2008

The Role of Matrix Metalloproteinases in Axon Guidance and Neurite Outgrowth

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The Role of Matrix Metalloproteinases in Axon Guidance and Neurite Outgrowth

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Joint Degree of Doctor of Medicine and Master of Health Science

By

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2008

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THE ROLE OF MATRIX METALLOPROTEINASES IN AXON GUIDANCE AND NEURITE OUTGROWTH. Lu Anne Velayo Dinglasan (Sponsored by Charles Greer and Helen Treloar). Dept. of Neurosurgery, Yale University School of Medicine, New Haven, CT.

Axons navigating the complex environment of the developing CNS use extracellular guidance cues to help find their correct synaptic target. Matrix metalloproteinases (MMPs), a family of zinc-dependent proteolytic enzymes, have been shown to regulate axon guidance by degrading extracellular matrix (ECM) or by cleaving guidance cues and their receptors. The olfactory system is an excellent model for studying the role of MMPs in axon guidance due to its capacity for continuous nerve regeneration and topographic maintenance during synaptic targeting. I hypothesized that MMPs may play a role in guiding olfactory sensory neurons to their correct glomerular target by sculpting the ECM and influencing axon interactions with the environment. To investigate this, I used RT-PCR to screen 19 members of the MMP family and their four endogenous inhibitors (tissue inhibitors of metalloproteinases, TIMPs) and performed immunohistochemistry to localize candidate MMP and TIMP proteins. Two MMP sub-families, the gelatinases and the membrane-bound MMPs (MT-MMPs) showed distinctive spatio-temporal expression patterns across different stages of olfactory development, which were consistent with their having a role in axon pathway formation. To assess gelatinases in their active form, I performed *in situ* zymography and found restricted patterns of proteolytic activity within the developing olfactory nerve. Finally, to study the role of MMPs in pathway formation, I applied active recombinant MT-MMPs to common ECM molecules found in the developing olfactory system, such as tenascin and proteoglycans, and examined subsequent changes in neurite outgrowth. The inhibitory effects of these substrata were decreased with enzyme treatment, with MT-MMPs having different substrate specificities and degradation efficiencies that allow for increased neurite outgrowth in culture. Collectively, the data suggest that MMPs are active in the developing olfactory system and have a role in axon guidance and neuronal pathway formation.

Acknowledgements

On a rainy afternoon in the winter of 2003/04, I soggily walked into the office of Dr. Charles Greer, knowing that he had a lab in the department of neurosurgery, a very long and powerful-sounding title, and thinking that I had little chance of being accepted to do a summer research project. What transpired that afternoon and in the subsequent five years has been nothing short of amazing, as I was not only accepted into the lab, but welcomed into a wonderful family where I have not only received unparalleled academic and intellectual support, but have also made life-long friends.

To Charlie, thank you so much for giving me a chance. You have challenged me and guided me both academically and professionally, and your leadership and teaching inspire me to become the type of scientist and thinker that you are. I know your incredible support and faith in me has opened doors I never dreamed would be accessible to me. Thank you also to Helen, who guided me in the day-to-day minutia with extraordinary patience and provided wonderful insight and knowledge throughout my HHMI year. You saved my ship from sinking when disaster stuck, and blew wind into my sails when I was mightily discouraged; this project was built on the foundation of your support, and I thank you for seeing me through it.

To Arundhati, an unfailing friend and sounding board throughout my HHMI year. How I will miss seeing you in the mornings! You are my scientific co-conspirator, the person from whom I can ask any question without fear of judgment (no matter how stupid the question may be), you are my fashion guru, my cheerleader, and most of all, my friend. Wherever fate takes us, whether on the East Coast, Europe, or even India, I know that I can always count on you. Thank you also to Diego--- what an amazing person and teacher you are. You have provided me invaluable insight and allowed me to

laugh and persevere in times of disappointment. A word of thanks also to Alex, whose generous, hilarious, and supportive nature makes her a great colleague and friend. To Dolores, you are truly “The One and Only Professor”. Thank you to you and Chris for being so kind and taking care of the innumerable details that make lab life easier. Lorena and Fumi, you are amazing stalwarts whose work ethic inspires me and whose genuine concern made the lab an even more warm and inviting place to work. Mary and Ali, you guys were great to have as resources and as fellow medical students in the trenches. And to Arie and Marion, thank you for continuing to make lab a fun and interesting place to be.

I also want to thank my parents for their constant love and encouragement. Their incredible, innumerable sacrifices allow me to have a precious education and pursue my dreams. You inspire me and drive me to achieve greater heights, and I am where I am today because of you. To Kelvin, thank you for being an enduring life partner and an unwavering pillar of strength and support. You challenge me to be better as an academician, environmentalist, student, caregiver, future physician, and friend, and I am a better person for it.

And finally, to God be the glory and praise. In Him alone I find purpose and strength, and I know that there is something greater than myself guiding my footsteps and lighting my path.

1 Introduction

1.1 Introduction

During the establishment of the nervous system, axons traverse long distances to reach their eventual target. While many factors mediate this process, it is clear that the extracellular matrix and molecular guidance cues play an important role. Recently, it has been shown that a family of zinc-dependent enzymes, matrix metalloproteinases, contributes to axon extension and guidance and the establishment of the central nervous system.

1.2 Axon guidance

1.2.1 Growth Cones

As growing axons navigate the complex environment of the developing nervous system, the leading edge of the axon, known as the growth cone, detects and responds to different cues [1]. These environmental cues can attract or repel the growth cone, thereby changing the direction of growth [1] and causing the growth cone to accelerate, pause, collapse, retract or bifurcate as dictated [2]. At the tip of the growth cone are web-like lamellipodia [3], composed of a cross-linked network of actin filaments that are associated with microtubules in the distal shaft of the axon [1]. Depending on the signal, actin polymerization or depolymerization occurs, thereby reconfiguring the cytoskeleton and allowing the growth cone to advance or retract [1]. This actin assembly and disassembly is coordinated by the Rho family of GTPases [1]. Axon guidance cues and receptors play a direct role in the cytoskeletal dynamics, as it has been shown that guidance receptors can be directly or indirectly coupled to guanine nucleotide exchange

factors (GEFs) or GTPase activating proteins (GAPs), the molecules that regulate the activity of the Rho GTPases [1].

1.2.2 Guidance Cues

The cues that modulate cytoskeletal dynamics include long-range diffusible cues and local cues restricted to cell membranes or the extracellular matrix [4]. In 1979, Gunderson and Barrett first demonstrated that growth cones could project to a diffusible factor by showing chick dorsal-root axons turning toward a point source of nerve growth factor (NGF) [5]. Other *in vitro* studies have demonstrated neuronal growth toward specific tissue explants thought to contain chemoattractant diffusible factors. For example, trigeminal ganglia grow robustly toward explants of maxillary arch, whereas when grown alone they did not demonstrate any outgrowth [6]; similarly, when spinal commissural axons are cultured with alar plate and floor plate (FP) explants, they preferentially grow toward the FP, suggesting the presence of a diffusible chemoattractant from the FP [7]. However, the FP has also been shown to secrete chemorepellants [4]. Axons from the alar plate and basal plate in the mesencephalon maintain their ipsilateral projections after initially being directed towards the midline, suggesting a chemorepulsive effect of the FP near the dorsal midline [8]. This same chemorepulsive effect has also been demonstrated in trochlear motor axons, which are repelled by FP explants *in vitro* [9].

Apart from longer-range diffusible cues, local cues also play a role in growth cone guidance [4]. For instance, midbrain dopaminergic neurons grow rostrally under the regulation of polarized cues in the substratum; when grown with a substratum of reversed rostrocaudal polarity, these axons turn in the opposite direction and follow the polarity of

the substratum [10]. Potential polarizing molecules include the Ephrins, the ligands of Eph receptors [4]. Expressed along the rostrocaudal axis in the optic tectum [4], it aids in the formation of the retinotectal map via gradient expression of its receptors [11] and repulsion of retinal ganglion cells [12]. Other molecules, such as Netrins, class 3 Semaphorins, and Slits, are secreted molecules that appear to act locally in certain instances [4]. For example, Netrin-1 has been immunolocalized to the neuroepithelial cells surrounding retinal ganglion cells exiting the optic disc, suggesting that they act locally in guiding RGC's toward and through the optic disc [13]; likewise, it has been postulated that Sema3A may be bound to neural tissue or within the ECM [14, 15], while *slit2* expression within the indusium griseum and the glial wedge (located above and below the corpus callosum, respectively) allow for Robo+ cortical axons to extend to the other hemisphere while in between these cell populations [16].

Although such guidance cues have traditionally been classified as attractive or repulsive, it is worthwhile to note that recent studies have shown that the internal state of the growth cone plays an important role in the interpretation of the cue and subsequent signal transduction [3]. For instance, it has been shown that changing the levels of a second messenger within the growth cone could alter its response to guidance cues [17]. In particular, Song et al., demonstrated that the turning of a growth cone toward a normally chemoattractive guidance cue, brain-derived neurotrophic factor (BDNF), could be changed into a repulsive response by changing levels of cAMP [17]. By using a competitive analogue of cAMP or an inhibitor of protein kinase A in cell cultures of *Xenopus* spinal neurons, opposite turning behavior was induced, suggesting that cAMP-dependent neuronal activity modulates the response of a growth cone to guidance cues

[17]. An identical response was observed in the same system with the attractive molecule netrin-1; when its receptor, deleted in colorectal cancer (DCC), was blocked, turning responses were abolished, indicating a single receptor mediates both responses [18, 19].

Intracellular calcium concentration $[Ca^{2+}]_i$ can also help determine the nature of the growth cone response to a guidance cue [3]. Studies have shown that the netrin-1 attraction mentioned above is dependent on local Ca^{2+} signals from influx through plasma membrane Ca^{2+} channels and intracellular stores; pharmacological blockage of either Ca^{2+} source resulted in the conversion of netrin-1 attraction to repulsion, while total blockage of Ca^{2+} release completely eliminated growth cone turning [20]. Interestingly, exogenous ryanodine (which at low concentrations produces Ca^{2+}-induced Ca^{2+} release from internal stores) was sufficient to induce a growth cone response in the absence of guidance molecules [20]. In addition to these global Ca^{2+} changes, local Ca^{2+} changes can modulate growth cone responses, as Zheng demonstrated in 2000 [21]. When a small, 2μm area elevation of caged Ca^{2+} was released at the growth cone via focal laser-induced photolysis, the growth cone consistently turned toward the side of elevated Ca^{2+}, whereas this response was reversed when extracellular Ca^{2+} was removed [21]. Taken together, these studies suggest that local Ca^{2+} release, external Ca^{2+} entry, and resting $[Ca^{2+}]_i$ integrate to determine growth cone responsiveness to guidance cues [3].

One way second messengers such as cAMP and Ca^{2+} can be modulated is through changes in membrane potential, which has been shown to play an important role in axon navigation [3]. By manipulating membrane potential through the alteration of voltage-dependent potassium currents (K_V), Mc Farlane and Pollock were able to show that blockage of these channels through 4-aminopyridine resulted in aberrant optic projections

toward the telencephalon rather than their correct target, the optic tectum [22]. In *C. elegans*, overexpression of this channel resulted in altered axon morphology and incorrect targeting [23]. Taken together, the results above indicate that axon targeting requires not only a variety of guidance cues, but also the proper intracellular environment for proper signal processing.

1.2.3 Extracellular matrix

In addition to guidance cues and second messengers, another major player in neuronal development and axon guidance is the extracellular matrix (ECM), a dynamic, complex system defined by a collagen scaffold and consisting of the basement membrane and the interstitial matrix [24]. ECM molecules such as laminin, tenascin and proteoglycans, which are present in developing neural tissues [25], adhere to this scaffold and interact with neurons coursing through the matrix through integrin receptors [24]. These component proteins are mosaic proteins upon which the lamellipodia of the growth cone can anchor and create the traction needed for migration, stability, and differentiation [26]. However, the interaction between the growth cone and ECM is not merely mechanical; it can also be antiadhesive [27] and can modify second messenger pathways such as Ca^{2+} levels [26].

Laminins (LN), first discovered in murine sarcoma, are a major component of the basement membrane [26] and consists of 12 different family members comprised of different heterotrimer combinations [28]. LN is expressed in a wide variety of cells, including epithelium, smooth and cardiac muscle, retina [24], kidney, skeletal muscle, and developing brain [28]. LN is known to support neurite outgrowth and differentiation [29], and it is found in areas of neuroblast migration and the pia overlying the floor plate

of developing spinal cord [30]. Mutations of LN subunits result in result in major deficits in myelination, neuromuscular junction formation, and electrical activity [28]. It has been suggested, however, that its primary role is in forming the architecture of and lending stability to the ECM, while negotiating the communication between the cell surface and the ECM [31].

The tenascin (TNC) family is a glycoprotein family with five family members, each containing EGF-like repeats, FNIII-like modules, and fibrinogen β- and γ- chains [32]. Of the five family members, only three (TNC-C, TNC-R, and TNC-W) have been identified in the CNS [33, 34], while TNC has been found in dopamine-containing neurons of the developing mouse brain as early as embryonic day 10 [35]. Also expressed in other developing neural tissue, such as radial glia and immature astrocytes [35, 36], TNC appears to play an important role in creating specific boundaries during key developmental processes, such as the in the barrel-boundaries of the somato-sensory cortex [37], the patch-boundaries of the developing neostriatum [38], and in the olfactory bulb abutting the presumptive nerve layer/dendritic zone boundary of the developing olfactory system (Treloar et al., unpublished data).

Chondroitin Sulfate Proteoglycans (CSPGs) and Heparin Sulfate Proteoglycans (HSPGs) are part of the general family of proteoglycans characterized by a protein core and glycosaminoglycan side chains [24]. CSPGs are comprised of several members, including aggrecan, versican, neurocan, and brevican, and were assumed to have more of a structural role in the ECM, serving to provide structural integrity and resistance to compression [24]. However, several studies have shown that CSPGs play a varied role in axon guidance. For instance, CSPGs have been shown to be inhibitory to E9 chick neurite

outgrowth through an anti-adhesive effect between growing neurites and NCAM [39, 40] and cause pathfinding errors when added exogenously to retinal ganglion cells [41]; however, CSPGs can be growth-promoting under specific conditions, as with cortical neurons grown at low cell density on a grid of adhesive substrata, thereby suggesting cell-type specificity and spatiotemporal distribution are important factors in determining how CSPGs modulate neurite outgrowth [42]. Interestingly, CSPGs can modulate the interpretation of guidance cues such as semaphorin5A, in fact causing the opposite effect on extending axons when compared to Sema5A modulation by HSPG [43]. When Sema5A binds to HSPG through thrombospondin repeats, it acts as an attractant to fasciculus retroflexus axons, whereas when Sema5A is presented in the presence of CSPG, the cue becomes inhibitory [43].

HSPGs can also bind to various molecules in the ECM and are expressed in developing axon tracts of the embryonic brain [44, 45]. Comprised of several members, including transmembrane syndecans, GPI-linked glypicans, and the secreted perlecans and agrins, they can bind a variety of molecules, including growth factors like FGF2, morphogens such as Wnt/Wg, cell adhesion factors, and laminin [46]. They can also bind to guidance cues and their receptors such as Slits/Robos, and to date this is the only known cue/receptor pair that requires HSPGs to function properly *in vivo* [46]. Indeed, HSPGs can mediate Slit/Robo signaling: Johnson et al. showed the repulsive action of Slit at the *Drosophila* midline is diminished when syndecan (*sdc*) function is lost, causing anomalous midline crossing of longitudinal axons [47]. Furthermore, when another HSPG, the glypican Dallylike, is neuronally expressed, the *sdc* mutant is partially rescued [47]. Immunohistochemistry demonstrated that *sdc* strongly colocalized with Robo, and

immunoprecipitation experiments suggested that this HSPG forms a ternary complex with Slit/Robo [47], leading some authors to postulate that Syndecan may be acting as a co-receptor for Slit [46]. Other experiments have demonstrated that a perturbation of HSPG can result in mistargeting and gross morphological deficits. For instance, when EXT1, a key enzyme in HSPG biosynthesis, is mutated, retinal axons project incorrectly to the contralateral optic nerve [48]. In addition, when a CNS-specific HSPG knock-out is created, severe nervous system defects that include the loss of olfactory bulbs, cerebellum and corpus callosum tract, and a diminished cerebral cortex are noted, and these mice die on the first day of life [48].

Given the complexity of ECM composition and its own component contribution to axon guidance and modulation of guidance cues, it is no surprise that the ECM itself is the subject of remodeling and regulation. Although numerous proteolytic enzymes play a role in this process, matrix metalloproteinases (MMPs) appear to dictate most of these dynamic changes [24], and accumulating evidence demonstrates their ability to not only regulate axon outgrowth via the classical method of ECM degradation, but also through the cleavage of guidance cues and receptors [49].

1.3 Matrix metalloproteinases

1.3.1 MMP families

Martirix Metalloproteinases (MMPs) were discovered in 1962 by Gross and Lapiere, who first described it in tadpole tale metamorphosis as a “diffusible collagenolytic factor operating at neutral pH and physiologic temperature” [50]. Since then, at least 24 MMPs have been identified, which combined have the ability to degrade all components of the ECM [51]. Zinc-dependent endopeptidases of the Metzincin

superfamily [51], MMPs are broken down into families which were initially named according to the substrate the enzyme degraded [24]. Although these family groupings are now a misnomer because of the significant overlap in substrate specificity, the names have persisted [24]. Thus, although the collagenase family only includes MMPs 1, 8, and 13, and the gelatinase family only includes MMPs 2 and 9, the gelatinases are capable of degrading collagen IV, V and X [24]. Other families include the matrylisins (MMPs 7 and 26) [24], the stromelysins (MMPs 3, 10, 11) [51], which along with the collagenases and gelatinases, are all secreted [24]. In addition to these secreted MMPs, a subset, known as Membrane-Type MMPs (MT-MMP), are membrane-bound. MMPs 14, 15, 16 and 24 contain a transmembrane region and a cytoplasmic tail, while MMPs 17 and 25 are anchored at the cell membrane by a glycosylphosphatidyl link [51].

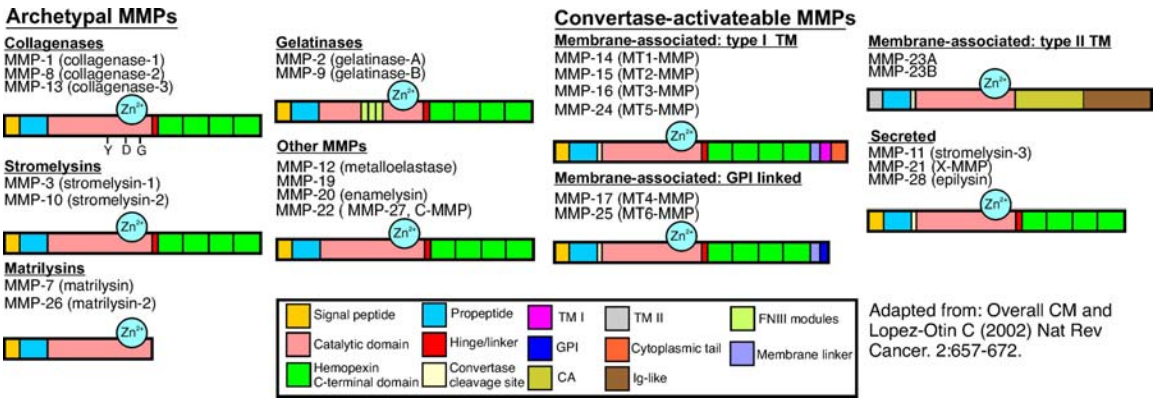

Figure 1: MMP Families [52]

1.3.2 Structure

Structurally, the MMPs generally consist of five major domains: a signal peptide that dictates its secreted or membrane-bound fate, a propeptide, a catalytic domain, a hinge region, and a hemopexin-like domain [53]. MMP 7 and 26 are the exception, consisting only of a propeptide and a catalytic domain [51]. The propeptide region, 10 kDA [54] or

80 amino acids in size, is responsible for maintaining the dormancy of the enzyme [55]. Consisting of a conserved PRCG(V/N)PD sequence, the propeptide contains a “cysteine switch” which interacts with the zinc in the catalytic domain to maintain inactivity [51, 55]. The catalytic domain consists of a HEXXHXXGXXH sequence, which coordinates the zinc between its three histidines and a water molecule (if there is no inhibitor or substrate present) in a trigonal pyramid [56]. This backbone also contains a conserved methionine, which forms a “met-turn” that brings backbone residues close to the substrate or inhibitor [57]. Further structural analysis has revealed secondary structures consisting of a 5-stranded β-pleated sheet, 3 α-helices, and multiple bridging loops [58], all of which require an additional zinc and 2-3 calcium ions for stability [55]. This overall catalytic domain structure is generally conserved among other members of the metzincin family, which include the astacins, the reprolysins (ADAMs) and serralysins [56]. The hemopexin-like domain, which is located on the C-terminal end, is approximately 30kDa [54] or 210 amino acids long and is shaped like an elliptical disk [59]. This disk contains 4 propeller-like blades, each with 4 antiparallel β strands and an α helix [59] stabilized by a disulfide bond [54]. The hemopexin domain is necessary for collagenases to bind native collagen, and removal results in ablation of collagenolytic activity, but not proteolytic activity for other substrates such as casein [60]. In other families, the hemopexin domain serves to bind tissue inhibitors of metalloproteinases (TIMPs), and in recombinant form has the ability to bind various molecules such as fibronectin and monocyte-attracting chemokines, and has been hypothesized to also sterically inhibit the binding of other potential substrates [60]. Lastly, the proline-rich hinge region links together the catalytic and hemopexin-like domains [55].

1.3.3 MMP regulation

Because MMPs have such powerful degradative capacities, constitutive activation would result in widespread destruction in any system. As a result, these enzymes are tightly regulated at each level of expression by a multitude of factors to ensure proper activation. For example, MMP regulation begins at the transcriptional level, with inducible factors including cell-cell interactions, and cell-matrix interactions [54]. MMPs 1, 2 and 3 expression is induced in fibroblasts by an immunoglobulin expressed on the surface of tumor cells, EMMPRIN [61], while MMP 2 can be induced in T-cells by vascular cell adhesion molecule-1 (VCAM-1)-mediated adhesion to endothelial cells [62]. MMP 9 gene expression can also be induced by a variety of cell-cell interactions: T lymphoma cell adhesion to endothelial cells via intercellular adhesion molecule-1 (ICAM-1)/leukocyte function-associated antigen-1 (LFA-1) upregulates MMP 9 [63]; monocyte expression of MMP 9 is increased via gp39-CD40 interaction with T cells [64]; and the interaction of $\alpha_5\beta_1$ integrin with fibronectin during macrophage differentiation also increases MMP 9 gene expression [65]. In addition, a three dimensional matrix culture system appears to induce greater expression of MMP 14 endothelial cells when compared to two dimensional cultures, indicating that cell-matrix interactions and dimensional structure play an important role [66].

Other ways MMPs can be induced at the transcriptional level include oncogenic cellular transformation, growth factors, cytokines, drugs, and physical stress [55]. Kidney cells rendered tumorigenic when transformed by v-src express MMP 14 and spontaneously metastasize to the lung after orthotopic implantation, suggesting that MMP expression may support invasiveness [67]. In addition, multiple cytokines and signaling

pathways induce the expression of MMP1. IL-1α is an autocrine inducer of MMP 1 expression in rabbit synovial fibroblasts when it is upregulated through the Rho-GTPase pathway [68]. This pathway increases IL-1α through the activation of GTP-binding protein Rac1, which creates reactive oxygen species that activate NF-κB, a transcriptional regulator of IL-1α [68]. Apart from autocrine induction of MMP 1, IL-1 can trigger the ceramide signaling pathway[69], which induces the expression of MMP 1 in human skin fibroblasts [70]. Human dermal fibroblasts can also express MMP 1 (and MMP 3 and 9) through ultraviolet B radiation [71], which increases the levels of AP-1 required for MMP transcription via ERK, JNK, and p38 MAP kinase pathways [72]. Clearly, a variety of molecules, intercellular interactions, and signaling pathways can influence MMP transcription and must coordinate in order to trigger enzyme expression.

Even though MMP expression can be induced in a variety of ways, these powerful enzymes are mostly secreted as inactive zymogens that must be proteolytically activated [55]. This initially occurs in an exposed region of the propeptide, located between the first and second helices [53, 73, 74]. This results in the generation of MMP intermediates and makes the propeptide more flexible, thereby allowing other bonds to become more susceptible to intramolecular catalysis by MMP intermediates [53, 73, 74]. This processing likely destabilizes the cysteine switch, and the final activation step involves proteolysis by an MMP [53, 73, 74]. MMP activation can occur in a step-wise manner *in vitro* via proteinases, low pH, heat, or chemical agents [53, 55], which include mercurial compounds, reactive oxygen, SH-modifying agents [55] ($HgCl_2$, 4-aminophenylmercuric acetate), oxidized glutathione, and SDS [53]. However, an example of chemical activation *in vivo* occurs during cerebral ischemia, in which NO activates pro-MMP 9

[75]. *In vivo*, MMPs are mostly activated by proteinases in the plasma, on the tissue, or from opportunistic bacteria [55]. Plasmin, generated from plasminogen by membrane-bound molecules such as tissue plasminogen activator and urokinase plasminogen activator, creates cell-surface localized MMP activity by activating pro-MMPs 1, 3, 7, 9, 10, and 13 [53, 76]. Intracellular activation can also occur, as with pro-MMP 11 activation by furin at its furin recognition site at the C-terminal region of the propeptide [77]. Pro-MMP 2 activation is unique in that its activation occurs at the cell surface by the membrane-bound MMPs, including MMPs 14, 15, 16, 24, and 25 [53]; MMP 17, however, does not activate pro-MMP 2 [53]. Interestingly, MMP 14 requires TIMP 2 to activate pro-MMP 2 [78-80]. TIMP 2 acts as a linker between pro-MMP 2 and MMP 14 expressed on the cell surface, using its C-terminal domain to bind pro-MMP2 and its N-terminal domain to bind MMP 14 [81]. Once pro-MMP 2 is bound to the cell surface, another MMP 14 that is not bound by TIMP 2 can then activate pro-MMP 2 [81]. TIMP 4 binding to pro-MMP 2, however, does not result in activation by MMP 14; this difference may be due to different binding of the TIMP to pro-MMP 2, although the actual mechanism has yet to be elucidated [82]. MMP 15 can activate pro-MMP 2 without the help of TIMPs [83].

1.3.4 Tissue Inhibitors of Metalloproteinases

A major level of MMP regulation involves inhibition by their endogenous inhibitors, the Tissue Inhibitors of Metalloproteinases (TIMPs). Although they can aid in activating pro-MMPs, their major function is restricting MMP activity. Binding to MMPs in a 1:1 stoichiometry, four TIMPs (TIMP 1, 2, 3, and 4) have been identified [53, 84] and in combination have the ability to inhibit the majority of MMPs tested so far [85].

Approximately 180 amino acids in length [84], TIMPs are shaped like wedges and bind the active-site cleft of MMPs via noncovalent bonds [53, 86]. Held together by six disulfide bonds [86], the N-terminal and C-terminal domains contain relatively flexible regions clamped to a more rigid five-stranded β-barrel and central helical region [84]. The N-terminal domain mediates the inhibitory action of the TIMP, while the C-terminal domain mediates the interaction with the hemopexin domain [79, 87]; deletion studies have demonstrated that the C-terminal domain is not necessary for the inhibitory function of TIMPs [88, 89]. While TIMPs 1, 2, and 4 are secreted, TIMP 3 is bound to sulfated glycosaminoglycans in the ECM [90]. As such, it has been postulated that this property better allows TIMP 3 to inhibit the membrane-bound MMPs [85, 91].

TIMP expression varies: TIMP 1 is highly expressed in heart, uterus, ovary, and muscle; TIMP 2 has broad expression in many tissues; TIMP 3 is found in the heart, kidney, ovary and thymus; and TIMP 4 has high expression in brain, heart, testes and thymus [92]. Knockouts have been produced for TIMPs 1, 2, and 3. Mice deficient in TIMP 1 have greater complement-dependent immune responses, conferring increased resistance to bacterial infections [93, 94]. Mice lacking TIMP 2 do not activate proMMP 2 as efficiently [80] and demonstrate changes at the neuromuscular junction, gross motor deficits, and increased neuronal branching and acetylcholine receptor expression [95]. TIMP 3-null mice have enlarged airspaces in their lung, resulting in premature death [96].

1.4 MMP roles

1.4.1 MMPs in development and physiology

Given their powerful degradative activity and tight regulation, MMPs are well suited to play prominent roles in development, common physiological processes and pathological states. Recent studies have highlighted the role of MMPs in cell migration and morphogenesis [97]. In particular, MMP 14 shifts toward the active migration front according to the movements of the actin cytoskeleton, forming homophilic complexes through their hemopexin domains to enhance degradation [81, 97]. One mechanism by which MMP 14 enhances cell migration is through processing of adhesion molecules such as CD44H [98]. Colocalizing at the lamellipodia of motile cells, MMP 14 and CD44H are dependent on each other for promoting cell migration [98]. MMP 14 must shed CD44H from the cell surface to generate migratory behavior; neither MMP 14 nor CD44H alone are sufficient to promote motility, and a mutant CD44H that was resistant to MMP processing resulted in stagnant MIA PaCa-2 cells [98]. MMP 14 can also affect cell migration and morphogenesis in the renal system. Expressed on the surface of MDCK cells, MMP 14 allows for cellular invasion into a three-dimensional matrix and stimulates morphogenesis into a branched tubular structure under the stimulation of hepatocyte growth factor [99]. When MMP 14 antisense RNA is expressed, tubular formation is reduced, demonstrating the essential role of MMP 14 in tubule formation [99]. However, while over-expression of MMP 14 on MDCK cells confers accelerated invasion and greater pit formation, these cells display disrupted morphogenesis and are unable to form branched networks [100]. It appears that the degradative properties of MMP 14 are dependent on its attachment to the cell membrane, as active soluble MMP

14 with a deleted transmembrane domain cannot induce a tubulogenic response in MDCK cells [100].

In other systems, such as the skeletal system, MMPs also play a crucial role. During bone development, MMPs 9, 13, and 14 play important roles in endochondral and intramembranous ossification, bone remodeling and triggered apoptosis during bone formation [101]. In particular, mice lacking MMPs 9 and 13 had problems with trabecular bone formation, inappropriate chondrocyte survival, delayed bone vascularization [102], while MMP 14 null mice showed severe deficits at the growth plate, including hypertophic zones, decreased chondrocyte populations, and delayed formation of secondary ossification sites, leading to extremely shortened bones [103]. MMPs are also present in the human endometrium, playing an important role in endometrial degradation after leukocyte release in the uterine stroma and epithelium [104], and some have suggested that MMPs are a critical element in creating the proper endometrial milieu for embryo implantation [105]. Wound repair is also mediated by MMPs, with MMP 1 appearing to play a prominent role in cutaneous wound healing [106, 107]. Expressed by basal keratinocytes, MMP 1 aids in degradation of the ECM, keratinocyte migration at the wound edge, proper re-epithelialization, and remodeling of granulation tissue [106, 107]. Similarly, MMP 7 plays an important role re-epithilialization, particularly in the migration of airway epithelial cells in incised mouse tracheas [106, 108]. However, apart from aiding in healing the airway, MMP 7 is upregulated in lungs with a heavy bacterial load, such as in cystic fibrosis [106]. Studies have suggested that MMPs aid in immunity by activating prodefensins, which mediate bacterial killing through membrane disruption [109]. This is especially true in Paneth

cells, which comprise the immune defense of the digestive system; MMP 7-null mice succumbed more easily to *Salmonella typhimurium,* with an oral 50% lethal dose only one-tenth of that necessary to kill wild-type mice [109].

1.4.2 MMPs in general pathology

While these enzymes are essential for proper development, homeostasis, and repair, their degradative capacity also lends itself to several pathologic states. For example, active MMP enzymes contribute to atherosclerotic plaque formation and instability [110, 111]. Increased expression of gelatinase, stromelysin, and interstitial collagenase have been found in the shoulder, core, microvasculature, and foam cell sites of plaques, whereas non-diseased arteries do not display focal overexpression [110]. Endogenous expression of these MMPs, such as MMP 9, is found in the coronary arteries, macrophages and smooth muscle cells [110]; however, this expression is greatly increased by inflammatory cytokines TNF-α and IL-1 β in inflammatory environments [112, 113], and very likely in the inflamed plaque of the arterial wall[106]. *In situ* zymography of fresh frozen sections demonstrated that the MMPs expressed in atherosclerotic tissue are indeed active, degrading gelatin and casein much more readily than frozen sections from normal arteries [110]. The presence of these activated MMPs facilitates vessel remodeling and cell migration, ultimately weakening the arterial wall [106, 114, 115]. Apart from MMP expression in human atheromas, MMPs 2 and 9 also contribute to the development of aortic aneurysms [116], while MMPs 13 and 14 are upregulated during the left ventricular remodeling of congestive heart failure [117].

The destruction of cartilage and bone proteins in synovial joint diseases is mediated primarily by MMPs [106]. In rheumatoid arthritis (RA), stromelysin (MMP 3)

and the gelatinases (MMPs 2 and 9) contribute to joint damage [106], while the collagenases (MMPs 1 and 13), primarily expressed in the cartilage-pannus junction and in synovial lining cells [118], specifically degrade the collagen isotypes found in articular cartilage and bone [111]. In addition, MMPs are secreted by T-lymphocytes in the T-cell mediated phase of RA [119] and they play an important role in the prominent angiogenesis that degrades the microvascular basement membrane and interstitium in RA [120]. In osteoarthritis (OA), IL-1, IL-6, IL-17 and TNF-α modulate the expression of MMPs, an interaction thought to be responsible for joint ECM degradation [121], while MMPs also play a role in dysfunctional apoptosis with in arthritic joints [122].

MMPs have also been widely studied in cancer, from tumorigenesis to tumor growth and metastases [106, 111]. In the early stages of tumor growth, MMPs mediate the degradation of ECM and basement membrane [106], and can specifically localize in the tumor and stromal cells at the periphery of the tumor as it invades the surrounding tissue [123]. As the tumor grows, it is supported by MMPs, which can process cell adhesion molecules [52] and release mitogenic factors in the surrounding ECM [106]. For example, MMP 9 facilitates the release of tissue-bound fibroblast growth factor (FGF) and vascular endothelial growth factor (VEGF), which assist tumor growth [111]. For growth beyond 1-2mm^3, a new blood supply must be recruited [124, 125], a process that is aided by the MMP-induced release or activation of βFGF, VEGF, or transforming growth factor-β [52, 126]. However, this blood supply not only sustains the increasing tumor bulk, it allows for metastases through MMP-mediated tumor cell intravasation and seeding of distant sites [123]. Indeed, MMPs not only facilitate metastases, they also support tumor survival by proteolytic inactivation of chemokines expressed by the host to

attract immune cells to the region [106, 127, 128]. With MMPs playing such vital roles in the genesis, growth and survival of nearly all common cancers, such as lung, breast, colon, prostate, gastric, and squamous cell carcinoma of the head and neck [106], it is no wonder that they have been a particularly tantalizing target for cancer therapy.

1.4.3 MMPs in nervous system pathology

The nervous system is also not immune to MMP-mediated disease processes. In Alzheimer's Disease (AD), amyloid-β (Aβ) proteins deposit in the neocortex, forming characteristic plaques [129]. Aβ is derived from the proteolysis of a larger membrane protein β-amyloid precursor protein (APP) by α-, β-, and γ- secretase, yielding Aβ peptides of differing lengths [130]; while the α-secretase pathway is non-amyloidogenic, β- and γ-secretase combined are thought to cleave APP into the amyloidogenic form [131]. Because Aβ stimulates MMPs 2, 3, and 9 expression in glial and hippocampal cultures, it was postulated that MMPs might mediate the plaque progression of AD [132]. The role of MMP 2, in particular, has been hotly debated, as initial reports indicated that it had protective, non-amyloidogenic α-secretase activity [133], while others have contended that MMPs demonstrate β-secretase activity [134]. While the role of MMPs may still be unclear, it does appear that select MMPs are increased in AD brains (e.g., MMP 1) [135] and specifically colocalize with senile plaques (e.g., MMP 24) [136] , suggesting that MMPs do play some role in the course of the disease.

In 1978, Cuzner et al. first reported the presence of proteases capable of degrading myelin in the CSF of multiple sclerosis patients [137], with more recent studies identifying some of these proteinases as MMPs [129, 138]. In particular, MMP 9 has been identified as having unique upregulation in the CSF of patients with MS, in

comparison to its absence in the CSF of healthy controls [139]. Indeed, MMP 9 levels in serum were shown to be elevated during periods of MS exacerbation [140], while MRI showed that MS patients with periods of high MMP 9 and low TIMP 1 levels had a tendency to develop new gadolinium-positive lesions in the following month [141]. In the animal model of MS, experimental autoimmune encephalomyelitis (EAE), synthetic inhibitors such as GM6001 are able to inhibit the development of EAE, maintain normal blood-brain-barrier permeability in EAE animals treated at onset of disease compared to increased permability in non-reated animals, and reverse already established EAE [142]. Antagonism of MMP activity in MS patients is also efficacious, as one of the mainstays of clinical therapy, interferon-β, works in part by downregulating the expression of MMP 9 by inflammatory cells [143].

1.4.4 MMPs in nervous system development

While it is clear that MMPs are present and play some role in the pathogenesis of AD and MS, there is emerging evidence that indicates MMPs are also present in other neurologic processes, such as amyotrophic lateral sclerosis, Parkinson's Disease, and in the edema following ischemic or hemorrhagic brain injury [106, 129]. However, MMPs do not always play a deleterious role in the CNS. Instead, they are also key players in the development and maturation of the nervous system, particularly active in myelin formation, [131], the formation of dendrites and synapses, and long-term potentiation (LTP) [144]. During myelinogenesis, oligodendrocytes express MMP 9 at sites where myelin basic protein is found, with the most gelatinolytic activity found at the tip of the extending process; furthermore, in MMP 9-null mice, oligodendrocyte process formation is severely inhibited [145]. It also appears that MMP 7 can affect the morphology of

dendrites [146]. *In vitro*, MMP 7 induces actin reorganization from intense puncta in the dendrites to a more homogenous distribution in the shaft; this is accompanied by the transformation of mushroom-shaped dendrites into immature-looking dendrites with long filopodia [146]. Interestingly, such morphological changes mirror the changes induced with NMDA channel activation and are inhibited when calcium influx through NMDA channels is blocked [146]. Likewise, MMP 9 can induce morphologic changes in dendrites, while inhibiting this enzyme improves the stability of the dendrite and its synapse both *in vitro* and *in vivo* [144]. It has also been suggested the MMP 24 can also regulate the formation of synapses, as it localizes to synapses in cultured hippocampal neurons and is enriched in synaptosomes from mature brain [147]. In LTP studies, MMP 9 protein synthesis and proteoytic activity increase with stimuli that induce LTP, and MMP 9 knockout mice not only have a smaller LTP magnitude and shorter duration than wild-type mice (which is restored by the application of active MMP 9 enzyme), they also exhibit deficits in hippocampus-dependent learning during context fear conditioning but not in amygdala-dependent cued fear conditioning [148].

1.5 MMPs in axon extension and guidance

1.5.1 MMPs, ECM, and neurite outgrowth

While the MMP roles reviewed thus far encompass multiple organ systems, disease states and developmental processes, an emerging area of particular relevance involves MMPs in axon outgrowth and guidance. Early culture studies by Muir demonstrated metalloproteinase activity as a critical component of neurite extension, supporting the classical view that MMPs degrade ECM components to physically clear a pathway for growing neurites [149]. Using chick dorsal root ganglion neurons (DRGs) cultured in a

three-dimensional reconstituted extracellular matrix, Muir showed that neurite extension potentiated by NGF could be decreased by a synthetic peptide designed to inhibit MMP activity [149]. Indeed, the mean neutrite length decreased by 48% when treated with MMP inhibitory peptide, reaching an average length of 128 μm/24h compared to 248 μm/24h under NGF plus control peptide conditions [149]. Proteinase activity was confirmed using zymographic analysis, which revealed a 72-kDa band that was immunologically similar to type-IV collagenase [149].

Two years later, Zuo et al. showed neurite outgrowth was dependent upon MMP 2 actively degrading inhibitory CSPG to unmask growth-promoting laminin [150]. Embryonic chick DRGs seeded on a laminin-CSPG substratum initially lag in neurite outgrowth in comparison to DRGs grown on laminin alone; however, over the course of 48 hours, neurite outgrowth on CSPG nearly reaches the same levels of outgrowth on laminin alone, suggesting the presence of a compound that allows for the neurites to overcome the inhibition of CSPG [150]. This compound was determined to be a metalloproteinase, as the effect was virtually abolished by treatment with an MMP inhibitor: neurite outgrowth remained relatively stagnant when grown on CSPG-laminin, rather than catching up with those grown on only laminin [150]. When DRGs were cryocultured on fresh-frozen sections of rat sciatic nerve, growth was potentiated by pre-treatment with recombinant MMP 2, increasing neurite length by 50% [150]. Zuo et al. determined MMP 2 was “deinhibiting” CSPG to reveal growth-promoting laminin by showing neurite outgrowth potentiated with chondroitinase pretreatment was equal to outgrowth with MMP 2 pretreatment, and neurite outgrowth on nerve pre-treated with MMP 2 was reduced to baseline in the presence of laminin antibodies [150]. This study

showed that MMPs not only clear physical boundaries in the ECM, but they can specifically degrade inhibitory components to reveal/release growth-promoting molecules that are otherwise bound in the matrix.

This idea of pretreatment with recombinant MMP to degrade inhibitory substrates was also used by Hyashita-Kino et al. In their study, they demonstrated MMP 24 is spatially and temporally expressed in the CNS, with expression during cellular migration and induction during cellular differentiation into neurons [151]. Expressed on the leading edge of the growth cone, MMP 24 was found to effectively degrade inhibitory proteoglycans when used to pretreat CSPG-LN or HSPG-LN coated coverglass [151]. This degradation was accompanied by increased neurite extension, an effect that was lost when MMP inhibitors were added to the pretreatment condition [151]. Taken together with evidence of MMP 24 expression in developing cerebrum, and zymography evidence that showed active MMP 24 in cerebellar neurons migrating to the granular layer, the authors suggested that the degradation capacity of MMP 24 on inhibitory molecules in the ECM could be active in axonal outgrowth during the period of neural network formation [151].

In a different study, Fambrough et al. showed that a metalloproteinase containing a disintegrin domain, *kuzbanian* (*kuz*) was necessary for axon extension through the nerve cord of *Drosophila* [152], one of the first studies to demonstrate *in vivo* evidence for MMP activity in neurite extension. Expressed throughout the embryonic *Drosophila* nervous system, *kuz* expression remains constant throughout the period of axon extension; in mutants, longitudinal connective axon tracts in the intercommisural region have greatly reduced thickness in comparison to wild-type counterparts, with axons

stalling and accumulating as clumps in the commissural region [152]. Fambrough proved the effects were due to the *kuz* mutation by creating a transgenic fly that carried a *pUAST-kuz* construct, which expressed *kuz* mRNA panneurally in embryos chromosomally mutant for *kuz* [152]. This effectively rescued the mutant phenotype, resulting in a wild-type axonal pattern [152].

1.5.2 MMP modulation of guidance cue/receptor interaction

While the above studies focused on MMPs acting in a degradative capacity in the ECM to facilitate axon extension, it became apparent that the picture was much more complex than initially thought. Apart from merely clearing physical barriers for extending neurites, MMPs were found to actually influence axon guidance through their interaction with guidance cues and their receptors. Instead of examining metalloproteinases in axon extension and their direct effects on the ECM, Schimmelpfeng and Klambt used *kuzbaninan* to understand its effect on guidance cue/receptor interaction [153]. In *Drosophila*, slit protein is expressed in the CNS midline where it repels longitudinally-projecting axons expressing its receptor, roundabout (robo), to keep them from crossing the midline [153]. *Kuzbanian* appears to mediate the interaction between slit-robo, as axons that normally stay ipsilateral instead crisscross the midline in *kuz* mutants [153]. Furthermore, in a dominant negative version of *kuzbanian* expression at the midline, both axons and muscle fibers cross the midline, suggesting *kuz* may cleave the slit-robo complex to drive axons from the midline [153]. In the *kuz* mutants, commisure-crossing axons express robo (which never occurs in wild type), yet these axons are able to cross the midline, suggesting the perception of slit is somehow incomplete and/or the signaling pathway is dysfunctional due to the *kuz* mutation [153].

These phenotypes show that *kuz* affects axon outgrowth beyond merely digesting ECM, but can also process guidance cue/receptor interactions to facilitate pathway formation [153].

Hattori et al. also examined the role of *kuzbanian* in ligand-receptor signaling in the CNS, focusing their efforts on ephrin-A2 and Eph receptor interactions in the mouse brain [154]. Ephrin-A2 is cleaved from the cell membrane upon binding of Eph receptor, a process that could be blocked with the addition of a synthetic metalloproteinase inhibitor *o*-phenanthroline [154]. Through coimmunoprecipitation experiments, the authors were able to show that kuzbanian was the metalloproteinase mediating this cleavage, as it forms a stable complex with ephrin-A2 that was necessary for ectodomain shedding [154]. In ephrin mutations that specifically block proteolysis, axon withdrawal is greatly delayed: when axons encounter wild-type ephrin-A2, growth cone collapse occurred and was followed by an average axon withdrawal time of 26 minutes, compared to a 72 minute axon withdrawal time for ephrin-A2 mutants [154]. The authors suggest that blocking proteinase activity incompletely blocks signaling, interfering with the ability to cease ephrin signaling and terminate the ligand/receptor interaction [154].

While Hattori demonstrated metalloproteinase activity mediated cue-receptor interaction by cleaving the guidance cue, Galko and Tessier-Lavigne showed metalloproteinase processing of receptors could regulate the movements of axons [155]. Netrin-1 attracts spinal commissural axons when binding its receptor, DCC (Deleted in Colorectal Cancer) [155]. When MMP inhibitors are applied to rat embryonic dorsal spinal cord explants, proteolytic processing of DCC is blocked, thus increasing the amount of receptor protein on axons [155]. This increase in DCC results in a potentiated

effect of Netrin-1 activity, leading to increased neurite outgrowth [155]. Indeed, neurtie outgrowth was greater with treatment containing both netrin-1 and metalloproteinase inhibitor than by high concentration of netrin-1 alone [155]. The authors conclude that metalloproteinase activity could mediate axon guidance by controlling the amount of guidance cue receptors found on the surface of growing axons through cleavage and shedding of receptor protein ectodomains [155].

This idea was further reinforced by Walmsley et al., who demonstrated MMPs could cleave human Nogo-66 receptor (NgR) [156]. Expressed in human neuroblastoma cells, NgR sheds a soluble N-terminal fragment into culture media, a process that could be blocked specifically by MMP inhibitor but not by inhibitors of proteases from unrelated families [156]. Interestingly, this shed fragment could bind Nogo-66 and prevent it from associating with its receptor NgR, suggesting a mechanism by which this inhibitory protein could be regulated [156].

In vivo evidence for MMPs having a role in axon guidance first came with a study done by Webber et al., which examined defects in retinal ganglion cell (RGC) axon projections following the application of MMP inhibitors on exposed brain preparations of *Xenopus laevis* [157]. When MMP inhibitors were applied at different decision points, RGCs made guidance errors, failing to turn properly or grow into their target, the optic tectum [157]. Although axon extension was only affected at higher doses, turning was altered even at low dose: when applied early, MMP inhibitors cause RGC axons to grow straight and into the mid-diencephalon rather than making a caudal turn, and when applied late, RGCs do not enter the optic tectum but rather grow around its anterior border [157]. The authors propose several substrates the MMPs may be acting on to

regulate axon guidance, including fibroblast growth receptors, DCC, and ephrins, all of which are found in the developing visual system [157]. The above studies demonstrate that MMPs are indeed active players in the developing nervous system. Their role is not simple or one-dimensional, for while it is true that they can degrade the ECM and clear a pathway for growing axon, they can process the ligands and receptors that affect axon behavior during targeting. Clearly, more studies are needed to further elucidate the subtleties of these interactions and uncover more of the mechanisms by which MMPs influence nervous system development.

1.6 Olfactory System

1.6.1 Organization and function

Given the accumulating evidence that matrix metalloproteinases are active in variegated developmental processes, and in particular axon extension and guidance in the developing nervous system, it seemed reasonable to examine MMPs in the olfactory system--- a superb model for studying developmental axon guidance and targeting in the central nervous system. Of note, the olfactory system is uniquely attractive because of the continuous turnover and regeneration of its sensory neurons and interneurons within the bulb [158]. Olfaction begins when an odorant molecule binds to an odorant receptor, which is expressed on the cilial portion of the olfactory sensory neuron lining the nasal epithelium [159]. The morphologically homogenous sensory neurons then project their axons from the epithelium and fasciculate as they pass through the basal lamina and cribiform plate, eventually forming the olfactory nerve layer (ONL), which is the outermost layer of the laminar olfactory bulb [158]. OSNs with different sensory receptors are intermingled in a randomly dispersed fashion within one of four broad

zones in the olfactory epithelium (OE) [159]. Projections are essentially organized around orthogonal axes [160, 161], with axons originating from the dorsal OE projecting to the dorsal OB, and those from the ventral OE projecting to the ventral OB [161-163]. However, the fascicles immediately arising from the epithelium are phenotypically heterogeneous because these axonal projections group together in a manner which reflects their point of origin in the OE [158]. Once these OSN bundles reach the bulb, however, defasciculation and reorganization occur [158] as OSNs expressing the same receptor converge upon a glomerulus [164-167]. The glomeruli, spherical neuropil containing excitatory synapses between sensory neuron axons and mitral/tufted output cells, therefore receive convergent input from one type of receptor arising from disparate sites in the OE [159]. With more than 2 million sensory neurons in the mouse [164] each expressing only one gene [168-171] of a repertoire of approximately 1100 functional olfactory receptor (OR) genes [172, 173], each glomerulus therefore receives several thousand axonal inputs emerging from a topographically dispersed origin [174]. Remarkably, though sensory neurons undergo degeneration and regeneration every few weeks [174-177] glomerular targeting and specificity remain conserved [158].

The underlying mechanisms by which such specificity is established and maintained are presumably complex and multi-faceted; while several groups have established that the OR itself contributes to glomerular targeting, it alone cannot fully determine the precise mapping [164]. Among the plethora of clues presumably influencing glomerular targeting, extracellular matrix molecules (ECM) may potentially play a role in establishing the pathway. Because ECM molecules have been demonstrated to be crucial in the development of other nervous system regions, such as cerebellar

circuitry and microorganization [178] and neural crest cell migration and somite formation [179], it seems possible that similar cues may be acting on the developing olfactory bulb.

1.6.2 Development

The development of the olfactory system as sensory axons project from the nasal epithelium to the bulb can be divided into three phases:

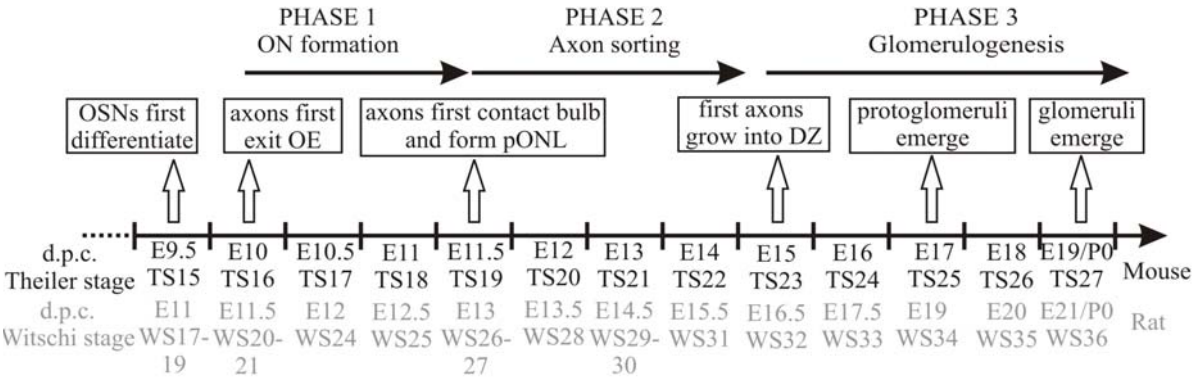

Figure 2: Timeline of early mouse olfactory development. OSN- olfactory sensory neuron, OE- olfactory epithelium, pONL- presumptive olfactory nerve layer, DZ- dendritic zone, d.p.c.- days of gestation.

OSNs derive from the placodal neuroepithelium [180] sending projections to the rostral telencephalon to form a simple olfactory nerve during phase one [180, 181]. In the second phase, these axons pierce the marginal zone of the telencephalon through fenestrations in the basement membrane, forming a presumptive nerve fiber layer of the bulb [180] directly apposed to a dense mass of mitral and tufted cell dendritic fibers in the dendritic zone [182]. The nerve remains restricted to the marginal layer of the telencephalon for the approximate four day duration of phase two [182], and the axons begin to reorganize and sort themselves into similar groupings [183]. Finally, in phase

three axons actually grow into the mitral/tufted cell dendritic zone, associating into protoglomeruli, which continue to mature into glomeruli until birth [182].

1.6.3 Axon guidance and targeting in the developing olfactory system

Mombaerts et al. and Wang et al. have shown that OR expression is required, yet insufficient, for specific glomerular convergence by OSNs; thus additional guidance cues are necessary to ensure correct targeting [164, 184]. Indeed, the complex events of neurite extension, axon guidance, bundling, appropriate targeting and synaptogenesis involved in pathway formation necessitate a constellation of cues from within the neuron and from the local environment [181]. The interaction of growth cones with the extracellular landscape via cell adhesion molecules and extracellular matrix receptors mediates axonal pathfinding [181] and can be broken down into four mechanisms: contact- mediated attraction (e.g., laminin secreted by Schwann cells that support axonal growth); contact-mediated repulsion (e.g., chondroitin sulfate proteoglycans in glial scars); chemoattraction (e.g., nerve growth factor (NGF), shown to advance nerve survival and differentiation); and chemorepulsion (e.g., semaphorins known to act in the collapse of growth cones) [185]. A fine-tuned spatio-temporal balance between such factors contribute to the complex interplay involved in ensuring proper axonal maneuvering in the central nervous system and may play a significant role in the developing olfactory system. Of particular interest to this project is the regulation of these processes by proteases, for which there is increasing evidence in various systems as being a key mechanism underlying pathway establishment during development.

2 Statement of Purpose (Hypothesis and Specific Aims)

Given the manifold roles of matrix metalloproteinases in a variety of physiological processes, such as bone formation [101], wound healing [107], and cancer metastases [106, 111], it is not surprising that these powerful degradative enzymes are also found in the nervous system [131]. While some focus has been on the roles of MMPs in neural pathology (e.g., Alzheimer's disease [132], ischemia [106], multiple sclerosis [137, 138]), an emerging area of interest is the role that MMPs play in nervous system development. They have been implicated in mediating neurodevelopmental processes such as myelination [145], neuron morphogenesis [146], and synapse formation [147]. More recently, MMPs have also been implicated in neurite outgrowth and axon guidance [49]. It is known that axonal growth and targeting is an extremely complex process mediated by the growth cone's interaction with guidance cues and the extracellular matrix [1]. Given that MMPs are widely expressed in the developing nervous system [131] and have the ability to degrade the ECM [149] and cleave guidance cues and their receptors [154, 155], these enzymes may play a significant role in establishing precise axonal connectivity during development. While others have examined MMPs in the *Drosophila* CNS [152] and in chick retino-tectal pathway [157], I have chosen to examine MMPs in the olfactory system because of its stereotyped pattern of axonal pathfinding and capacity for regeneration. Therefore, I hypothesize that MMPs may play a role in guiding olfactory sensory axons to their target by sculpting the extracellular matrix and influencing axon interactions with the environment. To elucidate whether MMPs are present in specific spatio-temporal patterns in the developing olfactory system

and whether MMP activity could modulate olfactory sensory neuron outgrowth, the study, therefore, has the following four specific aims:

1. Characterize mRNA expression profile of 19 members of the MMP family and their 4 endogenous inhibitors (TIMPs) in the developing olfactory system using an RT-PCR based approach.
2. Determine the spatio-temporal distribution of membrane-bound MMPs in the developing olfactory pathway using immunohistochemistry.
3. Localize MMP activity in tissue sections using *in situ* zymography.
4. Investigate MMP regulation of axon outgrowth using *in vitro* neurite outgrowth assays.

3 Materials and Methods

3.1 RT-PCR

3.1.1 RNA extraction

Studies were undertaken in CD-1 mice (Charles-River, Wilmington, MA). Timed pregnant dams that were pregnant 13, 15, and 17 days (day of positive vaginal plug = E0) were sacrificed via CO_2 inhalation and embryos were harvested. E13, E15, and E17 embryos and pups from postnatal days 0 and 2 (P0 and P2) were rapidly decapitated. Olfactory bulbs and epithelium were dissected and immediately frozen in dry ice. For positive controls (see Table 1), tissue was harvested from adult female mice and immediately frozen. RNA was extracted using RNeasy Plus Mini kit (Qiagen) following manufacturer's instructions. Briefly, 20-30mg frozen tissue was homogenized in Buffer RLT Plus with β-mercaptoethanol. DNA was removed through gDNA Eliminator column, and extracts were washed with 70% ethanol in RNeasy spin column. After several washes with Buffer RW1 and Buffer RPE, RNA was eluted with water. Total RNA concentrations was determined using spectrophotometry. To verify quality, cDNA was produced from each sample and RT-PCR was performed with β-actin primers (see below).

3.1.2 cDNA synthesis

cDNA was produced for each RNA sample via SuperScript III First-Strand Synthesis SuperMix (Invitrogen) following manufacturer's instructions. Briefly, RNA sample was mixed with random hexamers, annealing buffer, and water, which was then incubated at 65°C for 5 minutes. Subsequently, 2X First-Strand Reaction Mix and SuperScript III Enzyme Mix was added and incubated at 25°C for 10 minutes. The mix was then

incubated at 50°C for 50 minutes, and the reaction was terminated by incubation at 85°C for 5 minutes.

3.1.3 PCR reaction

Primers for 19 members of MMP family and 4 endogenous inhibitors (see Table 1) were designed using PerlPrimer primer design software, ranging in length from 19 to 24 bp (Marshall OJ. PerlPrimer: cross-platform, graphical primer design for standard, bisulphite and real-time PCR. *Bioinformatics* 2004 20(15):2471-2472). PCR was performed for each primer using cDNA from E13, E15, E17, P0, and P2 olfactory bulb and epithelium using Platinum BluePCR SuperMix (Invitrogen). MMP primers and template cDNA was mixed with the Platinum BluePCR SuperMix, which was activated at 94°C for 1 minute. cDNA was denatured at 94°C for 30 seconds, annealed at 55°C for 30 seconds, and extended at 72°C for 1 min; this cycle was repeated 30 times. Final extension was performed at 72°C for 10 minutes, and maintained at 4°C after cycling. Bands were visualized by gel electrophoresis with 2% agarose gel and ethidium bromide, run at 100mV and visualized under UV light.

Table 1: MMP Primers

Enzyme	Forward Primer	Reverse Primer	Positive Control
MMP1	GACAGTCTGGAAATACCTGGA	ACAATATCGCCTTCCTCCTC	Placenta
MMP2	GTTCTGGAGATACAATGAAGTG	CACCCTCTTAAATCTGAAGTC	Placenta
MMP3	TTGAAGCATTTGGGTTTCTC	ACCATCTACACAGTTCAGAC	Endothelial Cells
MMP7	CTCTTCTGTTCCCGGTACT	TGTTGATGTCTCGCAACTTC	Ileum
MMP8	ACTGCTGAGAATTACCTACGA	ATGTTGATGTCTGCTTCTCC	Jaw/Joint
MMP9	GTACCAAGACAAAGCCTATTTCTG	GCTGATTGACTAAAGTAGCTGG	Ovary
MMP10	CTTTAAAGACAGGTACTTCTGG	ATCATCTGTTATCTGTCTTGGG	Placenta
MMP11	CTCACCTATAGGATCCTCCG	GAACATGGCCAAATTCATGAG	Placenta
MMP12	TTAAAGACTGGTTCTTCTGGTG	ACCTCCAGTAGTGTTTATCCA	Placenta
MMP13	ATCATACTACCATCCTGCGA	AGTCACCATGTTCTTTAGTCC	Jaw/Joint
MMP14	CTTCAAAGGAGATAAGCACTG	GTTTCCCTTGTAGAAGTATGTG	Endothelial Cells
MMP15	GTCTTCTTCAAAGGTAACCGC	CCTTGTAGAAGTAGGTGTAGGC	Liver
MMP16	CAAAGGTGACAGGTATTGGA	CAATACAAGGAGGCATAAGG	Placenta
MMP17	TGTATGAGCGTACCAGTGAC	GAAATAGGATGCACCATCAGAC	Ovary
MMP19	TCTTCAAGGGAAACAAGGTG	GGTTGATGAGTTAGTGTCTGG	Liver
MMP20	TCCTGATGTGGCTAACTACC	TAAATTGAACCCATTCGTTCCC	Jaw/Joint
MMP23	ACATACAGAGTTCTTTCCTTCC	CAAACACCTTTCTTCCAACTG	Jaw/Joint
MMP24	CAGCAAGGAAGGATATTACACC	TTATAGTAGGTGACGGGCTG	Placenta
MMP25	GAGCCTGACATCATTATCCAC	GACACTCTCCCATAGAGCTG	Lung
TIMP1	CAGAAATCAACGAGACCACC	GGGATAGATAAACAGGGAAACAC	Ovary
TIMP2	ATGCAGACGTAGTGATCAGAG	AGATGTAGCAAGGGATCATGG	Endothelial Cells
TIMP3	AGCAGATGAAGATGTACCGA	CCAGAGACACTCATTCTTGG	Placenta
TIMP4	TGAAGCTAGAAACCAACAGTC	CTACTAGGGCTGGATGATGTC	Ovary

3.2 Immunohistochemistry

3.2.1 Tissue preparation

E13, E15, E17, P0, and P2 heads were rapidly immersion fixed in 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS; 0.1 M phosphate buffer and 0.9% NaCl, pH 7.4) at 4°C overnight. For tissue cryoprotection, heads were immersed in 30% sucrose in PBS at 4°C until the tissue sank. Tissue was equilibrated in equal parts 30% sucrose in PBS and OCT for 30 minutes at 4°C. Heads were embedded in OCT compound and frozen at -80°C until cryosectioning. All procedures undertaken in this study were approved by Yale's Animal Care and Use Committee and conform to NIH guidelines.

3.2.2 Cryosectioning

Tissue was serially sectioned at 20μm in the coronal plane using a Reichert-Jung 2800 Frigocut E cryostat. Sections were thaw mounted onto Superfrost Plus microscope slides (Fisher Scientific), air dried, and stored at -20°C until use.

3.2.3 Staining

Sections were washed in 0.3% Triton-X 100 in Tris-buffered saline (TBST; 0.1M Tris buffer and 0.9% NaCl, pH 7.4) and then incubated with Image-iT FX signal enhancer (Invitrogen) for 30 minutes at room temperature. After washing 3 times with TBST, they were preincubated in a blocking solution of 2% bovine serum albumin (BSA; Sigma, St. Louis, MO) in TBST for 30 minutes at room temperature to block nonspecific binding. Sections were then incubated with primary antibodies (see Table 2) at 4°C overnight. After incubation, sections were washed 3 times with TBST. Tissue was then incubated in the appropriate secondary antibody cocktail for 1 hour at room temperature (see Table 3). Sections were then washed twice in TBST and once more in TBS before being coverslipped in Gelmount (Biomeda, Foster City, CA) and stored at 4°C.

Table 2: Primary Antibodies used for Immunohistochemistry

Antibody	Host	Dilution	Source
MMP-2	Rabbit	1:1000	Chemicon
MMP-9	Rabbit	1:100	Affinity BioReagents
MMP-14	Rabbit	1:100	AbD Serotech
MMP-15	Rabbit	1:250	Chemicon
MMP-16	Rabbit	1:250	Chemicon
MMP-17	Rabbit	1:100	Biovision
MMP-24	Rabbit	1:100	Biovision
MMP-25	Rabbit	1:250	Chemicon
Doublecortin	Goat	1:1000	Santa Cruz Biotechnology
MAP-2	Chicken	1:1000	Chemicon
NCAM	Rat	1:300	Chemicon
NCAM	Mouse	1:1000	Sigma

Table 3: Secondary Antibodies used for Immunohistochemistry

Antibody	Host	Dilution	Source
Rabbit-Alexa 488	Donkey	1:1000	Molecular Probes
Goat-Alexa 555	Donkey	1:1000	Molecular Probes
Mouse-Alexa 555	Donkey	1:1000	Molecular Probes
Rat-Alexa 647	Chicken	1:1000	Molecular Probes
Chicken-Alexa 647	Goat	1:1000	Molecular Probes

3.2.4 Image acquisition and preparation

Staining was analyzed with a Leica TCS LS laser scanning confocal microscope. CorelDraw 12.0 and Adobe Photoshop 6.0 were used to process the images for contrast and brightness consistency, but further adjustments or alterations were not performed.

3.3 *In Situ* Zymography

3.3.1 Tissue preparation and sectioning

E13, E15, E17, P0, and P2 heads were harvested via rapid decapitation and frozen without fixation at -80°C in OCT compound. Tissue was then sectioned at 20μm in the coronal plane and thaw mounted onto Superfrost Plus microscope slides (Fisher Scientific) and air-dried. Sections were then stored at -80°C until use.

3.3.2 *In Situ* Zymography assay

DQ Gelatin (Molecular Probes), was reconstituted in water according to manufacturer's instructions. DQ-gelatin was mixed in a 1:5 ratio with zymography buffer (pH 7.6, 2.5 ml of 1M Tris, 7.5 ml of 1M $NaCl_2$, 250ul of 1M $CaCl_2$, and 200ul of 0.5M NaN_3) and then mixed with equal part 1% low melting point agarose. For controls, DQ-gelatin/buffer mixture was prepared as above, with 10mM 1,10-phenanthroline added in a 1:25 ratio. DQ-gelatin mixture was then overlaid on fresh frozen sections and incubated in humid

chamber at 37°C overnight. Fluorescence was then visualized with a Leica TCS SL confocal microscope.

3.4 Cell culture- neurite outgrowth with MMP inhibitors

3.4.1 Culture well preparation

Culture wells were treated with 20 μg/ml PDL (Sigma) for one hour and then rinsed with water.

3.4.2 Dissociated cell cultures

For dissociated cells incubated with inhibitor, epithelium was dissected from P1-P4 pups and treated with Papain Dissocation System (Worthington Biochemical) for one hour. Cells were resuspended in NeuroBasal media (Gibco) supplemented with vitamin B27, pen-strep antibiotic, and L-glutamine, but specifically made without L-cysteine, as it was discovered by Muir (1994) that cysteine (but not cystine) strongly inhibits metalloproteinase activity. After incubation for 30 minutes, cultures were flooded with either regular media or media plus GM6001 (Chemicon) at a final concentration of 20 μM. This was then incubated for a total of 48 hours.

3.4.3 Fixation and staining

Following the 48 hour incubation period, cells were washed 3 times in PBS and then fixed in 4% PFA/4% sucrose in PBS for 30 minutes at room temperature. Cultures were then washed 3 times in PBS. For staining, cultures were washed in TBST and incubated in Image-iT FX signal enhancer (Invitrogen) for 30 minutes at room temperature. Afterwards, cultures were washed 3 times in TBST and blocked with 2% BSA in TBST for 30 minutes to block nonspecific binding. NCAM antibody (see Table 4) was incubated for 1 hour at room temperature, then rinsed 3 times in TBST. Secondary

antibodies were applied for 30 minutes, rinsed 3 times with TBST, 1 time in TBS, and coverslipped in Gelmount.

Table 4: Antibodies and nuclear stains used for immunocytochemistry

Antibody/Nuclear Stain	Host	Dilution	Source
NCAM	Rabbit	1:1000	Chemicon
Rabbit- Alexa555	Donkey	1:1000	Molecular Probes
DRAQ5		1:1000	Alexis Biochemicals
DAPI		1:1000	

A subset of dissociated cells grown only in media were fixed and stained (with protocol above) for MMPs (see Tables 2 and 3 for antibodies and dilutions).

3.4.4 Image acquisition and analysis

Staining was visualized using an OlympusBX51 light microscope. Analysis was performed with MetaMorph software (Molecular Devices), which measured maximum process length, total neurite outgrowth, cell branching, and number of processes per cell through its Integrated Morphometry Analysis program.

3.5 Cell culture- neurite outgrowth on ECM substrates treated with MT-MMPs

3.5.1 Culture well preparation- predigestion assay

Culture wells were coated with PDL (see Table 5) overnight and rinsed with water. Slides were then coated with either Laminin (LN), tenascin-C (TNC), chondroitin sulfate proteoglycans (CSPGs) or heparan sulfate proteoglycans (HSPG) for one hour, then rinsed with water. Each set of slides was then digested with active MMP 14, MMP 15, MMP 16, or MMP 24 enzyme diluted in MMP digestion buffer (50mM Tris-Hcl, 150mMNaCl, 5mM $CaCl_2$) overnight, then rinsed with digestion buffer. For controls, MMP enzyme was mixed with a broad-spectrum MMP inhibitor, GM6001, which inhibits all MMPs as it is a potent zinc chelator.

Table 5: Substrates used for Cell Cultures

Substrate	Concentration	Source
Poly-D-Lysine (PDL)	50 μg/ml	Sigma
Laminin (LN)	10 μg/ml	Invitrogen
Tenascin-C (TNC)	20 μg/ml	Chemicon
Chondroitin Sulfate Proteoglycan (CSPG)	40 μg/ml	Chemicon
Heparin Sulfate Proteoglycan (HSPG)	40 μg/ml	Sigma
MMP-14 Enzyme	10 μU	Chemicon
MMP-15 Enzyme	10 μU	Chemicon
MMP-16 Enzyme	10 μU	Cal Biochem
MMP-24 Enzyme	10 μU	Cal Biochem
GM-6001	25 μM	Chemicon

3.5.2 Explant cultures

For explants used in the predigestion assay, epithelium was dissected from P1-P4 pups and treated with Papain Dissocation System (Worthington Biochemical) for 5 minutes. Explants were resuspended in NeuroBasal media (Gibco) supplemented with vitamin B27, pen-strep antibiotic, and L-glutamine and plated in each culture well. After incubation overnight, cultures were flooded with media and allowed to incubate for one more day.

3.5.3 Fixation and staining

Following the 48 hour incubation period, cultures were washed 3 times in PBS and then fixed in 4% PFA/4% sucrose in PBS for 30 minutes at room temperature. Cultures were then washed 3 times in PBS. For staining, cultures were washed in TBST and incubated in Image-iT FX signal enhancer (Invitrogen) for 30 minutes at room temperature. Afterwards, cultures were washed 3 times in TBST and blocked with 2% BSA in TBST for 30 minutes to block nonspecific binding. NCAM antibody (see Table 4) was incubated for 1 hour at room temperature, then rinsed 3 times in TBST. Secondary

antibodies were applied for 30 minutes, rinsed 3 times with TBST, 1 time in TBS, and coverslipped in Gelmount.

3.5.4 Image acquisition and analysis

Staining was visualized using an OlympusBX51 light microscope. Analysis was performed with MetaMorph software (Molecular Devices), which measured explant area (stained by DAPI) and total explant plus neurite area (stained by NCAM) through its Integrated Morphometry Analysis program.

PCR, immunohistochemistry, and *in situ* zymography experiments were all performed and analyzed by the author; cell culture experiments and analyses were performed by the author with the assistance and technical expertise of Helen Treloar.

4 Results

4.1 MMP and TIMP mRNA expression in the developing olfactory system

To determine whether matrix metalloproteinases and their inhibitors are present in specific spatio-temporal patterns in the developing olfactory pathway, I used RT-PCR to look for mRNA expression in the OB and OE at select developmental time points. Although there are some reports of selected MMPs acting in the developing olfactory system, no comprehensive expression screen exists. Using a PCR-based approach has allowed me to catalog which genes are present in the OB and OE at specific developmental time points, allowing for a more focused investigation of those particular MMP family members. Expression of 19 MMPs and their 4 known endogenous inhibitors were examined in five ages, E13, E15, E17, P0 and P2 (Figure 3). To ensure the efficacy of primers, mRNA from various tissue known to express individual MMPs were used as positive controls.

Among the collagenases, MMP 1 mRNA was not detected in the OE or OB at any age. MMP 8 and MMP 13 (collagenase-2 and-3, respectively) were not expressed in the OB, but did display differential expression in the OE. MMP 8 is expressed at E17 in the OE, with decreasing expression through P0 and P2. In contrast, MMP 13 mRNA is faintly detected at E15 OE and increases during development, showing robust expression at P2.

The stromelysins, MMPs 3, 10, and 11 also showed differential expression. Neither MMP 3 nor 10 were detected at any age in the OE or OB. MMP 11, however,

was highly expressed in the OE at E13 and E15, then expression decreased at E17 through P2. In the OB, MMP 11 expression increased during development, beginning at E13 and gradually increasing through P2.

Other MMPs tested included MMP 7, a member of the matrilysin family, MMP 12 (metallolastase) and MMP 20 (enamelysin). None of these 3 MMPs was detected in either the OE or OB at any age examined. MMP 19, which like MMP 12 and 20 are categorized in the "Other MMP" group, was expressed in the OE at P0 and P2, and was found in the OB beginning at E17 and persisting through P2.

The gelatinases, MMP 2 and MMP 9, were both found in the OE and OB. Expression was robust in the OE throughout all five ages tested, while in the OB, MMP 9 expression increased from E13 through P2 but MMP 2 had a delayed onset of expression at P2,

The membrane-bound MMPs also showed robust expression throughout the developing olfactory system. MMP 14 was highly expressed in the OE beginning at E13 and remained strongly expressed through P2. In the OB, MMP 14 expression was also found at E13, and steadily increased through P2. MMP 15 and MMP 16 were both strongly expressed in both the OE and OB at all ages. MMP 17 and 23 were also detectable at all ages in both the OE and OB. MMP 24 was detected in the OE at E13 and increased throughout development, while in the OB its expression was steadier throughout each age. MMP 25 expression in the OE was detected at E13 and decreased at each subsequent age, whereas its expression in the OB was steady throughout all ages examined.

The Tissue Inhibitors of Metalloproteinases, TIMPs, also had differing expression in the OE and OB. TIMP1 was found only at E13 and E15 in the OE, and only at E13 and E17 in the OB. TIMPs 2 and 3 were robustly expressed at all ages in both the OE and OB. TIMP 4 expression was found at all ages in the OE, peaking at E17, while its expression was only found from E17 through P2 in the OB.

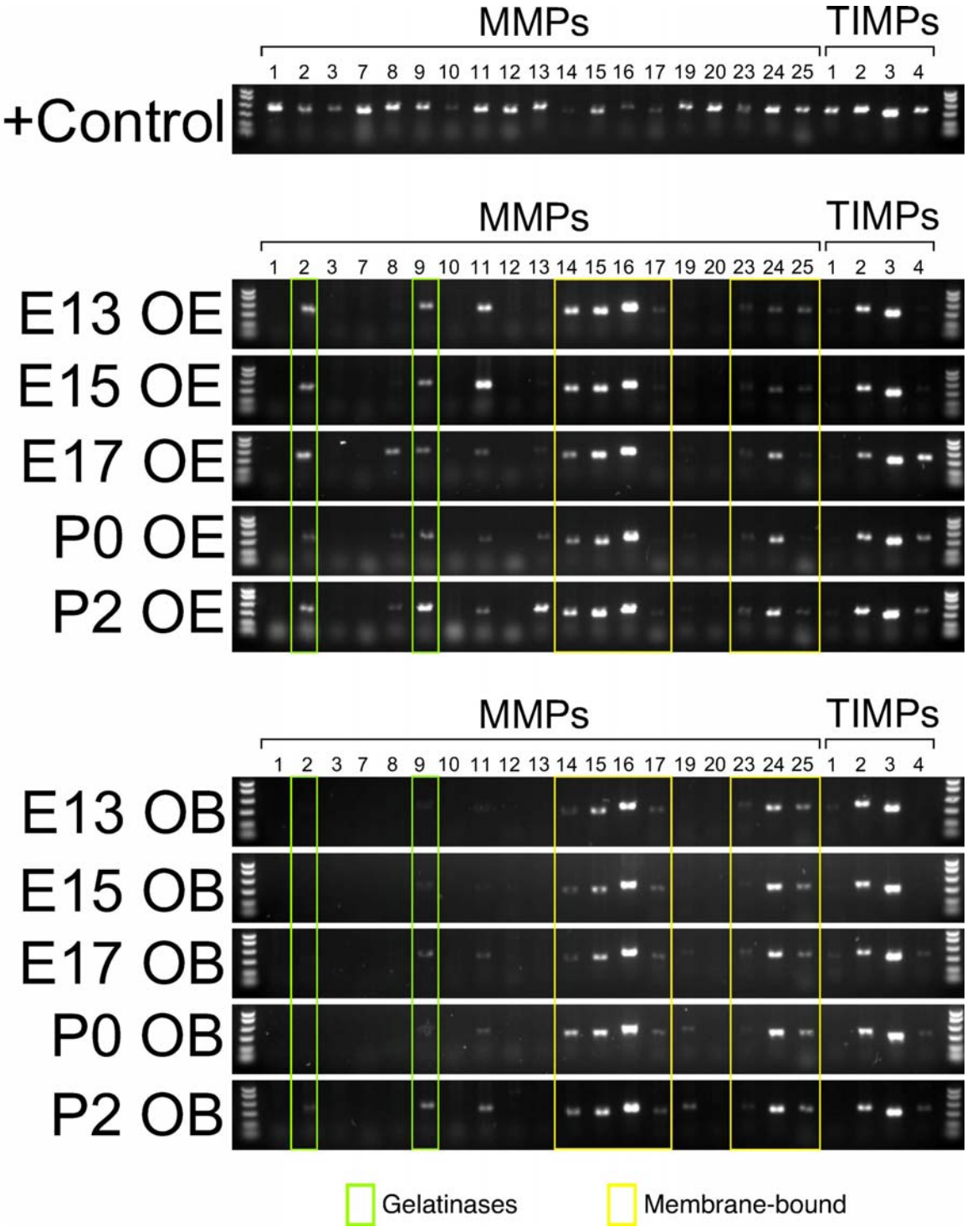

Figure 3: RT-PCR of 19 members of the MMP family and their 4 endogenous inhibitors (TIMPs) in the developing olfactory system. Members of the gelatinase family are highlighted in green, while members of the membrane-associated MMP family are highlighted in yellow. Positive control bands for each primer pair confirmed specificity.

4.2 Spatial Distribution of MT-MMPs in the developing olfactory system

Because the RT-PCR screen demonstrated that certain MMP families were more highly represented in the developing olfactory pathway, I next focused on characterizing their protein expression. In particular, because Membrane-Type MMPs (MT-MMPs) were particularly highly represented as a family throughout development, I elected to focus my studies on examining the spatio-temporal protein expression patterns of these family members in the olfactory system. For this study, five developmental ages were used (E13, E15, E17, P0, and P2), to complement my PCR screen, and stained with antibodies raised against six of the seven members of the MT-MMP family (MMP 14, MMP 15, MMP 16, MMP 17, MMP 24, MMP 25). As seen in Figure 4, MT-MMP expression (green) in E13 tissue was prominent in the olfactory epithelium. Each of the MT-MMP antibodies stained a subset of cells in the OE, which had a bipolar morphology, extending a single apical dendrite up to the lumen of the nasal cavity and a single axon from the basal pole of the cell body. This morphology is suggestive of these cells being olfactory sensory neurons (OSNs). To confirm this observation, I double stained with NCAM antibodies (purple, Figure 4), and observed colocalization of NCAM with each of the MT-MMP antibodies (white, Figure 4), indicating that these MT-MMP-expressing cells were indeed OSNs. However, not all NCAM-positive OSNs were observed to express MT-MMPs. I hypothesized that perhaps only immature OSNs were expressing MT-MMPs, using these proteinases to aid in axon extension and/or guidance. To test this hypothesis, I also stained with Doublecortin (Dcx), a marker of immature neurons (red, Figure 4). Interestingly, all MT-MMP expressing OSNs also expressed Dcx (yellow, Figure 4), confirming our hypothesis that newly generated OSNs express

metalloproteinases at their cell surface. Interestingly, all DCX expressing OSNs were observed to express each MMP, as no red (i.e., DCX^{+}/MMP^{-}) OSNs were observed (e.g., Figure 4). Thus, each DCX^{+} OSN expresses at least six MT-MMPs. MT-MMP expression in immature OSNs was also observed at the other developmental ages (E15 and E17, Figs. 5 and 6, respectively), although there was a gradual decrease in the MT-MMP expression by OSNs as development progressed to P0 and P2 (Figs. 7 and 8). In addition, MT-MMP expression can also be seen in the bundles of olfactory nerves as the sensory neuron axons fasciculate and travel to the olfactory bulb. Of note, there were no gross differences in MT-MMP distribution from rostral to caudal epithelium, nor were there any zonal differences in each epithelial section.

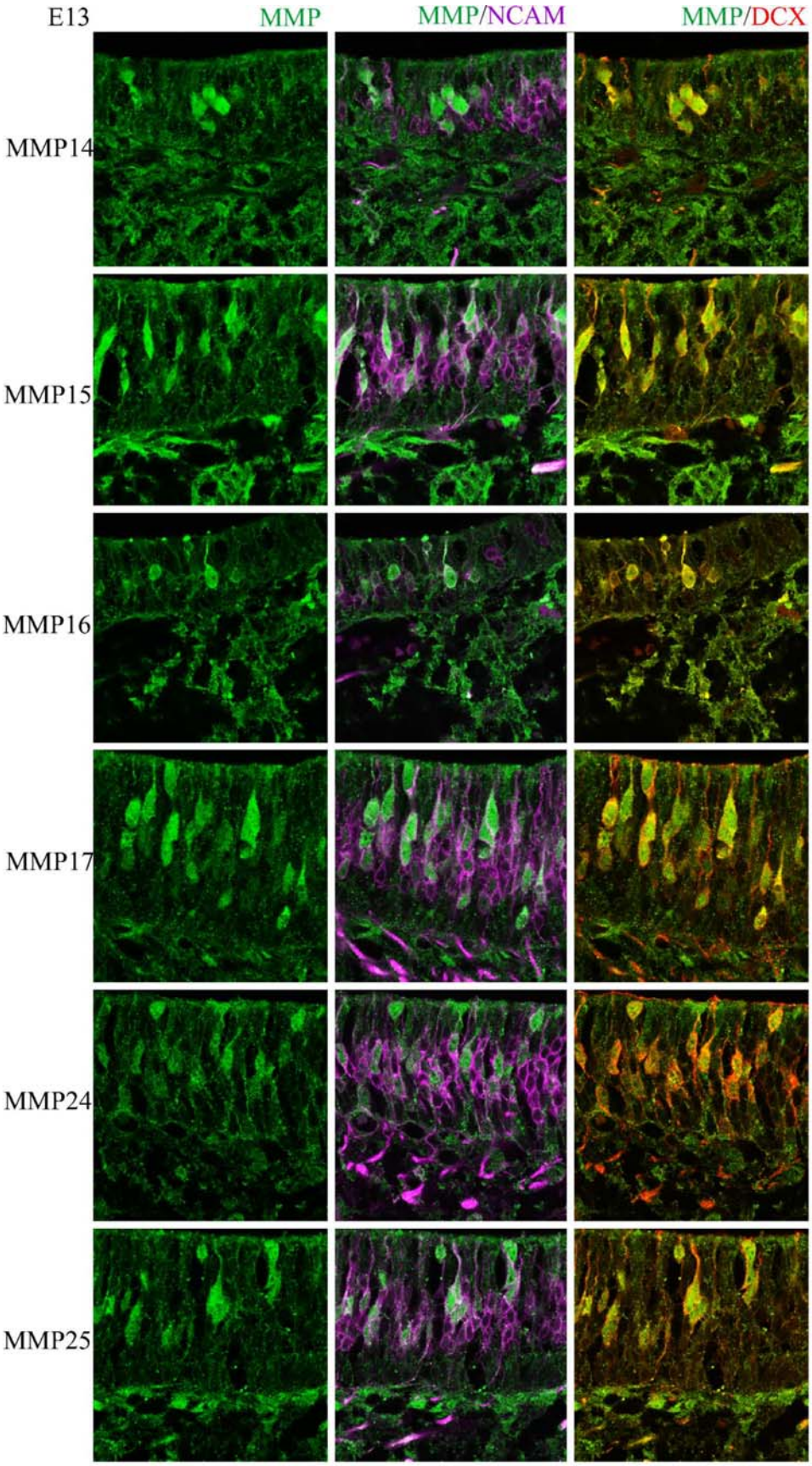
E13
MMP
MMP/NCAM
MMP/DCX
MMP14
MMP15
MMP16
MMP17
MMP24
MMP25

Figure 4: Immunohistochemistry of E13 olfactory epithelium. MMPs are shown in green, NCAM is purple, and Doublecortin is in red. All MT-MMPs are expressed in the OE, specifically in a small subset of NCAM-positive cells (colocalization appears white). Doublecortin labeling identified these cells as newly generated neurons, indicating that immature neurons express MMPs at their cell surface (colocalization appears yellow). MT-MMPs are also seen in the NCAM-stained axon bundles traversing the mesenchyme.

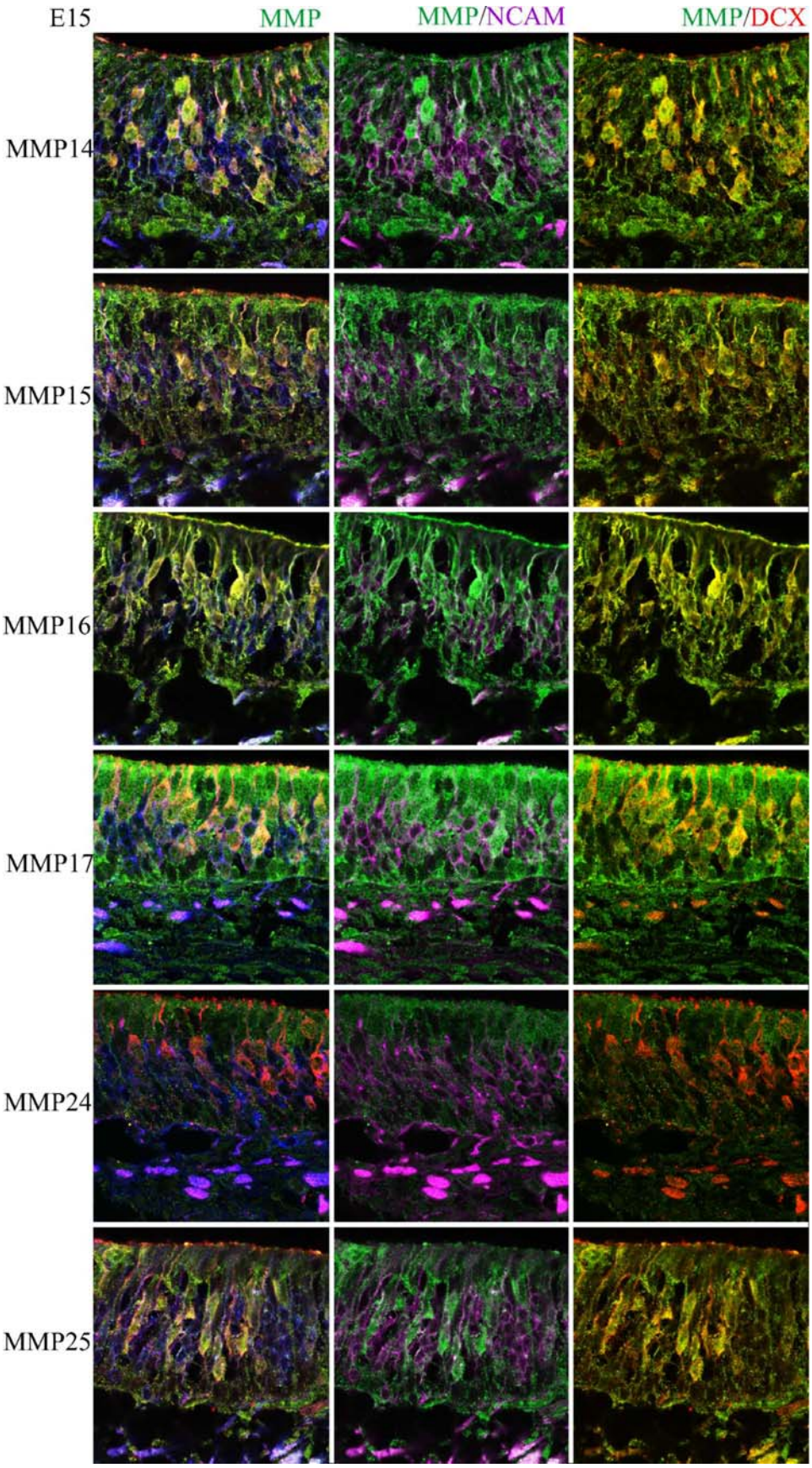
E15
MMP
MMP/NCAM
MMP/DCX
MMP14
MMP15
MMP16
MMP17
MMP24
MMP25

Figure 5: Immunohistochemistry of E15 olfactory epithelium. MMPs are shown in green, NCAM is purple, and Doublecortin is in red. Again, MT-MMPs are expressed in a subset of developing olfactory sensory neurons (yellow).

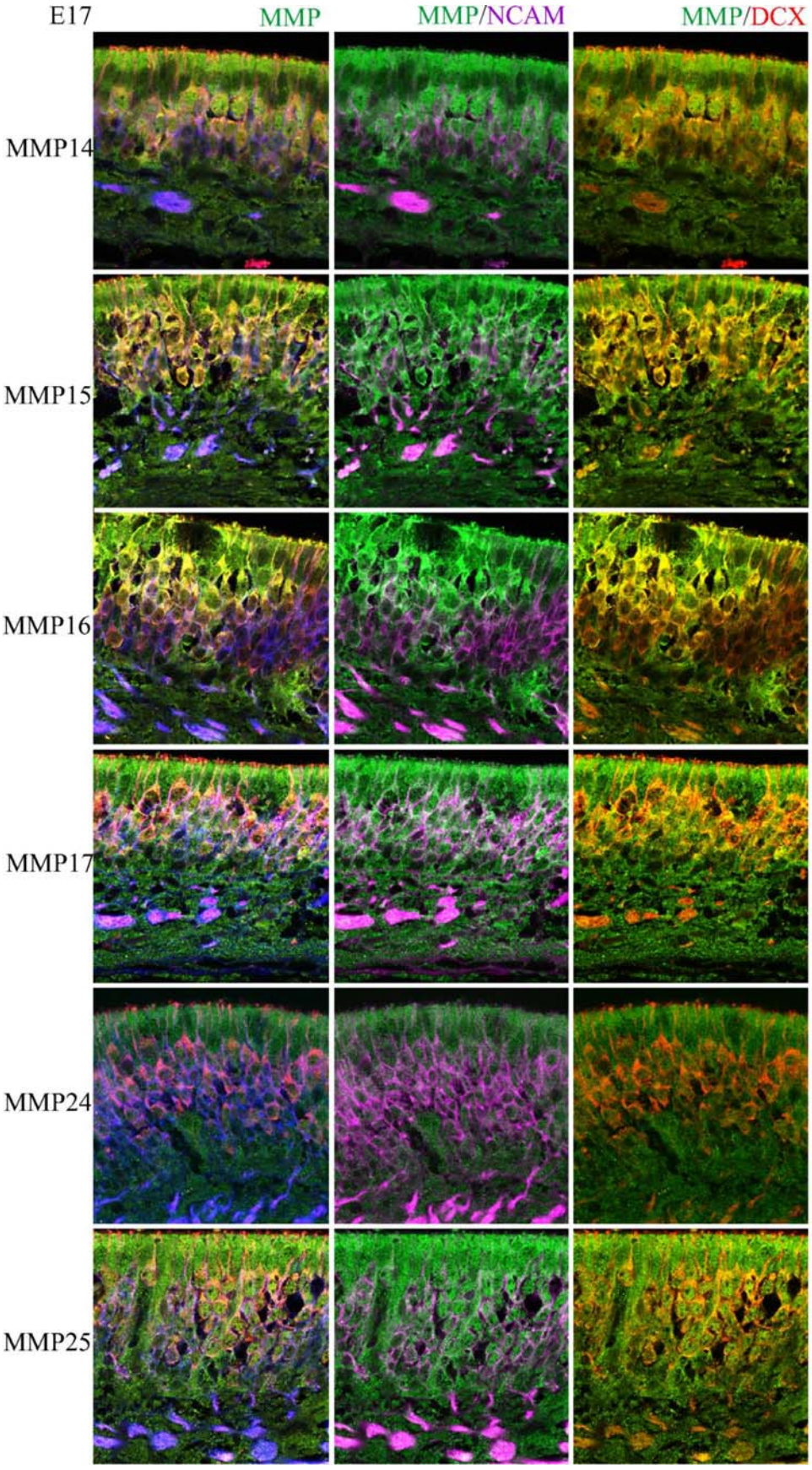
E17
MMP
MMP/NCAM
MMP/DCX
MMP14
MMP15
MMP16
MMP17
MMP24
MMP25

Figure 6: Immunohistochemistry of E17 olfactory epithelium. MMPs are shown in green, NCAM is purple, and Doublecortin is in red. Again, MT-MMPs are expressed in a subset of developing olfactory sensory neurons (yellow).

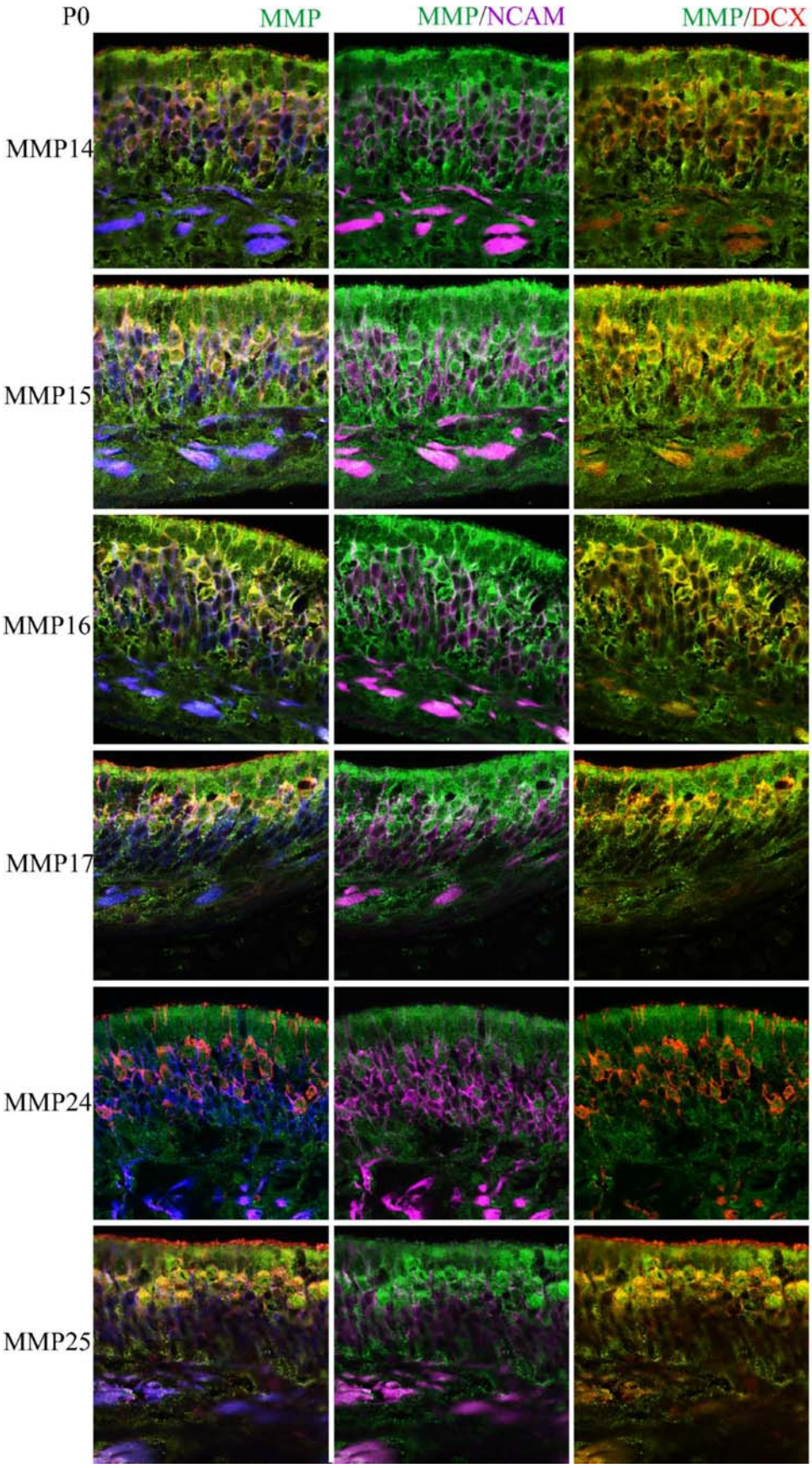
P0
MMP
MMP/NCAM
MMP/DCX
MMP14
MMP15
MMP16
MMP17
MMP24
MMP25

Figure 7: Immunohistochemistry of P0 olfactory epithelium. MMPs are shown in green, NCAM is purple, and Doublecortin is in red. Again, MT-MMPs are expressed in a subset of developing olfactory sensory neurons (yellow), although at this age expression is not as robust as in the embryonic ages.

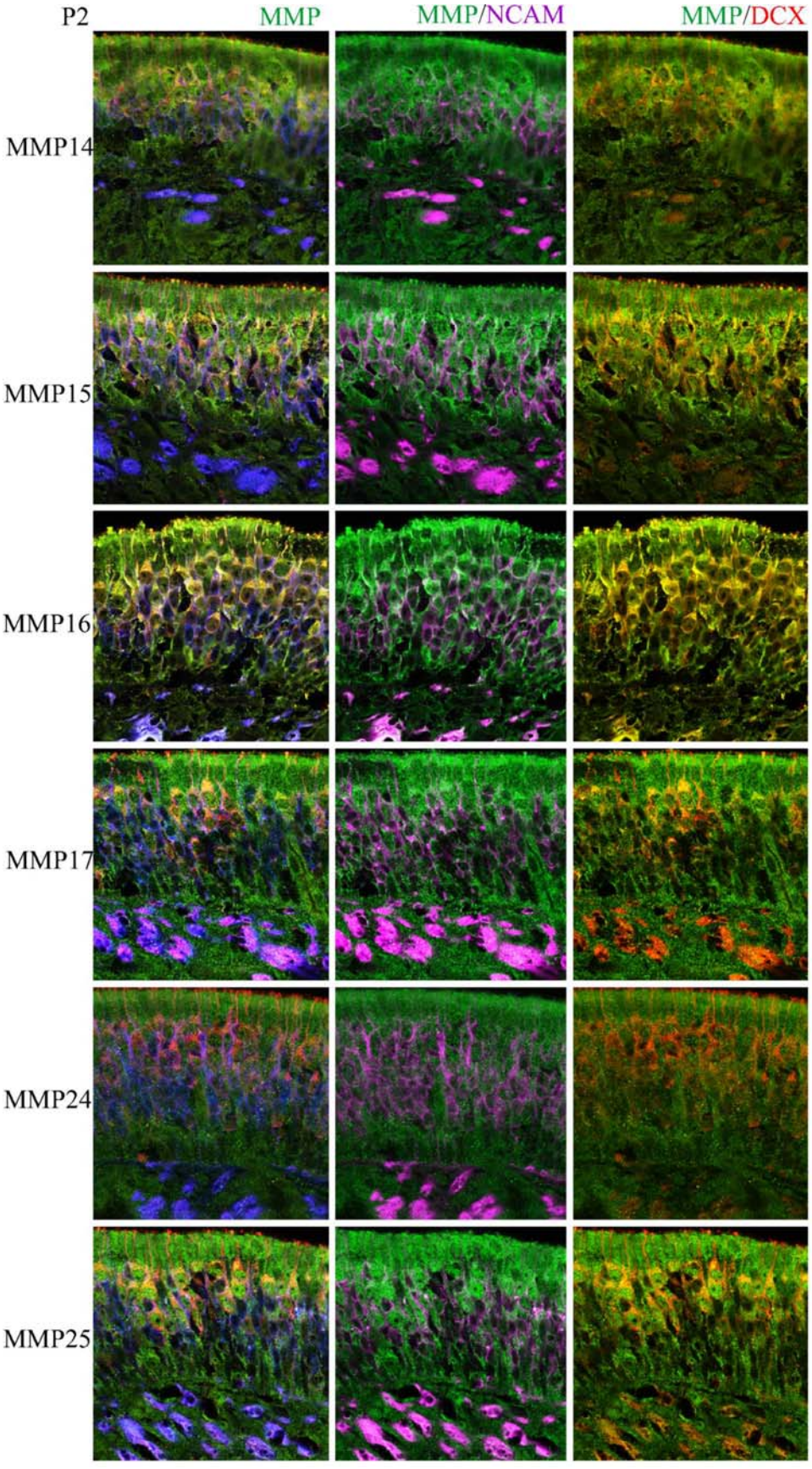
P2
MMP
MMP/NCAM
MMP/DCX
MMP14
MMP15
MMP16
MMP17
MMP24
MMP25

Figure 8: Immunohistochemistry of P2 olfactory epithelium. MMPs are shown in green, NCAM is purple, and Doublecortin is in red. Again, MT-MMPs are expressed in a subset of developing olfactory sensory neurons, although expression is decreased at this age.

In the olfactory bulb, MT-MMPs localize to the developing mitral cells at all developmental ages. In particular, MMPs 14, 15 and 16 (green, Fig. 9) and MMP 17, 24, and 25 (green, Fig. 10) show staining in the developing OB. The staining appears in immature tangentially oriented cells at E13. These cells become progressively radially oriented as development continues and eventually thin out into a distinct band at P0 and P2. MT-MMPs can also been seen in the olfactory nerve layer, labeled by NCAM (purple). At each age, MT-MMPs and NCAM colocalized (white), indicating that as the axons traveled from the olfactory epithelium and entered the bulb, MT-MMPs were present on their axonal surface.

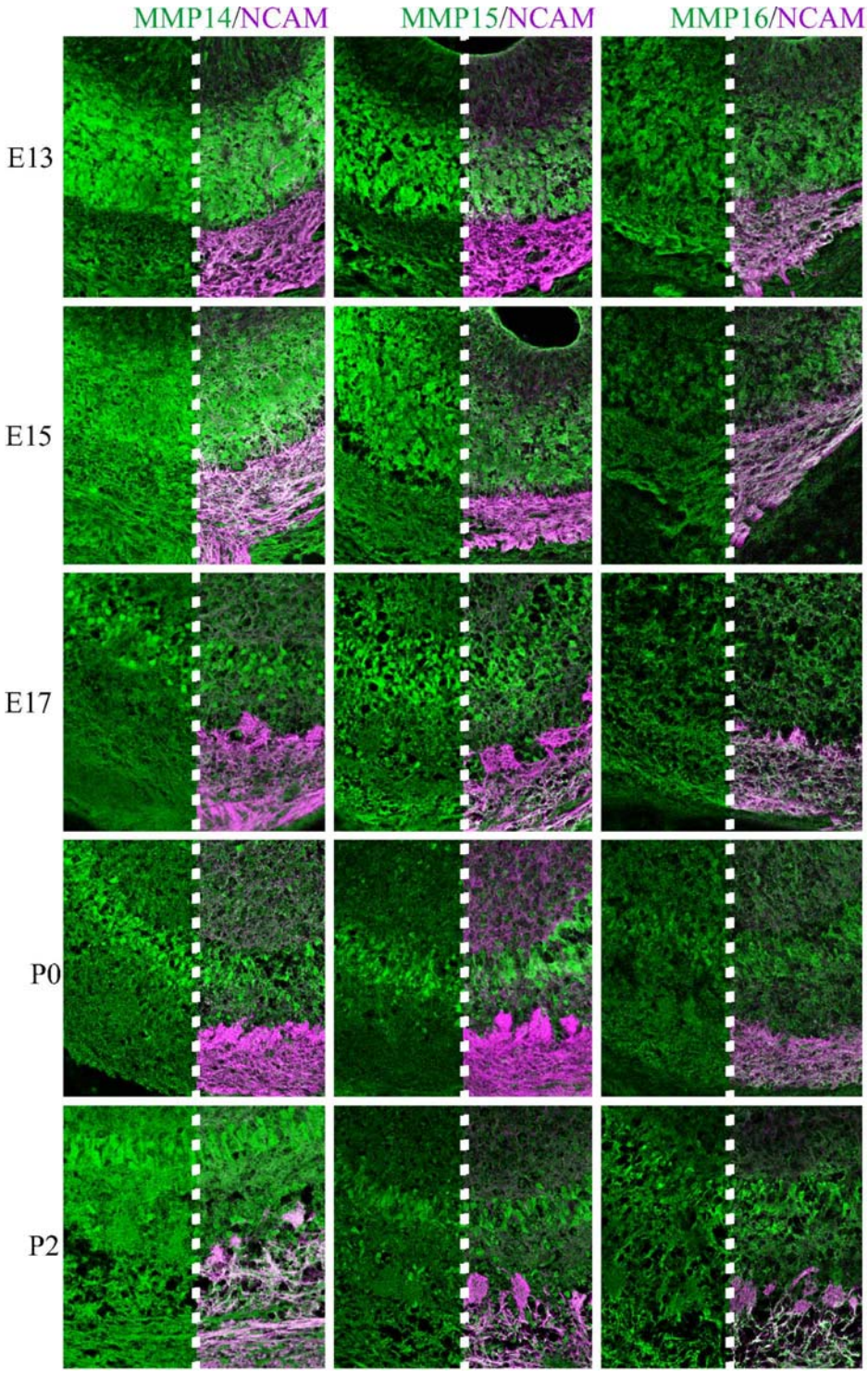

Figure 9: Immunohistochemistry of MMPs 14, 15, and 16 in olfactory bulb. MMPs are shown in green, NCAM is purple. MT-MMPs localize to the mitral cell layer inside the bulb, especially with MMPs 14 and 15. In addition, MT-MMPs localize to the NCAM-positive olfactory nerve layer, specifically within axon bundles.

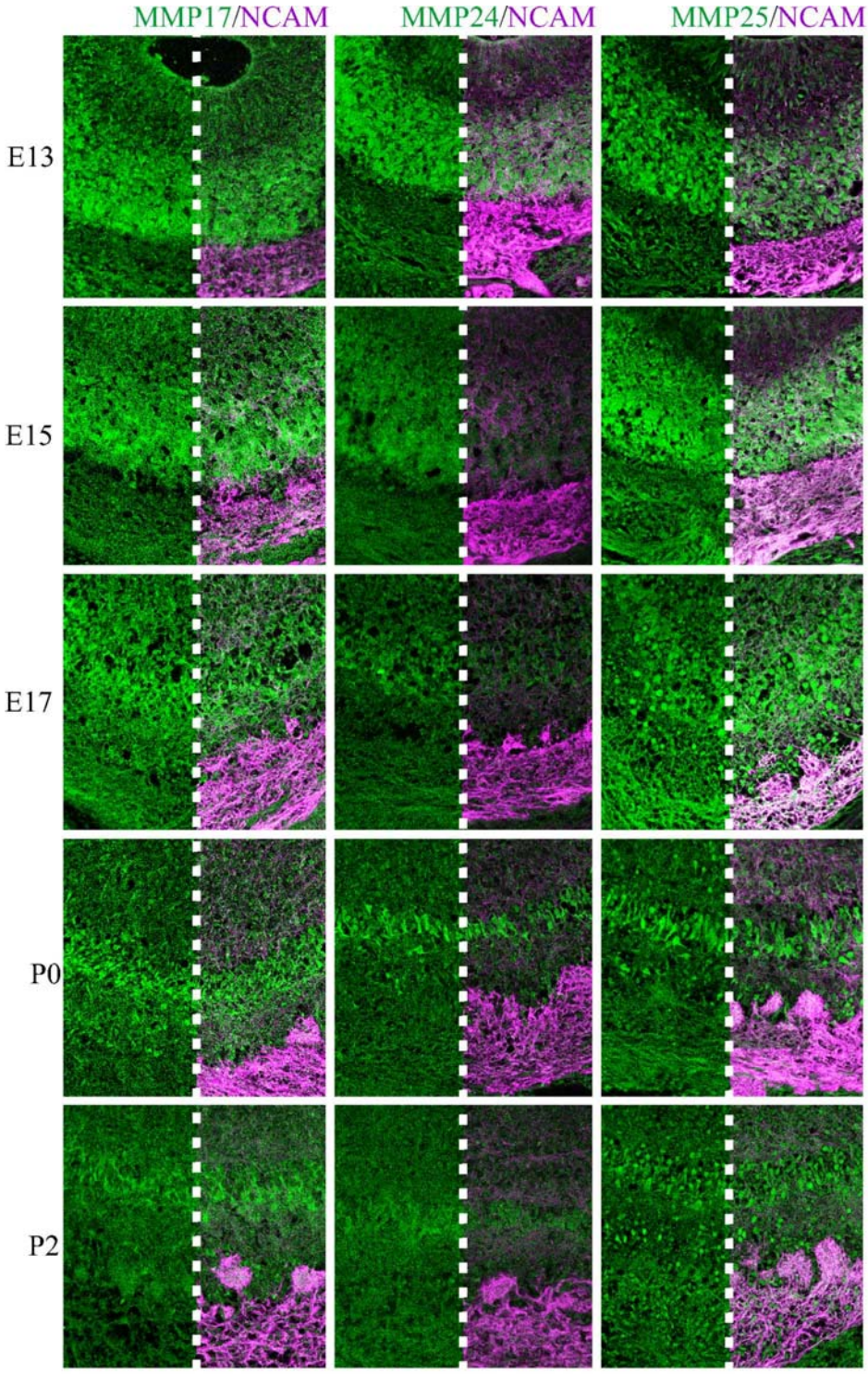

Figure 10: Immunohistochemistry of MMPs 17, 24, and 25 in olfactory bulb. MMPs are shown in green, NCAM is purple. MT-MMPs localize to the mitral cell layer inside the bulb. In addition, MT-MMPs localize to the NCAM-positive olfactory nerve layer, specifically within axon bundles.

4.3 Localization of gelatinase proteins and proteolytic activity

Immunohistochemistry was also used to reveal the protein distributions of the gelatinases (MMPs 2 and 9) in the developing olfactory pathway. As seen in the left panel of Figure 11, MMPs 2 and 9 are expressed in the bulb and epithelium. In particular, both proteins are found in the mitral cell layer and the nerve layer of the OB, and in the axons arising from the OE as well. In addition, MMP 9 appears to have relatively higher expression in the areas surrounding the nerve, particularly in the olfactory ensheathing cells.

Although the antibodies allowed for visualization of expressed gelatinase enzyme in the system, they were not able to distinguish whether the enzymes were present in their active or inactive pro-form. To determine whether active MMPs were present in the olfactory pathway, I turned to a different technique, *in situ* gelatin zymography. *In situ* zymography involves the breakdown of a non-fluorescent substrate (in this case gelatin) into a fluorescent product wherever active enzyme is present. Caged fluorescein-labeled gelatin (DQ-Gelatin, Molecular Probes) was overlaid on fresh tissue, and when cleaved by proteolytic enzymes, fluorescent peptides at the anatomic site of enzyme activity were released. The fluorescence detected is proportional to proteolytic activity.

Gelatinase activity was observed (as green fluorescence in the middle panel of Figure 11) around the olfactory nerves as they arise from the epithelium, and within the olfactory nerve layer, suggestive of expression of this enzyme by olfactory ensheathing cells. However, no activity was detected in the deeper layers of the olfactory bulb. Gelatinase activity was confirmed in the nerve with the addition of 1, 10-phenanthroline,

a synthetic inhibitor of metalloproteinases. This addition effectively abolished fluorescent peptide release by MMPs in the tissue.

MMPs 2 and 9 can cleave gelatin, as can the MT-MMPs. However, MMPs 2 and 9 have much higher affinities for this substrate than do the MT-MMPs (Fredericks and Mook, 2004). In my PCR screen MMPs 2 and 9 were found to be highly expressed in the developing olfactory system, both in the OE and OB. Similarly at the protein level, as seen in Figure 11, both MMPs 2 and 9 are found in the mitral cell layer in the olfactory bulb, and in the axon bundles of the olfactory nerve in both E17 and P0. Thus, the gelatinase activity revealed by the *in situ* zymography is likely due to cleavage by active MMP 9, which was found expressed in the ensheathing cells. It is possible that MMP 2 and MMP 9 in the olfactory bulb are being expressed in their pro-form (inactive) or their activity is being suppressed by TIMPs. Expression of active MT-MMPs may not be detectable using *in situ* zymography due to their lower affinity for gelatin and/or their hypothesized restricted activation within growth cones, which are such small cellular sub-compartments that activity may not be able to be detected using this technique.

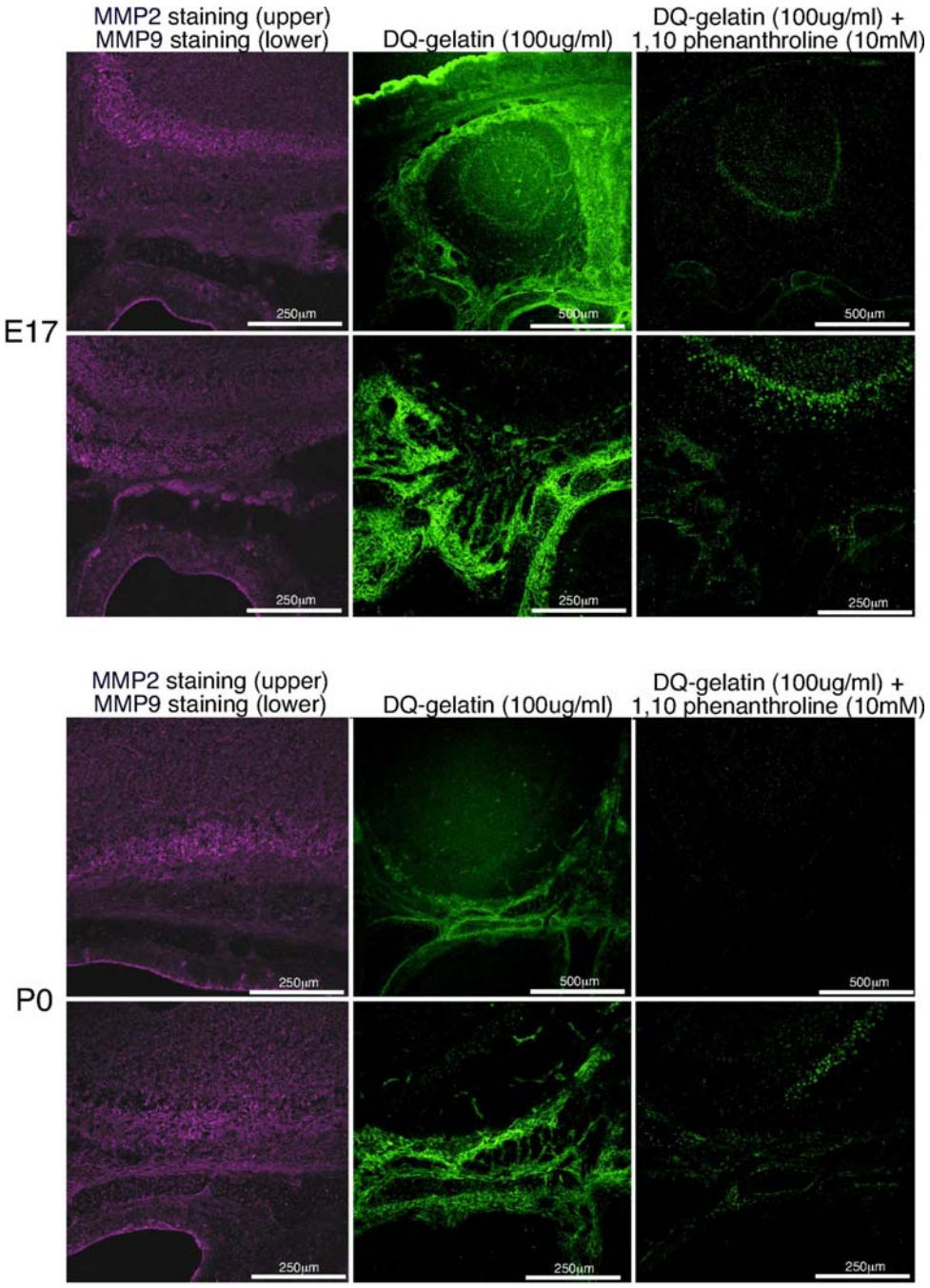

Figure 11: Gelatinase protein expression and enzyme activity in E17 and P0 olfactory system. In the left panel, MMP 2 and 9 proteins are localized in the olfactory system. As seen in the left panel, in both E17 and P0 the gelatinase proteins localize to the mitral cell layer of the olfactory bulb, as well as the axons arising from the olfactory epithelium and the olfactory nerve layer. In the middle panel, gelatinase activity is localized to the olfactory nerves arising from the epithelium and forming the nerve layer. However, no gelatinase activity was seen in the bulb. In the right panel, all MMP activity is abolished with the addition of synthetic inhibitor, confirming that the fluorescence in the gelatin is due to metalloproteinase activity.

4.4 Cell cultures

4.4.1 Axon outgrowth with synthetic inhibitors

To assess the function of MMPs in OSN neurite outgrowth, dissociated OSNs were grown in the presence and absence of GM6001, a zinc chelator and general MMP inhibitor. Because it was previously demonstrated by Muir (1994) that a common component of tissue culture media, cysteine, is a potent MMP inhibitor, I used specially designed media that replaced cysteine with its oxidized counterpart, cystine. This prevented any known inhibition from the media components, allowing for full metalloprotease activity under normal conditions and controlled inhibition solely from GM6001 (a general zinc chelator). When dissociated cells were analyzed for growth, there were consistent, significant decreases in all outgrowth parameters analyzed for OSNs grown in the presence of the MMP inhibitor.

Initial results indicated decreased growth when MMP activity was abolished by inhibitor: average maximum process length decreased from27.99 ± 2.57 μm to 20.08 ± 1.97 μm, and total neurite outgrowth (the sum of all neurites extending from a single cell body) decreased from 42.75 ± 3.60 to 27.43 ± 2.35, which was also reflected in the decrease in average number of processes per cell (3.0 ± .23 processes/cell to 2.1 ± .15) and a decrease in cell branching (from an average of 4.4 ± .72 branches per cell to 1.7 ± .32 per cell).

However, the synthetic inhibitor utilized comes from the manufacturer dissolved in DMSO. As a control, we added plain DMSO to the culture media to ensure the decrease in outgrowth was solely due to the effects of the GM6001. Unfortunately, we observed the same decrease in all parameters neurite outgrowth when DMSO was added.

Although we attempted several dose-response scenarios to minimize the amount of DMSO needed to deliver the GM6001, even small amounts of DMSO in the media caused an effect. Because the chemical properties of GM6001 do not allow us to utilize another solvent that could potentially have less of an effect on neurite outgrowth, the challenge now is to find a good MMP inhibitor whose vehicle has no effect. We are currently doing trials using antibiotics (e.g., minocycline) that can be dissolved in water and have been previously shown to inhibit MMP activity.

4.4.2 Growth on MMP-treated ECM substrates

Given the expression of multiple MT-MMPs on the surface of developing neurons, I examined their degradation properties to determine if this apparent redundancy of expression may be due to differing substrate specificities. For preliminary studies, I first tested olfactory sensory neuron (OSN) explant outgrowth on different substrates commonly found in the extracellular matrix (ECM), including tenascin-C (TNC), chondroitin sulfate proteoglycans (CSPGs), and heparin sulfate proteoglycans (HSPGs). Laminin (LN), known to be growth promoting, was also used as a reference. OSN explants were thus plated on each ECM substrate. Explants and their neurites were measured after a 48 hour incubation. Because explant size (and therefore total number of neurites) can vary, I took the ratio of total outgrowth (area of explant + neurites) and explant size (area of explant cell bodies) to normalize neurite outgrowth. As seen in Figure 12, LN is indeed growth-promoting, with a ratio of neurite outgrowth that was over 4 times that of the explant size. This can be seen in Figure 13a, in which the explant grown on LN has multiple branched neurites sprouting from the explant core. In contrast, TNC, CSPGs and HSPGs were all inhibitory; their outgrowth ratios all hovered around 1.0 (see Figure 12), indicating that neurite outgrowth did not extend very far beyond the explant itself. This effect is also seen in Figure 13a, in which each explant grown on the inhibitory substrates do not display any neurite expansion beyond the explant. However, treatment of these inhibitory substrates with active MMP enzymes alters neurite outgrowth. As seen in Figure 13b, when TNC is treated with MMP 14, neurite outgrowth is increased and multiple axons are able to extend from the explant. Presumably, MMP 14 degrades TNC, thus allowing the neurites to overcome the inhibitory nature of TNC

and grow accordingly. Similarly, MMP 15 treatment of CSPGs and MMP 24 treatment of HSPGs allow for increased neurite outgrowth due to the proteolytic degradation of these inhibitory proteoglycans.

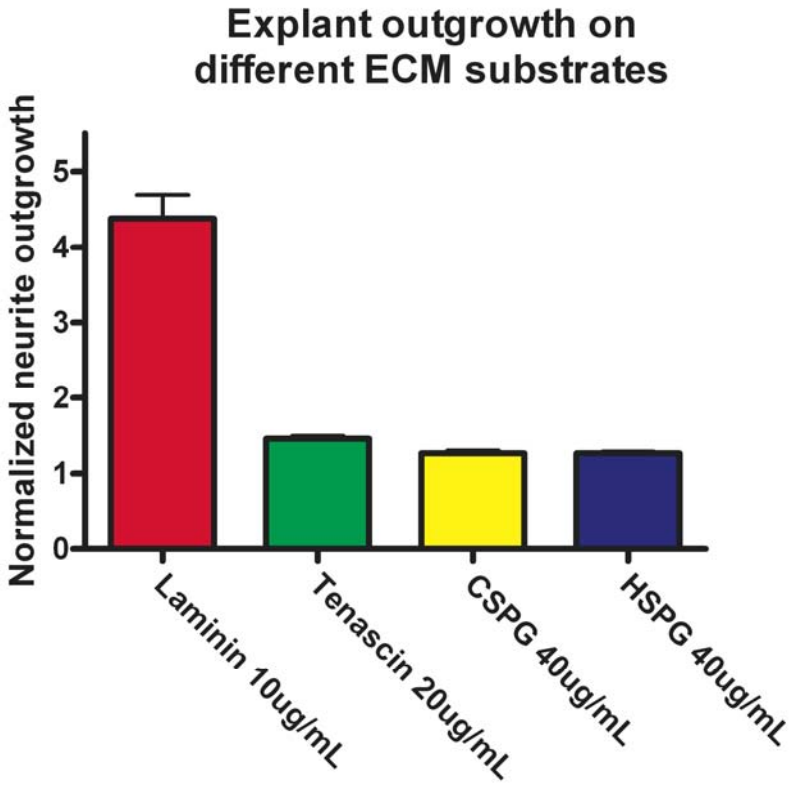

Figure 12: Neurite outgrowth on various ECM substrates. Laminin is growth promoting, with an outgrowth ratio of over 4. Tenascin, CSPGs and HSPGs are all inhibitory, as their outgrowth ratios remain around 1.0

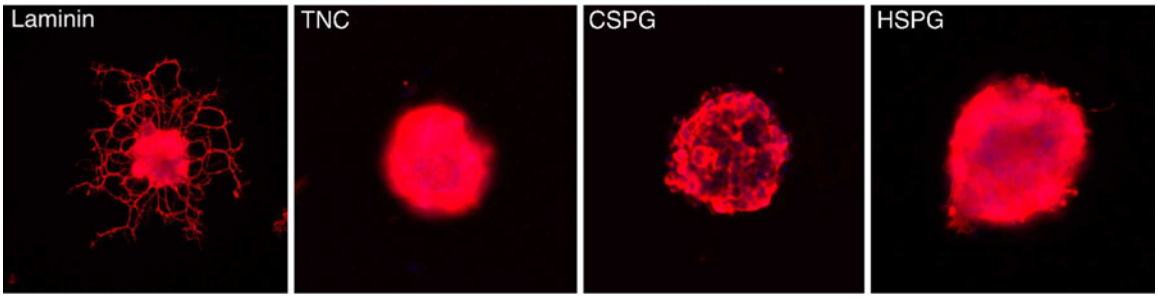

Figure 13a: Explants grown on different ECM substrates. The explant grown on laminin displays multiple branched neurites extending from the explant. The explants grown on TNC, CSPG and HSPG do not have any axons extending from the explant.

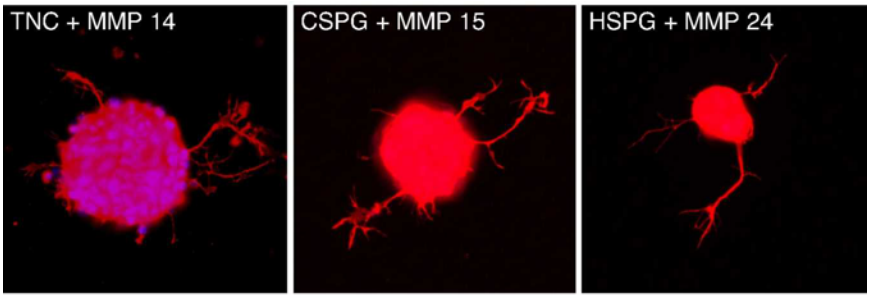

Figure 13b: Explants grown on ECM substrates treated with MMP enzymes. With MMP treatment, explants grown on inhibitory substrates are able to extend neurites.

I therefore tested each commercially available active MT-MMP enzyme on LN and the common inhibitory ECM substrates identified above. Treatment groups included a “No Treatment” group, in which the explants were grown on the substrate alone (i.e., LN only, CSPGs only, etc.); a “10μU” group, in which each ECM substrate was treated with selected MT-MMP enzyme at a concentration of 10μUnit (μU) of activity prior to plating of explants; and a “10μU + Inhib” group, in which each ECM substrate was treated with 10μU of selected MT-MMP enzyme mixed with a general zinc chelator, GM6001, at 25μM. Again, the ratio of total outgrowth to explant size was used to normalize for varying explant sizes, and comparisons were made in reference to the “No Treatment” group.

As seen in Figure 14, treatment of LN with MMP 14 appeared to have a decrease in outgrowth when compared to the No Treatment control. However, MMP 15 + Inhib and MMP 16 + Inhib also appeared statistically significant, although we would have expected these groups to be comparable to the No Treatment group. Importantly, however, the statistical trends we observed as more iterations were added lead us to believe that these significant values will drop out in the end. We predict that there will be no difference in neurite outgrowth between explants grown on LN alone versus those grown on LN treated with recombinant MMPs.

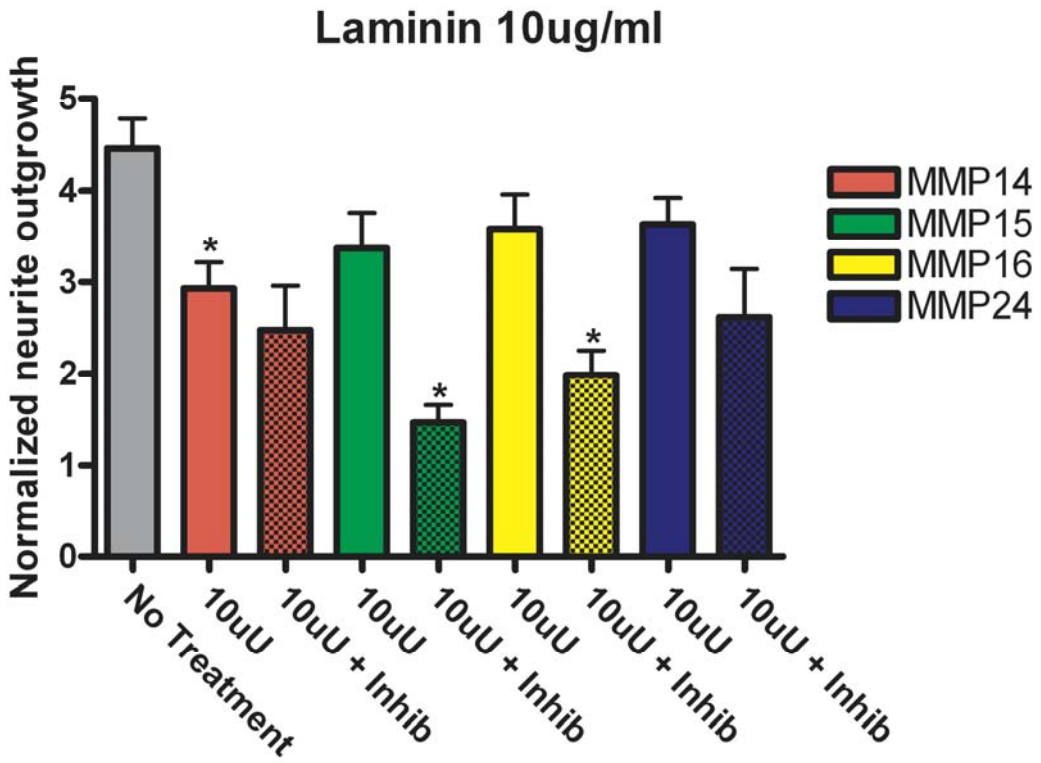

Figure 14: Measured outgrowth on laminin. MMP 14, MMP 15 + Inhibitor, and MMP 16 + Inhibitor showed decreases in outgrowth, although we expect these values to drop out as sufficient power is achieved.

When TNC was treated with active MT-MMP enzyme, neurite outgrowth was increased in some, but not all, conditions, indicating substrate specificity on the part of the enzymes (see Figure 15). In particular, the application of MMP 14 to TNC decreased the inhibitory effects of this substrate, allowing for a significant increase in neurite outgrowth ($p<0.01$). In contrast, MMPs 15, 16, and 24 treatment did not have any statistically significant effects on neurite outgrowth, indicating that these enzymes are not active on TNC. None of the enzyme + inhibitor combinations resulted in significant differences with the No Treatment group, proving to be effective controls.

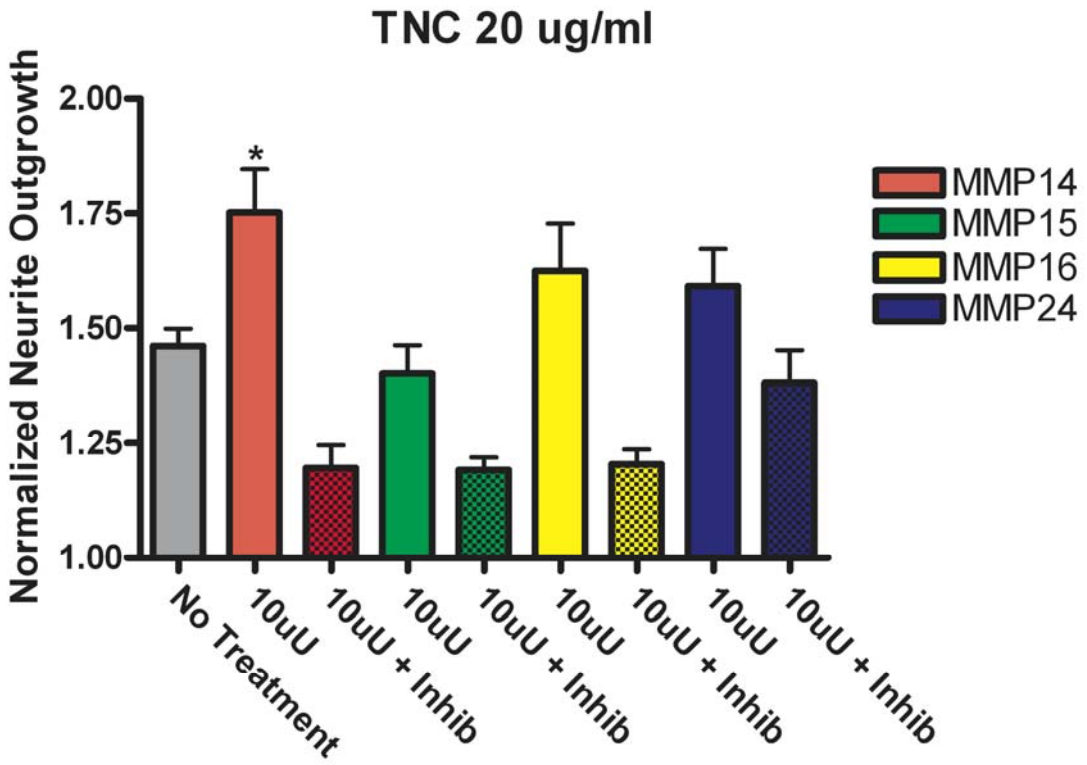

Figure 15: Measured outgrowth on tenascin. MMP 14 at 10μU was effective in degrading TNC and increasing neurite outgrowth ($p<0.01$). MMPs 15, 16, and 24 used on TNC had no effect on neurite outgrowth.

When compared to MMP treatment of TNC, the results for CSPGs differed. Unlike TNC, MMP 14 had no effect on CSPGs, and thus neurite outgrowth continued to be inhibited by this substrate (see Figure 16). However, MMP 15 had robust effects on CSPGs, overcoming its inhibitory effect through degradation and increasing neurite outgrowth nearly twice as much as its control ($p<0.01$). In addition, MMPs 16 and 24 treatment did not have any statistically significant effects on neurite outgrowth, indicating that these enzymes are not active on the CSPGs. None of the enzyme + inhibitor combinations resulted in significant differences with the No Treatment group, also proving to be effective controls.

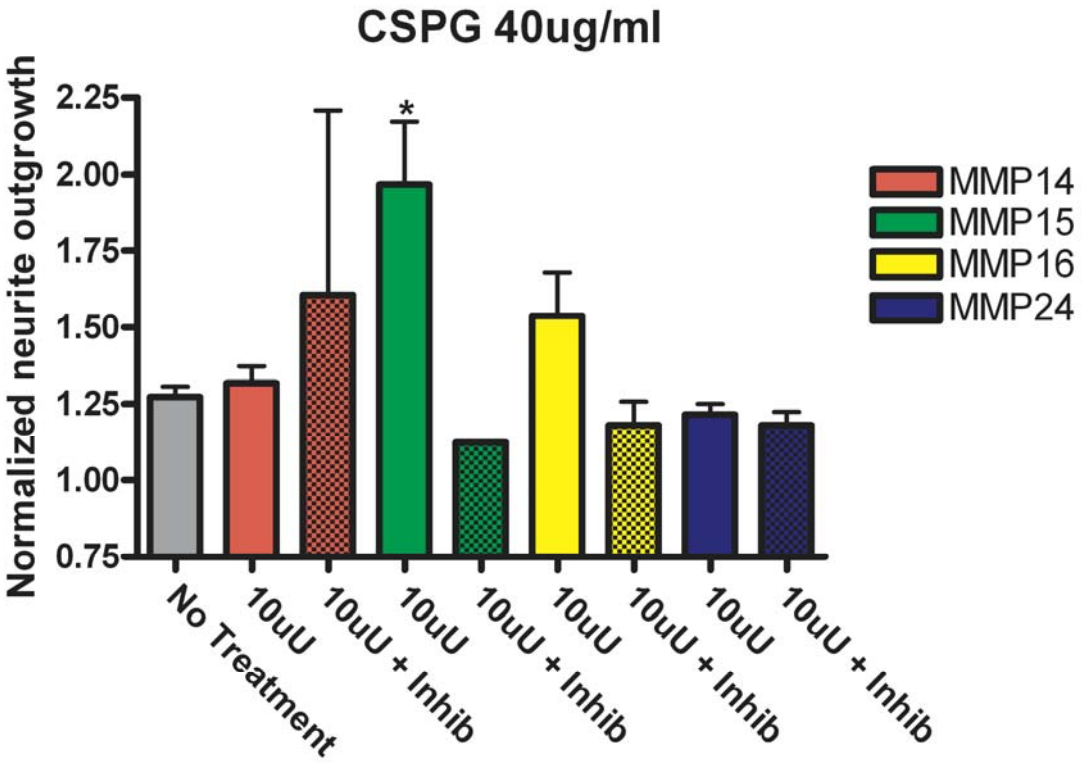

Figure 16: Measured outgrowth on CSPGs. MMPs 14, 16, and 24 were ineffective in degrading CSPGs, and outgrowth continued to be inhibited. MMP 15 had robust effects on CSPGs, and increased outgrowth to nearly two times as much as control.

Like the CSPGs, HSPGs were unaffected by MMP 14 and MMP 16 enzyme treatment, and the outgrowth ratio reflected that of the No Treatment group (see Figure 17). However, in contrast to the CSPGs, MMP 15 treatment was ineffective in increasing neurite outgrowth on HSPG. However, the data demonstrates that MMP 24 alone is active on HSPGs, degrading this substrate and allowing for increased neurite outgrowth in culture. Once again, none of the enzyme + inhibitor combinations resulted in significant differences with the No Treatment group, proving to be effective controls.

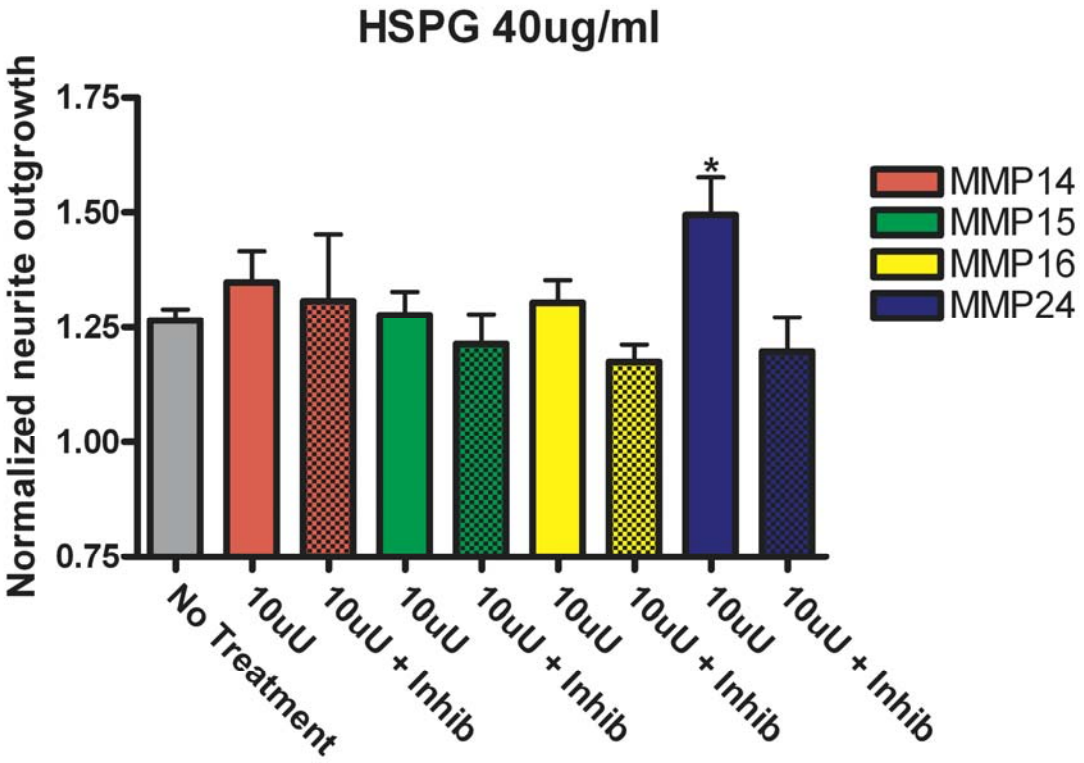

Figure 17: Measured outgrowth on HSPG. Enzymatic treatment of HSPGs by MMP 24 resulted in significant neurite outgrowth ($p<0.01$), while MMPs 14, 15, and 16 have no effect.

Table 6 summarizes the significant results of this study. From this, we can conclude that multiple MT-MMPs may be present on the cell surface of a developing neuron because they have differing substrate specificities and degradation efficiencies. In our assays, MMP 14 is only effective at increasing neurite outgrowth when used to treat inhibitory TNC, while MMP 15 increases outgrowth when applied to CSPGs. MMP 16, on the other hand, does not appear to increase neurite outgrowth when used to treat any of the ECM molecules tested, indicating that this enzyme may not be specific enough for these particular substrates. Finally, MMP 24 has the most potent degradative effects on HSPGs, increasing neurite outgrowth when used to treat this particular group of proteoglycans.

Substrate	MMP 14	**MMP 15**	**MMP 16**	**MMP 24**
Tenascin	*			
CSPG		*		
HSPG				*

Table 6: Summary of enzyme effectiveness on different substrates. * Denotes significant increase in neurite outgrowth, $p<0.01$.

5 Discussion

As axons navigate the complex milieu of the developing CNS, their growth cones encounter numerous signals in the extracellular environment that help guide them to their eventual synaptic target [1]. These signals, or guidance cues, can influence axon extension, retraction, or turning through cytoskeletal reconfiguration mediated by Rho-GTPases [1]. Although traditionally classified as attractive or repulsive [185], guidance cues can be interpreted differently by growing axons depending on the internal state of the growth cone [3] (e.g., second messenger signaling [17] or intracellular calcium concentrations [20, 21]) such that cues that are repulsive in one environment or situation can be attractive in another. It has also been recognized that the extracellular matrix (ECM) plays a role in axon guidance [25]. Apart from acting as a physical anchor for the lamellipodia of axons [26], the ECM acts as a scaffold for many important proteins, including laminins, tenascins, CSPGs and HSPGs [24, 25]. While much focus has been on identifying guidance cues and elucidating their effects on the nervous system, an emerging area of interest involves the regulation of these guidance cues. Matrix metalloproteinases (MMPs), a family of zinc-dependent proteolytic enzymes, have been shown to affect axon guidance by degrading the ECM or by regulating guidance cues through ectodomain shedding or receptor cleavage [49]. Collectively, these powerful degradative enzymes have the capacity to digest every component of the ECM [51]. Therefore, it is no surprise that they are tightly regulated by their endogenous inhibitors (tissue inhibitors of metalloproteinases, TIMPs) [84, 85] and have been implicated in numerous processes, ranging from osteogenesis [101] to cancer metastases [123].

5.1 Gelatinases are expressed and are proteolytically active in the developing olfactory system

Because of the increasing evidence that MMPs are a previously unrecognized mechanism in pathway formation during development, I examined the role of MMPs in the developing nervous system. Using the olfactory system as a model because of its complex axonal topography and capacity for regeneration, I first performed a comprehensive RT-PCR screen of 19 members of the MMP family and their TIMPs. This allowed for quick identification of relevant family members in the olfactory system, therefore streamlining further investigation and eliminating many genes from additional examination. From the RT-PCR screen, I was able to identify two major sub-families that were consistently upregulated throughout development in both the olfactory epithelium and olfactory bulb: the gelatinases and the membrane-bound MMPs (MT-MMPs).

In the olfactory epithelium, both gelatinases (MMPs 2 and 9) mRNA were expressed throughout all ages tested. In the bulb, MMP 9 mRNA was present throughout, while MMP 2 appeared at later ages. It is known that MMPs 2 and 9 are among the most highly expressed MMPs in the brain, playing roles in a variety of developmental processes and pathologic conditions [186]. For example, the proliferation and lineage specification of neural stem cells are mediated by EGF ligands [187], which are regulated by MMPs [186]. Because MMP 2 is expressed by neural stem cells *in vitro* [188, 189], it is in a position to help determine the eventual fate of neural stem cells. In addition, both gelatinases are instrumental in cerebellar morphogenesis [190], while MMP 9, in particular, aids with cerebellar granule cell migration [191]. It is possible that the gelatinases present in the developing OS also aid in cellular migration, for instance

facilitating the influx of mitral and periglomerular cells as they populate the bulb or contributing to the migration that establishes its laminar organization. In addition, because MMP 2 expression increases in the presence of growth factors and growth-promoting molecules such as laminin [192], MMP 2 in the epithelium may work synergistically with the laminin also present there [180] to promote OSN axon outgrowth toward the bulb. Finally, MMPs are also known to process cell adhesion molecules [186], a variety of which are expressed in the olfactory axon bundles and can regulate their fasciculation [193]. Therefore the gelatinases that are expressed at ages E13-E15, during the axonal sorting period, may cleave cell adhesion molecules present in the olfactory nerve and thus play a role in defasciculation and homotypic sorting of axons.

Immunolocalization of the gelatinases also confirmed their expression in the developing OS, in which MMPs 2 and 9 proteins were localized to both the olfactory bulb and epithelium. In addition, MMP 9 was found on olfactory ensheathing cells. Because immunohistochemistry does not distinguish between MMP zymogens versus active enzymes, *in situ* zymography was employed to further elucidate the function of gelatinases in the OS. Although gelatinase proteins were immunolocalized to both the OB and OE, *in situ* zymography gelatinase activity was restricted to the lamina propria around the olfactory nerve, especially in the olfactory ensheathing cells and surrounding the bundles of axons fasciculating in the nerve. It may be that their strong activity in the OSNs may be important in axon sorting and outgrowth from the neuroepithelium to the brain, and their activity on the ensheathing cells may allow those cells to be a conductive substrate for the elongating nerves. However, even though there was protein expression detected in the bulb, no gelatin was cleaved by MMPs at that site, indicating that MMPs 2

and 9 present in the bulb may be in pro-form or being inhibited by TIMPs. It is also possible that proteolytic activity may be present in the OB, but at such low levels that it is undetectable by the zymography assay. For example, the MMP activity needed in processes like synaptogenesis in the developing bulb may be relatively low when compared to the proteolysis necessary to mediate axon guidance in the olfactory nerve. However, it is also possible that MMPs are dormant in the bulb because their activity is only required in case of injury. Perhaps the necessity for rapid proteolysis during an acute insult requires their constitutive presence in the bulb so as to avoid potential damage during the time lag associated with enzymatic recruitment or the process of expression. Indeed, MMP 9 levels increase within five hours of olfactory nerve transection, suggesting its involvement in reactive processes such as inflammation [194]. Some have suggested that this timing is associated with the compromise of vascular integrity and leukocyte infiltration, especially since MMP 9 can act upon components of the basal lamina of the vasculature [194]. Furthermore, the rapid increase of MMP 9 coincides with neutrophil infiltration [186, 194], in which the activity of MMP 9 protects antimicrobial neutrophil elastase from degradation, therefore contributing to innate immunity to an infectious insult [195]. Similarly, the presence of dormant MMP 2 may be necessary to mediate healing after an insult, as it has been shown that MMP 2 can attenuate the recruitment of inflammatory cells [127], regulate gliosis, and aid in white matter sparing [196].

5.2 Membrane-bound MMPs are expressed in a subset of neurons and increase neurite outgrowth on inhibitory substrates

Another interesting sub-family that was strongly expressed was the MT-MMP family. Six of the seven members of the MT-MMP family (MMPs 14, 15, 16, 17, 24, and 25) had mRNA expression throughout development, both in the epithelium and the olfactory bulb. This spatio-temporal pattern was confirmed with immunohistochemistry, in which MMPs 14, 15, 16, 17, 24, and 25 were localized to the mitral layer directly apposed to the ONL in the OB, and in OSNs throughout the OE. Interestingly, however, the MT-MMPs were not found on all NCAM-positive OSNs in the epithelium; they were expressed only in a subset of neurons, which were determined to be immature neurons via Doublecortin staining. Although no gross zonal differences were noted, it appeared that all of the immature OSNs expressed all six MT-MMPs on their surface. It is possible that the transmembrane anchoring of these enzymes ideally suits them for action at the cell surface, especially at the growth cone of immature OSNs traveling from the periphery into the CNS. Because the growth cone constantly encounters and interprets the rapidly changing extracellular environment of the developing nervous system, it is possible that MT-MMPs play a crucial role in signal perception and transduction as they are well suited to cleave the ECM molecules and/or guidance cues the growth cone encounters, or cleave the receptors anchored at the growing tip. Furthermore, it has been suggested that the clustering of MMPs at the cell surface can enhance proteolytic efficiency [186]; if so, the simultaneous presence of multiple MT-MMPs might be needed on a single immature neuron because of their differing substrate specificities. Although there have been some reports of specific MMPs degrading inhibitory

components of the ECM (e.g., MMP 2 degrading CSPG [197]), many of the specific substrates upon which MT-MMPs act have not yet been identified, nor has their role in axon guidance been thoroughly elucidated. To investigate this intriguing possibility, I studied the effects of applying MT-MMPs on common growth-promoting and non-permissive substrates of the ECM.

Laminin-1 (LN) is generally growth promoting in the nervous system, and was shown to be expressed in the developing olfactory system between E12.5 and E 16.5 [180]. Although previous studies have shown that LN is a substrate of various MMPs, including MMPs 2, 3, 7, 9, 11, 12, and 19 and 25 [198], the results were so variable that it may be that the selected MT-MMPs in our assay do not have an effect on LN. Although significance was reached with MMP 14, MMP 15 with inhibitor and MMP 16 with inhibitor also appeared significant. Given statistical trends as more iterations were added, we believe that with increasing explants approaching sufficient power, there will be no difference in neurite outgrowth between explants grown on LN alone versus those grown on LN treated with recombinant MMPs. This would suggest that LN is not a substrate of the MT-MMPs chosen. It is possible that the interaction of MMPs and LN is more important in other areas of the nervous system, including synapse formation and remodeling, NMDA-R activity, and hippocampal LTP [144] rather than axon guidance.

Tenascin (TNC) was chosen as a culture substrate because it is expressed in the developing olfactory system [199]. When grown on TNC alone, cultured OSNs do not extend neurites, suggesting an inhibitory function of TNC in the olfactory system. It has been suggested that the presence of TNC in the developing olfactory bulb prevents the invasion of OSNs into the bulb during the early stages of development (E13-E17),

therefore creating a critical waiting period prior to glomerular formation (Treloar et al., unpublished data). This waiting period has been postulated to give the sensory axons a chance to reorganize their heterogeneous bundles and refasciculate in order to find their specific glomerular targets within the bulb (Treloar et al., unpublished data). However, as the mouse OB progresses beyond this waiting period, the inhibitory effect of TNC expressed in the bulb and glomeruli decreases (Treloar et al., unpublished data), perhaps due to a change in TNC expression or TNC receptor expression by OSN axons. The MMP immunohistochemistry data correlate with this pattern, as the most robust expression of MT-MMPs in the bulb occurs in the dendritic zone during E13 and E15, slightly tapering off as glomeruli appear. Of the recombinant MT-MMPs used to treat the TNC substratum, only MMP 14 appears to increase neurite outgrowth, likely through degrading the inhibitory substrate. Therefore, MT-MMPs may be in the OB to degrade inhibitory TNC once this critical waiting period is over, allowing for permissiveness through this macromolecular wall and subsequent axonal invasion. It is also possible that MMPs modulate the inhibitory effects of TNC by acting upon one of its receptor, F3/contactin, which is expressed by OSN axons concurrent with the expression of TNC in the bulb (Treloar et al., unpublished data). F3/contactin contains fibronectin repeats [200], which can be bound by the hemopexin domain of MMPs [60]. It is possible that MMP 14 regulates the inhibitory nature of TNC by acting upon the fibronectin domains of its receptor, perhaps by degradation of the receptor itself or by ectodomain shedding. If this were the case, then OSNs expressing this receptor would become functionally non-reactive to the inhibitory effects of any TNC that is present, thus allowing for growth into the olfactory bulb.

In contrast to their role in the degradation of TNC or its receptor to facilitate neurite outgrowth, MT-MMPs have a more complex function in axon outgrowth and guidance when proteoglycans are involved. In particular, CSPGs have been shown to be inhibitory to growing axons [39, 40, 201]. After injury to the CNS, regenerating axons are inhibited by the glycosaminoglycans (GAGs) of CSPGs; these GAGs are therefore tantalizing pharmeceutical targets for CNS injuries [201]. Apart from their inhibitory effects in glial scars, CSPGs have a varying role in the developing nervous system. They can be inhibitory, as with embryonic chick neurites [39, 40] or growth promoting to cortical neurons [42]. In the developing olfactory system, CSPGs are expressed as early as E12.5, particularly in the marginal zone next to the presumptive OB and in the mesenchyme surrounding OSNs [180]. It has been postulated that CSPGs are inhibitory to these axons, either restricting their entry into the brain early in development or shepherding these axons to a strict pathway between the epithelium and the bulb [180]. The inhibitory effect of CSPGs was confirmed in culture, where explants did not extend neurites on this substrate. Although MMP 14 was effective in overcoming the inhibition of TNC, it had no effect on neurite outgrowth when used to treat CSPG, suggesting substrate specificity on the part of these enzymes. MMPs 16 and 24 also did not affect outgrowth; only MMP 15 was successful in overcoming the inhibitory effects of CSPGs. It is possible that MMP 15 acts in a straightforward manner, degrading CSPGs present in the marginal zone to allow for formation of the presumptive nerve layer. However, MMP 15 may also be regulating axon guidance by modulating the interaction between CSPGs and other molecular guidance cues, such as semaphorins.

It is known that CSPGs can interact with Sema5A through thromobospondin repeats [202] and can switch Sema5A from an attractive guidance cue to a repulsive one for habenula nucleus axons [43]. Because of its effects on CSPGs, MMP 15 may modulate the interaction between CSPGs and Sema5A, perhaps by terminating their interaction. Without CSPGs acting on semaphorin to make it repulsive, axons would then be able to grow into a previously inhibitory region. Indeed, this may be the case in the developing OS, since MMP 15, CSPG [180], and semaphorins are expressed in the developing OS, where they play a direct role in the glomerular convergence of P2 axons [203].

The regulation of this interaction may also be important in other systems, since CSPGs and semaphorins are present in the motor pathway and optic tract [204]. For example, chondroitin sulfate (CS) removal in the zebrafish results in abnormal ventral motor nerve growth into the posterior portion of a somite, where Sema3A2 would normally repel such axons [205, 206], suggesting that the removal of CS may affect the localization of Sema3A2 into the appropriate region [204]. If CSPGs do help with the localization of semaphorins, then MMP 15 may add another layer of regulation, whereby the enzyme may affect the binding of CSPGs to semaphorins and either catalyze of terminate the interaction. Similarly, CSPGs can bind another guidance molecule, netrin [204]. It has been suggested that CSPGs can displace netrins in the ventral commissure of the *Xenopus* optic tract and may expose repulsive cues (possibly Sema3A) that cause inappropriate bypassing of the commissure by axons in the postoptic tract [207]. Again, MMP 15 may help determine the localization of netrins or cause the unmasking of an inhibitory guidance cue through its activity on the CSPG-netrin interaction. Perhaps it is

this sort of guidance cue modulation that makes MMPs critical elsewhere in the developing visual system, whereby their presence is necessary at select decision points so that retinal ganglion cell axons can properly turn into their target [208].

MMPs may similarly modulate the interaction between HSPGs and their binding partners in the developing CNS. Slit proteins act as axonal repellants in several areas, such as the motor, olfactory, and visual systems[209]. It has been shown that Slit proteins purified from brain can bind to heparin columns [210], while heparinase treatment of an HSPG, glypican, causes it to unbind from Slit2 [211]. In the developing OS, an HSPG appear in a punctate distribution on olfactory axons in the nerve pathway as early as E12.5 [180]. This HSPG has been shown to be a splice variant of perlecan [212], which when coupled with FGF-1 can promote neurite outgrowth of OSNs and can induce a growth-promoting phenotype in olfactory ensheathing cells [213]. However, it has also been shown that HSPGs are necessary for Slit's repulsive effect on olfactory bulb axons, in which the heparin sulfate chains are necessary for Slit2 to bind to its receptor, Robo [209]. In our assay, HSPGs are inhibitory to neurite outgrowth, likely due to the absence of FGF-1. MMPs 14, 15, and 16 do not appear to have any effect on the inhibitory nature of HSPGs in this system, whereas MMP 24, previously ineffective with TNC and CSPG, does allow for increased neurite outgrowth when used to treat HSPGs. In the olfactory system, OSNs express *robo2* mRNA just after sending their first axons into the OB, while its ligand *slit3* is expressed postnatally in axons ending in glomeruli [214]. Because Slit, Robo [214], and HSPG [180] are present in the nascent OS, and this assay shows that HSPG is an MMP 24 substrate, MMP 24 may be in a position to influence axon outgrowth. MMP 24 may degrade HSPG to functionally inactivate the repellant activity

of the Slits, or they can release growth-promoting factors such as laminin, which has been shown to colocalize with HSPG in a punctate distribution along primary olfactory axons [180]. Alternatively, MMP 24 may remove HSPGs from the pathway, thereby regulating the effects of FGFs on OSNs.

Unlike slits, netrins do not require HSPG to bind to its receptor, DCC [215]. However, heparin does bind to the receptor complex, either directly with netrin through its C-terminal domain, or on DCC itself [204, 215, 216]. MMP 24 may therefore modulate the activity of netrins and DCC by activity on HSPGs. In fact, it has been previously shown that MMPs are necessary in processing DCC to mediate netrin activity on spinal commissural axons [155]; while this study suggested cleavage of DCC ectodomains by MMPs regulates netrin activity, it is possible that MMP action on the HSPG portion of the HSPG/DCC complex also contributes to netrin regulation. Because netrin [217] and HSPGs [180] are expressed along the trajectory of olfactory axons during development, MMP 24 is poised to mediate these interactions and therefore affect axon outgrowth of the newly generated sensory neurons which express it.

Apart from the guidance cues and receptors already identified as having a specific association with the CSPG or HSPG, there are several candidate molecules present in the developing OS that could also underlie the outgrowth increase in the MMP-treated cultures. For instance, Nogo-A is preferentially expressed in immature OSNs extending their axons from the epithelium to the bulb and is highly enriched in the growth cones in the developing rat olfactory system [218]. Because this is the same population of cells that express MMPs on the cell surface, and others have shown that MMPs can specifically cleave Nogo receptor [156], it is possible that MMPs could be regulating this

guidance cue/receptor interaction in the olfactory pathway. Another possible candidates that is expressed in the developing OS include ephrin and its receptor, Eph [219], which was shown to be directly regulated by MMPs [154]. In fact, because eph and Ephrin have been postulated to codetermine glomerular positioning with ORs [220, 221], it is possible that MMP regulation of this interaction can mediate more that just axonal guidance, but also regulate targeting or positional coordinates in the CNS.

6 Conclusions and future directions

It is evident that MMPs have an important role in the developing CNS, acting on many different levels to regulate axon guidance and pathway establishment. For the first time, we have identified relevant MMP sub-families in the developing olfactory pathway, which include the gelatinases and the membrane-bound MMPs. Furthermore, we have demonstrated active proteolysis in the growing olfactory nerve through *in situ* zymography, showing that gelatins found in the pathway can be degraded by the MMPs expressed there. In addition, our *in vitro* studies have identified previously unrecognized specific substrates for different MT-MMPs, hinting at their possible function in matrix degradation and neurite outgrowth. However, future studies are needed to further clarify the role of MMPs in this model system. First, explant numbers should be increased to sufficient power to confirm outgrowth significance on inhibitory substrates. Also, instead of general MMP inhibition by synthetic zinc chelators, the addition of TIMPs to cultured neurons would block specific MMPs and may demonstrate different effects on neurite outgrowth by particular metalloproteinases. An *in vivo* approach can also be used, in which an ethylene vinyl acetate 40W implant (Elvax polymer) impregnated with MMP inhibitors could be implanted in the developing OS to see what kind of targeting defects

occur. And finally, MMP knockouts have already been produced and could be used to examine what kind of deficiencies result in the OS of these mice, and if exogenously applied MMPs could rescue this phenotype. Clearly, there is much left to do to fully elucidate the role of MMPs in nervous system development. However, the work I present here provides a solid foundation for future studies on their role in developing axon tracts and, moreover, insights made from such studies may help us understand their roles in pathologic processes and eventually lead to mechanistic manipulation of these enzymes to allow for therapeutic benefit.

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