MMP2 REGULATES THE MATRIX MOLECULE FAULTY ATTRACTION TO PROMOTE MOTOR AXON TARGETING IN DROSOPHILA

by

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DEDICATION

To all of those who have made me smile and laugh along the way, especially my husband, Derek.
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Mmp2 Regulates the Matrix Molecule Faulty Attraction to Promote Motor Axon Targeting in Drosophila

Abstract

by

CRYSTAL MARIE MILLER

During development, motor axons must navigate to their correct muscle targets in order to correctly wire the nervous system. The repertoire of receptors and adhesion molecules expressed on individual axons, combined with the unique milieu of extracellular cues the nerve encounters on muscle and glia, allows each axon to reach its particular destination. However, as in other developmental paradigms, guidance cues are only one aspect of the regulatory mechanism of axon pathfinding. In this thesis, I demonstrate that both proteolysis and the regulation of growth factors by an extracellular matrix molecule combine to direct nerves to their targets.

Using Drosophila as a model system, I examined the role of the two fly matrix metalloproteinases (MMPs) in axon guidance. Misexpression of either Mmp1 or Mmp2 results in an increase in bundling of motor axons. In contrast, Mmp1 and Mmp2 mutants both display loosely bundled and frayed axons, indicating that both MMPs promote fasciculation during axon guidance. The
catalytic domain of these proteases is necessary for this function, implying that they must act on a substrate to affect pathfinding.

A yeast two-hybrid screen identified the structural ECM molecule Faulty attraction (Frac) as an interaction partner and potential substrate for Mmp2. Frac is expressed in the embryonic mesoderm in the path of extending axons and adjacent to Mmp2-expressing exit glia. Both frac mutant embryos and embryos overexpressing frac phenocopy Mmp2 mutant guidance defects, indicating Frac also acts in axon guidance. Lastly, overexpression of Mmp2 results in smaller Frac fragments on Western blots and abrogates in vivo accumulation of Frac protein in embryos.

These data suggest that Mmp2 cleavage of Frac promotes fasciculation during guidance in the Drosophila embryo. Frac provides more than structural support to the ECM, also modulating the spatiotemporal concentrations of growth factors. At an even higher level of regulation, Mmp2 proteolysis controls the balance of full-length versus cleaved Frac. Similar elaborate mechanisms have been uncovered in other developmental processes and it seems likely that ECM molecules like Frac, controlled by post-translation modifications, may create multiple levels of regulation for seemingly simple signaling pathways in many physiological and pathological contexts.
CHAPTER 1: Introduction

Summary

During Drosophila embryogenesis, motor neurons make highly stereotypic connections with their synaptic targets, the bodywall musculature. The simplicity of this system has been utilized by a number of labs to gain insight into the roles of some of the most well-known classes of molecules required for pathfinding and target recognition. In almost all cases, signaling pathways are conserved across evolution, thus the power of Drosophila genetics and screening techniques are an unparalleled method for discovering new players involved in guidance. It is conceivable that this identification and characterization of cues required for the proper wiring of the nervous system during development may eventually lead to novel therapeutic approaches for the regeneration of nerves after injury.

Drosophila model system

Much of the pioneering work on guidance molecules in invertebrates was first performed in the grasshopper (Goodman et al., 1984). However, focus transferred to Drosophila upon realization that they share homology at the single cell level, that embryos are easily accessible, and that the genetics add a powerful dimension (Sanchez-Soriano et al., 2007). Study of guidance during Drosophila embryogenesis is also facilitated by short generation time and the extremely stereotyped connectivity between motor axons and their muscle targets.
The central nervous system (CNS), known as the ventral nerve cord (VNC) in Drosophila, is composed of longitudinal axon fibers that form three connectives on each side of the VNC and transverse fibers that form sets of two commissures per hemisegment that cross between the connectives. Most axons cross the midline once at a commissure and project on the contralateral side of the CNS, while some remain on their own side entirely. The motor neurons that innervate the peripheral nervous system (PNS) begin migrating out of the VNC at stage 13. There are ~36 motor neurons per abdominal hemisegment, composing three main nerve branches, including the intersegmental nerve (ISN), the segmental nerve (SN), and the transverse nerve (TN) (Landgraf and Thor, 2006). The ISN and SN main branches divide into smaller subbranches, termed the SNa, ISNb, SNc, and ISNd, and are named for their final positions from lateral to medial. Each branch and subbranch is defined by a specific stereotyped morphology that is exceedingly well defined (Fig. 1.1). The anti-FasII antibody, or 1D4, which recognizes Fasciclin II, the Drosophila homolog of vertebrate NCAM (Van Vactor et al., 1993), recognizes all motor axons and has been essential in characterizing their branching patterns. Perturbations to this simple system can easily be identified and facilitate the identity of molecules involved in guidance decisions.

**Axon guidance**

Correct wiring of the nervous system requires an interplay of signaling between the different tissue types involved in axon pathfinding. Axons must express the proper signaling cues and receptors that can respond to their target tissues.
Similarly, muscle targets must express complementary molecules in order to respond. In addition to these two tissue types, surrounding glia are also involved in signaling as well as acting as guidepost cells (Hidalgo, 2003; Chotard and Salecker, 2004). While previous studies have revealed many of the molecular mechanisms at play, in some cases down to the intracellular cues that regulate the actin cytoskeleton, questions remain as to how individual axons reach their particular muscle targets (Ayoob et al., 2004; Terman and Kolodkin, 2004). Here, I focus on axon pathfinding in the Drosophila model system; however, most molecules and signaling pathways are conserved across evolution with homologous proteins functioning similarly in vertebrate systems (Dickson, 2002; Huber et al., 2003).

Axons

Motor axons are fasciculated, or bundled, together as they exit the CNS and begin to enter the periphery during stage 13 of embryogenesis. A careful balance of fasciculation versus exploration must be maintained so that axons stay bundled together until they reach choice points, or particular locations at which individual axons or subgroups of axons separate to go on to innervate different targets (Landgraf and Thor, 2006). Therefore, axons must express and receive guidance cues that regulate their attraction to each other and their attraction to their muscle targets.

Fasciclin II (FasII) is an NCAM-like adhesion molecule expressed on motor axons that is responsible for keeping axons bundled together (Landmesser et al.,
One signaling system that overcomes this adhesion is the Semaphorin/Plexin pathway, which causes axons to become repulsive to one another and incites branching in order to innervate their target muscles (Yu et al., 1998; Yu et al., 2000; Ayoob et al., 2006). This pathway has been dissected to a great degree with multiple receptors identified, including PlexA, PlexB, and Off-track (Winberg et al., 1998b; Winberg et al., 2001; Cafferty et al., 2004; Ayoob et al., 2006). Additionally, downstream factors in this system have been identified such as MICAL, an oxidoreductase thought to link Semaphorin signaling to the cytoskeleton, as well as Nervy and Gyc76c, two proteins that can influence axonal response to guidance cues by regulating cyclic nucleotides (Terman et al., 2002; Ayoob et al., 2004; Terman and Kolodkin, 2004).

The family of receptor protein tyrosine phosphatases (RPTPs) and associated molecules Abl and Ena also influence guidance at certain choice points. Dlar, Ptp19D, Ptp69D, Ptp99a, and Ptp52f, expressed by motor axons, regulate extension of the ISNb and SNa through a combination of competition and collaboration (Desai et al., 1996; Krueger et al., 1996; Desai et al., 1997; Schindelholz et al., 2001; Sun et al., 2001). Interestingly, multiple ligands for Lar have been identified, and it has been hypothesized that Lar extracellular domain shedding may change the receptor’s ability to bind its different interactors, suggesting protease activity may confer ligand specificity (Serra-Pages et al., 1994; Fox and Zinn, 2005; Woo et al., 2009). Abl and Ena couple RPTPs to actin cytoskeleton dynamics so that guidance decisions are linked to axonal
movement (Wills et al., 1999). RPTPs also function in developing vertebrate neurons (Drosopoulos et al., 1999; Wang and Bixby, 1999; Burden-Gulley et al., 2002).

These stereotypical axon guidance molecules function mainly in the axons themselves in order to influence correct guidance decisions. But axons cannot reach their targets alone – they depend on interactions with other tissues they encounter along the way, including glia and their target tissue, the muscle.

**Muscle**

Sidestep (Side) is a target-derived attractant expressed by muscles to steer motor axons to their final destinations (Sink et al., 2001; de Jong et al., 2005). Interestingly, high levels of Side are found ahead of extending motor axons until they make contact, at which time Side is downregulated. Through this mechanism, Side expression in muscle guides axons through their target tissue and facilitates navigation of choice points (Siebert et al., 2009). Beaten path (Beat), a protein secreted by motor axons, allows axons to respond to the Side signal (Fambrough and Goodman, 1996; Pipes et al., 2001). However, Side/Beat signaling is one of the few known pathways active in the muscle to attract axons. While Side clearly functions to guide axons to their general muscle targets, how individual axons recognize and innervate specific muscle clefts remains unknown.

**Glia**

As in vertebrates, axons are supported by glia in Drosophila. The four types of
glia in Drosophila include the cortex, neuropil, surface, and peripheral glia – all of which perform many of the same functions as glia in vertebrates and share similar morphologies (Freeman, 2006). Neuropil glia are found near the longitudinal fascicles and commissures and serve functions similar to oligodendrocytes, including providing trophic support and ensheathing axons. Ablation of some types of neuropil glia can affect axon pathfinding. When longitudinal glia are ablated the formation of the longitudinals is disrupted (Hidalgo and Booth, 2000). Similarly, ablation of the midline glia results in the failure of axons to cross the midline (Jacobs, 2000). These data suggest that glia not only act as intermediate targets, but could be the source of important guidance molecules (Auld, 1999).

In the PNS, the peripheral glia ensheath motor and sensory axons. While sensory neurons are completely wrapped by the end of embryogenesis, it is not until larval stages that the glial cytoplasmic processes envelop the motor neurons all the way to the neuromuscular junction (Sepp et al., 2000). This wrapping of glia is similar to that performed by Schwann cells in vertebrates, although peripheral glia do not myelinate axons (Freeman, 2006). Most peripheral glia are born in the CNS and migrate into the PNS following after pioneer axons that form the axon tracts of the peripheral nerves and the incoming sensory neurons (Sepp et al., 2000). There are approximately 12 peripheral glia per hemisegment, which eventually spread throughout the periphery along neurons. Three to four of these glia have been termed exit glia and reside at the transition zone (TZ) between the CNS and PNS boundaries (Sepp et al., 2001). The TZ has also been termed the
exit junction as it is the location at which the peripheral nerves leave the CNS. It has been proposed that these glial cells in particular may play a specific initial role in facilitating the bundling of nerve branches into the periphery (Auld, 1999). However, removal of all peripheral glia initially stunts the extension of motor axons as they exit from the CNS into the PNS at the TZ, but these defects are largely corrected during later stages. While the innervation pattern of the nerve branches do exhibit some defasciculation, it is surprising that the loss of peripheral glia has such little effect on overall motor axon targeting (Sepp et al., 2001). A possible explanation could lie in the complex array of molecules required to achieve the balance of attractive and repulsive forces necessary for proper guidance. If peripheral glia express different guidance cues that normally act in opposition to one another, ablation of all glia may result in no net effect on targeting.

It is well established that glia do express molecules that can significantly affect axon pathfinding. For example, the midline glia (MG) express Netrin, Slit, and Commissureless (Comm), three guidance molecules essential for proper wiring of the midline (Jacobs, 2000). The Netrins are secreted from MG and attract axons that express the receptor Frazzled (Harris et al., 1996; Mitchell et al., 1996). However, these axons also express the receptor Roundabout (Robo) which acts as a repellent when it binds its ligand, Slit, another molecule expressed by MG, which normally keeps axons from crossing the midline randomly (Kidd et al., 1998b; Kidd et al., 1999). In order for these axons to be attracted to the midline by Netrin/Frazzled signaling at correct locations while
ignoring the Robo/Slit repellent signaling, another molecule must be involved. Comm is a transmembrane protein expressed by MG that causes Robo to be endocytosed on axons crossing the midline, allowing them to attend to the attractive signaling while ignoring the repulsive signaling (Kidd et al., 1998a). A similar mechanism controls midline guidance in vertebrates (Kennedy et al., 1994; Zou et al., 2000). This complex interplay of guidance molecules demonstrates the importance of glia to proper wiring through expression of multiple signals as well as the potential of individual cells to express cues acting in opposition to one another (Jacobs, 2000).

**Matrix metalloproteinases**

While many of the classical guidance molecules described above are fairly well-characterized and their downstream signaling pathways are slowly being elucidated, new players in guidance continue to be identified. Recent evidence has recognized the roles of metalloproteinases in axon guidance and pathfinding. The metalloproteinases were originally thought to degrade the extracellular matrix in order to clear a path for extending axons (Muir, 1994; Zuo et al., 1998), but it has become increasingly evident that metalloproteinases actually modulate guidance through more precise mechanisms (McFarlane, 2003).

The metzincin metalloproteinases comprise a large family of enzymes that require a zinc ion in their active site to function proteolytically. Based on their structures, they are subdivided into four subfamilies including matrix metalloproteinase (MMPs), astacins, serralysins, and adamlysins, which include
the ADAM (a disintegrin and a metalloproteinase) family (Sternlicht and Werb, 2001). In the last ten years, evidence has accumulated that ADAM family members particularly play very specific roles in a number of axon guidance decisions. For example, repulsive signaling occurs when ephrin-A2 and its receptor EphA bind, but it was not understood how binding could favor repulsion. Further, how would this binding be terminated so repulsion could occur? Work in cell culture revealed that Kuzbanian (Kuz)/ADAM-10 can end the ephrin-A2/EphA interaction by cleaving ephrin-A2. Interestingly, cleavage requires binding and clustering of the receptor, indicating that this proteolytic mechanism is tightly regulated (Hattori et al., 2000). Kuz has also been shown to genetically interact with Slit/Robo signaling where it is thought to participate in the down-regulation of Robo on commissural axons so that they may cross the midline (Schimmelpfeng et al., 2001). Finally, demonstrating the conservation of this protease in guidance, axon targeting in Xenopus retinal ganglion cell axons is disrupted when ADAM-10 is pharmacologically inhibited in vivo (Chen et al., 2007).

While the ADAMs are certainly now widely recognized as players in guidance, roles for other metalloproteinases are still beginning to be uncovered. The astacin-family metalloproteinase, Tolloid-related-1 (Tlr), has been reported to act as a permissive signal in axon guidance decisions during Drosophila embryogenesis. Tlr can process several TGF-β-type molecules in cell culture, including the activin-like protein Dawdle, and both tlr mutants and dawdle mutants display similar errors in motor axon guidance (Parker et al., 2006; Serpe and O'Connor, 2006). MMPs, another subfamily of the metzincin
metalloproteinases, have also been implicated in guidance. Work in Xenopus retinal ganglion cells showed that misrouting defects occur at specific choice points when an MMP-specific pharmacological inhibitor is applied \textit{in vivo} (Hehr et al., 2005). While this study was the first to demonstrate that MMPs function in guidance, it left many questions unanswered. Which of the vast number of MMPs in vertebrates could be responsible for this guidance decision? How is this proteolytic event regulated? Is this function redundant – can it be performed by many different MMPs? And lastly, what substrate is the MMP cleaving to promote its effect?

There are 24 mammalian MMP family members with a shared domain structure. They are initially expressed as zymogens with an autoinhibitory pro-domain that blocks the binding site of the zinc ion, rendering the protease inactive until the pro-domain is removed (Page-McCaw, 2008). The catalytic domain contains a conserved Met residue (Bode et al., 1993; Rudolph-Owen et al., 1997). Most MMPs also have a hemopexin domain at the C-terminus that is attached by a flexible hinge. It is thought that this region is responsible for protein-protein interactions as well as protease localization, substrate recognition, and degradation (Overall, 2002). In vertebrates, six membrane-type MMPs are attached to the cell through transmembrane domains or GPI-anchors, while the rest of the MMP family members are secreted (Page-McCaw, 2008).

The first MMP was discovered for its role in frog metamorphosis as an enzyme in the tail of the tadpole which could degrade fibrillar collagen (Gross and Lapiere,
While these enzymes seem to have the main function of remodeling the extracellular matrix (ECM), they perform a variety of functions in cell survival, differentiation, inflammation, and pathological conditions (Page-McCaw et al., 2007). Often they are involved in systematic chains of activation whereby MMPs are responsible for releasing the pro-domain of downstream MMP family members resulting in proteolytic cascades involving multiple MMPs – the complicating the understand of single MMP genes (Overall, 2002; Chen, 2004). Additionally, MMPs have garnered much attention for their role in disease states. MMPs have been implicated in cardiovascular disease from aneurysms (Kadoglou and Liapis, 2004; Barbour et al., 2007) to atherosclerotic plaque rupture (Newby, 2005) to cerebral ischemia (Cunningham et al., 2005). They have also been in the spotlight because of a prominent role in metastasis in a variety of cancers (Marshall et al., 1998). Their expression is raised in many different tumor types and often correlates with low survival rate (Fingleton, 2003). MMPs have also been linked to auto-immune disorders such as rheumatoid- and osteo-arthritis (Milner and Cawston, 2005; Burrage et al., 2006) as well as respiratory disorders such as asthma, emphysema, and acute respiratory distress syndrome (Suzuki et al., 2004). Based on the prevalence of MMPs in pathological disorders, inhibitors of these proteases were the focus of many clinical trials. However, results were disappointing when MMP inhibitors were either not efficacious at the doses given or resulted in side-effects, such as musculoskeletal syndrome (King et al., 2003; Peterson, 2006). It became increasingly clear that MMP actions could not all be considered detrimental, but
that these proteases also served developmental roles and functioned in positive ways during disease states as well as the negative ones given more attention (Fingleton, 2007). While MMPs are still being considered as targets for different therapies, many pharmaceutical companies have begun focusing on small molecules or antibodies that can be more precisely controlled rather than broad-spectrum MMP inhibitors (Sabeh et al., 2004; Baragi et al., 2009).

In light of the negative consequences revealed by therapy with MMP inhibitors, it is critical to elucidate the normal physiological functions of MMPs before additional clinical trials are performed. However, with 24 MMPs, redundancy and compensation have complicated the assessment of the role of individual MMPs. Many mouse knockouts have been made, but few display phenotypic abnormalities and none are embryonic lethal (Page-McCaw, 2008). Only one knockout, Mmp14, dies 3-12 weeks after birth (Holmbeck et al., 1999; Zhou et al., 2000). Double MMP knockouts confirm the theory of redundancy and compensation between MMPs, for example Mmp2-Mmp14- null mice die at birth and Mmp14-Mmp16- null mice are perinatal lethal (Lambert et al., 2003; Oh et al., 2004; Shi et al., 2008). However, it will be impossible to knockout all 24 MMPs simultaneously and even double and triple knockouts will not definitively rule out involvement of additional MMPs in particular processes or define overlapping substrates. For this reason, many have turned to in vitro approaches of identifying MMP substrates in order to apply that knowledge to those molecules that are actually cleaved in vivo. Identifying substrates in this manner has proven difficult, as the catalytic domain alone is often expressed without the
specificity-inducing hemopexin domain. This renders the proteases non-specific, yielding hundreds of substrates that may have no physiological relevance (Page-McCaw et al., 2007).

In Drosophila, there are only two MMPs, Mmp1 and Mmp2 (Llano et al., 2000; Llano et al., 2002; Page-McCaw et al., 2003). While the nomenclature can be confusing, these do not correspond directly to any of the particular MMPs in vertebrates, but do represent the different families of vertebrate MMPs. Mmp1 is secreted while Mmp2 is GPI-anchored (Page-McCaw et al., 2003). Loss of Mmp1 results in lethality around the third instar larval stage while Mmp2 mutants die during metamorphosis, indicating that both MMPs are mainly required for remodeling rather than development (Page-McCaw et al., 2003). Mmp1 mutants display defects in the larval tracheal system. During larval stages, the trachea must expand drastically in size, but without Mmp1, the trachea stretch inappropriately, rather than expanding - seemingly due to their attachment to the ECM. Further work has shown that Mmp1 is responsible for cleaving the signaling protein Ninjurin that signals cells to release adhesion, thus allowing the trachea to grow normally (Zhang et al., 2006). Mmp2, Mmp1 double mutants as well as germline clones live to the third instar larval stage and exhibit similar defects to those observed in Mmp1 mutants (Page-McCaw et al., 2003).

The first reported phenotypes in Mmp2 mutants included a melanized lump on the thorax along with a cleft bisecting the notum along the midline, both indications that Mmp2 is involved in the fusion of dorsal hemisegments during
differentiation of the wing imaginal disc (Page-McCaw et al., 2003). More recently, Mmp2 has been reported to participate at multiple levels in controlling formation of the air sac primordium (ASP). Characteristic of the nature of MMPs, Mmp2 can remodel the ECM by regulating levels of collagen IV and Perlecan in order to mediate the interaction between disc cells and the trachea, from which the ASP arises (Guha et al., 2009). Interestingly, Wang et al. (2010) recently reported on a second function for Mmp2 in the developing ASP, where Mmp2 controls the spatial pattern of FGF signaling as the ASP is initially budding. Restricted expression of Mmp2 at the forming tip functions in a lateral inhibition pathway to control tip versus stalk cell fate. Another recent example of Mmp2 acting locally demonstrated that Mmp2-expressing epithelial cells control dendrite reshaping in adult flies. Using clonal analysis, Yasunaga et al. (2010) showed that epithelial cells homozygous for mutant Mmp2, but not Mmp1, failed to modify the local basement membrane to allow dendrite reshaping. However, nearby clones heterozygous for Mmp2 displayed reduction in the basement membrane reporter and resulted in terminal dendritic branches properly aligned with muscles. Together, these data indicate that Mmp2 plays roles outside of simple tissue remodeling, engaging its membrane-association in order to control local signaling and modifications (Wang et al., 2010; Yasunaga et al., 2010).

As one might expect, proteolytic activity must be tightly regulated. If it is not, the likely results include the spreading of cancer cells and other pathological states for which MMPs have the most notoriety (Fingleton, 2003; Yong, 2005). Hence, it seems logical that normal developmental and physiological processes involving
MMPs must utilize mechanisms to control MMP activity. Accordingly, an endogenous inhibitor for MMPs is present in both vertebrates and insects. TIMPs, or tissue inhibitor of metalloproteinases, function by binding to the active site of MMPs and preventing substrates from being processed (Gomis-Ruth et al., 1997). There are four TIMPs present in vertebrates, but only a single Timp in Drosophila, which is most similar to vertebrate TIMP-3 (Page-McCaw et al., 2003; Wei et al., 2003). Interestingly, the single fly Timp can inhibit mammalian MMP-1,-2,-3, and -14 (Wei et al., 2003) and fly Mmp1 can be inhibited by mammalian TIMP-2 and TIMP-4 (Llano et al., 2000). These data demonstrate the conservation of this mechanism across evolution.

**The extracellular matrix: a higher order regulation**

The extracellular matrix (ECM) is thought to function primarily as a structural support for cells. It is composed of a variety of proteins, many of which are preserved through evolution; however, many are also novel proteins. When the Drosophila genome was first published it became possible to compare the full complement of vertebrate ECM proteins to those present in the fly (Adams et al., 2000; Rubin et al., 2000). It was quickly realized that the fly genome encodes many of the basic ECM proteins that are common to vertebrates. Laminins, collagens, proteoglycans, and integrins were found to be present; however, other notable molecules such as intermediate filaments, fibrillin, fibrillar collagens, elastase, fibrinogen, and fibronectin seemed to be absent (reviewed in Goldstein and Gunawardena, 2000; Hynes and Zhao, 2000). If the main functional role of
these proteins is simply to act as structural supports, what pressure is being exerted during evolution for so many new molecules to be developed and others to be very carefully conserved?

Over the past 15 years it has become apparent that structural ECM components are strategically positioned to also act as regulators of growth factor signaling. These large molecules harbor the potential to contain numerous specific binding sites for different growth factors in order to protect ligands from proteolysis, act as sponges to sequester them in the ECM, restrict diffusion, or prevent aberrant signaling to incorrect cell types. As intermediate filament proteins and type I collagen demonstrate that large size is not required to provide structural support, it is possible that the repeating and varied domains are instead utilized for the binding and regulation of many different soluble growth factors. Similarly, alternative splicing of some of these proteins in different cell types suggests that formation of slightly modified protein structures may expose new binding sites specialized for particular functions/tasks (Hynes, 2009). Many examples of ECM molecules functioning as a “higher order of regulation” have already been reported.

Fibrillins are large glycoproteins that are the major structural protein of microfibrils, a building block of the ECM (Dietz et al., 1991). In addition to giving tensile strength to connective tissues, they also regulate the bioavailability of growth factors such as TGFβ and promote cell-matrix interactions (Dietz and Mecham, 2000; Charbonneau et al., 2004; Chaudhry et al., 2007). Three
Fibrillins exist in vertebrates with partially overlapping expression profiles (Corson et al., 2004; Carta et al., 2006). However, the importance of Fibrillin is evident as mutations in Fibrillin-1 result in the hereditary congenital disorder, Marfan Syndrome (MFS) (Dietz et al., 1991). MFS is characterized by scoliosis, arachnodactyly, and aortic aneurysm, all resulting from connective tissue defects. Initially, these symptoms were thought to arise from the loss of the structural integrity Fibrillin affords to the ECM (Ramirez and Dietz, 2004; Dietz et al., 2005). More recently, evidence has indicated that many of these problems could arise from the dysregulation of TGFβ signaling (Dietz and Mecham, 2000; Neptune et al., 2003). Normally, it is thought that TGFβ binds LTBP (latent TGFβ binding protein) and forms an inactive complex which is then sequestered in the ECM by Fibrillin-1. Upon cleavage of Fibrillin-1, Fibrillin-1 fragments compete against the TGFβ/LTBP complex for binding with full-length Fibrillin and when TGFβ/LTBP is dislodged, downstream signaling can more readily occur (Chaudhry et al., 2007). However, in MFS, mutations in Fibrillin-1 can lead to both positive and negative derivations from the normal concentration of TGFβ as well as other growth factors, providing an additional explanation for the defects that characterize this disorder (Ramirez and Dietz, 2004).

Fibrillin is not the first ECM molecule capable of regulating the concentrations of growth factors. Heparan sulfate proteoglycans (HSPGs) are ECM macromolecules with multiple glycosaminoglycan (GAG) chains attached to a protein core. In Drosophila, two glypicans (HSPGs attached with a GPI-anchor), Dally and Dally-like (Dlp), have been well-characterized for their role in the
regulation of wingless, hedgehog and dpp morphogen gradients in the wing disc and other organs during development (reviewed in Yan and Lin, 2009). Signaling of these molecules is dependent on proper HSPG expression and biosynthesis. For example, the defects in the eye, genitalia, and antenna observed in dally mutants are enhanced when Dpp levels are also reduced in these mutants and wingless signaling is defective in sulfateless and sugarless mutants, in which HSPGs are not properly modified (Hacker et al., 1997; Jackson et al., 1997; Fujise et al., 2001; Akiyama et al., 2008). Clearly, the regulation of growth factors by ECM molecules is an established mechanism utilized for normal development.

In a neuronal context, HSPGs have also been implicated in the ability to regulation of Sonic hedgehog (Shh) during the specification of ventral neuronal subtypes (Danesin et al., 2006). Shh is required for both neuronal specification of motor neurons followed by an increase in concentration of Shh to trigger the switch in differentiation to oligodendrocyte precursors (OLPs) (Agius et al., 2004; Park et al., 2004). HSPGs are responsible for modulation of Shh concentration and for the sudden increase in Shh. Specifically, Sulfatase 1 is expressed just before the neuronal to OLP transition, and is attributed with the modification of HSPGs that leads to the increase in apical concentration of Shh (Danesin et al., 2006). This cascade is fairly complex, with multiple layers of regulation ultimately controlling Shh signaling. However, this sophisticated mechanism allows a single molecule to control different cellular identities in a linear pathway.
Other ECM proteins have also been reported as regulators of growth factors. In Drosophila, the type IV collagens, Viking and Dcg1, can bind Dpp to establish its proper gradient in the embryo and the gerarium. In the ovary, collagen restricts the signaling range of Dpp, preventing the formation of an overabundance of germline stem cells. Throughout the embryo, collagen binding of Dpp/Scw can either promote formation of an inhibitory complex composed of additional proteins, or lead to Dpp/Scw-receptor interactions. In this way, collagens function to appropriately distribute and present the growth factor Dpp to target cells (Wang et al., 2008a). Lastly, ECM molecules could be imagined to act as signaling molecules themselves. During synapse formation in the developing mammalian brain, astrocytes express thrombospondins (TSP) – large ECM proteins that can interact with membrane receptors and matrix proteins. However, TSP1/2 can also function to promote CNS synaptogenesis by binding to the α2δ1 calcium channel subunit mediated by its EGF domains. This interaction is thought to change the conformation of α2δ1 to allow the formation of a “synaptogenic signaling complex” of assorted proteins that assist in the development of a functioning synapse (Christopherson et al., 2005; Eroglu et al., 2009). Here, it is not only demonstrated that nearby glia secrete a molecule that is necessary for axonal synapse formation, but that an ECM molecule itself acts a signaling molecule to generate downstream responses, rather than acting as a regulator of other growth factors.

The cases above all demonstrate that ECM molecules can function as much more than structural supports. Their positioning between cells and their
composition of numerous and varied domains lends them as optimal molecules to bind and regulate soluble growth factors. Additionally, their size and complexity facilitates tissue-specific isoform expression as well as multiple protein conformations to reveal new and diverse binding sites. The examples mentioned above are only the beginning of a number of hypothetical roles ECM molecules could play in signaling during normal physiological as well as pathological conditions (Hynes, 2009). These cases establish ECM molecules as distributors of growth factors, as in the morphogen gradients of Dpp created by binding to HSPGs or collagens (Jackson et al., 1997). Collagen also acts as a presenter of Dpp by distributing it in the correct region of the embryo and positioning it to properly bind its receptor (Wang et al., 2008a). Fibrillins function as a sink or receptacle to control the amounts of active TGFβ and TSPs can themselves act as ligands to produce downstream events (Chaudhry et al., 2007; Eroglu et al., 2009). While it is not known exactly how TSPs bind and signal to the α2δ1 calcium channel subunit, one could imagine that they are capable of folding in such a way that the full-length protein is bound by its specific EGF-repeat regions, which act as a the ligand (Eroglu et al., 2009). Conversely TSP could first be cleaved by a protease, thereby releasing TSP fragments that could bind α2δ1 with less steric hindrance. Similarly, known functions of these proteins could be combined. Growth factors bound to structural proteins to control distribution or activation state could then be released with a piece of the matrix molecule, and together could function as a signaling complex. Lastly, one could
imagine that ECM molecules function together synergistically or alone as signal integrators.

If the ECM is truly composed of such a plethora of structural molecules operating to control signaling, it is possible that a single ECM molecule could be coordinating an assortment of growth factors at any one time. On a larger scale, neighboring ECM molecules could also be performing the same balancing act. If the specificity of binding, releasing, and signaling is dependent on the interplay of which growth factors are already present on a single molecule or in relation to those close by, a highly complex signaling system begins to arise. This type of regulation has been metaphorically described as chords, composed of the individual growth factors as the single notes (Hynes, 2009).

Regulation by ECM molecules has already been reported to occur in a number of organisms and processes. It would be unsurprising to find it at work in many more, if not most signaling mechanisms. If it is truly that widespread, axon pathfinding would be an ideal paradigm for ECM molecules to control spatiotemporal signaling of growth factors and guidance cues.

Aims of thesis

Despite the large number of guidance molecules already identified, many questions still remain about how axons reach their targets. Particularly, while general attraction and repulsion mechanisms have been shown to control axon bundling versus branching to targets, the precision required to generate the stereotyped paths axons follow remains unknown. These decisions must require
distinct spatial and temporal regulation to occur. One mechanism that would yield this kind of fidelity would require different complements of proteins expressed by each set of axons and their targets. However, proteins specific to particular muscles or the axons composing single nerves have yet to be identified. An alternative approach could potentially utilize guidance molecules that are already known and expressed more broadly. In this case, guidance cues could be activated, restricted, or distributed by post-translational modifications and/or ECM molecules.

In order to discover additional proteins that could be contributing to the process of axon pathfinding, a gain-of-function (GOF) screen was performed. One resulting gene identified was Drosophila Mmp1. Since there are only two MMPs in Drosophila, I characterized the overexpression phenotypes of Mmp1 and Mmp2 as well as loss-of-function (LOF) phenotypes of for Mmp1, Mmp2 and Mmp2, Mmp1 double mutants. Indeed, both Drosophila MMPs are required to promote axon fasciculation in the embryo. While only Mmp1 was originally identified in the GOF screen, Mmp2 appears to play the principal role, at least in the ISNb, based on penetrance and strength of phenotype. Next, I found that Mmp2 RNA is in glia at the edge of the CNS and that glial misexpression results in a stronger bundling phenotype than neuronal misexpression. Finally, to address whether MMP proteolytic activity is required for guidance, I misexpressed Drosophila Timp as well as catalytically inactive forms of Mmp1 and Mmp2. The resulting phenotypes mimic the MMP LOF phenotypes, suggesting that MMP enzymatic activity is necessary for promoting fasciculation.
These data also imply that MMPs influence guidance through cleavage of a substrate.

As discussed earlier, several metalloproteinases have been shown to play roles in axon guidance. For example, Kuz/ADAM10 can cleave the guidance molecule EphrinA2 and interact with Slit/Robo signaling (Hattori et al., 2000; Schimmelpfeng et al., 2001). As Mmp2 genetically interacts with Sema1a, it seemed likely that Mmp2 might regulate a member of the Semaphorin pathway or possibly act by modulating another known guidance molecule. If this was the case, Mmp2 could function in one of two ways. If Mmp2 cleaves its substrate in order to inactivate, the LOF phenotype of the substrate would be a stall, or increased bundling – opposite to that of the Mmp2 mutant phenotype. However, if Mmp2 cleaves its substrate to activate a latent molecule, the substrate’s LOF phenotype would phenocopy the Mmp2 mutant phenotype (Fig. 1.2).

To identify potential substrates of Mmp2, a yeast interaction screen was performed using catalytically-inactive Mmp2 as bait. Out of the seven positives identified from the screen, only one, CG7526, later named Faulty attraction or Frac, was expressed in a manner consistent with regulation by Mmp2. However, Frac is not a typical guidance molecule, as it is very large and its sequence and domain structure are reminiscent of several ECM molecules such as the fibrillins and fibulins. To address whether Frac is regulated by Mmp2, I examined its expression in embryos with different Mmp2 levels. Both immunoblot results and the in vivo expression pattern of Frac protein indicate that its expression is
dependent on changes in Mmp2 levels. To confirm that this matrix molecule could play a role in guidance, I analyzed the GOF phenotype and generated frac mutant alleles to characterize the LOF phenotype. Both resulted in axon guidance errors similar to those in Mmp2 mutants. However, the GOF phenotype suggested that another signaling molecule, controlled by Frac, could be involved, since overexpression of Frac alone was not sufficient to drive axon fasciculation. To identify the potential cue I tested whether frac genetically interacts with TGFβ/BMP family members and found that a constitutively-active Sax receptor could rescue the frac LOF phenotype.

Together, these data indicate that Mmp2 proteolysis modulates the amount of full-length Frac versus Frac fragments. The balance of these forms of Frac may then control BMP signaling or the availability of other guidance cues. It is conceivable that these events could be localized to areas with Mmp2 expression to create spatial regulation of Frac and downstream molecules. Future studies focused on elucidating the mechanism of axon targeting should consider “higher order forms of regulation” such as proteolysis and involvement of ECM molecules. These could modify broadly expressed cues so that their distribution or signaling capacity is specific and spatially-defined for particular muscles or nerve bundles - thus contributing to the stereotyped pattern of axons established during pathfinding.
Figure 1.1
Figure 1.1: Axonal projection pattern in Drosophila

(A) Three main branches of motor axons exit the VNC (solid lines): the transverse nerve (TN), intersegmental nerve (ISN) and segmental nerve (SN). These divide into subbranches and form synapses on their muscle targets (ovals). The SN (green) divides into the SNa (light green) and the SNc (dark green) while the ISN maintains a main branch (red, yellow) as well as two subbranches, the ISNb (light blue) and ISNd (dark blue). The TN (brown) meets sensory axons (black) extending toward the CNS in the periphery. Muscles are represented by gray boxes. (B) Micrograph of two hemisegments labeled with anti-FasII to mark all motor axon projections. Anterior to left. Scale bar: 20 µm. Adapted from *Seminars in Cell & Developmental Biology*, 17, Landgraf and Thor, “Development of Drosophila motoneurons: Specification and morphology,” 3-11, 2006, with permission from Elsevier.
Figure 1.2

A

Mmp2 cuts to inactivate - LOF mutant phenotype is stall

\[\rightarrow\]

B

Mmp2 cuts to activate - LOF mutant phenotype is defasciculation

\[\rightarrow\]
Figure 1.2: Potential LOF phenotypes of an Mmp2 substrate

Mmp2 could function by cleaving to (A) inactivate its substrate or (B) activate its substrate. If Mmp2 inactivates, the substrate LOF phenotype would be expected to be a stall, or the opposite of the Mmp2 mutant phenotype. If Mmp2 activates, the substrate phenotype should result in defasciculation just like in the Mmp2 mutant.
CHAPTER 2: Matrix metalloproteinases promote axon fasciculation in the Drosophila embryo (from Miller et al., 2008)

Abstract

Matrix metalloproteinases (MMPs) are a large conserved family of extracellular proteases, a number of which are expressed during neuronal development and upregulated in nervous system diseases. Primarily on the basis of studies using pharmaceutical inhibitors, MMPs have been proposed to degrade the extracellular matrix to allow growth cone advance during development and hence play largely permissive roles in axon extension. Here we show that MMPs are not required for axon extension in the Drosophila embryo, but rather are specifically required for the execution of several stereotyped motor axon pathfinding decisions. The Drosophila genome contains only two MMP homologs, \( Mmp_1 \) and \( Mmp_2 \). We isolated \( Mmp_1 \) in a misexpression screen to identify molecules required for motorneuron development. Misexpression of either MMP inhibits the regulated separation/defasciculation of motor axons at defined choicepoints. Conversely, motor nerves in \( Mmp_1 \) and \( Mmp_2 \) single mutants and \( Mmp_1 \ Mmp_2 \) double mutant embryos are loosely bundled/fasciculated, with ectopic axonal projections. Quantification of these phenotypes reveals that the genetic requirement for \( Mmp_1 \) and \( Mmp_2 \) is distinct in different nerve branches, though generally \( Mmp_2 \) plays the predominant role in pathfinding. Using both an endogenous MMP inhibitor and MMP dominant negative constructs, we demonstrate that MMP catalytic activity is required for motor axon fasciculation.
In support of the model that MMPs promote fasciculation, we find that the defasciculation observed when MMP activity is compromised is suppressed by otherwise elevating interaxonal adhesion—either by overexpressing FasII or by reducing Sema1a dosage. These data demonstrate that MMP activity is essential for embryonic motor axon fasciculation.

**Introduction**

Motor axons navigate an extracellular environment rich with potentially competing attractive and repulsive cues. Remarkably, motor axon growth cones are able to both interpret and integrate the signals present in this complex environment en route to their individual muscle targets. The particular axonal trajectory taken by any given motorneuron depends on the nature of the extracellular cues encountered by the extending axon as well as the complement of receptor or adhesion molecules expressed on its growth cone. In addition, several molecules required for either the activation or distribution of extracellular guidance molecules have recently been implicated in axon guidance (Johnson et al., 2004; Steigemann et al., 2004; Meyer and Aberle, 2006; Parker et al., 2006; Serpe and O’Connor, 2006).

The number and diversity of molecules implicated in motor axon pathfinding argue that work in genetic model systems will continue to be essential to identify and tease apart the relative contributions of proteins involved in this process. In particular, the Drosophila embryo provides an important model for the study of motor axon pathfinding as a result of the small number of motorneurons, their
defined trajectories and invariant muscle targets (Sink and Whittington, 1991; Landgraf et al., 1997; Schmid et al., 1999). Work by a number of groups has led to the identification and characterization of molecules critical for pathfinding and target recognition by Drosophila motor axons (Van Vactor et al., 1993; Terman et al., 2002; Fox and Zinn, 2005). An underlying principle to emerge from these studies is that in order for axons to reach their muscle targets, the activity of adhesion molecules that promote the fasciculation/bundling of motor axons must be precisely balanced with repulsive signals that trigger the defasciculation/separation of the extending axons (Winberg et al., 1998a; Yu et al., 2000).

Although the mechanisms responsible for limiting defasciculation to defined choicepoints in the periphery are not clear, a number of molecules necessary for proper defasciculation have been identified. In particular, repulsive signaling mediated by the Semaphorin/Plexin (Sema/Plex) pathway is essential for motor axon defasciculation (Winberg et al., 1998a; Yu et al., 2000; Terman et al., 2002; Ayoob et al., 2006). In wild-type embryos, axons of the intersegmental nerve branch b (ISNb) defasciculate from the primary ISN pathway and innervate the ventrolateral muscle (VLM) field. In embryos with reduced Sema/Plex pathway activity, however, ISNb axons fail to reach their targets and often remain bundled with the primary ISN branch—a phenotype consistent with diminished interaxonal repulsion. Furthermore, embryos with loss-of-function (LOF) mutations in nervy and protein kinase A RII, two genes that have been proposed to antagonize Sema/Plex signaling, exhibit premature and excessive motor axon
defasciculation (Terman and Kolodkin, 2004). In contrast, LOF mutations in the cell adhesion molecules *Fasciclin II (FasII)* or *Connectin (Conn)* suppress LOF mutations in *Sema1a* and *PlexA*, arguing that *Sema1a* and *PlexA* stimulate defasciculation by overcoming axon-axon adhesion maintained by FasII and Conn (Winberg et al., 1998a; Yu et al., 2000). These genetic interaction studies demonstrate the importance of balancing attractive and repulsive forces to enable correct fasciculation and pathfinding.

To understand how the precise balance of attraction and repulsion is achieved, the roles of additional molecules capable of modulating fasciculation of extending motor axons must be characterized. A number of studies have investigated the roles of metalloproteinases in axon extension and guidance. The metzincin metalloproteinases are zinc-dependent extracellular proteases that are subdivided into four subfamilies based on structure: astacins, serralysins, matrix metalloproteinases (MMPs), and adamlysins—a subfamily that includes the ADAMs (a disintegrin and a metalloproteinase) (Sternlicht and Werb, 2001). Classic models of metalloproteinase function in neuronal development proposed that they acted to degrade extracellular matrix (ECM) in order to clear a path for advancing axons (Muir, 1994; Zuo et al., 1998). Recently, the roles of metalloproteinases in axonogenesis have been revisited in a number of experimental systems (McFarlane, 2003). These studies indicate that relevant neuronal metalloproteinase substrates include molecules directly involved in mediating axon pathfinding, including guidance receptors and their ligands. Among the metalloproteinases, the ADAM family is most strongly implicated in
the regulation of axon guidance. For instance, ADAM10 terminates the interaction between ephrin-A2 and EphA by cleaving ephrin-A2, thereby facilitating axon retraction in vitro (Hattori et al., 2000). Analyses of Drosophila embryos mutant for the ADAM family homolog kuzbanian (kuz) further support the idea that ADAMs regulate particular guidance events, as kuz mutations display genetic interactions with mutations in the repulsive midline factor slit (Schimmelpfeng et al., 2001). Interestingly, independent work from several groups has recently provided evidence that toloid-related-1 (tlr1), a Drosophila astacin-family metalloproteinase, acts through its TGF-β ligand Dawdle to regulate motor axon guidance in the embryo (Meyer and Aberle, 2006; Parker et al., 2006; Serpe and O'Connor, 2006).

As a family, MMPs are able to cleave nearly every component of the ECM, as well as numerous signaling molecules and cell surface receptors (Sternlicht and Werb, 2001). In the CNS, investigations of MMP function have largely centered on the roles of these proteases in nervous system disease, as MMPs are known to be dramatically upregulated in a host of CNS diseases, as well as following nervous system injury (Yong et al., 2001; Yong, 2005). However, in large part due to issues of redundancy and compensation among the twenty-four vertebrate MMP family members, the normal physiological roles of MMPs in the nervous system have remained largely elusive. Notably, a number of vertebrate MMPs display neuronal expression patterns in the embryo, suggesting that they may be involved in normal nervous system development (Hayashita-Kinoh et al., 2001; Hehr et al., 2005; Gonthier et al., 2006). In support of this model, studies of
Xenopus retinal ganglion cell axon guidance using MMP pharmaceutical inhibitors suggest that MMPs are required for specific pathfinding decisions (Hehr et al., 2005). Drosophila affords an attractive genetic model system in which to study MMP function since there are only two MMP family members in the fly, \textit{Mmp1} and \textit{Mmp2} (Llano et al., 2000; Llano et al., 2002; Page-McCaw et al., 2003). Whereas Mmp1 is a secreted protein, Mmp2 contains a GPI-anchor sequence and has been shown to be membrane-bound in tissue culture cells.

In this work, we present an analysis of MMP function during Drosophila embryonic neuronal development. Both LOF and gain-of-function (GOF) analyses support the model that MMP activity promotes motor axon fasciculation in the embryo. Misexpression of either \textit{Mmp1} or \textit{Mmp2} drives excessive motor axon fasciculation. In contrast, we find aberrant defasciculation in MMP LOF mutants. While \textit{Mmp1} mutants display relatively mild pathfinding defects, many motor axons separate prematurely and aberrantly in \textit{Mmp2} single mutants and \textit{Mmp1 Mmp2} double mutants, indicating that \textit{Mmp2} plays a primary role in motor axon fasciculation. We have analyzed the embryonic expression of both MMPs and find that while \textit{Mmp1} exhibits a limited embryonic expression profile, \textit{Mmp2} is expressed in neurons and glia—supporting a primary role for \textit{Mmp2} in embryonic neuronal development. Importantly, we find aberrant motor axon defasciculation in embryos misexpressing the endogenous MMP inhibitor \textit{Timp} and in embryos misexpressing MMP dominant negative constructs, indicating that MMP catalytic activity is essential for pathfinding. Finally, we show that the defasciculation phenotype exhibited by MMP LOF mutants are dominantly
suppressed by LOF mutations in Sema1a, arguing that MMP activity normally acts to promote fasciculation by antagonizing Sema1a function. Together, our results indicate that MMPs are not required for motor axon extension per se, but instead may modulate the responses of the axons of defined neuronal populations to specific guidance cues.

Materials and Methods

Gene Misexpression Screen

To identify novel factors required for embryonic CNS development, we conducted a GOF screen for genes whose misexpression disrupted motorneuron fate or pathfinding. We screened a collection of 2800 Drosophila lines harboring P elements containing the yeast GAL4 binding site UAS (Rorth et al., 1998; Toba et al., 1999; Viquez et al., 2006). Males from these P element insertion lines were crossed to elavGAL4 virgin females to drive misexpression of the gene adjacent to the P element insertion in all post-mitotic neurons (Yao and White, 1994; DiAntonio et al., 2001). We first selected those lines in which elavGAL4-dependent misexpression was lethal, as we reasoned this would enrich for genes with neuronal misexpression phenotypes. As a secondary screen, we crossed males from the 114 elavGAL4-dependent lethal lines to elavGAL4 virgins and collected embryos to screen for neuronal phenotypes. We analyzed CNS cell fate by staining with antibodies labeling specific neuronal populations, including anti-Even-skipped, anti-Hb9, and anti-Nkx6 (Landgraf et al., 1999; Broihier and Skeath, 2002; Broihier et al., 2004). For lines in which neuronal fate appeared
normal, we screened for motor axon guidance phenotypes using anti-Fasciclin II (anti-FasII) (Van Vactor et al., 1993) to label motor axon projections.

Fly stocks

Stocks used in this work include: Mmp2\textsuperscript{W307*}, Mmp2\textsuperscript{Df(2R)Uba1-Mmp2}, Mmp1\textsuperscript{Q112*}, Mmp1\textsuperscript{2}, Mmp2\textsuperscript{W307*} Mmp1\textsuperscript{Q112*}, Mmp2\textsuperscript{Df(2R)Uba1-Mmp2} Mmp1\textsuperscript{2}, UAS-TIMP, UAS-Mmp2, UAS-Mmp1 (Page-McCaw et al., 2003), UAS-Mmp2\textsuperscript{E258A} (below) UAS-Mmp1\textsuperscript{E225A} (Zhang et al., 2006), UAS-FasII from A. Kolodkin, elavGAL4 from A. DiAntonio, gcm\textsuperscript{4P1} from M. Freeman, repoGAL4 from J. Simpson, Hb9\textsuperscript{GAL4} (Broihier and Skeath, 2002). The Sema1a\textsuperscript{P1} Mmp2\textsuperscript{W307*}, Sema1a\textsuperscript{P1} Mmp1\textsuperscript{Q112*} recombinant chromosomes were generated by standard genetic techniques. All other stocks were obtained from Bloomington Stock Center.

Transgenic MMP constructs

UAS-Mmp1\textsuperscript{E225A} contains a missense mutation in the conserved catalytic core that renders the enzyme catalytically inactive; in cell culture Mmp1\textsuperscript{E225A} acts dominantly to inhibit Mmp1 function (Zhang et al., 2006). Similarly, UAS-Mmp2\textsuperscript{E258A} disrupts the conserved catalytic core of Mmp2 and is expected to function as a dominant negative; the PCR-generated mutant cDNA was cloned into pUAST and injected into flies by standard methods. For the misexpression analysis with elavGAL4 and repoGAL4, similar results were observed with each of two independent transgenic lines for both UAS-Mmp1 and UAS-Mmp2 (Page-McCaw et al., 2003). This similarity argues that the observed phenotypic
differences are unlikely to be the result of expression level differences between the UAS responder lines.

Antibodies

Drosophila embryos were fixed by gentle rocking for 4 minutes in 2 ml heptane and 2 ml 37% formaldehyde followed by 30 seconds of shaking in 6 ml methanol to devitellinize. The following primary antibodies were used: mouse anti-FasII/1D4 at 1:10 (generated by C. Goodman and obtained from the Developmental Studies Hybridoma Bank (DSHB), rabbit anti-GFP at 1:100 (Invitrogen), mouse anti-Wrapper at 1:10 (generated by C. Goodman and obtained from the DSHB), mouse anti-Repo at 1:10 (generated by C. Goodman and obtained from the DSHB), mouse anti-βgal at 1:1000 (Promega), rat anti-Islet at 1:100 and rabbit anti-Hb9 at 1:500 (Broihier and Skeath, 2002), and an anti-Mmp1 monoclonal cocktail (a 1:1:1 mixture of 3B8, 5H7 and 23G1) at 1:50 (generated by A. Page-McCaw and obtained from the DSHB). Species-specific biotinylated secondary antibodies were used at 1:300 in concert with the ABC Elite kit for immunohistochemistry (Vector Labs). Species-specific Alexa-488 and Alexa-568 (Molecular Probes) were used for immunofluorescence. Embryos stained with anti-GFP were fixed for 40 minutes in 3 ml heptane and 3 ml 4% paraformaldehyde. For these embryos, incubation with ABC was followed by treatment with the TSA Biotin System kit (PerkinElmer), followed by another incubation with ABC before developing.
**In situ hybridization**

An antisense digoxigenin-labeled *Mmp2* RNA probe was generated with T7 polymerase from a full-length cDNA. Sense probes generated with T3 polymerase did not result in specific hybridization. Embryos were incubated with riboprobe at 57°C overnight. RNA probe hybridization was visualized with an alkaline phosphatase-conjugated anti-DIG antibody (Roche) followed by NBT and BCIP treatment. For double labeling with in situ probe and antibody, the in situ hybridization protocol was followed by storage in 70% ethanol overnight and standard antibody staining. For fluorescent labeling, an anti-DIG-POD antibody (Roche) was used to recognize the probe and was amplified using the TSA Plus Fluorescence system (PerkinElmer).

**Microscopy and data analysis**

Embryos were filleted in 70% glycerol under a Leica MZ125 dissecting microscope. Specimens were analyzed on a Zeiss Axioplan 2 microscope with a 63X or 100X oil-immersion objective using Nomarski optics, and images were captured with an AxioCam MRc camera. Brightness and contrast were adjusted using Adobe Photoshop. Fluorescent images were obtained on a Zeiss Axio Imager.Z1 confocal microscope and edited with LSM 5 Image Browser. Statistical analyses were performed using Fisher's exact test.
Results

Pan-neuronal misexpression of either Mmp1 or Mmp2 inhibits motor axon defasciculation

We conducted a misexpression screen to identify genes required for embryonic neuronal fate and axon pathfinding (see Materials and Methods). In this manner, we identified several P element lines in which the wild-type pattern of motor axon projections was disrupted. In wild type, ISNb axons defasciculate/separate from the primary ISN nerve at their first choice point proximal to the ventrolateral muscle (VLM) field. Within the VLM field, ISNb axons continue to extend dorsally, with axonal subsets defasciculating from the ISNb at secondary choicepoints when they contact their muscle targets (Fig. 2.1A, D). Embryos from one of the lines isolated in the screen, GS2402, exhibited strongly stalled ISNb motor axons (termed a “stall” phenotype; data not shown). Inverse PCR analysis indicated that this line contained a P element inserted directly upstream of Matrix Metalloproteinase 1 (Mmp1).

To confirm that Mmp1 misexpression was responsible for the motor axon phenotypes observed in GS2402, we used a UAS-Mmp1 transgene (Page-McCaw et al., 2003) to drive Mmp1 throughout the embryonic CNS via elavGAL4. Consistent with the identification of Mmp1 in our screen, neuronal misexpression of Mmp1 interferes with embryonic motor axon guidance (Table 2.1; Fig. 2.1A,B). ISNb morphology was disrupted in elavGAL4/UASMmp1 mutant embryos in 74% of hemisegments scored (n=138). The majority of
affected hemisegments displayed phenotypes indicative of increased motor axon fasciculation, ranging from relatively mild defects to a complete block of proper ISNb defasciculation. Specifically, aberrant hemisegments often exhibited ISNb stall phenotypes, in which ISNb axons separated from the ISN at their first choice point, but subsequently failed to defasciculate from each other at their individual muscle targets, instead stalling in the ventral longitudinal muscle field (33%; Table 2.1; Fig. 2.1B,E). In 14% of hemisegments we observed a stronger “fusion bypass” phenotype, in which ISNb axons failed to defasciculate from the ISN at the first ISNb choice point and remained bundled with ISN axons (e.g. Fig. 2.3C).

To investigate whether the effects of \textit{Mmp1} misexpression were restricted to the ISNb branch, we evaluated segmental nerve branch a (SNa) guidance in embryos with \textit{Mmp1} expressed pan-neuronally. In wild type, axons within SNa exit the CNS and extend dorsally past the ventral longitudinal muscles to innervate the lateral muscle field. Just dorsal to the ventral longitudinal muscles SNa axons reach their first choice point and separate into dorsal and posterior branches. Axons in the posterior branch innervate muscle 8, while axons in the dorsal branch extend dorsally between muscles 22 and 23 before subdividing again at their second choice point with a posterior sub-branch turning to innervate muscle 24 (Fig. 2.1G,J; Table 2.1). We find that neuronal misexpression of \textit{Mmp1} disrupted SNa pathfinding in 50% of hemisegments, with most hemisegments (39%) containing SNa nerves that failed to branch appropriately or stalled in the lateral muscle field. Typically, axons in the dorsal SNa branch failed to separate at the second choice point resulting in a failure to
innervate muscle 24 (Fig. 2.1H,K; Table 2.1). These data demonstrate that neuronal \textit{Mmp1} misexpression drives axon hyperfasciculation in multiple embryonic motor nerve pathways.

Since vertebrate MMPs often display overlapping substrate specificities (Page-McCaw et al., 2007), we wanted to determine whether neuronal misexpression of \textit{Mmp2} would have similar phenotypic consequences to \textit{Mmp1} misexpression. We find that \textit{elavGAL4/UASMmp2} embryos also exhibit axonal phenotypes consistent with increased motor axon fasciculation (Fig. 2.1; Table 2.1). In these embryos, ISNb pathfinding is aberrant in 72% of hemisegments, with 50% of all hemisegments displaying either ISNb stall or fusion bypass phenotypes (n=147; Fig. 2.1C,F). Furthermore, neuronal misexpression of \textit{Mmp2} interfered with SNa pathfinding in 80% of hemisegments, with axons in 68% of hemisegments either stalling or failing to branch appropriately (Fig 2.1I,L). While \textit{Mmp1} and \textit{Mmp2} misexpression have comparable effects on ISNb pathfinding, SNa appears particularly sensitive to \textit{Mmp2} levels since the penetrance of SNa phenotypes is significantly higher with \textit{Mmp2} misexpression (p<0.0001; Table 2.1). Together, these data indicate that pan-neuronal misexpression of either MMP inhibits the ability of motor axons in both ISNb and SNa to appropriately defasciculate en route to their muscle targets and raises the possibility that matrix metalloproteinases contribute to proper motor axon guidance in the embryo.
MMPs exhibit distinct and spatially restricted expression profiles in embryogenesis.

To further investigate the possibility that MMP activity plays a role in neuronal development, we characterized the embryonic expression patterns of \textit{Mmp1} and \textit{Mmp2}. Previous studies have established that both genes are embryonically expressed (Llano et al., 2000; Llano et al., 2002; Page-McCaw et al., 2003).

Using anti-Mmp1 antibodies, we find Mmp1 protein to be expressed in essentially the same spatiotemporal expression profile as has been described for \textit{Mmp1} RNA. The most prominent embryonic expression of Mmp1 is in the proventriculus and hindgut (data not shown). Consistent with previous studies (Llano et al., 2000; Page-McCaw et al., 2003), we find Mmp1 CNS expression to be restricted to small clusters of segmentally repeating cells at the CNS midline (data not shown). We also detect Mmp1 expression in the chordotonal organs of the peripheral nervous system (Fig. 2.2A) and in two cells situated in the ventral mesodermal region (Fig. 2.2B). This expression is undetectable in \textit{Mmp1} null mutant embryos, \textit{(Mmp1}^{2}/\textit{Mmp1}^{Q112*}), confirming antibody specificity (data not shown).

We next characterized the expression pattern of \textit{Mmp2} via whole-mount RNA in situ hybridization. In contrast to \textit{Mmp1}, \textit{Mmp2} is widely expressed in the embryonic CNS (Fig. 2.2C). To identify the neuronal cells, we double labeled wild-type embryos with \textit{Mmp2} RNA and markers for specific neuronal and glial populations. We find that \textit{Mmp2} is expressed in midline glia as \textit{Mmp2} RNA is co-
expressed with Wrapper in these cells (Fig. 2.2D) (Noordermeer et al., 1998). We next tested whether Mmp2 is expressed in additional glial populations by co-labeling embryos with Mmp2 RNA and the glial marker anti-Repo (Xiong et al., 1994). At stage 15, Mmp2 and Repo are co-expressed in approximately three glial cells per hemisegment situated at the base of motor nerve roots (circled cells in Fig. 2.2C, arrows in Fig. 2.2E). The position and morphology of these cells suggest they correspond to exit glia, a group of peripheral glia originating within the CNS before migrating into the periphery along extending motor axons (Klambt and Goodman, 1991; Sepp et al., 2000). To confirm that these Mmp2-expressing cells are glia, we asked whether they are absent in embryos mutant for glial cells missing (gcm), in which the number of glial cells is greatly reduced (Jones et al., 1995). In support of this conclusion, gcm mutant embryos specifically lack the Mmp2-expressing cells situated at the boundary between the CNS and periphery (arrows in Fig. 2.2F).

The observation that Mmp2-positive cells within the CNS do not co-express Repo suggested that they are likely neurons. To determine whether they correspond to well-characterized subsets of motoneurons or interneurons, we double labeled embryos with Mmp2 RNA and antibodies specific for particular neuronal populations. We detect co-expression between Mmp2 and Islet, a marker for distinct motoneuron and interneuron populations (Thor and Thomas, 1997), in three neurons per hemisegment in the lateral CNS (arrows in Fig. 2.2G). We next asked whether these Mmp2-expressing neurons are Hb9-positive motoneurons. We do not detect co-expression between Hb9 and Mmp2 RNA (Fig. 2.2H),
suggesting that the Mmp2-positive neurons in the lateral CNS are Islet-positive interneurons. In sum, while Mmp1 exhibits a limited neuronal expression pattern, Mmp2 is expressed in stereotyped populations of neurons and glia, consistent with a role for Mmp2 in neuronal development.

**MMP misexpression in glia blocks motor axon defasciculation**

Mmp1 is a secreted protein, while Mmp2 contains a GPI-anchor and is membrane-associated (Llano et al., 2000; Llano et al., 2002; Page-McCaw et al., 2003)—suggesting that the distribution of Mmp2 may be critical for Mmp2 to have access to its substrate. Although we do not detect endogenous Mmp2 RNA expression in the mesoderm or in motorneurons, Mmp2 is expressed in a subset of peripheral glia that are closely associated with extending motor axons. To test if glial expression of Mmp2 might play a role in motor axon pathfinding, we analyzed the phenotypic consequences of Mmp2 misexpression in glia using a repoGAL4 driver. For comparison, we also quantified motor axon pathfinding in repoGAL4>Mmp1 embryos. We first visualized glia in repo>Mmp1 and repo>Mmp2 embryos with anti-Repo since MMP misexpression might interfere with the migration of peripheral glia along the motor nerves (Sepp et al., 2000) and thereby indirectly influence motor axon pathfinding. We find that the number and position of Repo-positive glia are unaltered with glial misexpression of either Mmp1 or Mmp2 (data not shown).

We then analyzed motor axon pathfinding in repo>MMP embryos. Glial misexpression of either MMP leads to phenotypes that are qualitatively similar to
those observed with neural misexpression. In these embryos, axons in both ISNb and SNa stall prematurely and fail to branch appropriately (Table 2.1; Fig. 2.3). For \textit{Mmp1}, the frequency of ISNb hyperfasciculation decreases from 47\% in \textit{elav}>Mmp1 embryos to 26\% in \textit{repo}>Mmp1 embryos, suggesting that the level of secreted Mmp1 may be higher with neuronal vs. glial misexpression. In contrast, glial misexpression of \textit{Mmp2} significantly increases the frequency of motor axon fasciculation defects compared to neural misexpression of \textit{Mmp2} in the ISNb (63\% vs. 50\%; p<0.05; Table 2.1; Fig. 2.3B,E,H,K). This is particularly striking for the ISNb fusion bypass phenotype—the strongest class of ISNb hyperfasciculation. We observe a fusion bypass phenotype in 11\% of hemisegments in \textit{elav}>Mmp2 embryos, compared to 30\% in \textit{repo}>Mmp2 hemisegments (Table 2.1). Hence, glial misexpression of \textit{Mmp1} does not enhance the phenotypes above those observed with \textit{elavGAL4}; in contrast, \textit{Mmp2} misexpression in glia yields phenotypes significantly stronger than those induced by neuronal misexpression.

Since MMP misexpression in neurons or glia increases motor axon fasciculation, we next wanted to determine whether MMP misexpression in other embryonic tissues is also sufficient to interfere with motor axon pathfinding. Hence, we analyzed motor axon guidance in embryos misexpressing either \textit{Mmp1} or \textit{Mmp2} in mesoderm (using \textit{24B-Gal4} and \textit{dmeF2GAL4} drivers) or hemocytes (using \textit{HeGAL4} and \textit{CrqGAL4} drivers). We do not detect motor axon phenotypes in embryos with MMP misexpression using any of these GAL4 drivers (data not shown) indicating that motor axon pathfinding is not affected by MMP
misexpression in mesoderm or hemocytes, but is sensitive to elevated MMP levels in neurons and glia. For Mmp2 in particular, the penetrance and expressivity of motor axon phenotypes observed with glial Mmp2 misexpression as well as the endogenous expression of Mmp2 in a subset of peripheral glia suggest that Mmp2 expression levels in peripheral glia are critical for proper motor axon guidance.

MMP LOF mutants display inappropriate motor axon defasciculation

To determine if MMP function is necessary for neuronal development, we quantified motor axon pathfinding defects in Mmp1 and Mmp2 single mutant embryos as well as Mmp1 Mmp2 double mutants. In contrast to the hyperfasciculation phenotypes observed with MMP misexpression, MMP homozygous LOF mutants exhibit reduced fasciculation of the ISNb nerve (Table 2.2; Fig. 2.4). For Mmp1, we scored embryos homozygous mutant for a null allele, Mmp1Q112*, as well as embryos heterozygous for Mmp1Q112* and Mmp12, a deletion removing almost all coding sequence (Page-McCaw et al., 2003). In embryos carrying either allelic combination of Mmp1, we find similar, if relatively mild, ISNb phenotypes. In affected hemisegments, ISNb pathfinding was roughly wild-type, though the nerve was less tightly bundled and had a frayed appearance (compare Fig. 2.4A, B). While 50% of Mmp1Q112*/Mmp1Q112* hemisegments displayed a loosely fasciculated ISNb morphology, the penetrance fell to 28% for Mmp1Q112*/Mmp12 embryos (Table 2.2), suggesting
that a second-site mutation in the \textit{Mmp1}\textsuperscript{Q112*} line contributes to the observed ISNb phenotype.

For \textit{Mmp2}, we analyzed embryos homozygous for the null allele, \textit{Mmp2}\textsuperscript{W307*}, and embryos heterozygous for \textit{Mmp2}\textsuperscript{W307*} and \textit{Mmp2}\textsuperscript{Df(2R)Uba1-Mmp2 (Mmp2\textsuperscript{Df})}, a deletion removing the first three coding exons of \textit{Mmp2} (Page-McCaw et al., 2003). Embryos of both of these genotypes exhibited marked defects in ISNb fasciculation. The \textit{Mmp2} LOF phenotypes included not only the loosely-bundled morphology observed in \textit{Mmp1} homozygotes, but also exuberant branching of the ISNb—with ectopic axonal projections splintering off inappropriately and extending improperly within the ventral longitudinal muscle field (compare Fig. 2.4A,C). Quantifying these phenotypes, we find that 85\% of hemisegments from \textit{Mmp2}\textsuperscript{W307*} homozygotes and 74\% of \textit{Mmp2}\textsuperscript{W307*}/\textit{Mmp2}\textsuperscript{Df} hemisegments displayed either a frayed appearance or excessive projections (n=187 and 238; Table 2.2). The ISNb phenotypes apparent in both \textit{Mmp1} and \textit{Mmp2} homozygotes argue that both MMPs contribute to proper bundling of the ISNb motor nerve branch, with \textit{Mmp2} playing the major role.

Analyses of MMP double mutants have provided evidence for redundancy between MMPs in vertebrates (Oh et al., 2004; Stickens et al., 2004). To determine if the incomplete penetrance observed in \textit{Mmp1} and \textit{Mmp2} single mutants might be explained by genetic redundancy between Drosophila MMPs, we quantified motor axon pathfinding defects in \textit{Mmp1 Mmp2} double mutant embryos. We scored ISNb pathfinding in two different allelic combinations of
Mmp1 Mmp2 double mutants—Mmp2^{W307*} Mmp1^{Q112*} and Mmp2^{W307*} Mmp1^{Q112*}/Mmp2^{Df} Mmp1^{2}. The guidance defects in the double mutants mirrored the phenotypes observed in Mmp2 single mutants both qualitatively and quantitatively (Fig. 2.4D; Table 2.2). We observed both loosely-associated ISNb axons and ISNb axons separating prematurely and ectopically from the main nerve branch. The frequency of the hypofasciculation phenotypes is roughly 75% in both double mutant allelic combinations. This penetrance is nearly identical to that observed for Mmp2 single mutants, arguing that Mmp1 activity does not substantially compensate for the loss of Mmp2 function in promoting ISNb fasciculation.

To assess the LOF phenotypes in a second independent manner, we used Hb9^{GAL4} to drive expression of membrane-bound GFP in MMP mutant embryos, thereby limiting visualization to axonal projections of ventrally and laterally projecting Hb9-positive motoneurons (Fig. 2.4I-P) (Broihier and Skeath, 2002). By focusing on the projections of Hb9-expressing neurons, we can exclude the possibility that the ectopic ISNb projections observed in MMP mutants are the result of misrouting of motor axons that normally extend in different pathways, such as Eve-positive dorsally projecting motor axons (Landgraf et al., 1999). Hb9^{Gal4}>GFP Mmp1 single mutant embryos stained with anti-GFP displayed comparable fraying of the ISNb to those stained with FasII antibody (compare Fig. 2.4B,J). Similarly, Hb9^{Gal4}>GFP Mmp2 single mutants and Hb9^{Gal4}>GFP Mmp1 Mmp2 double mutant embryos continued to exhibit loosely bundled axons and ectopic branching of the ISNb when stained with anti-GFP (Fig. 2.4C,D,K,L).
As ectopic ISNb projections are still frequently observed in these embryos, they likely correspond to misguided ISNb axons rather than motor axons misrouted from other pathways. These data support our quantitative FasII analysis and indicate that MMP activity is necessary for ISNb fasciculation.

To determine if MMP LOF phenotypes are limited to the ISNb branch, we quantified SNa morphology in single and double mutant MMP embryos. Similar to the phenotypes observed for ISNb, we find evidence of decreased SNa fasciculation in MMP mutant embryos. Embryos of all six single and double mutant allelic combinations displayed SNa phenotypes consistent with decreased axonal fasciculation (Table 2.2; Fig. 2.5). In most cases, axons branched prematurely or inappropriately from either the dorsal or posterior SNa secondary branches (arrowheads in Fig. 2.5B,C,D). While \( Mmp2 \) is primarily responsible for promoting ISNb fasciculation, we do not observe a statistically significant difference in the frequency of SNa defasciculation in \( Mmp1 \) (36%) compared to \( Mmp2 \) (44%) single mutants (\( p=0.1 \)). These data argue that \( Mmp1 \) plays a relatively more significant role in SNa pathfinding than in ISNb pathfinding. Consistent with an increased genetic requirement for \( Mmp1 \) activity in SNa fasciculation, mutant analysis indicates that \( Mmp1 \) activity alone can promote substantial SNa fasciculation. In double mutant embryos, the penetrance of SNa defasciculation is significantly increased relative to that of either single mutant (Table 2.2). Specifically, 57% of double mutant hemisegments displayed ectopic SNa branches, relative to 36% for \( Mmp1 \) mutants and 44% for \( Mmp2 \) (\( p<0.05 \) for both). These data indicate that in contrast to the ISNb, \( Mmp1 \) and \( Mmp2 \) serve
partially redundant functions in SNa fasciculation. Together, our analyses indicate that MMP activity promotes motor axon fasciculation of multiple motor nerves in the embryo. Whereas *Mmp2* contributes significantly to fasciculation of both ISNb and SNa, we find that the role of *Mmp1* in pathfinding is largely specific to SNa.

Although our data suggest that MMPs are directly involved in pathfinding, in vertebrates MMPs have been shown to regulate processes as diverse as cell proliferation, survival, and differentiation (Page-McCaw et al., 2007). To address the possibility that MMPs might indirectly influence axon pathfinding by affecting earlier aspects of neurogenesis, we analyzed neuronal fate in MMP LOF and GOF mutants using a battery of molecular markers for distinct neuronal populations including anti-Eve, anti-Hb9, and anti-Nkx6 (Broihier and Skeath, 2002; Broihier et al., 2004). We do not detect any aberrations in neuronal fate specification in any MMP mutant background (C.M. Miller and H.T. Broihier, unpublished). The finding that MMPs do not appear to play a role in neural development prior to axon outgrowth is consistent with our expression analysis (see above) indicating that the MMP expression in the CNS initiates at stage 14 in post-mitotic neurons and glia.

**Inhibition of MMP catalytic activity disrupts motor axon guidance**

Our studies indicate that the level of MMP expression is a critical determinant of the degree of motor axon bundling. To test whether MMP catalytic activity regulates axon pathfinding, we specifically interfered with MMP catalytic activity
by several different means. We first used a UAS-Timp construct to misexpress Timp (tissue inhibitor of metalloproteinase) in embryonic neurons and glia via elavGAL4 and repoGAL4, respectively. TIMPs are secreted protein inhibitors that interfere with MMP catalytic activity by binding to the active site of the enzyme (Gomis-Ruth et al., 1997). The Drosophila genome contains a single Timp gene that inhibits both Mmp1 and Mmp2 function in vivo (Page-McCaw et al., 2003).

We find that ISNb morphology is aberrant in Timp misexpression embryos (Table 2.1; Fig. 2.6B,C,E,F). Whereas ISNb axons are tightly bundled in wild type, the ISNb appeared disorganized with Timp misexpression. Individual axons were often apparent, suggesting the nerves are more loosely associated than in wild type. Additionally, axons separated inappropriately from the ISNb and extended over the VLM field, similar to the phenotype observed in Mmp2 single and Mmp1 Mmp2 double mutant embryos. To test the extent of the Timp misexpression phenotypes, we assayed SNa motor axon guidance. We find that SNa pathfinding is sensitive to Timp expression levels as SNa morphology is aberrant with Timp misexpression. Most commonly, we observed ectopic branches extending from either the dorsal or posterior SNa branches (Table 2.1). Thus, Timp misexpression gives phenotypes roughly comparable to those observed in MMP LOF mutants in two distinct motor pathways, suggesting that MMP enzymatic activity is required for proper motor axon fasciculation.

While TIMPs are best characterized as MMP inhibitors, Drosophila Timp has been shown to interfere with the activity of other metalloproteinases (Wei et al.,
2003), raising the possibility that *Timp* misexpression inhibits metalloproteinases other than MMPs in embryonic neuronal development.

Additionally, vertebrate MMP-MT1 regulates cell migration independent of its catalytic domain (Cao et al., 2004), suggesting that Drosophila MMPs could have proteolysis-independent functions. Therefore, we evaluated whether the neuronal overexpression of catalytically inactive MMPs impaired axon pathfinding.

Overexpression of a catalytically inactive form of Mmp1, *Mmp1*\(^{E225A}\), in cell culture acts as a dominant-negative (Zhang et al., 2006), presumably by competing with wild-type Mmp1 for substrate binding. To determine whether overexpression of catalytically-inactive forms of *Mmp1* and *Mmp2* interfered with MMP function *in vivo*, we tested whether misexpression of *Mmp1*\(^{E225A}\) and an *Mmp2* mutant predicted to be catalytically-inactive, *Mmp2*\(^{E258A}\), disrupted motor axon pathfinding. We find that misexpression of either *Mmp1*\(^{E225A}\) or *Mmp2*\(^{E258A}\) had comparable effects on ISNb morphology as did *Timp* misexpression (Table 2.1; Fig. 2.6G,H,I,J). Namely, the ISNb in these embryos exhibited defasciculation with loose bundling and ectopic branches. Interestingly, for *Mmp2*\(^{E258A}\), we find that glial misexpression results in a significantly higher frequency of ISNb defects than does neural misexpression (50% vs. 37%, respectively; p<0.5). This increased penetrance may be explained by the fact that Mmp2 is thought to be both membrane-tethered and is normally expressed in glia, raising the possibility that a glial-derived catalytically inactive form of Mmp2 is better positioned to compete with the endogenous enzyme. It is also noteworthy that the defasciculation phenotypes observed with *Mmp1*\(^{E225A}\) are
stronger than those observed in Mmp1 single LOF mutants (Tables 2.1 and 2.2), raising the possibility that the MMPs have overlapping substrate specificities (see Discussion). Together, the analyses of MMP LOF mutants and the MMP enzymatic inhibitor studies demonstrate that MMP catalytic activity is necessary for motor axon fasciculation.

**MMPs promote FasII-dependent motor axon adhesion and antagonize Sema1a signaling**

Motor axon pathfinding is regulated by the interplay of factors that promote axon bundling such as cell adhesion molecules, and factors that antagonize motor axon adhesion to enable motor axon defasciculation at defined choice points. In the Drosophila embryo, motor axon defasciculation is controlled in part by the action of the Sema1a/PlexA signaling pathway (Winberg et al., 1998b; Yu et al., 1998). A number of classic genetic interaction studies have demonstrated that the relative strength of attractive and repulsive cues is critical for axon guidance. For example, the ISNb phenotype of Sema1a mutant embryos is dominantly suppressed by mutations in the cell adhesion molecule FasII (Yu et al., 2000). The FasII suppression of Sema1a LOF mutants provides strong support for the hypothesis that the balance of forces promoting and inhibiting motor axon adhesion is precisely regulated to ensure that defasciculation is tightly controlled. Our phenotypic analysis suggests that MMP activity promotes motor axon fasciculation and thus acts in concert with FasII and in opposition to the repulsive signaling mediated by Sema1a and PlexA. This model predicts that the
excessive axon defasciculation displayed by MMP mutants would be suppressed by otherwise elevating interaxonal adhesion. We first tested this hypothesis by asking if TIMP misexpression could counteract the motor axon hyperfasciculation observed with pan-neuronal overexpression of FasII. elav>FasII overexpression embryos display a high degree of ISNb hyperfasciculation, with many hemisegments displaying either a “bypass” or “detour” phenotype (Lin and Goodman, 1994). The “detour” phenotype resembles the “bypass” phenotype in that ISNb motor axons fail to exit the ISN at their first choicepoint. In “detour” hemisegments, however, some ISNb axons go on to separate from the ISN at more dorsal positions and enter the VLM field (arrowhead in Fig. 2.7B). We find that the extent of ISNb hyperfasciculation induced by FasII overexpression is significantly suppressed by co-overexpression of TIMP (Table 2.3; Fig. 2.7B,C). The frequency of “bypass” phenotypes decreases from 27% in elav>FasII embryos to 11% in elav>FasII, TIMP embryos. Similarly, the frequency of “detour” phenotypes is reduced from 28% in elav>FasII embryos to 15% in elav>FasII, TIMP embryos (p<0.05 for both phenotypic classes). The TIMP-mediated suppression of ISNb hyperfasciculation observed with FasII overexpression indicates that MMP activity normally promotes FasII-dependent motor axon adhesion.

To provide additional evidence that MMPs are required for motor axon fasciculation, we asked whether they normally act in opposition to the Sema1a/PlexA pathway by testing whether MMP LOF mutant phenotypes are dominantly suppressed by a null allele of Sema1a. In fact, we find that the
penetrance of ISNb defasciculation in Mmp2 mutants is significantly reduced in a Sema1a heterozygous background (Table 2.3; Fig. 2.7D-F). In particular, the frequency of loose bundling/ectopic branches for ISNb is decreased roughly two-fold: from 74% in Mmp2<sup>W307*/*</sup>Mmp2<sup>Df</sup> heterozygotes to 39% in Sema1a<sup>Pr</sup> Mmp2<sup>W307*</sup>/<sup>+</sup> Mmp2<sup>Df</sup> embryos. The Sema1a suppression of Mmp2 LOF mutations demonstrates that Mmp2 activity normally counteracts Sema1a-mediated repulsive signaling to regulate defasciculation of the ISNb motor projection. In contrast, the mild ISNb defects observed in Mmp1 single mutants are not dominantly suppressed by Sema1a (Table 2.3), in agreement with our phenotypic analysis indicating that Mmp1 does not contribute significantly to ISNb fasciculation. Since the aberrant motor axon defasciculation observed in MMP mutants is not limited to the ISNb, we wanted to determine whether the pathfinding defects apparent in the SNa pathway were also suppressed by reducing Sema1a dosage. Therefore, we compared the penetrance of SNa defects in Mmp1 and Mmp2 single mutants to those observed in MMP mutants in a heterozygous Sema1a background. The excessive defasciculation apparent in SNa in Mmp1 and Mmp2 mutants is also suppressed by reducing Sema1a dosage (Table 2.3), though the suppression is not as strong as was observed for Mmp2 mutants in ISNb. The modest suppression observed is consistent with published data demonstrating that the relatively large number of adhesion molecules promoting fasciculation of SNa decreases the strength of genetic interactions between any two factors (Yu et al., 2000). Combined with the strong dominant suppression observed for ISNb, these data demonstrate a critical and
widespread role for MMPs in achieving the required balance between attractive and repulsive factors underlying proper axon pathfinding.

**Discussion**

This work demonstrates that the level of MMP catalytic activity dictates the degree of motor axon fasciculation in the Drosophila embryo. While MMP misexpression is sufficient to inhibit separation of motor axons during outgrowth, both of the primary embryonic motor nerve branches display striking defasciculation in MMP LOF mutants. The opposing axonal phenotypes observed in MMP LOF and GOF embryos indicates that the level of MMP activity is critical for pathfinding and further suggests that the relevant MMP substrate(s) plays an instructive role in motor axon guidance. In support of the hypothesis that MMPs influence axon outgrowth by modulating the activity of established guidance cues, we show that *Mmp2* LOF mutants are dominantly suppressed by a null mutation in *Sema1a*, arguing that MMP function is tightly coupled to guidance decisions. Here we discuss possible substrates for *Mmp2* in motor axon pathfinding and put our findings in the context of proposed neural functions for metalloproteinases in vertebrates and invertebrates.

*Mmp1* and *Mmp2* are required for Drosophila embryonic CNS development

Both fly MMPs were previously shown to be expressed in the embryonic CNS (Llano et al., 2000; Llano et al., 2002; Page-McCaw et al., 2003), suggesting that they regulate aspects of neuronal development. However, the finding that both MMP single mutants and the *Mmp1 Mmp2* double mutant survived
embryogenesis called into question the extent of any possible roles for the MMPs in embryogenesis (Page-McCaw et al., 2003). In this work we present genetic evidence that MMP catalytic activity is essential for motor axon fasciculation.

While $Mmp1$ mutants display subtle fasciculation errors, we find that motor axons in $Mmp2$ mutants are markedly defasciculated, with many embryonic nerves appearing frayed and poorly organized. Consistent with this phenotypic analysis, the CNS expression profile of $Mmp2$ is considerably broader than that of $Mmp1$. While $Mmp2$ is expressed in midline glia, in clusters of interneurons and in peripheral/exit glia, CNS expression of $Mmp1$ is limited to the midline. The prominent expression of $Mmp1$ and $Mmp2$ at the CNS midline prompted us to examine whether either MMP might be required for proper guidance there. However, we do not find any alterations in the behavior of axons at the midline in either MMP LOF or GOF mutant backgrounds or any genetic interactions between $Mmp2$ and $Slit$ (C.M. Miller and H.T. Broihier, unpublished). These data argue that MMPs do not contribute significantly to embryonic midline guidance in the fly.

Although the $Mmp1$ and $Mmp2$ LOF phenotypes are distinct, several pieces of evidence suggest that they have overlapping substrate specificities and can cleave the same guidance cue(s). First, misexpression of either $Mmp1$ or $Mmp2$ yields qualitatively indistinguishable guidance phenotypes with many motor axons remaining inappropriately bundled together. Second, misexpression of an $Mmp1$ dominant negative transgene gives phenotypes nearly identical to those observed with an $Mmp2$ dominant negative. Furthermore, the phenotypes
observed with these constructs are stronger and more penetrant than the phenotypes of \( \text{Mmp1} \) LOF mutants (Tables 2.1 and 2.2), suggesting that the \( \text{Mmp1} \) dominant negative transgene affects motor axon pathfinding by interfering with \( \text{Mmp2} \) function by binding to the relevant \( \text{Mmp2} \) substrate(s). Lastly, if \( \text{Mmp1} \) and \( \text{Mmp2} \) cleave the same substrate(s), they might be expected to be genetically redundant, as removal of one would be compensated for by the presence of the other. In fact, we have shown that \( \text{Mmp1} \) and \( \text{Mmp2} \) play partially redundant roles in SNa pathfinding, as the double mutant phenotype is significantly stronger than the phenotype observed in either single mutant. These results are in agreement with analyses of enzymatic activity of vertebrate MMPs that suggest that there is overlap between the substrates cleaved by individual MMPs (Page-McCaw et al., 2007).

\( \text{Mmp2} \) contains a predicted GPI-anchor and is membrane-associated in Drosophila tissue culture cells (Llano et al., 2002). Thus, the expression pattern of \( \text{Mmp2} \) in the embryo would be expected to reflect the locations of Mmp2-dependent proteolysis. We find \( \text{Mmp2} \) RNA to be expressed in restricted populations of interneurons and peripheral glia, but not in motorneurons (Fig. 2.2). Peripheral glia originate at the lateral edge of the CNS and migrate into the periphery along elongating motor axons. By the end of embryogenesis, they extend cytoplasmic processes and wrap axon bundles in a manner similar to vertebrate non-myelinating Schwann cells (Jacobs and Goodman, 1989; Sepp et al., 2000, 2001). We propose that peripheral glial-derived \( \text{Mmp2} \) modulates the activity of factors required for pathfinding. This model implies that peripheral glia
play a significant role in regulating motor axon fasciculation. This finding contrasts slightly with the results of Sepp et al. (2001) who find more subtle errors in the motor axon projection pattern when peripheral glia are genetically ablated. One possible explanation for the weaker phenotypes in the peripheral glia-ablated embryos relative to Mmp2 LOF mutants is that peripheral glia express several factors that influence axon pathfinding in opposing directions—for example, proteins that both inhibit and stimulate fasciculation. In this way, peripheral glia would somewhat resemble midline glia which express both an axonal attractant (Netrin) and repellent (Slit) (Dickson, 2002). Therefore, ablation of the entire cellular population would be expected to yield different phenotypes than mutating individual molecules. Another possibility is that although Mmp2 is likely to act locally, its substrate might be secreted and could regulate motor axon guidance at a distance. In this case, Mmp2 need not be expressed at the site of fasciculation decisions, and either midline or interneuron-derived Mmp2 might provide the relevant proteolytic activity.

What guidance cues could be MMP targets in Drosophila motor axon pathfinding?

In principle, since MMP cleavage might either activate or inhibit the function of a molecule required for axon guidance, the motor axon phenotypes observed in MMP mutants could be expected to be identical to or opposite of the phenotypes displayed by substrate mutations. Based solely on phenotypic considerations, several guidance molecules could be considered candidate MMP substrates. For
example, LOF mutations in a number of genes give hyperfasciculation/stalled motor axon phenotypes. These include beaten path (beat) and sidestep (side), two immunoglobulin superfamily proteins required for proper defasciculation of both ISNb and SNa (Fambrough and Goodman, 1996; Sink et al., 2001). There are also five CNS-expressed receptor protein tyrosine phosphatases (RPTPs) that have combinatorial roles in the regulation of motor axon pathfinding. A number of these RPTPs, in particular LAR, are involved in ISNb defasciculation decisions (Desai et al., 1997; Schindelholz et al., 2001). Additionally, Plexin proteins and their receptors, the Semaphorins, are critical regulators of motor axon fasciculation. Sema/Plex pathway activity promotes inter-axonal repulsion so that LOF mutations in Sema/Plex pathway components yield ISNb stall phenotypes (Winberg et al., 1998a; Yu et al., 1998; Terman et al., 2002; Ayoob et al., 2006). Importantly, it has also been shown that for axons to remain tightly bundled during normal axon outgrowth, Sema/Plex signaling must be actively antagonized, as LOF mutations in two downstream inhibitors, Nervy and Protein Kinase A, give aberrant defasciculation phenotypes similar to that observed in MMP mutations (Terman and Kolodkin, 2004). Hence, levels of Sema/Plex activity must be tightly controlled to ensure that defasciculation occurs properly at guidance choicepoints. And similar to what we describe here for MMPs, reciprocal GOF and LOF mutations in the pathway can yield opposing hyper- and hypo-fasciculation phenotypes.

The MMP family as a whole does not cleave a conserved amino acid sequence in their targets, meaning that Drosophila substrates must be determined
empirically, not computationally. One identified Mmp1 substrate, Ninjurin A (NijA), represented an appealing candidate in motor axon guidance as it is a Drosophila homolog of a vertebrate protein required for cell adhesion that is upregulated in response to nerve injury (Zhang et al., 2006). However, we do not detect any aberrations to motor axon pathfinding in either NijA LOF or GOF mutants (C.M. Miller and H.T. Broihier), indicating that NijA is unlikely to be a relevant substrate in this context. While few other Drosophila MMP substrates have been identified, the Drosophila homologs of several putative vertebrate MMP substrates make appealing candidates for MMP targets in embryonic CNS development. For instance, vertebrate membrane type MMP-1 (MT1-MMP), has been shown to interact with the transmembrane heparan sulfate proteoglycan Syndecan-1 and triggers Syndecan-1 ectodomain shedding (Endo et al., 2003). Syndecan-1 processing stimulated cell migration on collagen, suggesting that this cleavage has functional consequences in vivo. Interestingly, Fox and Zinn (2005) identified Drosophila Syndecan (Sdc) as a ligand for the LAR RPTP. Accordingly, genetic interaction studies indicate that Sdc and LAR act in concert to regulate ISNb pathfinding. As it is currently unknown whether LAR binds membrane-bound or soluble Sdc, MMP activity could potentially regulate the LAR/Sdc interaction. In addition, MT1-MMP has also recently been shown to be required for ectodomain shedding of Semaphorin 4D in a model of tumor-induced angiogenesis—a processing event required for the induction of blood vessel growth in vivo (Basile et al., 2007). As discussed above, Semaphorin signaling plays a well-documented role in regulating motor axon behavior. Furthermore,
since we have found that \textit{Sema1a} mutations display strong genetic interactions with \textit{Mmp2} mutations in this system, it is conceivable that MMPs directly modulate Sema/Plex signaling activity.

\textbf{Metalloproteinases serve constructive functions in the CNS}

MMP expression levels are highly elevated in a number of neuronal pathologies and after nervous system injury. MMP upregulation in CNS disease states raises the issue of whether MMP induction has an overall positive or negative effect on disease outcome. There is substantial evidence that the net effect of high MMP expression in some diseases is detrimental (Yong et al., 2001; Yong, 2005). For example, treatment with broad-spectrum metalloproteinase inhibitors is able to alleviate or prevent experimental autoimmune encephalomyelitis (EAE), a mouse multiple sclerosis model (Chandler et al., 1997; Yong et al., 1998). There is also, however, growing recognition of beneficial functions for MMPs following CNS injury. The diverse functions for MMPs in disease states have become increasingly apparent as investigators have moved beyond the use of general metalloproteinase inhibitors to the study of particular MMPs. For example, increased expression of individual MMPs has been shown to correlate with periods of regeneration and repair following nervous system injury (Demestre et al., 2004; Shubayev and Myers, 2004; Ahmed et al., 2005). The functional significance of elevated MMP expression on regenerating axons has not been established, though in some regeneration models treatment with active MMPs promotes axon outgrowth (Siebert et al., 2001; Heine et al., 2004). In
regeneration, it is thought that MMPs influence axon growth by degrading chondroitin sulphate proteoglycans (CSPGs), which normally inhibit regrowth beyond the glial scar.

In the context of neuronal development, there is substantial support for the idea that metalloproteinases, and in particular the ADAM subfamily, regulate axon outgrowth and pathfinding (McFarlane, 2003). Early work in the field suggested that metalloproteinases play a largely permissive role in axon outgrowth—by degrading the ECM in order to clear a path for extending axons (Muir, 1994; Nordstrom et al., 1995; Zuo et al., 1998). Supporting a role for MMPs in outgrowth, it has been shown that a number of MMPs are expressed on the growth cones of vertebrate neurites extending in vitro (Nordstrom et al., 1995; Zuo et al., 1998; Chambaut-Guerin et al., 2000; Hayashita-Kinoh et al., 2001). More recent work has demonstrated that in vitro, metalloproteinases are capable of modulating the interactions between guidance cues and their receptors (Galko and Tessier-Lavigne, 2000; Hattori et al., 2000). For example, the interaction between ephrin-A2 and Eph receptor is terminated by ephrin-A2 cleavage via ADAM10/Kuz. Functionally, this cleavage allowed growth cone withdrawal of hippocampal neurons in culture, as a cleavage-inhibiting mutation delayed axon retraction (Hattori et al., 2000). Metalloproteinases have also been implicated in DCC (Deleted in Colorectal Cancer) receptor activity as broad-spectrum metalloproteinase inhibitors inhibit ectodomain shedding of DCC and potentiate netrin-mediated axon outgrowth (Galko and Tessier-Lavigne, 2000). In vivo support for the role of ADAM proteases in axon outgrowth and pathfinding comes
from work in Drosophila (Fambrough et al., 1996; Schimmelpfeng et al., 2001). 

_kuz_ mutant embryos display ectopic axon crossing at the midline suggesting that _kuz_ is required for repulsive signaling mediated by Slit/Roundabout (Robo).

Supporting this idea, _kuz_ and _slit_ mutations genetically interact, and Kuz appears to be required for the clearance of the Robo receptor from commissural axons (Schimmelpfeng et al., 2001).

While a number of vertebrate MMPs display neuronal expression patterns in the embryo, (Jaworski, 2000; Hayashita-Kinoh et al., 2001; Sekine-Aizawa et al., 2001; Gonthier et al., 2006), until relatively recently there was little direct evidence supporting a role for this metalloproteinase subclass in axon pathfinding. Studies of retinal ganglion cell (RGC) pathfinding in frogs argue that MMP activity is required for axon guidance at several defined choice points. Hehr et al. (2005) used an MMP-specific inhibitor to demonstrate that MMPs were required for RGC guidance decisions both at the optic chiasm and tectum. This work suggested that MMPs are normally required for axon guidance during vertebrate development, though the particular MMPs involved in RGC pathfinding remain to be identified. Exploiting the relative simplicity of the Drosophila model system, we have now established that individual MMPs play critical and distinct roles in well-defined axon pathfinding decisions during development. To extend this work to more complex vertebrate systems, it will be critical to analyze axon outgrowth and pathfinding in MMP single and compound mutant mice.
Figure 2.1
Figure 2.1: Pan-neuronal misexpression of either *Mmp1* or *Mmp2* inhibits motor axon defasciculation

In each micrograph, two abdominal hemisegments of Stage 17 dissected embryos stained with α-FasII to label the motor projections are shown with anterior left and dorsal up. Below each image are schematics diagramming the observed phenotypes with motor axons in brown and muscles represented by gray boxes. (A,D) In wild type, ISNb axons defasciculate from the ISN at a choicepoint proximal to the VLM field (arrowheads in A,D), then continue to extend dorsally to innervate muscles 6/7 and 12/13. (B,C,E,F) In *elav>*Mmp1 and *elav>*Mmp2 embryos, ISNb axons appear to defasciculate correctly from the ISN at their first choicepoint, but fail to defasciculate their appropriate muscle targets, a phenotype referred to as “parallel bypass”. (G,J) In wild type, the SNa branch innervates the lateral musculature and is comprised of dorsal and posterior branches. The posterior branch extends posteriorly to innervate muscle 8, while the dorsal branch makes two stereotyped turns en route to muscles 23 and 24. (H,K) In *elav>*Mmp1 embryos, the dorsal branch of the SNa stalls (arrowhead) before reaching its final target. (I,L) In *elav>*Mmp2 embryos, the dorsal and lateral branches of the SNa stall (arrowheads) before reaching their synaptic targets. Scale bar equals 15 µM.
Figure 2.2
Figure 2.2: Embryonic expression profiles of *Mmp1* and *Mmp2*

(A-E,G,H) Stage 15 wild-type embryos stained with indicated markers. (F) Stage 15 *gcm<sup>ΔP1</sup>* homozygous mutant embryo labeled with *Mmp2* RNA and anti-Repo. (A) *Mmp1* is expressed in PNS neurons in the chordotonal organs and (B) two cells in the ventral longitudinal muscle field (arrows). (C) Expression of *Mmp2* RNA indicates that *Mmp2* is expressed in a subset of CNS neurons and glia (circled in red). (D) *Mmp2* RNA (green) co-localizes with anti-Wrapper (red) in midline glia. (E) Nuclear anti-Repo (brown) and cytoplasmic *Mmp2* RNA (purple) in a subset of exit glia (arrows). (F) In *gcm<sup>ΔP1</sup>* embryos, which lack most glia, *Mmp2* RNA is no longer expressed at the lateral edge of the CNS where the exit glia are located (arrows). (G) Anti-Islet (brown) co-localizes with *Mmp2* RNA (purple) in approximately three neurons per hemisegment (arrows). The location of these neurons is consistent with an interneuron identity. (H) Anti-Hb9 (brown) does not co-localize with *Mmp2* RNA (purple). Insets in G and H show high-magnification view of *Mmp2* expression. Dotted white line marks the CNS boundary. Anterior is up in all panels. Dorsal is right in A,B,E, and F. Scale bar equals 10 μM in A,B,E, F and 20 μM in C, D, G, H.
Figure 2.3
Figure 2.3: Pan-glial misexpression of Mmp1 or Mmp2 blocks motor axon defasciculation

In each micrograph, two abdominal hemisegments of Stage 17 dissected embryos stained with α-FasII to label the motor projections are shown with anterior left and dorsal up. Below each image are schematics diagramming the observed phenotypes with motor axons in brown and muscles represented by gray boxes. (A,D) In wild type, axons in ISNb defasciculate from the ISN and extend dorsally to their synaptic targets. (B,C,E,F) In repo>Mmp1 and repo>Mmp2 embryos, ISNb axons fail to defasciculate appropriately and instead remain tightly bundled together, exhibiting either a “parallel bypass” (B,E); or a stronger “fusion bypass” phenotype (C,F). In “fusion bypass”, ISNb axons fail to separate from ISN axons at their first choice point and bypass the VLM field. (G,J) In wild type, SNa axons bifurcate into dorsal and posterior SNa branches and innervate lateral muscle targets. (H,K) repo>Mmp1 embryos exhibit mild stalls of the dorsal SNa branch (arrowheads). (I,L) repo>Mmp2 embryos display strongly truncated dorsal branches (arrowheads) and mildly-stalled posterior branches. Scale bar equals 15 μM.
Figure 2.4: MMP LOF mutants display inappropriate ISNb defasciculation

In each micrograph, two abdominal hemisegments of Stage 17 dissected embryos stained with α-FasII (A-D) or α-GFP (I-L) to label the motor projections are shown with anterior left and dorsal up. Below each image are schematics diagramming the observed phenotypes with motor axons in brown (E-H) or black (M-P) and muscles represented by gray boxes. In addition to carrying mutations in the indicated MMP genes, embryos in (I-L) are heterozygous for both Hb9Gal4 and UAS-mCD8GFP to visualize Hb9-positive axons with α-GFP. (A,E,I,M) Wild-type embryos have tightly bundled axonal projections. (B,F,J,N) Mmp1Q112*/Mmp12 mutant embryos exhibit moderate ISNb defasciculation. (C,G,K,O) ISNb-projecting axons in Mmp2W307*/Mmp2Df embryos are loosely bundled and frequently project aberrantly within the VLM field (arrowheads). (D,H,L,P) Mmp2W307*Mmp1Q112*/Mmp2Df Mmp12 double mutants are indistinguishable from Mmp2 single mutants with frequent ectopic projections (arrowheads). Scale bar equals 15 µM.
Figure 2.5
Figure 2.5: MMP LOF mutants exhibit excessive SNa defasciculation

In each micrograph, two abdominal hemisegments of Stage 17 dissected embryos stained with α-FasII to label the motor projections are shown with anterior left and dorsal up. Below each image are schematics diagramming the observed phenotypes with motor axons in brown and muscles represented by gray boxes. (A) In wild type, the two branches of the SNa stay tightly bundled after dividing into dorsal and posterior branches and go on to correctly innervate their lateral muscle targets. (B,F) In Mmp1Q112*/Mmp12 embryos, axons within the dorsal and posterior SNa branches project ectopically (arrowheads). (C) Mmp2W307*/Mmp2Df mutants display aberrant SNa defasciculation phenotypes similar to those observed in Mmp1 mutants. (D) SNa-projecting axons in Mmp2W307*/Mmp1Q112*/Mmp2Df Mmp12 double mutant embryos branch aberrantly and are less tightly bundled (arrowheads). The penetrance of SNa defasciculation observed in MMP double mutants is increased relative to either MMP single mutant. Scale bar equals 15 μM.
Figure 2.6

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Figure 2.6: MMP catalytic activity is required for motor axon pathfinding

In each micrograph, two abdominal hemisegments of Stage 17 dissected embryos stained with α-FasII to label the motor projections are shown with anterior left and dorsal up. Below each image are schematics diagramming the observed phenotypes with motor axons in brown and muscles represented by gray boxes. (A) In wild type, the ISNb exhibits a highly stereotyped morphology as its axons innervate their muscle targets. (B,E) In elav>Timp embryos, ISNb axons appear loosely bundled. (C,F) In repo>Timp embryos, ISNb axons are loosely bundled and project ectopically. (G,I) elav>Mmp2E258A embryos exhibit aberrant ISNb projections with axons failing to innervate their appropriate targets. (H,J) In repo>Mmp2E258A embryos, ISNb axons are loosely bundled and often branch ectopically. Scale bar equals 15 µM.
Figure 2.7

A

B

C

D

E

F

G

H

I

J

K

L

wild type

elav>Fas2

elav>Fas2, Timp

Mmp2^{W307}/Mmp2^{0}

Sema1a^{1}/Sema1a^{P1}

Sema1a^{P1} Mmp2^{W307}/+ Mmp2^{0}

Mmp2^{W307}/Mmp2^{0}

Sema1a^{1}/Sema1a^{P1}

Sema1a^{P1} Mmp2^{W307}/+ Mmp2^{0}
Figure 2.7: The inappropriate defasciculation observed in mutants with reduced MMP activity is suppressed by increasing interaxonal adhesion

In each micrograph, two abdominal hemisegments of Stage 17 dissected embryos stained with α-FasII to label the motor projections are shown with anterior left and dorsal up. Below each image are schematics diagramming the observed phenotypes with motor axons in brown and muscles represented by gray boxes. (A,D) Wild-type embryo exhibiting normal ISNb morphology. (B,E) elav>Fas2 mutant embryos have increased motor axon adhesion. (C,F) elav>Fas2, Timp embryos exhibit phenotypes consistent with weakened interaxonal adhesion relative to elav>Fas2 embryos. (G,J) Mmp2^{W307*}/Mmp2^{Df} mutant embryos have loosely bundled ISNb axons and ectopic branching. (H,K) The ISNb of Sema1a^{P1}/Sema1a^{P1} mutant embryos exhibits hyperfasciculation and does not properly innervate its muscle targets. (I,L) In Sema1a^{P1} Mmp2^{W307*}+/Mmp2^{Df} mutant embryos, ISNb morphology resembles that of wild-type embryos. Note that these embryos have more tightly bundled nerves than do the Mmp2 homozygous mutants shown in (G). Scale bar equals 15 µM.
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*Includes hyperfasciculated, hypofasciculated and loss/reduction of the synapse in the 6/7 muscle cleft which could be attributed to either hypo- or hyperfasciculation. The number of hemisegments scored for each genotype is listed as n.
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*Includes hyperfasciculated, hypofasciculated and loss/reduction of the synapse in the 6/7 muscle cleft which could be attributed to either hypo- or hyperfasciculation. The number of hemisegments scored for each genotype is listed as \( n \).
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*Includes hyperfasciculated, hypofasciculated and loss/reduction of the synapse in the 6/7 muscle cleft which could be attributed to either hypo- or hyperfasciculation.

The number of hemisegments scored for each genotype is listed as $n$. 
Abstract

Matrix metalloproteinases (MMPs) are widely hypothesized to regulate signaling events through processing of extracellular matrix (ECM) molecules, though in vivo evidence is limited. We previously demonstrated that membrane-associated Mmp2 is expressed in exit glia and contributes to motor axon targeting. To identify possible substrates, we undertook a yeast interaction screen for Mmp2-binding proteins and identified the novel ECM protein Faulty Attraction (Frac). Frac encodes a multi-domain extracellular protein rich in EGF and cbEGF domains, related to the vertebrate fibrillin and fibulin gene families. It is expressed in ventral mesodermal domains situated immediately adjacent to Mmp2-positive glia at the edge of the ventral nerve cord. The juxtaposition of Mmp2 and Frac is consistent with the hypothesis that Frac is a proteolytic target of Mmp2. Indeed Mmp2 cleaves Frac. To test whether frac is necessary for axon targeting, we characterized guidance in frac LOF mutants. Motor axons in frac LOF embryos are loosely associated and project ectopically—a phenotype essentially equivalent to that of Mmp2 LOF. The phenotypic similarity between enzyme and substrate suggest that Mmp2 may cleave and activate Frac to promote proper targeting. However, frac overexpression experiments indicate that frac is unlikely to possess intrinsic signaling activity. In light of this, we asked...
if \( \text{frac} \) genetically interacts with known signaling pathways. We find genetic interactions between \( \text{frac} \) and the BMP pathway—raising the possibility that \( \text{frac} \) contributes to BMP pathway activation in motorneurons. Together, these results identify a novel \textit{in vivo} substrate for \textit{Mmp2} in motor axon guidance.

\textbf{Introduction}

The mechanisms that confer spatial and temporal precision to signal transduction by axon guidance receptors remain largely elusive. While substantial progress has been made in identifying proteins required for axon pathfinding (Dickson, 2002), the regulatory systems that modulate their activity to give rise to the stereotyped paths that axons follow are unclear. Localized proteolysis of receptors or their ligands is an attractive mechanism to contribute to the spatiotemporal specificity of axon pathfinding (Meyer and Aberle, 2006; Serpe and O'Connor, 2006; Miller et al., 2008).

The genetic advantages Drosophila affords have proven essential to the characterization of conserved pathways and proteins. Relevant to the work presented here, compensation and redundancy among the 24 vertebrate matrix metalloproteinase (MMP) family members have necessitated \textit{in vitro} approaches to characterizing their functions. MMP family members can degrade structural components of the ECM and are of substantial clinical interest, as they are often dramatically upregulated in inflammatory and metastatic contexts. However, their non-pathological actions, and in particular the identities of their substrates, remain poorly understood (Page-McCaw et al., 2007; Kessenbrock et al., 2010;
Rodriguez et al., 2010). Drosophila provides a greatly simplified in vivo paradigm for the characterization of MMP function, as the fly genome contains only two MMP homologs – Mmp1, a secreted protein, and Mmp2, which is membrane-associated (Llano et al., 2000; 2002; Page-McCaw et al., 2003). An analysis of Drosophila MMP mutants demonstrated that MMPs are required for proper motor axon pathfinding. In particular, Mmp2 is expressed in motor axon-associated exit glia and promotes axon fasciculation (Miller et al., 2008). It is reasonable to consider that the Drosophila system could also facilitate MMP substrate identification.

In this work, we demonstrate that faulty attraction (frac) is an Mmp2 substrate essential for motor axon targeting during embryogenesis. We identified Frac as a potential Mmp2 substrate in a yeast interaction screen. Frac is predicted to encode an extracellular matrix (ECM) protein containing EGF and calcium-binding EGF (cbEGF) repeats, related to the vertebrate fibrillin and fibulin gene families (Downing et al., 1996). Fibrillins are large matrix-associated glycoproteins that comprise the major structural protein of microfibrils (Sakai et al., 1986; Zhang et al., 1995). In addition to giving structural support to connective tissue, they play a major functional role in the regulation of TGFβ bioavailability (Charbonneau et al., 2004; Dietz et al., 2005).

Here, we provide evidence that Frac is an Mmp2 substrate via biochemical, expression, and genetic analysis. Our data indicate that Mmp2 cleaves and activates Frac to promote motor axon bundling during outgrowth, and in this
capacity, acts in opposition to the Semaphorin1A/PlexinA pathway. We demonstrate that Frac is expressed in the mesoderm flanking extending axons and immediately adjacent to Mmp2-positive exit glia. Hence, the Mmp2-dependent activation of the matrix molecule Frac provides a novel mechanism to precisely control the local activation of a guidance cue.

Materials and Methods

Fly Stocks

Stocks used in this work include: Mmp2\textsuperscript{W307*}, Mmp1\textsuperscript{Q112*}, UAS-Mmp1, UAS-Mmp2, tubulinGAL4 (Page-McCaw 2003), viking::GFP (a kind gift from A. Prokop), UAS-Sax\textsuperscript{act} (a kind gift from K. Wharton), elavGAL4 (A. DiAntonio), frac\textsuperscript{Δ1} (see below) and frac\textsuperscript{Δ2} (see below). UAS-frac was generated from a full-length cDNA plasmid template (RE24628), cloned into pUAST and injected into flies by standard methods. Similar results were observed with at least two independent transgenic lines to control for position and copy number effects. All remaining stocks were obtained from the Bloomington Stock Center. Double mutants were generated using standard genetic techniques.

Minos element excision

Minos imprecise excision was performed as described (Metaxakis et al., 2005). Briefly, flies heterozygous for a single Minos transposon insertion, Mi\{ET1\}CG7526\textsuperscript{MB05690}, (marked by EGFP) in the first intron of the frac gene region were crossed to flies carrying the Minos transposase (marked by white).
Two days after setting up the cross, adults were transferred to new vials and the old vials were heat-shocked daily for 1 hour in a 37° water bath until pupariation. Adults carrying both the transposon and transposase were then crossed to a chromosome 3 balancer stock. Progeny with transposon excisions were identified as carrying the correct balancers but lacking EGFP and white markers. These flies were crossed individually to a chromosome 3 balancer and analyzed for imprecise excision events using PCR.

**Immunohistochemistry**

Embryo fixation, antibody staining, and RNA *in situ* hybridization were performed as described (Miller 2008). The following primary antibodies were used: mAb1D4 (Fasciclin II) at 1:10 (generated by C. Goodman and obtained from the Developmental Studies Hybridoma Bank [DSHB]), mAb myosin heavy chain (MHC) at 1:500 (a kind gift from Erika Geisbrecht), gpAb kakapo at 1:300 (a kind gift from Talila Volk), mAb repo at 1:10 (generated by C. Goodman and obtained from the DSHB), rbAb pMad/pSmad3 (Epitomics) at 1:100 and rbAb β-gal (Immunology Laboratory Consultants, Inc) at 1:1000. For RNA *in situ* hybridization, the digoxigenin (DIG)-labeled probe was made using the Roche DIG RNA Labeling Kit (SP6/T7) using the full-length Frac cDNA plasmid as the template. Guinea pig and rabbit anti-Frac antibodies were generated against a peptide composed of residues 1216-1500 of the Frac protein. Antibodies generated in different animals from both species all recognized the same protein expression pattern on whole mount embryos. For immunohistochemistry, Frac
antibodies were affinity-purified using the AmminoLink Plus Immobilization Kit (Pierce) and were not diluted for use on embryos. All microscopy and data analysis was performed as described (Miller et al., 2008).

**Western blotting**

Western protein samples were prepared by boiling 60 embryos (stage 15-17) in 36 µL Laemmli sample buffer. A total of 25 µL of lysate, equivalent to 42 embryos, was loaded into each lane. Protein was transferred to Millipore Immobilon-P 0.45 µM membrane. Guinea pig anti-Frac whole serum was used at a 1:100 dilution and goat anti-GAPDH (Imgenex) was used as a loading control at 1:5000. HRP-labeled goat anti-guinea pig and rabbit anti-goat (MP Biomedicals) were diluted at 1:10000. Bands were detected using the Supersignal West Pico Chemiluminescent Substrate kit (Thermo Scientific).

**Yeast two-hybrid screen**

The catalytically-inactive Mmp2 plasmid was engineered by splice overlap extension (Vallejo et al., 1994) from an Mmp2 cDNA (Page-McCaw et al., 2003). The bait plasmid pGBD-C1.Mmp2CAT was constructed by inserting catalytically-inactive Mmp2 into pGBD-C1 (James et al., 1996). We screened 1.8X10⁵ cDNAs from a Drosophila embryo library and 2.3X10⁵ cDNAs from a third instar larval library (Durfee et al., 1993) using the methods of James et al., 1996 as described (Zhang et al., 2006). Catalytically-inactive Mmp2 positively interacted with six predicted extracellular or transmembrane proteins including the hyalin domain region of Frac. A parallel screen for Mmp1 interactors successfully identified
Ninjurin A as an *in vivo* substrate for Mmp1 illustrating the effectiveness of this method (Zhang et al., 2006).

**Results**

CG7526 interacts with Mmp2 and is related to vertebrate cbEGF-domain containing proteins

We previously demonstrated that the catalytic activity of Matrix Metalloproteinase 2 (Mmp2) promotes motor axon fasciculation (Miller et al., 2008), implying that its substrate also functions in axon targeting. As a route to substrate identification, we performed a yeast interaction screen using a substrate-trapping variant of Mmp2 as bait (see Materials and Methods). The screen yielded seven transmembrane or extracellular proteins representing potential substrates. We examined their RNA expression profiles via *in situ* hybridization to determine if any were expressed in a manner consistent with regulation by Mmp2. Mmp2 is GPI-anchored (Llano et al., 2000; Llano et al., 2002; Page-McCaw et al., 2003), and expressed by exit glia, which are tightly associated with motor axons (Sepp et al., 2001; Sepp and Auld, 2003; Freeman, 2006; Miller et al., 2008). Hence, we anticipated relevant substrates would be expressed in a pattern consistent with cleavage by exit glia-associated Mmp2—namely the glia themselves, neurons, or mesoderm. We identified one gene, CG7526, with salient mesodermal expression (see below and Fig. 3.2).

Sequence analysis indicates that CG7526 codes for a predicted extracellular protein including three EGF and seven calcium-binding EGF (cbEGF) repeats.
CG7526 shares a conserved domain structure with extracellular proteins containing arrays of cbEGF repeats (Fig. 3.1A). Calcium binding is thought to impart structural rigidity to these proteins, and the cbEGF domains also participate in protein-protein interactions (Handford et al., 1995; Downing et al., 1996). Best characterized among related vertebrate proteins are Fibrillins, which serve both structural and signaling functions in the ECM. Fibrillins are modular glycoproteins that compose microfibrils and thus function as structural components of the ECM (Sakai et al., 1986; Zhang et al., 1995). They are characterized by arrays of cbEGF domains interspersed by TB (TGFβ-binding) domains. In addition to their structural role, Fibrillins regulate TGFβ superfamily bioavailability (Charbonneau et al., 2004; Ramirez and Rifkin, 2009).

Underscoring the major functional relationship between Fibrillins and TGFβ signaling, human Marfan Syndrome results from haploinsufficiency of Fibrillin-1, and is strongly linked to elevated levels of active TGFβ (Ramirez and Dietz, 2007). While CG7526 is structurally related to Fibrillins, the presence of CCP and hyalin domains, coupled with the absence of TB domains, argue that it is not orthologous (Hynes and Zhao, 2000). Based on the axon guidance phenotype described here, we have named CG7526, faulty attraction (frac).

To ask whether frac plays a role in axon guidance, we generated loss-of-function (LOF) alleles via imprecise excision of a Minos transposon situated in the first intron (Metaxakis et al., 2005; Material and Methods). Two alleles were generated with significant deletions of the frac locus (Fig. 3.1B). frac^17 is a 1.2 kb deletion removing part of the second exon. Anti-Frac antibodies fail to recognize
Frac protein in homozygous mutant embryos, thus confirming it as a protein null (Fig. 3.1C,D). The second allele, frac\(^{\Delta 2}\), is a 2.1 kb deletion removing the first two exons and half of the third exon. However, while the start is deleted, a truncated protein is translated, as frac\(^{\Delta 2}\) is not a protein null (data not shown). Both frac alleles are homozygous viable and display no overt behavioral defects.

**Drosophila Frac is expressed in the embryonic mesoderm**

We characterized frac’s expression profile to ask whether it is expressed in a pattern consistent with regulation by Mmp2. At embryonic stage 13, frac RNA is expressed strongly in ventral mesoderm adjacent to the CNS and at lower levels in more dorsal mesoderm (Fig. 3.2A). At stage 15, the frac RNA expression domain expands to include all embryonic musculature (Fig. 3.2B). To characterize Frac protein expression, we generated anti-Frac antibodies (Fig. 3.1A). Confirming antibody specificity, anti-Frac antibodies do not recognize protein in frac\(^{\Delta 1}\) mutant embryos (Fig. 3.1C,D). The dynamic pattern of frac RNA expression in the mesoderm is recapitulated by Frac protein. At stage 14, Frac expression is apparent in wedges of ventral mesoderm immediately adjacent to the nerve cord and then expands to encompass all embryonic muscle fibers at stage 15 (Fig. 3.1C,D). Frac continues to be expressed throughout the embryonic musculature until late stage 17, when its expression concentrates to slivers at hemisegment boundaries in a pattern characteristic of muscle attachment sites (Fig. 3.2E; Becker et al., 1997). Confirming that Frac is mesodermal, it is co-expressed with myosin heavy chain, a well-characterized muscle marker (Fig. 3.1F; Kiehart and Feghali, 1986). Furthermore, we verified that Frac expression
in late stage 17 embryos is restricted to muscle attachment sites by double labeling with Shortstop/Kakapo (Fig. 3.2G; Strumpf and Volk, 1998). In addition to its mesodermal expression, Frac is expressed in the stomatogastric nervous system (Fig. 3.2H; Gonzalez-Gaitan and Jackle, 1995).

Since the wedges of Frac-positive ventral mesoderm appear to be in close proximity to Mmp2-positive exit glia, Frac may represent an Mmp2 substrate. Hence, we analyzed the relative positions of Frac protein, Mmp2-positive glia, and motor axons in more detail. To compare the position of Frac protein to extending motor axons, we double-labeled wild-type embryos with anti-Frac antibodies and anti-FasII antibodies, which marks motor projections (Van Vactor et al., 1993). Motor axons are extending through the periphery at stage 14, a time when high levels of Frac are expressed in the muscles through which these nerves traverse (Fig. 3.2I). Since Mmp2 is expressed by exit glia and is membrane-bound (Llano et al., 2002; Miller et al., 2008), if mesodermal Frac is an in vivo substrate, it is predicted to be present immediately adjacent to these glia. In support of this hypothesis, Repo-positive glia align along the edge of the Frac-positive wedge of ventral mesoderm (arrows point to exit glia in Fig. 3.2J). This striking juxtaposition of Frac-positive mesoderm and Mmp2-positive glia argues that Mmp2 is poised to cleave Frac during embryogenesis.

**Frac is processed in an Mmp2-dependent manner**

Frac was identified in a yeast two-hybrid screen as an Mmp2 interactor, and its embryonic expression domain is in line with regulation by Mmp2. Hence, we
investigated whether Frac represents a proteolytic substrate of Mmp2. Frac has a predicted size of 172 kDa. We find that Frac runs as a doublet at 170 and 250 kDa on Western blot of wild-type embryo extracts (Fig. 3.3A). The larger molecular weight band likely reflects a post-translationally modified form of Frac, as many matrix molecules are heavily glycosylated (Comer and Hart, 2000). These two bands are not present in extracts from frac deletion embryos, demonstrating specificity if the antibodies (Fig. 3.3A). To determine if Mmp2 is responsible for processing Frac, we analyzed Frac expression in Mmp2 LOF and GOF embryo extracts (Fig. 3.3A). In Mmp2 misexpression embryos (tub>Mmp2), full-length Frac is nearly absent, arguing that Mmp2 can process the full-length form. Consistent with this model, three small molecular weight Frac fragments are observed in extracts from Mmp2 misexpression embryos (arrowheads). Conversely, in Mmp2W307* embryo extracts, we do not observe the small molecular weight bands and we consistently detect a slight increase in the amount of full-length Frac (asterisk), arguing that Frac is not appropriately processed in the absence of Mmp2. The alterations in Frac processing observed in Mmp2 mutant embryonic extracts provide evidence that Frac is sensitive to Mmp2 levels.

The marked reduction in full-length Frac in Mmp2 GOF protein extracts indicated that we might detect a visible reduction in Frac expression in these embryos. In stage 14 wild-type embryos, Frac is expressed strongly in wedges of ventral mesoderm. We compared Frac levels in Mmp2 GOF embryos and wild-type embryos at stage 14, with the observer blinded to genotype. We find a 2.5 fold
reduction in fluorescence intensity in embryos with Mmp2 misexpression relative to wild type ($P<0.0001$; Fig. 3.3B,C), demonstrating that Mmp2 is sufficient to block accumulation of Frac. As controls, we scored Frac intensity in embryos with Mmp1 overexpression or in embryos mutant for sidestep, a guidance molecule expressed in muscle (Sink et al., 2001). Neither of these genotypes show significant modifications to Frac levels (Fig. 3.3D). MMPs often display overlapping substrate specificities; however, this result indicates that Frac is unlikely to be cleaved by Mmp1. These expression data support our biochemical data and demonstrate that Frac is processed in an Mmp2-dependent manner. Taken with the interaction between Frac and Mmp2 in yeast, we conclude that Frac likely represents an Mmp2 substrate.

frac LOF mutants display ectopic motor axon projections and loosely bundled axons

Complementary LOF and GOF analyses established that Mmp2 promotes motor axon fasciculation during outgrowth (Fig. 3.4D,H; Miller et al., 2008). Identification of Frac as an Mmp2 substrate prompted us to investigate if frac was involved in motor axon targeting. We generated two frac deletion alleles via imprecise excision of a Minos transposon in the first intron (see above and Materials and Methods).

We first assayed embryonic development in frac LOF mutants. Since frac is a component of the mesodermal ECM, frac mutants might display gross defects that preclude an interpretation of any axon pathfinding errors. However, our
phenotypic analyses argue that this is not the case. First, both \( \text{frac} \) alleles are homozygous viable, demonstrating that it is not an essential gene. Second, both alleles have an intact basement membrane as visualized with \( \text{viking}::\text{GFP} \) (Morin et al., 2001; Urbano et al., 2009). Collagen IV, or \( \text{viking} \), is expressed by macrophages of the basement membrane. These cells surround most tissues including the VNC, brain, and gut (Yasothornsrikul et al., 1997). Both \( \text{frac} \) alleles display normal localization of \( \text{viking}::\text{GFP} \), indicating the basement membrane is not perturbed (Fig. 3.8A-C). Third, appreciable defects in muscle size, number, or epidermal attachment are not observed in \( \text{frac} \) mutants as visualized with myosin heavy chain (MHC) antibody (Fig. 3.8D-F; Kiehart and Feghali, 1986). Thus, we conclude that morphological defects in \( \text{frac} \) LOF mutants do not interfere with an analysis of motor axon guidance.

We next analyzed motor axon development in \( \text{frac} \) mutants. In wild type, the intersegmental nerve branch b (ISNb) extends from the CNS bundled with the main branch of the ISN. ISNb axons defasciculate, or separate from, the ISN before the ventrolateral muscle field (VLM). Subsets of ISNb axons innervate the clefts between VLM muscles 7, 6, 13, and 12 (Fig. 3.4A,E). Embryos mutant for either \( \text{frac} \) allele have prominent guidance errors. 64% of ISNb axons in \( \text{frac}^{\Delta 1} \) embryos do not stay tightly bundled and display targeting errors in which individual axons circle back or project to neighboring nerve bundles. These were further classified as defasciculation and/or misprojection errors. 51% of ISNb nerves in \( \text{frac}^{\Delta 1} \) mutant embryos display hypofasciculated or frayed axons, and 29% include axons that make connections with other nerve branches or other
ISNb axons (Fig. 3.4B,F; Table 3.1). Similarly, in frac\(^{\Delta 2}\) mutants, 57% of hemisegments have aberrant ISNb branches. The same phenotypic classes are present as in frac\(^{\Delta M}\), with 44% of ISNb nerves exhibiting ectopic splintering of axons, and 22% displaying erroneous projections (Fig. 3.4C,G; Table 3.1). Both frac alleles are homozygous viable and fertile, so we tested whether maternal contribution of Frac contributes to motor axon behavior. The penetrance of ISNb guidance defects does not increase in the absence of both maternal and zygotic Frac protein (\(P \geq 0.3\) for all; Table 3.1), indicating that maternal Frac does not contribute to motor axon pathfinding.

Transposon excisions can result in second site mutations if the transposon excises only to briefly integrate elsewhere. Hence, we analyzed frac\(^{\Delta 1}/frac^{\Delta 2}\) embryos and found that the penetrance remains at about 61% (Table 3.1). Similarly, we tested each allele over a frac deficiency. Neither frac\(^{\Delta 1}/Df\) or frac\(^{\Delta 2}/Df\) were statistically different from the respective homozygous mutants (\(P \geq 0.7\); Table 3.1). These data indicate that both alleles are genetic nulls, though frac\(^{\Delta 2}\) is not a protein null. The ISNb phenotypes observed in frac LOF mutants strongly resemble those in Mmp2 LOF mutants and support the hypothesis that Mmp2-dependent regulation of Frac contributes to motor axon targeting.

Mmp2 mutants display defects in the segmental nerve a (SNa) projection in addition to ISNb. To determine if frac is necessary for SNa projections, we scored SNa guidance in frac mutants. In wild type, the SNa extends dorsally from the CNS to innervate the lateral muscle field. SNa axons divide into dorsal and
posterior branches. The dorsal branch extends between muscles 22 and 23 and divides a second time to innervate muscle 24. The posterior branch extends to innervate muscle 8 (Fig. 3.4I,M). In control embryos, 11% of hemisegments display hypofasciculation or misprojection defects in the SNa (Table 3.1). In contrast, 45% of hemisegments in frac\textsuperscript{A1} embryos and 49% in frac\textsuperscript{A2} embryos contain axons that separate inappropriately and project ectopically (P<0.0001 for both compared to wild type; Fig. 3.4J,K,N,O; Table 3.1). The majority of defects are hypofasciculation errors in which axons inappropriately branch away from the SNa, occurring 46% in frac\textsuperscript{A1} and 43% in frac\textsuperscript{A2} (Table 3.1). Most SNa nerves fray near choice points, where nerve bundles divide and axons follow different pathways to innervate their specific targets. The SNa hypofasciculation phenotypes observed in frac mutants resemble those in Mmp2 mutants and indicate that Mmp2 regulates Frac processing to modulate motor axon fasciculation in multiple motor tracks (Fig. 3.4L,P; Table 3.1). A priori, Mmp2-dependent cleavage could lead to either substrate activation or inactivation. Since Mmp2 and frac mutants both display loosely bundled axons with ectopic projections, we propose that Mmp2-dependent cleavage activates Frac to promote motor axon bundling.

The pathfinding phenotype in frac LOF mutants argues that frac promotes motor axon fasciculation. Appropriate levels of motor axon bundling require a balance between interaxonal adhesion and attraction between motor axons and their targets (Winberg et al., 1998b; Yu et al., 1998; Yu et al., 2000). The Sema1a/PlexA repulsive signaling pathway plays a central role in this process.
LOF mutants in the pathway display increased motor axon bundling and fail to properly innervate their targets (Fig. 3.8G; Yu et al., 1998) - a phenotype roughly opposite to that displayed by fbn LOF mutants. We asked whether frac displays genetic interactions with Sema1a to obtain additional evidence that frac enhances fasciculation. Heterozygosity for Sema1a suppresses the pathfinding phenotypes from 64% in frac homozygotes to 18% in frac mutants that are also heterozygous for Sema1a (P<0.0001; Table 3.1; Fig. 3.8G-J). This genetic interaction argues that frac regulates pathfinding by influencing the degree of attraction between motor axons during guidance. Furthermore, it provides evidence that the defects observed in frac mutants reflect a specific role for frac in guidance and are not the consequence of morphological defects.

**Frac overexpression promotes defasciculation and suppresses Mmp2 overexpression**

These studies argue that Mmp2 cleaves Frac to promote motor axon fasciculation and raise the question of the identity of the Mmp2-generated pro-fasciculation signal. A matrix molecule such as Frac may influence signaling by a variety of means (Hynes, 2009). We can envision three likely relationships between Frac and the guidance signal. First, it is possible that full-length Frac sequesters a soluble guidance cue. In this case, Mmp2 cleavage liberates the signaling molecule from full-length Frac. frac LOF would then be characterized by elevated guidance factor signaling (no Frac to sequester cues), and Mmp2 LOF would be characterized by decreased guidance factor signaling (increased Frac to sequester cues). The finding that the guidance phenotypes displayed by
Mmp2 LOF and frac LOF mutants strongly resemble each other argues against the model that Frac is simply a sink for an active guidance factor. Alternatively, the Frac fragment may have intrinsic signaling activity and act as a repulsive mesoderm-based cue to keep motor axons tightly bundled. As a third possibility, the Frac cleavage fragment may be a co-factor in a signaling complex with a guidance cue.

To distinguish between the second and third signaling mechanisms, we overexpressed Frac throughout the mesoderm. If the Frac cleavage fragment has intrinsic signaling activity then we would expect frac overexpression to lead to elevated levels of axon bundling, as the axons are exposed to more pro-fasciculation signal. However, if the Frac cleavage fragment represents only part of a signaling complex, then frac overexpression would not lead to hyperfasciculation, as the Frac fragment is insufficient to signal alone. We find that frac overexpression does not promote hyperfasciculation - in fact the motor axons in 24B>frac embryos are poorly fasciculated and project ectopically (Fig. 3.5B,F,J,N; Table 3.1). The hypofasciculation observed in these embryos suggests that Frac overexpression overwhelms normal regulation by Mmp2 and attenuates an active Frac fragment/signaling cue complex. This phenotype demonstrates that Frac is not sufficient to promote fasciculation and hints at the existence of a signaling cue associated with the Frac cleavage fragment.

The frac overexpression phenotype suggested that elevated Frac levels interfere with normal Mmp2-dependent regulation. Hence, we looked for genetic
interactions between *frac* and *Mmp2*. In particular, we asked whether *frac* levels modify the *Mmp2* overexpression phenotype. *Mmp2* overexpression is sufficient to drive an inappropriately high degree of motor axon fasciculation (Fig. 3.5C,G,K,O; Miller et al., 2008). We interpret this phenotype to indicate that *Mmp2* levels normally limit the extent of motor axon fasciculation and that elevated *Mmp2* activates inappropriately high amounts of a pro-fasciculation signal. This GOF phenotype is not modified by altering the dosage of any other guidance molecules we have tested, including *Sema1a* and *sidestep* (data not shown; Miller et al., 2008) - arguing that the phenotype is not sensitive to mutants that affect axon targeting via other pathways. We reasoned that if *frac* overexpression disrupts pathfinding by interfering with, or diluting out, an active signaling complex, then the hyperfasciculation triggered by *Mmp2* overexpression may be suppressed by *frac* overexpression. Consistent with this hypothesis, *frac* overexpression strongly suppresses the guidance defects in *Mmp2* overexpression embryos (Fig. 3.5D,H,L,P; Table 3.1). For example, SNa hyperfasciculation drops from 61% in *Mmp2* overexpression embryos to 13% in embryos co-overexpressing *Mmp2* and *frac*; whereas ISNb hyperfasciculation decreases from 63% to 23% (P<.0001 for both). This genetic interaction provides additional support for a close relationship between *Mmp2* and *frac*. Furthermore, the defasciculation phenotype displayed by *frac* GOF embryos raises the possibility of a Frac-regulated guidance cue.

**Frac may regulate a non-canonical BMP signaling pathway**

The motor axon phenotypes associated with *frac* overexpression indicate that
frac is not sufficient to promote fasciculation and suggest the presence of a frac-regulated pathfinding cue. Studies in vertebrate systems provide ample evidence that the related Fibrillin family controls the bioavailability of growth factors, most notably TGFβ/BMP family members (Neptune et al., 2003; Charbonneau et al., 2004; Chaudhry et al., 2007; Sengle et al., 2008). To test if frac modulates BMP signaling, we investigated genetic interactions between frac and the BMP pathway. With the exception of the activin-like protein Dawdle (Parker et al., 2006; Serpe and O’Connor, 2006), there is a lack of published evidence for BMP involvement in motor axon guidance. However, many of the BMP pathway mutants have pleiotropic embryonic defects, complicating LOF analysis (Khalsa et al., 1998; Wharton et al., 1999). Thus, we asked whether an activated form (Q263D mutation) of the type I receptor saxophone (sax) modulates the frac LOF phenotype (Haerry et al., 1998). Pan-neuronal overexpression of saxact does not yield appreciable motor axon phenotypes (Fig. 3.6B,E; Table 3.1); yet saxact strongly suppresses the frac LOF phenotype, reducing the defasciculation from 64% to 19% (P<.0001; Fig. 3.6A,D,C,F; Table 3.1). The suppression of the frac pathfinding phenotype via neuronal activation of the BMP pathway argues that frac activity normally promotes BMP pathway activation in motoneurons. In line with this view, overexpression of the BMP ligand gbb partially suppresses the phenotype observed with frac overexpression (data not shown), providing evidence that frac overexpression attenuates signaling via a Frac/BMP complex. However, the incomplete suppression in both assays indicates that frac likely regulates additional guidance cues.
Classical BMP signaling results in phosphorylation of the transcription factor Mad, which translocates to the nucleus and activates gene transcription (Marques et al., 2002). BMP signaling is required for NMJ growth in Drosophila, and pMad has been shown to accumulate in motorneurons. Motorneuronal pMad is apparent during embryogenesis, raising the possibility that this pathway plays a role in targeting. To test whether Mad-mediated BMP signaling is regulated by frac, we examined pMad expression in frac LOF and GOF embryos. We do not detect an obvious difference in the number of pMad-positive neurons, or in pMad intensity, in either LOF or GOF frac mutants (Fig. 3.6G-I). These results suggest that frac may regulate a non-canonical BMP signaling pathway.

**Discussion**

Signaling downstream of axon guidance receptors is tuned precisely to generate proper connectivity. Elucidating the mechanisms allowing for stereotyped local activation of these pathways remains a major challenge. The ECM is perfectly situated to provide extracellular cues that could impinge on targeting—and provide the spatiotemporal signals to regulate events such as fasciculation, branching, or synaptogenesis. However, the interactions between matrix molecules and guidance receptors remain poorly defined. Here we demonstrate that the novel ECM molecule Frac regulates motor axon guidance in Drosophila. Furthermore, Frac itself is regulated via proteolysis, adding another layer of regulation to ensure local activation of guidance receptor pathways.
Faulty attraction, (Frac), an ECM molecule structurally related to vertebrate Fibrillins and Fibulins, was identified in a yeast interaction screen for Mmp2-binding proteins. Frac is expressed in the embryonic mesoderm concurrent with axon pathfinding and directly adjacent to Mmp2-expressing exit glia. We further show that Frac processing in embryos is Mmp2-dependent. frac LOF alleles display marked defects in axon pathfinding, which are tantamount to those displayed by Mmp2 LOF mutants, providing evidence that Mmp2 cleaves and activates Frac (Fig. 3.7). We undertook genetic interaction analyses to elucidate the mechanism of Mmp2-Frac signaling. These studies argue that (1) the Frac fragment generated by Mmp2 does not have inherent signaling activity, and (2) frac may contribute to the activation of a non-canonical BMP signaling pathway in motorneurons. To our knowledge, these data are the first to demonstrate that proteolysis of an ECM molecule is involved in regulating the distribution or activation of a signaling cue during axon guidance.

Motor axons in Drosophila selectively defasciculate at guidance choicepoints as they follow individual routes to their synaptic targets. Repulsive signaling driven by the Semaphorin1a-PlexinA pathway acts in motor axons to promote inter-axonal repulsion essential for axon separation (Yu et al., 1998; Terman and Kolodkin, 2004). However, the mechanism by which defasciculation is normally confined to axon choicepoints is unknown. We have presented evidence that an Mmp2-Frac pathway is necessary to limit axon defasciculation. Together, our data argue that a Frac cleavage product acts in the mesodermal ECM to signal axons to remain bundled. Because Mmp2 regulates Frac processing, signal
activation may be precisely modulated both spatially and temporally. We hypothesize that the Mmp2-Frac pathway provides a cue to axons to remain bundled during outgrowth and overcomes the interaxonal repulsion driven by the Sema1a-PlexA pathway. This antagonistic relationship receives strong support from the finding that both Mmp2 and Frac are dominantly suppressed by Sema1a mutations (Fig. 3.8G-J; Miller et al., 2008). We speculate that Mmp2 is inactive at choicepoints, allowing the Sema1a-PlexA pathway to promote motor axon separation at these locations.

Metalloproteinase function in the developing nervous system

Among the metalloproteinases, the ADAM family has been most intimately linked to the regulation of axon targeting to date. In particular, the transmembrane protein ADAM10/Kuzbanian (Kuz) regulates the ectodomain shedding of a number of neural substrates including the Roundabout receptor, Notch, and GPI-linked ephrin-A2 (Hattori et al., 2000; Mumm and Kopan, 2000; Coleman et al., 2010). Given the central role that proteolysis is likely to play in sculpting nervous system connectivity and function, why are there few examples of MMP activity in this process? Analysis of the MMP family in neuronal development in vertebrates has likely been largely obscured by the functional redundancy among the 24 MMP family members. Broad-spectrum MMP inhibitors and compound mouse mutants are beginning to resolve their functions. For example, Hehr et al. (2005) used MMP inhibitors in an analysis of RGC guidance in Xenopus and found evidence for MMP function at two distinct choicepoints. There is also emerging evidence that MMP-9 elicits stable modifications of spine structure in LTP via...
ECM remodeling (Bozdagi et al., 2007; Wang et al., 2008b), arguing that synaptic plasticity requires MMPs. These examples indicate that neural MMP functions are unlikely to be defined exclusively by ECM degradation in the contexts of inflammation and metastasis, but rather that they are likely to play more subtle roles in neuronal development and physiology.

In Drosophila, Mmp1 is secreted, while Mmp2 is anchored to the membrane via a GPI-link. Mmp2’s membrane association may position it well to regulate defined aspects of neuronal development. In addition to the work presented here, Mmp2 is required for dendritic reshaping of sensory neurons in the adult (Yasunaga et al., 2010). These authors found that a pulse of Mmp2 expression in epithelial cells directly apposed to a class of adult sensory neurons is coincident with the dendritic remodeling. Furthermore, dendritic reshaping is blocked in Mmp2 mutants, and clonal analysis indicates that Mmp2 acts locally in dendritic morphogenesis, and reshapes only those dendrites in direct contact with Mmp2-positive epithelia. Hence, this work provides a second example of a local function of Mmp2 defining neuronal morphology in development. Given the specialized microenvironments likely present in the neuronal ECM, the spatiotemporal control of proteolysis provided by a membrane-associated MMP is predicted to be of fundamental importance.

An Mmp2-Frac signaling pathway

This study has uncovered an Mmp2-Frac signaling module that promotes proper motor axon targeting. Motor axons have a precisely modulated attraction for their
mesodermal ECM substrate. They must be sufficiently attracted to the mesodermal ECM to leave the ventral nerve cord and initiate migration on it; however, they need to limit exploration on this substrate and maintain strong interaxonal adhesion. Our data argue that an Mmp2-dependent Frac cleavage fragment keeps motor axons on track. Since Mmp2 is expressed by motor-axon associated exit glia, this processing event is positioned only in the vicinity of extending axons (Fig. 3.7). In general terms, the ECM is positioned to regulate the distribution, activation, and presentation of growth factors—raising a number of possibilities for the role of Frac in axon guidance.

A relatively straightforward model based on the role of the related Fibrillin family in vertebrates is that Frac acts as a reservoir or sink for a guidance cue, which is released to signal via Mmp2-dependent cleavage. Vertebrate Fibrillins play major functional roles in limiting the bioavailability of TGFβs (ten Dijke and Arthur, 2007; Ramirez and Rifkin, 2009). TGFβs are targeted to Fibrillin scaffolds by latent TGFβ-binding proteins (LTBPs) and are released from this latent Fibrillin-associated complex via proteolysis and integrin-mediated activation. Support for the inhibitory role for Fibrillins is the TGFβ pathway comes from work on human Marfan Syndrome (MFS), which is a connective tissue disorder caused by mutations in human Fibrillin-1. Recent work demonstrates that the aortic root dilation associated with MFS in both mice and humans can be attenuated or reversed with losartan, a drug with anti-TGFβ signaling activity (Habashi et al., 2006; Brooke et al., 2008). The implication from these studies is that Fibrillin normally sequesters TGFβ, so that a decrease in Fibrillin dosage results in
excessive TGFβ signaling. If a related mechanism is at play in Mmp2-Frac signaling, we would predict that (1) frac LOF embryos would phenotypically resemble Mmp2 GOF, since in both scenarios too much signal is released, and that (2) frac LOF embryos would exhibit the opposite phenotype of Mmp2 LOF, since in the first case excess signal is released, whereas in Mmp2 LOF mutants, too little signal is generated. The finding that frac LOF and Mmp2 LOF mutants display essentially identical phenotypes, which are opposite to those displayed by Mmp2 GOF—suggests that Frac does not simply act to sequester or store a guidance cue.

A second model is that the Frac cleavage product has intrinsic signaling activity. Until recently, there have been few compelling examples of the direct action of matrix molecules in signaling in neuronal development. However, the Barres lab has demonstrated that Thrombospondin (TSP), another EGF-domain containing matrix protein, is secreted by astrocytes to promote synaptogenesis (Christopherson et al., 2005). They further demonstrated that EGF domains in TSP directly bind to the neuronal α2δ-1 calcium channel subunit to increase synapse formation (Eroglu et al., 2009). If Frac were solely responsible for providing the pro-fasciculation signal, the simplest prediction is that increasing Frac levels would increase axon bundling. In fact, we find that motor axon separate prematurely and inappropriately in 24B>frac embryos, arguing against this model. We interpret the pathfinding phenotypes in these embryos to result from a “dominant negative-like” effect of elevated Frac. If the addition of excess Frac to the signaling system effectively dilutes out the guidance cue (Factor X in
Fig. 3.7), then decreased fasciculation is expected to result. To provide additional support for this genetic argument, it will be critical to define additional components of this signaling pathway.

Frac is structurally related to the vertebrate Fibrillin family, making the interaction between the Fibrillins and TGFβ signaling discussed above of interest. Furthermore, BMPs in Drosophila have been shown to be under complex regulation by Type IV Collagens (Wang et al., 2008a), underscoring the role matrix molecule regulation plays in this conserved signaling pathway. In addition, phospho-Mad is present in motorneuron nuclei as motor axons leave the ventral nerve cord (Marques et al., 2002), demonstrating that the pathway is active during guidance. In light of these data, we analyzed genetic interactions between frac and the BMP pathway. Strikingly, activation of BMP signaling in motorneurons via overexpression of an activated type I receptor (saxact) strongly suppresses the guidance defects associated with frac LOF, arguing that frac may regulate a BMP cue. These data are also in line with our genetic data arguing that frac acts in concert with another factor to promote targeting. This model requires further support, though, as the BMP pathway has not yet been implicated in motor axon guidance. It remains possible that a guidance function for this pathway has been masked by a combination of maternal contribution of pathway components and early morphological defects (Dorfman and Shilo, 2001; Marques et al., 2002; McCabe et al., 2004; O'Connor et al., 2006). In conclusion, we have described a novel pathway controlling motor axon targeting. We propose that Mmp2 expressed on the surface of exit glia controls the processing
of the matrix molecule Frac, which sends a pro-fasciculation signal to extending axons. These findings highlight the complex interactions between motor axons, glia, and the mesodermal matrix during motor axon targeting—and lay the groundwork for future investigations into \textit{Mmp2-frac} pathway function.
Figure 3.1
Figure 3.1: *frac* gene structure and allele generation

(A) Domain structure of Drosophila Frac and *Homo sapiens* Fibrillin-1, Fibulin 1, and Fibulin 7. Two Fibulins were included to account for the variation between family members. Domains pictured include: EGF-like repeats (green), cbEGF (orange), CCP/SUSHI (blue), hyalin (yellow pentagon), TB (gray hexagon), fibulin (gray circles). (B) Schematic of the *frac* gene region and span of *frac* LOF alleles. Exons are represented by rectangular black boxes and introns by thin lines. Position of Minos insertion is displayed. Stage 14 (C) wild-type and (D) *frac*Δ1 embryos labeled with Frac antibody. (C,D) Anterior up. Scale bar: 100 µM.
Figure 3.2
Figure 3.2: *frac* RNA and Frac protein are expressed in the mesoderm and muscle attachment sites during embryogenesis

(A,B) Stage 13 and stage 15 wild-type embryos hybridized with anti-sense *frac* RNA probe. (C) Stage 14, (D) stage 15, and (E) stage 17 wild-type embryos labeled with anti-Frac. (F,G,I,J) Wild-type embryos co-labeled with Frac in green and indicated antibodies in red. (F) At stage 15, Frac co-localizes with the muscle marker MHC (red). (G) During late stage 17, Frac protein co-localizes with Kakapo/Shortstop, marking muscle attachment sites (red). (H) Stage 16 whole-mount wild-type embryo with ventral side down. Frac is expressed in the esophageal ganglion 1, part of the stomatogastric nervous system. (I) At stage 14, Frac protein is directly adjacent to the VNC from which axons, marked with 1D4 (red), are extending (dashed white lines mark the border of the VNC). (J) Frac borders the Mmp2-expressing exit glia (arrows), a subset of glia marked by anti-Repo (red). (A-E, H) Anterior up, (F,G,I,J) anterior left and midline at the bottom. Scale bar: 100 µM.
Figure 3.3: Frac protein processing is Mmp2-dependent

(A) Western blot with Frac antibody. (Lower) shows GAPDH loading control. Full-length Frac is detected in wild-type embryo extracts at 170 and 250 kDa. Non-specific bands are indicated by brackets in the frac deficiency column. In Mmp2 GOF embryos, cleavage products are present at 17 kDa, 30 kDa, and 33 kDa (arrowheads). Stage 14 (B) wild-type and (C) tub>Mmp2 embryos labeled with Frac antibody. Anterior is up. Scale bar: 50 µM. (D) Quantification of Frac protein expression in indicated genotypes. Stage 14 embryos were dissected and scored blindly on a scale of 0-3 for strength of Frac protein expression. The number of embryos analyzed is indicated below the histogram. (*) signifies that $P<0.0001$. 

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Figure 3.4: *frac* LOF mutants display misprojections and defasciculation defects similar to *Mmp2* mutants

In each micrograph, two abdominal hemisegments of stage 17 dissected embryos stained with anti-FasII to label the ISNb (A,B,C,D) or SNa (I,J,K,L) motor projections, are shown. Below each image are schematics of the branching patterns with the motors axons in brown and the muscles represented by gray boxes. (A,E) In wild type, the ISNb defasciculates from the main branch of the ISN and then innervates four muscle clefts. (B,C,F,G) *frac*\(^{\Delta 1}\) and *frac*\(^{\Delta 2}\) homozygous mutants display frayed ISNb axons and axons that separate at incorrect sites (arrows). (I,M) In wild type, the SNa extends dorsally to lateral muscles and then bifurcates into posterior and dorsal branches. (J,K,N,O) In *frac*\(^{\Delta 1}\) and *frac*\(^{\Delta 2}\) homozygous mutants, axons make projections to aberrant muscle targets and are loosely bundled. (D,H,L,P) *Mmp2*\(^{W307*}\) mutants display both defasciculation and targeting errors. Anterior left and dorsal up in all. Scale bar: 15 µM.
Figure 3.5: Inhibition of motor axon defasciculation in embryos misexpressing Mmp2 is suppressed by overexpression of frac

Two abdominal hemisegments of stage 17 embryos labeled with anti-FasII are shown with a schematic of the phenotype shown below. (A,E,I,M) Wild-type embryos have tightly bundled axonal projections. (B,F) repo>Mmp2 embryos exhibit ISNb axons that are hyperfasciculated and fail to reach their targets. (C,G) In 24B>frac embryos, the ISNb displays extra projections and misrouted axons (arrows). (D,H) Embryos misexpressing Mmp2 and overexpressing frac display normal ISNb innervations with some defasciculation of individual axons. (I,M) The SNa in wild-type embryos divides into stereotyped posterior and dorsal branches. (J,N) SNa axons fail to extend to their final targets in Mmp2 misexpression embryos. (K,O) The SNa nerve branch in 24B>frac embryos displays premature and extraneous branching. (L,P) In repo,24B>Mmp2,frac embryos the SNa innervates its proper muscle targets. Anterior left and dorsal up in all. Scale bar: 15 µM.
Figure 3.6
Figure 3.6: Frac signals downstream through a Mad-independent mechanism

(A-C) Two abdominal hemisegments of stage 17 embryos stained with anti-FasII to mark motor axon projections. (A,D) fracΔ1 mutant embryos display hypofasciculation and misprojection errors. (B,E) In embryos expressing constitutively-active Sax the ISNb innervates its proper targets. (C,F) Homozygous fracΔ1 mutant embryos expressing elav>Saxact reach their targets with some defasciculation errors. Stage 16 (G) wild type, (H) fracΔ1 homozygous mutant, and (I) 24B>frac embryonic nerve cords labeled with pMad antibody. (A-F) Anterior left and dorsal up. Scale bar: 15 µM. (G-I) Anterior up. Scale bar: 20 µM.
Figure 3.7
Figure 3.7: Model of the interaction between Mmp2 and Frac during axon guidance

(A) In wild type, both full-length Frac and Frac protein fragments bind a “factor X.” Cleaved Frac fragments form a signaling complex with available factor X and signal axons to stay bundled. (B) In Mmp2 LOF mutants there is no protease to generate Frac fragments and therefore a signaling complex cannot form, resulting in increased defasciculation. (C) In frac LOF mutants there is no full-length or cleaved Frac present to bind growth factor, preventing downstream signaling from occurring and resulting in increased defasciculation.
Figure 3.8
Figure 3.8: The phenotypes displayed in *frac* LOF mutants are not a result of structural defects indicating that Frac functions as a guidance molecule

(A,B,C) Lateral view of stage 16 embryos marked with anti-FasII, labeling all motor axon projections, and anti-GFP. In addition to the marked genotypes, embryos carry *viking::GFP* to visualize the cadherin-positive basement membrane. (A) Wild-type, (B) *frac*\(^{Δ1}\) homozygous mutant, and (C) *frac*\(^{Δ2}\) homozygous mutant embryos all have Collagen IV (green) expression at the basement membrane surrounding the ventral nerve cord (red) and midgut.

(D,E,F) MHC antibody was used to visualize the segmentally repeated pattern of muscles in each embryo. In (D) wild-type embryos, muscles are properly formed and attached to the epidermis at muscle attachment sites (arrow). (E) *frac*\(^{Δ1}\) and (F) *frac*\(^{Δ2}\) homozygous mutant embryos both exhibit normal muscle formation and attachment of muscles at their muscle attachment sites (arrows). (G-J) Each micrograph displays two hemisegments of a stage 17 embryo labeled with anti-FasII, labeling motor axons. (G,I) Homozygous *Sema1a\(^P1\)* LOF mutant embryos display axons that remain fasciculated and do not innervate their target muscles. (H,J) In *+/Sema1a\(^P1\); frac*\(^{Δ1}\)/*frac*\(^{Δ1}\) embryos the ISNb correctly innervates its target muscles. Anterior left and dorsal up in all. (A-C) Scale bar: 100 µM. (D-F) Scale bar: 50 µM. (G-J) Scale bar: 15 µM.
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^a^ the number of hemisegments scored for each genotype

^b^ hemisegments may have defects in both misprojection and hypofascitulation

^c^ data reported in Miller et al., 2008
CHAPTER 4: Discussion

Summary

During development, axons must navigate from the CNS into the periphery, finding their proper targets so that the nervous system can be correctly established. Many of the molecules involved in this process have already been elucidated through the utilization of a variety of model systems (reviewed in Tessier-Lavigne and Goodman, 1996; Dickson, 2002). However, several questions still remain concerning the exact mechanism each axon uses to arrive at its stereotyped destination. Specifically, how do axons separate from each other and follow their own correct path at each choice point if the guidance molecules already known are expressed similarly throughout the pathway? It seems reasonable to hypothesize that additional players are involved in this system or higher orders of regulation that control the spatiotemporal signaling profile of guidance cues or growth factors may be responsible for specificity in axon pathfinding.

Previous reports have already implicated a variety of metalloproteinases in axon guidance decisions. While many of these instances focus on the ADAM subfamily (Hattori et al., 2000; Schimmelpfeng et al., 2001), there is also evidence indicating the MMPs may also function to regulate specific guidance decisions (Hehr et al., 2005). Given that MMPs play such a profound role in pathological conditions like cancer, it is essential that we determine the normal physiological functions of these proteases so that future therapeutic options can
target the detrimental effects of MMPs rather than those of benefit (Fingleton, 2003; Agrawal et al., 2007; Fingleton, 2007).

Here, Drosophila *Mmp1* and *Mmp2* were studied for their roles in axon guidance. Using LOF and GOF analysis it was determined that both MMPs promote fasciculation of axons during axon outgrowth, with *Mmp2* playing the more significant role. Importantly, the catalytic activity of these proteases is essential for their function in guidance, indicating that they enact their effects on fasciculation through one or more substrate molecules. The *Mmp2* substrate *Frac* was identified through a yeast interaction screen and confirmed through biochemical analysis and *in situ* changes in Frac protein expression in embryos with varying levels of *Mmp2*. As *frac* LOF and GOF phenotypes also exhibit axon pathfinding errors and genetically interact with other known guidance molecules, it seems likely that Frac is a bona fide *Mmp2* substrate acting in guidance during Drosophila embryogenesis. The combined actions of a membrane-tethered protease and an ECM molecule functioning together in the glia and mesoderm, respectively, should yield insight into how individual axons are imparted the specificity to make spatially-regulated axon guidance decisions.

**A model for the regulation of Frac by Mmp2 in axon targeting**

*Mmp2* promotes fasciculation, as *Mmp2* mutants exhibit axons with increased fraying and ectopic projections. A similar phenotype is displayed in both *frac* GOF and LOF embryos. While this *frac* LOF phenotype would indicate that Frac functions in the same direction as *Mmp2*, (Fig. 1.2) the *frac* GOF phenotype
would then be expected to present an increased bundling phenotype if Frac is cleaved by Mmp2 and signals directly to promote fasciculation. However, this is not the case and therefore, Mmp2 and Frac must function in a different manner.

Besides direct signaling, which is not consistent with the frac GOF phenotype, the most straightforward model to describe the regulation of Frac by Mmp2 seems to involve downstream signaling by a Frac-regulated guidance cue. If cleaved Frac fragments and the cue function as a complex to signal, then in Mmp2 GOF embryos, the increase in Frac fragments available to form the complex is sufficient to lead to a bundling phenotype, indicating the cue is not limiting. If this is the case, then in the frac GOF phenotype it would seem that Mmp2 is the ultimate bottleneck in that faster processing of full-length Frac would result in normal signaling. However, it is likely that in frac GOF embryos the cue is also being sequestered by the increase of full-length Frac, thus diluting its availability and decreasing the complex formation.

If indeed this is the mechanism through which Mmp2 and Frac promote fasciculation, it would be consistent with other modes of regulation displayed by ECM molecules previously discussed (Chapter 1 and references therein). Of particular note is the neuronal to OLP fate transition controlled by the shift in Shh concentration. The HSPG controlling Shh concentration is itself regulated by Sulfatase 1, which can modify sulfur residues on HSPGs directly affecting its sequestration of Shh (Danesin et al., 2006). A similar multilevel program could be envisioned for axon pathfinding with involvement of molecules from all tissues.
required for proper guidance. The expression of Mmp2 from exit glia would ultimately regulate the balance of full-length versus cleaved fragments of Frac in the mesoderm. These changes would affect the spatiotemporal concentration of any growth factors dependent on Frac as a structural regulator thus influencing downstream signaling to receptors expressed on axons.

Although this model is in accordance with all our phenotypic analysis as well as biochemical data, it is difficult to imagine Mmp2 impeding downstream complex formation simply because it cannot function quickly enough in the frac GOF embryos. MMPs normally seem to have the inherent characteristic of being very active to the point of functioning indiscriminately – hence their involvement in so many pathological disorders and the need for endogenous inhibitors to ensure their tight regulation. However, in this case, it is possible that growth factor binding functions to slow down complex formation in two ways. The first, already mentioned, is that less of the growth factor or cue could be available to form the signaling complex if more is bound to full-length Frac. Additionally, it is possible that when the growth factor binds Frac it obstructs access to the Mmp2 cleavage site. Thus, even when more full-length Frac is available for Mmp2 to cleave, it is not actually accessible. Unfortunately, this explanation alone does not adequately account for the defasciculation phenotype, but a combination of reduced growth factor availability and impeded cleavage of Frac may retard complex formation enough to significantly reduce the signal to “stay bundled.”
Alternatively, Frac could be functioning through a different mechanism than the model proposed (Fig. 3.7). While I have found that Frac genetically interacts with members of the BMP family (Fig. 3.6), and it is possible that Factor “X” is a BMP, these molecules have generally not been linked to axon guidance. Instead of functioning exclusively through one molecule such as a BMP, it seems likely that Frac may modulate a number of different growth factors and cues. This type of regulation would be congruent with the metaphorical “chords” described by Hynes (2009). In this case, it could be imagined that an increase in Mmp2, and thus more cleavage of Frac, would change the active state or concentrations of several molecules – overall culminating in an increase in bundling of axons. However, an increase in full-length Frac could affect the balance in a different way and due to the variety of molecules affected the final result could be an increase in defasciculation. Finally, as Frac is a large ECM molecule potentially functioning in structural support of muscle cells, overexpression of this molecule throughout the mesoderm may simply interfere with normal cytoskeletal dynamics. Closer examination of the ECM using electron microscopy would yield insight into the effect of Frac overexpression.

**Developmental functions of MMPs**

Both *Mmp1* and *Mmp2* mutants survive embryogenesis but die later during periods of exceptional growth, arguing that MMPs function predominantly in remodeling rather than development (Page-McCaw et al., 2003). This is further established by numerous studies in both mammals and flies. For example, Mmp1
and Mmp2 are involved in the tissue remodeling that occurs in the development of the imaginal tissue that becomes the wing. In vivo monitoring of the basement membrane during this process revealed that MMPs are necessary for tissue invasion during disc eversion and tumor progression. MMPs are required to degrade the basement membrane which appears to be the first step in either of these cases (Srivastava et al., 2007). Instances such as these that contribute to our understanding of how MMPs function and promote metastasis are perfectly valid, but unfortunately feed the notion that MMPs simply degrade everything in their path and do not contribute to specific signaling events. Such a narrow scope makes the Drosophila MMPs role in axon guidance more interesting.

As mentioned previously, other metalloproteinases have been found to contribute to guidance decisions, indicating that MMPs can be involved in specific signaling events as well as local instructive degradation of the ECM. Another elegant example of the developmental role of MMPs in vertebrates is the involvement of MMP-9 in synaptic plasticity. The Huntley lab has recently provided evidence indicating that MMP-9 is necessary and sufficient for both spine expansion and synaptic potentiation in the hippocampus of rats (Bozdagi et al., 2007; Wang et al., 2008b). Induction of LTP leads to increased local MMP-9 proteolysis linked to actin remodeling and protein synthesis. The authors hypothesize that proteolysis likely reveals latent recognition sequences that activate downstream integrin-mediated signaling (Wang et al., 2008b). These data indicate that MMPs function normally during developmental paradigms to contribute to major events such as learning and memory.
MMPs are also known to degrade ECM components with the result of releasing growth factors such as FGF and TGFβ (McCawley and Matrisian, 2001). For example, MMP-1 and -3 can degrade the proteoglycan perlecan in vitro, thus releasing tightly bound FGF (Whitelock et al., 1996). Similarly, the ECM molecule decorin functions as a reservoir for active TGFβ which can be released by MMP-2, -3 and -7 (Imai et al., 1997). Although both of these examples are based on in vitro evidence, they demonstrate the capacity for MMPs to function as part of a signaling system that utilizes multiple levels of regulation in order to control the concentration of growth factors.

Despite the negative publicity that MMPs have garnered, ever increasing reports have demonstrated that these proteases are essential for a number of normal physiological functions. Not only can membrane-bound MMPs function in a local environment rendering spatial specificity (Wang et al., 2010; Yasunaga et al., 2010), they can also remodel the ECM in preparation for new organs and systems (Page-McCaw et al., 2003; Srivastava et al., 2007). Additionally, precedence for degradation of ECM molecules by MMPs has already been established based on in vitro models of perlecan and decorin (Whitelock et al., 1996; Imai et al., 1997). Therefore, it should be unsurprising that Mmp2 can function locally to regulate an ECM molecule such as Frac to affect downstream signaling in guidance rather than acting indiscriminately to degrade all components of the ECM.
Frac function in the ECM

As noted previously, ECM molecules vary from being highly conserved to completely novel between branches of the evolutionary tree (Hynes, 2009). For example, collagens and laminins are present in both vertebrates and metazoa, although their families have been largely expanded in vertebrates. Other proteins are completely novel such as fibronectin, thrombospondin, and fibrinogen (Goldstein and Gunawardena, 2000; Hynes and Zhao, 2000). In this thesis, I have described how the ECM molecule Frac is regulated by Mmp2 and modulates growth factor signaling. However, Frac does not have a clear vertebrate homolog, but it appears to clearly share some of the distinct domains that normally characterize vertebrate ECM proteins (Hynes, 2009).

According to domain structure and sequence alignments, Drosophila Frac is most similar to vertebrate fibulins and fibrillins, but when the Drosophila genome was first sequenced and reported, analysis against vertebrate adhesion proteins revealed no metazoan homologs for these two families of ECM molecules (Goldstein and Gunawardena, 2000; Hynes and Zhao, 2000). Although Drosophila proteins, including Frac, were identified that contained the repeating cbEGF domains characteristic of fibrillins, there were no strong candidates that also contained the TB domains. Hynes and Zhao did however categorize Frac as “fibrillin-like” (2000). Similarly, a BLAST search of the Drosophila genome using vertebrate Fibrillin-2 identifies dumpy and notch as the most closely related fly genes before Frac. Notch has been shown to be homologous to a protein of the
same name in vertebrates (Weinmaster et al., 1991), while dumpy is also a structural protein that has been compared to vertebrate fibrillins (Wilkin et al., 2000). The homology that is present between the fibrillins and Frac most likely arises from the cbEGF domains that are responsible for organizing fibrillin into rigid linear structures to form microfibrils by binding calcium (Handford et al., 1995). The TB domains, on the other hand, seem to function as specific binding sites for growth factors, such as the pro-peptide containing TGFβ (Handford, 2000; Hubmacher et al., 2006). Additionally, Frac is about half the size of vertebrate fibrillins and aligns to two parts of each vertebrate fibrillin. It is conceivable that a smaller ancestral homolog may have had strictly structural functions utilizing the cbEGF repeats while additional binding domains, such as the TB domains in vertebrates and the CCP and hyalin domains in Drosophila, evolved divergently to function in growth factor regulation. Whether or not Frac and fibrillins are truly homologous is less important than the fact that their similarities argue that Frac is truly an ECM molecule.

The fibulin family is composed of seven members divided into two classes with widely varying size and domain structure. These ECM proteins also aid in elastic fiber assembly, and do not have direct homologs in Drosophila (Hynes and Zhao, 2000). All are composed of cbEGF repeats as well as a unique C-terminal fibulin module (Yanagisawa et al., 2009). Class I fibulins are longer than Class II fibulins and contain additional N-terminal domains. Fibulin-7, a Class II fibulin, is the newest family member and is the only fibulin to contain a SUSHI/CCP domain such as is found at the C-terminus of Drosophila Frac. This type of domain is
common to complement proteins and often mediates protein-protein interactions (Yanagisawa et al., 2009). Obviously fibulin domain structure varies widely and Frac aligns more closely to some of these family members than to others. However, the similarities between Frac and fibulin domains indicate that these ECM proteins could function comparably or share a common ancient connection.

ECM molecules found in metazoan and vertebrates may also diverge due to functional differences as vertebrate proteins are responsible for providing support between connective tissues while similar proteins in Drosophila would function to attach muscle cells to the chitin exoskeleton (Goldstein and Gunawardena, 2000; Hynes and Zhao, 2000). In support of this hypothesis, muscle attachment sites, at which some of these connections occur, are enriched for a number of characterized structural molecules (Fogerty et al., 1994; Gregory and Brown, 1998; Subramanian et al., 2003). Frac is also present at these sites at the end of embryogenesis (Fig. 3.2). While Frac cannot be directly aligned to any known vertebrate ECM proteins, it is apparent that it contains the proper domains and characteristics that define ECM proteins and would likely function as a structural support and a regulator of growth factors as do other proteins of similar nature (Hynes, 2009).

The identification of Frac as an ECM protein initially created apprehension that the guidance defects displayed in frac LOF embryos were simply secondary effects of the loss of an important structural molecule in the muscle. To refute this notion, the myosin heavy chain antibody was used to view muscle architecture
and a *viking::GFP* transgene allowed visualization of the basement membrane in *frac* mutants (Yasothornsrikul et al., 1997). While muscles in *frac* mutant embryos are slightly thinner than in wild type embryos, all muscles are present and properly attached to muscle attachment sites and the basement membrane surrounds proper embryonic tissues (Fig. 3.8). However, by the third instar larval stage phalloidin and bright field microscopy reveal that some muscles have detached from attachment sites (data not shown). Specifically, the VLM’s have disconnected, although more lateral muscles, such as muscle 4, appear normal. Nevertheless, the embryonic axon guidance events we are interested in have already taken place before detachment of these muscles occurs. It may be that Frac is only needed to maintain muscle integrity in larval stages after it has localized to muscle attachment sites during late embryonic stage 17, or that the vast increase in muscle size during larval development overloads already weak attachment sites. In either case, *frac* mutants have normal muscles during axon pathfinding in embryos indicating Frac plays a direct role in pathfinding.

**Non-canonical BMP pathway in axon guidance**

The vertebrate TGFβ/BMP family is represented by three distinct subfamilies in *Drosophila* including activins, prototypical TGFβs, and Bone Morphogenetic Proteins (BMPs). These molecules play a wide variety of roles in the nervous system throughout development including neural patterning, cell fate, and synaptogenesis (reviewed in Moustakas and Heldin, 2009; Wharton and Derynck, 2009). Yet the activin-like molecule, Dawdle, functioning through
Baboon and Smad2 is the only family member that has been reported to participate in axon guidance (Parker et al., 2006; Serpe and O’Connor, 2006). Sax is not known to function in guidance, nor did I see any changes in axon pathfinding in elav>\textit{sax}$^{\text{act}}$ embryos, but overexpression of constitutively-active receptor alone may not outcompete wild-type receptor to yield defects. In addition, while zygotic sax mutants live to larval stages, maternal clones have not been analyzed (Twombly et al., 2009). Similarly, mutants for \textit{thick veins}, \textit{punt}, and \textit{wishful thinking}, other receptors in this pathway, also lack evidence for a role in guidance, but maternal clones have not been thoroughly examined despite significant known maternal contributions (Dorfman and Shilo, 2001; Marques et al., 2002; McCabe et al., 2004). Therefore, further evidence is needed to support the finding that sax$^{\text{act}}$ partially suppresses the guidance defects in frac LOF mutants including careful study of maternal clones of the different TGFβ/BMP family members. It seems likely that more than one of these signaling molecules may play a role or that other growth factors and guidance cues are involved. As discussed previously, ECM proteins like Frac may modulate the distribution and signaling capacity of several molecules simultaneously and may even function in concert with other matrix proteins.

However, if TGFβ/BMP molecules are involved in guidance, they may not be signaling through the “normal” pathway. Canonical TGFβ signaling occurs through ligand-specific type-I and type-II receptors that form heteromeric complexes of serine/threonine kinases. Ligand binding activates a receptor-specific downstream effector Smad, which then transduces to the nucleus to
regulate gene expression (Wharton and Derynck, 2009). However, the finding that sax\textsuperscript{act} genetically interacts with frac is not consistent with this pathway as changes in Frac expression do not affect phospho-Mad levels in the CNS, indicating that Frac cannot be utilizing the canonical Smad pathway. Instead, it may be that Frac is acting in a Smad-independent pathway.

Recent evidence has confirmed that TGFβ/BMPs do signal by way of Smad-independent pathways such as LIM kinase 1, thus affecting actin dynamics through Rho GTPases (Lee-Hoeflich et al., 2004; Wen et al., 2007; Ng, 2008). In Drosophila, work in the mushroom body neurons has indicated that this pathway can function through the receptors Baboon and Wishful Thinking to regulate axon guidance (Ng, 2008). In motor axons, LIMK1 is required for synaptic stability during larval stages through binding to Wishful Thinking (Eaton and Davis, 2005); however, a role in guidance has not yet been examined. While a connection between LIMK1 and Sax, the receptor that genetically interacts with frac, has not been reported, future work could focus on whether Frac downstream signaling could be mediated by LIMK1. This would be an attractive fit in the context of axon guidance where pathfinding at the final level must direct cytoskeletal reorganization.

**Conclusions**

In this thesis, I have demonstrated that the proteases Mmp1 and Mmp2 are involved in axon guidance during embryogenesis in *Drosophila melanogaster*. Specifically, both MMPs are required to promote fasciculation of multiple nerves.
as a loss of either \textit{Mmp1} or \textit{Mmp2} results in defasciculated axon branching and misexpression of either MMP yields axons that are more bundled than in wild-type embryos. The catalytic activity is also necessary for this role in guidance demonstrated by misexpression of the endogenous inhibitor of MMPs, Timp, or by catalytically-inactive \textit{Mmp1} or \textit{Mmp2}. While it was originally envisioned that the MMPs could be affecting guidance by cleaving a known guidance molecule, a yeast interaction screen revealed the ECM molecule Frac as a potential substrate for Mmp2. Protein and RNA expression patterns of Frac confirmed that its presence in the mesoderm could not only be regulated by Mmp2-expressing exit glia at the CNS/PNS transition zone, but that it was ideally situated to play a role in axon guidance. Subsequent biochemical analysis demonstrated that indeed Frac expression was dependent on Mmp2 levels. Assessment of axon guidance in both \textit{frac} LOF and GOF embryos established a role for Frac in pathfinding as axons in these embryos displayed defasciculation and misprojection defects. Importantly, genetic interactions between \textit{frac} and \textit{Sema1a} demonstrate that Frac affects the balance of attraction and repulsion, just as Mmp2 can, and indicates that guidance errors are not simply a consequence of morphological defects. Finally, as a means of identifying potential Frac-regulated growth factors, a constitutively-active Sax transgene was able to partially suppress the defasciculation phenotype in \textit{frac} LOF mutants. However, this prospective downstream pathway would need to utilize a non-canonical signaling mechanism as the normal effector of this system, Mad, is not affected in \textit{frac} LOF and GOF embryos.
The evidence presented here suggests that Mmp2 regulates the amount of cleaved and full-length Frac protein. Frac, as an ECM molecule with multiple domains that could function as binding sites, may act as a modulator of growth factor signaling. Based on our data, I propose that Frac functions both as a sink, in its full-length form, and as a co-factor that properly positions growth factor as an Mmp2-cleaved fragment. The growth factor is likely to be a member of the TGFβ/BMP family as supported by the genetic interaction data provided here; however, it is possible that multiple growth factors or guidance cues may be regulated by Frac or other ECM molecules.

Future work should focus on providing additional support for the downstream proteins involved in the Mmp2-Frac signaling pathway. The analysis of maternal clones from the genes in the TGFβ/BMP family could provide insight into whether these molecules play roles in guidance in addition to their essential roles in earlier developmental paradigms. Additionally, genetic interactions between Frac and the LIMK1 pathway could uncover insights into non-canonical Smad signaling in motor axon guidance. Finally, involvement of the ECM molecule Frac in axon guidance raises the question of whether other ECM molecules also regulate cues that affect pathfinding decisions, and if so, whether a complex interaction of multiple ECM molecules and guidance cues impart the spatial instructions necessary to direct axons to their specific muscle targets.

Understanding the complexity involved in a relatively simple part of development such as axon guidance in Drosophila provides a basis for grasping the biological
mechanisms that underlie more complicated processes. First, this work
demonstrates that post-translational modifications coupled to regulation by ECM
molecules may be a prevalent method to control growth factor distribution and
activation in various signaling paradigms. Second, the finding that both Mmp1
and Mmp2 contribute to axon pathfinding advances our understanding of MMPs
and their roles in nonpathological conditions. These discoveries are essential in
order that future therapies targeting MMPs can avoid affecting these functions.
And finally, the elucidation of new molecules involved in axon guidance moves
the field ever closer to developing therapies for individuals with injuries to the
nervous system.
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