaction of water molecules and zinc ions with histidines is fundamental for mediating protein hydrolysis. Lastly, the hemopexin domain is located in the carboxy-terminus and is attached by a flexible hinge. The hemopexin is composed of a 4-bladed β-propeller fold, important for protein-protein interactions; substrate recognition; enzyme activation; protease localization; protease internalization or inactivation by protease degradation (Figure 1) (37).

Metalloproteinases are initially expressed as inactive zymogens, where the pro-peptide domain forms hydrogen bonds with the catalytic subunit and prevents interaction with water and zinc ions (39). Destabilization, or degradation of the pro-peptide domain converts metalloproteinases from inactive zymogens to their mature active form (40,41). A number of mechanisms release or destabilize the cysteine-histidine binding interaction, termed cysteine switch, to induce proteolytic activity. Furin, plasmin, mechanical stress, changes in pH, growth factors, intracellular calcium and processing by other metalloproteinases can all interfere with the auto-inhibitory activity of the pro-peptide structure (40,42,43). For example, MT-MMPs have the ability to cleave the pro-peptide domain of soluble metalloproteinases. MT1-MMP associates with proMMP2, releasing

the repressive cysteine switch (44). Furthermore, the presence of a signal sequence in the pro-peptide domain (KX(R/K)R) triggers intracellular cleavage by furin, resulting in metalloproteinase activity. Plasmin, a serine secretase activated by tissue plasminogen activator and urokinase plasminogen activator, can also activate proMMPs. proMMP-1, proMMP-3, proMMP-7, proMMP-9, proMMP-10, and proMMP-13 are examples of metalloproteinases activated by Plasmin (45). There is also evidence that ADAM-8 and ADAM-28 can remove the cysteine-switch through an autocatalytic mechanism (46). Furthermore, the cysteine switch can also be disrupted by amino phenyl mercuric acetate (APMA) (47).

At the transcript sequence, metalloproteinases share cis-regulatory elements (CRE), regions of non-coding DNA that serve as binding-sites for transcription factors, thus most metalloproteinases are co-expressed or co-repressed through various stimuli. The most common metalloproteinase promoter contains a TATA box, an activation protein (AP)-1 binding site and an upstream polymavirus enhancer activator (PEA)-3-binding site (48). Although, metalloproteinase expression is tightly regulated at the transcriptional level, post-transcriptional mechanisms that affect mRNA stability have recently been described as contributing repressive factors (49-52).

1.1.1 Collagenases, gelatinases, stromelysins and matrilysins

Collagenases (MMP-1, MMP-8, MMP-13 and MMP-18), the initial metalloproteinase subgroup discovered by Gross and Lapierre, are characterized for their ability to cleave interstitial collagen-II, -III, and -I at sites located in the target N-terminus (37). Gelatinases (MMP-2 and MMP-9) process type-IV, -V, -XI collagens, laminin, gelatin and aggrecan core proteins. Similar to collagenases, MMP-2 digests collagen-I, -II, and -III (53,54). MMP-2 and MMP-9 knockout mice exhibit delays in bone growth and development (55). The stromelysin subfamily (MMP-3, MMP-10 and MMP-11) processes ECM components and activate proMMPs. For example, MMP-3 processes proMMP-1 to generate fully active MMP-1 (56). Matrilysins (MMP-7 and MM-26) are characterized as metalloproteinases that lack the hemopexin domain (57). They target cell surface molecules such as pro-α-defensin, Fas-ligand, tumor necrosis factor (TNF)-α and

E-cadherin (58). MMP-26 also digests several ECM molecules, and unlike most metalloproteinases, it is largely stored intracellularly (59).

Almost all collagenases, gelatinases, stromelysins and matrilysins are secreted as inactive proteases, and thus, activation requires extracellular processing of the pro-peptide domain. However, recent studies have identified MMP-1, MMP-2 and MMP-11 as proteases acting intracellularly (60-62).

1.1.2 Membrane-type metalloproteinases

MT-MMPs are a specific subtype of matrix metalloproteinases sequestered to the cell surface by a GPI-anchor moiety (MT4-MMP and MT6-MMP), or a type-I transmembrane domain (MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP) (63). The specific cell surface localization of MT-MMPs provides a unique set of membrane-anchored and ECM targets, distinct interaction with endogenous metalloproteinase inhibitors and non-conventional regulatory mechanisms; involving enzyme internalization, processing and ectodomain shedding (37). These enzymes can also digest a number of ECM molecules. For instance, MT1-MMP has a collagenolytic activity on type-I, -II, -III collagens and complete MT1-MMP KO mice exhibit skeletal abnormalities during postnatal development (63). MT-MMPs can also interact and anchor secreted metalloproteinases to the cell surface, such as the case of proMMP-2 (43). Another important difference between MT-MMPs and secreted metalloproteinases is intracellular activation. A furin recognition motif present in all six MT-MMPs is recognized and cleaved by pro-protein convertases in the trans-Golgi network; ensuring activity when they reach the cell membrane (64). In the case of MT5-MMPs, the C-terminal tail interacts with AMPA-receptor binding protein (ABP) and glutamate receptor interacting protein (GRIP)-1, providing synaptic membrane localization (65). Furthermore, MT3-MMP null mice display growth inhibition, while double MT3-MMP and MT1-MMP knockouts in mice leads to embryonic lethality due to strong defects in palatogenesis and bone formation (66).

1.1.3 Adamalysins

ADAM proteases also belong to the metzincin subgroup of zinc metalloproteinases (67). Their name derives from structural components (A Disintegrin and Metalloproteinase) and the initial identification in sperm proteins associated with fertility (68). The main difference between ADAMs and matrix metalloproteinases lies in the structure and substrate specificity. ADAM family members have a unique integrin receptor binding disintegrin domain and have a preference for cell surface proteins rather than ECM targets (69). They are a family of zinc transmembrane glycoproteins with essential functions in cell-cell interaction, cell signaling and proteolysis of important cytokines, cytokine receptors and other cell surface proteins. With the exception of ADAMDEC-1 that lacks a transmembrane domain, the vast majority of ADAM family members are type-I transmembrane proteins whose pro-peptide domain is generally removed intracellularly (69). In addition, some ADAM family members (ADAM-11, -12, -15, -17 and -28) have alternatively spliced forms that generate secreted isoforms (64). Recently, a new secreted family member, ADAMTS, ADAM with thrombospondin motifs, has been identified to bind to the ECM and degrade several CSPGs, including aggrecan, brevican and versican (5,6,70). Studies from transgenic mice have provided new insights into the role of ADAM family members in development. For instance, ADAM-1, ADAM-2 and ADAM-3 null mice are infertile (68,71-74). ADAM-10 knockout mice die at embryonic day (E9.5) due to major defects in development (75). Similarly, ADAM-17 deficient mice exhibit perinatal lethality due to multiple defects in lungs, heart and skin development (76-79). Lastly, ADAM-22 and ADAM-23 develop ataxia and tremor and die at postnatal day 21 and 14, respectively (80,81).

In addition to a pro-peptide and catalytic subunit, the ADAM structure comprises a disintegrin domain for adhesion, a cysteine-rich region for substrate recognition, epidermal-growth factor (EGF) repeats, a transmembrane module and a cytoplasmic tail (67). Mutations in the pro-domain of ADAM-17 and ADAM-10 have determined that, similar to matrix metalloproteinases, the pro-domain is necessary for maintaining enzyme latency, assisting in the proper folding of the catalytic subunit and in trafficking of ADAMs throughout the secretory pathway (82-84). However, it is important to note that

although most human matrix metalloproteinases contain the zinc-binding motif, only twelve ADAMs have the catalytic sequence and are enzymatically active (69). For example, ADAM-22 is as intermediary synaptic adhesion protein that facilitates protein-protein interactions between leucine-rich glioma-inactivated protein 1 (LGI-1) and NgR1 (85,86). Also, ADAM-23 and ADAM-22 can bind to integrins and have been reported to play a crucial role in synapse formation and myelination (80,87,88). Therefore, ADAM proteases are unique cell-surface proteins having both adhesive and proteolytic activities.

The disintegrin-like domain, also known as the disintegrin loop facilitates interactions with integrins, while the cysteine-rich region can interact with proteoglycans (89,90). The last subunit is a cytoplasmic tail that contains specialized motifs to regulate proteolytic activity, cell signalling, and subcellular localization. For example, ADAM cytoplasmic domains have potential serine-threonine and/or tyrosine phosphorylation sites. The cytoplasmic domain of ADAM-9 and ADAM-12 contains proline-rich (PxxP) motifs serving as a binding site for Src-homology 3 (SH3) and protein kinase C (PKC) (69). They can also bind to other intracellular proteins such as endophilin-I, SH3PX1 (sortin nexin-9), ArgBP1 (Arg protein tyrosine kinase binding protein-1), beta-cop and ubiquitin (91,92). Finally, phosphorylation of ADAM-17 prevents dimerization with other ADAM-17 proteases and prevents inactivation by endogenous metalloproteinase inhibitors (93).

1.1.4 Metalloproteinase inhibitors

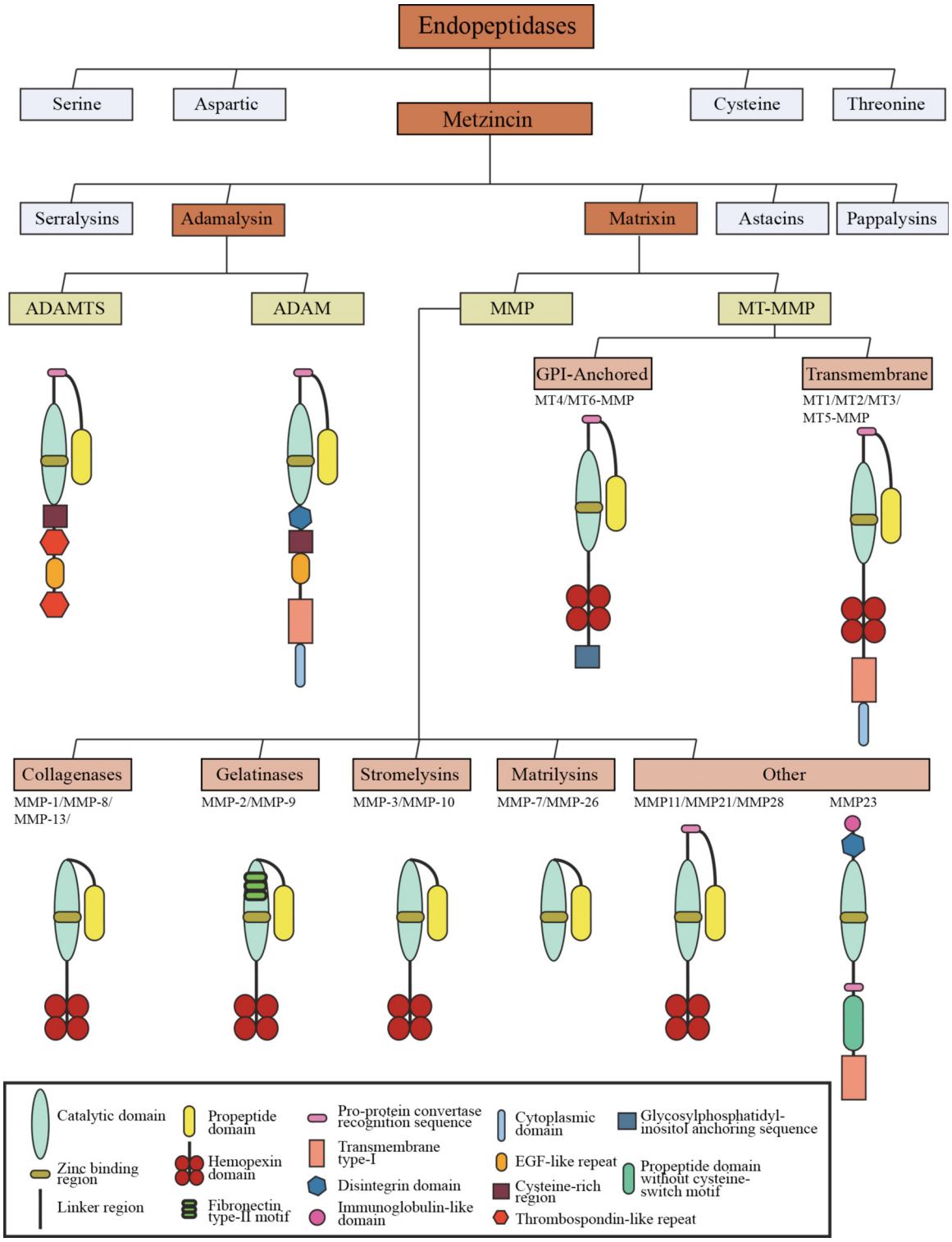
In addition to the existence of a cysteine switch, endogenous metalloproteinase inhibitors repress metalloproteinase activity. Tissue inhibitors of metalloproteinases (TIMPs) are small family of endogenous metalloproteinase inhibitors expressed in many tissues, alongside metalloproteinases (94). With exception of the membrane-anchored TIMP3, TIMPs are secreted metalloproteinase inhibitors. Presently, four TIMPs (1-4) have been identified in mammals that differ in their affinity towards metalloproteinases. TIMP1 inhibits secreted metalloproteinases, whereas TIMP2 inhibits secreted and MT-MMP family members. The membrane bound TIMP3 interacts with a larger array of metalloproteinases, including secreted, membrane-bound and ADAM family members. Interestingly, TIMP3 mRNA is highly expressed during development and after injury in

rat brains, suggesting a role in growth, survival and regeneration (95). TIMP2 and TIMP3 are vastly expressed during embryonic development and their presence persists throughout postnatal life (95). The most recently discovered TIMP family member, TIMP4, inhibits secreted and MT-MMPs similar to TIMP2 (96).

In addition to TIMPs, plasma α-macroglobulins, tissue factor pathway inhibitor-2, thrombospondin-1 and thrombospondin-2, the secreted form of membrane bound β-amyloid precursor protein, the C-terminal fragment of pro-collagen C proteinase, chlorotoxin, reversion-inducing cysteine-rich protein with Kazal motifs (RECK) and high concentration of reactive oxygen specifies (ROS) have all been shown to repress single or some metalloproteinases (97-101).

Furthermore, pharmacological metalloproteinase inhibitors have been developed to work through a similar mechanism as endogenous inhibitors. The first generation of inhibitors was designed to mimic the structure of collagen and comprise a chelating moiety, hydroxamate, which forms a complex with the zinc ion in the catalytic metalloproteinase domain and represses proteolytic activity. However, most hydroxamate-based metalloproteinase inhibitors exhibit low specificity between family members. Examples of collagen-based hydroxamates include marimastat, ilomastat (GM6001) and batimastat (BB-94) (102,103). Recently, non-hydroxamate inhibitors with various backbone structures have been designed to avoid several limitations associated with the first generation of inhibitors, such as, metabolic inactivation and chelation of metals of other metalloproteinases.

Figure 1: The family of metzincin metalloproteinases



1.2 The role of metalloproteinases in the nervous system

Fifty-four years after their discovery, the physiological function of metalloproteinases remains largely unknown because of the vast number of metalloproteinase subtypes with overlapping functions. Genetic knockout mice exhibit subtle phenotypes as a result of proteolytic redundancy, compensation and developmental adaptation (99,104-107). There is also a lack of reliable reagents to detect individual matrix metalloproteinases and to monitor their activity. Nonetheless, several metalloproteinases have been implicated in neurodevelopment and play roles in establishing neuronal architecture and facilitating neuronal communication (108-110). For instance, seven proteolytically active ADAM family members have been detected in the adult rodent brain primarily in neuroepithelial precursors and postmitotic neurons (111,112). ADAM-10 is expressed in the forebrain, dorsal root ganglia (DRG), sympathoadrenal and olfactory precursors, while ADAM-19 is expressed in the craniofacial ganglia, dorsal root ganglia (DRG) and the ventral horn (113,114). ADAM-17 is present in postnatal cerebellum and DRGs (115,116), whereas ADAM-33 is predominantly expressed in the central and peripheral nervous system (81,117). MMP-2, MMP-11, MMP-13, MT1-MMP, MT2-MMP and MT5-MMP have also been detected in the brain and spinal cord with highest expression after birth (118).

In the CNS, various ADAM proteases are expressed by neurons, astrocytes or oligodendrocytes (111,112,119). MMP-2 is detected in astroglia and some pyramidal neurons in the cortex and Purkinje cells in the cerebellum, whereas MMP-9 is expressed in the hippocampus, cerebellum and cortical neurons (109,118,120). Furthermore, several studies have revealed a strong matrix metalloproteinase expression in migrating population of neurons. MMP-8 is expressed in migrating neural crest cells and MMP-9 in radially migrating immature granule cells. Additionally, some matrix metalloproteinases are expressed in precursors cells (121,122). MMP-2 is present in human neuroepithelial CNS stem cells (108), and MMP-9 progenitors cells of the pituitary gland and the choroid plexus (123). Lastly, matrix metalloproteinases are expressed in structures such as the cerebellum during postnatal periods when cellular differentiation occurs (116,122,124).

1.3 Metalloproteinases in synapse formation

Synapses are macromolecular contacts that facilitate the vectorial flow of information between neurons. They are formed through a series of discrete steps, where a physical contact between a growing neurite and its target cell initiates the recruitment of synaptic components and differentiation into synaptic terminals (125). The presynaptic terminal contains hundreds of synaptic vesicles that reside in specialized regions in the plasma membrane, termed the active zone, and are densely packed with chemical neurotransmitters. Entry of an action potential to the presynaptic terminal generates an influx of extracellular calcium, followed by fusion of synaptic vesicles with the plasma membrane and culminating in neurotransmitter release. Across the synaptic cleft, a short distance of approximately 20nm, presynaptically released neurotransmitters will encounter receptors from the postsynaptic terminal, a structure involved in the reception and transduction of neurotransmitters, which will increase/decrease the neuronal electrical activity. Throughout life, synapses undergo structural modifications that correlate with memory and cognitive function, such as synapse density, morphology of dendritic spines and strength of synaptic signaling (126,127). Synaptic remodeling depends on a variety of ECM, receptors, cell adhesion molecules, and growth factors that undergo metalloproteinase-dependent proteolysis.

Several lines of evidence have implicated metalloproteinases as facilitators of interneuronal communication. Critically, metalloproteinase expression and activity is observed in synapses. In hippocampal slices processed for *in situ* zymography, a technique to observe proteolytic activity, gelatinolytic puncta were identified in close opposition to molecular markers of excitatory synapses, post-synaptic scaffolding, cytoskeletal proteins and pre-synaptic vesicle proteins (128,129). Interestingly, metalloproteinase activity is largely absent from GABAergic synapses, suggesting a specific role for metalloproteinases in excitatory communication (130). Furthermore several metalloproteinases have been identified at the synapse and to co-localize with peri-synaptic markers. High-resolution microscopy of immunolabelled hippocampal

tissue sections showed that MMP-9 co-localizes with pre-synaptic and post-synaptic molecular markers (128). Similarly, MT5-MMP co-localizes with synaptic markers in cultured rat hippocampal neurons and is enriched in synaptosomes from adult rat forebrain (65). At the cell surface, the active form of MT5-MMP is only visualized in the presence of metalloproteinase inhibitors, suggesting that duration of metalloproteinase activity is regulated by proteolytic degradation.

Electrophysiological experiments have further implicated metalloproteinases in the strength of synaptic communication. Long-term potentiation (LTP) and Long-term depression (LTD) are experimental models of learning and memory formation, where a series of high or low frequency stimulations alters the electrical state of chemical synapses (131,132). MMP-9 immunoreactive puncta and gelatinolytic activity are detected in hippocampal neurons after late-phase LTP (L-LTP) induction protocols (133). Experiments with broad metalloproteinase inhibitors or anti-MMP-9 antibodies have reported impaired synaptic stability in long-term potentiation (LTP) and impaired spine enlargement (128,134). Diminished pre-synaptic vesicle release has also been reported following long-term exposure of hippocampal cultures to the broad-metalloproteinase inhibitor, GM6001. Furthermore, hippocampal slices exposed to FN439, a broad-metalloproteinase inhibitor, exhibit deficits in both paired-pulse facilitation (PPF), a form of presynaptic plasticity, and long-term depression (LTD) (128,134). Therefore, metalloproteinases might facilitate structural synaptic remodeling, necessary for memory and learning behavior.

Several synaptic proteins have been identified to be subject to metalloproteinase-mediated proteolysis and to affect synaptic remodelling. For instance, ICAM-5, a transmembrane protein localized in dendrites and fillopodia, can be processed by MMP-3, MMP-7 and MMP-9 (135). Following LTP stimulation protocols, an Ecto-ICAM-5 fragment can be detected in the media of dissociated neurons that is dependent on metalloproteinase activity (136,137). In biochemical experiments, Ecto-ICAM-5 fragments interact with β1-integrins and stimulate cofilin phosphorylation (138). In addition, ADAM-10 and MMP-9 mediate neuroligin (NLG)-1 shedding. NLG-1 cleavage

occurs through NMDA receptor activation, and destabilizes its presynaptic partner neurexin (NRX)-1β. A cleavage resistant NLG-1 construct increases dendritic spines in neuronal cultures (139), while NLG-1 cleavage depresses synaptic transmission (140). Furthermore, activity-dependent processing of the membrane glycoprotein SIRP-α by metalloproteinases induces pre-synaptic differentiation and maturation in hippocampal neurons (141). SLITRK; N-Cadherin; β-dystroglycan; NCAM; TNF-α; neurotrophins, thrombospondin, hevin; SPARC; ephrins and Eph receptors are a small number of synaptic proteins that undergo proteolytic cleavage and could regulate the process of synaptogenesis through metalloproteinase activity (142,143). The role of MT3-MMP in excitatory synapse development through surface Nogo-66 receptor (NgR1) proteolysis will be further discussed in Chapter 2.

1.3.1 The Nogo-66 Receptor

Nogo-66 receptor (NgR1) was the first member of the reticulon-4 receptor family identified through a screen for proteins interacting with the growth inhibitory factor, Nogo-66 (144). To this day, three members of the reticulon-4 receptor family have been described, comprising NgR1, NgR2 and NgR3. The NgR1 crystal structure reveals an elongated banana-shaped protein composed of a GPI-anchored segment, eight leucine-rich repeat domains (LRR) and flanking N-terminal (NT) and C-terminal (CT)-LRR regions that are rich in cysteine residues (145). The GPI domain provides a specific surface localization for Nogo receptors within signaling platforms, known as lipid rafts (146,147). The eight LRR domain exhibit a 55% sequence homology with the three NgR family members, while the stalk region, a domain that connects CT-LRR to the GPI-anchored protein, is less conserved.

Immunohistochemical analyzes of the mouse cerebrum have identified a prominent and broad distribution of NgR1 in the CNS, including the cerebral cortex, the hippocampus, the amygdala, the thalamus, the olfactory bulb, the cerebellum and the spinal cord (148). In the developing mouse brain, NgR1 expression increases during the first week of postnatal development and retains a relatively high level in the adult mouse (148-150). In the cerebral cortex, NgR1 is present in multiple layers and is enriched in axons

surrounded by myelin (148). In the hippocampus, NgR1 is highly expressed in pyramidal cells and the dentate gyrus, while in the spinal cord NgR1 is present in many neuronal cell bodies and the neuropil. Furthermore, electron microscopy, immunocytochemistry and biochemical studies reveal a prominent expression of NgR1 in pre-synaptic and post-synaptic sites (150-152). These findings indicate that NgR1 may contribute to axonal track stability and synaptic plasticity during postnatal development.

Several groups have described a potential role for NgR1 in psychiatric disorders. For instance, Nogo-A rand NgR1 knockout mice acquire a schizophrenia-like behavioral phenotype (153,154). There are also genetic associations between certain families and patients suffering from Schizophrenia and NgR1 mutations. Deficits in MAI signaling though NgR1 may lead to deregulation of circuit functions in adulthood and disease.

1.3.2 NgR1-tripartite receptor complex

Like many other GPI-anchored proteins, Nogo receptor proteins lack a transmembrane domain, and thus, require a signaling-capable co-receptor for intracellular communication. It is now established that a member of the tumor necrosis factor receptor superfamily, $P75^{NTR}$, associates with NgR1 to form a complex that remodels the actin cytoskeleton (155). TROY or TAJ is another member of the tumor necrosis factor able to substitute for $p75^{NTR}$ in the NgR1 receptor complex (156,157). Subsequently, a third component of the complex, a member of IgSF termed Lingo-1 interacts with ligand-bound NgR1 and $p75^{NTR}$/TROY in order to bring the two-receptor components together (158). The tripartite-NgR1 receptor binds to proteins expressed on the surface of myelin that have been extensively characterized as inhibitors of neurite outgrowth *in vitro*, such as: Nogo-A, myelin-associated glycoprotein (MAG) and oligodendrocyte myelin-glycoprotein (OMgp) (159). NgR2 also binds to MAG, with a higher affinity than NgR1 (160), and NgR3 forms a receptor complex with NgR1 to bind chondroitin sulphate proteoglycans (CSPGs) (161). CSPGs are proteoglycans composed of a core glycoprotein and covalently attached glycosaminoglycan chains (162). They are components of the extracellular matrix, the glial scar and perineuronal nets and are known to inhibit axon regeneration after spinal cord injury and synaptic plasticity. Upon MAI/CSPG-binding,

the cytoplasmic region of $P75^{NTR}$ associates with RhoGDI, releasing the small GTPase RhoA. The RhoA/ROCK signaling pathway activates Sling Shot and LIM kinase (LIMK)-1, followed by phosphorylation of the depolymerizing factor cofilin. As a result, the MAI/CSPG-NgR1 signaling axis collapses the growth cone, represses neurite outgrowth and reduces excitatory synapse formation through regulation of actin dynamics (156,157).

In addition, NgR1 binds to amyloid-β (163), to B-lymphocyte stimulator (BLys) (164), to fibroblast growth factor (FGF)-1, FGF-2 (150) and to LGI-1 (86). The NgR1 receptor complex has also been observed to interact with integrins, resulting in decreased integrin activity and decreased in cell-adhesion (165,166).

1.3.3 NgR1 in synaptic plasticity

The discovery of Nogo-66 receptor proposed a novel target to neutralize the vast number of inhibitory cues present in the extracellular milieu, with potential therapeutic applications for axon regeneration (144,167). Surprisingly, NgR1 deficient mice exhibited a limited regrowth of injured fibers, but a robust sprouting and rearrangement of fiber connections (168-170), suggesting a physiological role in neuronal plasticity.

In the last decade, mounting evidence confirmed a role for NgR1 in repressing synaptic plasticity in the adult brain. For instance, experiments conducted by the Strittmatter group identified NgR1 to be necessary for the closure of the critical period in the postnatal mouse visual cortex (171). A timed-lapse examination of neuronal connections in adult mice further revealed that downregulation of NgR1 increased dendritic spine and axonal varicosity turnover to a similar rate observed in pre-adolescent mice (172). Furthermore, NgR1 overexpression interfered with the formation of long-lasting memories and limited the number of excitatory synapses in hippocampal neurons (151,173), categorizing NgR1 as en endogenous repressor of excitatory synaptic connections. Several groups have also reported changes in electrical activity in synapses from NgR1 deficient mice. Loss of NgR1 enhanced FGF-dependent LTP and attenuated LTD in hippocampal slices without changes in basal synaptic transmission (150). Acute administration of antibodies against

Nogo-A or NgR1 strongly enhanced LTP in cortical slices from wild-type animals (152). Acute treatments with Nogo-66 or OMgp suppressed LTP in an NgR1-dependent manner (149). Recently in conditional NgR1 knockout mice, NgR1 restricted surface trafficking of AMPA receptors in the barrel cortex (174), further illustrating a potential role for NgR1 in limiting the strength of excitatory synaptic communication.

1.3.4 NgR1 proteolytic cleavage

The role of NgR1 as a principal mediator of MAI and CSPG-outgrowth inhibition stirred great interest in the generation of NgR1 peptide antagonists. Through binding assays with NgR1 deleted or replaced constructs, different groups examined the importance of NgR1 subdomains to interact with MAI, CSPGs and surface NgR1 proteins. Critically, the LRR and the NT/CT-LRR subdomains (human NgR1 residues 27-310) bind to Nogo-66, MAG and OMgp, while only the CT-LRR component (human NgR1 residues 311-446) interacts with $P75^{NTR}$ and CSPGs (146,161). Subsequently, several NgR1 antagonists were generated with a certain level of success in attenuating outgrowth inhibition by Nogo-66, MAG and whole CNS myelin *in vitro*, such as*:* Nogo-A extracellular peptide residues 1-40 (NEP1-40), soluble full length NgR1 or residues 1-310 of NgR1 (NgR1-310) (175-179). Furthermore, administration of the function blocking peptide NgR1-310 or a Lingo-1 antagonist (LINGO-1-Fc) promoted sprouting of various axonal tracts after spinal cord injury (180,181).

A soluble NgR1 (Ecto-NgR1) fragment comprising the entire ligand-binding domain has been identified in the media and lysates from human neuroblastoma cells (25). A zinc metalloproteinase inhibitor blocked the presence of soluble Ecto-NgR1 fragment, suggesting that metalloproteinases are most likely responsible for NgR1 proteolytic cleavage. Recently, the Fournier laboratory further identified MT3-MMP, as the endogenous sheddase for surface NgR1 in embryonic cortical neurons and postnatal DRG neurons (26). Ecto-NgR1 fragments, analogous to those generated in human neuroblastoma cells, were also identified within human brain cortex and cerebrospinal fluid (CSF) (25), inferring the existence of a physiological role for NgR1 cleavage. In

immunoprecipitation assays, the Ecto-NgR1 failed to associate with $p75^{NTR}$, but retained the ability to bind Nogo-66, suggesting a potential role in antagonizing Nogo-66 function (25). Therefore, MT3-MMP-dependent shedding of NgR1 might provide a novel mechanism to remove the NgR1 repressive break in synaptic plasticity, and this mechanism will be further discussed in Chapter 2.

1.4 Metalloproteinases in neurite outgrowth

The wiring of the nervous system depends on the ability of newly formed fibers to navigate, sometimes over long distances, and encounter their correct signaling targets (1). Molecular cues present in the extracellular environment confer growth promoting, or repressive signals important for directionality. Furthermore, a repertoire of cell surface proteins present in growth cones, motile structures at the tip of growing fibers, greatly coordinates neuronal interactions with the surrounding microenvironment (182).

A great number of surface receptors have been characterized to regulate outgrowth and guidance decisions through proteolytic cleavage. For instance, cleavage of the Slit receptor Roundabout (Robo) by Kuzbanian (KUZ), the drosophila ortholog for ADAM-10, was identified to prevent longitudinal axons from crossing the midline (27). In rat embryonic spinal cord explants, treatment with broad metalloproteinase inhibitors, IC-3 and GM6001, accumulates DCC on the neuronal surface and potentiates the outgrowth response to acute treatments with Netrin-1 (183). In the frog's visual system, ADAM-10 was identified to cleave surface ephrinA2, thus enabling release of the growth cone during its collapse (28). In the vertebrate spinal cord, temporal downregulation of Neuropilin-1 by ADAM-10 decreases sensitivity to Sema3A and allows proprioceptive neurons to reach the ventral side of the spinal cord (29).

In neurite outgrowth, other important targets of metalloproteinase activity are members of the immunoglobulin superfamily of cell adhesion molecules (IgSF-CAM). Cell adhesion molecules (CAM) facilitate axonal targeting through axon pathfinding and fasciculation (184,185). They undergo protein-protein interactions with identical (homophilic) or

different (heterophilic) adhesion proteins, providing a tracking force that progressively attaches the protruding growth cone to the substrate surface (186,187). On the neuronal cell surface (Cis), cell adhesion molecules dimerize, trimerize or oligomerize forming protein clusters that increase their adhesive properties and/or activate intracellular signaling pathways (188). Adhesion molecules do not possess any catalytic activity in their intracellular domain, and thus, depend on signaling-capable co-receptors for eliciting intracellular signaling cascades. For example, L1 interacts with integrins and activates a kinase Src, small GTPase Rac, PI3 kinase and extracellular-signal regulated kinase (Erk), thereby promoting neurite growth (189-191). Similarly, Neuronal cell-adhesion molecule (NCAM) promotes neurite outgrowth by recruiting the fibroblast growth factor receptor (FGFR) and activating the small GTPase Rho and kinases Fyn, Fak, and Erk (192,193).

Different adhesion molecules are targeted by metalloproteinases in order to change the adhesive properties of individual growth cones. Ectodomain shedding of CAMs releases adhesive fragments into the growth cone vicinity that could provide a growth permissive substrate, could interact with cognate surface receptor to regulate signaling cascades, or could reduce cell-surface adhesive interactions. For examples, in cerebellar granule cells, ADAM-8 was identified to process the cell adhesion molecule L1-like (CHL1), a shedding mechanism that promotes growth and survival *in vitro* (194). ADAM-10 and ADAM-17 process L1 to activate αvβ5-integrin receptors and promote neuronal cell adhesion and growth (195). Other members of the immunoglobulin superfamily (IgSF), such as NCAM (196-198) and TAG-1 (199-201), are metalloproteinase targets known to promote neurite growth. In primary cortical neurons, the metalloproteinase inhibitor GM6001 decreases NCAM ectodomain shedding and significantly increased NCAM-dependent neurite branching and growth (202). Most notably, among the IgSF-cell adhesion proteins is the IgLON subfamily. In Chapter 3 and Chapter 4, we will discuss the role of ectodomain shedding of IgLONs by members of the ADAM family in mature cortical neurons and embryonic sensory neurons.

1.4.1 IgLON cell adhesion proteins

IgLONs are characterized as the earliest and most abundant glycosylphosphatidyl-inositol (GPI)–anchored proteins expressed in the nervous system (203). They are members of the immunoglobulin (Ig) superfamily of cell adhesion molecules, composed of three Ig-like domains and a GPI-anchor moiety required for extracellular surface localization. Following their initial discovery in soluble fractions from crude membrane extracts pre-treated with phospholipase C, an enzyme that cleaves GPI-anchored proteins, four members of this family have been identified in rodents, chick, monkey and humans, including Neurotrimin (NTM or CEPU (chick ortholog)); Opioid-binding cell adhesion molecule (OBCAM); Limbic system associated membrane protein (LSAMP); and Neuronal growth regulator 1 (NEGR1, Neurotractin (chick ortholog) or Kilon (Rat ortholog)) that exhibit significant sequence homology (203-205). Lachesin, a cell adhesion molecule that is involved in the formation of the blood-brain barrier has been defined as the IgLON ortholog in *Drosophila melanogaster* (206). In addition a secreted IgLON isoform has been identified in chick (CEPU-se) (207).

Genetic studies have determined a strong correlation between IgLON family members and neurological disorders. For instance, LSAMP gene expression correlates with mood and anxiety disorders in humans (208). Other genetic studies have identified altered NEGR1 expression in autism, schizophrenia and dyslexia, suggesting that normal levels of NEGR1 expression are required for proper neuronal functioning (209-212). Finally, LSAMP and NEGR1 have been detected in the cerebrospinal fluid of psychiatric patients, raising the possibility that IgLONs might be targeted by proteases and soluble LSAMP and NEGR1 may be pathologically relevant (213,214).

1.4.2 IgLON expression in the nervous system

GPI-anchored cell adhesion proteins have previously been described to provide guidance to projecting neurons (215-217). In the nervous system, IgLON family members are distinguished by a complex spatial distribution in distinct neuronal populations. For instance, immunohistochemical analysis of developing rat brains determined that LSAMP

is expressed in cortical and subcortical populations of the limbic system and regulates neuronal projections at the earliest stages of development (204). *In situ* hybridization experiments from the rat brain revealed high expression of NTM in layers IV, V and VI of the cerebral cortex, hippocampus, thalamus, pontine nucleus, cerebellum, olfactory bulb, neural retina, dorsal root ganglia, spinal cord and basal ganglia (203). Contrary to LSAMP and NTM, mRNA expression studies determined a more restricted distribution for OBCAM, with highest expression in the cortical plate and hippocampus (218). Similar to OBCAM, the mRNA expression profile for NEGR1 revealed robust expression in the cerebral cortex and the dentate gyrus of the hippocampus (219). In rat brain homogenates a wider distribution for NEGR1 was identified, with high levels in the olfactory bulb, the cerebral cortex, the diencephalon, the hippocampus, the cerebellum and low expression in the medulla oblongata and spinal cord (219). Therefore, different patterns of expression for IgLON family members might provide an identity tag that differentiates neuronal subpopulations and guide their projections towards their correct signaling target.

Furthermore, the presence of IgLON family members gradually increases during late stages of development and acquires a restricted subcellular localization. OBCAM and NEGR1 increase their expression during postnatal development and are maintained until adulthood, as shown by western blotting from different stages of the cerebral cortex, diencephalon, hippocampus and cerebellum (219). Immune electron microscopy studies determined that NTM expression is also increased during postnatal development, peaking by the time of myelination (203). In light and electron microscopy studies, LSAMP is present in the developing rat brain in a restricted and transient manner on neuronal somata, dendrites, axons and even growth cones (204). During maturation of the nervous system, LSAMP expression becomes restricted to the neuronal somata and dendrites in regions of the limbic system (204).

In addition to neuronal expression, IgLON members are present in oligodendrocytes and Schwann cells, suggesting a potential role in myelination. A NTM knockdown in Schwann cells decreases proliferation and migration (220). Through a high-resolution

mass spectrometry-based proteomics, IgLONs were recently identified as negative regulators of myelination in specific fiber tracts in the CNS (221).

1.4.3 IgLON homophilic and heterophilic-complex interactions

Members of the immunoglobulin super family of cell adhesion proteins have the ability to form homophilic and heterophilic interactions (222,223). Similarly, IgLONs form adhesive complexes with members of the IgLON subfamily (224). Aggregation assays using IgLON coated polystyrene beads determined that IgLON family members form homodimer complexes. Binding assays using soluble recombinant IgLON proteins on Chinese Hamster Ovary (CHO) cell lines expressing single or different IgLON family members confirmed that IgLONs could form heterodimers on the cell surface. These experiment also determined that NTM might be both a homophilic and a heterophilic cell adhesion molecule, whereas LSAMP and OBCAM only mediate heterophilic interactions (224). In addition, CHO cells expressing IgLON family members adhere to corresponding IgLON-Fc family members immobilized onto nitrocellulose substrates (225). All together, IgLONs interact with members of the IgLON subfamily expressed on the cell surface or with IgLONs present on the peri-cellular microenvironment.

1.4.4 IgLONs in neurite outgrowth

The effect of IgLON family members in neurite outgrowth depends on the surface repertoire of IgLONs and IgLON-complex interactions. For instance, CHO cell lines co-expressing NTM and OBCAM, but not single NTM and OBCAM transfections, inhibit growth from cerebellar granule cells (224). CHO monolayers co-expressing NTM and OBCAM, or OBCAM and LSAMP repress growth in dissociated embryonic (E7-8) chick forebrain neurons, but not at embryonic day 6 (226). Removal of GPI-anchored protein by pre-treating embryonic (E8) chick DRG neurons with PI-PLC attenuates the inhibitory effect of CHO cells co-expressing NTM and OBCAM (226), suggesting that IgLONs expressed on the neuronal surface might be interacting with immobilized IgLON substrates to repress growth. Furthermore, soluble and immobilized NTM have the ability to increase neurite extension in embryonic (E16) rat hippocampal and embryonic DRG

neurons (227). Recently, soluble NEGR1 has been reported to retain the ability to promote dendritic growth and increase the complexity of dendritic arbor at mature stages of cortical neurons *in vitro* (228,229). However, other groups have reported no outgrowth defect in embryonic (E9-10) chick DRG neurons overexpressing surface NTM and seeded on immobilized NTM substrates (225). The neuronal population, the developmental stage and surface repertoire of IgLON complexes could provide an explanation for discrepancies in outgrowth assays reported by different groups.

It remains to be determined the mechanistic function of IgLON family members to promote growth. One possible model relies on the adhesive interactions between IgLONs present on the neuronal surface and peri-cellular IgLONs. This model was elegantly described in the subdomain-specific targeting of the habenula. In a paper by Schmidt et al, efferent projections from the lateral subnucleus of the habenula (LHB) express surface LSAMP, thus providing a growth-permissive substrate and directionality for dopaminergic afferents to the LHB (230). However, IgLONs are GPI-anchored proteins unable to transmit growth promoting signals, and thus, a signaling-capable co-receptor is indispensable for this mechanism. In another scenario, IgLON family members might interact with receptors present on the cell surface, forming a complex that represses neuronal extension. Immobilized IgLON substrates or treatment with soluble IgLONs could destabilize inhibitory surface IgLON-receptor complexes to promote growth.

THESIS RATIONALE AND OBJECTIVES

The vast accumulation of growth inhibitory molecules in the adult CNS prevents neurite outgrowth, synaptic connectivity and functional regeneration after injury. Proteolysis of extracellular proteins is an important mechanism that rapidly changes neuronal sensitivity to environmental cues and releases biological active and dominant negative fragments into the pericellular microenvironment. We hypothesized that proteolytic activity, by the action of membrane-anchored metalloproteinase, might release or destabilize surface inhibitory complexes and alter the cell activity and response to the environment. In the present thesis, I sought to characterize the function of membrane-anchored metalloproteinases in the process of neurite outgrowth and synapse development. In chapter 2, I evaluated the function of membrane-type metalloproteinases in the establishment of the brain connectivity. Metalloproteinase expression and activity has previously been reported to localize at the site of excitatory synaptic connections. However, the physiological role of metalloproteinase in excitatory synapse development remains unknown. Through in situ hybridization and genetic mutations, I identified MT3-MMP, a membrane-bound metalloproteinase, and NgR1, as a potential downstream MT3-MMP proteolytic target. Accordingly, surface NgR1 proteolysis and Eco-NgR1 fragments promote the formation of excitatory synapses. Therefore, MT3-MMP and NgR1 proteolysis might confer levels of plasticity important for higher cognitive processes and memory formation in the CNS (manuscript in preparation). In chapter 3 and 4, I characterized members of the metalloproteinase subfamily to be important for neuronal outgrowth in cortical and dorsal root ganglion neurons. In chapter 3, I identified the IgLON family of cell adhesion molecules, as novel metalloproteinase substrates processed from the cell surface to facilitate neuronal extension. In chapter 4, I reported IgLON family members to be expressed and shed from the surface of embryonic DRGs to promote growth (manuscript in preparation), supporting a conserved mechanism where IgLON proteolysis titrates growth by releasing an outgrowth inhibitory signal from the cell surface.

RESULTS

Preface

Synapses are the sites of communication between neurons. They are macromolecular junctions that facilitate the exchange of information within neuronal networks. Originally formed during the early stages of development, synapses maintain the ability to change their density, size and electrical conductance throughout development. In the present manuscript, we explore the role of metalloproteinases in the formation of excitatory synapses during cortical development. We characterize the spatiotemporal expression of membrane-type metalloproteinases in the mouse brain. We identify MT3-MMP as a metalloproteinase subtype predominantly expressed throughout development, and characterized its role in excitatory synapse formation.

CHAPTER 2

2. MT3-MMP promotes excitatory synapse formation by promoting ectodomain shedding of Nogo-66 receptor

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Abbreviated title: NgR1 shedding in excitatory synapse formation

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Manuscript in preparation

2.1 ABSTRACT

The connectivity of the mammalian brain depends on extracellular matrix molecules, synaptic cell adhesion proteins and growth factors that are all substrates of metalloproteinase-dependent proteolysis. In the present study, we evaluated the role of membrane-type metalloproteinases (MT-MMPs) in excitatory synaptogenesis. We find that MT3-MMP, a member of the metzincin metalloproteinase subfamily, is broadly expressed in the mouse cerebral cortex. In dissociated cultures of cortical neurons MT3-MMP loss-of-function interferes with excitatory synapse development. We find that Nogo-66 receptor (NgR1) is an MT3-MMP substrate in isolated synaptosome preparations and that NgR1 is required for MT3-MMP-dependent synapse formation. Further, introduction of a constitutively shed NgR1 protein (CE-NgR1) or treatment with soluble Ecto-NgR1 (1-358) fragments are sufficient to accelerate excitatory synapse formation. Together, our findings support a role for MT3-MMP-dependent shedding of NgR1 in regulating excitatory synapse development in cortical neurons.

SIGNIFICANCE

In this study we identify MT3-MMP, membrane-bound zinc protease, to be necessary for the development of excitatory synapses in cortical neurons. We identify NgR1 as a downstream target of MT3-MMP proteolytic activity. Furthermore, processing of surface Nogo-66 receptor (NgR1) by MT3-MMP generates a soluble Ecto-NgR1 (1-358) fragment that accelerates the formation of excitatory synapses. Therefore, MT3-MMP activity and NgR1 shed fragments could stimulate circuitry remodeling in the adult brain and enhance functional connectivity after brain injury.

2.2 INTRODUCTION

The functionality of the mammalian central nervous system depends on the formation of a precise network of synaptic contacts that actively change their strength, morphology and density throughout life (231,232). Extracellular matrix and synaptic components are subject to proteolysis, a mechanism that alters protein function and allows circuit remodelling (233,234). A better understanding of the molecular and cellular mechanisms that dictate how synapse formation is regulated could lead to therapies to promote functional synaptic recovery after injury, treat neurological disorders and increase cognitive function.

In the nervous system, members of the metalloproteinase subfamily, Matrix metalloproteinases and Adamalysins, mediate proteolytic processing of membrane-anchored precursors and the subsequent release of biologically active, or dominant negative fragments. Membrane-type metalloproteinases (MT-MMPs) are members of the zinc endopeptidase subfamily of matrix metalloproteinases (MMPs). They are extracellular membrane-bound proteases that release surface proteins through a mechanism termed ectodomain shedding. Metalloproteinase activity is controlled through removal of a repressive pro-peptide domain and the expression of endogenous inhibitors called Tissue Inhibitor of Metalloproteinases (TIMPs). At the subcellular level, MT-MMPs are present in dendritic spines and proteolytic activity is largely absent from GABAergic synapses, suggesting a potential role in excitatory synaptogenesis. Ectodomain shedding targets inhibitory or permissive membrane-anchored substrates that alter synapse rearrangements (139-141,235-238). Several factors that restrict synapse formation are present in myelin and glial cells. Nogo-A, myelin associated glycoprotein (MAG), oligodendrocyte glycoprotein (OMgp) and chondroitin sulphate proteoglycans (CSPGs) converge onto signaling pathways that restrain axon regeneration and neuronal connectivity (149,152,239,240). The Reticulon-4 receptor (Nogo-66 receptor or NgR1) is as an endogenous negative regulator of synaptic plasticity. NgR1 signaling restricts experience dependent plasticity in the visual cortex, induces long-term depression (LTD) and blocks FGF2-mediated LTP in hippocampal slice preparations (151,171-173). Loss

of NgR1 expression in hippocampal neurons leads to an increased number of excitatory synapses and impedes dendritic spine maturation. We previously reported a decrease in neuronal sensitivity to myelin-associated inhibitors (MAI) associated with NgR1 surface proteolysis (26). A deficiency in metalloproteinase activity could impair excitatory synapse formation by preserving the integrity of inhibitory NgR1 at the synapse.

In the present manuscript, we evaluated the role of MT-MMPs in the development of excitatory synapses. By *in situ* hybridization, we identify MT3-MMP, a member of the membrane-bound MMP subfamily, in embryonic and postnatal stages of the cerebral cortex. In dissociated cultures, pan-metalloproteinase inhibitors and MT3-MMP loss-of-function reduce excitatory synapse formation and expression of structural synaptic components. We find that Nogo-66 receptor (NgR1) is an MT3-MMP substrate in isolated synaptosome preparations and that NgR1 is required for MT3-MMP-dependent synapse formation. MT3-MMP activity generates soluble Ecto-NgR1 (1-358) and CT-NgR1 fragments that gradually increase in the postnatal cortex and correlate with periods of excitatory synapse development. Expression of a constitutively shed NgR1 construct, or treatment with soluble Ecto-NgR1 (1-358) fragments accelerates excitatory synaptogenesis. Our results demonstrate that MT3-MMP plays a role in the formation of excitatory synapses through regulated NgR1 cleavage.

2.3 EXPERIMENTAL PROCEDURES

2.3.1 Animals -

Timed pregnant (embryonic day 18-19) female Sprague Dawley and CD1 mice were purchased from Charles River Laboratories (Senneville, QC). C57/BL6 NgR1 null mice and cortices from C57/BL6 MT3-MMP null mice were kindly provided by Dr. Mark Tessier-Lavigne and Dr. Kenn Holmbeck, respectively. Brain areas from WT CD1 mice were isolated from embryonic (E18) and postnatal day 5 to 20 (P10-60). E18, P10 and P60 mice brains were embedded in Tissue-Tek O.C.T. compound (Miles, Elkhart, IN) and flash frozen with 2-methylbutane. All animal care and use was in accordance with the McGill University guidelines and approved by the University Animal Care and Use Committee.

2.3.2 Antibodies and reagents -

For immunofluorescence, the following antibodies were used: mouse anti-PSD95 (1,1000, Millipore, Ectobicoke, ON), rabbit anti-Synapsin-1 (1:1,000, Millipore), mouse anti-PSD95 (1:200, NeuroMAB, UC Davis, CA), Guiney pig anti-VGLUT-1 (1:400, Synaptic Systems, Burlington, NC) and mouse anti-Myc (1:500, Sigma-Aldrich, St. Louis, MO). Alexa-fluor secondary antibodies were purchased from Invitrogen Life Technologies (1:500, Burlington, ON). For western blot analysis, the following antibodies were used: goat anti-NgR1 (1:200, R&D systems, Minneapolis, MN); rabbit anti-NgR1 (Dr. Roman Giger, University of Michigan); mouse anti-N-Cadherin (1:10000, Takeichi, M. and Matsunami, H., Developmental Studies Hybridoma Bank); mouse anti-MMP16/MT3 (1:200, Millipore); goat anti-Lingo-1 (1:1,000, R&D); mouse anti-Synaptophysin (SynPhy, 1:10000, Sigma-Aldrich); mouse anti-PSD95 (1:100,000, NeuroMAB); mouse anti-Myc (1:1000, Sigma-Aldrich); rabbit anti-GLUR1 (1:1000, Millipore); mouse anti-VGLUT1 (Synaptic systems, 1:1000); mouse anti-Flag (1:5000, Sigma-Aldrich) and mouse anti-GAPDH (1:200, Santa Cruz, Dallas, Texas). HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch (West Grove, PA).

2.3.3 Primary cell culture -

Mouse and rat cortical neuron dissections were described previously (241). Briefly, cortical neurons were prepared from embryonic day 17-19. Cerebral cortices were dissected; 0.25% trypsin-EDTA digested; mechanically dissociated and cultured for 14 days on 100μg/mL Poly-L-Lysine (PLL, Sigma-Aldrich) coated dishes. Neurons were grown in Neurobasal media (Gibco, Burlington, ON) supplemented with 2% B27 (Gibco), 1% N2 (Gibco), 1% penicillin/streptomycin (Gibco) and 1% L-glutamine (Gibco). Neuronal culture media were refreshed every 4 days. For immunocytochemical experiments, 8.750 x 10^3 cells per cm^2 were plated on coverslips. For biochemical experiments, 50 x 10^3 cells per cm^2 were plated on plastic plates and analyzed 14 days *in vitro* (DIV).

2.3.4 Plasmids and cloning -

Constructs and preparations for soluble human MT1-MMPs, lentivirus rat shRNAmir (MT3-MMP and MT5-MMP), WT-NgR1 and constitutively cleaved (CE)-NgR1 were described previously (26,242). The following primers were used to generate mouse shRNAmir for MT3-MMP lentivirus: top: 5'TGCTGTTATCAA GTCATGAGGGTAACGTTTTGGCCACTGACTGACGTTACCCTTGACTTGATAA'3 and bottom: 5'CCTGTTATCAAGTCAAGGGTAACGTCAGTCAGTGGCCAAAACGTTACCCTC ATGACTTGATAAC'3.

The following primers were used to validate MT3-MMP knockdown in mouse cortical neurons: Mouse MT3-FW: 5'CAGCTCTGGAAGAAGGTTGG'3, and Mouse MT3-RV: 5'GAGCTGCCT GTCTGGTC'3.

To generate Myc-tagged CT-NgR1 construct, the CT-fragment of human NgR1 was subcloned into a Psectag2B vector by PCR. Then, the IgK-signal sequence and the CT-NgR1 were subcloned into the pRRL-sinPPT vector by PCR. The following primers were used to generate a Myc-tagged CT-NgR1 fragment: Forward: 5'GAA

GGATCCGAACAAAAACTCATCTCAGAAGAGGATCTGCGCGTGCCGCCCGGT‘3 , Reverse: 5’GAACTCGAGTCAGCAGGGCCCAAGCAC ‘3.

The following primers were used to subclone the IgK-signal sequence and CT-NgR1 into the pRRL-sinPPT vector: Forward: 5’GGAGGCCGGCCATGGAGACAGACACACTCCTG‘3 and reverse: 5’GAACTCGAGCTAGCTACTAGCTAGTCGAGATCTGAGTCCGG‘3.

To generate soluble 358-Fc NgR1, the ectodomain of rat NgR1 up to amino acid 358 was subcloned into a Psectag2B vector by PCR. Then, the IgK-signal sequence and the ectodomain of rat NgR1 was fused to a human Fc segment by subcloning into the PFUSE vector (Invitrogen) at the C-terminal end. The following primers were use to subclone 358-Fc NgR1 into the Psectag2b vector: 358-Fc NgR1 Forward: 5’GCTCAAGCTTCCTGGTGCCTGTGTGTG C‘3 and 358-Fc NgR1 Reverse: 5’GCTCGGATCCTCATTTACCCGGAGACAGG‘3.

2.3.5 Recombinant protein purification -

Generation of Fc tagged recombinant proteins was extensively described previously (241). Briefly, HEK293T cells were transfected with calcium phosphate, incubated in OptiMEM (GIBCO) media and purified by affinity chromatography with protein A Sepharose beads. Protein concentration was estimated by protein assay and visualized by Coomassie-brilliant blue stain next to a BSA curve.

2.3.6 Immunochemistry -

For shedding experiments in dissociated neuronal cultures, the culture medium was replaced with Neurobasal media and supplemented with pan-metalloproteinase inhibitors, Batimastat (BB-94, 5μM, Tocris Bioscience, Bristol, United Kingdom); Ilomastat (GM6001, 20μM, Tocris); inactive GM6001 analogue (20μM, Tocris) and phospholipase C (PI-PLC, 1U/mL, Invitrogen). After 4-6hrs, supernatants were collected and briefly centrifuged to dispose of residual cell debris. Supernatants were concentrated using column centrifuge filters (10K, Amicon Ultra-4; Millipore), resolved on 10% SDS-PAGE gels and analyzed by western blot.

2.3.7 Membrane extracts and synaptosome preparations -

Membrane extract preparations were performed in WT and MT3-MMP null mice. Cerebral cortices were dissected and homogenized in 1mM $NaHCO_3$, 0.2mM $CaCl_2$ and 0.2mM $MgCl_2$ (Homogenization buffer, pH 7.9). All steps were performed at 4^{o}C. Debris was removed by centrifugation at 600g for 15min. The supernatant was centrifuged at 25,000g for 45min and the pellet was lysed in RIPA lysis buffer.

Synaptosomal preparations were performed as previously described (150). Briefly, the cortex was dissected and homogenized in 0.32M Sucrose, 1mM EDTA, 5mM Tris, pH 7.4. Buffer was supplemented with Complete-EDTA free protease inhibitor mix (Roche Diagnostics, Laval, Canada). All steps were performed at 4^{o}C. Homogenate was centrifuged at 1,000g for 15min. The supernatant (S1 fraction) was overlayed on a Percoll discontinuous gradient, which consisted of the following layers (from top to bottom): 3%, 10%, 15% and 20% Percoll. Synaptosomes were collected at the 10/15% and 15/20% interfaces and washed twice in homogenization buffer. For shedding experiments, pellet was resuspended in Neurobasal media and incubated with pan-MMP inhibitors. Samples were centrifuged at 20,000g for 15min, the pellet was lysed in RIPA buffer and the supernatant was concentrated with centrifugal filter units (Millipore).

2.3.8 Riboprobe Synthesis and In Situ Hybridization –

MT-MMP probes were synthesized from mouse cDNA clones of the full coding sequence (Open Biosystems, Huntsville, AL); MT1-MMP (MMM1013-9498156), MT2-MMP (MMM1013-98477873), MT3-MMP (5292478) and MT5-MMP (5687204). Riboprobes were synthesized as described previously (243). Briefly, digoxigenin (DIG)-labeled cRNA riboprobes with sense or antisense orientation were synthesized by *in vitro* transcription using DIG labelling mix (Roche, Mannheim, Germany) followed by partial hydrolysis with 10mM DTT, 200mM $NaHCO_3/Na_2CO_3$, and pH 11. Probed were stored in diethylpyrocarbonate (DEPC)-treated water at -80^{o}C. Fresh frozen brains were cryosectioned at 20mm at -17^{o}C and thaw mounted on microscope slides (Fisher Scientific, Hampton, NH). Sections were fixed in 4% paraformaldehyde/0.1M phosphate-

buffered isotonic saline (pH 7.4) then rinsed in PBS and DEPC-treated water. Sections were incubated for 10min with 0.25% acetic anhydride in 1% triethanolamine, washed twice in PBS, rinsed in 1x standard saline citrate (SSC), and prehybridized for 3hrs in 50% formamide, 5x Denhardt's solution, 5x SSC, 200 mg/mL baker's yeast tRNA. Sections were hybridized overnight at 60^{o}C with 100ng/mL DIG-labeled riboprobe. Sections were washed for in 5x SSC, followed by in 2x SSC then in 50% formamide containing 0.2x SSC and finally in 0.2x SSC. Sections were then washed in Tris-buffered saline and blocked for 1hr in a 1% solution of blocking reagent (Roche, Mannheim, Germany). Sections were incubated with anti-DIG Fab fragments conjugated to alkaline phosphatase (1:3,000) for 3hrs followed by washes in TBS. The color reaction was performed overnight at room temperature. Sections were rinsed extensively in PBS and coverslipped with Mowiol 4-88 (Calbiochem, San Diego, CA). Each *in situ* hybridization experiment was repeated a minimum of three times to eliminate any variability in expression between animals.

2.3.9 Synaptic puncta analysis -

For experiments with soluble recombinant treatments, 13DIV cortical neurons were treated with 5µg/mL of 358-Fc NgR1 and Fc control construct every 24hrs for 2days. For expression of MT3-MMP and MT5-MMP shRNAmir, cortical neurons 3DIV were infected with designated lentivirus at a multiplicity of infection (MOI) 10 for 4hrs in Neurobasal media. For overexpression of WT-NgR1, CE-NgR1 and CT-NgR1, cortical neurons were infected at an MOI of 0.3 or 3. At 14-15 DIV, cortical neurons were fixed in 4% PFA and 20% sucrose in PBS for 30min. Neurons were blocked in 5% BSA and 0.2% Triton X-100 in PBS solution for 1hr and stained for PSD95 (Millipore), VGLUT1 (Synaptic Systems) or Synapsin-1 (Millipore).

On the basis of previously described methods (244), all image acquisition; analysis and quantification were performed in a blinded fashion. Cell culture images were acquired on a confocal microscope, Zeiss 710 and Leica SP8, using a 63x, 1.4 NA oil objective. Images were acquired and prepared for presentation using Adobe Photoshop.

For quantification, cells were stained simultaneously and imaged with identical settings. Synaptic puncta were delineated by the perimeter of the transduced designated neuron.

Three dendrites per neuron were randomly selected and the number of synaptic puncta (Synapsin-1 or VGLUT1, PSD95 and colocalized Synapsin-1 or VGLUT1 /PSD95) per 20μm of dendrite length was measured using the Puncta Analyzer plugin from the ImageJ software. A total of 30-45 cells per condition were analyzed in at least 3 independent experiments.

2.3.10 In utero electroporation -

Pregnant mice were deeply anaesthetized with isoflurane (4-5% for initial anesthesia, ~2-3% for maintenance). Midline incision was performed through the skin and the abdominal wall to expose the uterine horns. 2μl of 2.7μg/μl plasmid mixture (18 μg/μL pCAG_MT3-GFP/control-GFP construct; 0.9 μg/μl pCAG_Lck-mCherry) were injected into the lateral ventricles of E13-14 embryos using a glass micropipette. Immediately after injection (as opposed to injecting all embryos and then pulsing them) 5 square pulses of current were applied (39-40 V; 50 ms followed by 950 ms intervals) using an electroporator (Harvard Apparatus) and 3-pronged tweezer-electrodes. Two electrodes connected to the negative pole were placed on the side of the head with a single positive electrode on the top, above the ventricles. The uterine horns were subsequently replaced in the abdominal cavity. The abdominal cavity was filled with warm PBS, and silk sutures were used to close the overlying abdominal muscle and skin. Pregnant mice received multiple subcutaneous injections of the non-steroidal anti-inflammatory analgesic carprofen (10%): 50 μl on the day of surgery, and 30 μL every 24 hours for the next 48 or 72 hours or until birth.

2.3.11 Spine counts -

Mice were perfused at P24. Brains were collected, fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4 ^{o}C, and immersed consequently in 30% sucrose in PBS at 4 ^{o}C. The brains were then embedded in O.C.T compound, and stored at -20 ^{o}C until analysis. Frozen brains were cut into 40μm thick coronal sections by cryostat. Dendritic spines from the cerebral cortex were visualized using a spinning-disc microscope at 100x magnification. Neurons from the layer VI cortex expressing both GFP and Lck-mCherry

were randomly selected. 3D spine counts were performed using the Imaris software. 10 images from brain section were compiled and analyzed.

2.3.12 Statistics -

Analyses were performed using Microsoft Excel and GraphPad Prism5. Statistical comparisons were made using 1-way and 2-way ANOVA, followed by Bonferroni post hoc's test and unpaired two-tails T-test with Welch's correction, as indicated in figure legends. All data are reported as the mean + standard error of the mean (SEM) from at least three independent experiments. Statistical significance was defined as: * $P< 0.5$, * * $P<0.01$ and *** $P<0.001$.

2.4 RESULTS

2.4.1 MT3-MMP and MT5-MMP are expressed during periods of synaptogenesis -

To evaluate the role of MT-MMPs at synapses, we examined the spatiotemporal expression of transmembrane MT-MMPs in the mouse cerebrum by in situ hybridization (Fig. 1a). The critical period of synaptogenesis occurs during the first month of murine life (P5-35), peaking at the second postnatal week (245). Prior to synapse formation at E18 (246), robust expression of MT3-MMP and MT5-MMP is detected in the cortex and hippocampus, while MT2-MMP and MT1-MMP are weak or absent (Fig. 1a). At P10, when synapses begin to abruptly increase in density, MT3-MMP and MT5-MMP are expressed in layers II-IV and layer VI of the cerebral cortex and in areas CA1/CA3 and dentate gyrus (DG) of the hippocampus, suggesting a role in neuronal connectivity. After synaptogenesis, neuronal networks undergo continuous remodeling of synaptic connections, possibly through focal expression and activity of metalloproteinases (231,247). Interestingly at P60, MT3-MMP and MT5-MMP are highly present in regions of synaptic plasticity. MT5-MMP is expressed in the granular layer of the cerebellum and the CA1/CA3 and DG of the hippocampus, while MT3-MMP is specifically enriched in the DG. Similarly, MT3-MMP and MT5-MMP are expressed in most cortical layers, except for layer I and layer V (Fig. 1b). Together, we conclude that MT3-MMP and MT5-MMP expressions correlate with the process of synaptogenesis and are maintained in regions that exhibit high levels of synaptic plasticity.

2.4.2 MT3-MMP loss of function decreases excitatory synapse formation-

To evaluate the contribution of MT-MMP activity in synaptogenesis, we examined the number of excitatory synapses in mature cortical neurons treated with the pan-metalloproteinase inhibitor, BB-94. We focused on the cerebral cortex due to the expression profile of both MT3-MMP and MT5-MMP mRNAs in the mouse cerebrum (Fig. 1). PSD95 and Syn-1 puncta were used to delineate excitatory synapses from mature cortical neurons *in vitro*. At 14DIV, the presence of BB-94 significantly decreased the

levels of Syn-1 by ≈40%, PSD95 by ≈30% and co-localized puncta by ≈49% signifying that metalloproteinases promote excitatory synaptogenesis (Fig. 2a, b). We then examined the number of excitatory synapses in neurons expressing MT3-MMP and MT5-MMP shRNA with a microRNA stem (shRNAmir) (Fig. 2c, d). Knockdown of MT3-MMP, but not MT5-MMP, phenocopied the repressive effect of BB-94 on synapse formation, significantly decreasing the levels of Syn-1 by ≈30%, PSD95 by ≈50% and co-localized Syn-11/PSD95 puncta by ≈50% (Fig. 2e, f). This supports a role for MT3-MMP in promoting excitatory synaptic development.

2.4.3 MT3-MMP loss-of-function inhibits synaptogenesis in vivo -

To examine the role of MT3-MMP in synaptogenesis *in vivo,* we analyzed the expression of synaptic markers in brain membrane extracts from MT3-MMP null mice (Fig. 3a-e). The levels of Synaptophysin (SynPhy) and the excitatory pre-synaptic vesicle-bound protein, VGLUT-1, were significantly reduced in the absence of MT3-MMP suggesting a pro-synaptogenic role for MT3-MMP (Fig. 3c, d). We then performed experiments to knockdown MT3-MMP expression selectively in neurons *in vivo* to explore the physiologic role of neuronal MT3-MMP in excitatory synaptogenesis. MT3-MMP shRNAmir was introduced in the mouse brain at E13 by in utero electroporation and the number of dendritic spines was quantified in layer VI of the cerebral cortex (Fig. 3h, j). At P24, the loss of MT3-MMP activity significantly decreased the density of dendritic spines (Fig. 3i, j), confirming a role for MT3-MMP in synaptic development.

2.4.4 MT3-MMP mediates synaptic NgR1 shedding from cortical neurons -

NgR1 is an endogenous repressor of synaptic plasticity. Overexpression of MT1-MMP, MT3-MMP and MT5-MMP are able to cleave the ectodomain of NgR1 relieving myelin-dependent outgrowth inhibition (26). In loss-of-function experiments in healthy cortical neurons, it has been shown that MT3-MMP mediates endogenous NgR1 shedding (26). We therefore asked if NgR1 is a synaptic MT3-MMP substrate that is shed to relieve a brake on excitatory synapse formation. We examined the expression of NgR1 and MT3-

MMP in synaptosomes. NgR1 shedding generates two fragments, a soluble shed 50KDa fragment and a 30KDa carboxy-terminal (CT) fragment that remains anchored to the cell surface by the GPI moiety (Fig. 4a). We identified a commercial antibody that detects both FL-NgR1 and CT-NgR1 in crude membrane extracts (Fig. 4b). Both NgR1 bands were absent in lysates from NgR1 knockout mice. By treating cortical neurons with recombinant MT1-MMP to cleave NgR1 and performing cell surface biotinylation, we demonstrated an increase in CT-NgR1 levels at the expense of FL-NgR1 (Fig. 4c). Furthermore, PI-PLC treatment to remove GPI-anchored proteins including NgR1 resulted in a loss of FL-NgR1 and CT-NgR1 from cell surface biotinylated fractions (Fig. 4c). Using this antibody we assessed expression of NgR1 and MT3-MMP in synaptosomes isolated from the mature cerebral cortex and separated into extra-synaptic, pre-synaptic and post-synaptic fractions (Fig. 4d). Consistent with previous reports FL-NgR1 was present in all synaptic subdomains, with highest expression in extra-synaptic and post-synaptic subfractions (150) (Fig. 4d). The CT-NgR1 fragment is strongly enriched in the post-synaptic subfraction suggesting that it is cleaved on the post-synaptic membrane. MT3-MMP is present in all synaptosome subfractions and is enriched in extra-synaptic and post-synaptic compartments. To directly assess NgR1 shedding, we probed conditioned media from isolated synaptosomes. A 50KDa NgR1 fragment corresponding to the shed NgR1 Ecto domain (1-358) was detected in the conditioned media and treatment of synaptosomes with pan-metalloproteinase inhibitors BB-94 or GM6001 blocked the release of the NgR1 ectodomain fragment (Fig. 4e). The NgR1 shedding profile was similar to that of N-Cadherin, a previously identified metalloproteinase substrate (Fig. 4e). To test if MT3-MMP mediates synaptic NgR1 cleavage, we performed shedding experiments from dissociated 14DIV cortical neurons expressing MT3-MMP shRNAmir and assessed NgR1 ectodomain levels in the conditioned media (Fig. 4f, g). Knockdown of MT3-MMP expression, but not MT5-MMP as a control, significantly decreased the levels of NgR1 shed fragment by ≈70% (Fig. 4f). Finally, we examined NgR1 shedding in cortices from an MT3-MMP deficient mouse. The CT/FL-NgR1 ratio, but not N-Cadherin as a control, significantly decreased by threefold in the absence of MT3-MMP activity (Fig. 4h, i). Together, we conclude that MT3-MMP is responsible for synaptic NgR1 shedding from cortical neurons and that

NgR1 is primarily cleaved on the post-synaptic membrane releasing a soluble fragment into the synaptic cleft.

2.4.5 Ecto-NgR1 (1-358) fragment is sufficient to promote excitatory synapses -

Previously, we reported that MT-MMPs decrease neuronal sensitivity to soluble MAI by diminishing NgR1 surface levels (26). Furthermore, NgR1 shed fragment retains the ability to bind Nogo-66, suggesting a dominant-negative function for NgR1 cleavage fragments (25). To characterize the mechanistic function of NgR1 shedding in excitatory synapse development, we exposed cortical neurons to the recombinant shed ecto-domain of NgR1 Ecto-NgR1 (1-358). Ecto-NgR1 (1-358) increases Syn-1 by ≈54%, PSD95 by ≈60% and Syn-1/PSD95 co-localized puncta by ≈73%, demonstrating that the soluble NgR1 cleaved fragment can enhance excitatory synapse formation (Fig. 5b, c). The 30KDa carboxy-terminal stub of NgR1 that is retained on the cell membrane (CT-NgR1) did not affect the formation of excitatory synapses (Fig. 5d-f).

To test if NgR1 shedding regulates synapse formation in a more physiological context, we infected neurons with a cleavage-enhanced NgR1 (26). Synaptic counts were performed in neurons expressing CE-NgR1 (Fig. 5g-j). High levels of CE-NgR1 expression were sufficient to mediate strong NgR1 cleavage in the cultures (Fig. 5g) and this was accompanied by a significant increase in Syn-1 puncta (≈62%), PSD95 puncta (≈59%) and co-localized puncta (≈71%) (Fig. 5h). Furthermore, the presence of BB-94 decreased the number of excitatory synapses in CE-NgR1 transduced neurons, implicating metalloproteinase activity in excitatory synapse potentiation by CE-NgR1 (Fig. 5i, j). All together, MT3-MMP promotes excitatory synapses through regulated surface NgR1 proteolysis.

2.4.6 MT3-MMP is necessary for excitatory synapses in the presence of NgR1 -

To test if the pro-synaptogenic effects of MT3-MMP can be attributed to its effect on NgR1 shedding, we suppressed MT3-MMP expression in neurons from NgR1 null mice

and quantified the number of excitatory synapses (Fig. 6a, b). In an NgR1-null background, loss of MT3-MMP activity failed to inhibit excitatory synaptogenesis (Fig. 6b). Together this demonstrates that MT3-MMP activity is necessary for the development of excitatory synapses and that this effect is dependent on the expression of NgR1 (Fig. 6c).

2.5 DISCUSSION

Several lines of evidence have suggested a role for metalloproteinases in structural remodelling of synaptic networks. Metalloproteinase expression and activity is observed in close opposition to molecular markers of excitatory post-synaptic scaffolding and pre-synaptic vesicle proteins (130). Furthermore, many constituents of synaptic connections are targeted by metalloproteinases, implying their importance in synaptic circuit remodeling. In the present study, we evaluated the role of membrane-type metalloproteinases in excitatory synapse development. Through *in situ* hybridization, we identified two members of the MT-MMP subfamily, MT3-MMP and MT5-MMP, abundantly expressed in the developing and mature cerebral cortex (Fig. 1). MT5-MMP has previously been reported to localize to synapses through interactions with proteins containing PDZ domains and the glutamate receptor interacting protein (GRIP), suggesting a function for MT-MMPs in synaptogenesis (65). Interestingly, loss of MT3-MMP activity, but not MT5-MMP, restricted the number of excitatory synapses in dissociated cortical neurons and reduced the expression of synaptic proteins in crude membrane extracts (Fig. 2, 3). Similar to MMP9, a well-characterized metalloproteinase important for synaptic physiology and plasticity (65), the loss of MT3-MMP expression decreased the density of dendritic spines *in vivo* (Fig. 3), implying a new function for MT3-MMP in excitatory synapse development.

Previously, we reported a role for metalloproteinases in the processing of surface NgR1 (26). NgR1 is an endogenous repressor of synaptic plasticity that gradually increases in expression in the neonatal brain, inhibits the formation of excitatory synapses and the turnover of dendritic spines (150,151,172). MT3-MMP might have a pro-synaptic effect by targeting synaptic NgR1 proteins. In here, we identified MT3-MMP as the major sheddase for synaptic NgR1 at baseline levels in cortical neurons. MT3-MMP is present in synaptosomes and co-localizes with NgR1 cleave fragment (Fig. 4). Loss of MT3-MMP activity decreases NgR1 shedding in dissociated cortical neurons and in crude membrane extracts from the cerebral cortex (Fig. 2-4). Furthermore, the absence of MT3-MMP activity inhibits excitatory synapse formation only in the presence of NgR1 (Fig.

6), implying NgR1 as the downstream effector of MT3-MMP-dependent proteolysis important for excitatory synapse development. Accordingly, ectopic expression of a constitutively cleaved NgR1 (CE-NgR1) construct and treatment with Ecto-NgR1 (1-358) are sufficient to accelerate excitatory synaptogenesis (Fig. 5). Therefore, we propose that NgR1 shed fragment might be acting as a dominant negative entity to attenuate inhibitory cues in the synaptic environment and promote excitatory synaptogenesis.

NgR1 has recently been described as a molecular-break that titrates synaptic plasticity and is responsible for the repressive adult-like state in the brain connectivity (150,151,172). Furthermore, NgR1 restricts surface trafficking of AMPA receptors in the barrel cortex (174) illustrating a potential role for NgR1 in limiting the strength of synaptic communication.. Interestingly, NgR1 shedding is present during postnatal development and in the mature cerebral cortex (unpublished data), implying its importance in ongoing cognitive processes. Surface NgR1 expression might be important for the stability of neuronal networks, while NgR1 shedding might provide a permissive microenvironment to facilitate changes in synaptic strength, morphology and density that continue to appear throughout life (231,232). It remains to be determined whether synaptic activity triggers MT3-MMP expression and NgR1 shedding. Glutamatergic receptors, intracellular calcium, as well as Ca^{+2}/calmodulin-dependent protein kinase II (CAMKII) activity have been reported to promote trafficking and activation of metalloproteinases (139-141). In addition, metalloproteinase expression is upregulated in response to sensory deprivation and damage to the brain circuitry. ADAM-10 and MT5-MMP increase synaptic expression in response to traumatic brain injury (248), while rapid changes in ECM composition following monocular deprivation are inhibited in MMP9 deficient mice (249). Therefore, focal expression and activity of MT3-MMP might increase NgR1 proteolysis to promote synaptic plasticity.

Several groups have targeted the NgR1 signalling pathway as a potential strategy to promote synaptic plasticity. Functional blocking antibodies against Nogo-A and NgR1 promote spine formation and long-term potentiation in organotypic slice cultures from the cerebral cortex (152). Recently, delivery of (1-310) Ecto-NgR1 fragment promotes

erasure of fear memories by increasing plasticity of inhibitory synaptic connections (250). Furthermore, a soluble ecto-domain fragment of paired-immunoglobulin-like receptor B (PirB) increases visual acuity and spine density in mice following long-term monocular deprivation (251). Here, we report that NgR1 proteolysis is an endogenous mechanism necessary for excitatory synapse development that promotes excitatory synapse formation. MT3-MMP activity or Ecto-NgR1 (1-358) fragments could enhance circuitry remodelling during development, as well as during cognitive processes in the adult brain.

2.6 FIGURE LEGENDS

2.6.1 Figure 1: MT3-MMP and MT5-MMP are expressed during periods of synaptogenesis

A. In situ hybridization experiment from coronal section of embryonic (E18) and early postnatal (P10) mouse cerebrum with antisense cRNA probes for members of the transmembrane membrane-type metalloproteinase (MT-MMP) subfamily (MT1-MMP, MT2-MMP, MT3-MMP and MT5-MMP). B. Sagittal sections from mouse cerebrum at postnatal day 60, probed for MT3-MMP and MT5-MMP expression. Mouse cerebral areas form postnatal day 60, hippocampus and cerebral cortex, probed for MT3-MMP and MT5-MMP mRNA expression. (H) Hippocampus, (c) cortex and (DG) dentate gyrus. The expression of membrane-type metalloproteinases was assessed in 3 independent brains from developmental stages (E18, P10 and P60). The ability of MT-MMP probes was validated by comparison to sense control probes. Scale bar, 1mm

2.6.2 Figure 2: MT3-MMP loss-of-function decreases excitatory synapse formation

A. 14DIV cortical neurons infected with a control lentivirus and treated with DMSO or pan-metalloproteinase inhibitor, BB-94, for 7days. B. Synaptic counts from DMSO and BB-94 treated cortical neurons stained for Synapsin-1 (Syn-1) and PSD95. C. RT-PCR from rat cortical neurons 14DIV infected with a lentivirus encoding shRNAmir-MT3-MMP, shRNAmir-MT5-MMP or a control empty vector. GAPDH was used as a loading control. D. A densitometry analysis for MT3-MMP and MT5-MMP mRNA expression in cortical neurons infected with shRNAmir MT-MMPs relative to GAPDH. Densitometry analysis was normalized to control empty vector. E. Cortical neurons 14DIV infected with a lentivirus encoding shRNAmir-MT3-MMP, shRNAmir-MT5-MMP or a control empty vector. F. Synaptic counts from MT3-MMP and MT5-MMP knockdown experiments in cortical neurons stained for Synapsin-1 (Syn-1) and PSD95. Data are mean + SEM, * $P<0.05$, ** $P<0.01$ by Bonferroni Post-hoc's test. Scale bar, 12μm and 4μm.

2.6.3 Figure 3: MT3-MMP loss-of-function inhibits synaptogenesis in vivo

A-E. Membrane extracts and quantification from postnatal stage P2 and P33 from WT and MT3-MMP knockout cerebral cortices. Membrane extracts were probed for N-Cadherin (N-Cad), Synaptophysin (SynPhy), VGLUT-1 and GLUR1. F-G. RT-PCR and densitometry analysis from mouse cortical neurons infected with control empty vector or shRNAmir-MT3-MMP. H. Coronal sections of mouse brain in utero co-electroporated with control GFP or MT3-MMP shRNAmir and Lck-mCherry. I. Dendritic spines in layer VI of the cerebral cortex in utero co-electroporated with control GFP or MT3-MMP shRNAmir and Lck-mCherry. J. Number of dendritic spines in layer VI of the cerebral cortex, expressing MT3-MMP shRNAmir or GFP control by in utero electroporation. 100 dendrites from 3-4 independent brains. Scale bar, 500μm and 5μm.

2.6.4 Figure 4: MT3-MMP mediates synaptic NgR1 shedding from cortical neurons

A. Schematic representation of soluble Ecto-NgR1 (1-358) and CT-NgR1 (359-410) fragments generated by surface NgR1 proteolysis. B. Crude membrane extract from WT and NgR1-KO mice probed with commercially available mouse anti-NgR1 polyclonal antibody. C. Cell surface biotinylation of mouse dissociated cortical neurons treated with 0.75μm rec-MT1-MMP and PI-PLC, probed with commercially available mouse anti-NgR1 polyclonal antibody. D. Synaptosomes from mouse cerebral cortex fractionated into extra-synaptic (Extra), pre-synaptic (Pre) and post-synaptic (Post) compartments. Post-synaptic density 95 (PSD95), Synaptophysin (SynPhy) and Lingo-1 were used as markers for synaptic subfractions. E. Conditioned media and lysates from cortical synaptosomes treated with pan-metalloprotease inhibitors, BB-94 and GM6001, DMSO and GM6001-inactive control (GM-I). N-Cadherin (N-Cad) expression in media and lysates was used as a control MMP-substrate. F-G. NgR1 shed fragments in the conditioned media and quantification (protein densitometry) from MT3-MMP and MT5-MMP knockdowns in cortical neurons aged for 14DIV. H-I NgR1 expression and FL/CT-NgR1 ratio in cerebral cortex from MT3-MMP null mice and WT animals. N=3-7 from

independent brains, or dissociated cortical cultures. Data are mean + SEM, * P<0.05, ** P<0.01, *** P<0.001, by Bonferroni Post-hoc's test.

2.6.5 Figure 5: Ecto-NgR1 (1-358) fragments are sufficient to promote excitatory synapse formation

A. Recombinant Fc and 358-Fc generated in Hek293T cells. B. Cortical neurons aged for 14DIV and treated with soluble 358-Fc or Fc control for 48hrs. C. Synaptic counts for cortical neurons treated with recombinant proteins for 48hrs and stained for Synapsin-1 (Syn-1) and PSD95. D. Lysates from cortical neurons expressing CT-NgR1 at different MOI. E. Cortical neurons infected with lentivirus encoding CT-NgR1 or empty vector at MOI 0.3. F. Synaptic counts from cortical neurons overexpressing CT-NgR1 stained for VGLUT-1 and PSD95. Synaptic counts were normalized to corresponding vector control. G. Lysates from cortical neurons expressing WT-NgR1 and CE-NgR1 at different MOI (0.3 and 3). H. Synaptic counts from cortical neurons overexpressing CE-NgR1 at MOI 0.3 and 3 and stained for Synapsin-1 (Syn-1) and PSD95. Synaptic counts were normalized to corresponding Vector control. I. Lysates from cortical neurons expressing CE-NgR1 and exposed to BB-94. J. Synaptic counts from cortical neurons overexpressing CE-NgR1 in the presence or absence of BB-94. Synaptic counts were normalized to DMSO control. N=3-4 from independent cultures. Data are mean + SEM, * P<0.05, ***P<0.001 by Bonferroni Post-hoc's test. Scale bar, 12μm and 4μm.

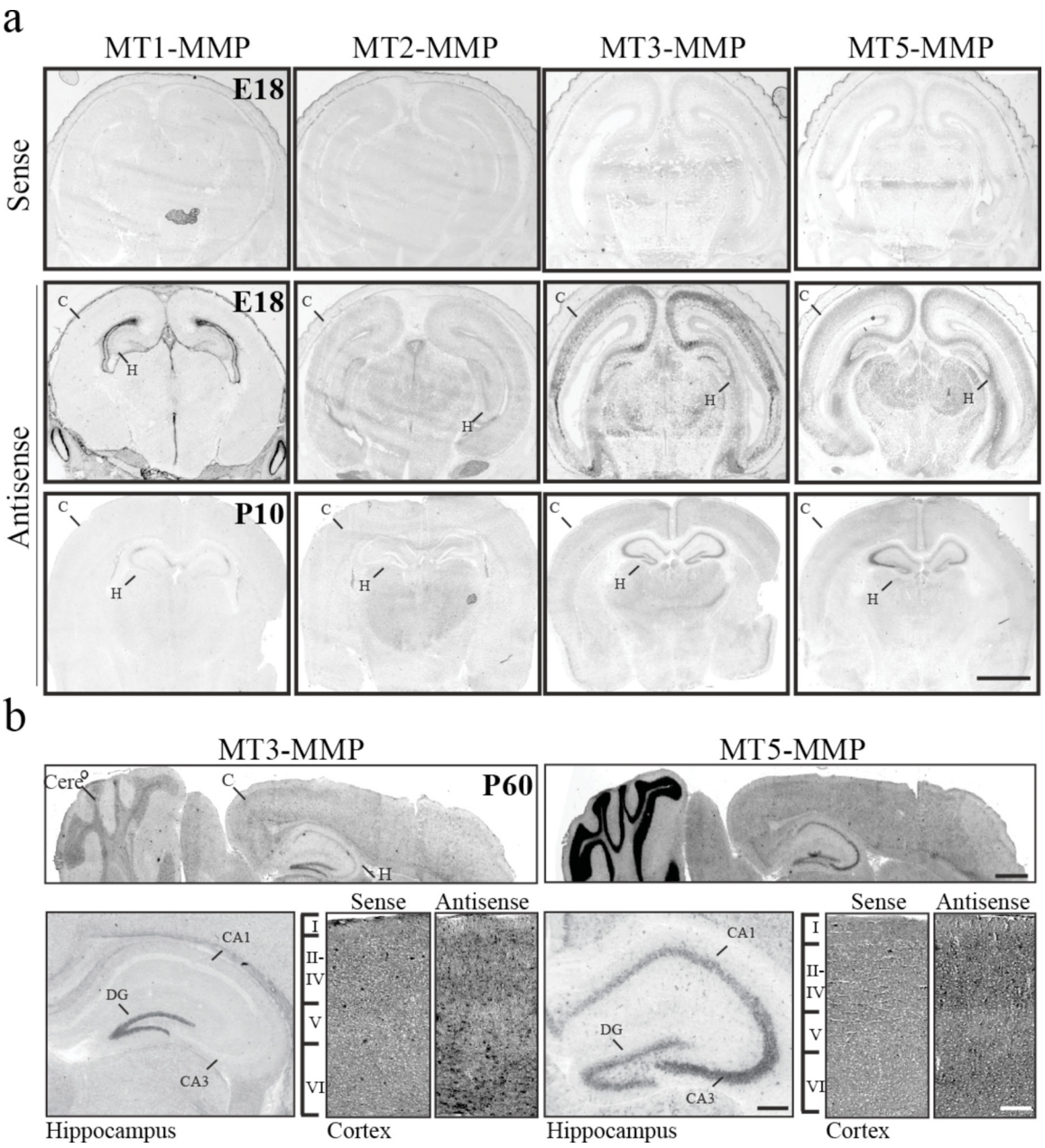
2.6.6 Figure 6: MT3-MMP is necessary for excitatory synapses in the presence of NgR1

A. Cortical neurons from WT-NgR1 or NgR1 deficient mice infected with control empty vector or shRNAmir-MT3-MMP. B. Synaptic counts from MT3-MMP knockdown experiments in WT and NgR1 null cortical neurons. Synaptic counts were normalized to corresponding vector control. C. Model for MT3-MMP and NgR1 shedding in excitatory synaptogenesis. Cortical neurons express FL-NgR1 in the extra-synaptic, pre-synaptic and post-synaptic terminal subdomains. FL-NgR1 is processed by MT3-MMP activity, releasing an ecto-NgR1 fragment (1-358) into the pericellular microenvironment. Surface FL-NgR1 proteolysis and release of Ecto-NgR1 (1-358) promote the formation of

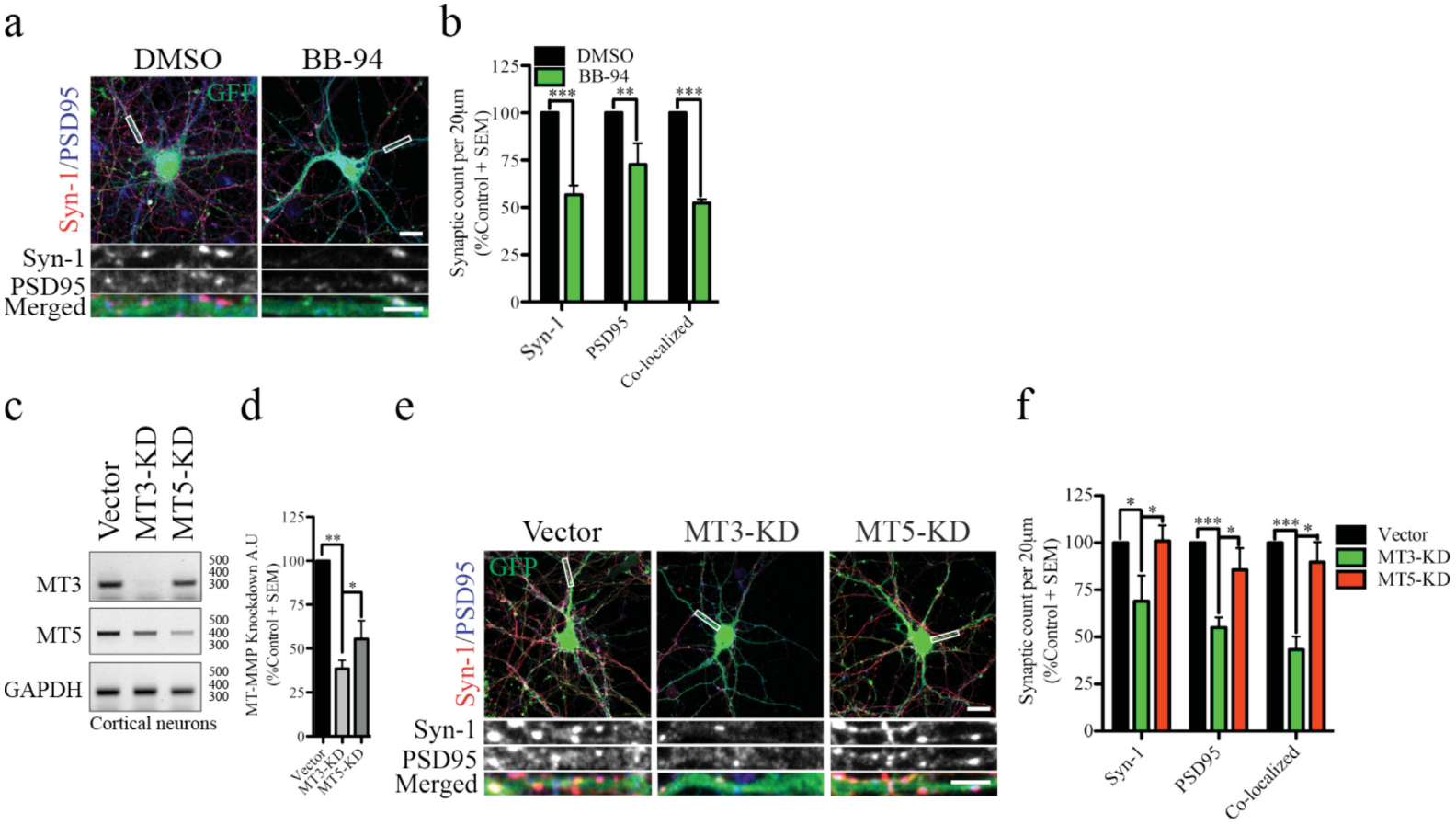
excitatory synapses. All data corresponds to N=3 from independent cortical cultures. Data are mean + SEM, * $P<0.05$, ** $P<0.01$, *** $P<0.001$, by Bonferroni Post-hoc's test. Scale bar, 12μm and 4μm.

2.7 FIGURES

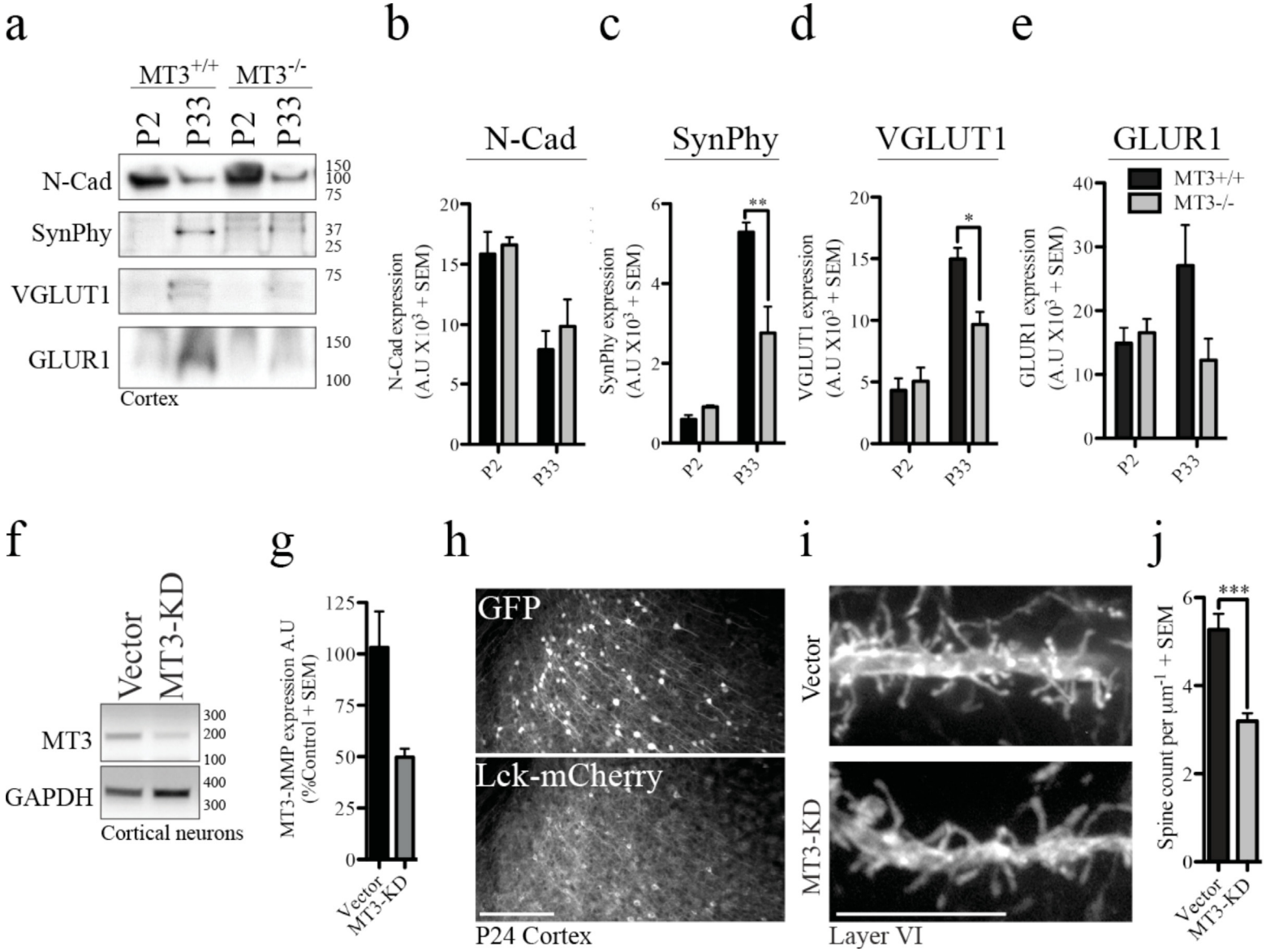
2.7.1 Figure 1: MT3-MMP and MT5-MMP are expressed during periods of synaptogenesis



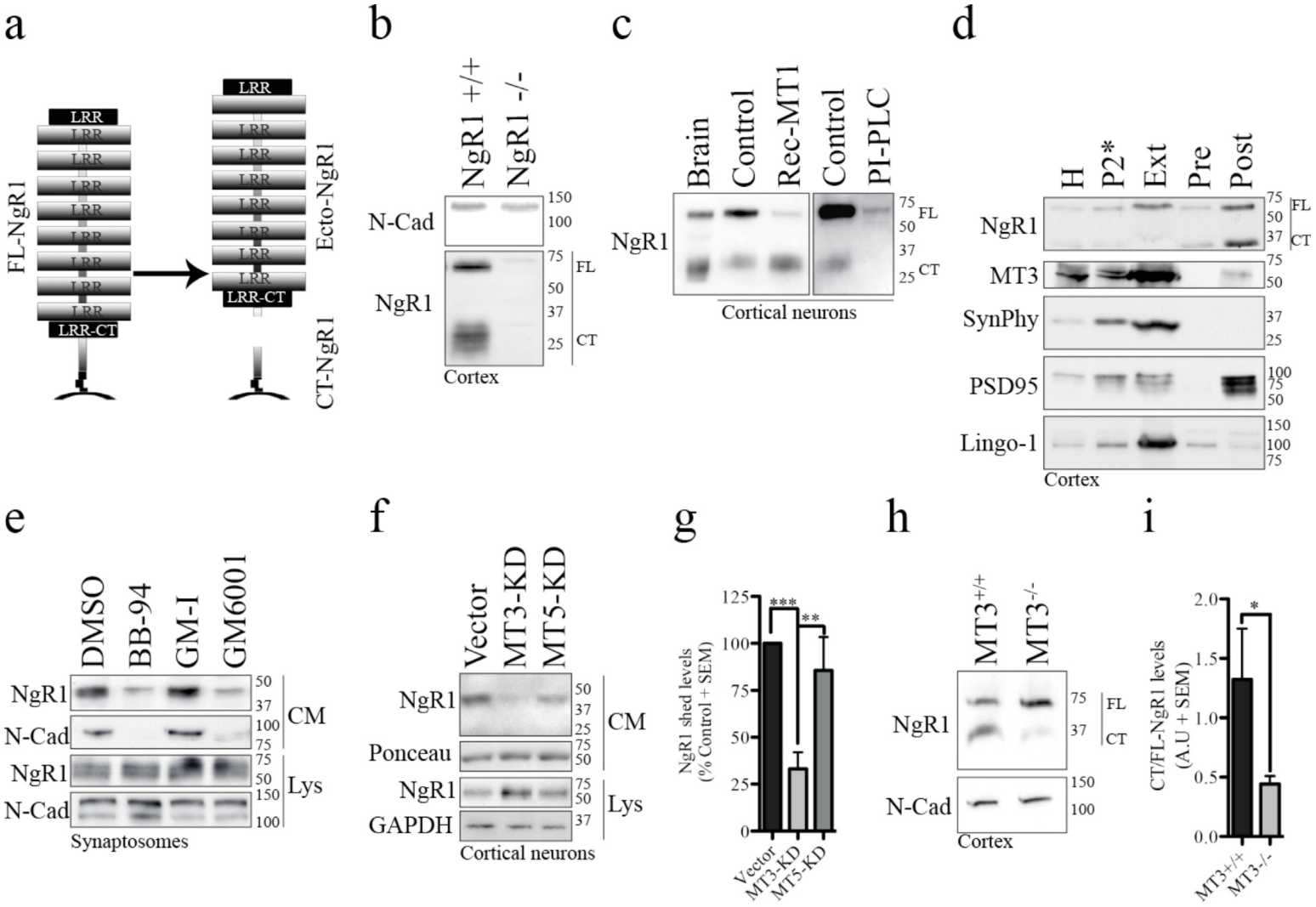
2.7.2 Figure 2: MT3-MMP loss-of-function decreases excitatory synapse formation



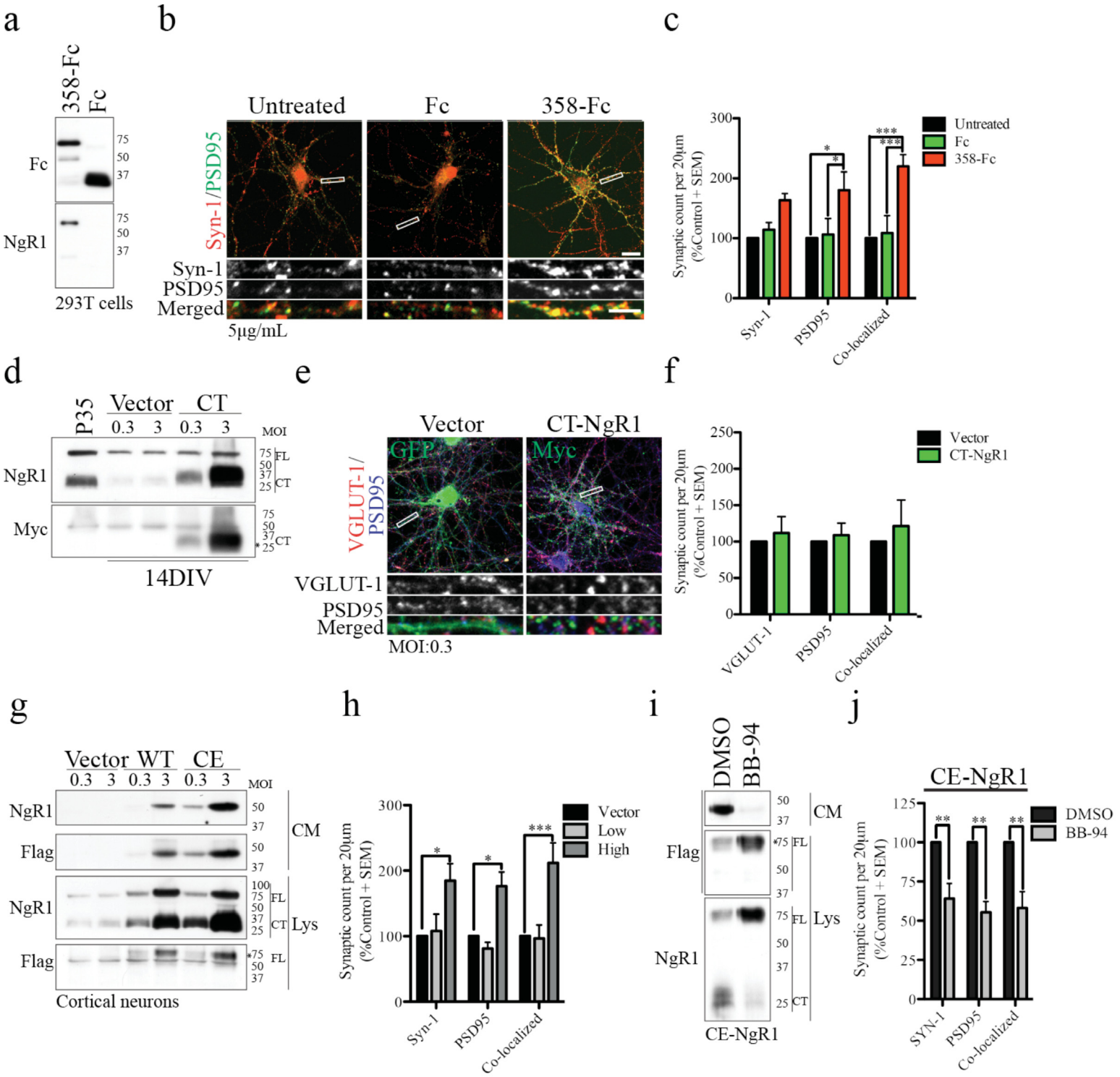
2.7.3 Figure 3: MT3-MMP loss-of-function inhibits synaptogenesis *in vivo*



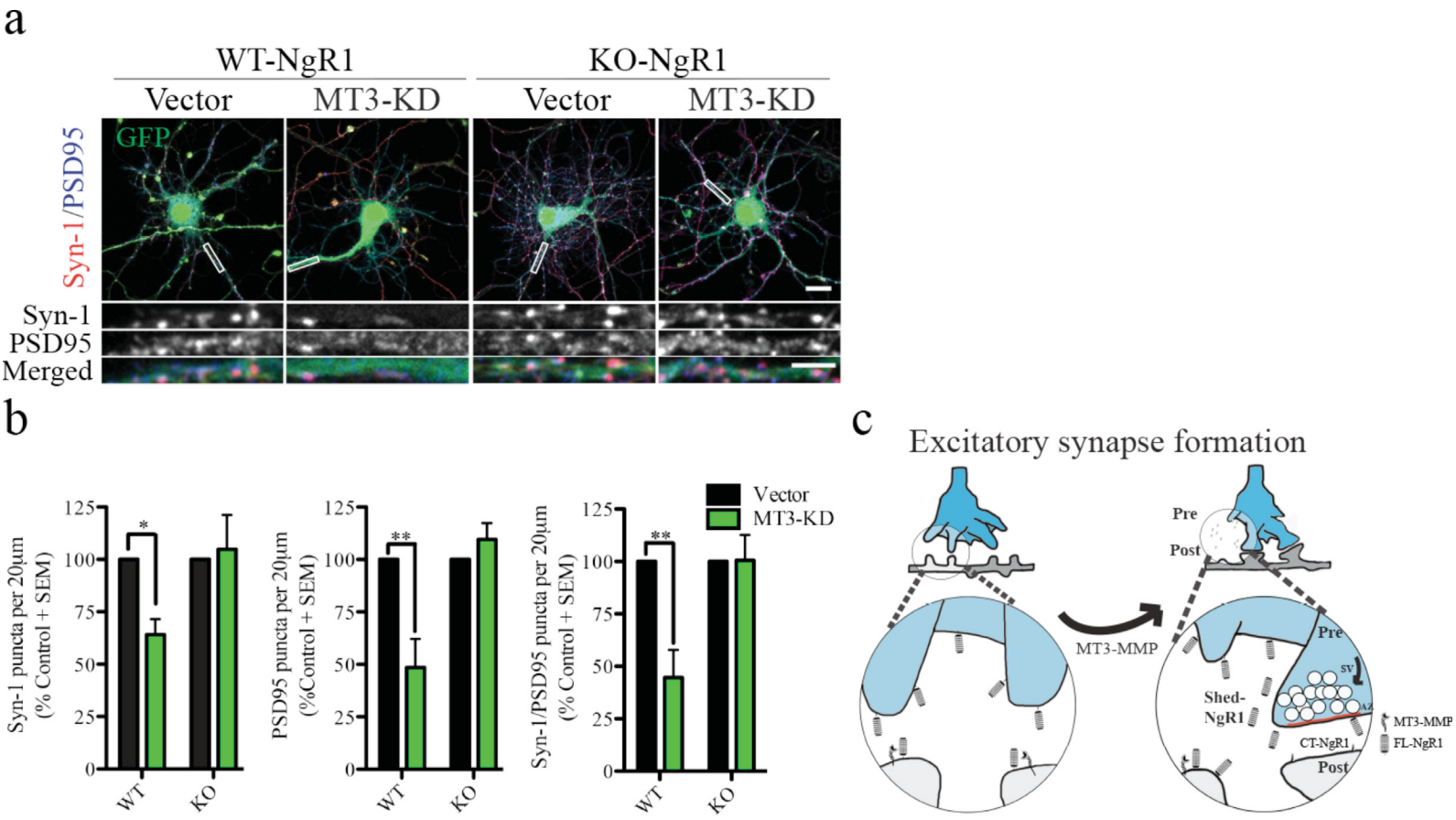
2.7.4 Figure 4: MT3-MMP mediates NgR1 shedding from cortical neurons



2.7.5 Figure 5: Ecto-NgR1 (1-358) fragment is sufficient to promote excitatory synapses



2.7.6 Figure 6: MT3-MMP is necessary for excitatory synapses in the presence of NgR1



Preface

The inability of damaged mature CNS axons to reestablish functional connectivity lies in the combined inhibitory effect of many repulsive guidance cues expressed during development; a decline in the expression of growth promoting genes; the formation of a the glycolytic scar; the inflammatory response and diverse inhibitory cues deposited at the injury site (252). In the present manuscript, we sought to determine the potential role of metalloproteinases in neurite outgrowth by destabilizing inhibitory neuronal complexes present in the mature CNS. Through a proteomic analysis, we identified the IgLON family of adhesion proteins, as previously uncharacterized metalloproteinase substrates. Ectodomain shedding of IgLONs by members of the ADAM family promotes neurite outgrowth in mature cortical neurons by providing a permissive substrate in the vicinity of the neuronal growth cone and through the relief of a neurite outgrowth inhibitory signal from cell surface IgLONs.

CHAPTER 3

3. IgLON cell adhesion molecules are shed from the cell surface of cortical neurons to promote neuronal growth

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*****Running title: IgLON Ectodomain shedding

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3.1 ABSTRACT

Matrix metalloproteinases and A disintegrin and metalloproteinases are members of the zinc endopeptidase, which cleave components of the extracellular matrix as well as cell surface proteins resulting in degradation or release of biologically active fragments. Surface ectodomain shedding affects numerous biological processes including survival, axon outgrowth, axon guidance and synaptogenesis. In the present study, we evaluated the role of metalloproteinases in regulating cortical neurite growth. We found that treatment of mature cortical neurons with pan-metalloproteinase inhibitors or with tissue inhibitor of metalloproteinases-3, TIMP3, reduced neurite outgrowth. Through mass spectrometry we characterized the metalloproteinase-sensitive cell surface proteome of mature cortical neurons. Members of the IgLON family of glycosyl-phosphatidylinositol-anchored neural cell adhesion molecules were identified and validated as proteins that were shed from the surface of mature cortical neurons in a metalloproteinase-dependent manner. Introduction of two members of the IgLON family, Neurotrimin and NEGR1, in early embryonic neurons was sufficient to confer sensitivity to metalloproteinase inhibitors in neurite outgrowth assays. Outgrowth experiments on immobilized IgLON proteins revealed a role for all IgLON family members in promoting neurite extension from cortical neurons. Together our findings support a role for metalloproteinase-dependent shedding of IgLON family members in regulating neurite outgrowth from mature cortical neurons.

3.5 INTRODUCTION

Axon outgrowth is a fundamental process required for proper wiring of the developing brain and for axon regeneration following injury in the adult. During development, gradients of guidance cues bind to cognate receptors on the cell surface to initiate intracellular signals that regulate axon growth (253). Following CNS injury, the environmental cues are mainly repellent, posing an impediment to axon repair, and the intrinsic capacity of the cell to grow is diminished compared to embryonic neurons (254). Therefore, understanding how axonal projections modulate their responses to environmental cues will provide new insights into how the brain achieves functional connectivity and new strategies to promote regeneration following CNS injury. The nervous system has evolved numerous strategies to dynamically regulate how axonal projections respond to the environment. One such mechanism is the regulated proteolytic cleavage of cell surface receptors in a process termed ectodomain shedding (255). Ectodomain shedding can result in insensitivity to ligands in the environment or lead to the formation of biologically active or dominant negative receptor fragments. Shedding has been implicated in a variety of biological processes within the nervous system including neuronal differentiation, cell migration, neuronal survival, synaptic plasticity, axon guidance and outgrowth (29,122,195,256).

Ectodomain shedding is primarily achieved through the activity of Matrix metalloproteinases and A Disintegrin and Metalloproteinase (ADAMs) of the zinc metalloproteinase family (109). These are ubiquitously expressed proteases that include intracellular, cell surface and secreted family members. Their activity is controlled through removal of a propeptide domain to convert them from inactive zymogens to their mature active form and through the expression of endogenous inhibitors called Tissue Inhibitors of metalloproteinases (TIMPs) (257). Application of metalloproteinase inhibitors to developing *Xenopus laevis* results in defects in retinal ganglion cell outgrowth and guidance (258). Similarly, in *Drosophila melanogaster,* mutations in the ADAM family member Kuzbanian result in severe defects in central nervous system axon guidance (9). Proteolytic cleavage of a number of receptors and cell adhesion

molecules affect axon guidance and outgrowth including L1 cell adhesion molecule and the axon guidance receptors EphA2, Neuropilin-1, Robo and DCC (21,28,29,183). Moreover, Nogo Receptor-1 (NgR1) is a receptor for multiple repellent molecules in the adult CNS that is processed by Membrane-Type 3 MMP (259).

In this manuscript, we identify members of the IgLON protein family as metalloproteinase substrates. IgLONs are members of the immunoglobulin (Ig) superfamily of cell adhesion molecules and are the most abundant GPI–anchored proteins expressed in neurons (203). The IgLON proteins contain three immunoglobulin domains followed by a GPI-anchor protein and possess 6-7 potential glycosylation sites (260). IgLON family members include Neurotrimin (NTM), Opioid-binding cell adhesion molecule (OBCAM), Limbic system associated membrane protein (LSAMP), and Neuronal growth regulator 1 (NEGR1) (203-205). IgLON proteins form homophilic and heterophilic complexes along the cell surface and with juxtaposed cells to modulate adhesion and neurite outgrowth. Individual IgLON family members can promote or inhibit growth of different types of neurons in part dependent on the complement of IgLON surface expression (227,261). IgLONs may also play a role in the formation and maintenance of excitatory synapses (262). Our results identify a new family of proteins that are subject to metalloproteinase cleavage and raise the interesting possibility that regulated cleavage of these proteins may play important roles in axon extension and synaptic plasticity.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 Animals -

Timed pregnant (embryonic day 18-19) female Sprague Dawley rats were purchased from Charles River Laboratories (Senneville, QC). All animal care and use was in accordance with the McGill University guidelines and approved by the University Animal Care and Use Committee.

3.3.2 Antibodies -

For immunofluorescence, the following antibodies were used: mouse and rabbit anti-tubulin βIII from Covance (1:1000, Princeton, NJ) and mouse anti-Myc from Sigma-Aldrich (1:1000, St. Louis, MO). Alexa-fluor secondary antibodies were purchased from Invitrogen Life Technologies (1:1000, Burlington, ON). For western blot analysis, the following antibodies were used: anti-NTM (1:100, R&D systems, Minneapolis, MN), anti-LSAMP (1:100, R&D systems), anti-OBCAM (1:100, Santa Cruz, Dallas, Texas), anti-NEGR1 (1:100, Santa-Cruz), mouse and rabbit anti-Myc (1:500, Sigma-Aldrich), anti-GAPDH (1:10000, Abcam, La Jolla, CA) and anti-Human IgG (1:1000, Jackson Immunoresearch, West Grove, PA). HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch.

3.3.3 Plasmids and cloning -

Full-length human cDNA sequences for NTM, OBCAM, LSAMP (OpenBiosystems, Ottawa, ON) and NEGR1 (SinoBiological, Beijing, P.R. China) were cloned into the PsecTag-2B vector (Invitrogen) in frame with the IgK-chain leader sequence at their N-terminal ends. The following primers were used to subclone IgLON proteins into the Psectag-2B vector and introduce a Myc (EQKLISEEDL) epitope tag at the N-terminus: NTM forward: 5'- GAA AAG CTT GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AGC GGA GAT GCC ACC TTC-3', reverse: 5'- GAA CTC GAG TCA AAA TTT GAG AAG CAG GTG C-'3'; OBCAM forward: 5'- GAA GAT ATC GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG AGC GGA GAT GCC ACC TTC-3', reverse: 5'-

GAA CTC GAG TCA AAA CTT GAT GAA GAA GTG GG-3'; LSAMP forward: 5'-GAA GATATC GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG CGC AGC GTG GAT TTT AAC C-3', reverse: 5'- GAA CTC GAG TTA ACA TTT GCT GAG AAG GCA G-3'and NEGR1 forward: 5'-GAA AAG CTT GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG GTG GAC TTC CCC TGG GCG-3', reverse: 5'-GAA GAA TTC TTA TTG TAG AAT GGC ATT CTT CAG GT-3'.

To generate soluble IgLON proteins, the three Ig domains of NTM, OBCAM, LSAMP and NEGR1 were fused to a human Fc segment encoded by the PFUSE vector (Invitrogen) at their C-terminal ends. The constructs, corresponding to the sequence encoding the ectodomain of NTM, OBCAM, LSAMP and NEGR1 subcloned into the PFUSE vector were transiently transfected into HEK293-T cells with Calcium phosphate (263). Transfected cells were incubated in the serum-free medium, OptiMEM (Gibco, Burlington, ON), and the media were collected 4 days post-transfection. IgLON-Fc proteins were purified by affinity chromatography with protein A Sepharose beads. After purification, protein concentration was assayed by protein assay and visualized on an 8% SDS-polyacrylamide gel by Coomassie-brilliant blue stain.

3.3.4 Cortical culture -

Embryonic day 18-19 (E18-19) rat cortical neurons were dissected in ice-cold Leibovitz (L-15) medium (Gibco). Cortical tissue was dissociated in 0.25%Trypsin-EDTA (Invitrogen) and gently triturated. Cortical neurons were aged for 8 DIV in culture plates pre-coated with Poly-L-Lysine (100 μg/mL, Sigma-Aldrich,). Culture medium consisted of Neurobasal (Gibco), 1%B27 (Gibco), 1%N2 (Gibco), 50μg/mL penicillin-streptomycin (Gibco) and 2mM L-glutamine (Gibco).

3.3.5 Outgrowth assays -

For outgrowth assays with pan-metalloproteinase inhibitors or TIMPs, cortical neurons from E18-19 rats and cortical neurons aged for 8 DIV were seeded in a 96-well plate previously coated with poly-L-lysine (PLL) and/or Aggrecan (Sigma-Aldrich). To reseed aged cortical neurons, cells were detached from the culture plate with 0.125%Trypsin-

EDTA. Dissociated aged cortical neurons were washed and seeded in Dulbecco's Modified Eagle Medium (DMEM) (Gibco) supplemented with 10%FBS (Gibco). After 4hrs, the medium was replaced to culture medium enriched with pan-metalloproteinase inhibitors, Batimastat (BB-94; 5μM, Tocris, Ellisville, Missouri), Ilomastat (GM6001; 20μM, CALBIOCHEM, San Diego, CA), Ilomastat-negative control (20μM, CALBIOCHEM), or endogenous metalloproteinase inhibitors (TIMP1, TIMP2, TIMP3; 20μg/mL, R&D). To block neuronal apoptosis, cortical neurons were pre-incubated with the pan-Caspase inhibitor z-VAD-fmk (50μM, R&D) for 1 hour. The neurons were reseeded in a 96-well plate and exposed to BB-94 in the presence of the pan-Caspase inhibitor for 48 hours. After 48 hours, cortical neurons were fixed in 4% PFA, 20% sucrose in PBS for 30 minutes. Neurons were blocked in 5%BSA and 0.2%Triton X-100 in PBS solution for 1hour and stained for βIII-tubulin antibody and Hoechst 33342 stain (Sigma-Aldrich). For outgrowth assays with immobilized recombinant IgLON proteins, IgLON-Fc proteins in PBS solution were coated in a 96-well plate for 3 hours. Aged cortical neurons were seeded in IgLON-Fc coated wells, fixed and immunostained after 48 hours. Fluorescent images were automatically acquired and analyzed through the MetaXpress software on an ImageXpress system.

3.3.6 Apoptosis assay -

DNA fragmentation was detected by labeling free 3'-OH DNA strands with a TMR Red fluorescent marker using a commercially available In Situ Cell Death Detection Kit (Roche Applied Science, Laval, QC). Cortical cultures were counterstained with anti-βIII-tubulin and Hoechst 33342 and imaged using the ImageXpress system microscope. The percentage of TUNEL positive neurons was determined using the MultiWavelength Cell Scoring module of MetaXpress.

3.3.7 Proteomics -

Cortical neurons from E18-19 rats were grown for 8 DIV on PLL-coated plates. Cortical neurons were exposed to DMSO vehicle control or BB-94 for 16hrs. The following day, cortical neurons were washed with 1mM $MgCl_2$, 0.1mM $CaCl_2$ in cold PBS solution and

incubated with EZ-link-Sulfo-NHS-LC-biotin (0.5mg/mL, Thermo Scientific) on ice for 30mins. Two washes with 10mM glycine and PBS were performed to remove any unreactive and unbound biotin. Finally, cells were lysed in 1 x RIPA buffer, supplemented with 1x Complete EDTA-free proteinase inhibitor cocktail (Roche Applied Science, Laval, QC). The lysate-containing buffer was transferred into eppendorf tubes for 30mins, and cell debris was removed by centrifugation. Biotinylated surface proteins were precipitated using streptavidin agarose beads (Thermo Scientific), resolved on 4-16% gradient SDS-PAGE gels and stained with Coomassie-brilliant blue. Upregulated bands in the BB-94 conditions were processed by trypsin digestion and the resulting peptide mixtures were analyzed by nanoscale liquid chromatography quadrupole time-of-flight tandem mass spectrometry. Mass spectrometry profiles were analyzed using Scaffold software.

3.3.8 RT-PCR -

To assay IgLON expression in cortical neurons, RNA was isolated from 2 DIV and 8 DIV cortical neurons followed by a reverse transcriptase polymerase chain reaction (RT-PCR). The following primers were used to detect rat IgLON family members: NTM forward: 5'-CAA CCA CCC TAA GAC CTC CA-3', reverse: 5'-TGC ACT CAT ACT CGC CTG AC-3'; OBCAM forward: 5'-TCC TCT ACG CTG GGA ATG AC-3', reverse: 5'-GTT GGT TCT GGT CTG CCA AT-3'; LSAMP forward: 5'-ATC ACC AGG GAA CAG TCA GG-3', reverse: 5'-TCC CGG TAC CAC TCA AAG TC-3'; NEGR1 forward: 5'-ATG CAG TGC AGA GAA CGA TG-3', reverse: 5'-AGT GCT CCT GTG TCA CGT TG-3'.

3.3.9 Immunochemistry -

COS-7 cells were seeded on PLL-coated 6-well or 12-well plates and cultured in DMEM with 10%FBS. The cells were transiently transfected with Myc-tagged IgLON constructs, using Lipofectamine-2000 (Invitrogen). After 24 hours, the medium was refreshed and supplemented with pan-metalloproteinase inhibitors, phorbol 12-myristate 13-acetate (TPA; 200nM, Sigma-Aldrich) or PI-PLC (1U/mL, Invitrogen). After another 24hr, cell

surface biotinylation was performed. For cortical neurons, $3x10^6$ neurons were seeded in 60mm dishes. Biotinylated cell surface proteins and cell lysates were resolved on 8%SDS-PAGE gels and analyzed by western blot.

For shedding analysis, the culture medium was replaced with serum-free medium (OptiMEM) and supplemented with TPA, pan-metalloproteinase inhibitors or PI-PLC. After 24 hours, supernatants were collected and briefly centrifuged to dispose of residual cell debris. Supernatants were concentrated using column centrifuge filters (10K, Amicon Ultra-15; Millipore, Etobicoke, ON), resolved on 8% SDS-PAGE gels and analyzed by western blot. For IgLON shedding analysis in cortical neurons, $5x10^6$ neurons were seeded in 10 cm plates. The medium of mature cortical neurons was replaced with Neurobasal medium supplemented with DMSO, BB-94 or PI-PLC. After 6 hrs, the medium was collected, briefly centrifuged, concentrated and analyzed by western blot. Shedding experiments were also conducted on brain extracts, prepared from rat at postnatal day 6. Briefly, cortical tissue was homogenized in ice-cold homogenizing solution (1mM $NaHCO_3$, 0.2mM $CaCl_2$, 0.2mM $MgCl_2$ at pH7) and centrifuged to dispose of any residual tissue. Supernatants were collected and centrifuged for 45 min at 25000g. The pellet was resuspended in Neurobasal medium supplemented with DMSO, BB-94 or PI-PLC. After 5 hrs, the medium was centrifuged for 1hr at 100000g. The supernatant and pellet were collected and analyzed by western blotting.

3.3.10 Electroporation -

To express IgLON family members in immature cortical neurons, dissociated cortical neurons were co-electroporated using the Amaxa nucleofector system (Amaxa Kit: VPG-1003, Myrus Kit: MIR5011, Program: O-003; Lonza, Allendale, NJ and Myrus, Madison, WI) according to the manufacturer's protocol. Myc-tagged IgLON family members and a plasmid encoding an eGFP cassette were introduced in a 3:1 ratio, respectively. Surface IgLON expression was assessed through immunostaining of non-permeabilized cells with mouse or rabbit anti-Myc antibodies (Sigma-Aldrich).

Nucleofected cortical neurons were incubated with an anti-Myc antibody for 1 hour at 37ºC and fixed for 15 minutes with 4% PFA, 20% sucrose in PBS solution. Neurons were blocked in 5% BSA in PBS solution for 1 hour and incubated with corresponding

fluorophore-conjugated secondary antibody and Hoechst 33342 stain. The length of the longest neurite in neurons expressing GFP was measured using the NeuronJ plugin from the ImageJ software.

3.3.11 Statistics -

For multiple comparisons, unpaired two-tailed Student T-test, 1-way ANOVA and 2-way ANOVA followed by a Bonferroni post-hoc's test were performed using GraphPad Prism software.

3.4 RESULTS

3.4.1 Inhibitors of zinc metalloproteinases repress neurite outgrowth of cortical neurons

Ectodomain shedding of a number of receptors and adhesion molecules affects axon growth. To further investigate the role of ectodomain shedding in regulating outgrowth, we measured the length of neurites projecting from rat cortical neurons upon exposure to pan-inhibitors of zinc metalloproteinases. We assessed responses of both embryonic cortical neurons and cortical neurons aged for 8 DIV to sensitize them to repellents in the adult CNS (264). Outgrowth of embryonic cortical neurons was not affected by treatment with BB-94 or GM6001, two broad inhibitors of zinc metalloproteinases (Fig. 1A, B; (265,266). In contrast, outgrowth from cortical neurons aged for 8 DIV and re-seeded on a permissive PLL substrate was inhibited by both BB-94 and GM6001 (Fig. 1A, C). The metalloproteinase inhibitors also mildly attenuated the neurite outgrowth inhibitory effect of the chondroitin sulfate proteoglycan (CSPG) aggrecan (Fig. 1D). Neurons treated with DMSO or the inactive analogue of GM6001 (GM-I) were significantly inhibited by 1μg/mL or 5μg/mL of aggrecan, respectively, whereas those treated with BB-94 or GM6001 only became responsive to aggrecan at 25μg/mL, the highest dose tested (Fig. 1A, D). This suggests that zinc metalloproteinase substrate/s are important for promoting the intrinsic growth capacity of mature cortical neurons on permissive substrates and also contribute to neurite outgrowth inhibition on CSPGs. We investigated the molecular mechanism that is responsible for promoting the intrinsic growth capacity of aged cortical neurons.

To determine if metalloproteinase inhibitors suppress neurite extension in mature cortical neurons through indirect effects on cellular adhesion or viability, we quantified the total number of neurons and the number of apoptotic neurons following treatment with BB-94 or GM6001. The metalloproteinase inhibitors had no effect on the number of neurons that adhered to the substrate (Fig. 2A, B). To assay for cell viability, TUNEL TMR-red staining was performed and the cultures were co-stained with a nuclear and a neuronal marker (Fig. 2A). Both BB-94 and GM6001 had a small but significant effect on the number of TUNEL positive neurons (Fig. 2B). In control conditions, we found 3-

4% of apoptotic neurons while BB-94 increased the number to 10% and GM6001 increased the number to 9%. We therefore tested the effects of BB-94 in the presence of the pan-caspase inhibitor z-VAD-fmk to block apoptosis (267). Z-VAD-fmk blocked the BB-94-dependent increase in cell death but did not attenuate the neurite outgrowth inhibitory effect of BB-94 leading us to conclude that the neurite outgrowth inhibitory effect of the metalloproteinase inhibitors is not an indirect effect of reduced cell viability (Fig. 2C).

3.4.2 An ADAM family member promotes neurite outgrowth of mature cortical neurons

We then determined the type of metalloproteinase responsible for promoting cortical neurite outgrowth by treating cells with TIMPs, endogenous inhibitors of zinc metalloproteinases. Individual TIMPs vary in their selectivity (268). TIMP-1 inhibits ADAM-10 and MMPs except for most membrane type MMPs. TIMP-2 inhibits all metalloproteinases while TIMP-3 inhibits the metalloproteinases and some ADAM family members. When mature cortical neurons were exposed to recombinant TIMPs, TIMP3 selectively and significantly inhibited neurite outgrowth (Fig. 3A, B). Similar to the pan-metalloproteinase inhibitors, TIMP3 had no effect on cell adhesion and a modest effect on cell survival (Fig. 3C, D). The selective effect of TIMP3 implicates an ADAM family member in enhancing outgrowth in mature cortical neurons.

3.4.3 Members of the IgLON family of cell adhesion molecules are shed from the surface of cortical neurons by metalloproteinases

In the interest of identifying metalloproteinase substrates that may be responsible for the intrinsic ability of cortical neurons to extend neurites, we identified proteins that are shed from the surface of cortical neurons in a metalloproteinase-dependent manner. We biotinylated cell surface proteins in mature cortical neurons cultured in the presence or absence of the BB-94. We then washed the cells and precipitated cell surface biotinylated proteins with streptavidin beads, separated them by SDS-PAGE and stained with Coomassie-brilliant blue stain. Seven bands were markedly more intense in samples treated with BB-94 suggesting that they contained metalloproteinase substrates that were

enriched on the cell surface upon metalloproteinase inhibition (Fig. 4). These bands were excised from the gel and analyzed by tandem mass spectrometry. Peptides from 44 proteins were identified, 13 of which have been previously described to undergo ectodomain shedding (Table 1). A survey of the proteins revealed functions in cell adhesion, ion conduction and cell signaling. Numerous proteins have roles in regulating synaptogenesis. Notably, four of the proteins identified belong to the IgLON subgroup of the immunoglobulin superfamily cell adhesion molecules.

To validate that IgLON family members are indeed processed by metalloproteinase activity, we transfected COS-7 cells with myc-tagged IgLON constructs and analyzed both the conditioned media and biotinylated cell surface proteins. All IgLON family members could be detected on the cell surface and in the conditioned media from the transfected COS-7 cells (Fig. 5A). The bands that we detect correspond to the glycosylated forms of the IgLONs, which migrate with molecular weights between 50 and 68 kDa, demonstrating that transfected IgLONs are properly glycosylated and traffic to the cell surface. When the cells were exposed to BB-94 or GM6001, IgLON family members accumulated on the cell surface and were weak or undetectable in the conditioned media. Further, treatment of cells with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA), which increases levels of metalloproteinase activity, resulted in a loss of the IgLONs from the cell surface and accumulation in the conditioned media (269). The effect of TPA was blocked with BB-94 confirming that the enhanced shedding was metalloproteinase-dependent (Fig.5B). We also treated neurons with phosphatidylinositol phospholipase C (PI-PLC), an enzyme that cleaves GPI-anchored proteins. IgLONs in the conditioned media following PI-PLC treatment migrate slightly higher than the metalloproteinase-dependent shed product (Fig. 5A). The distinction between the product generated by metalloproteinases and PI-PLC was even more apparent when the deglycosylating agent PNGase F was added to the conditioned media (Fig. 5C) (270). Following treatment with PNGase, the IgLONs migrate near their predicted molecular weights of 37 kDa and the endogenously cleaved products migrate more rapidly than full length IgLONs released by PI-PLC. It was also apparent that the product in the conditioned media migrated more rapidly than the product from cell lysates

(Fig. 5D). We conclude that IgLON proteins are cleaved close to the membrane to shed an ectodomain fragment that is near full-length.

Metalloproteinase activity in any given cell type is a function of the expression of mature metalloproteinases as well as the expression of TIMPs. We next characterized the shedding profile of IgLONs from cortical neurons to determine if they were good candidate substrates to promote the intrinsic growth capacity of the aged cortical neurons. We analyzed the expression pattern of IgLON family members in cortical neurons cultured for 2 DIV and 8 DIV. mRNA for all four IgLON family members was detectable in both stages of cortical neurons by RT-PCR (Fig. 6A). We then acquired commercial antibodies to individual IgLON family members and confirmed that they detected recombinant protein (Fig. 6B, C). The anti-NTM antibody showed some cross-reactivity to OBCAM while the other anti-IgLON antibodies were specific. Using these antibodies to probe cortical lysates we found that IgLON proteins were weakly expressed or absent in 2 DIV cortical neurons both in the cell lysate and at the cell surface and were upregulated at 8 DIV (Fig. 6D). The upregulation in IgLON protein expression was also apparent in cortex isolated from rat brain at embryonic day (18-19) and postnatal day six, ruling out the possibility that this is an effect of the cell culture (Fig. 6E). The expression profile of the IgLONs correlated with the metalloproteinase-sensitivity of the cortical neurons in neurite outgrowth assays (Fig. 1).

We then used these antibodies to probe conditioned media from cortical neurons cultured for 8 DIV. When cortical neurons were treated with PI-PLC, all IgLON family members were enriched in the conditioned media indicating that they are all expressed on the cell surface of these neurons (Fig. 6F). In the conditioned media from untreated cortical neurons, NTM and NEGR1 were present and levels were reduced by treatment with BB-94 demonstrating that these two family members undergo constitutive shedding in mature cortical neurons (Fig. 6F). LSAMP and OBCAM were not detectable in the conditioned media from resting cortical neurons leading us to conclude that these IgLON family members undergo little shedding in these conditions or that they are unstable upon their release. To validate the processing of NTM and NEGR1 by metalloproteinases *in vivo*, we collected the conditioned media from brain extracts. All IgLON family members

were detected in the conditioned media and similar to the culture experiment, NTM and NEGR1 were most robustly shed (Fig. 6F).

3.4.4 NTM and NEGR1 sensitize immature cortical neurons to BB-94

We next asked if introducing IgLONs into 2 DIV cortical neurons would be sufficient to sensitize the neurons to a metalloproteinase inhibitor in the neurite outgrowth assay. 2DIV cortical neurons were nucleofected with individual IgLON constructs. We confirmed that myc-tagged IgLON constructs were expressed at the cell surface of the neurons by performing immunofluorescence staining on cortical neurons prior to permeabilization (Fig. 7A). Introduction of NTM or NEGR1, but not OBCAM or LSAMP was sufficient to sensitize these neurons to the outgrowth inhibitory activity of the metalloproteinase inhibitor BB-94 implicating shedding of these IgLON family members in regulating the intrinsic growth capacity of neurons (Fig. 7B). Introduction of the IgLONs in the absence of BB-94 did not impact outgrowth suggesting that BB-94 leads to an accumulation of NTM and NEGR1 on the cell surface that impedes neurite extension.

3.4.5 Immobilized full-length IgLON family members promote neurite outgrowth from 8DIV cortical neurons

IgLON family members act through homophilic and heterophilic interactions and through interactions with other receptors to promote adhesion and to promote or repress growth (226,271). The effects of IgLON family members vary depending on the cell type and stage (224,227). We coated IgLONs as substrates and tested their effects on neurite outgrowth from 8 DIV cortical neurons (227). We observed a dose dependent increase in neurite outgrowth with recombinant NTM, OBCAM, LSAMP and NEGR1 compared to control substrate (Fig. 8A). This finding supports a model whereby IgLONs shed from the surface of mature cortical neurons could generate a growth permissive substrate for outgrowth. We then treated mature cortical neurons grown on IgLON substrates with BB-94. We found that outgrowth of cortical neurons grown on IgLON substrates was insensitive to BB-94 suggesting that the substrate can overcome inhibitory effects of cell

surface IgLONs (Fig. 8B). Together, our data demonstrates that IgLON shedding promotes the growth of cortical neurons by forming a growth permissive substrate for cortical neurons and by relieving a neurite outgrowth inhibitory signal transduced by a NTM- or NEGR1-containing cell surface complex (Fig. 8C).

3.5 DISCUSSION

Ectodomain shedding is an important regulatory mechanism that can desensitize a cell to a ligand and generate biologically active fragments. Here we report that members of the IgLON family of cell adhesion molecules are shed from the surface of mature cortical neurons through ADAM-dependent proteolysis. Release of IgLON proteins from the surface of mature cortical neurons promotes their outgrowth likely by providing a permissive substrate at the leading edge of the neuronal growth cone and through the relief of a neurite outgrowth inhibitory signal from cell surface IgLONs.

Ectodomain shedding of IgLON family members in neurite outgrowth - Previous studies have uncovered important aspects of metalloproteinase activity in neurite outgrowth. One major aspect is the enrichment of the extracellular matrix with functionally active fragments to enhance neurite outgrowth. For example, cleavage of L1-CAM proteins promotes neurite outgrowth in cerebellar granule cells (195) while cleavage of Neuronal cell adhesion molecule (NCAM) promotes neurite outgrowth in hippocampal neurons (196). The growth promoting effect of the L1-CAM and NCAM shed products is mediated through homophilic interactions and activation of the integrin-receptor (21,202,272). While we have not characterized the site of metalloproteinase cleavage, our experiments reveal that near full length IgLON family members are processed in a metalloproteinase-dependent manner, similar to L1-CAM and NCAM, to facilitate neuronal extension in cortical neurons. The ability of substrate-bound IgLONs to promote outgrowth of mature cortical neurons supports the idea that shed IgLONs signal through homophilic-/heterophilic-IgLON interactions or alternative receptor molecules to promote growth (Fig. 8). IgLON overexpression did not promote neurite growth on its own suggesting that insufficient concentrations of IgLONs were generated in the vicinity of the growth cone to generate a growth permissive substrate. By mass spectrometry we found that all IgLON family members were enriched on the cell surface following treatment with metalloproteinase inhibitors. In contrast, only NTM and NEGR1 could be detected in conditioned media from cortical neurons by Western

blotting. This may reflect limited stability of OBCAM and LSAMP upon their release from the cell surface.

Our data also supports the idea that shedding of NTM and NEGR1 acts to relieve an outgrowth inhibitory signal transduced from the cell surface. Introduction of NTM and NEGR1 into 2DIV cortical neurons, which do not express endogenous IgLON at the cell surface, sensitized neurons to outgrowth inhibitory effect of BB-94 but did not affect outgrowth in the absence of BB-94 (Fig. 7). If the sensitivity to BB-94 resulted from a failure to present growth promoting fragments in the vicinity of the growth cone then it follows that transfection of IgLONs themselves should have had a growth promoting effect. Since they did not, we reason that BB-94 leads to the enrichment of a cell surface complex that limits the extension of cortical neurons. This complex could be formed by IgLON homodimers or heterodimers or by IgLON proteins in complex with a co-receptor (226,271). IgLON overexpression likely fails to inhibit outgrowth in the absence of BB-94 because it is constitutively shed. Mature cortical neurons grown on IgLON substrates were insensitive to BB-94 suggesting that the growth promoting activity of shed IgLONs overcomes the influence of any cell surface inhibitory signals. A tempting speculation is that substrate bound IgLONs may complex with cell surface IgLONs to displace a co-receptor that is required for transducing neurite outgrowth inhibitory signals intracellularly.

IgLON shedding by the ADAM family of metalloproteinases – The selective effect of TIMP3 on neurite extension implicates a member of the ADAM family of metalloproteinases as likely mediators of NTM and NEGR1 processing. NTM has also been identified as a potential BACE-1 substrate raising the possibility that it is a substrate of multiple proteases (273). Thirty-seven ADAM family members are expressed in mice, twelve of which contain proteolytic activity (274). Among the best-characterized ADAM proteases, ADAM-10, ADAM-17 and ADAM-8 are observed to regulate axon extension. Proteolytic cleavage of NCAM and L1-CAM by ADAM10 and ADAM-17 is involved in axon growth and ectodomain shedding of CHL1 by ADAM-8 promotes neurite outgrowth in cerebellar granule cells (194-196). While we have not conducted a

comprehensive analysis of all ADAM family members, we did find that knockdown of ADAM-10 had no impact on IgLON shedding (data not shown).

Physiological role of IgLON shedding – It is noteworthy that metalloproteinase inhibitors selectively affected the outgrowth of aged cortical neurons while immature neurons were unaffected (Fig. 1). The developmentally regulated response of the cortical neurons correlates with an increase in expression of IgLON proteins at the cell surface. The developmental increase in IgLON expression is consistent with previous reports analyzing the expression of several IgLON family members in tissue from several regions of the rodent brain (203,219). In the course of postnatal development, axons continue to grow in complexity and length to establish connections and form the laminated cortical structure. The delayed expression of IgLON family members raises the possibility that IgLON shedding may play roles in late stages of cortical neuron outgrowth or synaptogenesis rather than the initial outgrowth of cortical neurons. Axonal LSAMP expression plays an important role in the specific targeting of dopaminergic axons to the lateral subdomain of the habenula (230). Further, downregulation of NEGR1 expression both *in vitro* and *in vivo* was observed to decrease the overall complexity of neurite arborization suggesting a role in axon targeting and stability rather than overall outgrowth (229). IgLON family members are also expressed at synaptic sites (219,262,275). Overexpression of individual IgLON family members differentially affect the number of dendritic synapses that form in cultured hippocampal neurons while NEGR1 and OBCAM loss of function results in a reduction in synapse formation on dendrites (229,276,277). Proteolytic cleavage of IgLON family members could thus have critical roles in specific targeting and synaptogenesis of cortical neurons similar to roles of other synaptic cell adhesion molecules including NCAM, L1-CAM and N-Cadherin (278-280). The physiological role of IgLON shedding is a particularly interesting question in the context of the recent classification of IgLONs as potential candidate genes underlying aspects of autism spectrum disorder and cancer susceptibility (281-284)

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3.6 FIGURE LEGENDS

3.6.1 Figure 1: Pan-metalloproteinase inhibitors repress neurite outgrowth in mature cortical neurons

(A) Rat cortical neurons from two developmental stages (2DIV and 8DIV) seeded on a PLL and an aggrecan (5µg/mL) substrate and treated with a vehicle control (DMSO), metalloproteinase inhibitors (BB-94 and GM601) and a GM6001-Inactive control (GM-I). Neuronal projections were visualized with βIII-tubulin staining. (B-C) Outgrowth quantification from 2DIV (B) and 8DIV (C) cortical neurons. Outgrowth was normalized to the DMSO control for each experiment. (D) Outgrowth quantification from 8DIV cortical neurons seeded on PLL and aggrecan substrates (1, 5 and 25µg/mL) and treated with DMSO, BB-94, GM-I and GM6001. Outgrowth data was normalized to DMSO control on PLL substrate. N=4-8 from independent cultures. Data shown as mean + S.E.M *P<0.05, **P<0.01, ***P<0.001 by 1-WAY and 2-WAY ANOVA, followed by Bonferroni post-hoc's test. Scale bar, 100µm.

3.6.2 Figure 2: Metalloproteinase inhibitors inhibit growth independently of adhesion or cell death

(A) A TUNEL assay was performed in aged rat cortical neurons (8DIV) seeded on a PLL substrate and treated with a vehicle control (DMSO), metalloproteinase inhibitors (BB-94 and GM6001) and GM-I. Neurons were stained with anti-βIII-tubulin, Hoechst and TUNEL. As a positive control for the TUNEL assay, neurons were pretreated with a DNA-nuclease. (B) Total number of neurons and apoptotic neurons present after indicated treatments. For TUNEL assays, the data is shown as a percentage of TUNEL positive neurons. For total number of neurons, data has been normalized to control DMSO. (C) TUNEL assay and neurite outgrowth quantification of mature cortical neurons treated with DMSO and BB-94 in the presence of the pan-caspase inhibitor (Z-VAD-fmk). N=3-4 from independent cultures. Data shown as mean + SEM ** P<0.01, *** P<0.001 by (B) 1 WAY-ANOVA followed by Bonferroni post-hoc's test, and (C) Student T-test. Scale bar, 100µm.

3.6.3 Figure 3: An ADAM family member promotes neurite outgrowth in aged cortical neurons

(A) Aged cortical neurons (8DIV) seeded on a PLL substrate and treated with control (H_2O) or endogenous metalloproteinase inhibitors (TIMP1, 2 and 3). Neuronal projections were visualized with βIII-tubulin staining. (B) Neurite outgrowth quantification of aged cortical neurons exposed to soluble recombinant TIMPs and control condition. Data has been normalized to the control condition. (C) A TUNEL assay was performed in aged cortical neurons treated with TIMPs or H_2O control. DNA nuclease was used as a positive control. Data is shown as a percentage of TUNEL positive neurons. (D) Number of neurons present on the PLL substrate after treatment with soluble TIMPs and control condition. N=3-4 from independent cultures. Data are mean + SEM ** $P<0.01$, *** $P<0.001$ by 1 WAY-ANOVA followed by Bonferroni post-hoc's test. Scale bar, 100μm.

3.6.4 Figure 4: A proteomic analysis in cortical neurons identifies IgLON family members as novel metalloproteinase substrates

A cell surface biotinylation was performed on mature cortical neurons treated with a control condition (DMSO) and metalloproteinase inhibitor (BB-94). Cells were washed and cell surface proteins biotinylated proteins were precipitated with streptavidin beads, separated by SDS-PAGE and stained with Coomassie-brilliant blue stain. Seven upregulated bands (arrowheads) in the BB-94 treatment compared to control condition were excised and sent for a proteomic analysis. Arrows define protein bands that are insensitive to metalloproteinase activity.

3.6.5 Figure 5: IgLON family members are shed by metalloproteinases

(A) To validate the processing of IgLON family members, myc-IgLON constructs were expressed in COS-7 cells. Biotinylated cell surface proteins (CSB) and conditioned medium (CM) of transduced COS-7 cells were analyzed by Western blotting after treatment with DMSO, GM-I, BB-94, GM6001, TPA or PI-PLC. (B) To validate the processing of IgLON family member by metalloproteinase activity, the medium of COS-7 cells expressing the myc-tagged IgLON family member NEGR1 was analyzed after

treatment with TPA in the presence or absence of BB-94. (C) To identify the size of the cleaved IgLON fragment, the medium of COS-7 cells, transduced with myc-IgLON constructs and treated with DMSO and PI-PLC, was concentrated and deglycosylated with PNGase-F. (D) Conditioned media and lysates of COS-7 cells transduced with myc-IgLON constructs were analyzed by SDS-PAGE and western blotting with IgLON-specific antibodies. Western blots are representative of 3-6 independent experiments.

3.6.6 Figure 6: IgLON expression and processing is developmentally regulated in cortical neurons

(A) Reverse transcription PCR (RT-PCR) was performed in cortical neurons from two developmental stages (2DIV and 8DIV). (B) Recombinant IgLON-Fc proteins were purified from the conditioned media of transfected HEK-293T cells and analyzed by SDS-PAGE and Coomassie-brilliant blue stain. (C) The specificity of commercially available IgLON antibodies was validated by Western blot against soluble recombinant IgLON-Fc proteins. (D-E) The developmental expression of IgLON family members was assayed in cortical neurons. Lysates and biotinylated cell surface proteins were collected from 2DIV and 8DIV cortical neurons, and lysates from the cortex of embryonic (e18-19) and postnatal (P6) stage rats. The expression of IgLON family members was determined by western blot using commercially available IgLON antibodies. (F) To validate processing of IgLON family members by metalloproteinase activity in cortical neurons, the medium of aged cortical neurons and the medium of membrane extracts from postnatal stages of brain cortex were collected. Commercially available IgLON antibodies were used to detect cleaved IgLON fragments in the medium. Anti-GAPDH and Tubulin antibodies were used as loading controls. Western blots are representative of 3-4 independent experiments.

3.6.7 Figure 7: Surface IgLON expression sensitizes 2DIV cortical neurons to metalloproteinase inhibitors

(A) Myc-IgLON family members were co-electroporated with eGFP in dissociated e18-19 rat cortical neurons. Surface myc-IgLON expression was detected with anti-myc antibody in the absence of permeabilization. (B) An outgrowth assay was performed in

eGFP positive cortical neurons transduced with myc-IgLON constructs treated with DMSO or BB-94. N=3-4 from independent cultures. Data are mean + SEM, * P<0.05, by Student T-test. Scale bar, 50μm.

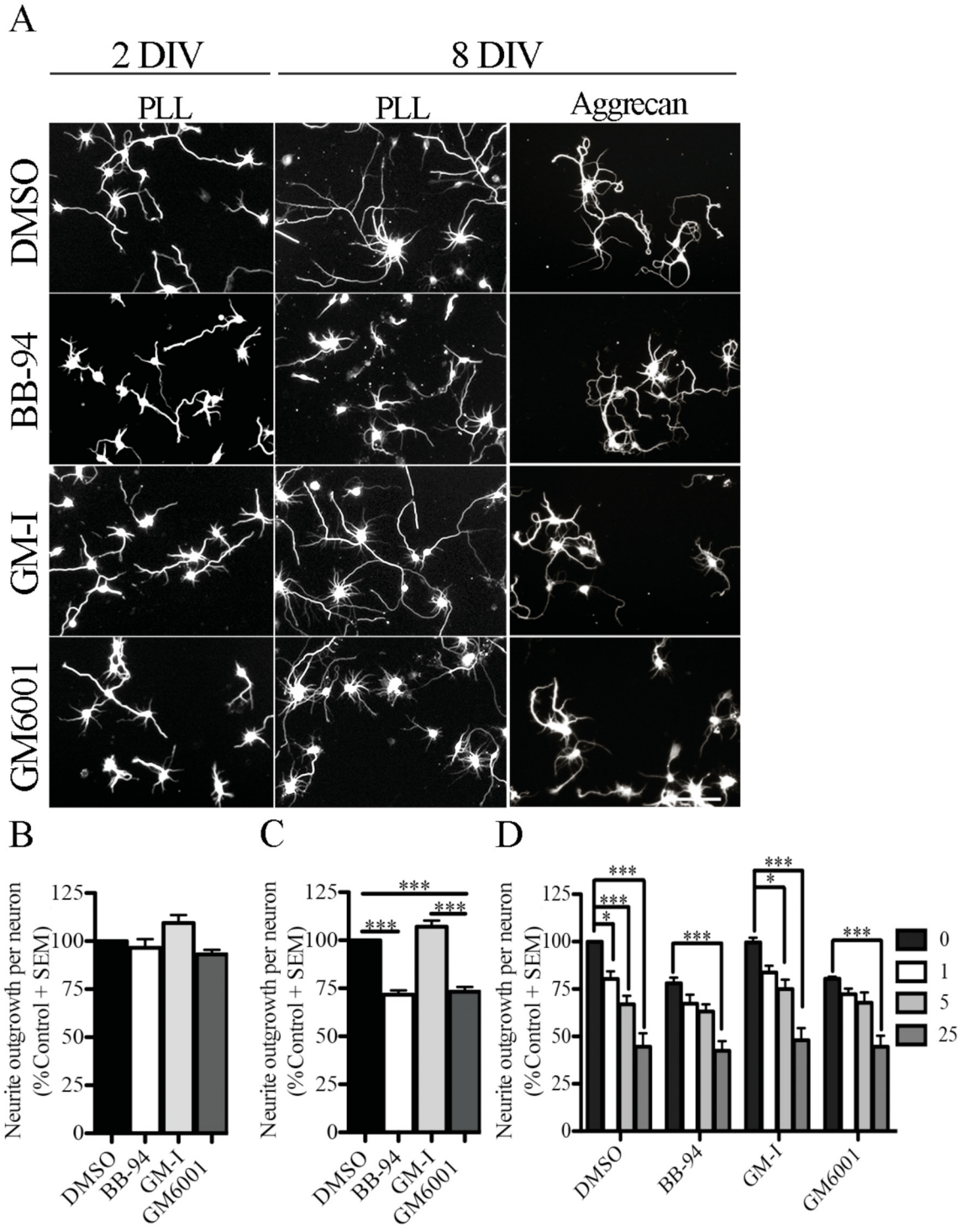
3.6.8 Figure 8: Immobilized IgLON family members promote neurite outgrowth in cortical neurons

(A) Outgrowth quantification of mature cortical neurons seeded on Fc-IgLON substrates (10, 50 and 100μg/mL). Data normalized to control Fc substrate. (B) Outgrowth quantification of mature cortical neurons seeded on IgLON substrates (100μg/mL) and exposed to DMSO or BB-94. Data shown as mean + SEM, * P<0.05, **P<0.01, ***P<0.001 by (B) 1-WAY and (C) 2-WAY ANOVA, followed by Bonferroni post-hoc's test. (C) A schematic representation of the role of IgLON processing in neurite outgrowth.

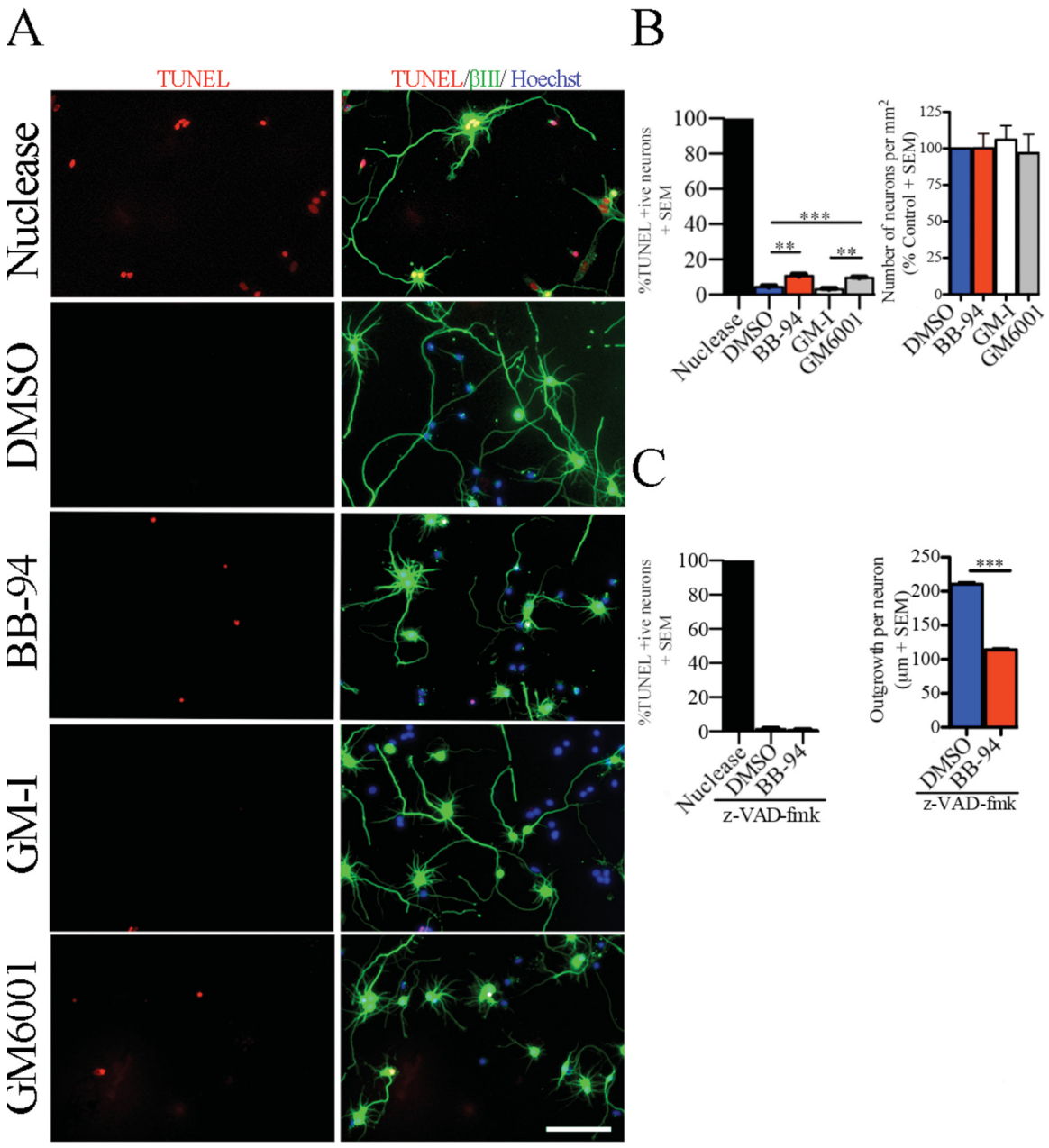
3.6.9 Table 1 - Mass Spectrometry Results

(A) A list of potential metalloproteinase substrates from a proteomic analysis performed on seven-sample bands upregulated in a cell surface biotinylation (Fig. 4). A list of 44 potential metalloproteinase substrates obtained from a proteomic analysis. From this list, 13 peptides were previously identified as metalloproteinases substrates (red), and 4 members of a family of adhesion proteins (IgLONs), as potential metalloproteinase substrate candidates (blue).

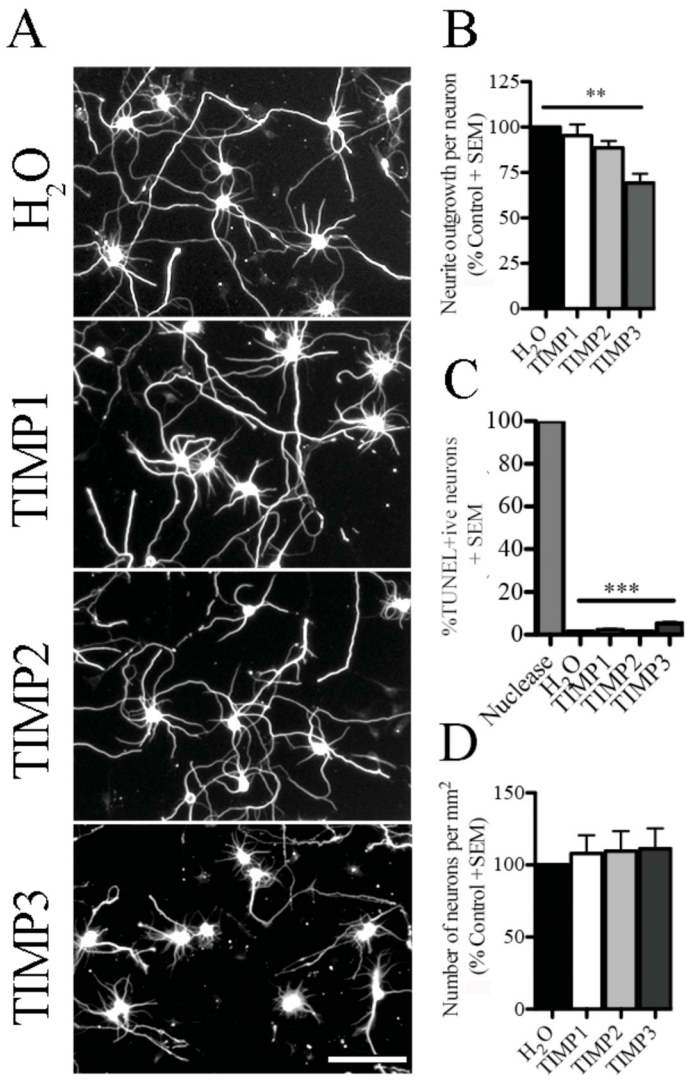
3.7.1 Figure 1: Pan-metalloproteinase inhibitors repress neurite outgrowth in mature cortical neurons



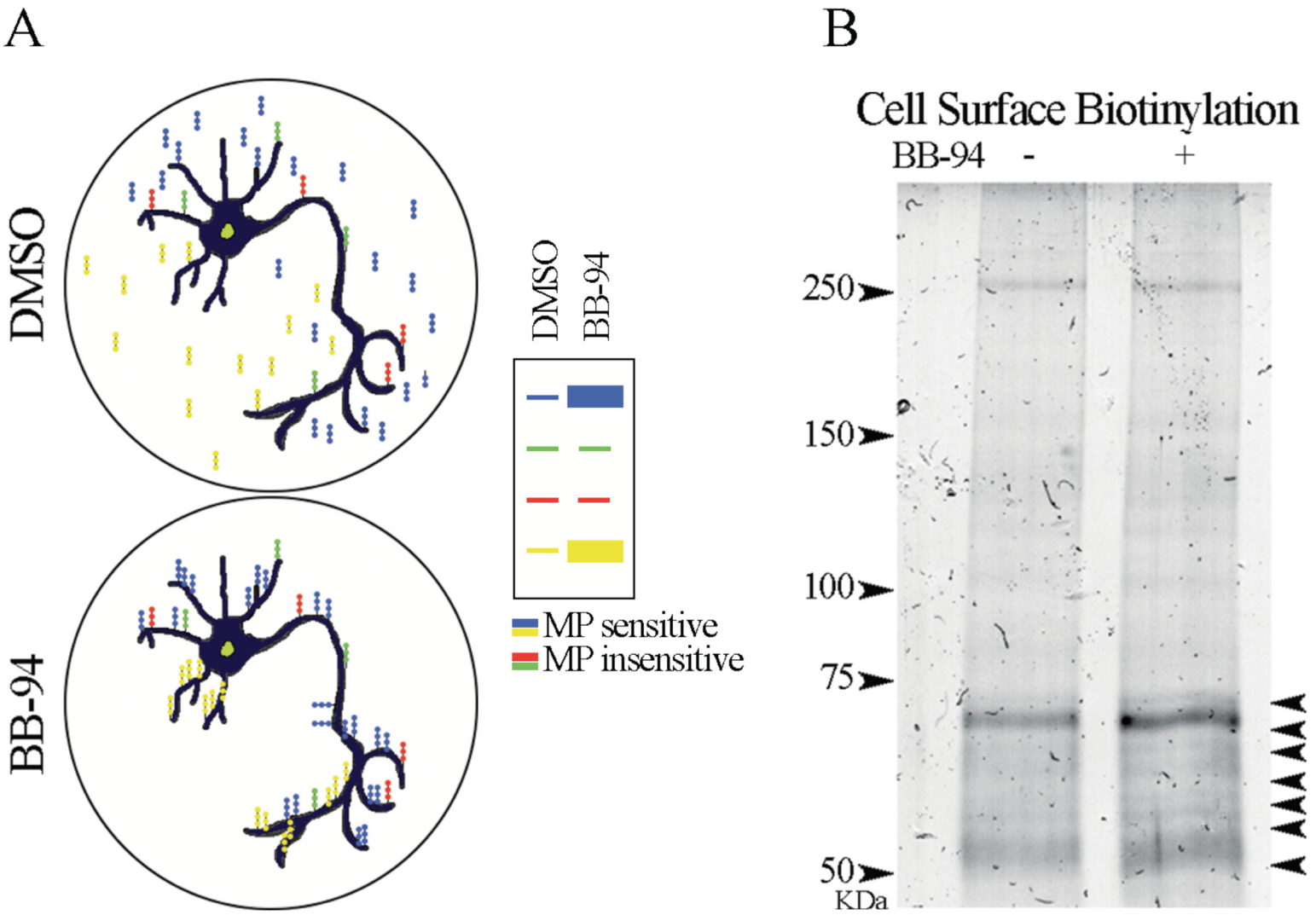
3.7.2 Figure 2: Metalloproteinase inhibitors inhibit growth independently of adhesion or cell death



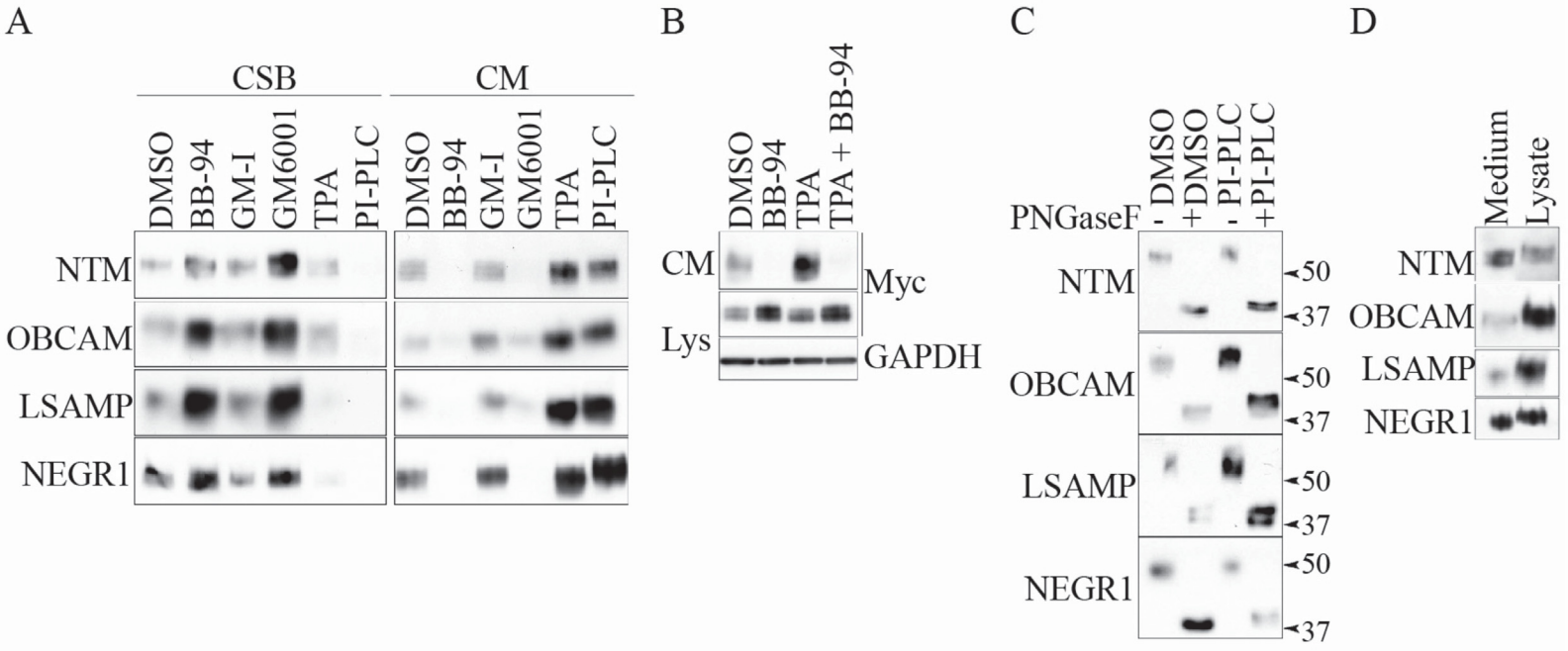
3.7.3 Figure 3: An ADAM family member promotes neurite outgrowth in aged cortical neurons



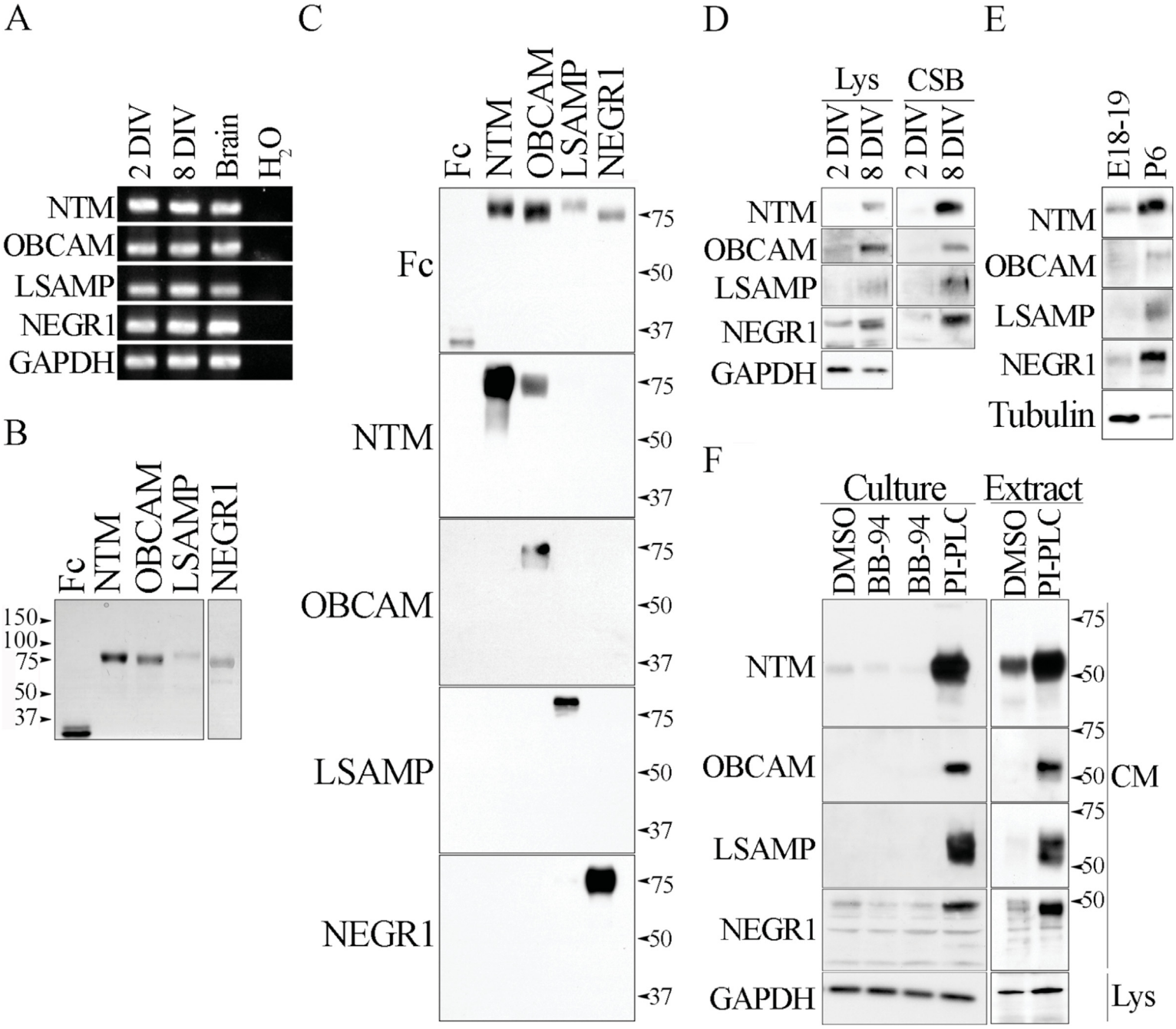
3.7.4 Figure 4: A proteomic analysis in cortical neurons identifies IgLON family members as novel metalloproteinase substrates



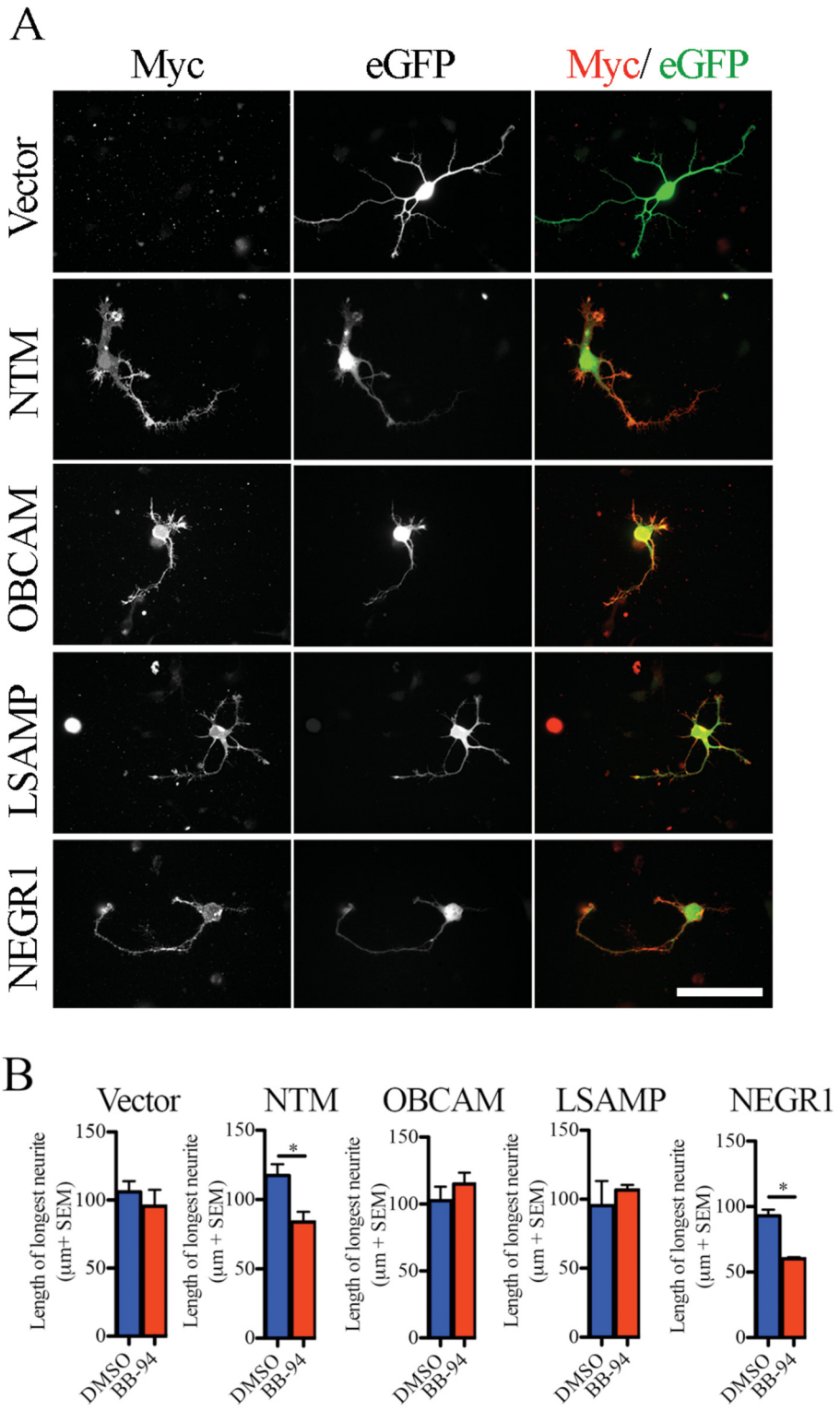
3.7.5 Figure 5: IgLON family members are shed by metalloproteinases



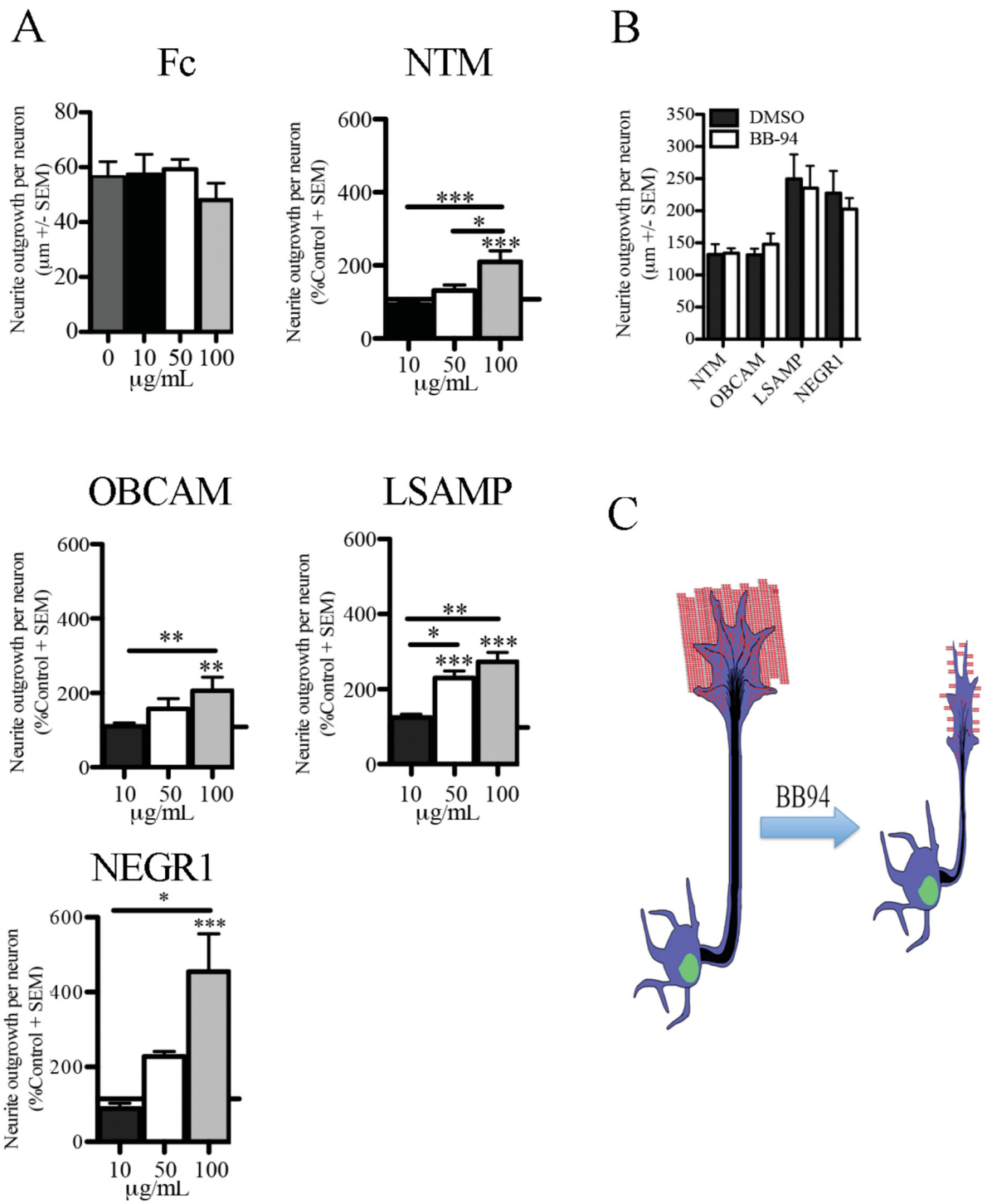
3.7.6 Figure 6: IgLON expression and processing is developmentally regulated in cortical neurons



3.7.7 Figure 7: Surface IgLON expression sensitizes 2DIV cortical neurons to metalloproteinase inhibitors



3.7.8 Figure 8: Immobilized IgLON family members promote neurite outgrowth in cortical neurons



3.7.9 Table1. Mass Spectrometry results

Accession #	Protein name	Unique Peptide #	Reference #
Cell adhesion			
O54800	Cadherin-8	1	
Q63198	Contactin-1	10	
Q9R066	Coxsackievirus and adenovirus receptor homolog	10	(285)
Q6RKB2	Fasciclin II GPI-linked protein isoform	3	
D3ZPC5	Immunoglobulin superfamily member 8	6	(286)
P0CC10	Leucine-rich repeat-containing protein 4B	2	
Q5M960	Limbic system-associated membrane protein	13	
Q1WIM3	Nectin-like protein 1	6	(287)
Q1WIM1	Nectin-like protein 4	10	(288)
Q3T1H3	Neural Cell Adhesion Molecule 1	14	(289)
Q63372	Neurexin-1-alpha	6	(290)
D3ZAJ5	Neuroligin-3	2	(291)
Q9Z0J8	Neuronal growth regulator 1	7	
D3ZDF0	Neuroplastin	9	
Q62718	Neurotrimin	5	
P32736	Opioid-binding protein/cell adhesion molecule	8	
D3Z8D8	SLIT and NTRK-like family, member 1	2	(292)
Q1WIM2	Synaptic cell adhesion molecule 2	8	(142)
Ion conduction			
P24942	Excitatory amino acid transporter 1	6	
P23576	Gamma-aminobutyric acid receptor subunit alpha-2	4	
P19969	Gamma-aminobutyric acid receptor subunit alpha-5	4	
P63138	Gamma-aminobutyric acid receptor subunit beta-2	2	
D3ZW84	Gamma-aminobutyric acid A receptor, beta 3	6	
Q76GL9	Neutral amino acid transporter ASCT1	6	
D3ZE88	Sodium/potassium-transporting ATPase subunit alpha-3	5	
Cell signaling			
P41740	Atrial natriuretic peptide receptor 3	10	
Q78EA7	Bone morphogenetic protein receptor type-1A	5	
Q08406	Ciliary neurotrophic factor receptor subunit alpha	5	
Q10743	Disintegrin and metalloproteinase domain-containing protein 10	2	(293)
Q6P7B6	Ephrin B1	3	(294)
Q8K3V3	G-protein coupled receptor 56	5	
Q80ZC7	GPI-anchored cell-surface protein hyaluronidase 2	3	
Q8K4Y5	Leucine-rich glioma-inactivated protein 1	12	
D3ZJ42	Netrin G2	4	
C6K2K4	Neuropilin and tolloid-like protein 2	8	
Q5XIA1	Nicalin	5	
D3ZES7	Plexin A4	3	
D3ZQ57	Plexin B2	2	
D3ZWP6	Plexin A2	9	
Q8CIN0	Receptor-like protein tyrosine phosphatase gamma S-type isoform	3	
Q99M75	Reticulon-4 receptor	3	(26)
Q80WD1	Reticulon-4 receptor-like 2	7	
Q6IE50	SPARC-related modular calcium binding protein	2	(295)
Q5PQV5	Trophoblast glycoprotein	5	

Preface

IgLONs are members of the immunoglobulin (Ig) superfamily of cell adhesion proteins implicated in the process of neuronal outgrowth, cell adhesion and subdomain target recognition. In the present study, we sought to identify the role of IgLON shedding in the growth of Dorsal Root Ganglion (DRG) sensory neurons. We identify Neurotrimin and LSAMP as members of the IgLON family that are expressed by DRGs and are shed from the surface of DRG neurons in an ADAM-10-dependent manner. Ectopic expression of surface IgLONs and immobilized LSAMP substrates repress neurite extension from DRG neurons. Furthermore, LSAMP loss of function desensitizes DRG neurons to a selective ADAM-10 inhibitor. Together our findings support a mechanism where ADAM10-dependent shedding of surface LSAMP promotes growth in embryonic DRG neurons.

CHAPTER 4

4. Ectodomain shedding of LSAMP by ADAM-10 promotes neurite outgrowth in DRG neurons

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*Running title: IgLON shedding promotes embryonic DRG growth

Keywords: Dorsal root ganglion cell, neurite outgrowth, ADAM10, Neurotrimin

Manuscript in preparation

4.1 ABSTRACT

IgLONs are members of the immunoglobulin (Ig) superfamily of cell adhesion proteins implicated in the process of neuronal outgrowth, cell adhesion and subdomain target recognition. IgLONs form homophilic and heterophilic complexes on the cell surface that repress or promote growth depending on the neuronal population, the developmental stage and surface repertoire of IgLON family members. Previously, we described metalloproteinase-dependent shedding of IgLONs as a mechanism that promotes neuronal outgrowth from cortical neurons. In the present study, we identified a metalloproteinase-dependent mechanism necessary to promote growth in embryonic dorsal root ganglion cells (DRGs). Treatment of embryonic DRG neurons with pan-metalloproteinase inhibitors, tissue inhibitor of metalloproteinase 3, or a selective ADAM-10 inhibitor reduces outgrowth from DRG neurons without affecting cell adhesion or cell survival. The IgLON family members Neurotrimin and LSAMP were identified as two metalloproteinase substrates that are expressed by DRG neurons and are shed from the cell surface. LSAMP alone suppresses outgrowth from DRG neurons when overexpressed or presented as a substrate. Furthermore, LSAMP loss of function decreases the outgrowth sensitivity to a selective ADAM-10 inhibitor. Together our findings support a role for ADAM10-dependent shedding of surface LSAMP in promoting growth from embryonic DRG neurons.

4.2 INTRODUCTION

During development, membrane associated and soluble proteins drive the direction and extent of growing projections. Cell adhesion molecules of the immunoglobulin and cadherin family, integrin, ephrin, neurexin and neuroligin largely modulate axonal growth through growth-promoting and growth-repressive signalling events (296-304). Members of the immunoglobulin superfamily (IgSF), such as NCAM, L1 and TAG-1, support growth and guidance for correct axon targeting (199-201). Most notably, among the IgSF-cell adhesion proteins is the IgLON subfamily. IgLONs are the earliest and most abundant glycosylphosphatidylinositol (GPI)-anchored proteins expressed in the nervous system. Through trans-membrane interactions, they are implicated in the process of neuronal growth, cell adhesion and subdomain target recognition (203,219,275). The IgLON subfamily, comprising Neurotrimin (NTM), Opioid binding cell adhesion molecule (OBCAM), Limbic system-associated membrane protein (LSAMP), and Neuronal growth regulator 1 (NEGR1), is characterized by three Ig-like domains that are attached to the cell membrane through a GPI-anchor moiety. IgLONs exist as dimeric structures capable of repressing or promoting growth, depending on the neuronal population, the developmental stage and surface repertoire of IgLON family members (224,226,230,262,271). Although, IgLONs has been implicated in the growth and adhesion of different neuronal populations, their physiological role remains incompletely understood.

Previously, we identified IgLON members as novel metalloproteinase substrates that are shed from the surface of cortical neurons to promote neurite outgrowth (241). Ectodomain shedding is a key post-translational mechanism necessary for the disassembly of receptor complexes and promoted by members of the metzincin family of metalloproteinases, including matrix metalloproteinases (MMPs) and a disintegrin and metalloproteinases (ADAMs). Genetic and pharmacological manipulations have demonstrated a requirement for metalloproteinases for guidance of axons during development (9,183). Metalloproteinases control receptor levels, activate signaling upon ligand binding and release biologically active, or dominant negative receptor fragments.

By remodeling their receptor levels, axons can switch responsiveness to environmental cues and modulate their outgrowth response.

In the present study, we evaluated the role of IgLON ectodomain shedding in the growth of dorsal rot ganglion cells (DRGs). Through our work, we identify a novel metalloproteinase-dependent mechanism for neurite outgrowth in embryonic DRGs. Treatment of embryonic DRG neurons with pan-metalloproteinase inhibitors, tissue inhibitor of metalloproteinase 3 (TIMP3) or a selective ADAM-10 inhibitor reduces outgrowth from DRG neurons without affecting cell adhesion or cell survival. We identify IgLON family members, Neurotrimin and LSAMP, as two known metalloproteinase substrates that are expressed by DRG neurons and that are shed from the cell surface. IgLONs demonstrate a repressive activity on outgrowth of DRG neurons when overexpressed or presented as a substrate. Furthermore, loss of LSAMP expression attenuates the sensitivity of DRG projections to an ADAM-10 inhibitor. We thus define a role for ADAM10-dependent shedding of surface LSAMP in promoting growth from embryonic DRG neurons.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 Animals -

Timed pregnant (embryonic day 18-19) female and postnatal (P4-6) Sprague Dawley rats were purchased from Charles River Laboratories (Senneville, QC). All animal care and use was in accordance with the McGill University guidelines and approved by the University Animal Care and Use Committee.

4.3.2 Antibodies -

For immunofluorescence, the following antibodies were used: mouse and rabbit anti-tubulin βIII from Covance (1:1000, Princeton, NJ) and mouse anti-Myc from Sigma-Aldrich (1:1000, St. Louis, MO). Alexa-fluor secondary antibodies were purchased from Invitrogen Life Technologies (1:1000, Burlington, ON). For western blot analysis, the following antibodies were used: anti-NTM (1:100, R&D systems, Minneapolis, MN), anti-LSAMP (1:100, R&D systems), anti-OBCAM (1:100, Santa Cruz, Dallas, Texas), anti-NEGR1 (1:100, Santa-Cruz), mouse and rabbit anti-Myc (1:500, Sigma-Aldrich), anti-GAPDH (1:10000, Abcam, La Jolla, CA) and anti-Contactin-2 (TAG-1, 1:500, R&D systems). HRP-conjugated secondary antibodies were purchased from Jackson Immunoresearch.

4.3.3 Plasmids and cloning -

Full-length human cDNA sequences for NTM, OBCAM, LSAMP (OpenBiosystems, Ottawa, ON) and NEGR1 (SinoBiological, Beijing, P.R. China) were subcloned from a Psectag2B vector into an HSV-viral vector, including the IgK leader sequence and the Myc epitope tag. The following primers were used to subclone IgLON proteins: NTM and NEGR1 forward: 5'-GAATCTAGAATGGAGACAGACACACTC -'3'; OBCAM and LSAMP forward: 5'- GAAGTCGACATGGAGACAGACACACTC-3', NTM reverse: 5'- GAAGAGCTCTCAAAATTTGAGAAGCAGGTGCAAGAC-3'. OBCAM reverse: 5'- GAAGAGCTCTCAAAATTTGAGAAGCAGGTGCAAGAC-3', LSAMP reverse: 5'- GAATCTAGATTAACATTTGCTGAGAAGGCAGAG-'3, NEGR1 reverse:

5’- GAAGAGCTCTTATTGTAGAATGGCATTCTTCAGGT-‘3.

The following primers were used to generate GFP-shRNAmir-NTM: NTM-A forward: 5’-TGCTGATAGCTTTGGGAAAGGTGGCAGTTTTGGCCACTGACTGACTGCCACCTCCCAAAGCTAT-3’, NTM-A reverse: 5’-CCTGATAGCTTTGGGAGGTGGCAGTCAGTCAGTGGCCAAAACTGCCACCTTTCCCAAAGCTATC-3’, NTM-B forward: 5’-TGCTGCATTCTGGATCTCAATGCTGTGTTTTGGCCACTGACTGACACAGCATTGATCCAGAATG-3’, NTM-B reverse: 5’-CCTGCATTCTGGATCAATGCTGTGTCAGTCAGTGGCCAAAACACAGCATTGAGATCCAGAATGC-3’, and GFP-shRNAmir LSAMP: LSAMP-A forward: 5'-TGCTGTCTTCTACCACACACCTGAGGGTTTTGGCCACTGACTGACCCTCAGGTGTGGTAGAAGA -3', LSAMP-A reverse: 5'-CCTGTCTTCTACCACACCTGAGGGTCAGTCAGTGGCCAAAACCCTCAGGTGTGTGGTAGAAGAC -3', LSAMP-B forward: 5'-TGCTGTTTGCCTGACTGTTCCCTGGTGTTTTGGCCACTGACTGACACCAGGGAAGTCAGGCAAA -3', LSAMP-B reverse: 5'-CCTGTTTGCCTGACTTCCCTGGTGTCAGTCAGTGGCCAAAACACCAGGGAACAGTCAGGCAAAC -3'.

4.3.4 Recombinant IgLON production -

Generation of IgLON constructs has been previously described (241). Briefly, IgLON constructs were transiently transfected into HEK293T cells (263). Transfected cells were incubated in the serum-free medium, OptiMEM (Gibco, Burlington, ON), and the media were collected 4 days post-transfection. IgLON-Fc proteins were purified by affinity chromatography with protein A Sepharose beads.

4.3.5 DRG neurons -

Embryonic day 18-19 (E18-19) and postnatal day 4-6 (P4-6) rat DRG neurons were dissected in ice-cold Leibovitz (L-15) medium (Gibco). DRG tissue was dissociated in

0.25%Trypsin-EDTA (Invitrogen) and gently triturated. DRG were seeded in culture plates pre-coated with Poly-L-Lysine (100 µg/mL, Sigma-Aldrich,). Culture medium consisted of Neurobasal (Gibco), 1%B27 (Gibco), 50µg/mL penicillin-streptomycin (Gibco), 2mM L-glutamine (Gibco) and 50nM NGF.

4.3.6 Outgrowth assays -

For outgrowth assays with pan-metalloproteinase inhibitors, ADAM10 inhibitor, TIMPs and virus infection, DRG neurons (20 000 neurons/ cm^2) were seeded in a 96-well plate previously coated with poly-L-lysine (PLL). After 2hrs, the medium was replaced to culture medium enriched with pan-metalloproteinase inhibitors, Batimastat (BB-94; 5µM, Tocris, Ellisville, Missouri), Ilomastat (GM6001; 20µM, CALBIOCHEM, San Diego, CA), Ilomastat-negative control (GM-I; 20µM, CALBIOCHEM), ADAM10 inhibitor (GI254023X, 5µM, Tocris), endogenous metalloproteinase inhibitors (TIMP1, TIMP2, TIMP3; 20µg/mL, R&D) and HSV viruses at an MOI of 1. After 24hrs, DRG neurons were fixed in 4% PFA, 20% sucrose in PBS for 30min. Neurons were blocked in 5%BSA and 0.2%Triton X-100 in PBS solution for 1hour and stained for βIII-tubulin antibody and Hoechst 33342 stain (Sigma-Aldrich).

Outgrowth assays with immobilized recombinant IgLON proteins were previously described (241). Briefly, IgLON-Fc proteins in PBS solution were coated in a 96-well plate for 3hrs. DRG neurons were seeded in IgLON-Fc coated wells, fixed and immunostained after 24hrs. Fluorescent images were automatically acquired and analyzed through the MetaXpress software on an ImageXpress system.

For NTM land LSAMP loss-of-function, DRG neurons at 3DIV were infected with corresponding lentivirus at an MOI 10 for 4hrs. After 8DIV, DRG neurons were reseeded on PLL coated 96-well plate for outgrowth assays.

4.3.7 Apoptosis assay -

Apoptosis was detected as previously described using a commercially available In Situ Cell Death Detection Kit (Roche Applied Science, Laval, QC), following the manufacturer's instructions. DRG neurons were counterstained with anti-βIII-tubulin and

Hoechst 33342 and imaged using the ImageXpress system microscope. The percentage of TUNEL positive neurons was determined using the MultiWavelength Cell Scoring module of MetaXpress.

4.3.8 RT-PCR -

To assay IgLON expression in DRG neurons, RNA was isolated from dissociated embryonic and postnatal DRGs cultures, followed by a reverse transcriptase polymerase chain reaction (RT-PCR). Rat IgLON primers have been previously described (241).

4.3.9 Immunochemistry -

Shedding experiments were conducted on brain and DRG extracts prepared from rat at embryonic E18 and postnatal day 4-6. Briefly, dissected tissue was homogenized in ice-cold homogenizing solution (1mM $NaHCO_3$, 0.2mM $CaCl_2$, 0.2mM $MgCl_2$ at pH7) and centrifuged to dispose of any residual tissue. Supernatants were collected and centrifuged for 45min at 25000g. The pellet was resuspended in Neurobasal medium supplemented with DMSO, PI-PLC and pan-metalloproteinase inhibitor (BB-94). After 5hrs, the medium was centrifuged for 1hr at 100000g. Supernatants were collected and analyzed by western blotting.

4.3.10 Statistics -

For multiple comparisons, 1-way ANOVA followed by a Bonferroni post-hoc's test were performed using GraphPad Prism software.

4.4 RESULTS

4.4.1 Pan-metalloproteinase inhibitors repress growth in embryonic DRG neurons

Metalloproteinases are a family of zinc endopeptidases ubiquitously expressed during embryonic development and capable of remodeling the extracellular matrix and the cell surface proteome. Previously, we identified ADAM family members to be necessary for the growth of mature cortical neurons (241). In the present study, we evaluated the role of metalloproteinases in the growth of dorsal root ganglion cell projections. We first examined the outgrowth performance of sensory DRG cells from embryonic (E18-19) and postnatal (P4-6) stages in the presence of pan-metalloproteinase inhibitors for 24hrs. BB-94 and GM6001 mediate a ≈50% reduction in the growth of embryonic DRG neurons (Fig. 1a-c). The outgrowth inhibition observed with pan-metalloproteinase inhibitors decreases at postnatal day 4-6, indicating a potential developmental switch in the ability of metalloproteinases to promote DRG growth. To determine whether pan-metalloproteinase inhibitors indirectly repress growth through a decrease in cell viability or cell adhesion, we quantified the number of neurons and apoptotic neurons following treatment with BB-94 and GM6001. The presence of metalloproteinase inhibitors did not affect the ability of neurons to adhere to the substrate. Further analysis with TUNEL TMR-red staining revealed that neither BB-94 nor GM6001 affected neuronal viability (Fig. 2a-d). Metalloproteinases therefore mediate a pro-neurite outgrowth function from embryonic DRG neurons that is directly suppressed by pan-metalloproteinase inhibitors.

4.4.2 ADAM-10 protease activity is necessary to promote growth in embryonic DRG neurons

To investigate the type metalloproteinase responsible for promoting embryonic DRG growth, we performed outgrowth assays in the presence of endogenous zinc-metalloproteinase inhibitors. Tissue inhibitors of metalloproteinases (TIMPs) are a family of endogenous metalloproteinase inhibitors that differ in their substrate selectivity. TIMP1 represses the activity of secreted MMPs, TIMP2 represses all matrix metalloproteinases and TIMP3 represses metalloproteinases and some ADAM family

members. When embryonic DRG neurons were exposed to recombinant TIMP3, but not TIMP1 nor TIMP2, the DRG outgrowth was significantly reduced. Similar to the pan-metalloproteinase inhibitors, TIMP3 had no effect in cell adhesion or in cell viability (Fig. 3a-d). Previous reports have identified ADAM-10 as an ADAM family member predominantly expressed in embryonic DRG neurons and relatively absent in aged DRGs (305). To examine ADAM-10 proteolytic activity in the growth of DRG projections, we performed outgrowth experiments in embryonic DRG neurons treated with GI254023X, a selective ADAM-10 inhibitor (305), and BB-94 as a positive control. As previously reported, GI254023X phenocopies the repressive effect of BB-94, without affecting cell adhesion or cell survival (Fig. 3f-h). We conclude that ADAM-10 is a protease that promotes growth of embryonic DRG neurons.

4.4.3 NTM and LSAMP are expressed and predominantly shed from the surface of embryonic DRG neurons by ADAM-10

Previously, we identified IgLON family members as novel metalloproteinase substrates that promote growth in mature cortical neurons (271). To evaluate the role of IgLON shedding in DRG outgrowth, we first examined the presence of IgLON family members. We assayed the RNA from embryonic (E18-19) and postnatal (P4-6) DRGs followed by reverse-transcription polymerase chain reaction (PCR) and detected NTM, OBCAM, LSAMP and NEGR1 in both developmental populations (Fig. 4a). Similarly, NTM, LSAMP and NEGR1 proteins were present in embryonic and postnatal DRG lysates, whereas OBCAM was undetectable (Fig. 4b).

To examine the processing of IgLON family members, the conditioned media from DRG membrane extracts was analyzed for deposition of IgLON shed fragments. Membranes were treated with PI-PLC to cleave all GPI-anchored proteins as a positive control. Shedding of all IgLON family members was detectable following treatment with PI-PLC, whereas NTM was the sole family member that was detected in neurons in the control condition (DMSO treated). The weak OBCAM, LSAMP and NEGR1 bands detected in the PI-PLC condition raised the possibility that these family members are in fact shed but are below the level of detection for these antibodies. To explore this issue, we analyzed

conditioned media from dissociated DRGs infected with Myc-tagged IgLONs. All four IgLON family members were detected with an anti-Myc antibody in conditioned media following PI-PLC treatment (Fig. 4d). NTM and LSAMP were released from untreated membranes (DMSO) and this release was reduced upon treatment with the ADAM-10 inhibitor GI254023X (Fig. 4d). Shedding of OBCAM and NEGR1 was weak to undetectable unless membranes were treated with PI-PLC. When membranes were probed with anti-IgLON antibodies we found that the anti-NTM and anti-NEGR1 antibodies were robust whereas anti-OBCAM and anti-LSAMP antibodies weakly detected their target antigens and are likely not sensitive enough to detect endogenous shedding (Fig. 4d). We conclude that NTM and LSAMP are endogenously shed from the surface of embryonic DRG neurons.

4.4.4 LSAMP represses growth in embryonic DRG neurons

IgLON family members form cis- and trans-membrane IgLON-complex interactions. In mature cortical neurons, IgLONs are shed from the cell surface depositing a growth permissive IgLON substrate (241). To test if shed NTM and LSAMP may also promote the outgrowth of DRG neurons, embryonic DRG neurons were exposed to immobilized recombinant IgLON proteins. Contrary to their role in cortical neurons, immobilized NTM failed to promote neurite outgrowth, while immobilized LSAMP represses growth and adhesion in a dose-dependent manner (Fig. 5a, b). The repressive role of LSAMP led us to ask if cell surface IgLON proteins may limit outgrowth of DRG neurons and if metalloproteinase-dependent outgrowth inhibition could be ascribed to stabilization of IgLON complexes on the cell surface. To test this scenario, IgLONs were introduced into embryonic DRG neurons. We analyzed transduced neurons in the absence of permeabilization by immunofluorescence to validate cell surface expression of the IgLONs (Fig. 6a). The expression of NTM, LSAMP and NEGR1, but not OBCAM, restricted the outgrowth of DRG neurons without affecting their ability to adhere to the substrate (Fig. 6b-d). Together, this suggests that metalloproteinase inhibitors may suppress DRG outgrowth by stabilizing inhibitory IgLON complexes on the cell surface.

4.4.5 LSAMP loss of function desensitizes DRG neurons to ADAM10 inhibitor

To evaluate the implication of surface IgLONs to the repressive outgrowth phenotype with pan-metalloproteinase inhibitors, we knockdown NTM and LSAMP, which are strongly expressed in DRGs, and assayed the outgrowth response in the presence or absence of GI254023X. NTM and LSAMP shRNA with a microRNA stem (shRNAmir) were introduced in embryonic DRG neurons. After 8 days, transduced DRGs were reseeded and exposed to DMSO or GI254023X for 24hrs. We validated the efficiency of NTM and LSAMP knockdown by western blotting (Fig. 7a-d). The loss of LSAMP expression, but not NTM, desensitized DRG projections to GI254023X treatment (Fig. 7h). All together, LSAMP is necessary to mediate the repressive outgrowth effect of ADAM-10 inhibitor in DRG neurons.

4.5 DISCUSSION

Ectodomain shedding is an important post-translational mechanism that can destabilize a surface receptor complex, could desensitize a cell to a ligand, or could generate biologically active fragments. In the present study, we identified a member of the ADAM metalloproteinase subfamily, ADAM-10, as an important mediator of neurite outgrowth in sensory DRG neurons during embryonic development (Fig. 1). Other groups have reported ADAM-10 to promote growth in retinal ganglion cells and cerebellar ganglion cells by processing cell adhesion proteins N-Cadherin (306) and L1 (195), respectively. Previously, we reported the ADAM family of metalloproteinases as potential mediators of IgLON processing (241). In here, we characterized the IgLON family of cell adhesion proteins to be shed from the surface of embryonic DRGs by ADAM-10 to facilitate neurite outgrowth. Through reverse transcription polymerase chain reaction and analysis of protein lysates from embryonic DRG ganglia, we identified two IgLON family members, NTM and LSAMP, robustly expressed in DRG neurons (Fig. 4a, b). In shedding experiment from transduced DRGs, only NTM and LSAMP fragments were strongly detected in the conditioned media and reduced in the presence of GI254023X (Fig. 4d). This may reflect limited stability for NEGR1 and OBCAM upon their release from the cell surface, or a limited affinity for ADAM-10 proteolytic activity.

In a previous publication, we proposed two potential mechanisms for IgLON proteolysis in neurite outgrowth: IgLON shed fragments could provide a growth permissive substrate, or could relieve an outgrowth inhibitory signal from the cell surface (241). In DRG neurons, our data supports the latter mechanism. The outgrowth sensitivity to GI254023X could result from enrichment of a cell surface complex that limits the extension of DRG neurons (Fig. 1, 3). Accordingly, NTM, LSAMP and NEGR1 introduced in embryonic DRG neurons mimic the outgrowth inhibition with GI254023X (Fig. 6), implying that accumulation of surface IgLONs represses neurite outgrowth. In addition, substrate-bound LSAMP, but not NTM, inhibits DRG outgrowth (Fig. 5). A hierarchy for IgLON interactions has previously been reported. In a cell-based ELISA assay, LSAMP expressing CHO cells exhibit the strongest binding with soluble NTM-Fc

and OBCAM-Fc (224), suggesting that immobilized LSAMP may have a unique capacity to form IgLON heterophilic complexes and stabilize IgLONs expressed on the neuronal surface to limit growth. Similarly, loss of surface LSAMP expression, but not NTM, desensitized DRG projections to GI254023X, supporting a mechanism where an outgrowth inhibitory signal is generated by the accumulation of LSAMP at the cell surface. Other groups have reported no outgrowth defect in E16 chick DRG neurons overexpressing NTM, or seeded on immobilized IgLON substrates (225). Furthermore, cortical neurons exhibit a growth promoting effect, while the growth of cerebellar ganglion cells is repressed in the presence of immobilized IgLON substrates (224,241). Discrepancies between experiments might result from the stage of neuronal development, different neuronal populations and a different repertoire of surface IgLONs. Alternatively, ectodomain shedding of surface IgLON could have different outgrowth mechanisms in different animal or cellular models.

In the nervous system, IgLONs are important mediator of axon patterning and fine connectivity. Anti-LSAMP antibodies have been reported to induce aberrant Timm-stained fibers throughout the CA3 region of the hippocampus (307), and to significantly decrease the outgrowth of dopaminergic neurons towards the habenula (230). Human genetic studies have found associations between LSAMP allelic polymorphisms and mood dysfunction, panic disorder, male suicide and schizophrenia (214,308), implying its importance in the formation of the brain circuitry. Furthermore, LSAMP deficient mice display hyperactivity in novel environments and reduced anxiety-like behaviors (309). IgLONs are also characterized as tumor suppressor genes. NEGR1, OBCAM and LSAMP expressions are absent in clear renal cell carcinoma, epithelial ovarian cancer and in osteosarcomas, while restoring their expression decreases the rate of cell proliferation and tumor cell growth *in vivo* (309,310). Based on our experiments, we hypothesize that IgLONs act as endogenous repressors of neurite outgrowth in DRG neurons and IgLON ectodomain shedding might relieve this outgrowth inhibition. It is noteworthy that loss of LSAMP expression did not affect the baseline DRG outgrowth (Fig. 7g). We reason that at embryonic stages, the expression levels of endogenous LSAMP might be insufficient to repress neurite outgrowth, and only in the presence of

metalloproteinase inhibitors, surface accumulation of unprocessed LSAMP might attain levels that limit growth. Future directions will evaluate the outgrowth performance of postnatal DRG neurons deficient of LSAMP expression.

Furthermore, metalloproteinase inhibitors selectively inhibited the outgrowth of embryonic DRG neurons, while postnatal DRG neurons were unaffected (Fig. 1a-c). The developmentally regulated response of DRG neurons is consistent with previous reports analyzing the expression of several ADAM family members in DRG ganglia (305). In the course of development, embryonic day 18-20 peripheral DRG projections have reached their target destination in the hindlimb and forelimb, while central DRG projections have entered the spinal cord. Proteolytic cleavage of IgLON family members could titrate DRG outgrowth and could have critical roles in subdomain targeting and synaptogenesis. In addition, ADAM-10 activity decreases during the first week of neonatal life preceding the process of myelination. Intriguingly, LSAMP has recently been described as a negative regulator of CNS myelination (221). Of great interest would be to examine role of IgLON proteolysis and soluble Ecto-IgLON fragment in the proliferation, maturation and migration of Schwann cells.

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Conflict of interests: The authors declare no competing financial interests.

4.6 FIGURE LEGENDS

4.6.1 Figure 1: Pan-metalloproteinase inhibitors repress neurite outgrowth in embryonic rat DRG neurons

A. Dorsal root ganglion cells from two developmental stages (E18-19 and P4) seeded on Poly-L-lysine (PLL, 100µg/mL) coated 96-well plate and treated with a vehicle control (DMSO), metalloproteinase inhibitors (BB-94 and GM6001), and a GM6001-Inactive (GM-I) control for 24hrs. Neuronal projections were visualized with βIII tubulin. B. Outgrowth quantification from E18-19 and P4 DRG neurons. Outgrowth was normalized to DMSO control for each experiment. N=4 from independent cultures. Data shown as mean + S.E.M. ***, $p<0.001$ by one-way ANOVA, followed by Bonferroni post hoc test. Scale bar, 100µm.

4.6.2 Figure 2: Pan-metalloproteinase inhibitors inhibit growth independently of adhesion or cell death

A. TUNEL assay was performed in embryonic DRG neurons (E18-19) seeded on PLL substrate and treated with a vehicle control (DMSO), metalloproteinase inhibitors (BB-94 and GM6001) and GM-I. Neurons were stained with anti-βIII tubulin, Hoechst and TUNEL. As a positive control for the TUNEL assay, neurons were pretreated with a DNA nuclease. B-D. Total number of neurons and apoptotic neurons present after indicated treatments. For TUNEL, the data are shown as a percentage of TUNEL-positive neurons. For total number of neurons, data have been normalized to control DMSO. N=4 from independent cultures. Data are shown as mean + S.E.M. Scale bar, 100µm.

4.6.3 Figure 3: ADAM-10 proteolytic activity is necessary for embryonic DRG growth

A. Embryonic DRG neurons seeded on a PLL substrate and treated with control (H_2O), or endogenous metalloproteinase inhibitors (TIMP1-3). Neuronal projections were visualized with βIII-tubulin staining. B. Neurite outgrowth quantification of embryonic DRG neurons exposed to soluble recombinant TIMPs and control conditions. Data have

been normalized to control condition. C. TUNEL assay was performed in embryonic DRG neurons treated with TIMPs or H_2O control. DNA nuclease was used as a positive control. Data are shown as a percentage of TUNEL-positive neurons. D. Number of neurons present on the PLL substrate after treatment with soluble TIMPs and control condition. E. Embryonic DRG neurons exposed to DMSO as a control and a selective ADAM-10 inhibitor, GI254030X. F-H. Neurite outgrowth, cell adhesion and percentage TUNEL-positive neurons exposed to GI254030X and BB-94. For TUNEL, the data are shown as a percentage of TUNEL-positive neurons. For total number of neurons, data have been normalized to control DMSO or H_2O. N=3-4 from independent cultures. Data are shown as mean + S.E.M. **, $P<0.01$ by one-way ANOVA followed by Bonferroni post hoc test. Scale bar, 100μm.

4.6.4 Figure 4: NTM and LSAMP are expressed and shed from the surface of embryonic DRG neurons by ADAM10

A. Reverse transcription PCR (RT-PCR) was performed in DRG neurons from two developmental stages (E18-19 and P4). Brain RNA was used as a positive control and H_2O as a negative control. GAPDH RNA expression was used as a loading control. B. Lysates from embryonic (E18) and postnatal (P4) DRG neurons probed for IgLON family members. Expression of IgLON family members was determined by western blot using commercially available IgLON antibodies. GAPDH was used as a loading control. C. Conditioned media from membrane-extracts from (E18-19) DRG exposed to PI-PLC, as a positive control. Commercially available IgLON antibodies were used to detect cleaved IgLON fragments in the media. D. Specificity of commercially available IgLON antibodies to detect shed IgLON fragments was validated by western blot. Conditioned media from dissociated embryonic (E18-19) DRG neurons expressing Myc-tagged IgLON constructs were probed with commercial anti-IgLON antibodies and anti-Myc antibodies.

4.6.5 Figure 5: Immobilized LSAMP substrate represses neurite outgrowth and cell adhesion in embryonic DRG neurons

A-C. Outgrowth and cell adhesion quantification of embryonic DRG neurons seeded on

immobilized Fc-IgLON substrates (10, 50 and 100µg/mL). Data were normalized to control Fc substrate. N=4 from independent cultures. Data are shown as mean + S.E.M. *, P<0.05, ***, P<0.001 by one-way ANOVA followed by Bonferroni post hoc test.

4.6.6 Figure 6: Ectopic surface NTM, LSAMP and NEGR1 repress neurite outgrowth in embryonic DRG neurons

A. Embryonic (E18-19) DRG neurons seeded on a PLL substrate and expressing HSV-GFP and HSV-Myc-tagged IgLON constructs. Surface Myc-IgLON expression was detected with anti-Myc antibody in the absence of permeabilization. B. Number of infected neurons present on the PLL substrate. C. Total number of neurons present on the PLL substrate. D. Outgrowth quantification of embryonic (E18-19) DRG neurons expressing HSV-GFP or HSV-Myc-IgLON constructs on PLL substrates. Data were normalized to control HSV-GFP. N=6 from independent cultures. Data are shown as mean + S.E.M. *, P<0.05, ***, P<0.001 by one-way ANOVA followed by Bonferroni post hoc test. Scale bar, 50µm.

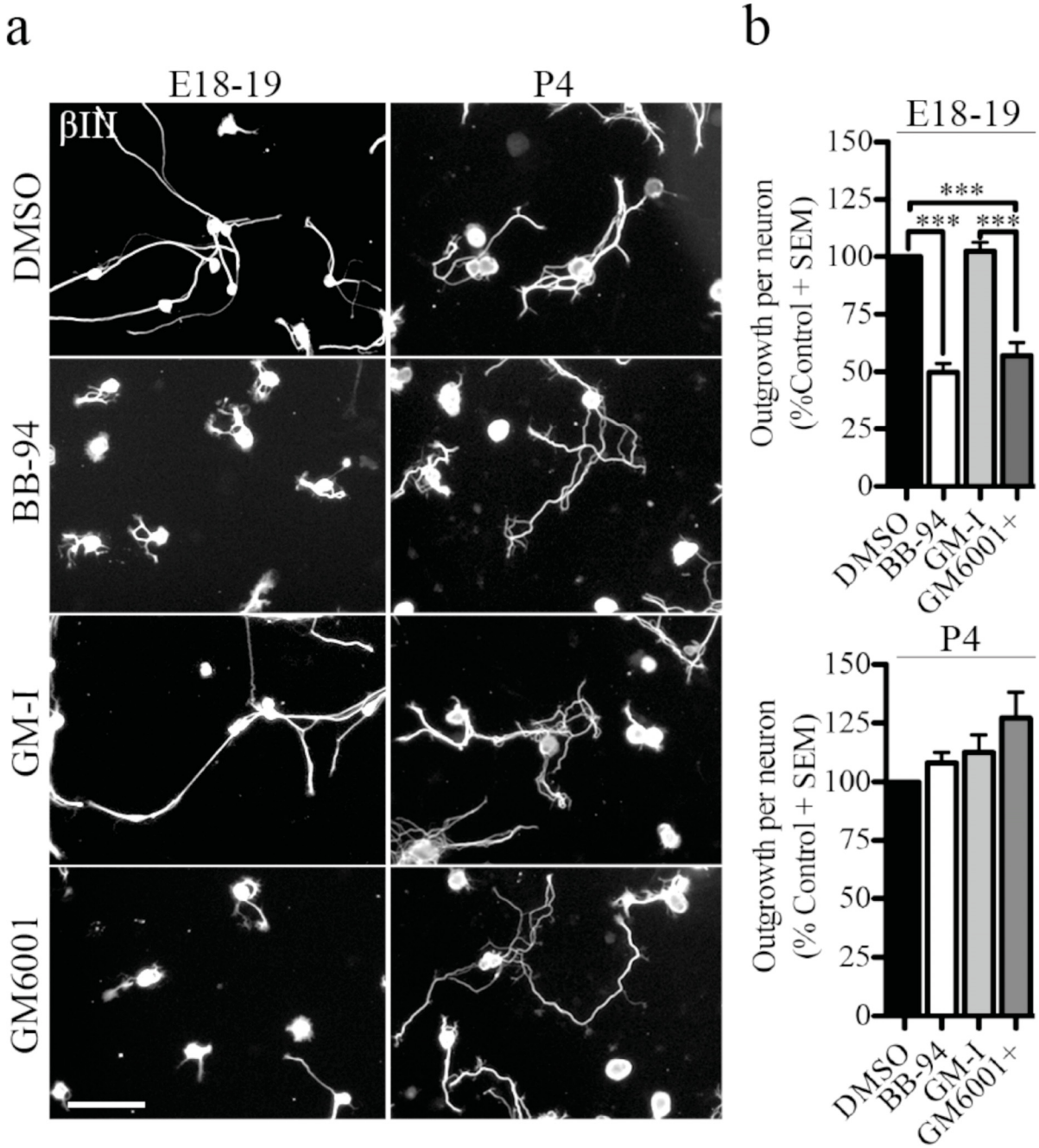
4.6.7 Figure 7: LSAMP loss of function desensitizes DRG neurons to ADAM-10 inhibitor

A-D. Conditioned media from embryonic cortical neurons expressing control lentivirus, NTM-shRNAmir (NTM-A and NTM-B) or LSAMP-shRNAmir (LSAMP-A and LSAMP-B) for 8days and treated with PI-PLC for 1hr. Commercially available IgLON antibodies were used to validate the knockdown efficiency by western blotting. E. Embryonic DRG (e18-19) neurons infected with control GFP-lentivirus, NTM-shRNAmir or LSAMP-shRNAmir for 8days and reseeded on PLL coated plates. Neuronal projections were visualized with βIII tubulin and nuclear stain Hoechst. F-H. Mean outgrowth measurements from reseeded embryonic DRG neurons expressing GFP, NTM-shRNAmir or LSAMP-shRNAmir and exposed to DMSO or GI254023X. In figure H, outgrowth was normalized to DMSO control for each experiment. N=3 from independent cultures. Data shown as mean + S.E.M. **, p<0.01, ***, p<0.001 by one-way ANOVA, followed by Bonferroni post hoc test. Scale bar, 100µm.

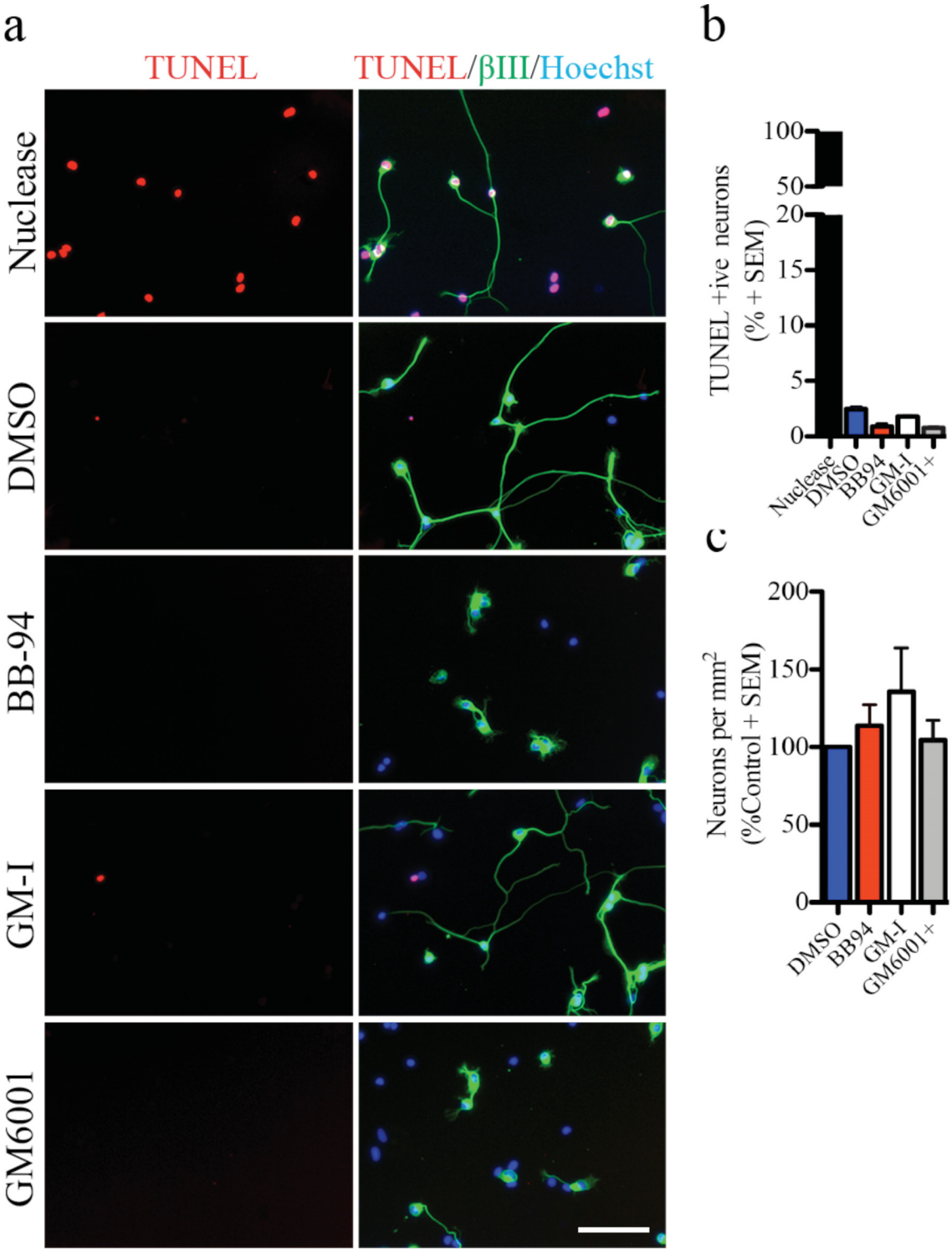
4.6.8 Figure 8: Ectodomain shedding of surface LSAMP promotes growth

A schematic representation of IgLON function in DRG neurite outgrowth. Surface IgLON shedding by ADAM-10 releases a growth inhibitory signal and promotes neurite extension. Metalloproteinase inhibitors accumulate unprocessed IgLON family members at the cell surface and possibly stabilize and activate unknown IgLON receptors, resulting in outgrowth repression.

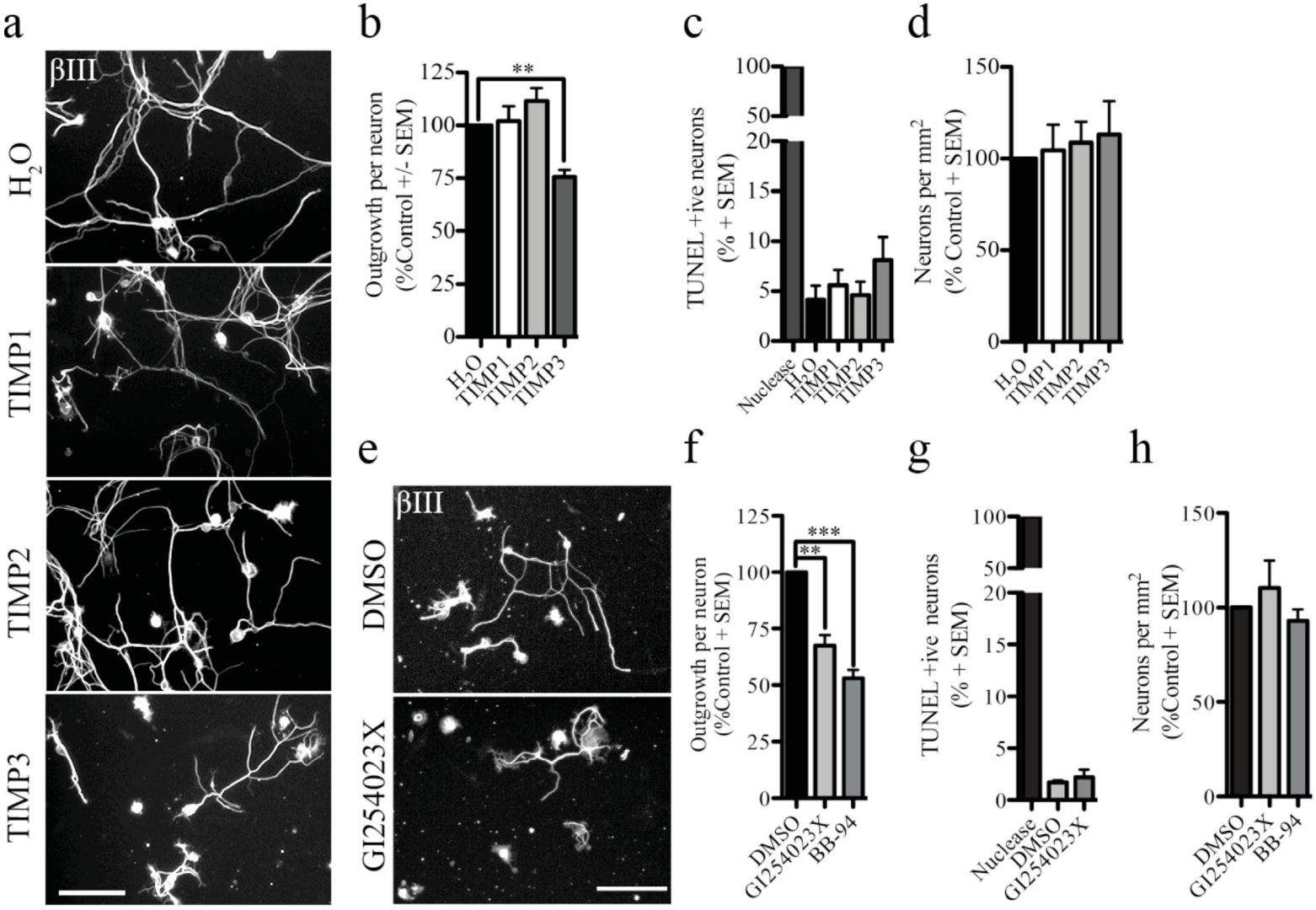
4.7.1 Figure 1: Pan-metalloproteinase inhibitors repress neurite outgrowth in embryonic rat DRG neurons



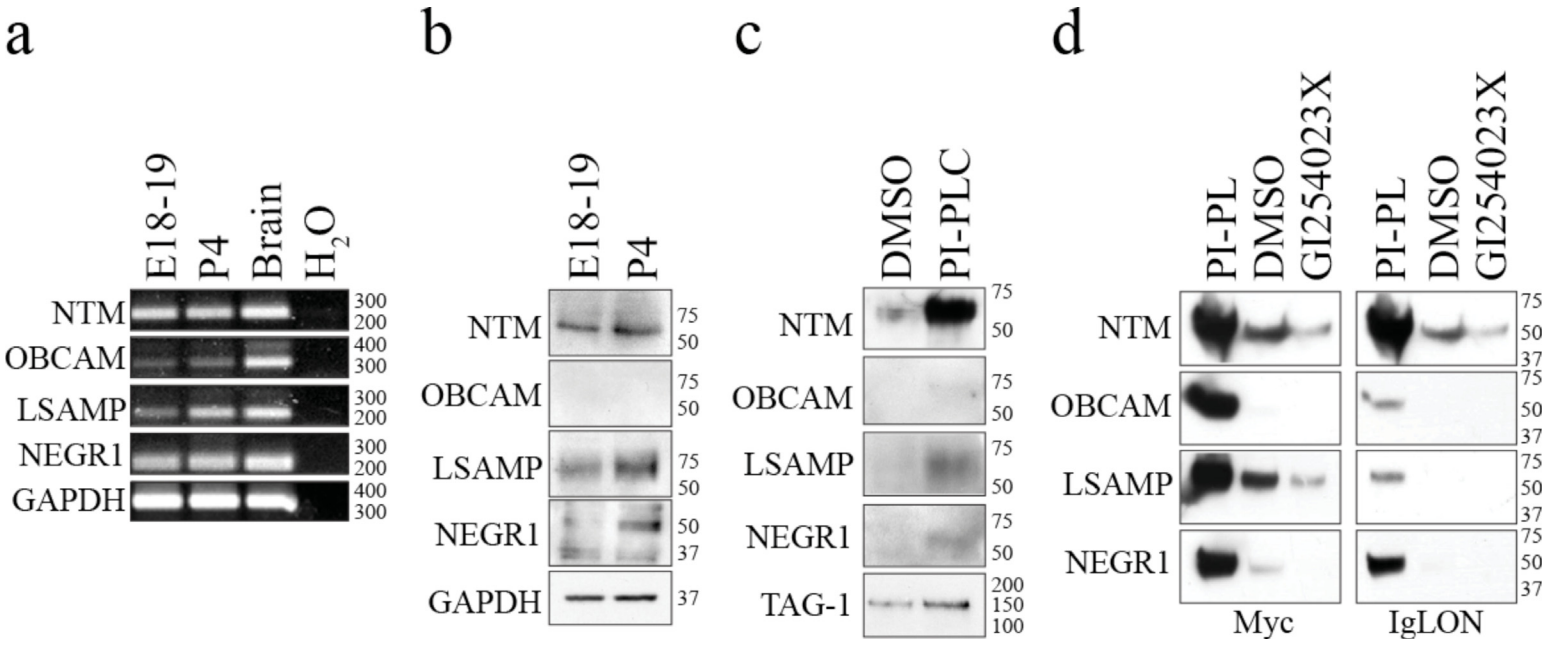
4.7.2 Figure 2: Pan-metalloproteinase inhibitors inhibit growth independently of adhesion or cell death



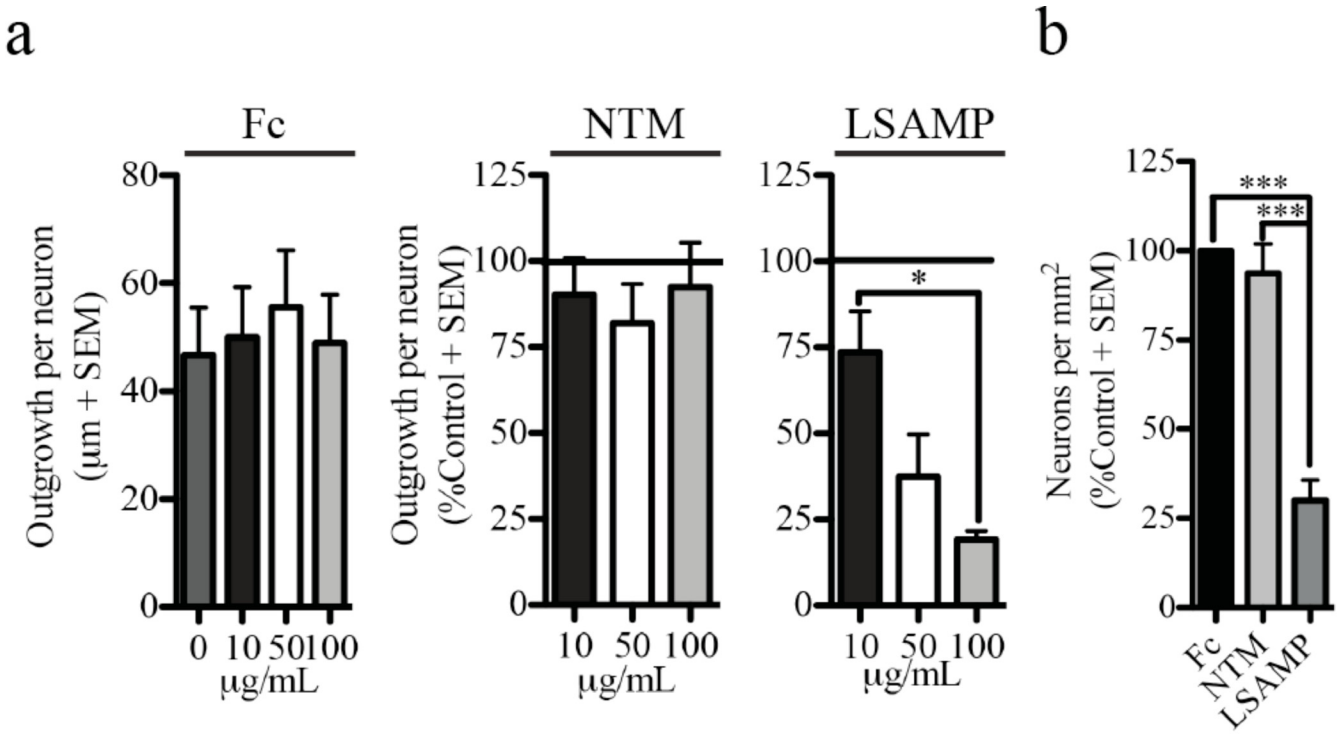
4.7.3 Figure 3: ADAM-10 proteolytic activity is necessary for embryonic DRG growth



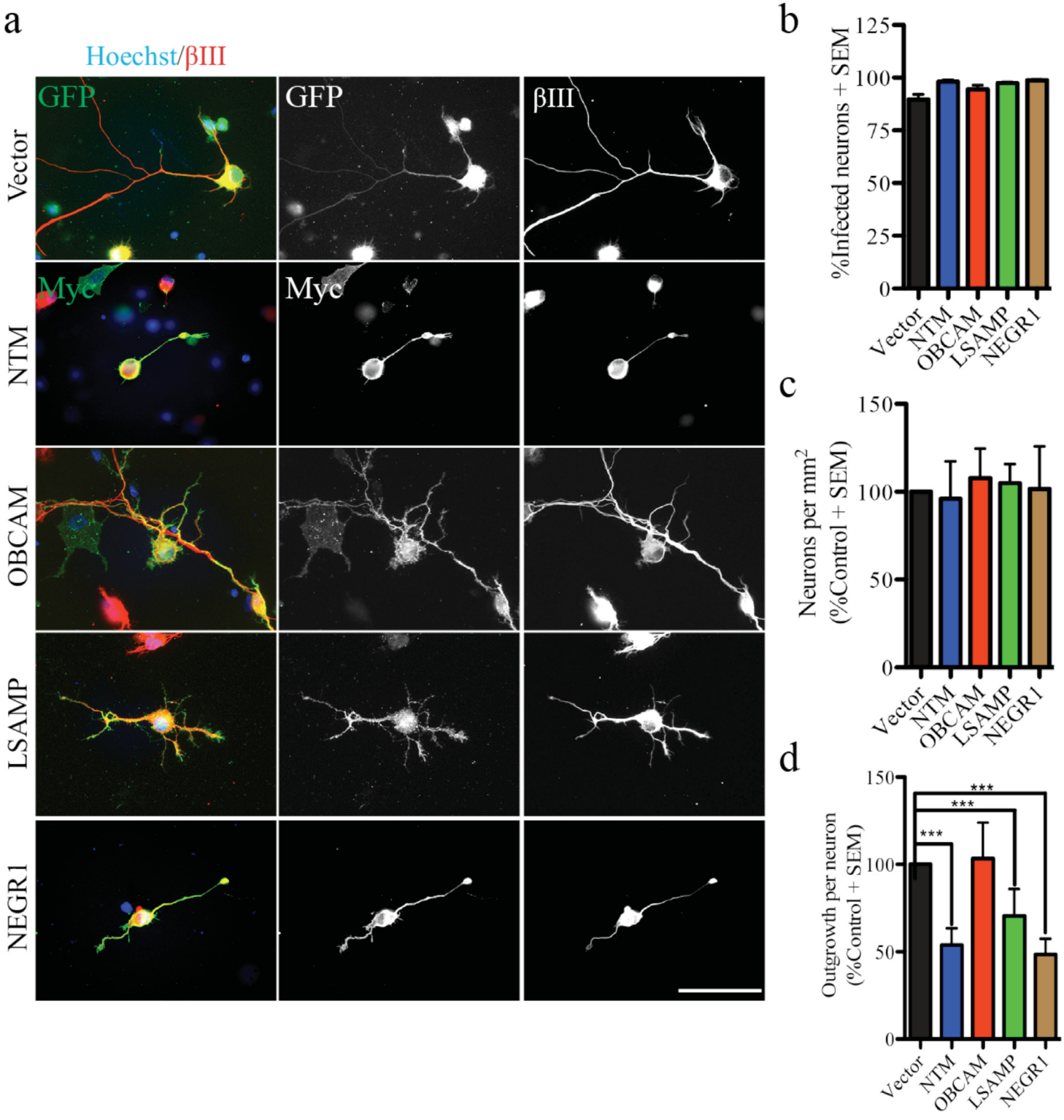
4.7.4 Figure 4: NTM and LSAMP are expressed and shed from the surface of embryonic DRG neurons by ADAM-10



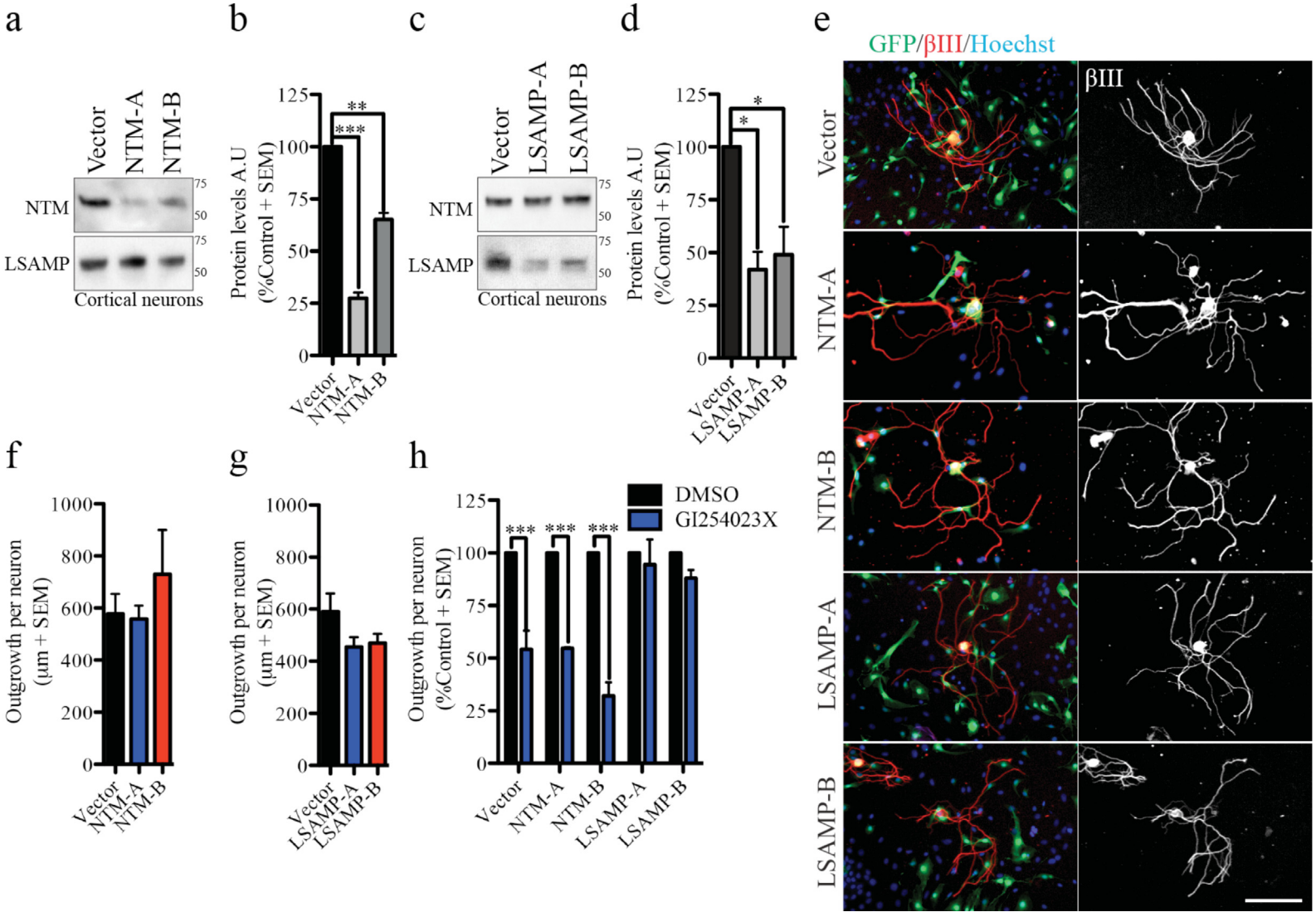
4.7.5 Figure 5: Immobilized LSAMP substrates repress neurite outgrowth and cell adhesion in embryonic DRG neurons



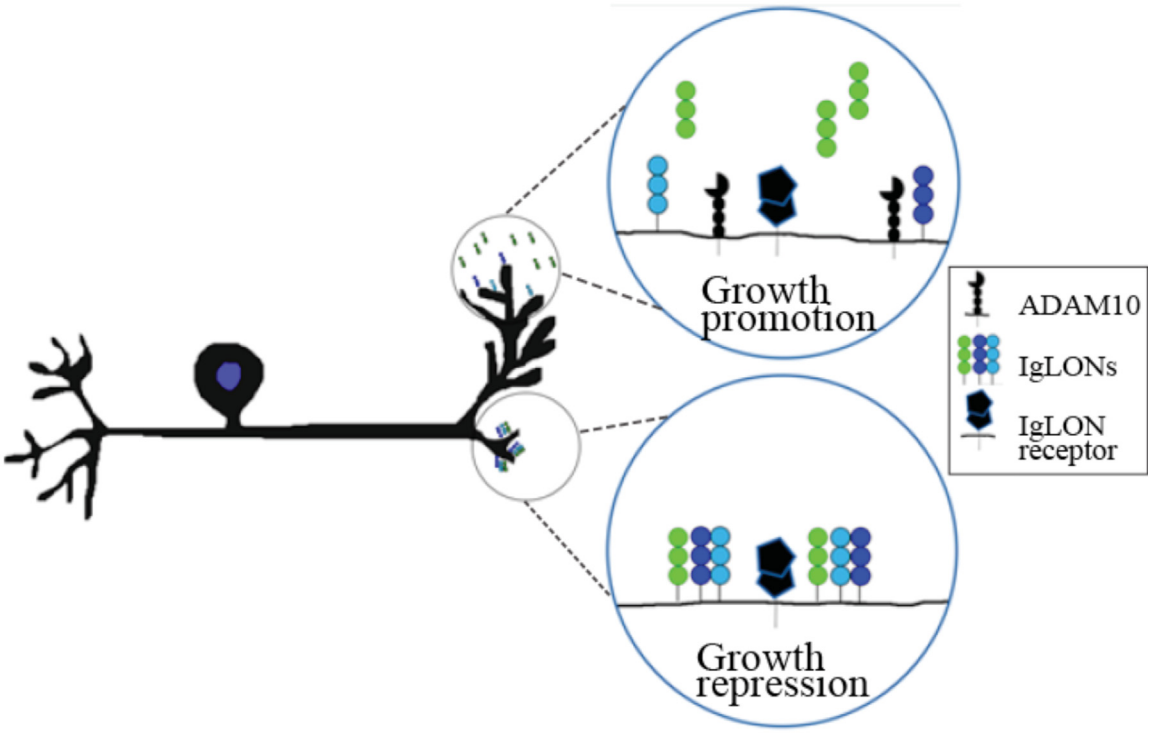
4.7.6 Figure 6: Ectopic surface NTM, LSAMP and NEGR1 repress neurite outgrowth in embryonic DRG neurons



4.7.7 Figure 7: LSAMP loss of function desensitize DRG neurons to ADAM-10 inhibitor



4.7.8 Figure 8: Ectodomain shedding of surface LSAMP promotes DRG growth



DISCUSSION AND CONCLUSION

5.1 Summary of results

In the present thesis dissertation, we sought to characterize novel roles for metalloproteinases in neuronal development. Metalloproteinases target components of the extracellular matrix and cell surface proteome and release biologically active or dominant negative receptor fragments that remodel the brain circuitry. In the chapters presented, we described members of the membrane bound metalloproteinase subfamily and previously uncharacterized metalloproteinase substrates, implicated in the process of synaptogenesis and neurite outgrowth.

5.1.1 Nogo receptor shedding -

In Chapter 2, we evaluated the spatiotemporal expression of transmembrane MT-MMPs in the mouse cerebrum and identified MT3-MMP and MT5-MMP as proteinases present during the process of synaptogenesis and in regions that exhibit synaptic plasticity in the adult brain. Metalloproteinases and proteolytic activity have previously been observed in close apposition to synaptic structures. In here, the absence of proteolytic activity and the selective loss of MT3-MMP expression strongly decreased the density and structural components of excitatory cortical synapses, suggesting a novel function in excitatory synaptic development. What could be the contribution of MT3-MMP activity in the development of excitatory synapses? Temporal downregulation of MT3-MMP during different stages of postnatal development, followed by synaptic counts from electron micrographs would distinguish the function of MT3-MMP in synapse formation, synapse maturation or synapse maintenance. Furthermore, a detailed examination of the number and morphology of dendritic spines in MT3-MMP deficient mice will further define the physiologic role for MT3-MMP in the formation of excitatory synaptic connections.

Previously, the Fournier group explicitly identified MT3-MMP as the physiological sheddase for surface NgR1 in cortical and DRG neurons. A growth cone collapse assay, in which recombinant MT3-MMP was exposed to postnatal (P4) rat DRG neurons

demonstrated that NgR1 cleavage is an endogenous mechanism that attenuates the response of growing axons to soluble myelin (259). Through shedding experiments from synaptosomes, neuronal cultures and membrane extracts, we presently identified synaptic NgR1 as a downstream effector of MT3-MMP proteolytic activity. While NgR1 cleavage increases the density of excitatory synapses, the necessity of NgR1 processing in synapse formation, synapse stabilization, synapse strengthening, or spine turnover remains unknown. Introduction of cleavage resistant NgR1 species into the NgR1 knockout background would further contribute to our understanding of NgR1 proteolysis in the development of excitatory synapses. Time-lapse imaging of dendritic spines expressing thrombin-cleavage NgR1 species will provide insight into the role of NgR1 shedding in dendritic spine turnover. In the barrel cortex, NgR1 has recently been observed to limit GLUR1 surface trafficking (174). Furthermore, in acute hippocampal slices loss of NgR1 inhibits LTD, while the NgR1-MAI signalling axis has been reported to restrict LTP (150). Of great interest would be to challenge MT3-MMP deficient mice to similar electrophysiological experiments. The lack of MT3-MMP activity, and consequently an increase in unprocessed synaptic surface NgR1, could sensitize neurons to myelin-associated inhibitors and could alter neuronal communication by decreasing surface GLUR1 and LTP. Other groups have reported a deficit in long-term memory formation in an overexpressed NgR1 mouse model (173). Based on our experiments, we speculate that synaptic MT3-MMP deficiency could phenocopy the defects of NgR1 overexpression in memory formation, by increasing unprocessed NgR1 surface levels. Furthermore, MT3-MMP is strongly expressed in the cerebellum and the dentate gyrus of the hippocampus, supporting roles for MT3-MMP in memory formation and motor coordination. Challenging MT3-MMP deficient mice to the Morris water maze, or to a rota-rod would shed some light in the contribution of MT3-MMP in spatial memory and motor learning.

In addition, sensory deprivation experiments in MT3-MMP deficient mice would provide some insights into the contribution of MT3-MMP activity and NgR1 shedding in activity-dependent circuitry remodelling. Several groups have identified NgR1 as an important mediator for the closure of the critical period and the transition to more restricted adult-like connections (171,172). In crude membrane extracts from the cerebral cortex, we

observed NgR1 shedding during the first week of neonatal life and a gradual increase throughout postnatal development (P5-P30) (Unpublished data). Late NgR1 shedding was also observed in the primary visual cortex, as well as in mature cultures of dissociated cortical neurons (Unpublished data). Why would NgR1 shedding increase so late in development? One possible explanation is the late expression of MAIs and CSPGs that signal through NgR1 to refrain synaptic plasticity (250). MT3-MMP proteolytic activity might provide a neuronal mechanism to remodel inhibitory complexes present on the cell surface. Furthermore, NgR1 shedding might compensate for the inhibitory cues that accumulate in the synaptic microenvironment. Therefore, the presence of synaptic NgR1 might be important for the stability of the brain connectivity, while NgR1 proteolysis might confer levels of plasticity important for higher cognitive processes and memory formation. An interesting experiment would be to examine whether adult mice infused with Ecto-NgR1 (1-358) fragments recover from monocular deprivation. The success of the experiment would provide evidence for the role of NgR1 shedding in synaptic plasticity and support a new treatment for neurological disorders, such as Amblyopia.

Recently, NgR1 has been observed to regulate the plasticity of inhibitory synapses. Conditional NgR1 knockouts in Parvalbumin interneurons increase the ability to suppress fear memories through a rise in inhibitory synaptic connections (250). While in Chapter 1 we focused on excitatory synapses, the contribution of surface NgR1 remodelling in inhibitory synaptic plasticity remains to be determined. The Strittmatter group recently reported the ability of NgR1 (1-310) to promote erasure of fear memories in mice (250). The presently described Ecto-NgR1 fragment (1-358) might enhance inhibitory synaptic plasticity and provide a new treatment for post-traumatic stress disorder by repressing fear memories. Synaptic counts from neuronal cultures labelled with inhibitory synaptic markers in the presence of Ecto NgR1 fragments might provide some preliminary insights into the function of NgR1 proteolysis in inhibitory synaptogenesis. Furthermore, fear-conditioning experiments in MT3-MMP deficient mice could elucidate the function of MT3-MMP and NgR1 shedding in the acquisition and erasure of fear memories.

Lastly, NgR1 shedding could have a function in other developmental processes. Myelination is one of the last processes that occur during development to ensure proper neuronal connectivity. Myelin is an insulating sheath produced by oligodendrocytes in the CNS and Schwann cells in the PNS that surrounds axons and nerve cells (311). In the mature CNS, the number of internodes and myelinated fibers continuously change in response to synaptic activity (312-315). Interestingly, components of the NgR1 signalling pathway, such as: NgR1, $p75^{NTR}$, Lingo-1 and MAIs, are strongly expressed on the surface of oligodendrocytes (316-322). Furthermore, different groups have reported changes in the myelogenic ability of oligodendrocytes by interfering with the NgR-MAI signalling axis. Polystyrene beads coated with Nogo-A reduced oligodendrocyte myelinating potential, while a soluble Lingo-Fc construct promoted oligodendrocyte differentiation and myelination (320,321,323). Hypermyelination and enhanced remyelination is also observed in Nogo-A deficient mice (317). Therefore, a development increase in NgR1 shedding, reported in Chapter 2, might interfere with NgR1-signaling on the surface of oligodendrocytes and strengthen the communication of newly formed synapses. The ability of Ecto-NgR1 fragments to enhance myelination could provide a potential therapeutic for neurodegenerative disorders, such as Multiple Sclerosis.

One of the most challenging aspects of neural repair is the abortive regenerative response of damaged axons within the adult mammalian CNS. It is now well established that a developmental decline in growth promoting genes and deposition of inhibitory factors at the injury site prevent outgrowth and functional CNS recovery (324-326). Soluble Ecto-NgR1 (1-358) fragments could neutralize the vast number of inhibitory cues present in the injured extracellular milieu and promote functional regeneration. However, the role of NgR1 in other cellular systems needs to be fully addressed before Ecto-NgR1 (1-358) could become a potential treatment for axon regeneration. For instance, immune cells that infiltrate the injury site to phagocyte myelin and axonal debris also express NgR1, NgR2 and TROY receptors. The NgR-MAI signaling axis restricts the entrance of immune cells into the injury site, preventing their infiltration into uninjured neighboring spaces. A combination of Ecto-NgR1 (1-358) and anti-inflammatory drugs would be required to preserve the benefit of repressing NgR1-MAI signaling in axon regeneration.

5.1.2 IgLON shedding -

In Chapter 3, we sought to characterize the role of metalloproteinase in the context of inhibitory cues present in the injured CNS. The potential of metalloproteinases to promote neurite regrowth was initially suggested for their ability to degrade inhibitory structural components of the ECM. For example, pre-treatment of CSPG-containing substrates with recombinant MT5-MMP alleviates the DRG neurite outgrowth inhibition *in vitro* (327). Furthermore, treatment of nerve sections with recombinant MMP-2 degrades CSPGs, exposing laminin and promoting regrowth of seeded DRG neurons *in vitro* (328). MMP-2 and MMP-9 increase their levels of expression during peripheral nerve degeneration, suggesting that degradation of CSPGs by metalloproteinase activity could promote neurite outgrowth (22). As a result, proteolysis of ECM components enhances neurite outgrowth by degrading areas of dense inhibitory molecules and exposing growth promoting signals (289,329,330). Metalloproteinases are also capable of processing inhibitory surface proteins modulating the interaction of growing axons with the environment. For instance metalloproteinase activity attenuates the inhibitory effect of MAIs. MT1-MMP and MMP-2 process Nogo-A, while MMP-7 and MMP-9 cleave MAG. Also, metalloproteinases are capable of downregulating the presence of NgR1 and $p75^{NTR}$ in neuronal populations including retinal ganglion cells (RGCs), cortical, hippocampal and DRG neurons, suggesting a potential mechanism to suppress neuronal sensitivity to inhibitory CNS environment (259,331,332).

Given the ability of metalloproteinases to remodel the cell surface proteome, we hypothesized that proteolytic cleavage should desensitize neurons to growth inhibitory substrates. Interestingly, we identified members of a family of cell adhesion proteins that regulate the extent of baseline neurite outgrowth through a metalloproteinase-dependent mechanism. How do IgLON family members repress neurite outgrowth? The field of cancer research has provided some insights into the molecular mechanism of IgLON receptor signaling. In cancer, IgLON are characterized as tumor suppressor genes that regulate surface expression of tyrosine kinase receptor (TKR), including FGFR-1, FGFR-3, EPHA-2, HER-2 and HER-4 (333-335). Normally, cell surface IgLONs form complexes with TKRs, inducing receptor-complex endocytosis and attenuating

downstream signaling from these receptors. In the nervous system, soluble NEGR1 was recently identified to promote neurite arborization by activating a pathway that requires FGFR-2 and ERK1/2 phosphorylation (228). As a potential mechanism, surface IgLON proteolysis might promote growth through regulated stability of FGF receptors and increased sensitivity to growth factors present in the environment. The levels ERK1/2 phosphorylation could be assayed in aged cortical neurons overexpressing or deficient of surface IgLONs. Furthermore, immunoprecipitation assays with recombinant IgLON family members, followed by mass spectrometry could identify potential IgLON targets that might regulate the process of neuronal outgrowth. Subsequently, the ability of immobilized IgLONs to promote growth could be challenged in aged cortical neurons from mice deficient of the IgLON receptor. Lastly, the sensitivity to metalloproteinase inhibitors could be evaluated in outgrowth assays from cortical neurons overexpressing mutant IgLON species unable to bind to an IgLON receptor.

In Chapter 4, we further examined the mechanistic function of IgLON proteolysis in neurite extension. Previous studies have uncovered the expression of NTM, and the growth promoting effect of NTM substrates in embryonic DRG projections (227). Based on previous findings, we sought to examine whether a metalloproteinase-dependent mechanism also regulated the outgrowth performance in DRG neurons. Similar to the cerebral cortex, we reported IgLON family members to be expressed and shed from the surface of embryonic DRGs. Other groups have recently identified ADAM-10 as a protease responsible for surface NEGR1 proteolysis in cortical neurons (228). In our experiments, the ability of GI254023X to repress growth and deposition of IgLON shed fragments in the conditioned media further confirmed ADAM-10 as the protease most likely responsible for DRG growth. Furthermore, the developmental decrease in response to metalloproteinase inhibitors correlated with the expression of ADAM family members. In DRG neurons, ADAM-10 activity decreases during the first week of neonatal life preceding the process of myelination (305). Why would ADAM-10 activity decrease during postnatal development? A temporal decline in ADAM-10 activity might provide a mechanism to enrich surface proteins that stabilize the neuronal circuitry. Interestingly, a decrease in ADAM-10 proteolytic activity correlates with a developmental decline in

NGF sensitivity and expression of the NGF receptor (TrkA) in DRG neurons (336,337). Loss of ADAM-10 activity and surface accumulation of IgLON family members might decrease surface TrkA, thus decreasing sensitivity to NGF and limiting neurite growth. Through immunoprecipitation assays, future directions should address the interaction between TrkA and IgLONs. In a gain or loss of IgLON function, the levels of surface TrkA could be examined in dissociated DRG neurons. These experiments could also be performed in the presence of pharmacological inhibitors of the endocytic pathway. Furthermore, IgLON species unable to interact with TrkA could be implemented to examine the role of IgLON proteolysis in neurite outgrowth in the presence of GI254023X. The effect of cleavage resistant IgLONs or mutant IgLONs unable to bind to TrkA could also be explored by measuring the length and trajectory of in-utero electroporated DRG projection in the spinal cord and their ability to reach and innervate the skin.

Noteworthy, several differences between cortical and DRG neurons were identified for the IgLON shedding mechanism in neurite outgrowth. For instance, only two IgLON family members, NTM and LSAMP were strongly expressed in the DRG cell surface and protein lysates. Metalloproteinase inhibitors selectively affected the outgrowth of embryonic DRG neurons, while embryonic cortical neurons were unaffected. In shedding experiment from transduced DRGs, only NTM and LSAMP were strongly detected in conditioned media by western blotting. Furthermore, ectopic expressions of surface NTM, LSAMP or NEGR1 and outgrowth assays on LSAMP substrates repress baseline growth in the absence of metalloproteinase inhibitors. Discrepancies in the outgrowth response might result from the repertoire of IgLON family members or IgLON receptors endogenously expressed in cortical and DRG neurons. Alternatively, ectodomain shedding of surface IgLON could have different outgrowth mechanisms depending on the neuronal population.

The findings reported in Chapter 3 and Chapter 4 support a conserved mechanism in the central and peripheral nervous system, where IgLON proteolysis titrates growth by releasing an outgrowth inhibitory signal transduced from the cell surface. Why would

surface IgLONs repress neuronal extension? Enrichment of IgLON family members at the cell surface might stabilize the brain circuitry by minimizing the growth of neuronal branches. Regulated surface IgLON proteolysis might selectively direct neuronal projections to subdomain regions. Furthermore, cleaved Ecto-IgLON fragments might provide a growth permissive substrate for mature cortical neurons by destabilizing surface IgLON receptor complexes. The generation of cleavage resistant IgLON family members, or a detailed examination of axonal projections in IgLON deficient mice would provide a better understanding into the role of IgLON family members in neurite growth.

Furthermore, IgLON proteolysis could regulate different developmental processes. For instance, IgLONs have previously been implicated in the process of synaptogenesis (277,338). While in our studies we did not characterize the synaptic function of IgLON proteolysis, surface accumulation of IgLONs or IgLON shed fragments might stabilize neuronal contacts necessary for establishing in novo synapses. Synaptic counts from neurons overexpressing or downregulating IgLON family members, treated with soluble IgLON fragments or co-cultured with IgLON-expressing cell lines might contribute to the function of IgLONs in synaptogenesis. In addition, IgLON family members are also present in Schwann cells and oligodendrocytes. Recently, IgLONs have been reported to repress myelination in CNS fibers and regulate Schwann cell migration and proliferation (220,221). Surface IgLON proteolysis might restrain glial cells from differentiating or myelinating neurite tracks, while enrichment of IgLON in the neuronal surface might provide a substrate for myelinating cells. Co-culture experiments with neurons and glial cells and treatment with soluble IgLON recombinant proteins would increase our understanding into the role of IgLON family members and IgLON proteolysis in myelination.

All together, metalloproteinases are capable of regulating the presence of extracellular proteins having a role in restricting neurite regrowth and synaptic plasticity. Furthermore, it is becoming clear that surface remodeling alters the communication between growing axons and their environment, becoming a potential mechanism to reform damaged connections and treating neurological disorders in the adult CNS.

5.2 Conclusions

Understanding the molecular pathways involved in the repressive nature of the mature the nervous system is critical to making targeted and effective interventions to facilitate recovery after injury, treat neurological disorders and enhance learning and memory in healthy individuals. Our work in the thesis established members of the metalloproteinase subfamily, MT3-MMP and ADAM-10, as novel regulators of synaptic plasticity and neurite outgrowth and demonstrate the important role they play in NgR1 and IgLON signaling activity. We demonstrate how surface proteolysis provides spatial and temporal control to mechanisms that are responsible for circuitry maturation. Our work provides new insights into the mechanisms that restrict excitatory synaptic connectivity and growth, and indicates new pathways of investigation for potential therapeutic interventions after nervous system damage, developmental or neurodegenerative disorders, or enhancing learning in the developing or adult nervous system.

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