SEMAPHORIN 5B FUNCTIONS AS A DIFFUSIBLE GUIDANCE CUE TO REGULATE SENSORY AXON PATHFINDING DURING DEVELOPMENT

by

RACHEL QIAN LIU

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Abstract

The centrally projecting sensory axons of the dorsal root ganglia follow a well established pattern that is conserved across many species and offers a robust model for the study of axonal guidance. When primary sensory axons leave the sensory ganglia and project to the embryonic spinal cord, they do not immediately extend into the spinal cord dorsal horn, but bifurcate and travel long the rostrocaudal axis of the animal to form the dorsal funiculus and Lissaur's tract. At a later stage they extend collateral fibres that enter the dorsal horn and target to specific laminae according to their sensory modality. The factors that prevent immediate entry into the dorsal horn or regulate the timing of sensory collateral formation and specificity of lamina innervation have not been clearly identified. Our lab previously showed that Semaphorin5B (Sema5B), a member of the semaphorin family of guidance molecules, is dynamically expressed in the embryonic spinal cord and correlates with these sensory axon targeting events. Using *in vitro* assays, I show systematically that Sema5B inhibits growth of both nerve growth factor-responsive and neurotrophin-3-responsive dorsal root ganglion neurites and that this inhibitory effect on the former is mediated in part through the cell adhesion molecule TAG-1. Using the technique of RNA interference, I show *in vivo* that a reduction-of-function of Sema5B in the spinal cord leads to cutaneous axons not only projecting prematurely into, but to erroneous targets within the dorsal horn of the spinal cord, while proprioceptive axons continued to pathfind correctly. Together, these results suggest that Sema5B acts as a repulsive barrier for centrally projecting primary sensory axons that first reach the spinal cord, and once collaterals form, Sema5B exerts a differential function on different types of sensory fibres to regulate their pathfinding. This is the first study to identify the specific cue that regulates sensory neuron entry and guidance into the spinal cord dorsal horn grey matter.

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Chapter 1: Introduction

Axon Guidance

During the development of the nervous system, billions of neurons send out axons to their appropriate targets in order to form a framework of connections from which functional circuits can be established. The process of pathfinding by an individual axon is accomplished by a highly dynamic, fan-shaped, actin-rich structure located at its tip, the growth cone. First described by Ramon y Cajal over a century ago, this structure has been extensively studied for the role it plays in axon guidance. It is widely accepted that growth cones respond to extrinsic cues from the surrounding tissue and actively steer the growing axon in the correct direction in response to those cues. A wealth of knowledge exists describing the guidance cues that instruct growth cones to undergo classic behaviours such as turning, fasciculation, collapse, retraction, and stalling (Suter and Forscher, 2000). These cues can be attractive or repulsive, and can act at short or long ranges. Short range cues are often membrane associated molecules anchored to stationary cells or tethered to a particular substrate and mediate contact attraction or repulsion. Meanwhile, long-range cues are often secreted molecules that establish concentration gradients and function in chemoattraction or chemorepulsion from a distance. Often, the expression of one guidance molecule can mediate different effects on different growth cones. This phenomenon has been shown to depend on the types of receptor molecules present on the growth cone and the downstream effecter pathways (Grunwald and Klein, 2002; Huber et al., 2003; Meyer and Feldman, 2002). Furthermore, it is common for a growth cone to respond sequentially to different guidance cues along the way to its target, thus the growth cone must have the ability to integrate

incoming signals, terminate its responsiveness to certain cues and begin responding to others so as to make the correct choices in direction (Kolodkin and Tessier-Lavigne, 2011).

In general, the downstream signalling of guidance cues converge on to the action of the Rho family of small GTPase proteins including Rho, Rac, and Cdc42 (Huber et al., 2003; Kolodkin and Tessier-Lavigne, 2011). Rho GTPases in turn govern the dynamic rearrangement of the cytoskeletal network of actin filaments and microtubules and ultimately orchestrate the movement of growth cones (Gallo and Letourneau, 2004; Kapfhammer and Raper, 1987; Kolodkin and Tessier-Lavigne, 2011). Rho-GTPases can be transformed into the active GTP-bound state and back to the inactive GDP-bound state by GTPase-activating proteins (GAPs) and guanine-nucleotide-exchange factors (GEFs), respectively (Kolodkin and Tessier-Lavigne, 2011). In general, the activation of Rac1 and Cdc42, often via the PI3-kinase and protein kinase A (PKA) pathway, and the inhibition of Rho A results from the signalling of attractive cues ((Kolodkin and Tessier-Lavigne, 2011; Meyer and Feldman, 2002). This leads to stabilization and extension of actin filaments (Kolodkin and Tessier-Lavigne, 2011). Inhibitory cues often confer the opposite changes to Rho-GTPases and result in the destabilization of actin filaments and collapse of the growth cone (Kolodkin and Tessier-Lavigne, 2011; Zhang et al., 2003).

Guidance Cues

Much work in the past decades has been focused on the identification of the molecules that guide neuronal processes to their targets, as well as the molecular signalling events underlying these events. A broad spectrum of molecules have been shown to be involved in neuronal guidance, among which four classic families of guidance cues, known as the canonical cues, have been best characterized: the netrins, slits, ephrins, and

semaphorins (Grunwald and Klein, 2002; Huber et al., 2003; Kolodkin and Tessier-Lavigne, 2011; Tran et al., 2007; Yu and Bargmann, 2001).

Briefly, netrins are a small family of glycoproteins characterized by an amino-terminal globular domain and three epidermal growth factor (EGF)-like repeats in the C terminus (Kolodkin and Tessier-Lavigne, 2011). Netrin was first identified in *C. elegans* and was shown to mediate its guidance effects on axons that extend to the worm ventral midline (Hedgecock et al., 1990). In vertebrates, netrins were found in the ventral midline floor plate cells and described for their chemoattractive activity for spinal commissural axons (Fazeli et al., 1997; Kennedy et al., 1994; Serafini et al., 1994; Tessier-Lavigne et al., 1988). Shortly after, netrins were found to be bifunctional in that they can confer different effects on different types of axons (Colamarino and Tessier-Lavigne, 1995). Attractive functions of netrins are mediated by the DCC receptor family (Deleted in Colorectal Carcinomas) while their repulsive effects are mediated by members of the UNC5 family (Chan et al., 1996; Keino-Masu et al., 1996; Kolodkin and Tessier-Lavigne, 2011; Kolodziej et al., 1996; Leonardo et al., 1997; Leung-Hagesteijn et al., 1992). Slits act through the Robo family of receptor molecules to mediate spinal commissural axon guidance (Ma and Tessier-Lavigne, 2007; Whitford et al., 2002). The signals conferred are inhibitory and axons that are attracted to the floor plate by netrins are then repelled in part by slits after crossing the midline (Kolodkin and Tessier-Lavigne, 2011; Long et al., 2004; Mambetisaeva et al., 2005; Zou et al., 2000). There are two subfamilies of the cell surface associated Ephrins (Kolodkin and Tessier-Lavigne, 2011). Class A ephrins are associated with the membrane by glycoprotein (GP) linkages while class B ephrins contain a transmembrane domain (Klein, 2004; Kolodkin and Tessier-Lavigne, 2011). The ephrin receptors are tyrosine kinases of the Eph

family (Kolodkin and Tessier-Lavigne, 2011). Ephrin/Eph interactions are extremely versatile. They can be attractive or repulsive, and can function through reverse signalling when ephrins act as the receptor molecules (Kolodkin and Tessier-Lavigne, 2011).

Other classes of molecules now recognized to function in neuronal guidance have been primarily known to function in different contexts. For example, immunoglobulin (Ig)-containing cell-adhesion molecules (CAMs), cadherin family members and canonical morphogens such as bone morphogenic protein (BMP) and sonic hedgehog (Shh) have received increased attention for their roles as guidance cues (Augsburger et al., 1999; Charron et al., 2003; Dickson, 2002; Kolodkin and Tessier-Lavigne, 2011; Okada et al., 2006; Serafini et al., 1996; Tessier-Lavigne and Goodman, 1996). Conversely, classic guidance cues have been increasingly recognized for their role as morphogens as a number have been shown to have the ability to pattern developing tissue (Hinck, 2004).

Semaphorin Guidance Cues

Semaphorins make up the largest and most diverse family of guidance cues (Figure 1-1, (Yazdani and Terman, 2006a)). There are eight classes of semaphorins, totalling more than 30 different members that are all conserved structurally as well as functionally across divergent animal phyla including invertebrates, vertebrates, and viruses (Yazdani and Terman, 2006a; Yazdani and Terman, 2006b). Secreted, transmembrane and glycosylphosphatidylinositol (GPI)-linked semaphorins have been identified, and are all characterized by a conserved N terminus extracellular domain of about 500 amino acids named the semaphorin (sema) domain (Yazdani and Terman, 2006a). The sema domain plays a critical role in mediating the effects of semaphorins (Behar et al., 1999; Eickholt et al., 1997; Koppel et al., 1997; Oster et al., 2003). Next to the sema domain on the C terminus

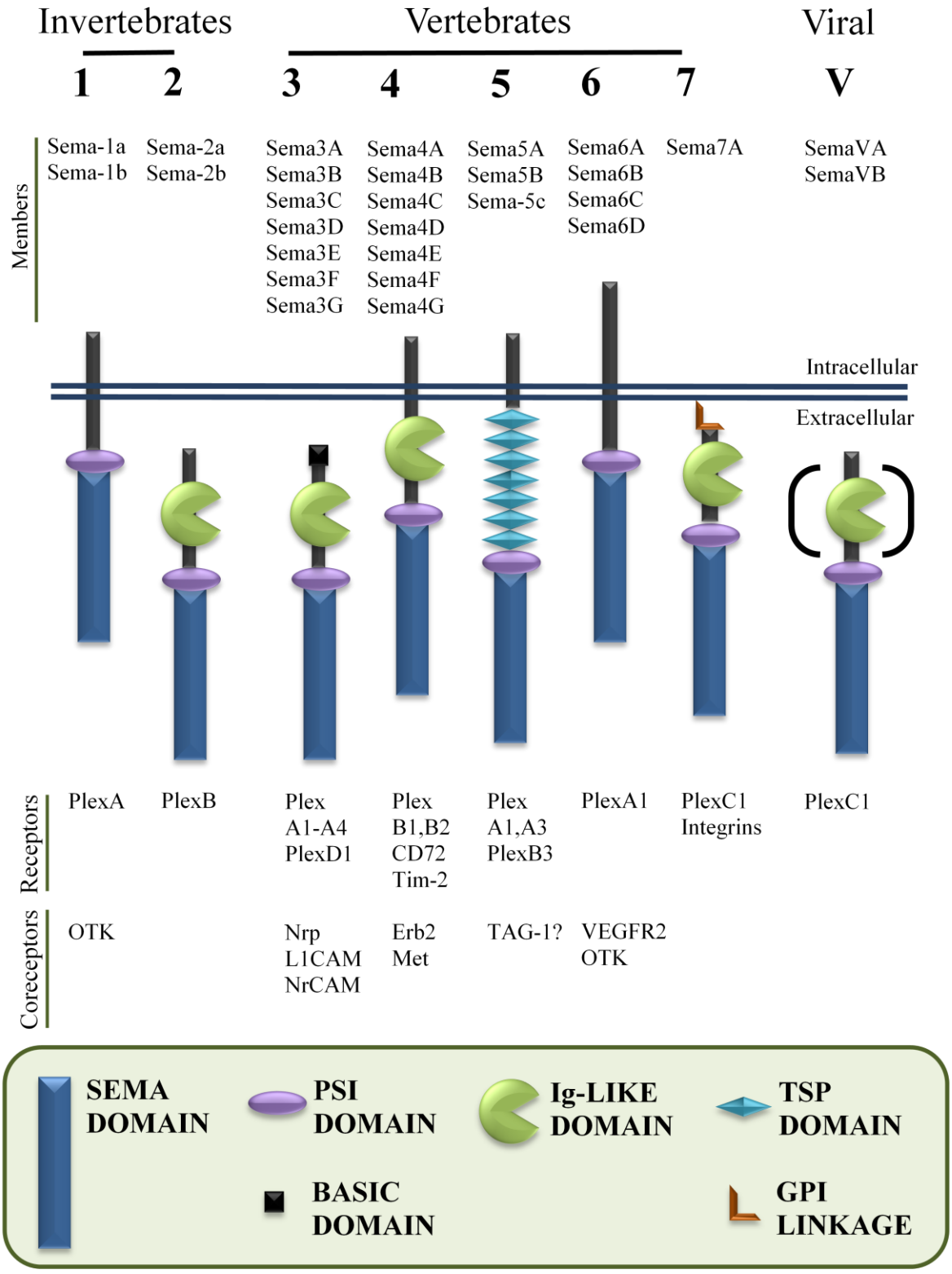

Invertebrates
Vertebrates
Viral
1
2
3
4
5
6
7
V
Members
Sema-1a
Sema-1b
Sema-2a
Sema-2b
Sema3A
Sema3B
Sema3C
Sema3D
Sema3E
Sema3F
Sema3G
Sema4A
Sema4B
Sema4C
Sema4D
Sema4E
Sema4F
Sema4G
Sema5A
Sema5B
Sema-5c
Sema6A
Sema6B
Sema6C
Sema6D
Sema7A
SemaVA
SemaVB
Intracellular
Extracellular
Receptors
PlexA
PlexB
Plex A1-A4 PlexD1
Plex B1,B2 CD72 Tim-2
Plex A1,A3 PlexB3
PlexA1
PlexC1 Integrins
PlexC1
Coreceptors
OTK
Nrp L1CAM NrCAM
Erb2 Met
TAG-1?
VEGFR2 OTK
SEMA DOMAIN
PSI DOMAIN
Ig-LIKE DOMAIN
TSP DOMAIN
BASIC DOMAIN
GPI LINKAGE

Figure 1- 1

Figure 1- 1

Schematic representation of primary structures of the Semaphorin family of guidance cues. Class 1 and 2 semaphorins and a member of the class 5 semaphorin, Sema5c, are found in invertebrates, class 3-7 semaphorins are found in vertebrates, and class V semaphorins have been identified in viruses. There are both secreted and membrane-associated semaphorins. Class 2, 3 and V semaphorins are secreted, class 1, 4-6 semaphorins contain a transmembrane region, and class 7 semaphorins are attached to the membrane via a glycosylphosphatidylinositol (GPI) linkage. All semaphorins contain a conserved 500 amino acid domain known as the sema domain near the N terminus of the protein. Class 5 semaphorins contain 7 type-1 thrombospondin repeats (TSP) near the C terminus. Class 2, 3 ,4 and 6 proteins have an immunoglobulin (Ig) like domain near the C terminus. SemaVA proteins also contain an Ig domain while SemaVB proteins do not. Known receptors and co-receptors are listed for each class of protein. Class 1 and 2 semaphorins function through Plexin-A (PlexA) and Plexin-B (PlexB) receptors. The Offtrack (OTK) co receptor is known to function with PlexA. Sema3s and Sema6s function through PlexA receptors and most Sema3s require neuropilins (Nrp) as obligate co-receptors. L1- and neuronal-cell adhesion molecules (L1CAM, NrCAM), vascular endothelial growth factor receptor2 (VEGF2), and OTK have been shown to to be important for PlexA function. Certain Sema4s bind to PlexBs in the nervous system and function with Met and ErbB2 as coreceptors. Some Sema4s bind directly to CD72 and Tim-2 in the immune system. Sema5A has been shown to bind to PlexB3, while Sema5A and Sema5B have been suggested to function through PlexA1 and PlexA3. The present work suggests the involvement of TAG-1 in Sema5B function. Sema7A and SemaVs both function through PlexC1.) Sema, semaphorin; PSI, plexin-semaphorin-integrin.

side, there is a stretch of cysteine-rich residues known as the plexin-semaphorin-integrin (PSI) domain which is also found in all semaphorins (Gherardi et al., 2004; Yazdani and Terman, 2006a). In contrast to these regions that define the family, the eight classes of semaphorins are distinguished from each other from the way they are associated with the cell membrane as well as specialized domains C terminal to the sema domain. Some C terminal domains include a single C2-class Ig-like domain, a sequence of basic amino acids, or seven type 1 and type-1 like thrombospondin repeats (TSRs) (Adams and Tucker, 2000; Kantor et al., 2004; Koppel et al., 1997; Yazdani and Terman, 2006a). The first semaphorin identified was the transmembrane protein semaphorin 1A (Sema1A) in grasshoppers (originally named Fasciclin IV). It was found to mediate the pathfinding of pioneer sensory axons in the embryonic limb (Kolodkin et al., 1992). Subsequent experiments showed that class 1 semaphorins show attraction to and permit the growth of axons (Cafferty et al., 2006; Godenschwege et al., 2002; Wong et al., 1999). In contrast, class 2 semaphorins have an Ig domain C terminal to the sema domain, are secreted and show strong repulsive activity in flies and grasshoppers (Bates and Whitington, 2007; Isbister et al., 1999; Zlatic et al., 2009). Whereas class 1 and 2 semaphorins are exclusively expressed in invertebrates, classes 3 to 7 semaphorins are found in vertebrates (Raper, 2000; Yazdani and Terman, 2006a). Sema3A (originally named Collapsin), a secreted protein found in chick brain membranes, was also identified as a potent growth cone collapsing factor to sensory and sympathetic neurites (Luo et al., 1993). Class 3 semaphorins, like those in class 2, all have an Ig domain C terminal to the sema domain followed by a highly basic domain (Luo et al., 1993). Class 4 semaphorins are transmembrane molecules largely associated with the immune system, especially on the surface of T lymphocytes (Bougeret et al., 1992; Kumanogoh, 2002; Yazdani and Terman,

2006a). Class 5 semaphorins are unique in that they contain seven type 1 and type-1 like TSRs on the extracellular portion of the molecule C terminal to the sema domain (Adams et al., 1996). They will be described in further detail below. Similar to class 4 semaphorins, class 6 semaphorins are also transmembrane proteins found in the immune system although they lack the Ig domain (Bron et al., 2007; Kerjan et al., 2005; Runker et al., 2008). The only member of the class 7 semaphorin identified to date is tethered to the plasma membrane via a GPI linkage and is known to be attractive (Pasterkamp et al., 2003; Suzuki et al., 2007). Finally, class V semaphorins are found exclusively in viruses (Yazdani and Terman, 2006a).

Evidence has shown that different classes of semaphorins have unique distribution patterns in different organisms and are implicated in a large number of physiological processes such as immune function, organogenesis (of lung, liver, heart, etc.), nervous system formation, oncogenesis and tumour suppression (Roth et al., 2009; Yazdani and Terman, 2006a). Expression patterns of semaphorins have been best characterized in the nervous system, and many are observed to be dynamic throughout development, after temporally associated with axonal tract formation (Fiore and Puschel, 2003; Yazdani and Terman, 2006a). Furthermore, expression levels have been described to change in the adult (increase in most cases) after an injury to the nervous tissue, as well as during tumorigenesis and other disease conditions (Yazdani and Terman, 2006a).

Based on their diverse expression patterns, it is easy to imagine that semaphorins play numerous roles during development. Past experiments using genetic analysis and functional assays show that semaphorins are involved in cellular adhesion and migration, cellular proliferation, embryonic patterning, neurite formation, and cytoskeletal rearrangement. The best described role of semaphorins is their involvement in neurite pathfinding and nervous

system development (Kruger et al., 2005). In the nervous system, semaphorins have been shown to act as repulsive molecular cues to direct growing axons to the right target and away from inappropriate ones (de Wit and Verhaagen, 2003; Kruger et al., 2005; Lett et al., 2009; Yazdani and Terman, 2006a; Zhou et al., 2008). It has been hypothesized that altered semaphorin function is linked to numerous nervous system disorders, including autism, epilepsy, retinal degeneration, Azheimer's disease, Parkinson's disease, cancer, and vascular disease (Autiero et al., 2005; Eastwood et al., 2003; Neufeld et al., 2005; Pasterkamp and Kolodkin, 2003; Sahay et al., 2005).

As mentioned above, Sema3A is recognized as the prototype semaphorin and consequently its repulsive effects have been extensively studied in a variety of systems (Antipenko et al., 2003; Castellani and Rougon, 2002; Luo et al., 1993). However, subsequent data has shown that a number of individual semaphorins can be bifunctional in that they can also exert attractive effects on neurons *in vitro* (Bagnard et al., 1998; de Castro et al., 1999). These observations were supported by experiments done by Song et al. (1998) and Polleux et al. (2000). These authors demonstrated that by increasing the cyclic nucleotide, cGMP, the repulsive activity of Sema3A on cortical neurons can be converted to attraction. This is an example of how intrinsic signalling can influence the effect of semaphorins on neuronal processes. In reality, both intrinsic and extrinsic modulatory factors can affect the attractive or repulsive activity of semaphorins (Falk et al., 2005; Law et al., 2008; Polleux et al., 2000; Song et al., 1998; Suzuki et al., 2007; Vizard et al., 2008). Molecules such as α1β1 integrin, Ig superfamily members such as L1-CAM and TAG-1, proteoglycans and receptor tyrosine kinases (RTKs) have also been shown to modulate the function of semaphorins (Barberis et al., 2004; Chauvet et al., 2007; Falk et al., 2005; Law et

al., 2008; Swiercz et al., 2008). For example, focal adhesion-associated protein kinases (FAKs) aid in integrin-induced signalling and in turn facilitate Sema3B-mediated attraction but not repulsion (Barberis et al., 2004). Furthermore, Sema5A has been shown to function either as an attractive or repulsive cue depending on whether it is bound by chondroitin sulphate proteoglycan (CSPG) or heparin sulphate proteoglycan (HSPG) , respectively (Kantor et al., 2004).

Semaphorin Receptors and Signalling

The two major families of receptors for semaphorins that have been identified are the plexins and neuropilins (Kolodkin and Tessier-Lavigne, 2011; Tran et al., 2007). Members of the Plexin family are large transmembrane proteins distantly related to semaphorins in that they also contain short sema domains that are important for binding semaphorin ligands (Tamagnone and Comoglio, 2000; Yazdani and Terman, 2006a). Neuropilins, however, are smaller in size, containing an extracellular domain, a single transmembrane domain, and a very short cytoplasmic tail (Fujisawa et al., 1997; Huber et al., 2003). Many semaphorins bind plexins directly and carry out their functions, but the secreted class 3 semaphorins must first bind to the obligate co-receptor, Neuropilin-1 or -2 (Huber et al., 2003; Kolodkin and Tessier-Lavigne, 2011). Tran et al., showed using cell culture and *in vivo* experiments that the type of class 3 semaphorin as well as the subtype of neurons determine the specific combination of Plexin and Neuropilin needed to signal the effect of the semaphorin in question (2007).

It is of interest to note the disparity between the severe axon targeting defects and lethality seen in neuropilin-1 knock-out mice (Kitsukawa et al., 1997) and the lack of

defective axon targeting in the non-lethal Sema3A knock-out mice (Catalano et al., 1998). This can be explained by the fact that each neuropilin is required to mediate the effect of more than one secreted semaphorin (Renzi et al., 1999; Takahashi et al., 1998). The redundancy of function of semaphorins and their receptors is discussion further in chapter IV.

Class 5 Semaphorins

Among the semaphorins found in vertebrates, class 5 semaphorins are unique in that the extracellular portion of these proteins contain seven type 1 and type 1-like TSRs (Adams et al., 1996; Oster et al., 2003). TSRs have been characterized for a wide range of functions. They are permissive to neurite outgrowth and promote neuronal adhesion through integrins (Adams and Tucker, 2000); they mediate interactions between carbohydrates and proteoglycans in the ECM (Krutzsch et al., 1999; Li et al., 2002); they serve to modulate synaptogenesis (Christopherson et al., 2005); and they also function in mitotic cycle arrest and differentiation of certain cells by binding to transforming growth factor β1 (TGF β1) (Adams and Tucker, 2000; Schultz-Cherry and Murphy-Ullrich, 1993). The presence of an outgrowth promoting domain along with the repulsive sema domain poses an intriguing question as to the functional role class 5 semaphorins play in the development of the nervous system. Specifically, there might be an innate bifunctionality to this class of molecules. Recently, much work has been focused on the class 5 semaphorin Sema5A in mice, and such bifunctionality has been demonstrated. Sema5A has an inhibitory effect on cultured fibroblasts as well as retinal ganglion cells (RGCs) and was shown to collapse neuronal growth cones (Artigiani et al., 2004; Goldberg et al., 2004; Oster et al., 2003). Conversely, Sema5A has been shown to be permissive to and enhance the migration of cultured epithelial and endothelial cells (Artigiani et al., 2004). Experimentation by Kantor et al. has illustrated

that the bifunctionality of Sema5A can be regulated by extrinsic factors, such as chondroitin sulphate proteoglycans (CSPGs) in the extracellular matrix (ECM) (2004). It has been hypothesized that CSPGs likely regulate the function of Sema5A by binding to the TSRs and exposing the sema domain to carry out its repulsive activity (Artigiani et al., 2004; Kantor et al., 2004; Tran et al., 2007). Overall, these findings suggest that class 5 semaphorins have the ability of to function both as an attractive and a repulsive guidance cue to growing axons.

Along with Sema5A (originally called SemF), Puschel and colleagues described another protein of the same class in rodents, Sema5B (originally SemG), which has 58% identity and 72% similarity to Sema5A (Adams et al., 1996; Puschel, 1996). In embryonic rats, Sema5B was shown to be broadly expressed in the neuroepithelium along the entire rostrocaudal axis of the developing neural tube and additionally in the emerging motor neurons in the ventral-lateral region of the spinal cord (Adams et al., 1996; Puschel et al., 1996). Its expression in the rat telencephalon was described subsequently to be in regions including the piriform cortex, insular cortex, and lateral portions of the amygdala (Lett et al., 2009; Skaliora et al., 1998). Given the widespread expression pattern of Sema5B in the germinal zones of the nervous system, our laboratory has been interested in and was the first to demonstrate the functional roles of Sema5B during development.

Previously, Legg (2003) completed a detailed investigation of the expression of chick Sema5B (cSema5B) in the developing embryo using RT-PCR, *in situ* hybridization, and immunohistochemical labelling methods (Figure 1-2). It was shown that cSema5B RNA was detectable as early as embryonic day 3 (E3) in the developing chick embryo. *In situ* hybridization studies revealed that cSema5B is expressed at E5 in a variety of tissues including epidermis, the gut, the retina, spinal cord, brain, olfactory and ventricular

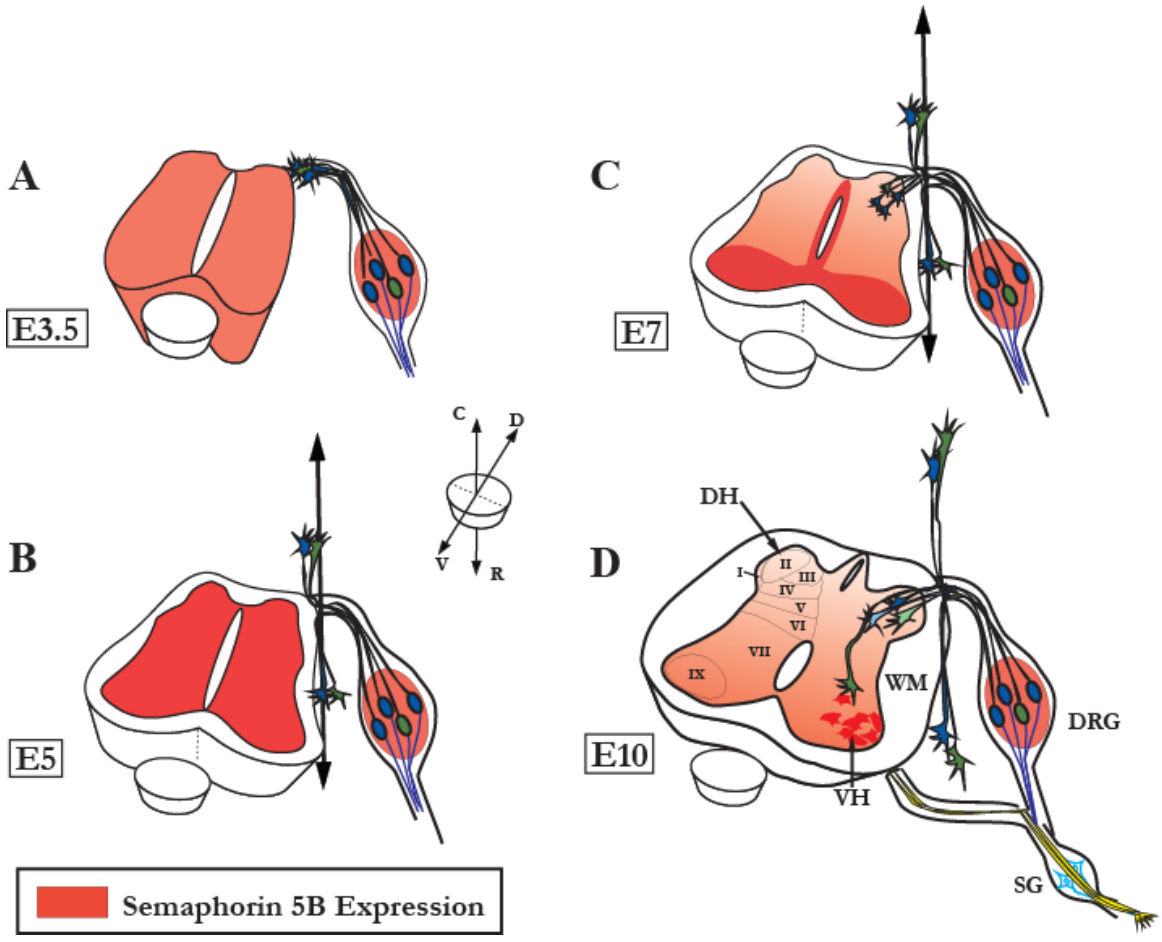

A
E3.5
C
E7
B
E5
C
D
V
R
D
DH
I
II
III
IV
V
VI
VII
IX
WM
DRG
E10
VH
SG
Semaphorin 5B Expression

Figure 1- 2

Figure 1-2

Dynamic expression of Sema5B in the chick spinal cord during development (adapted from Legg, 2003) and the projection pattern of sensory axons originating from the DRG. Sema5B is detected in the spinal cord at E3, 12 hours before sensory afferents arrive at the dorsal root entry zone (A). Sensory afferents bifurcate and travel along the rostral-caudal axis to form the dorsal funiculus while Sema5B expression is still present throughout the spinal cord grey matter at E5 (B). Sema5B expression decreases in the dorsal horns starting at E6 and becomes restricted to the ventral horns and the ventricular zone surrounding the central canal by E7 (C). Meanwhile, sensory collaterals start to form around E6-6.5 and extend away from the dorsal funiculus (C). By E10, Sema5B expression becomes localized only in large diameter neurons in the lateral motor column in the ventral horn while the projection patterns of cutaneous (blue) and proprioceptive (green) collaterals are clearly distinguishable (D). DH, dorsal horn; WM, white matter; DRG, dorsal root ganglia; SG, sympathetic ganglia; VH, ventral horn.

epithelium, with the highest expression in the nervous system. In the spinal cord, a broad expression pattern is observed throughout the grey matter, including both in the dorsal and ventral regions, but not in the surrounding white matter. Robust expression is found along the dorsoventral axis of the ventricular epithelium, where progenitor cells reside and undergo mitosis (Bellairs R, 1998). Interestingly, the expression pattern of cSema5B in the spinal cord was found to be dynamic during the time period of sensory system development. By E6, it was observed that cSema5B expression in the dorsal horns of the grey matter started to decrease, while expression was maintained in the ventral spinal cord. This change in expression pattern continued through E9, when cSema5B expression became restricted to the ventral horns and associated mainly with cells located in the region of the lateral motor column. This expression pattern is maintained until after E11, the last embryonic stages analyzed. This set of studies demonstrated that the expression of cSema5B closely resembles the expression previously described in rodents (Adams et al., 1996; Puschel et al., 1996). Also, given the prominent and dynamic expression of Sema5B in the spinal cord over the critical period of nervous system development, these findings suggested that it may function to guide the development of the circuitry in the spinal cord.

Central Projections of Sensory Neurons as a Model for Axon Guidance

During the development of the spinal cord, centrally projecting primary afferent axons of different classes of sensory neurons located in the dorsal root ganglia (DRG) enter the grey matter and establish distinct connection patterns in the dorsal and ventral horns. These stereotyped projection patterns are robust and conserved across many species and thus serve as an appropriate system through which axonal guidance mechanisms in higher vertebrates can be elucidated. This model is illustrated in Figure 1-2 and summarized in

Table 1-1. Following the migration of neural crest cells from the dorsal region of the neural tube, they differentiate into pigment cells or cells of the DRG, autonomic ganglia, and adrenal medulla (Bronner-Fraser, 1993; Masuda and Shiga, 2005). DRG neurons send axons bidirectionally both to peripheral targets as well as centrally to the spinal cord at around E3 in the chick (stage 18-20 of(Hamburger and Hamilton, 1951)). Central sensory afferents reach a region in the dorsolateral spinal cord called the "dorsal root entry zone" (DREZ) by E3.5-4 (stage 23) (Davis et al., 1989; Eide and Glover, 1997; Mendelson et al., 1992; Ozaki and Snider, 1997). Rather than penetrating the grey matter right away, these afferents bifurcate to travel along the dorsal surface of the grey matter rostrally as well as caudally in the primordium of the dorsal funiculus for the next 48 hours (Davis et al., 1989; Eide and Glover, 1995; Eide and Glover, 1997; Mendelson et al., 1992; Ozaki and Snider, 1997; Perrin et al., 2001; Shiga et al., 1997). The DREZ is considered an intermediate target of the primary sensory afferents (Masuda and Shiga, 2005). At E6-6.5 (stage 28-29), sensory afferent collaterals begin to extend and can be visualized in the gray matter (Davis et al., 1989; Eide and Glover, 1995; Eide and Glover, 1997; Mendelson et al., 1992; Ozaki and Snider, 1997; Perrin et al., 2001; Shiga et al., 1997). From this time on, sensory fibres innervating different peripheral targets begin to project to their central targets in the spinal cord and establish strikingly different projection patterns depending on their sensory modality (Davis et al., 1989; Eide and Glover, 1995; Eide and Glover, 1997). These projection patterns are distinct from each other with respect to the axon entry site into the grey matter, amount of branching, and the ventral extent of targeting (Davis et al., 1989; Eide and Glover, 1995; Eide and Glover, 1997; Perrin et al., 2001). For instance, small-calibre, unmyelinated cutaneous afferents enter the grey matter from the lateral half of the dorsal

Table 1- 1

Major events during sensory circuit development in the chick spinal cord.

Age	Event
E3.5	Earliest central projections of DRG neurons reach spinal cord (both muscle and cutaneous)
E6	Sensory afferent collaterals begin to form (both muscle and cutaneous, initial patterns similar)
E8-9	Muscle afferents grow ventrally through laminae V-VII without major branching Cutaneous afferents confined to dorsal horn, mostly in laminae I and II, with much branching
E14	Mature wiring pattern formed

Table 1- 2

Distinguishing factors of different classes of sensory afferents.

	Cutaneous		Muscle
Subclass	Nociceptive	Mechanoreceptive	Proprioceptive
Markers Expressed	TrkA+, TAG-1+, SP+, CGRP+	TrkB+, TAG-1 (before E6)	TrkC+, TAG-1+ (before E6)
Neurotrophin Required	NGF	BDNF	NT-3

funiculus and are confined within the dorsal horn where they extensively branch (Brown and Culberson, 1981; Davis et al., 1989; Eide and Glover, 1997; Perrin et al., 2001; Woodbury, 1992). These afferents form somatotopically precise connections with neurons that express substance P, calcinonin gene-related peptide, galanin and/or somatostatin (reviewed in (Fitzgerald, 2005). Large-diameter proprioceptive muscle afferents project from the medial half of the dorsal funiculus and extend past the dorsal laminae into the ventral horn to form synaptic connections with motoneurons in the ventral horn (Davis et al., 1989; Eide and Glover, 1997; Perrin et al., 2001). These sensory projection patterns persist and at E13 (stage 39), they appear identical to those in the mature animals (Davis et al., 1989; Eide and Glover, 1997). Subpopulations of sensory afferents can also be differentiated based on the neurotrophic factors they depend on for survival as well as the immunohistochemical markers they express (Huang and Reichardt, 2001). Namely, cutaneous fibres depend on nerve growth factor (NGF) for survival and growth and express the tyrosine kinase receptor TrkA while muscle fibres are neurotrophin-3 (NT-3) dependent and express TrkC (Snider, 1994) (Table I-2).

Class 5 semaphorins were first implicated in axon guidance after the examination of genetic mutations of human patients with the neurological disorder, Cris-du-chat syndrome (Simmons et al., 1998). A recent publication by our laboratory described the role of Sema5B to act as a repulsive cue in the germinal regions of the cortex to prevent inappropriate targeting by corticofugal axons (Lett et al., 2009). Therefore, Sema5B is a viable candidate as a guidance cue in the spinal cord, especially given the temporal correlation of the dynamic expression of cSema5B in the grey matter with key steps taken by the developing sensory axon (Figure 1-2). For instance, sensory afferents reach the spinal cord at E3.5, around 12

hours after cSema5B mRNA is detected throughout the grey matter (Eide and Glover, 1997; Legg, 2003). Between this time and the formation of sensory collaterals, there is a waiting period of approximately 2.5 days when sensory afferents are traveling along the dorsal funiculus and Lissaur's tract without penetrating the grey matter (Davis et al., 1989; Eide and Glover, 1997). Guidance cues are thought to develop in the spinal cord at this time, and the expression of cSema5B becomes increasingly prominent (Mendelson et al., 1992). It is possible that cSema5B acts as a repulsive cue at this time and prevents sensory afferents from invading the dorsal grey matter prematurely. Subsequently, the expression of cSema5B decreases in intensity in the dorsal horn at E6 (stage 28), at which time sensory collaterals begin to form in the dorsal grey matter (Davis et al., 1989; Eide and Glover, 1997; Perrin et al., 2001). If cSema5B acted as a repulsive cue to the sensory collaterals in the spinal cord, a decrease in this cue could facilitate premature entrance of collateral fibres into the dorsal horn. Finally, as the expression of cSema5B continues to recede into the ventral regions of the grey matter, cutaneous fibres are confined do the dorsal horn while muscle fibres target the motoneurons in the ventral horn. This observation could be explained by the inhibitory effects of cSema5B on cutaneous fibres, thus preventing them from leaving the dorsal horn, while the muscle fibres lose their responsiveness to cSema5B and target the ventral horn.

Hypothesis

Given the implication of semaphorins in a wide range of physiological processes including immunology, cancer biology, and neurobiology, it is of great importance to characterize the functions of these proteins and the mechanisms through which they signal. Here I plan to investigate the role of Sema5 as a guidance cue in the patterning of sensory afferent fibres in the embryonic chick spinal cord. I hypothesize that **Sema5B acts as a repulsive cue and regulates the timing and extent of sensory collateral targeting in the dorsal spinal cord by acting as a barrier in the grey matter**. To address this hypothesis I will address the following questions.

1. Does Sema5B inhibit growth of different classes of sensory axons *in vitro*?
2. Can Sema5B function as a diffusible guidance cue?
3. Can Sema5B act as a repellent cue *in vivo* to prevent premature entry of sensory axons in the dorsal spinal cord?
4. Can Sema5B mediate the correct targeting of sensory collateral fibres in the dorsal spinal cord *in vivo*?
5. What receptor protein can act as a mediator of Sema5B function?

Chapter 2: Materials and Methods

Full-length Chick Sema5B Constructs

A previous member of the lab subcloned the full-length chick Sema5B construct into the XmaI/SacII sites of the pDisplay expression vector (Invitrogen, Eugene, OR, USA) with an hemagglutin (HA) epitope tag to generate an N-terminal HA-tagged fusion protein. The construct has been described previously (O'Connor et al., 2009). Multiple stop codons were positioned before the platelet derived growth factor receptor (PDGFR) transmembrane region of the vector. Deletion constructs have been generated in order to examine the function of the two major domains of Sema5B – Sema domain and TSP repeats. The Sema construct was prepared by subcloning amino acids 20-554 (corresponding to the length of the Sema domain) of Sema5B into the pDisplay vector in frame with the PDGFR transmembrane region. The TSP construct was made by deleting amino acids 1-547 and subcloning the remaining sequence into the pDisplay vector. Sequencing was performed on all constructs to ensure the absence of errors.

Stable Cell Lines

Using Lipofectamine 2000 (Invitrogen), the above DNA constructs were transfected into HEK293 cell lines according to product protocol. Clonally derived stable cell lines were selected and maintained using 500ug/mL Geneticin (Gibco®, Life Technologies, Carlsbad, CA, USA) in Dulbecco's modified Eagle's Medium (DMEM)/F12 (Sigma-Aldrich, Inc., St. Louis, MO, USA) supplemented with 10% fetal bovine serum (FBS, Sigma), 50ug/mL penicillin/streptomycin (Pen/Str, Invitrogen) and 1X Glutamax (Gibco®). HEK293 cell lines

were kept in an incubator at 37℃ with 5% CO_2. Expression of constructs has been previously confirmed by immunocytochemistry (ICC) and Western analysis.

Immunocytochemistry

1cm circular glass cover slips (FisherScientific, Fairlawn, NJ, USA) were placed on the bottom of cell culture dishes prior to seeding with cells. To label cells, the culture media was removed and the cells were fixed for 30min with 4% (wt/vol) paraformaldahyde (PFA, Sigma) containing 4% sucrose in PBS at 37℃, washed 2x5min with PBS, then blocked for 1hr in blocking buffer (0.1M Glycine, 0.3% TritonX-100, 7%BSA in PBS) at room temperature. Primary antibody (rabbit anti HA, Cell Signaling Technology, Inc., Danvers, MA, USA) was diluted 1:500 in blocking solution and applied to cells for incubation overnight at 4℃ or for 1hr at room temperature. The cells were washed 3x15min in PBT (0.1% Tween-20 in PBS) followed by incubation in secondary antibody (goat anti rabbit IgG Alexa Fluor® 488, Molecular Probes®, Invitrogen) diluted 1:500 in blocking solution for 1hr at room temperature. The cells were washed in PBT as before, rinsed 2x5min in PBS, and mounted in ProLong® Gold plus DAPI (Invitrogen). Positive labelling was visualized using the fluorescent Axio Scope (Carl Zeiss MicroImaging GmbH, Germany).

Western Blot Analysis

HEK293 cells were rinsed in cold PBS on ice and incubated in Complete RIPA buffer (150mM NaCl, 50mM Tris pH 8.0, 1.0% NP-40, 0.5% sodium deoxycholate (DOC) and 0.1% sodium dodecyl sulphate (SDS), 1X Complete Protease Inhibitors (Roche)) on ice for 30min. Plate scrapers (Corning Inc., Costar, Corning, NY, USA) was used to harvest cell lysates which were first sonicated using the Branson Sonifier 250 and pelleted by centrifuging at

13,200 rpm for 10min. Total protein concentration of the supernatant was measured using the bicinchoninic acid (BCA) protein assay according to product protocol (Pierce, Thermal Fisher Scientific Inc., Rockford, IL, USA). Depending on protein concentration, 10-25ug of total protein from each sample was used for SDS-poly acrylamide gel electrophoresis (SDS-PAGE). Equal loading was confirmed by antibody labeling of γ-tubulin (1:10,000 Sigma) on the Western blot membrane as follows.

An equal volume of 2X SDS loading buffer (4.4% SDS, 3.5M Tris HCl pH6.8, 20% glycerol, 2% 2-mercaptoethanol (β-merc), Bromophenol Blue) was added to each protein sample and boiled at 95℃ for 5 min. Samples were added to SDS-PAGE gels (5% stacking and 8% separating gel made from 30% Acrylamide/Bis stock solution, Bio-Rad Laboratories, Inc., Hercules, CA, USA) and run in running buffer (25 mM Tris base, 190 mM glycine, 0.1% SDS) at 80V through stacking gel and 100V through separating gel. Protein samples were transferred to a nitrocellulose membrane pre-soaked in transfer buffer (25 mM Tris base, 190 mM glycine, 20% methanol) at 100V for 1hr. The membrane was blocked with 5% skim milk in TBST (0.3% Tween-20 (Sigma) in PBS) for 1hr at room temperature with rotation and probed with mouse anti-HA antibody (1:10,000, Sigma) in block solution overnight at 4℃. The next day, the blot was washed 3x5min in PBT at room temperature with rotation and incubated in horse radish peroxidase conjugated goat anti-rabbit secondary antibody (1:10,000) in block for 1hr at room temperature. After washing with PBT as before, the membrane was developed using Luminol Enhancer Detection Reagent (Thermo Scientific, Rockford, IL, USA).

Animals

Fertilized White Leghorn chicken eggs were obtained from the University of Alberta and incubated at 38℃. The embryos were staged according to Hamburger and Hamilton (1951) periodically during incubation and prior to use in each experiment.

3D Collagen Assay

HEK293 cells stably transfected with control or HA-tagged Sema5B plasmids were suspended in a 13:1 mixture of rat tail derived type I collagen (BD Biosciences, Franklin Lakes, NJ, USA) and 10X Minimum Essential Medium (Sigma) adjusted to pH 6.5 with 7.5% $NaHCO_3$. The suspension was plated onto the bottom of a petri-dish to form a block with a base area of 10mm x 10mm, and allowed to solidify at 37℃ in a humidified chamber. The cell blocks were cut into 2mm cubed cell aggregates. E7 chick DRGs were dissected into cold DMEM/F-12 medium. DRGs and cell aggregate cubes were embedded ~200um apart in 40uL of collagen mixture on top of a 1cm glass coverslip. The cultures were incubated at 37℃ at 5% CO2 in culture media (100 ng/ML NGF, DMEM/F-12) overnight.

Cultures were fixed for 2hrs with 4% PFA, washed 3 times with PBS, then blocked for 4hrs in blocking solution (10% heat inactivated horse serum in PBT solution made of 0.1% Bovine Serum Albumin (BSA, Fisher Scientific) and 0.1% Triton X-100 in BCS at pH7.2). Cultures were probed for HA and β-tubulin (Rabbit anti HA, 1:500, and mouse anti Tuj-1, 1:500, R&D Systems Inc., Cedar Lane).

Cultures were visualized using an upright ZEISS AxioScope and TIFF images were obtained using the AxioCam MRm camera and the AxioVision Documentation software. For quantification, neurite growth into the collagen gel was measured from the outer edge of the

cell bodies of the DRG explant to the perimeter of the bulk of stained neurites using the National Institutes of Health Image J software as described before (Messersmith et al., 1995). Measurements were taken of neurites perpendicular to the edge of the HEK cell aggregate on both the proximal and distal side to the aggregate (see Figure 3-3). A ratio of proximal to distal neurite length was calculated. Data were analyzed using one-way ANOVA followed by unpaired Student's t-test.

Dissociated DRG HEK293 Overlay Assay

DRG neurons were first dissociated and cultured on top of a confluent monolayer of HEK293 cells expressing control or full-length Sema5B constructs as previously described (Matsuoka et al., 2011). Stable cell lines were plated onto coverslips coated with mouse laminin at 50% confluence and allowed to grow overnight to achieve a confluent layer of cells on the day of experiment. DRG were dissected from E5, E6, E7 or E8 embryos into cold DMEM-F12 medium and spun into a pellet at 1,600 rmp for 6 min. The DRG pellet was resuspended in 0.25% Trypsin-EDTA (Gibco®) and incubated at 37℃ for 15min to allow dissociation of sensory neurons. Trypsin inhibitor was added to the suspension at a 1:1 volume to stop the enzyme function of trypsin. Dissociated neurons were spun as before into a pellet and rinsed in DMEM and counted using a haemocytometer. Neurons were added to the culture plates containing the confluent layer of HEK cells at a concentration of $8x10^4$ cells/well. The cultures were allowed to grow overnight at 37℃ and 5% CO_2. To select for the growth of cutaneous neurons, cell culture medium supplemented with 40 ng/mL 7S Nerve Growth Factor (NGF, Invitrogen) and to select for the growth of proprioceptive neurons, the same amount of Neurotrophin-3 (NT-3, PeproTech, Rocky Hill, NJ, USA) was used (Table 1-2). On the next day, cultures were fixed and immunolabelled as before. Mouse

anti Tuj-1 (1:500, Sigma) was used to label neurites. For quantification, TIFF images of positive immunolabelling were obtained and the length of the axons from each neuron was measured using ImageJ. Mean axon lengths of neurons in each condition were compiled and a standard error of the mean was calculated. Two tailed, unpaired T-tests were performed to establish statistical significance between treatments.

To block the function of Tag-1, HEK293 cells were plated at low density on coverslips and allowed to grow to 50% confluence overnight in supplemented DMEM-F12 media. Fresh transfections with control and Sema5B plasmids were performed with polyethylenimine (PEI, Polysciences, Inc.) according to product protocol. At 32 hours post transfection of HEK293 cells, dissociated DRG neurons were added to appropriate cell culture wells and incubated overnight. DRGs were obtained from E6 chicks and dissociated as above and incubated in either culture media alone or culture media containing mouse anti-TAG-1 antibody (23.4-5, Developmental Studies Hybridoma Bank, Iowa City, IW, USA) diluted to 170ug/mL for 1 hour. On the next day, cultures were fixed, immune-labelled, and quantified as above.

Preparation of shRNA Vectors and Validation

Sequences ideal for RNA interference targeting were analyzed using pSico oligomaker v1.5 software (http://web.mit.edu/jacks-lab/protocols/PSICOLIGOMAKER_manual.pdf) and the oligoduplex palindromes designed for hairpin loop formation were generated by Invitrogen (Burlington. ON). The following sequences were targeted: shRNA1 (1203) = 5' –GAAATCCCTTTCTATTATA and shRNA2 (3442) = 5' GGAGTTCAAGACACTTTAA. Oligoduplex palindromes were cloned into the *Xho*I/*Hpa*I restriction sites of the Lentilox 3.7 (pLL3.7) expression vector which contains an

enhanced green fluorescent protein (GFP) sequence driven by a CMV promoter located downstream of the cloning site and confers resistance to ampicillin (Reynolds et al. 2004).

Resulting shRNA plasmids were transfected into HEK293 cell lines expressing HA-tagged full-length cSema5B or mSema5B using polyethylenimine as above. The ability of the shRNA constructs to knockdown cSema5B expression and the lack of knockdown effect on mouse Sema5B (mSema5B) expression was confirmed by Western Blot analyses of cell lysates as above. β-tubulin was probed as a loading control.

***In ovo* Electroporation of Chick Spinal Cord**

In ovo unilateral electroporations of developing chick spinal cords were performed as previously described (Nakamura and Funahashi, 2001). Chicken eggs were incubated along their horizontal axis throughout their development. To prepare embryos for electroporation, 3mL of albumin was removed with a 20-g needed attached to a 3-cc syringe and a small window was cut into the top of the eggshell to allow visualization of the embryo for staging purposes. In order to facilitate closing and re-opening of the window, it was sealed with scotch tape to avoid dehydration of the embryo during incubation. At the appropriate time of electroporation (H&H stage 21 and H&H stage 27), the existing window on the egg was enlarged to expose the embryo and to ensure enough room for manipulation. Purified plasmid DNAs were re-suspended in TE buffer (Invitrogen) at a concentration of 4 ug/uL and mixed at a ratio of 25:1 with a volume of Fast Green for microinjection. 1 uL of the DNA/Fast Green mixture was injected into the developing chick neural tube using a glass micropipette. A few drops of Hanks' Balanced Salt Solution (HBSS) with calcium and magnesium were added on top of the embryo to facilitate electric field formation. Paddle electrodes (CUY650-P3 Platinum Plate Tweezers Electrode, Protech International Inc.) were

placed on either side of the neural tube such that the DNA will migrate toward the positive electrode into one side of the developing spinal cord. At st20, electroporation was performed with five 50ms pulses of 25V at one second intervals. At st27, electroporation was performed at 45V. The electrodes are moved along the length of the spinal cord to ensure sufficient electroporation of the entire region. For rescue experiments, equal amounts of the shRNA plasmids and full-length HA-tagged mouse Sema5B DNA constructs were co-injected into the spinal cord. As an additional control for the rescue experiments to confirm the lack of phenotype under the rescue treatment was not due to the dilution of the shRNA plasmids injected, I co-injected the shRNA plasmids and mouse Sema5B DNA constructs at different volume ratios (1:1, 1:0.5, 1:0.25), and co-injected the shRNA plasmids with an equal volume of a control pDisplay plasmid. After electroporation, the openings of the eggs were sealed and the embryos were allowed to grow further at 38℃ until the desired stage for analysis.

Cryosectioning and Immunohistochemistry of Electroporated Spinal Cords

When the desired stage has been reached, the embryonic spinal cords were dissected out into cold PBS and the presence of GFP was confirmed using a fluorescent dissection microscope. The spinal cords were fixed overnight at 4℃ in a solution of 4% PFA and 4% sucrose in PBS. On the next day, the spinal cords were washed in PBS and immersed in 15% followed by 30% sucrose solutions for cryo-protection. Tissues were embedded in optimal cutting temperature (O.C.T) compound (TissueTek) and 30um cryostat sections were obtained using a cryostat (MICROM HM525, Thermo Scientific) set at -25℃ and collected onto positively charged glass slides (Globe Scientific Inc., Paramus, NJ, USA). Sections were air-dried for 5 min and stored at -20℃ until immunohistochemical labelling.

Sensory axons were immunolabeled for analysis. Slides were rinsed in PBS for 10min at room temperature to remove any remaining O.C.T. compound, then blocked for 10min at room temperature in blocking solution (0.3% Tritron X-100 and 5% normal goat serum (NGS) in PBS). Primary antibody was diluted in antibody dilution solution (0.3% Triton X-100, 1% BSA, 0.3% NGS in PBS) and applied to the slide. Rabbit anti-TrkC (1:1000, Cell Signaling) and Mouse anti-TAG-1 (1:500) antibodies were used to label proprioceptive and nociceptive fibres, respectively. Rabbit anti-HA (1:500, Cell Signaling) antibodies were used to label mSema5B to confirm co-transfection with shRNA vectors for rescue control. Slides were incubated in primary antibody overnight at 4℃, washed in PBT (0.1% Triton X-100 in PBS) as follows: 3x1min, 3x5min, 1x15min. Secondary antibody was diluted in the same antibody dilution solution and applied to the slides for incubation at room temperature for 4hrs. The secondary antibodies used includes Goat anti-mouse IgG Alexa Fluor® 568, Goat anti-rabbit IgG Alexa Fluor® 568 and 488 (Invitrogen). The slides were washed as before plus 5min in PBS, and mounted in ProLong® Gold plus DAPI. Positive immunostaining was visualized under a confocal microscope (Leica DM6000CS) and images of the spinal cords were taken with the Leica TSC SP511 camera and TIFF files were created with the Leica Application Suite – Advanced Fluorescence software (2.5.2.6939). Aberrantly projecting sensory collaterals were counted per section and 5 to 15 sections were analyzed per animal. The average numbers of aberrant collaterals per section were calculated and averaged across the number of animals (n = 4 to 10 chicks) per treatment. SEM of the number of aberrant collaterals per section was calculated and one way ANOVA followed by unpaired t-tests were performed to establish statistical significance.

Chapter III: Results

Semaphorin 5B Inhibits Outgrowth of Chick Sensory Neurons *in vitro*

The previously established dynamic expression of Sema5B during the development of the chick spinal cord from E3 to E9 supports a potential role in guiding the growth of sensory axons from neurons in the DRG. In order to evaluate this potential role as a guidance cue, I asked if Sema5B can affect neurite outgrowth in dissociated embryonic sensory neurons. An *in vitro* assay was employed to determine whether Sema5B directly regulates neurite growth. Wild-type sensory neurons were obtained from chicks of different embryonic ages by dissociating DRG explants dissected from E5 to E8 chicks and cultured on top of a confluent monolayer of HEK293 cells stably expressing Sema5B or an empty expression vector (Figure 3-1). The culturing media were supplemented with different neurotrophic factors to select for the growth of different classes of sensory neurons in each treatment (Friedel et al., 1997). Cultures were labeled with the post-mitotic neuronal marker Tuj-1 which recognizes beta-III tubulin and has been previously used to identify terminally differentiated neurons and their axons (Matsuoka et al., 2001; Pimentel et al., 2000; Sakagami et al., 2003).

For all embryonic ages included in the analysis, the mean neurite lengths of both NGF-dependent nociceptive neurons and NT-3-dependent proprioceptive neurons were significantly shorter (ranging from ~30%-40%) when cultured on Sema5B-expressing HEK293 cells compared to when cultured on control HEK293 cells (Figure 3-2, $^{**}p < 0.05$ for all treatments, n=3 separate experiments with 64 – 228 neurons in total per each treatment). Thus exogenous Sema5B can significantly inhibit sensory neurite outgrowth in vitro. To investigate whether different subclasses of sensory neurons at different embryonic

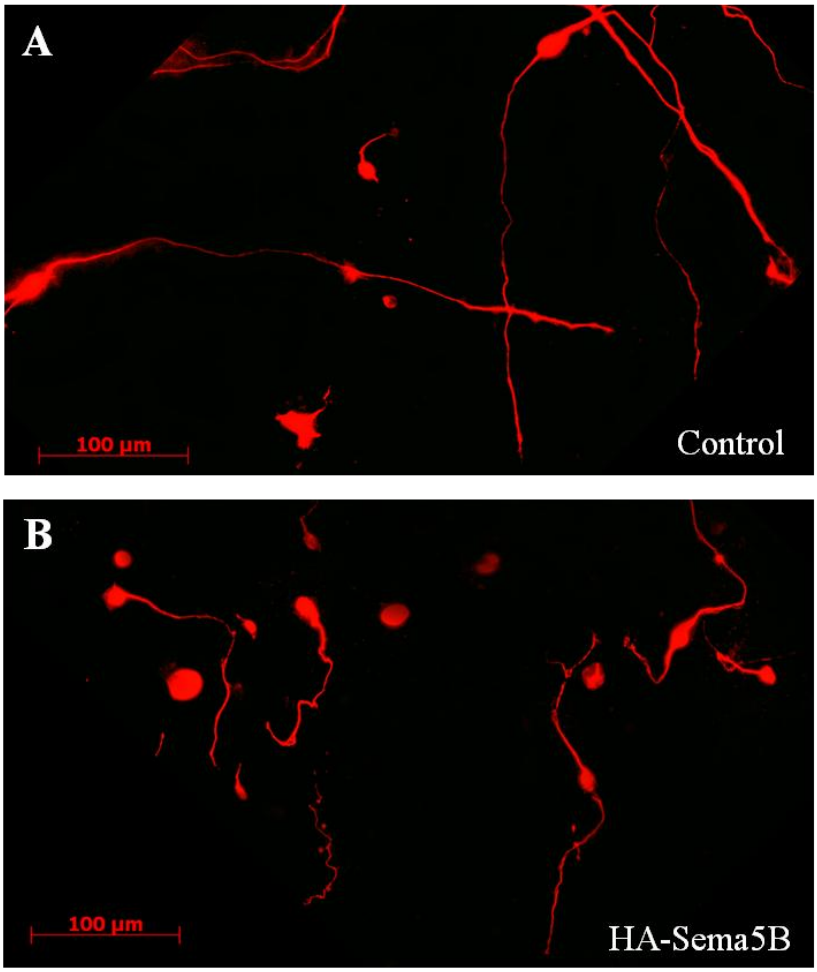
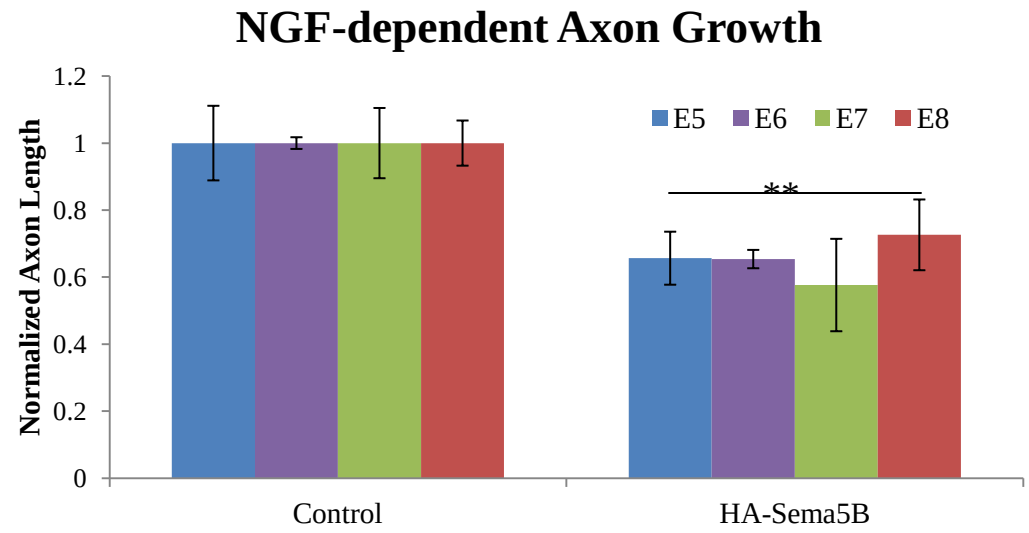


Figure 3- 1

HA-Sema5B inhibits sensory axon outgrowth in dissociated neuron/overlay assay. DRG explants were obtained at different ages and dissociated in trypsin. Sensory neurons (E6 + 1DIV neurons shown here) were cultured on top of a confluent mono-layer of HEK293 cells expressing control (A) or HA-Sema5B vectors (B) in the presence of 40ng/mL NGF (shown in above set of images) or 40ng/mL NT3 (not shown). Overnight cultures were fixed, sensory axons are labelled using mono-β-tubulin (anti-Tuj1) and HEK 293 cells with the HA epitope (anti-HA). For quantification, neurite lengths were measured for each treatment (see Figure 3-2).


NGF-dependent Axon Growth
Normalized Axon Length
1.2
1
0.8
0.6
0.4
0.2
0
E5
E6
E7
E8
**
Control
HA-Sema5B

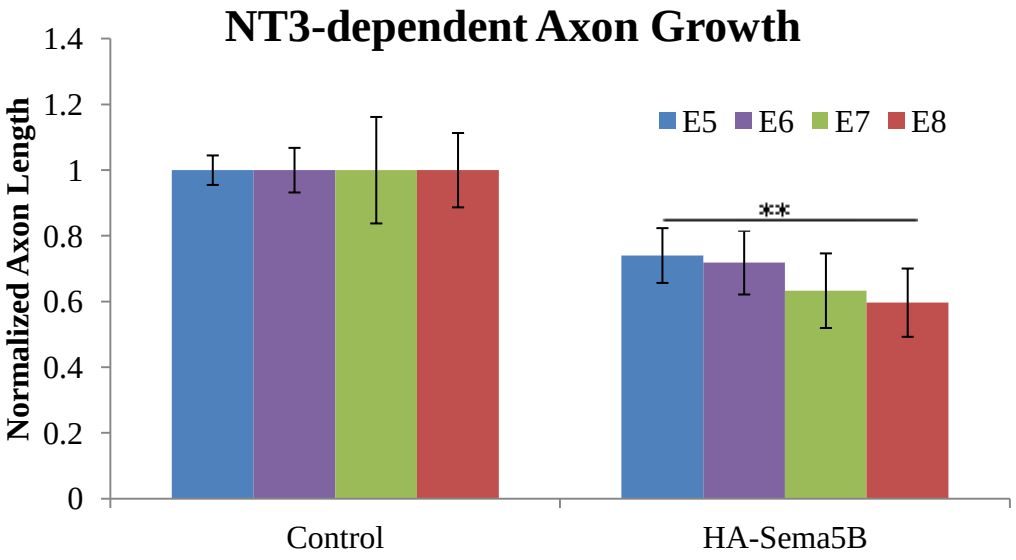

NT3-dependent Axon Growth
Normalized Axon Length
1.4
1.2
1
0.8
0.6
0.4
0.2
0
E5
E6
E7
E8
**
Control
HA-Sema5B

Figure 3- 2

Figure 3- 2

Quantification of dissociated neuron-overlay assay (Figure 3-1). (A) The growth of cutaneous nociceptive axons were selected for using NGF. The lengths of axons are significantly shorter when neurons were grown on HA-Sema5B expressing cells compared to control, indicating inhibition of axon growth at all time points tested (E5-E8). n=3 separate experiments with 107-217 neurons per treatment in total. (B) Proprioceptive axons were cultured with the addition of NT-3 in culture media. Axon lengths were significantly shorter when grown on top of HA-Sema5B expressing cells compared to control for all time points tested (E5-E8). n=3 separate experiments with 66-228 neurons per treatment in total. Error bars represent SEM. One way ANOVA followed by unpaired Student's T test: **$p<0.05$ for all days tested.

ages responded differentially to Sema5B, I compared the repulsive effect of Sema5B on the two subclasses of sensory neurons on each embryonic day tested. One way ANOVA followed by Student's T test revealed no statistically significant difference in neurite outgrowth when the two subclasses of neurons are cultured with control HEK293 cells in all ages tested (Figure 3-2, p=0.34 to 0.71). Lastly, I compared whether the inhibitory effect of Sema5B on each class of sensory neurite outgrowth was different at different embryonic ages. Statistical analysis revealed no significant difference in the extent of growth inhibition between any stages tested within each class of sensory neuron (Figure 3-2, p=0.35 to 0.84). Taken together, these data suggest that Sema5B acts as an inhibitory molecule on both types of sensory neurons tested and the *in vitro* inhibitory effect remains constant through the embryonic stages tested.

Semaphorin 5B can Act as a Diffusible Repulsive Guidance Cue for Chick Sensory Neurons

Previous studies have suggested the presence of a diffusible inhibitory cue (that is not Sema 3A) that originates from the embryonic spinal cord and guides early sensory axons exiting the DRG (Masuda and Shiga, 2005). My first series of experiments analyzed whether Sema5B can function as an inhibitory cue when it is expressed as a substrate for neuronal growth. In order to determine whether Sema5B can function as a possible diffusible guidance barrier, additional *in vitro* experiments were employed. Browne et al. has recently shown that Sema5B is proteolytically processed and released from cell membranes (Browne et al., submitted). In addition, conditioned media collected from cultures of HEK293 cells expressing Sema5B was able to collapse growth cones of cultured dissociated E7 DRG neurons (Browne et al., submitted). These assays demonstrated that cleaved Sema5B can be

released into the extracellular surroundings to inhibit axonal extension To extend these observations, I further investigated whether released Sema5B can create an inhibitory gradient to affect neurite outgrowth from a distance using a collagen gel assay (Lumsden and Davies, 1983). E8 DRG explants were co-cultured 200 um away from aggregates of HEK293 cells expressing Sema5B or a control vector in a collagen matrix. Using the 3-dimensional collagen matrix is important as it provides the appropriate substrate to establish a gradient of a putative guidance molecule (Lumsden and Davies, 1983; Messersmith et al., 1995) Neurite measurements were taken in the quadrant between the DRG explants and the HEK293 cells (proximal) and in the quadrant on the direct opposite (distal) side and the ratio of neurite lengths in the two quadrants were analyzed (Figure 3-3A,B). Consistent with the dissociated neuron culture data, there was a significant decrease in neurite outgrowth towards HEK293 cells expressing Sema5B compared to control, thus resulting in a significant decrease in the ratio of proximal:distal axon lengths (Figure 3-3C, $p<0.05$, n = 21 – 23 DRG explants per treatment over 3 experiments). Whereas neurites consistently entered the control cell aggregates, they were repelled from the boundary of cell aggregates expressing Sema5B. This result suggests that Sema5B can function as a diffusible repellent guidance molecule for DRG axons.

Function of Semaphorin 5B *in vivo*

Having shown that Sema5B is inhibitory to axon growth *in vitro*, a major focus of my project was to investigate the function of Sema5B in vivo during early spinal cord development. I hypothesized that early in development (E3), the uniform distribution of Sema5B throughout the gray matter acts as a barrier to prevent premature ingrowth of

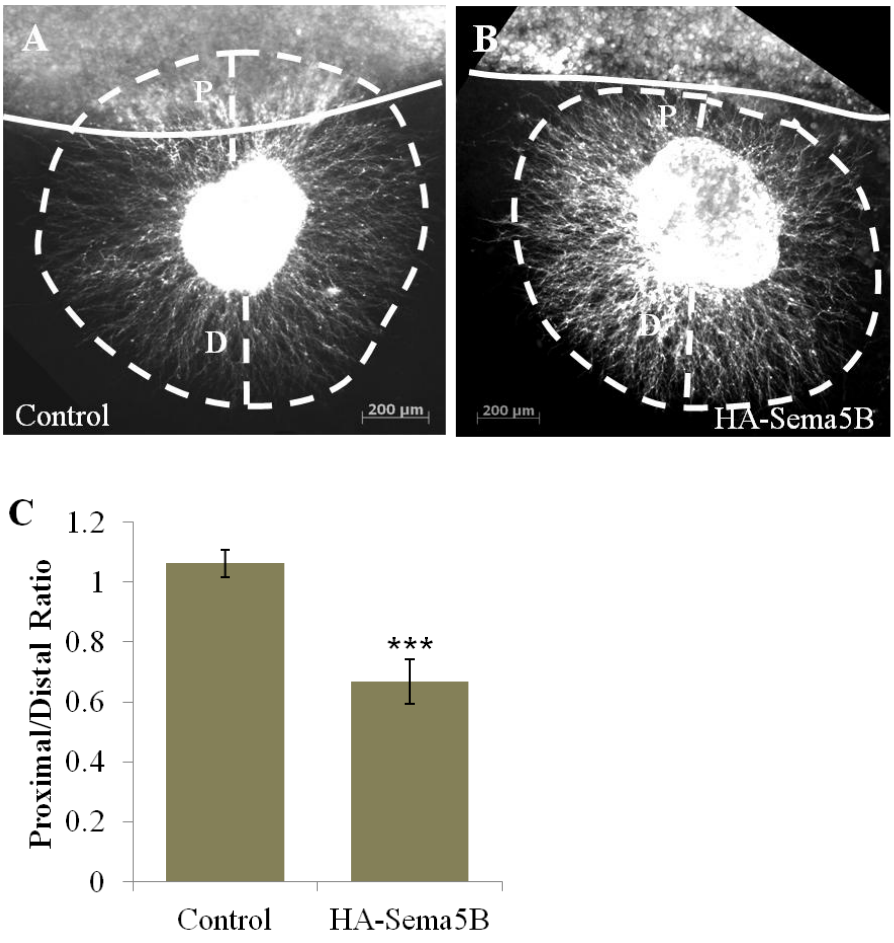

A
P
D
Control
200 µm
B
P
D
200 µm
HA-Sema5B
C
Proximal/Distal Ratio
1.2
1
0.8
0.6
0.4
0.2
0
***
Control
HA-Sema5B

Figure 3- 3

Figure 3-3

HA-Sema5B acts as a repulsive cue for chick sensory axons in collagen co-culture assay. Control (A) and HA-Sema5B (B) transfected HEK293 cell aggregates were co-cultured ~200um from E8 chick DRG explants in a suspension of collagen and supplemented with NGF. Neurons were labelled using anti-β-tubulin (anti-Tuj1) antibodies and HEK 293 cells were labeled with antibodies against the HA epitope (anti-HA). Measurements of axon length were taken from the border of the DRG to that of the neurite edge (outlined with dashed line) at sites proximal (P) and distal (D) to the cell aggregate (outlined with solid line). (C) The proximal:distal ratio of axon lengths were quantified for control and HA-Sema5B cultures to be 1.06 and 0.67, respectively, indicating repulsion from the HA-Sema5B cell aggregate through diffusible activity. Error bars represent SEM. Unpaired student's T test: ***$p<0.05$, n=21-23 DRGs over 3 separate experiments.

primary sensory axons. Furthermore, I hypothesized that as development proceeds, the restriction of Sema5B expression at approximately E6 to the ventricular zone and the ventral spinal cord serves to inhibit axons from invading these areas. Here I utilized the technique of RNA interference (RNAi) to knock-down the protein expression of Sema5B in vivo at two critical time points in sensory system axon guidance: E3.5 – when primary sensory axons reach the dorsal root entry zone, and E6– when collateral sensory fibres branch off the primary axons and enter the gray matter. The aim of Sema5B knockdown at E3.5 is to test whether Sema5B is required to block primary sensory axons from entering the gray matter, while that of Sema5B knockdown at E6 is to investigate whether Sema5B is required to facilitate the pathfinding of collateral fibres that have entered the dorsal horn.

Validation of shRNA Constructs

Two shRNA constructs targeting the chick Sema5B gene were generated previously in the laboratory and cloned into the lentilox3.7 (LL3.7) plasmid by Dr. Wenyan Wang. The plasmids used contained an enhanced green fluorescence protein (EGFP) sequence driven by a CMV promoter which allowed me to positively identify transfected cells.

In order to confirm the ability of these constructs to knock-down Sema5B, their effects were first tested *in vitro* by transfecting them individually or in combination into HEK293 cell cultures stably expressing an HA-tagged Sema5B. Cell lysates were collected and a Western Blot analysis was performed with antibodies against the HA-tag to examine the level of Sema5B protein expression (Figure 3-4). Because the transfection rate with the Sema5B-targeting shRNA plasmids was not 100%, some HA remained detectable in the cell lysates, but significant reduction was observed for each of the shRNA constructs (Figure 3-

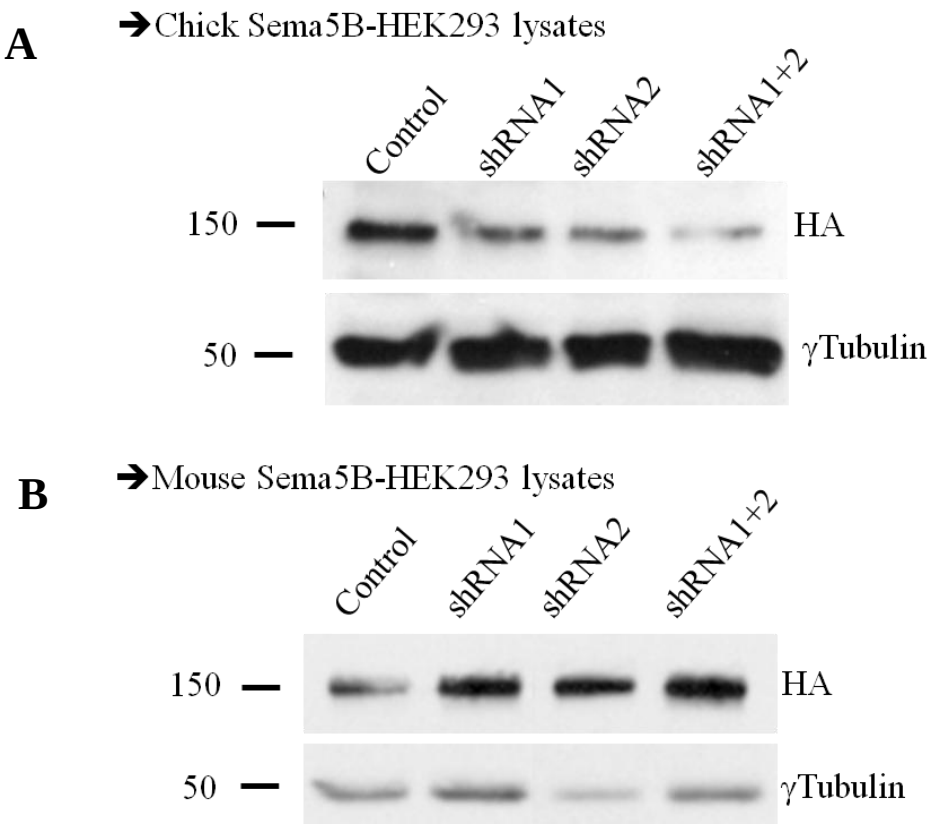


Figure 3- 4

RNA interference constructs effectively reduced expression of chick Sema5B but not mouse Sema5B protein. Two DNA sequences designed for the formation of short hairpin loops (shRNA1, shRNA2) and interfering RNA targeting chick Sema5B mRNA were previously generated and cloned into pLL3.7 plasmids, and transfected into HEK293 cells stably expressing chick Sema5B-HA or mouse Sema5B-HA. HEK293 cell lysates were analyzed 48 hours post transfection for the presence of Sema5B using Western blots and antibodies against the HA tag. Compared to cell lysates transfected with a control (empty pLL3.7) plasmid, both RNAi constructs were effective at reducing the amount of chick Sema5B-HA detected in cell lysates when transfected individually or in combination (A), but did not reduce the amount of mouse Sema5B-HA (B). γ-tubulin was detected as a loading control.

4A, compare lanes 2-4 to lane 1). Furthermore, the knock-down effect of the two shRNA constructs combined was more prominent than when the two constructs were transfected individually (lane 4 compared to lanes 2 and 3), therefore the two constructs were also used in combination (at equal concentration) in knock-down experiments.

As a functional control for potential off-target effects of the shRNA vectors, we constructed a HA-tagged mouse Sema5B vector that is resistant to RNAi by the shRNA sequences we used to knock down chick Sema5B expression. To examine whether the mouse Sema5B was resistant to chick shRNA knockdown, a similar Western Blot analysis was performed on lysates of HEK293 cells stably expressing HA-mSema5B and transfected with the same shRNA vectors (as above). No reduction of HA-mSema5B expression was observed after transfection with any of the chick shRNA vectors (Figure 3-4B), confirming the viability of mSema5B to be used as a functional rescue.

Nociceptive Sensory Axons Invade the Gray Matter Prematurely in the Absence of Sema5B

To examine whether Sema5B may function as a barrier for premature entry of sensory neurons into the spinal cord we knocked down its expression at the earliest stages when sensory axons fist extend into the dorsal root entry zone. Following transfection of control and shRNA constructs at E3.5 (st21), chicks were sacrificed at E6 (st29, just before collaterals invade the gray matter) (Mendelson et al., 1992; Perrin et al., 2001). Spinal cords were sectioned and nociceptive sensory axons were immunohistochemically labeled with anti-TAG-1, to examine the timing and extent of their entry into the gray matter. Control transfected spinal cords showed no TAG-1 labeled sensory axons outside of the Lissaur's

tract on either side of the spinal cord (Figures 3-5), indicating the control plasmid had no effect on the timing of sensory axon entry. In contrast, when Sema5B expression was knocked down using shRNA constructs, TAG-1 positive nociceptive afferents showed a striking change in their pathfinding pattern after reaching the DREZ. Instead of keeping with the longitudinal axon tracts in the Lissaur's tract, many of the axons directly invaded the gray matter from the point of entry via the dorsal root (Figure 3-6.1, 3-6.2). These early penetrating nociceptive axons did not appear to pathfind correctly as the majority of them extended beyond their normal sites of termination and reached the midline of the spinal cord where the ventricular zone is located. On average, 50 fibres were observed to enter the gray matter prematurely per 300 um analyzed compared to 2 fibres per 300 um in control spinal cords (ANOVA and one tailed, unpaired Student's T test showed $p<0.05$ (Figure 3-7A)). It was of interest to note that the majority of prematurely projecting fibres appeared to preferentially project aberrantly to the ventricular zone surrounding the cerebral aqueduct, the location at which Sema5B is prominently expressed in the developing spinal cord. This finding shows that Sema5B acts as a barrier in the gray matter for primary sensory axons and may prevent the premature entry of axons that first arrive via the dorsal roots. .

To examine this more closely, I analyzed where along the rostral-caudal axis the sensory axons prematurely extended into the grey matter of Sema5B knockdown animals. The number of fibres found in sections where the dorsal roots enter the spinal cord (root sections) were compared to the number of fibres in sections between dorsal root entry sites (non-root sections) (Figure 3-7B). In all experimental spinal cords analyzed, significantly more aberrant projections were found on root sections (50 per 300 um) on average compared to those found on non-root sections (2 per 300 um) (Figure 3-7C). Furthermore, 99% of

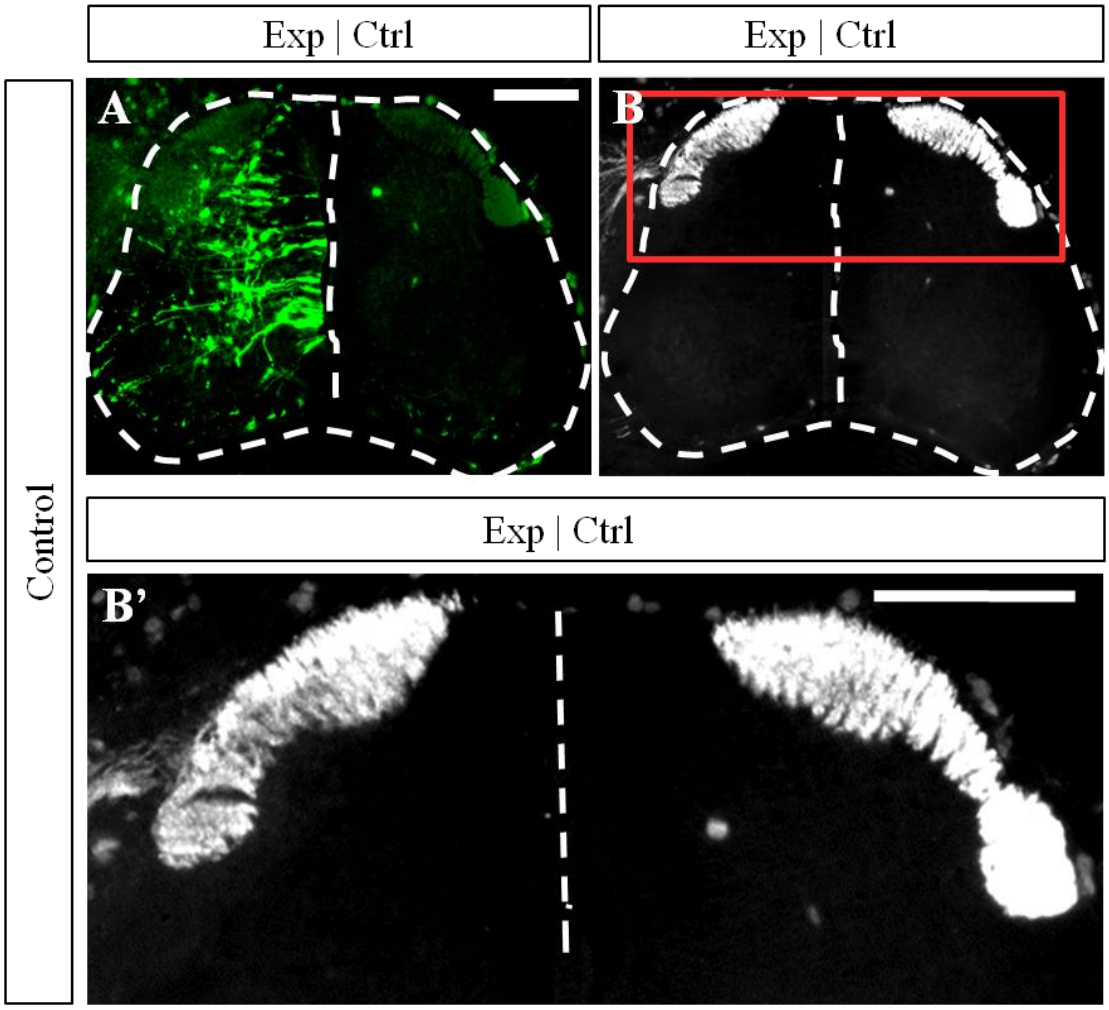


Figure 3- 5

Spinal cords transfected with control vectors at E3.5 show normal axon projection patterns at E6. Positive unilateral transfection is illustrated by the expression of GFP in E6 spinal cord cross section (A). Cryosections were labeled with anti-TAG-1 and show the localization of cutaneous nociceptive axons (B, B'). Only the axons entering the DREZ and travelling rostrocaudally in the dorsal funiculus were detected and no axons are observed entering the spinal cord grey matter at this stage. Region in red box in B is presented at a higher magnification in B'. Dashed lines outline the spinal cord and the location of the spinal cord midline. Scale bars = 100um.

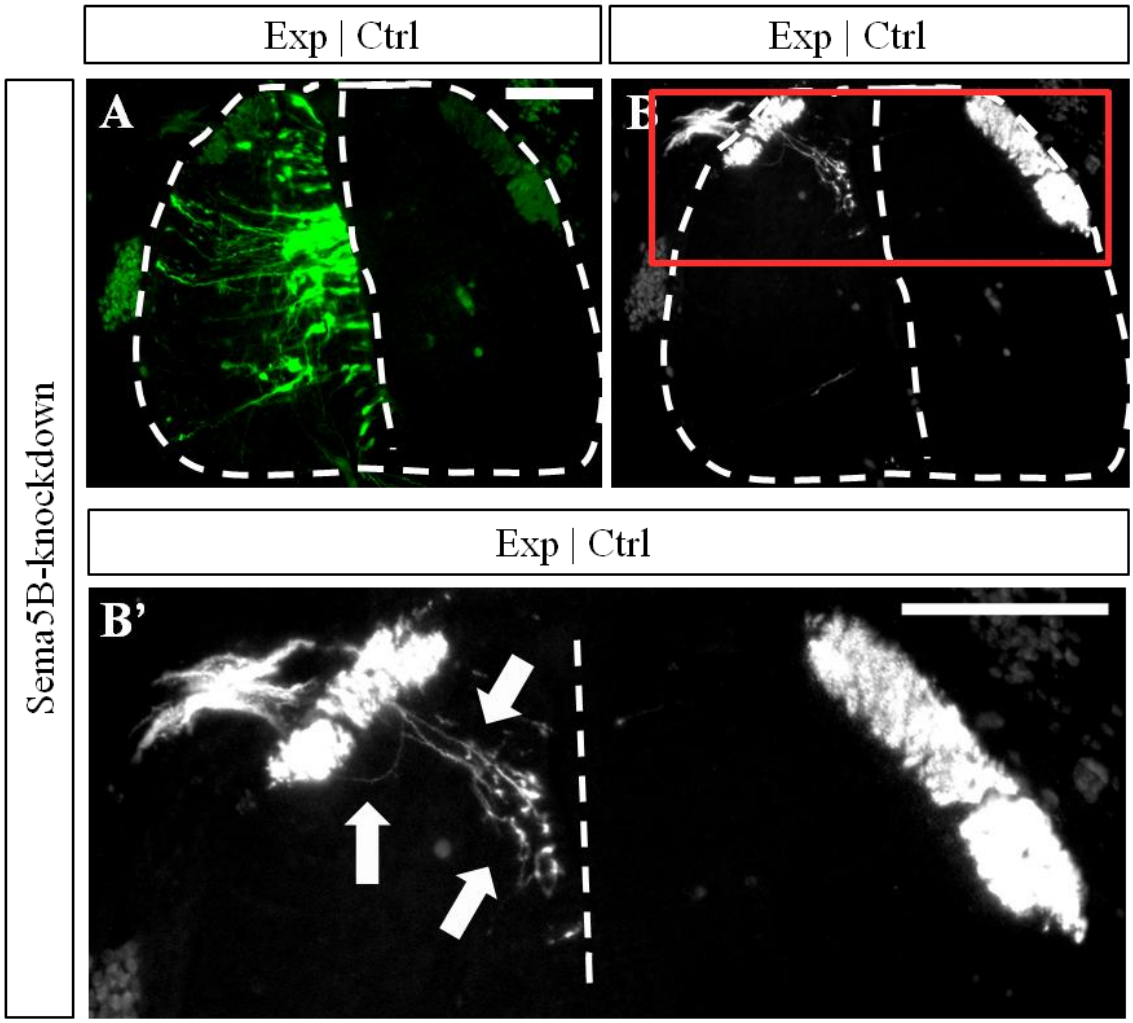


Figure 3- 6.1

Knockdown of Sema5B at E3.5 leads to premature entry of cutaneous afferents in the dorsal horn. Spinal cords electroporated with two RNAi constructs at E3.5 show positive unilateral transfection at E6 (A). TAG-1 expressing cutaneous afferents prematurely enter the spinal cord grey matter (B, B'). Boxed region in B is shown at higher magnification in B'. Arrows in B' indicate prematurely entering and mistargetting projections (B') originating from the dorsal roots and have already extended to the midline (dashed lines) at this stage. Scale bars = 100um.

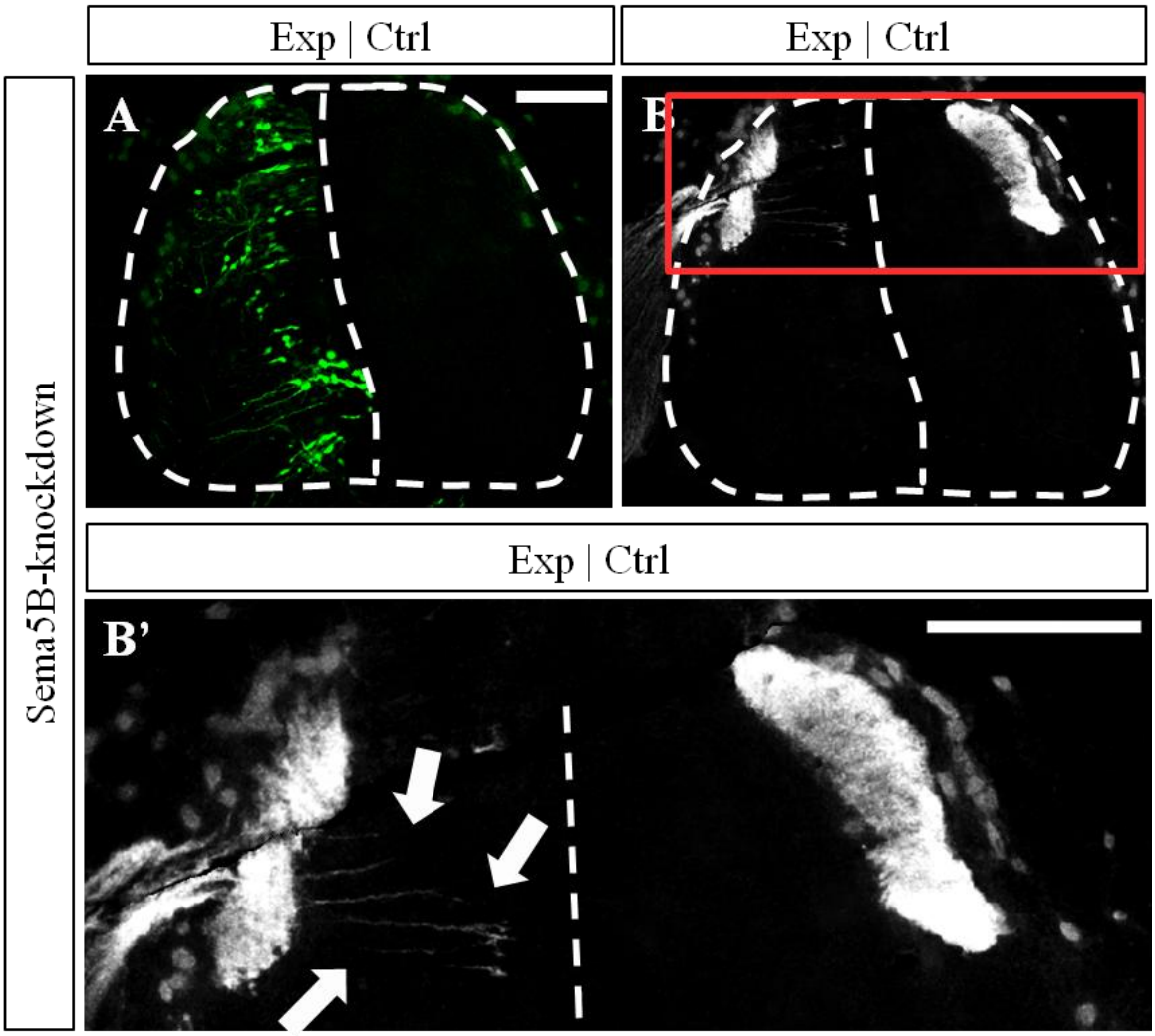
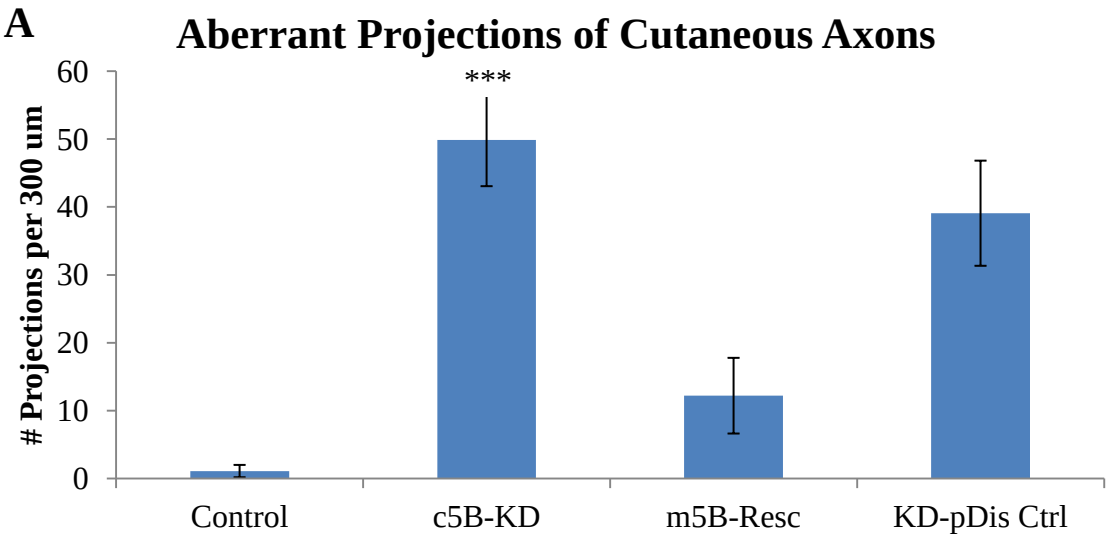
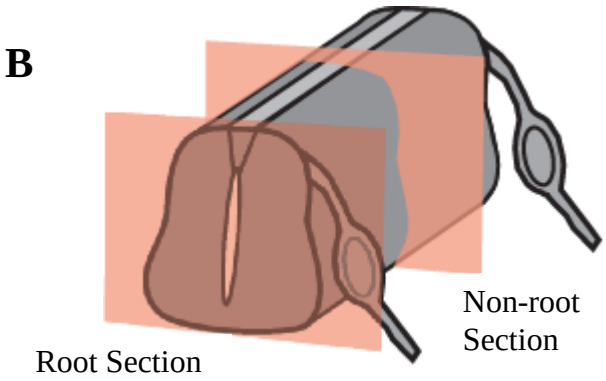


Figure 3- 6.2

Knockdown of Sema5B at E3.5 leads to premature entry of cutaneous afferents in the dorsal horn. Spinal cords electroporated with two RNAi constructs at E3.5 show positive unilateral transfection at E6 (A). TAG-1 expressing cutaneous afferents prematurely enter the spinal cord grey matter (B, B'). Boxed region in B is shown at higher magnification in B'. Arrows in B' indicate prematurely entering and mistargetting projections (B') originating from the dorsal roots and extending to the midline (dashed lines) at this stage. Scale bars = 100um.


A
Aberrant Projections of Cutaneous Axons
Projections per 300 um
60
50
40
30
20
10
0
***
Control
c5B-KD
m5B-Resc
KD-pDis Ctrl


B
Root Section
Non-root
Section

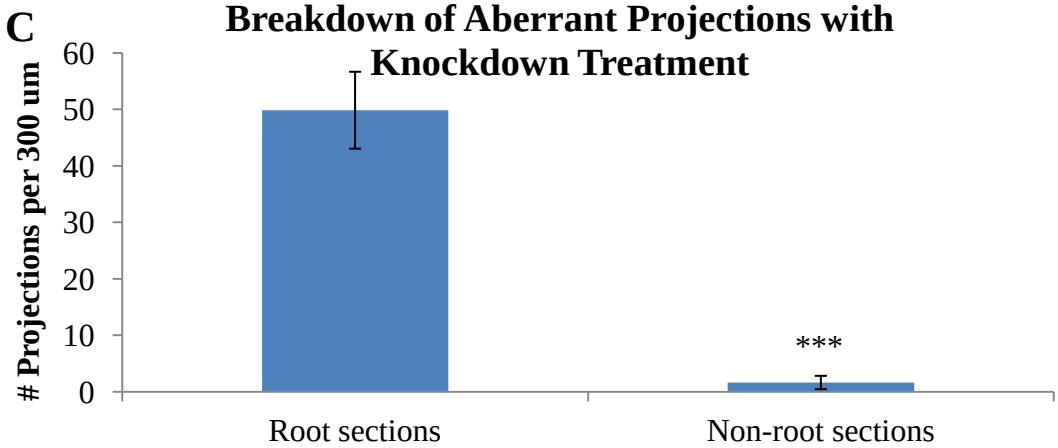

C
Breakdown of Aberrant Projections with
Knockdown Treatment
Projections per 300 um
60
50
40
30
20
10
0
***
Root sections
Non-root sections

Figure 3- 7

Figure 3-7

(A) Quantification of mean number of cutaneous axons in the dorsal grey matter show a significant increase in aberrant projections in spinal cords where the expression of Sema5B has been reduced compared to control. When the RNAi constructs were co-injected and transfected with mouse Sema5B DNA (c5B KD + m5B) into the spinal cord, the number of prematurely entering fibres returned to control levels. When knockdown constructs were co-injected and transfected with an empty pDisplay plasmid (c5B-KD + pDis), the knockdown phenotype was not rescued (n=4-10 animals per treatment over more than 3 independent experiments. Error bars represent SEM. Unpaired T-test: ***$p < 0.05$; $p=0.66$ between control and rescue treatments). Aberrantly projecting fibres were predominantly found in spinal cord sections where dorsal roots enter. (B,C) The number of aberrantly projecting axons in Sema5B-knockdown spinal cords were analyzed such that the number of fibres on sections where the dorsal roots enter the spinal cord (root sections in B) were compared to those on sections taken between dorsal roots (non-root sections in B). Significantly more fibres were found on root sections, suggesting that primary fibres are entering the grey matter without stalling or bifurcating. 88% of root sections show the presence of aberrantly projecting fibres compared to 15% in non-root sections (n=5 animals. Error bars indicate SEM. Unpaired T-test: ***$p<0.05$).

aberrant projections observed were located on root sections, whereas only 1% of aberrant projections were observed on non-root sections. These data suggest when axons first enter the DREZ they are normally inhibited to grow into the spinal cord by Sema5B expression.

When the shRNA constructs were co-transfected with HA-mSema5B constructs, the effect of prematurely targeting sensory fibres was eliminated. The rescued spinal cords showed a significant reduction in aberrant projections and were not significantly different than control spinal cords (Figure 3-7A, 3-8A,A'; $p=0.066$ when compared with control and $p<0.05$ when compared with shRNA transfected spinal cord). However, no rescue effect was observed when the shRNA constructs were co-transfected with an empty pDisplay vector (Figure 3-7A, 3-8-B,B'). These controls show the specificity of the shRNA constructs at reducing the expression of chick Sema5B, i.e., the observed phenotype was not due to off-target effects. Furthermore, the effect of rescue was not achieved as a result of dilution of knock-down constructs with the addition of another vector.

Proprioceptive Sensory Axons are not Affected by the Change in Sema5B Expression

To investigate whether Sema5B acts as a barrier to proprioceptive axon entry into the spinal cord, I labeled spinal cord section with anti-TrkC to examine whether proprioceptive axons aberrantly extended into the spinal cord after Sema5B knockdown. In contrast to the phenotype observed in nociceptive sensory fibres pathfinding, no TrkC labeled fibres were observed in the grey matter in either control or shRNA transfected spinal cords (Figure 3-9). This finding suggests that Sema5B is not necessary for directing the timing of proprioceptive sensory fibres entry into the grey matter.

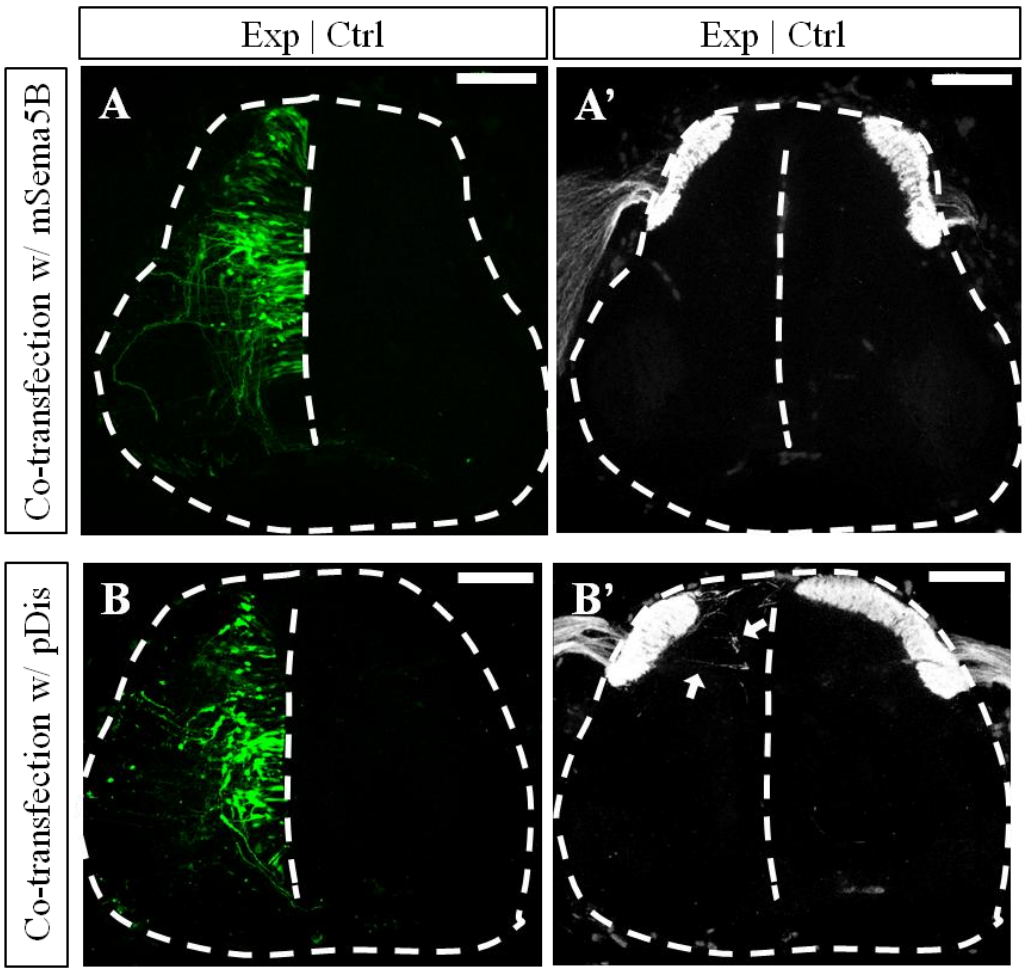


Figure 3- 8

Control experiments confirmed the specificity of the RNAi constructs used. When knock-down constructs were co-transfected with full-length mouse Sema5B DNA (A), the knock-down phenotype was eliminated in that no more prematurely entering cutaneous axons were observed in the transfected spinal cord (A'). To test whether the rescue effect observed was due to dilution of the amount of knock-down constructs, they were co-transfected with a empty pDisplay vector at a 1:1 ratio (B). Under these conditions I saw an aberrant phenotype with cutaneous fibres entering the dorsal horn before the normal time of entry (B'). Dashed lines outline the spinal cord and position of the midline. Scale bars = 100 um.

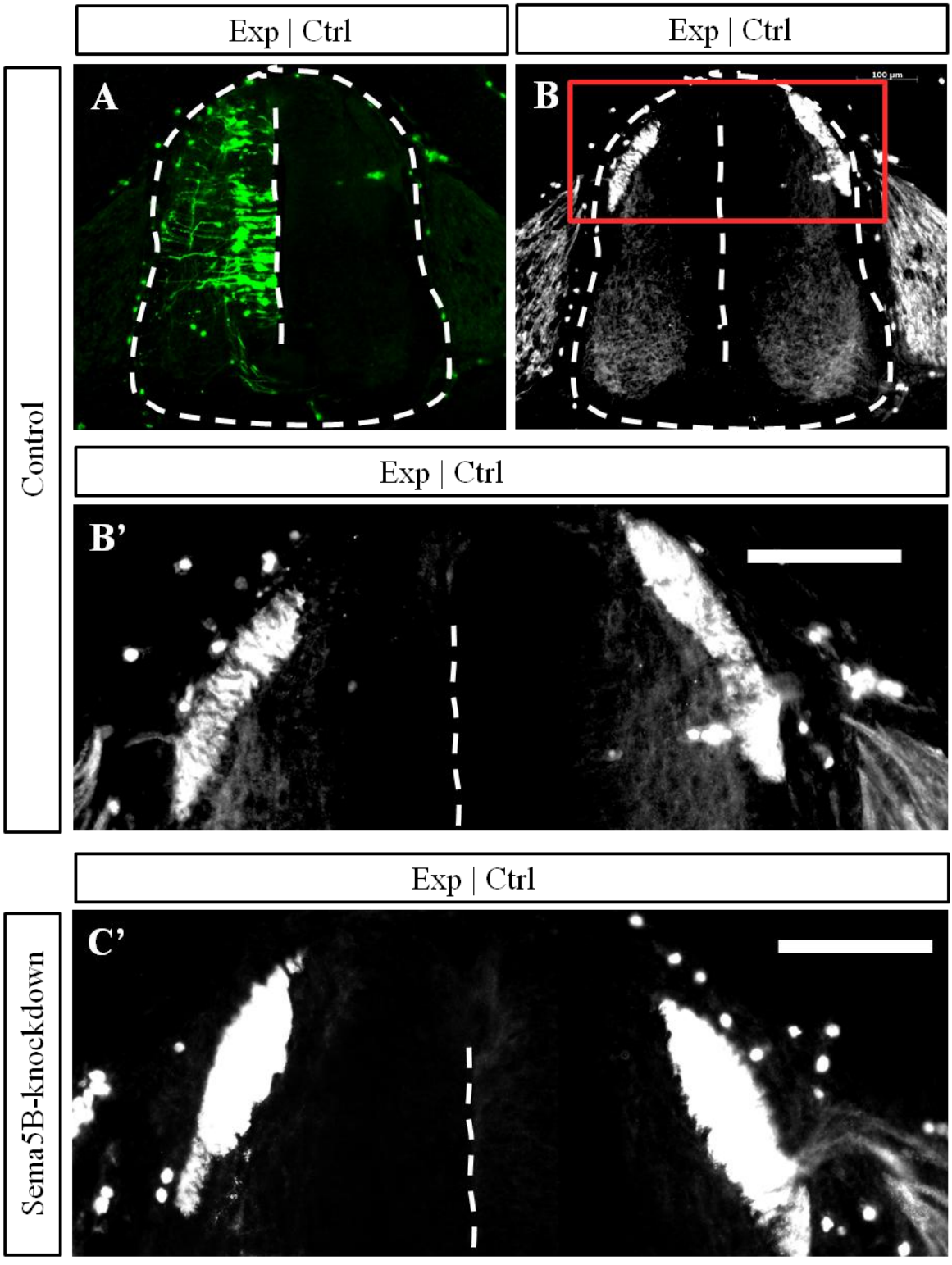

Exp | Ctrl
Exp | Ctrl
Control
A
B
100 µm
Exp | Ctrl
B'
Exp | Ctrl
Sema5B-knockdown
C'

Figure 3- 9

Figure 3-9

Knockdown of Sema5B at E3.5 does not affect the timing of proprioceptive afferent entry into the dorsal horn. Cryosections of spinal cords unilaterally electroporated with control (A-B') and Sema5B-knockdown (C') vectors at E3.5 and fixed at E6 are immunolabelled for TrkC (B, B',C') to show localization of muscle proprioceptive axons. In both control and Sema5B-knockdown transfected spinal cords, the dorsal funiculus is labeled but no fibres are observed in the dorsal horn grey matter. No distinguishable difference was observed in the position of axons at this time in the dorsal horn grey matter between the control and electroporated sides. Boxed region in B is shown at greater magnification in B'. Dashed lines outline the spinal cord and the midline. Scale bars = 100um.

Nociceptive Afferents Commit Pathfinding Errors in the Dorsal Horn in the Absence of Sema5B

In addition to acting as a barrier to early axon entry, I also tested whether Sema5B may function to correctly guide collateral fiber targeting once they have entered the spinal cord. To do this I reduced the level of Sema5B expression in the spinal cord at a later stage (E6) and examined the effect on nociceptive axons guidance at E8 when they are normally establishing contact with their correct targets. After decreasing the expression of Sema5B in the E6 spinal cord and analyzing TAG-1 labeled axons at E8 (st34), I saw pathfinding errors made by nociceptive collateral axons. In control spinal cords, TAG-1 positive collaterals extending from the Lissaur's tract remain confined to the lateral half of the dorsal horn (laminae I and II, Figure 3-10). In contrast, after transfection with shRNA targeting Sema5B, TAG-1 positive collaterals extend more medially and target the ventricular zone surrounding the central canal (Figure 3-11) similar to our observations when Sema5B was knocked down at earlier stages. Significantly more mistargeted fibres were found per 300 um when Sema5B was knocked down compared to control (Figure 3-12). These findings suggest that Sema5B is necessary for guiding the later arising nociceptive collateral afferents to their correct targets once they invade the grey matter.

Sema5B Functions Through TAG-1

Much experimental evidence from in vitro as well as in vivo systems has shown that there is a ventral spinal cord derived chemorepellent that functions though the immunoglobulin superfamily cell adhesion molecule (IgSF-CAM) TAG-1 (Law et al., 2008; Masuda and Shiga, 2005; Perrin et al., 2001; Sharma and Frank, 1998). Indeed, injection of TAG-1

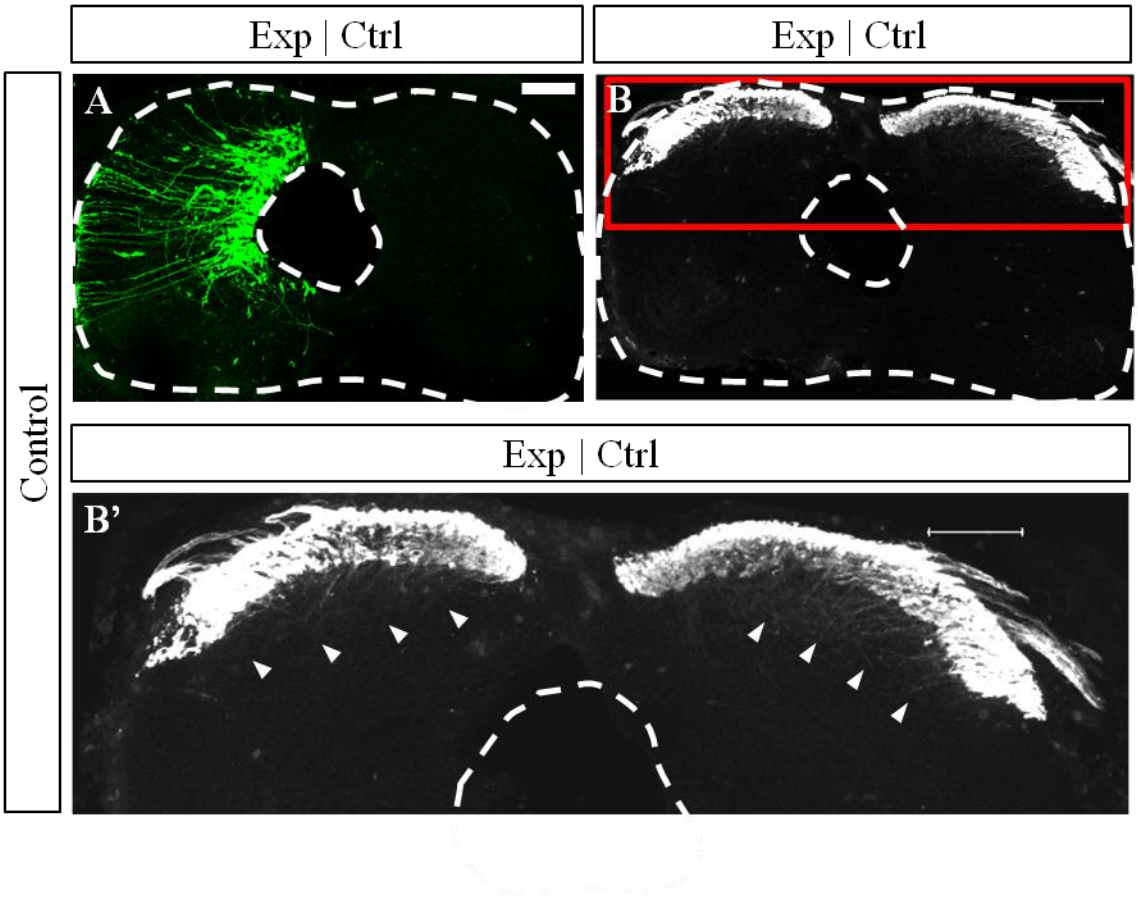


Figure 3- 10

Control-transfected spinal cords show normal sensory fibre growth at E8. Spinal cords electroporated with a control plasmid at E5.5-6 (st27) show positive unilateral transfection as per the expression of GFP in E8 (st34) spinal cord cross section (A). Cryosections are immunolabeled for TAG-1 for the visualization of cutaneous nociceptive fibres (B, B'). Normal short projections of nociceptive fibres were observed entering the lateral regions of the dorsal horn grey matter (arrow heads). No distinguishable differences in projection patterns in the transfected side compared to the untransfected side. Dashed lines outline the spinal cord and central canal. Scale bars = 100um.

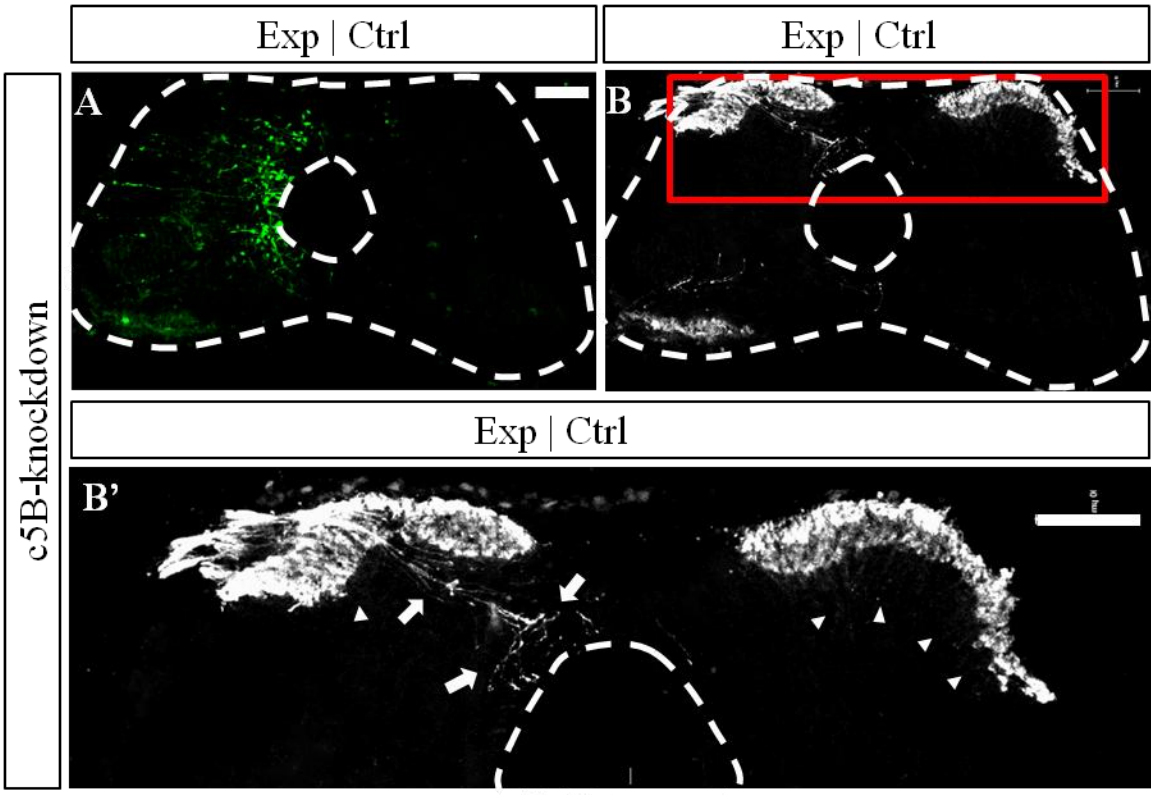


Figure 3- 11

Knockdown of Sema5B at E5.5-6 causes aberrant sensory fibre pathfinding in the dorsal horn at E8. Spinal cords were electroporated with RNAi constructs at E5.5-6 (st27) as shown by EGFP expression at E8 (st34) (A). Cryosections are immunolabeled for TAG-1 for the visualization of cutaneous nociceptive fibres (B, B'). Normal projection of nociceptive fibres were observed entering the lateral regions of the dorsal horn grey matter in the untransfected side, but aberrant projections are observed in the transfected side targeting to the ventricular zone surround the central canal (arrows in B'). Dashed lines outline the spinal cord and central canal. Scale bars = 100um.

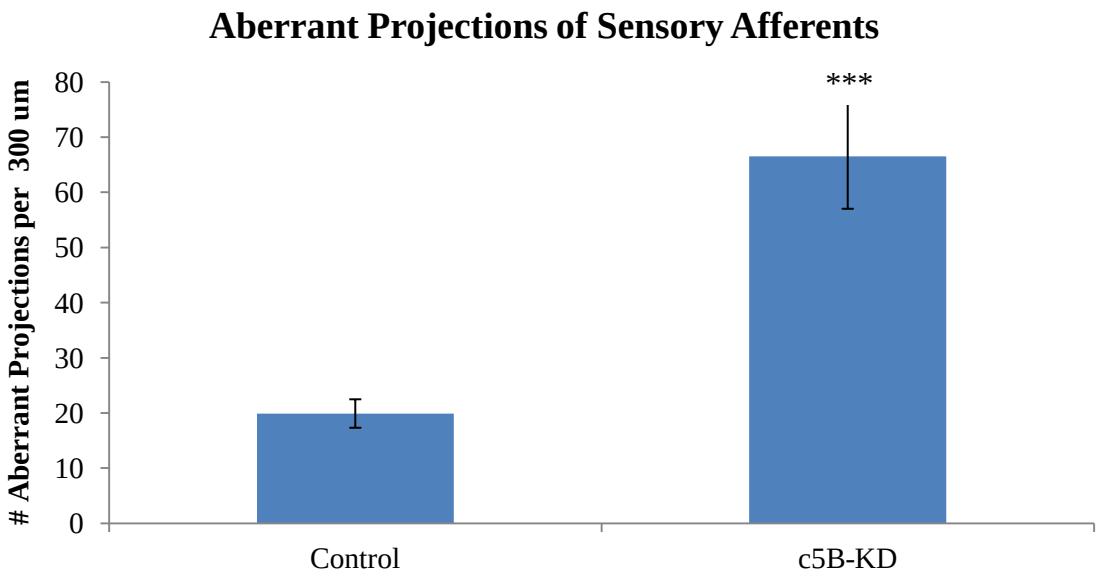


Figure 3- 12

Quantification of aberrant projections found in E8 spinal cords transfected at E5.5-6. Significantly more projections were found in spinal cords where Sema5B expression is reduced. n = 7 chicks for control and n =9 for Sema5B-knockdown. One tailed, unpaired student's T test: $p<0.05$.

function blocking antibodies into the central canal of the spinal cord resulted in aberrant pathfinding of sensory neurons similar to our observations (Perrin et al., 2001). Thus, based on Sema5B's expression pattern and the similarity between the phenotypes of TAG-1 knockout and that of Sema5B knockdown, I went on to test the possibility that Sema5B may function in part through the TAG-1 protein. I employed the same dissociated DRG overlay assay used previously but added a monoclonal mouse anti-TAG-1 antibody to some cultures in order to inhibit the function of TAG-1 on sensory axons. This antibody has been used previously to inhibit TAG-1 function (Lett, 2009). In both NGF-dependent nociceptive neuron cultures and NT-3-dependent proprioceptive neuron cultures, the inhibitory effect of Sema5B on axon outgrowth was observed as before; axons were around 50% shorter when cultured with Sema5B expressing cells. When the anti-TAG-1 antibody was added, the inhibitory effect of Sema5B on NGF-dependent fibres were completely eliminated (Figure 3-13A). However, when anti-TAG-1 antibody was added to NT-3-dependent cell cultures, no change in the inhibitory effect of Sema5B was observed (Figure 3-13B). These findings show that the inhibition of TAG-1 function is sufficient to relieve the inhibitory effect of Sema5B on TAG-1 positive nociceptive axons and support the hypothesis that Sema5B acts partly through TAG-1 to inhibit the outgrowth of these axons. Whereas TAG-1 is not expressed by NT-3-dependence proprioceptive axons (at the embryonic stage of these experiments), their repulsive effect to Sema5B was not rescued by the addition of an anti-TAG-1 antibody, suggesting the presence of a non-TAG-1 receptor complex to mediate the repulsive effect of Sema5B in vitro.

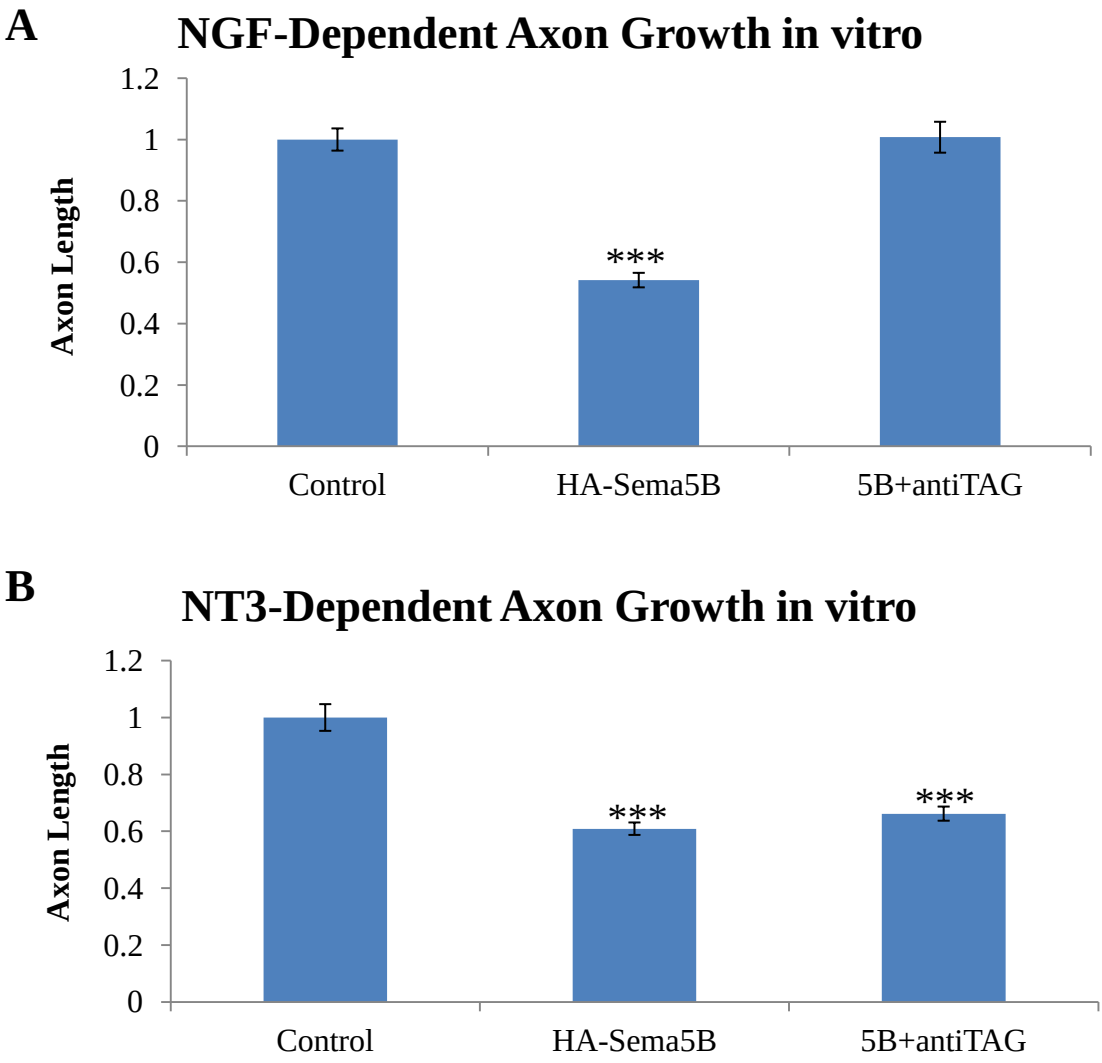


Figure 3-13

Comparison of axon lengths of cultured E6 DRG neurons in the presence of Sema5B and anti-TAG antibody. Inhibition of TAG-1 function eliminates the inhibitory effect of Sema5B on NGF-dependent cutaneous axons grown on Sema5B-expressing HEK293 cells (A) but does not affect Sema5B repulsion of NT3-dependent proprioceptive axons (B). n=57-135 axons measured per treatment over three experiments. *** Unpaired Student's T test: $p<<0.05$. (A) T test for control and antibody blocking experiments (5B+antiTAG) treatments: $p=0.475$.

Chapter IV: Discussion

Decades of research has begun to unveil the crucial role that guidance cues play in the process of axonal targeting during the development of the nervous system. Among the four canonical families of guidance cues, semaphorins make up the largest, consisting of membrane associated as well as secreted members, and have received increasing attention due to their implication in the large number of developmental processes and axon targeting disorders (Gherardi et al., 2004; Kruger et al., 2005; Neufeld et al., 2005; Pasterkamp and Kolodkin, 2003; Yazdani and Terman, 2006a). Previous data from our lab indicated that Sema5B, a member of the transmembrane class 5 semaphorins, is expressed in various nervous tissues of the developing chick embryo. In particular, the dynamic expression of Sema5B in the spinal cord bears a striking correlation with the timing and targeting of central sensory projections of DRG neurons (Davis et al., 1989; Eide and Glover, 1997; Legg, 2003; Masuda and Shiga, 2005; Mendelson et al., 1992). Based on these findings, I hypothesized that in the early stages of development, Sema5B acts as a repulsive cue and regulates the timing of sensory collateral formation by acting as a barrier in the grey matter; and once collateral fibres have entered the grey matter, Sema5B regulates their correct targeting in the dorsal horn of the spinal cord.

Results from the present series of experiments provide evidence for the ability of Sema5B to mediate sensory axon targeting in the developing spinal cord by acting as a guidance cue. *In vitro* assays show that different classes of sensory neurons can respond to the growth inhibitory effects of Sema5B over a significant time period during sensory system development and that Sema5B can act as a diffusible repulsive cue presumably through the establishment of a gradient. Furthermore, function analyses *in vivo* at two different time

points show Sema5B exerts its effect on both primary sensory axons and collateral fibres formed in the grey matter. Finally, an investigation into the mechanism by which Sema5B functions illustrates the likelihood that the immunoglobulin superfamily cell adhesion molecule TAG-1 is one component of the receptor complex and is necessary in modulating nociceptive axonal responses to Sema5B. Results presented here are the first evidence of the involvement of Sema5B in sensory circuit formation in the developing spinal cord.

Multiple Spinal Cord Derived Chemorepellent Cues act in Redundancy

The differential targeting of the central projections of different sensory neurons (despite their common origin from the DRGs), reflects the significance of guidance cues in orchestrating this process. It has been long known that the ventral spinal cord conveys repulsive effects on certain types of sensory afferents and it has been proposed that the differential effects of these cues guide axons to specific targets in the spinal cord (Fitzgerald et al., 1993). Subsequent studies have identified members of the semaphorin family to be the potential molecular mediators of such effects, particularly semaphorin-D in rodents, or its homologue semaphorin 3A (Sema3A) in chicks (Luo et al., 1993; Messersmith et al., 1995; Puschel et al., 1996; Wright et al., 1995). *In vitro* and *in vivo* work have shown that Sema3A is repulsive to nociceptive cutaneous fibres and exerts a dynamic repulsive effect on proprioceptive muscle afferents (Messersmith et al., 1995; Puschel et al., 1996; Shepherd et al., 1997). In addition, it was thought that both nociceptive and proprioceptive fibres enter the dorsal horns only after downregulation of Sema3A (Messersmith et al., 1995). Subsequently, while remaining Sema3A expression in the ventral horns prevents cutaneous fibres from entering the ventral spinal cord, proprioceptive muscle afferents downregulate a necessary component of the Sema3A receptor – neuropilin-1 (Nrp1) - and target to the

ventral horns of the grey matter owing to a loss in sensitivity to Sema3A (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997; Shepherd et al., 1997).

However, several lines of evidence show that additional repulsive cues exist in the spinal cord to modulate the process of sensory axon entry and targeting. First, Sema3A mRNA is not detected in the spinal cord during initial DRG axon growth and is expressed 12-24 hours after the arrival of DRG axons at the spinal cord (Masuda and Shiga, 2005; Masuda et al., 2003; Shepherd et al., 1996). The lack of entry of sensory fibres into the grey matter during this period suggests the presence of another chemorepellent. In addition, mice lacking Sema3A or components of its receptor complex (Nrp1, PlexA3/A4) show normal axon projection patterning into the CNS (Behar et al., 1996; Catalano et al., 1998; Gu et al., 2003; Kitsukawa et al., 1997; Taniguchi et al., 1997; Yaron et al., 2005). Last, when DRG explants are co-cultured with ventral spinal cords taken from Sema3A knock-out mice, the presence of a sensory axon repellent is still observed (Masuda et al., 2003). Together, these results all suggest that an additional repulsive cue exists in the spinal cord that may functionally combine with Sema3A to mediate sensory axon guidance and targeting.

Previously, Legg et al. have examined the expression of Sema5B in the chick and found that it is similar to the previously published expression patterns of Sema5B in rodents and suggested this protein plays complex roles during neural development (Adams et al., 1996; Legg, 2003; Medina et al., 2004; Skaliora et al., 1998). Of particular interest is that the dynamic expression of Sema5B in the chick spinal cord correlates with the temporal regulation of sensory axon collateral formation and thus suggests its involvement in the guidance of sensory axons into the spinal cord. Sema5B mRNA was detected in the chick spinal cord as early as E3, considerably earlier than Sema3A, and thus supporting its role as

the repellent cue preventing premature entry of sensory axons. Sema5B expression was seen as late as E17, corresponding to the time when majority of the spinal cord development is complete (Eide and Glover, 1997; Legg, 2003). In addition, considerable evidence suggested that Sema5B may function as a repulsive guidance cue to a variety of neuronal populations (Lett et al., 2009; Matsuoka et al., 2011; O'Connor et al., 2009; Wood, 2006). Thus, given the expression data and known functions of Sema5B, I proposed that Sema5B may function as a chemorepellent cue that regulates sensory axon guidance in the spinal cord.

Semaphorin 5B Shows Differential Effects *in vitro* and *in vivo*

Since the DRG harbors a heterogeneous population of sensory neurons that innervate different peripheral and central targets (Snider and Wright, 1996), it is likely that they respond differentially to different guidance molecules in order to reach their distinct final targets. To test if Sema5B can differentially regulate neurite development of different sensory neurons and whether there is a temporal dependence to this effect, I examined whether Sema5B can affect neurite outgrowth in dissociated DRG neurons of specific classes taken from chicks at different developmental stages. NGF was used to select for the survival and growth of TrkA- and TAG-1-expressing cutaneous nociceptive neurons in culture and NT-3 was used to select for the survival and growth of TrkC-expressing muscle proprioceptive neurons. This co-culture technique offers a robust and direct way of analyzing the effect of Sema5B on neurite development, and was used by Matsuoka et al. to study the effect of Sema5A and Sema5B on embryonic retinal neurons (Matsuoka et al., 2011). I found that Sema5B exerts a similar inhibitory effect on both types of DRG neurons tested, as it does on retinal neurons *in vitro*. The mean neurite length of both types of sensory neurons

cultured on stable Sema5B-expressing HEK293 cells was significantly shorter than those on control cells.

Interestingly, Sema5B had almost the exact same inhibitory effect on the outgrowth of the two types of sensory neurons tested when using the *in vitro* dissociated neuron assay (Figures 3-1 and 3-2). However, from my *in vivo* analysis of Sema5B function using reduction-of-function experiments employing RNA interference, cutaneous axons (labeled with anti-TAG-1) were observed to enter the spinal cord prematurely and display aberrant targeting after the reduction of Sema5B, whereas no such phenotype was observed for proprioceptive axons (labeled with TrkC). The observations for the former axon type is in agreement with the hypothesis and *in vitro* data suggesting Sema5B creates a barrier at the edge of the spinal cord and prevents entry of cutaneous axons at inappropriate times by acting as an inhibitory cue. Even though the growth of proprioceptive axons was inhibited *in vitro*, there was a lack of an *in vivo* phenotype after a decrease of Sema5B.

Why is there a reduction of proprioceptive fiber outgrowth when grown on Sema5B, but no phenotype is observed when Sema5B is knocked down? It is known that proprioceptive and nociceptive axons are repelled by numerous inhibitory cues including Sema5B. Thus, it is not surprising that culturing neurons on cells that over express an inhibitory molecule, in this case Sema5B, resulted in a reduction of outgrowth. In contrast, while reducing the levels of Sema5B may reduce the balance of inhibition sufficiently enough for nociceptive fibers to prematurely extend into the spinal cord, it is apparent that this reduction is not sufficient to allow proprioceptive fiber entry. This would suggest that other repellents are significantly more repulsive for proprioceptive fibers. This difference in nociceptive/proprioceptive sensitivity to Sema5B is also partly reflected in their receptor

components as Sema5B appears to signal through different receptor complexes on the two neuronal populations. The results of these experiments illustrate the importance of performing *in vivo* functional analyses to complement *in vitro* experiments.

TAG-1 Mediates Differential Effects of Sema5B on Cutaneous and Muscle Afferent in the Spinal Cord

The immunoglobulin superfamily cell adhesion molecule TAG-1 is an interesting candidate for mediating subpopulation-specific sensory axon guidance in the spinal cord (Perrin et al., 2001). As described by Zuellig et al., TAG-1 is linked to the cell membrane by a glycosylphosphatidylinositol (GPI) anchor (Zuellig et al., 1992), and can bind homophillically to other TAG-1 molecules on adjacent cells (Freigang et al., 2000; Rader et al., 1993). TAG-1 has also been shown to interact with a variety of other cell adhesion molecules such as L1 (Kuhn et al., 1991; Lemmon et al., 1989), NgCAM-related cell adhesion molecule (Nr-CAM) (Stoeckli et al., 1997; Suter et al., 1995), and neural cell adhesion molecule (NCAM) (Milev et al., 1996). In addition, TAG-1 has been implicated in neuronal developmental processes including cell adhesion, axon fasciculation and myelination, and has recently been shown to play a role in axonal signaling (Chatzopoulou et al., 2008; Law et al., 2008; Perrin et al., 2001). Friedel et al. were the first to show that Tag-1 was a differentially expressed gene DRG neurons in a screen comparing NGF- and NT-3-dependent sensory neuron expression profiles (Friedel et al., 1997). This finding was then supported by immunohistochemical analyses of chick sensory axons by Perrin et al., (and others) who showed that TAG-1 is expressed by all cell bodies and axons of DRG neurons during early stages of development but its expression then becomes restricted to the NGF-

dependent, TrkA-expressing nociceptive fibres after E4.5 (Perrin et al., 2001; Snider and Silos-Santiago, 1996). The timing of this dynamic TAG-1 expression by the two axon types is in accordance to the "waiting period" during which axons are kept out of the spinal cord grey matter. This temporal correlation provides support for TAG-1 as a component of the receptor complex for mediating the inhibitory effect of sensory axons to Sema5B.

Additional work by Perrin et al., has also shown that TAG-1 is required for correct nociceptive cutaneous axon targeting in the dorsal part of the spinal cord. They found that after injection of function blocking antibodies against TAG-1 into the cerebral aqueduct of the developing spinal cord that nociceptive axons projected aberrantly into the dorsal horn (Perrin et al., 2001). Strikingly, the phenotype observed when TAG-1 function is perturbed is extremely similar to the phenotypes I observed when Sema5B was knocked down in the developing spinal cord. Specifically, nociceptive axons projected prematurely into the dorsal horn grey matter (1 day before the normal time of collateral formation) and grew aberrantly toward the midline, dorsal to the central canal (lamina III region) instead of innervating laminae I and II as seen in control animals. In contrast, and similar to my findings, the proprioceptive fibres were not affected by the function blocking TAG-1 antibodies (Perrin et al., 2001).

In 2008, Law et al. examined the role of TAG-1 in regulating sensory axon responses to diffusible guidance cues in mice by following pathways taken by sensory afferents in TAG-null mice. Similar to observations in the chick, they first showed that TAG-1 is expressed in all DRG neurons and their axons until the time of axon arrival at the dorsal root entry zone (E10.5 in mice), but by E12.5 TAG-1 expression is restricted in TrkA expressing nociceptive fibres, 1 day before TrkA- (TAG-1-) proprioceptive collateral extension into the

dorsal horn (Law et al., 2008). It is of interest to note that in rodents, NT3-dependent proprioceptive collaterals form in the dorsal horn earlier and more medially than NGF-dependent nociceptive fibres (Mirnics and Koerber, 1995; Ozaki and Snider, 1997). Law et al., observed similar phenotypes in the TAG-1 null mice as were observed in the chick after TAG-1 antibody injections or Sema5B knockdown. They observed premature projections of cutaneous axons in TAG-1 null mice, particularly focused around points of dorsal root entry. These authors argued that TAG-1 is required on sensory axons to mediate their response to a non-Sema3A diffusible repellent guidance cue(s) found in the spinal cord, although they had not identified the specific cue(s) (Law et al., 2008). This argument was supported by two additional *in vitro* experiments. First, NGF-dependent axons from TAG-1-null mice completely lost their sensitivity to ventral spinal cord-derived chemorepellents (Law et al., 2008). Second, when wild-type NGF-dependent axons were cultured with ventral spinal cord in the presence of soluble neuropilin-1 (Sema3A receptor/function blocker), repellent activity was diminished as shown previously, but significant repulsion remained (Law et al., 2008). Together, these experiments provided clues to the presence of another chemorepellent in the ventral spinal cord that acts through TAG-1 and was yet to be described (Law et al., 2008).

Given these lines of evidence, I tested the hypothesis that Sema5B is the diffusible chemorepellent cue found in the ventral spinal cord and mediates its function in part through TAG-1. First, the ability of Sema5B to act as a diffusible cue was demonstrated using an assay where DRG explants were cultured in a three dimensional matrix at a distance from HEK293 cell aggregates expressing Sema5B. Overnight cultures showed that the growth of DRG axons on the side proximal to the cell aggregates were inhibited compared to those on the side distal to the cell aggregates. Next, using dissociated DRG neuron cultures, I

demonstrated that an anti-TAG-1 antibody eliminated a Sema5B-induced neurite outgrowth inhibition. In contrast, when added to NT3-dependent DRG neurons not expressing TAG-1, the anti-TAG-1 antibody was not sufficient to block the inhibitory effect of Sema5B further demonstrating that TAG-1 is required to mediate Sema5B function on nociceptive fibers. Finally, my *in vivo* data presented here provided further evidence that TAG-1 expressing cutaneous fibres are affected by the reduction of Sema5B in the spinal cord. It is of interest to note that compared to previous studies examining the TAG-1 regulation of sensory fiber entry into the spinal cord, my knockdown of Sema5B showed a considerably more robust phenotype. Namely, Perrin et al. found around 40 pre-mature projections per 600 um of examined spinal cords at E5 in chick (Perrin et al., 2001) and Law et al. found around 18 and56, fibers per 600 um of spinal cord of TAG-1 knockout mice at E12.5 and E13.5 respectively (Law et al., 2008)(roughly corresponding with E5 and E6, in the chick in terms of the timing of TAG-1 expression change and sensory axon entry in the spinal cord). In comparison, my results shown here were consistently more robust, showing around 100 pre-mature projections per 600 um of spinal cord at E6 in the chick. This difference could be due to a number of factors. First, even though the loss of TAG-1 function can completely eliminate the repulsive effects of Sema5B *in vitro*, it does not eliminate the fact that Sema5B could have other binding partners (receptors) *in vivo* through which its effects are exerted. Another binding partner/receptor component could act in redundancy with TAG-1 to mediate Sema5B function and allow partial function of Sema5B at the loss of TAG-1. Second, there is a difference in techniques used in the above mentioned studies. RNAi could be causing a more efficient knockdown of Sema5B compared to the blockage of TAG-1 using function blocking antibodies. Although this difference would have been eliminated in null mice used

in the Law et al. (Law et al., 2008) study, there may be subtle differences in the comparison between chick and mouse spinal that could explain the slight difference in effect. Finally, my analysis of prematurely projecting fibres at this stage was done at E6, 24 hours after the stage of analysis in Perrin et al. and 12 hours before the last stage of analysis in Law et al. (Law et al., 2008; Perrin et al., 2001). Should Perrin et al. have carried out their experiments for one more day, they may have observed more prematurely projecting axons. On the other hand, I observed twice the aberrantly projecting axons seen by Law et al. at the same age of analysis This may reflect the advantages of temporally controlling the knockdown of protein expression with RNAi as it does not allow for compensation of other proteins as might be observed in a mouse knockout.

Patterning of Initial Trajectories of Sensory Axons

In higher vertebrates, sensory axons of DRG neurons take a stereotyped path, which is conserved across different species, to reach the spinal cord at the dorsal root entry zone. Previous reports of DRG axon pathfinding changes after the neural tube has been rotated in ovo suggests that the DREZ plays a possible chemoattractive role for DRG axons (Stern et al., 1991; Tanaka et al., 1990). Although this chemoattractive effect has not been demonstrated using organotypic co-cultures of so far, the presence of a non-diffusible attractive cue at the dorsal root entry zone or along the pathway between that and the DRG has not been ruled out (Keynes et al., 1997; Masuda and Shiga, 2005; Nakamoto and Shiga, 1998). Another possibility for the orientation of the initial pathway of DRG axons is the presence of an unidentified attractive cue found near the midline of the spinal cord. Whatever makes up the attractive activity, the initial DRG axonal trajectories are shaped along with inhibitory guidance cues such as Sema3A, CSPGs and those yet unidentified, which are

derived from the dermatome, the ventral spinal cord, and the notochord (Keynes et al., 1997; Masuda et al., 2003; Nakamoto and Shiga, 1998). Based on work reviewed in Masuda and Shiga et al., it was argued that an unidentified diffusible repellent cue derived from the spinal cord mediates the early pathway formation of sensory axons through the function of TAG-1 (Masuda and Shiga, 2005).

Expression data discussed earlier shows that Sema5B is present early in the spinal cord (at E3) which can mediate the projection of initial sensory fibres targeting the DREZ. *In vivo* functional analysis data presented here suggest that Sema5B then acts as the barrier at the border of the dorsal grey matter for DRG primary axons that have reached the DREZ at a right angle to the length of the spinal cord, i.e., primary axons first reach the spinal cord along the lateromedial axis. At this time, sensory axons need to bifurcate and travel along the rostrocaudal axis of the animal in order to form the dorsal funiculus /Lissaur's tract to relay signals to the brain. The Sema5B barrier function presumably facilitates the turning of the growth cones at this time by preventing and further mediolateral growth and thus ensuring they turn 90 degrees onto their subsequent pathway of rostrocaudal growth. When this barrier is taken away or weakened by the reduction of Sema5B, cutaneous axons are observed to enter the grey matter prematurely at this first site of spinal cord entry and presumably lose the stimulus to bifurcate (Figure 4-1). This was evident from the data that showed that significantly more aberrant projections are present in root sections where the dorsal root entry zone can be traced back to the dorsal roots, whereas aberrant projections were rarely seen in sections taken between two adjacent dorsal roots. Last, in general the aberrantly projecting fibres were observed to target directly to the midline/ventricular zone, a region avoided by normally projecting cutaneous axons. This is consistent with the presence

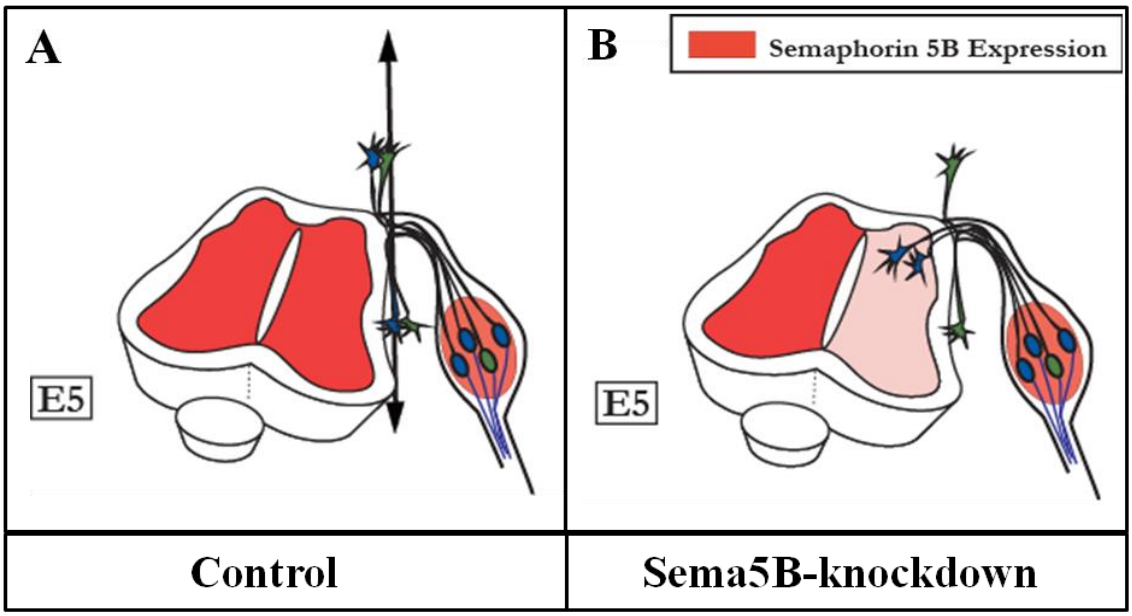


Figure 4- 1

Schematic representation of phenotype observed when Sema5B expression is knocked down in the chick spinal cord during development. Knockdown of Sema5B at E3.5 (right side of B) is sufficient to cause cutaneous afferents (blue) to enter the dorsal horn prematurely without bifurcation while proprioceptive afferents (green) are not affected.

of an attractive cue at the midline that is normally masked by the presence of Sema5B. Thus, Sema5B may provide the essential guidance needed for sensory axons in the developing spinal cord. First, it may act as a repulsive barrier for the first arriving sensory fibers in order to stimulate their bifurcation and extension along the rostral caudal axis and second, it may provide an inhibitory cue that is essential to provide the necessary balance between attraction and inhibition for nociceptive fibers such that they connect to their appropriate targets in the dorsal horn.

Conclusion

This study was the first to investigate the function of Sema5B in the developing spinal cord. *In ovo* reduction-of-function studies were supplemented with *in vitro* co-culture assays to establish the role of Sema5B as a repulsive barrier for centrally projecting sensory axons first arriving at the dorsal spinal cord. Sema5B has been shown to regulate the timing of sensory afferent entry in the dorsal horn and the targeting of sensory afferents to their appropriate targets post entry. An investigation into the mechanism has shown that the cell adhesion molecule TAG-1 mediates the function of Sema5B. Future studies should focus more on the identification of the receptor (receptor complex) and the antibody blockage experiment described in this thesis offers a good starting point experiment.

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