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Molecular Mechanisms of Axon Guidance in the Developing Spinal Cord

A dissertation submitted in partial satisfaction of the requirements for the degree
Doctor of Philosophy

in

Biology

by

Liseth M Parra

Committee in charge:

Professor Yimin Zou, Chair
Professor Marilyn G. Farquhar
Professor Anirvan Ghosh
Professor Eduardo Macagno
Professor Barbara Ranscht
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2009

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Chair

University of California, San Diego

2009

DEDICATION

I dedicate my dissertation work to my family and my many good friends. A very special feeling of gratitude to my loving parents, Pablo Parra and Edy Rojas for always believing in me and whose words of wisdom and encouragement help me get through everyday life. I hope I have made you proud. To my siblings, Daniel, Elly and Leicy for never leaving my side and for loving me no matter what, I appreciate what you have done for me and will always have you in a place very close to my heart. Because of all of you, I am truly blessed.

EPIGRAPH

“...La porción anterior del cuerpo celular de las células comisurales se prolonga en largo cono que se va paulatinamente adelgazando, hasta convertirse en fibra nerviosa y se termina por un engrosamiento ya simplemente redondeado y poco aparente ya representado por un grumo cónico de base periférica. Este grumo terminal, que llamaremos cono de crecimiento, presenta, a veces, finas expansions cortas, espinosas y divergentes, que el cromato de plata tiñe de amarillo de canela; otras ofrece prolongaciones triangulares, laminosas, que parecen insinuarse entre los demás elementos, fraguándose a viva fuerza un camino por el cemento intersticial”

Ramón y Cajal, 1890

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LIST OF ABBREVIATIONS

AKAP	A-kinase Anchoring Protein
ALS	Anterior Lateral System
AP	Alkaline Phosphatase
A-P	Anterior-Posterior axis
BCIP	5-Bromo-4-Chloro-3-Indolyl Phosphate
BDNF	Brain Derived Neurotrophic Factor
BMPs	Bone Morphogenic family of Proteins
Boc	Cdon binding protein
C	Central domain
cAMP	Cyclic Adenosine Monophosphate
CAMs	Cell Adhesion Molecules
DCC	Deleted in Colorectal Cancer
DIC	Differential Interference Contrast microscope image
DiI	1, 1'-Dilinoleyl-3, 3, 3'3'-tetramethylindocarbocyanine perchlorate
DMSO	Dimethyl Sulfoxide
dnPA3	dominant negative PlexinA3
DRG	Dorsal Root Ganglia
drl	Derailed
D-V	Dorso-Ventral axis
E	Embryonic day

ECM	Extracellular Matrix membrane
Ena/VASP	Enabled Vasodilator Stimulating Protein
FGFs	Fibroblast Growth Factors
FRET	Fluorescence Resonance Energy Transfer
GαI	G-coupled Inhibitory proteins
GDF7	Related Growth Differentiation Factor 7
GDI	Guanine nucleotide Dissociation Inhibitor
GFP	Green Fluorescent Protein
GPCR	G-protein Coupled Receptor
GTPases	Guanosine Triphosphatases
Hh	Hedgehog
Hip	Hedgehog-interacting protein
HLH	Helix-Loop-Helix
HNF-3β	Hepatocyte Nuclear Factor 3Beta
MTGs	Mammalian Myeloid Translocation Genes
NBT	Nitro Blue Tetrazolium
NP1	Neuropilin1
NP2	Neuropilin2
P	Peripheral domain
PA3	PlexinA3
PCR	Polymerase Chain Reaction
PI3K	phosphatidylinositol-3-Kinase

PKA	Protein Kinase A
PKA RIIα	Regulatory Subunit of Type II PKA
PTC1	Patched1
RGC	Retina Ganglion Cells
Robo	Roundabout
RYK	Atypical tyrosine Kinase receptor
SDF-1	Chemokine Stromal Cell-Derived Factor-1
sFRPs	secreted Frizzled-related Proteins
Shh	Sonic Hedgehog
Smo	Smoothened
TAG-1	Axonal surface glycoprotein
TGFβ	Transforming Growth Factor Beta
WASP	Wiskott-Aldrich Syndrome Proteins
WNTs	Wingless

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ABSTRACT OF THE DISSERTATION

Molecular Mechanisms of Axon Guidance in the Developing Spinal Cord

by

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Doctor of Philosophy in Biology
University of California, San Diego, 2009

Professor Yimin Zou, Chair

The formation of intricate neural circuits requires that nascent axons navigate the complex environment of the developing embryo and precisely determine the trajectories that would lead them to their final synaptic targets. Although many guidance cues and their receptors have been identified, the intracellular signaling cascades that control and direct axonal growth remained to be fully understood. Developing axons constantly reassess the immediate environment to distinguish between a myriad of guidance molecules that are simultaneously present along their trajectory. Exactly how the axon interpret these signals at a specific time and space are limited, thus understanding how guidance cues work in concert during axonal

pathfinding is a major goal in developmental neurobiology and in the regeneration of axonal connections after injury and disease.

In this dissertation we studied the pathfinding of spinal cord commissural axons to understand the molecular mechanisms underlying changes in axonal responses from attraction to repulsion during floor plate crossing at the spinal cord ventral midline. Here, we found that chemorepellent signals emanating from floor plate cells of the Slit and class 3 Semaphorin family of proteins synergize *in vitro* to repel precrossing commissural axons possibly through Neuropilin2 pathways. In addition, we propose that Sonic Hedgehog (Shh) plays a pivotal role in mediating commissural axon repulsive guidance during floor plate crossing. We provide evidence that Shh is a regulator of class 3 Semaphorin signals in precrossing axons and specifically show that knockdown of Shh pathway components, Patched1 (PTC1) and Smoothened (Smo) disrupted proper guidance of commissural axons during floor plate crossing. We also show that both Smo and PTC1 are expressed around the developmental stages we studied and are key determinants of this regulation. The third major finding of this dissertation is that the activation of Semaphorin3 signals by Shh might be induced through the alteration of cAMP/PKA levels via Smo dependent activation of inhibitory G alpha proteins.

Together the data presented in this dissertation propose new exciting models in which axon guidance regulatory pathways can induce profound changes in axonal navigational responses by successfully integrating and interpreting signaling inputs from external and internal axon guidance systems.

CHAPTER 1

The Development of the Nervous System: Molecular Mechanisms of Circuit Formation

Introduction

The mature nervous system controls the way we perceive and respond to our environment using single cell units known as neurons and the connections between them. In the late 1800's the pioneer neuroanatomist Santiago Ramón y Cajal revealed that the nervous system was composed of millions of individual neurons using a novel silver nitrate stain created by Camillo Golgi and for the first time in history showed a clear appreciation of the structure and the organization of the nervous system (de Castro, Lopéz-Mascaraque and De Carlos, 2007).

The human nervous system is composed of around 100 billion neurons and their extensions and over 100 trillion synapses organized into specialized functional anatomical circuits that control behavioral responses. This intricate structure is assembled with extraordinary precision during embryonic development and despite the great diversity in the animal kingdom the developmental programs that control the formation of neural circuits are highly conserved among species (Dickson and Gilestro, 2006).

The growth and plasticity of the young mammalian nervous system that enables it to become a highly specialized structure is heavily depended on the progressive regulation of molecular programs intrinsic to developing neurons and their successful responses to extracellular signals present in the embryonic environment

(Yu and Bargmann, 2001). In the early embryo, three germ layers are formed, the endoderm, the mesoderm and the ectoderm layer, which through complex cell interactions and growth, will arrange themselves into the tissues and organs to give rise to a whole organism. The outer most germ layer comprises the dorsal ectoderm, which later forms the entire nervous system by faithfully responding to signals present in the developing environment. In response to these signals, neural progenitors are first recruited into a structure known as the neural plate, which later folds to become the neural tube. As development progress, the most caudal region of the neural tube gives rise to the spinal cord, whereas the more rostral regions of the neural tube become the hindbrain, midbrain and most anteriorly, the brain.

Neural Specification

Proper neuronal specification during early development is essential for establishing highly ordered cellular organizations in the nervous system (Jessell, 2000). Immediately after neural tube formation, spinal cord neurons are segregated into specific dorsal-ventral domains of neural cells, which are induced dorsally by signals emanating from the roof plate and ventrally by signals emanating from the notochord and later the floor plate. The notochord is a transient mesodermal structure that secretes the Sonic Hedgehog (Shh) protein, a fundamental regulator of various aspects of development and responsible for directing the pattern of neurogenesis by conferring positional information to ventral neural progenitors (Dessaud, Mchanon and Briscoe, 2008). The notochord also regulates the development of floor plate cells,

a specialized class of ependymal-like glial cells located at the ventral midline of the developing spinal cord just above the notochord. Floor plate cells act as a second organizing center of the developing spinal cord by also secreting Shh, and together with the notochord help position and differentiate ventral progenitor cells into specific cell types of motor neurons (Jessell, 2000). Furthermore, at the dorsal midline of the spinal cord, a secondary signaling center is established, the roof plate, which induces the fate and position of dorsal progenitor cells dictated by signals of the transforming growth factor beta superfamily of proteins (TGF β), especially the actions of the bone morphogenic family of proteins, BMP4 and BMP7 (Butler and Dodd, 2003). Interestingly, Shh and BMP signals both induce the position and diversity of neural cell types in the developing spinal cord as a function of their concentration (as they diffuse away from signaling centers) in a gradient of activity, a special characteristic that classifies these proteins as morphogens (Dessaud, McMahon and Briscoe, 2008).

Axonal Navigation

Axonal navigation, just like the migration and specification of the entire neuron is guided by the interaction of the developing neuron with the embryonic environment. Shortly after acquiring their neural fate, postmitotic spinal cord neurons begin to send stereotypical axonal projections on defined pathways that will eventually lead to a final synaptic target (Song and Poo, 2001). Ramón y Cajal described the leading terminal of the growing axon as a “battering ram” on a journey, “opening up a path through the interstitial cement”, an axonal swelling he named, the growth cone

(“cono de crecimiento”) (Ramón y Cajal, 1890). It is now well recognized that the growth cone is a motile sensory structure located at the tip of the axon, that detects environmental signals and transduces them into a specific navigational response of axon guidance and elongation (**Fig. 1.1**) (Chilton, 2006). The growth cone is composed of cytoskeletal elements (microtubules, rich actin fibers), mitochondria and other organelles anatomically divided into two prominent compartments, a central core or central domain (C) and a peripheral domain (P) which is composed of a veil like structure known as lamellipodia and finger like protrusions known as filopodia. The central core of the growth cone is composed of microtubules from the distal end of the axon shaft along with other organelles and vesicles, whereas the lamellipodia and filopodia contain pools of highly dynamic networks of actin filaments, cross-linked actin fibers and rich bundled F-actin respectively, which constitute the leading edge actin-based cytoskeleton of the growth cone (Gallo and Letourneou, 2003; Huber, Kolodkin and Ginty, 2003). During axon guidance, the growth cone undergoes cytoskeletal changes that allows it to expand or retract according to the information received and appropriately navigate axons towards the favored direction. Not surprisingly, the growth cone’s cytoskeleton (e.g. actin fibers and microtubules) is known to be a major determinant of axonal behaviors and a major intracellular target for axon guidance signals (Gallo and Letourneou, 2003).

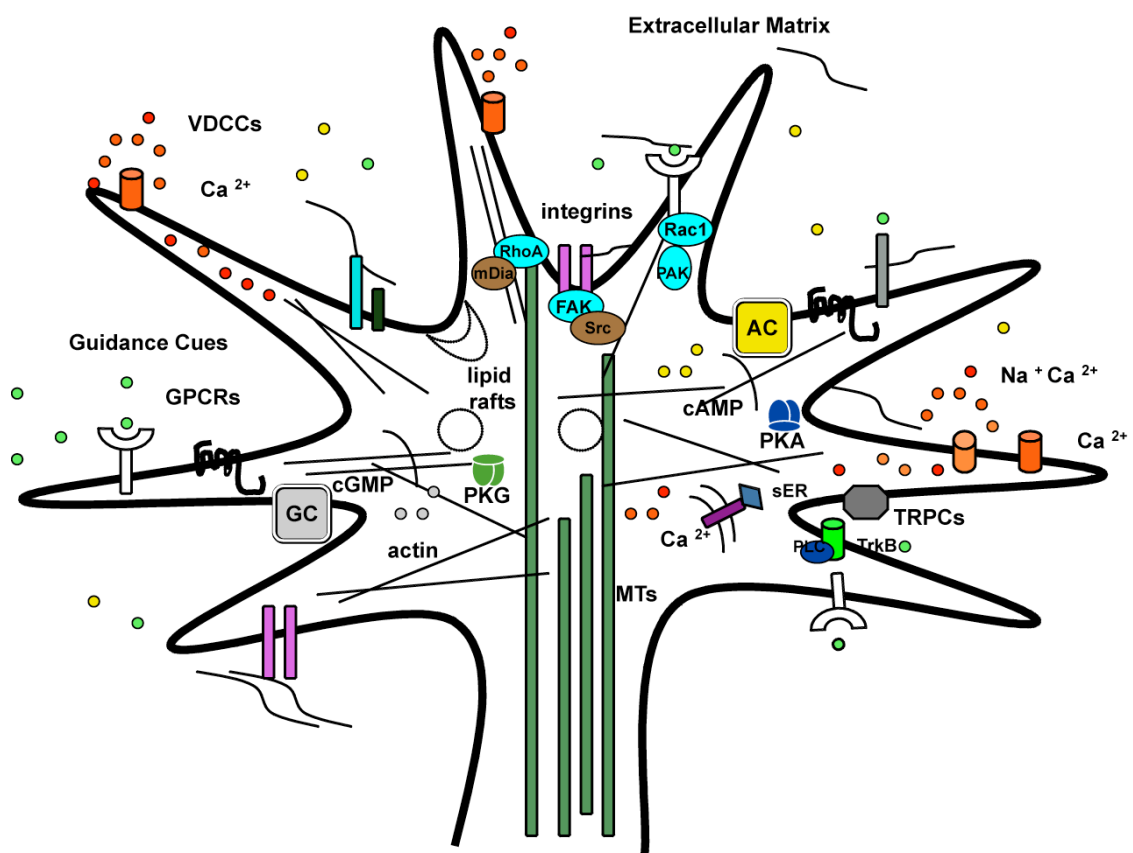


Figure 1.1 The growth cone is a sensory structure that navigates axons in response to molecular guidance cues.

The growth cone guides developing axon across the embryonic environment. The stability and growth dynamics of the growth cone depend on the regulation of the cytoskeleton, composed of rich actin fibers and microtubules, the major effectors of growth cone morphology. GPCRs: G-protein coupled receptors (GPCRs), microtubules (MTs), transient receptor potential channels (TRPCs), voltage calcium channels (VDCCs), guanylyl cyclases (GCs), adenylyl cyclases (ACs).

In his work, Ramón y Cajal also proposed that the migration of neuroblasts and axons might be guided by gradients of chemical substances secreted by target cells, “target derived factors that attract or repel axons at a distance” (Ramón y Cajal, 1890;

de Castro, López-Mascaraque and De Carlos, 2007). Direct evidence of Cajal's concepts in the mechanisms of axon guidance have begin to emerge in the last few decades with significant progress made in identifying axon guidance molecules and receptors that regulate axonal navigation (Chisholm and Tessier-Lavigne, 1999; Dickson and Gilestro, 2006). Several short-range surface bound or long-range diffusible secreted molecules that can either attract or repel developing axons have now been identified, which include the evolutionarily conserved molecules of the Netrin1, Slit, Semaphorin, and Ephrin family of proteins. These four families of proteins have are classical axon guidance cues, but their role extends to morphogenesis, neuronal migration, leukocyte chemotaxis, angiogenesis, among others (Yu and Bargmann, 2001), which supports the notion that similar guidance mechanisms can regulate the migration of either the entire neuron or its developing axon (Song and Poo, 2001). During axonal navigation, these cues are thought to form gradients of attractions as in the case of Netrin1 (Serafini, et al., 1996), repulsion zones, Slits and Semaphorins (Zou, et al., 2000), or act as bifunctional cues capable of mediating both attraction or repulsion effects depending on the context in which they are perceived such as in the case of Netrin1 (Seeger and Beattie, 1999) and Ephrins (Dickson, 2002). Netrin1 plays a crucial role in promoting axonal extension in the spinal cord and other several major commissures within the forebrain via interactions with the receptor deleted in colorectal cancer, DCC (Seraffini, et al., 1996). In addition, Netrin1 participates in neural migration of pontine cells of the hindbrain as well as the migration of mesodermal cells. Netrin1 has been well characterized as a

chemoattractant through DCC, however, Netrin1 has been also shown to interact with the guidance receptor UNC5 to induce a chemorepellent response (Bradford and Cooper, 2009). Moreover, the Ephrin family of axon guidance molecules can mediate either attraction or repulsion and are able to elicit bidirectional signaling (signal transduction is trigger for both the ligand and receptor expressing cells), for example Ephrin-B reverse signaling repels forebrain commissural axons away from regions of receptor EphB expression, while attracting them to regions of receptor EphA4 expression (Dickson, 2002). Slit1, 2 and 3 proteins acting via Roundabout (Robo) receptors are required to navigate commissural axons of the spinal cord in concert with class 3 Semaphorin proteins to prevent them from lingering or recrossing the floor plate (Zou, et al., 2000). In addition, Slit repels other axon populations including, the olfactory bulb, hippocampal, and spinal motor axons and has been implicated in the targeted migration of neuroblast of the olfactory bulb rostral migratory stream among others (Cooper, et al., 2002). Semaphorins are a large diverse family of proteins that signal through diverse multimeric receptor complexes beginning to be identified, including Neuropilins, Semaphorin3 binding proteins, Plexins, Semaphorin3 signal transducers and other essential receptors, such as the neural cell adhesion molecule, L1 (Castellani, et al., 2002; Dickson, 2002). Semaphorin proteins can act as chemorepellents to keep Semaphorin sensitive axons from innervating Semaphorin secreting regions, a phenomenon that is thought to induce axonal fasciculation to keep axons from extending in these regions (Dickson, 2002). In addition, Semaphorin signals have been reported to regulate axonal branching and pruning of hippocampal

mossy fibers and pyramidal axons, and can also regulate cell motility, attachment, vascular growth, immune cell regulation, tumor progression, among others (Kruger, et al., 2005).

Guidance cues can initiate signaling cascades that determine whether the growth cone of the developing axon will advance, change speed, turn or retract in response to a signal. These signals rely heavily on local changes to actin and microtubule motility processes of the cytoskeleton, which regulate the stability of the growth cone and dictates its response to the guidance information (Loverly and Vactor, 2009; Poo, 2001). The family of small Rho guanosine triphosphatases (GTPases) is of particular interest as major regulators of cell motility in migrating fibroblasts through their interactions with the cytoskeleton and key determinants in mediating axon guidance responses in both invertebrates and vertebrates (Loverly and Vactor, 2009). The GTPases, RhoA, Rac, and Cdc42 have been consistently shown to act as downstream signal convergence points of guidance cues to regulate cytoskeleton rearrangements with the notion that RhoA regulates contractile actin-myosin stress fibers, Rac, actin lamellipodia and Cdc42, F-actin rich filopodia. The overall consensus from experiments of dominant mutant, loss of function, and biochemical analysis is that attractive cues activate Rac and Cdc42, while repulsive cues activate RhoA. In addition, the active (GTP bound) and inactive (GDP bound) states of GTPases are regulated by GTPase activating proteins (GAPs), guanine nucleotide exchange factor proteins (GEFs), and guanine nucleotide dissociation inhibitor (GDIs), which can be recruited during intracellular signaling to act as catalysts for

GTPase activity (Guan and Rao, 2003). Rho GTPase signals regulate kinases and effector proteins that directly induce actin assembly and disassembly, including major Rac and Cdc42 effectors, actin nucleation factors Arp2/3, and actin polymerization factors, enabled vasodilator stimulating protein (Ena/VASP), and Wiskott-Aldrich syndrome proteins (WASP), which together control the preferential reorganization of the actin cytoskeleton after signal transduction essential for completing the navigational response (Loverly and Vactor, 2009).

More recently, highly conserved classical morphogens including fibroblast growth factors (FGFs), BMPs, wingless (WNTs), and Shh proteins, were shown to have consistent dual roles in neural patterning and axon guidance (Salie, et al., 2006; Farrar and Spencer, 2008). For example, the FGF family of proteins apart from their role in assigning anterior and posterior (rostrocaudal) identity to spinal motor neurons, also help establish trochlear motor neuron and retinotectal projections in the chick embryo (Salie, et al., 2006). BMPs emanating from the roof plate of the vertebrate neural tube form a dorsal gradient that determines the neural cell fate of spinal cord dorsal progenitor domains (Hogan, 1996). In commissural axon guidance, BMP7 and related growth differentiation factor 7 (GDF7) heterodimers were found to repel axons *in vitro* and BMP together with GDF7 was suggested to direct nascent commissural axons ventrally towards the floor plate (Augsburger, et al., 1999). More recently, the Wnt family of secreted proteins known for their patterning functions in development were shown to mediate axon guidance events in the developing spinal cord. In the *Drosophila* embryo, Wnt5a binds to the receptor Derailed (drl), an atypical tyrosine

kinase receptor was shown to induce repulsion of anterior commissural axons in the *Drosophila* central nerve cord (Yoshikawa, et al., 2003). In addition, Wnt proteins in *C. elegans* (Hillard, et al., 2006) and in mammals (Lyuksyutova, et al., 2003) were found to direct axonal navigation on the anterior-posterior axis of the embryo through the Frizzled receptor (Zou and Lyuksyutova, 2006). Shh another important morphogen involved in tissue patterning of the limb, spinal cord and forebrain structures, has been shown to regulate midline crossing of retina ganglion cells (RGCs) and direct commissural axon pathfinding through the ventral spinal cord (Charron, et al., 2003; Bovolenta, 2005; Bourikas, et al., 2005).

Two elegant studies in chick (Trousse, et al., 2001) and mammalian RGCs (Sanchez-Camacho and Bovolenta, 2008), explored the role of Shh in directing the navigation of visual axons. In the early stages of development of the chick eye, Shh mRNA was found to be expressed in the entire ventral midline of the preoptic/chiasmatic region (between the two eyes) of the embryo, but downregulated at the time when RGCs reached the optic chiasmatic midline, which suggested that Shh might play a role in regulating RGC midline crossing (Trousse, et al., 2001, Sanchez-Camacho and Bovolenta, 2008). Experiments that followed showed that *in vitro*, Shh induced retraction of newly formed E5 chick retina axons by downregulating cyclic adenosine monophosphate (cAMP) levels, suggesting a mechanistic role for Shh in channeling retinal axon growth across the chiasm (Trousse, et al., 2001). While in chick embryos most RGC projections cross to the contralateral side of the optic chiasm (panoramic vision), in mouse embryos, RGCs

can remain ipsilateral or cross the optic chiasm (binocular vision) depending on their intrinsic properties (Sanchez-Camacho and Bovolenta, 2008). Neutralizing Shh activity in mouse embryos resulted in abnormal accumulation of RGC axons at the optic chiasm where axonal fibers were enlarged and appeared less fasciculated with many wandered axons extending in aberrant posterior directions. Moreover, contralateral RGCs expression of a dominant negative form of the Shh receptor, Patched (PTC1) showed a decrease of contralateral RGC axons reaching the optic midline, with many axons wandering and turning away from the midline, projecting ipsilateral or turned back at the midline (Sanchez-Camacho and Bovolenta, 2008). A similar role for Shh in midline pathfinding was found in commissural axons of the spinal cord during the time frame of this study and will be discussed in **Chapters 2 and 3**.

Conversely, in mammals, while maintaining a ventral to dorsal gradient in the developing spinal cord, Shh was shown to attract commissural axons towards the floor plate ventral midline through PTC1 in cooperation with Shh binding receptor, Cdon binding protein (Boc) (Charron, et al., 2003; Okada, et al., 2006). Intriguingly, in the chick embryo, Shh appears to create a posterior to anterior gradient that repels spinal cord commissural axons from posterior regions of the spinal cord through the Hedgehog-interacting protein (Hip) after floor plate crossing (Bourikas, et al., 2005). Hip has been shown to be a direct transcriptional target of Shh signals and a negative feedback loop regulator of the signal (Jeong, et al., 2004; Lum, et al., 2004). The differential regulation of Shh signals at the floor plate might be necessary to induce a

change in the receptor composition to allow the axon to respond to new signals and as described here, consequently guide axonal projections on the different axes of the developing embryo.

Proposed over 100 years ago by Ramón y Cajal, graded signals are now established hallmarks in axon guidance (Poo, 2001; Zou and Lyuksyutova, 2007; Cooper, 2002). Complex guidance behaviors appear to be regulated by complex interactions between the relative concentrations of the guidance cues present and the sensitivity of the growth cone (e.g. intrinsic state of the neuron, receptor availability). In light of numerous studies of axon guidance in development, the more favorable model in axon guidance leads to a cooperative model where balances between growth promoting, attractive and repulsive forces across the growth cone surface activate intracellular signals that results in a desirable behavioral outcome (i.e. proper axonal pathfinding). The growth cone has been shown to be remarkably sensitive to concentration differences across its total cell surface ($\sim 10 \mu\text{m}$) and is able to respond reliably to small changes in concentration gradients, 1% changes of substrate bound gradients and 5-10% changes of diffusible attractant gradient (Song and Poo, 1998; Song, et al., 1997). These fine gradient detections can lead to the asymmetrical distribution of ligand-receptor complexes that can dictate the direction of movement and/or lead to the amplification or sensitization of the signal as the growth cone advances through the gradient (Poo, 2001). *in vivo* the concentration gradients of classical axon guidance molecules and morphogens are likely to overlap in time and space, so perhaps the convergence of gradients among guidance cues can create points

of specific information that can be transduced into a stereotypical behavior.

Alternatively, a morphogen as it functions earlier in development can help establish the expression gradient of other axon guidance molecules (Zou and Lyuksyutova, 2007).

As the case may be, it is obvious that simple attractive and repulsive responses are not sufficient to explain the highly temporal and spatial regulated axonal trajectories formed in development. In addition to complex concentration gradient signals axons respond to guidance cues also depending on the molecular composition of the environment (e.g. substrate bound molecules: integrins, cell adhesion molecules, laminin), strength and duration of the signal, crosstalk and regulations between signaling pathways, synchronous activation of signals, convergence of regulators of the cytoskeleton machinery and second messenger signals (e.g. cAMP, cGMP, Ca^{2+} , known to switch the directionality of the response). The latter cAMP signals will be discussed more precisely in **Chapters 3 and 4**. In addition, the availability of receptors on the plasma membrane to a given signal can be regulated by previous or present signals and/or in response to the signal itself (Poo, 2001). Given the intricateness of the system, further molecular dissection of axon guidance pathways and accurate understanding of their signaling dynamics will help gain a broader picture of the regulation of axon guidance in development. Future studies that could improve our understanding of axon guidance mechanisms at the floor plate ventral midline will be discussed in **Chapter 4**.

The mechanisms by which axons change their responses to the same guidance cue is better illustrated as a way for axons to advance along their trajectories, by ignoring the signals that guide them there in the first place, literally “moving on”. The realization that complex axon trajectories can be broken up into a series of segments connected by intermediate targets (e.g. the floor plate of the ventral midline), has significantly improved the understanding of axon guidance (Kaprielian and Runko 2001). Intermediate targets or “choice points” are composed of specialized cells that secrete long and short-range guidance cues. Strategically located at the end of a trajectory segment, intermediate targets are known to provide a molecularly rich environment where growing axons can undergo necessary changes in their molecular composition that will enable them to respond to directional cues on the next segment of the trajectory. This arrangement in the developing embryo can certainly afford more control over how axons respond to new changing environments and makes the study of axonal navigation more attainable. A perfect example is the trajectory of spinal cord commissural axons through the floor plate ventral midline (Chisholm and Tessier-Lavigne, 1999). A number of studies in commissural axons have paved the way for several of the axon guidance and development concepts discussed here and as a well-characterized model for axon guidance research, there are a number of techniques available for their study, making them more appealing for manipulation in a laboratory setting. The main characters of this dissertation are the commissural neurons of the developing spinal cord, the best understood model in axon guidance, which mechanisms are likely to be applicable general principles in axon guidance.

Commissural Neurons

Commissural neurons are born in the most dorsal regions of the developing spinal cord near the ventricular zone and the roof plate between embryonic day (E) 9.5-11.5 in the mouse and E11-13 in the rat. The domains of their progenitor cells are specified in the early neural tube by the actions of basic helix-loop-helix (bHLH) transcription factors regulated by the actions of TGF β signals (e.g. BMPs) emanating from the roof plate. Subsequently, transduction of TGF β signals lead rise to the differentiation of three types of dorsal relay interneurons dI1, dI2 and dI3. dI1, dI2 and dI3 neurons migrate ventrally away from the ventricular zone and settle in the intermediate spinal cord and express proneural bHLH transcription factors, Math1, Ngn1 and Mash1, respectively (Goulding, et al., 2002; Gowan, et al., 2001; Lee and Jessel, 1999; Gross, et al., 2002; Muller, et al., 2002).

In the mature central nervous system (CNS), commissural neurons are located in the deep layers of the dorsal horn and their projections join ascending somatosensory pathways (e.g. anterior lateral system pathways) that transmit signals from the periphery to higher order brain centers (e.g. thalamus) (Goulding, et al., 2002). Upon activation, commissural axons transfer sensory information of pain, touch, and temperature modalities from one side of the spinal cord to the other through spinal cord ventral commissures, a mechanism essential for the integration of sensory input in bilaterally-symmetric organisms (Kaprielian and Runko, 2000).

Commissural Axon Pathfinding

Commissural axons are guided in a stereotypical trajectory across the spinal cord, a pattern extensively studied in axon guidance, which principles are generally applicable to other cell and axon guidance systems.

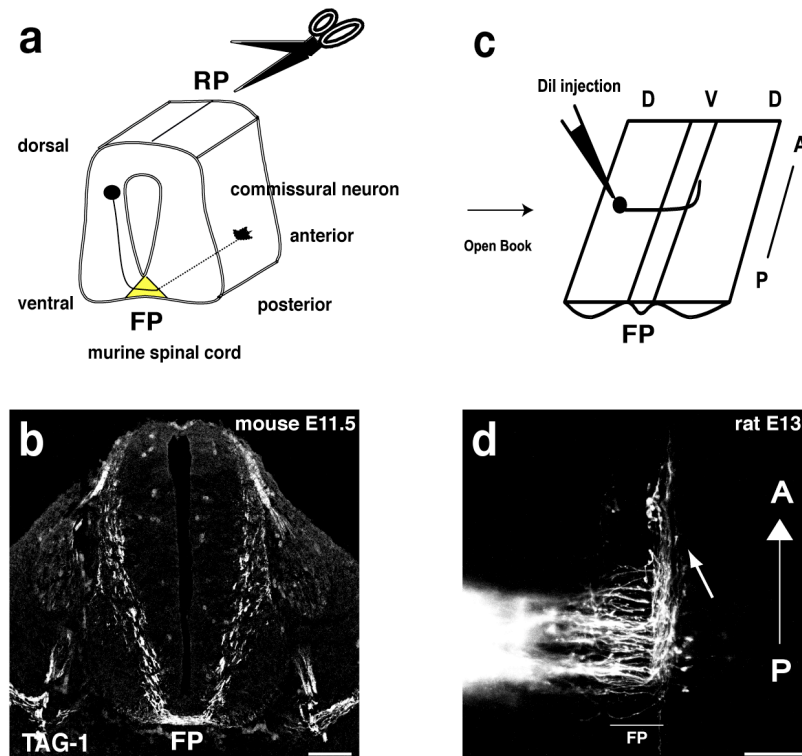


Figure 1.2 Commissural axon pathfinding.

(a) Schematic of a transverse section of mouse E11.5 or rat E13 spinal cord depicting the stereotypic trajectory of commissural axons in development on the dorso-ventral (D-V) and antero-posterior (A-P) embryonic axis (b) Mouse E11.5 spinal cord cross section labeling the D-V trajectory of commissural axons as they project towards the floor plate (FP), commissural axons are stained with TAG-1, a marker for precrossing axons. (c) Diagram of an open book explant dissected at the level of the roof plate (RP). In this preparation commissural axon projections can be visualized in both the D-V and A-P axis by labeling axons with the lipophilic dye, DiI (1'-Dilinoleyl - 3,3,3',3' - tetramethylindocarbocyanine methanesulfonate). (d) Commissural axons project towards the floor plate, cross the floor plate and turn anteriorly on the contralateral side of the spinal cord. Scale bars represent 100 μm (b and d).

The molecular mechanisms regulating commissural axon pathfinding have been extensively characterized, which makes commissural axons an excellent model for axon guidance studies (Chilton, 2006). In the beginning of their trajectory commissural axons project ventrally towards the floor plate in a circumferential pattern guided by the chemoattractant activities of Netrin1 (Serafini, et al., 1996) and Shh signals (Charron, et al., 2003), while being concomitantly repelled by proteins belonging to the TGF β family of proteins, GDF7 and BMP7 emanating from dorsal roof plate cells (Augsburger, et al., 1999). At this stage in their pathfinding before encountering the floor plate, commissural axons express the transiently axonal surface glycoprotein, TAG-1, and the Netrin1 receptor DCC and are often referred as precrossing commissural axons (Serafini, et al., 1996; Zou, et al., 2000). The concentrations of Netrin1 and Shh proteins are expected to be highest at the floor plate; however, once commissural axons reach the floor plate they lose responsiveness to the attractive properties of the floor plate and instead gain sensitivity to the activities of Slit and the Semaphorin3 family of secreted floor plate proteins, which act in conjunction to increase the repulsion away from the midline (Zou, et al., 2000). This allows commissural axons to enter the floor plate only once without recrossing it and prevents axons from lingering and stalling within the floor plate (Chilton, 2006; Zou et al., 2000). Thus, proper axon guidance through the floor plate relies heavily on the ability of growth cones to successfully prioritize and integrate signals from a variety of guidance cues.

The known repulsive activity of the developing spinal cord constitute the Slit family of proteins (Slit1, Slit2, and Slit3) signals through Robo receptors (Robo1 and Robo2) and a subset of the class 3 Semaphorin family of proteins (Semaphorin3F and Semaphorin3B) signals through the Neuropilin2-PlexinA3 receptor complex (Chilton, 2006; Zou, et al., 2000).

Slits are highly conserved proteins originally identified in the *Drosophila* developing nerve cord where they were found to repel axons expressing Roundabout receptors. In both the *Drosophila* slit and roundabout mutants, commissural axons remained insensitive to midline repulsive activities and were unable to project out of the floor plate on the contralateral side. In roundabout mutants, commissural axons crossed and recrossed the floor plate, whereas in mutants for Slit, axons stalled and were not able to leave the midline (Kidd, et al., 1996; Kidd, et al., 1998; Dickson and Gilestro, 2006). In the vertebrate counterparts, three Slit and Robos were identified; remarkably only in the *slit* triple knockouts commissural axons lingered and stalled at the midline and clearly not as severe as the *Drosophila* loss of repulsion phenotypes (Long et al., 2004) likely due to the presence of other chemorepellent signals in these mutants (Zou, et al., 2000). In both invertebrates and vertebrates, Robo proteins were found to be expressed at low levels during their initial extension towards the floor plate and upregulated after crossing consistent with their proposed repulsive role after midline crossing (Long et al., 2004). Conversely, a third Robo receptor, Rig1, highly expressed in commissural axons prior midline crossing, appears to regulate Slit repulsion by masking signals through Robo1 and Robo2 before crossing. In the

absence of R1g1, commissural axons were shown to prematurely respond to Slit/Robo1 and Slit/Robo2 repulsive signals failing to form ventral commissures (Sabatier, et al., 2004).

In addition, a crosstalk between Netrin1 and Slit signals was reported to induce silencing of Netrin1 by Slit/Robo activities without affecting Netrin1 growth-stimulatory effects. The signaling receptor for Slit, Robo and the signaling receptor for Netrin1, DCC, directly interact in the cytoplasm through their intracellular domains and abolish Netrin1 attraction of *Xenopus* spinal neurons providing a plausible model of hierarchical organization of repellent Slits and attractant Netrin1 signals (Stein and Tessier-Lavigne, 2001).

The activation of Semaphorin repulsive signals during commissural axon guidance is less understood. The Semaphorin family of glycoproteins is divided into eight classes, five of which are expressed in vertebrates, the most studied Semaphorins in axon guidance and the most extensively characterized in vertebrates are the Semaphorin3 family of proteins (Chilton, 2006).

Class 3 secreted Semaphorins were identified in chick dorsal root ganglia (DRG) growth cone collapsing assays *in vitro* and were named after the axonal behaviors they induced, collapsins (Fujisawa and Kitsukawa, 1998). Semaphorins signal through multi-molecular receptor complexes, which composition is not completely known (Dickson, 2002; Chilton, 2006; Castellani, et al., 2002). Nonetheless, numerous studies show that repulsive Semaphorin3 signals are mediated through Neuropilin1 (NP1) and Neuropilin2 (NP2) receptors, which are specific

Semaphorin3 binding subunits, in association with PlexinA receptors, the Semaphorin3 signal transducers (Castellani, et al., 2002). Among class 3 Semaphorins, the NP2 receptor has been shown to mediate the repulsive activities of Semaphorin3B, Semaphorin3C and Semaphorin3F, whereas NP1 binds and regulates Semaphorin3A function. These binding affinities are not exclusive between Semaphorin receptor complexes, as they have been shown to interrelate promiscuously and/or competitively, potentially allowing a wider range of subsequent Semaphorin responses (Kruger, et al., 2005). In addition, there are other proteins shown to form stable complexes with NP and PlexinA receptors; in particular the neural cell adhesion molecule, L1 have been consistently implicated in the regulation of Semaphorin3A repulsion by binding to NP1 receptors (Julien, et al., 2005; Castellani, et al., 2002). Similarly, a different member of the L1 family, NrCAM associates with NP2 and forms part of receptor complexes for Semaphorin3B and Semaphorin3F signals (Julien, et al., 2005). Precrossing commissural axons express axonin1/TAG-1, a receptor for NrCAM expressed in midline floor plate cells (Zou, et al., 2000). Inhibiting NrCAM prevented axons from crossing the midline, which suggested a model in which precrossing axons could be already sensitive to midline repellents, but masked at the receptor level by interactions with inhibitory receptor complexes. During midline crossing, commissural axons lose their TAG-1 expression and upregulate L1 proteins, suggesting a possible regulatory mechanism for restricting Semaphorin inhibitory activities, however, mammalian spinal cord commissural axons do not express NP1, the presumptive coreceptor for L1 (Castellani, et al., 2002).

These permutations in receptor interactions could indicate a layer of regulation for Semaphorin signals similar to the regulation of Slit signals by Rig1, however, the mechanisms underlying Semaphorin3 signal upregulation in commissural neurons are still not very well understood. As mentioned above, commissural axons are only sensitive to Slit and Semaphorins upon reaching the floor plate, a mechanism not well understood (Zou, et al., 2000). The differences in commissural axon responsiveness to repellents between the first segment of their trajectory (precrossing) and their next leg of the trajectory (postcrossing) can be studied by analyzing their axonal behaviors *in vitro* using explant assays. Responses to spinal cord chemorepellents, Slit2, Semaphorin3B and Semaphorin3F were found to be upregulated only in commissural axons cultured after floor plate crossing (postcrossing explants) but not prior (precrossing explants) (Zou, et al., 2000). Thus, the floor plate must house a mechanism to activate repulsion responses to Semaphorin3 signals. Alternatively, Slit and Semaphorin pathways can come together to elicit a robust signal that would be sufficient to activate repulsion; initial studies carried out in this dissertation were aimed towards investigating the crosstalk of these pathways and will be discussed in depth in **Chapter 2**. Furthermore, genetic analysis of Semaphorin3 function in mice suggested that Semaphorins can act primarily as short-range inhibitory cues that deflect axons away from inappropriate targets channeling axons through repulsive corridors (Dickson, 2002). Guidance of commissural axons by class 3 Semaphorins signals are proposed to channel postcrossing commissural axons in the ventral and lateral funiculus along the floor plate (Zou, et al., 2000).

Although, unclear how commissural axons are directed to turn abruptly on the contralateral side of the spinal cord after floor plate crossing, a mechanism by which these axons are attracted to anterior regions in the spinal cord was shown to involve axonal attraction to a Wnt4 anterior-high and posterior-low floor plate gradient (Lyuksyutova, et al., 2003). Commissural axons extending in this A-P trajectory are known as postcrossing axons. Intriguingly, a posterior-high anterior-low gradient of Shh was shown to regulate anterior turning of commissural axons in chick embryos through the receptor, Hip (Bourikas, et al., 2005). How Shh and Wnt signals integrate directional signals on the A-P axis of the spinal cord to orchestrate anterior pathfinding of commissural axons is not well understood.

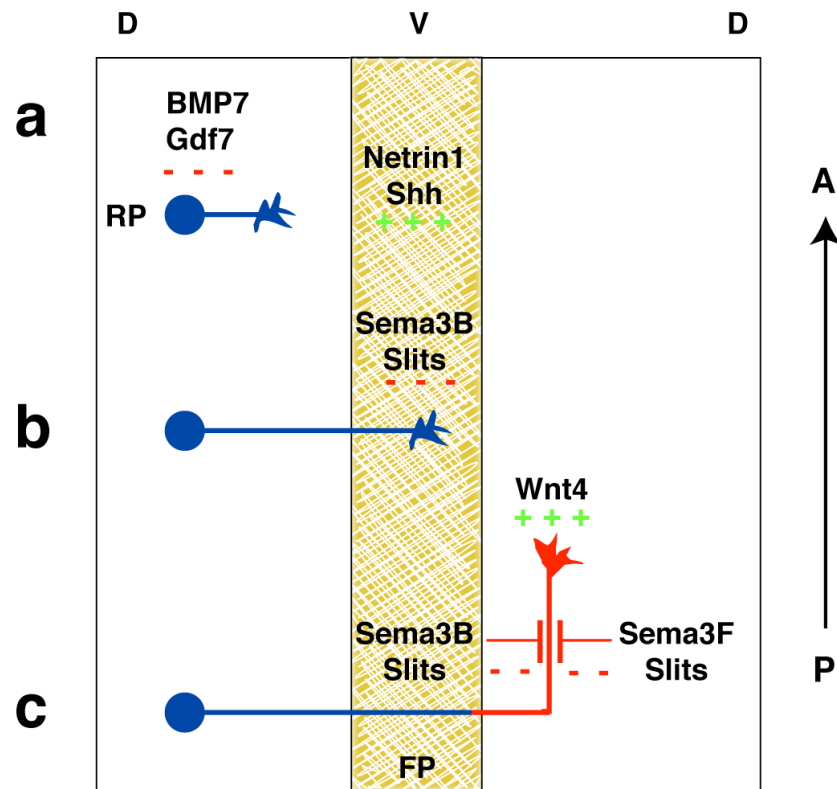


Figure 1.3 Molecular mechanisms of commissural axon guidance.

Schematic diagram depicting navigating commissural axons in an open book preparation. (a) At early stages of axonal pathfinding BMP7 and Gdf7 signals secreted from the roof plate (RP) repel commissural axons from dorsal regions, while combinatorial activities of Netrin1 and Shh attract axons to the floor plate (FP). (b) After crossing the FP, commissural axons change their responsiveness to attractants and are repelled out of the midline by Semaphorin3B and Slits signals also secreted by FP cells. (c) Losing responsiveness to midline attractants allows axons to escape the FP and concomitantly respond to Semaphorin3 and Slit repulsive signals from ventral regions that create repulsive corridors where commissural axons can now form axonal tracts on the contralateral side of the spinal cord. The newly formed tracts are guided anteriorly towards the brain by the chemoattractant properties of Wnt proteins.

Summary of Findings

The collection of data presented in this dissertation seeks to contribute useful information on the mechanisms underlying commissural axon pathfinding at the ventral midline of the developing spinal cord. In particular, the molecular mechanisms underlying changes in axonal responses to repulsive guidance cues and the signaling pathways that might be involved in the process.

The dissertation explores some of the molecular mechanisms underlying commissural axon behaviors during floor plate crossing and in particular will propose a new role for Shh signals in axon guidance. Much focus is given to the regulation of Semaphorin3 repulsive pathways and the mechanisms involved in keeping commissural axons from projecting back into the ventral midline.

Previous unpublished studies (Zou, et al., in preparation) suggested a crosstalk between Semaphorin3 and Slit2 signaling pathways that could activate repulsion of commissural axons via interactions with the Neuropilin2 receptor. In **Chapter 2**, we explore these ideas using precrossing explant assays and assess for premature repulsion in response to different combinations of Semaphorin3 and Slit protein gradients. Our results suggest a functional interaction between Semaphorin3F and Slit2 signals in precrossing commissural axons.

In addition, in search of regulators for Semaphorin3 signals we performed a screen of proteins expressed by floor plate cells during the time commissural axons cross the ventral midline. In this screen we found that Shh proteins can activate Semaphorin3 repulsion prematurely in precrossing commissural axons and that both

PTC1 and Smo were necessary to regulate commissural axon midline crossing. A manuscript in preparation summarizes these experiments in **Chapter 3**.

Moreover, based on the notion that cyclic nucleotide levels can switch an attractive response to repulsion and vice versa, we investigated the role of cAMP second messenger signals in mediating Shh induced Semaphorin3 repulsion and proposed that Smoothed signals through the G-coupled inhibitory proteins, G α i, can modulate cAMP levels in commissural axons, **Chapters 3 and 4**.

Shh role as a guidance molecule highlights the unusual yet intriguing and intricate signaling mechanisms of this morphogen. Shh is known to employ multifaceted roles to properly orchestrate important developmental events, which makes it an attractive candidate for axon guidance regulation. This dissertation discusses insights into the molecular mechanisms of commissural axon guidance during ventral midline pathfinding and presents compelling evidence on the regulatory role of Shh during floor plate crossing. Although studies of commissural axon pathfinding have revealed many insights into the mechanisms of axon guidance, it is evident that other non-classical signaling pathways are necessary to enhance the diversity of behavioral outcomes involved in axonal connectivity. Thus, it is not surprising to find intricate integration layers of signal regulation during axonal navigation, the goal here is to dissect out the key principles in a reliable system model such as the guidance of commissural neurons and determine more general and applicable mechanisms of nerve wiring. Future studies should focus on the spatiotemporal regulation and dynamics of Shh and Semaphorin3 signals and the

convergence of concentration gradients that set up points of specific information where a distinct behavioral response is needed. Advances in real time imaging techniques and mouse genetics could help determine intrinsic changes of protein localization, concentration, and receptor presentation discussed in more detail in **Chapter 4.**

Extrapolation of axon guidance studies to therapeutic approaches in spinal cord repair lies in successfully recapitulating the intrinsic state of the developing neuron and the instructional growth-promoting environment of the embryo. Understanding how axon guidance pathways are regulated in development is crucial in generating models and therapies for the amelioration of spinal cord injury and disease.

CHAPTER 2

Multiple Guidance Cues Control the Pathfinding of Spinal Cord Commissural Axons

Introduction

Developing axons navigate towards their specific targets and establish complex networks of projections by constantly assessing the surrounding environment and accurately choosing their pathfinding decisions. Commissural axons of the spinal cord must interpret multiple signals, both short range and membrane bound, which upon activation trigger a series of intracellular events and cytoskeletal rearrangements that promote movement along the desired trajectory (Stein and Tessier-Lavigne, 2001). At the floor plate, commissural axons lose responsiveness to the attractants Netrin1 and Shh, while simultaneously becoming repelled by the actions of the midline chemorepellents. These mutually related changes ensure that the axons find the once attractive floor plate repulsive and allow axons to move from this intermediate target to the next as it navigates further to their final destination. The upregulation of midline chemorepellent activity explains why axons do not stay at the floor plate or linger and instead leave and turn on the contralateral side of the spinal cord, forming a longitudinal tract that projects parallel to the floor plate (**Fig. 1.2b, d**) (Dickson and Gilestro, 2006). The best studied chemorepellents at the spinal cord ventral midline are proteins of the Slit and Semaphorin families. Slits are large secreted proteins expressed in the floor plate by ventral midline cells that signal through the

Roundabout (Robo) family of receptors. There are three mammalian Slit homologs (Slit1-3), initially identified in *Drosophila*, but highly conserved in vertebrates, Slits signal repulsion by activating Robo1 and Robo2 receptors (Long, et al., 2004). At the time of commissural axon pathfinding through the floor plate, Slit2 mRNA is expressed mainly in the floor plate and motor neurons and begins to expand dorsally at later stages. Slit1 mRNA is also expressed at the floor plate and dorsal regions of the spinal cord, whereas Slit3 mRNA is mostly expressed in restricted regions of the ventral spinal cord and the floor plate (**Fig. 2.1a-c**) (Wang, et al., 1999). Initially, commissural axons express low levels of Robo proteins (although uniformly express Robo mRNA) allowing axons to enter and cross the floor plate. However, upon crossing Robo1 and Robo2 receptors are upregulated on the postcrossing segments of commissural axons, enabling axons to respond to Slit repulsive signals, which presumably prevent axons from crossing the midline again (Stein and Tessier-Lavigne, 2001; Long, et al., 2004; Dickson and Gilestro, 2006).

Analysis of the Slit1 and Slit2 mutant embryos showed no obvious commissural axon guidance defects in the spinal cord, however knockout mouse embryos lacking all three Slits showed that even though normal projecting axons were still seen in these mutants many commissural axons stalled at the floor plate (~72%) with a small number of axons recrossing the floor plate, a phenotype never observed in the single mutants (Long, et al., 2004). Moreover, characterization of Robo1 mutants showed that some axons entered the dorsal floor plate and the ventral ventricular zone (commissural axons are restricted between the floor plate cells and the underlying

extracellular matrix basement membrane (ECM) (Yaginuma, et al., 1991), and although some axons stalled within the floor plate and exhibited enlarged growth cones indicative of loss of repulsion, the finding was not extensive (< 50% of axons) (Long, et al., 2004). Analysis of the Robo2 knockout embryos showed no commissural axon defects toward and across the floor plate, but axons did appear to extend closer to the contralateral border of the floor plate instead of extending to the medial longitudinal tract as their wild type counterparts indicating some loss of sensitivity to repulsive signals (Long, et al., 2004). Double Robo1 and Robo2 knockout mutants are yet to be analyzed, but the proximity of their chromosomal genetic loci (1.8 Mb apart) has made it difficult to generate (Long, et al., 2004). Nonetheless, the decreased severity of phenotypes in the Robo1 and Robo2 single mutants compared the Slit triple knockout mutants implies the existence of an additional receptor for sensing Slit repulsive activity at the floor plate.

Class 3 Semaphorins, Sema3B and Sema3F, are expressed in the spinal cord along with Slits during commissural axon guidance. Sema3B is found in the floor plate and ventral ventricular zone, whereas Sema3F is expressed widely in the spinal cord (**Fig. 2.1d-e**) (Zou, et al., 2000). Both Sema3B and Sema3F mediate their repulsive activities through the Neuropilin2 (NP2) receptor. The NP2 receptor binds to Sema3F and Sema3B and does not appear to have signaling function alone, but instead forms a receptor complex with PlexinA3 (PA3) family of proteins, which are the signal transducers for secreted Semaphorin3 in vertebrates (Chen, et al., 1997). NP2 is expressed in commissural axons both before and after crossing and Sema3F

and Sema3B inhibitory effects are thought to be mediated only by NP2/PA3 dependent mechanisms. In NP2 knockout embryos, commissural axons show severe defects during midline crossing, stalling and lingering in the floor plate with some axons failing to extend into the longitudinal tract after crossing (Zou, et al., 2000). It has been proposed that the acquired sensitivity to the repellent activity from both the floor plate and the ventral portion of the midline is not only necessary to push commissural axons out of the midline, but also keeps them in longitudinal tracts after floor plate crossing. Importantly, based on their expression patterns, all three Slits and Sema3B proteins are likely to contribute to the midline repulsive activity, whereas Slit2, Slit3 and Sema3F further prevent axons from entering ventral regions of the spinal cord allowing them to fasciculate on their respective fiber tracts along the A-P axis (**Fig. 2.1**) (Zou, et al., 2000).

The highly penetrant and robust phenotype of NP2 mutant embryos during midline crossing consistent with the inability to respond to axonal repellents was not compensated by the Slit/Robo pathway still active in these axons, which raised the possibility that Slits and Semaphorins might function as a common pathway through the NP2 receptor to mediate ventral repulsion in commissural axons. The NP2 loss of function phenotypes highly resemble the Slit triple mutant phenotypes and even though Slit triple mutants display particularly prominent stalling and recrossing phenotypes, Robo1 and Robo2 single mutant phenotypes are far less severe. Together, these observations suggest a broader function for the NP2 receptor in mediating

ventral midline repulsion possibly by orchestrating the chemorepellent responses of both Slits and Semaphorins in commissural axons during midline crossing.

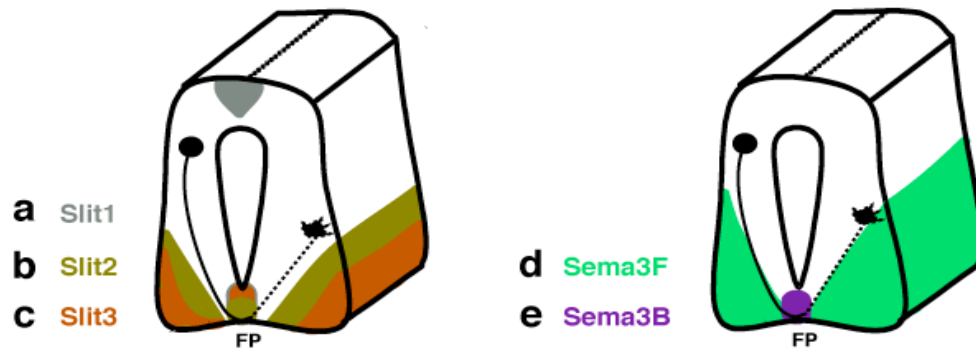


Figure 2.1 Expression patterns of Slits and class 3 Semaphorins in the mammalian spinal cord during commissural axon guidance.

Schematics of mRNA expression patterns from *in situ* hybridization studies of Slits and Semaphorins expressed in the spinal cord at the time of commissural axon guidance (mouse E11.5-12.5 and rat E13-14). (a) Slit1 is expressed in dorsal regions of the spinal cord and floor plate. (b) Slit2 is present mainly at the floor plate and motor neurons, whereas, Slit3 (c) is expressed most highly in restricted regions of the ventral spinal cord and floor plate (Wang, et al., 1999). (d) Semaphorin3B is found in the floor plate and ventricular zone of the spinal cord. (e) Semaphorin3F is expressed throughout the ventral regions of the spinal cord and mantle zone, but not in the floor plate or ventricular zone (Zou, et al., 2000).

The tight temporal and spatial regulation of Robo receptors ensures that commissural axons respond to Slits only after they have crossed the midline, but during floor plate crossing axons must also become expelled from the floor plate midline. NP2 receptors are always expressed in commissural axons and might function as a common pathway for repulsion for both Semaphorins and Slits during and after midline crossing.

Unpublished studies by Yimin Zou as a post-doctoral fellow in Dr. Marc Tessier-Lavigne's lab at the University of California, San Francisco, demonstrated an unexpected crosstalk between Slit and Semaphorin repulsive pathways. In particular, NP2 was found to be required for Slit2 repulsion. Postcrossing explants cultured as short segments of rat E13 spinal cords leaving the floor plate attached were found to become sensitive to Slits and Sema3B and Sema3F only after floor plate crossing (**Fig. 2.2b-c**) (Zou, et al., 2000). To address whether Slit2 mediated repulsion required the NP2 receptor, postcrossing explants were cultured in the presence of a NP2 functional blocking antibody. Postcrossing axons cocultured with Slit2 or Sema2F aggregates in the presence of the NP2 antibody failed to respond to Slit2 or Sema3F repulsive activities suggesting that blockade of NP2 function could completely block floor plate derived chemorepellents (**Fig. 2.2d**, Zou, et al. unpublished). In addition, epitope-tagged NP2 and Robo1 proteins expressed in COS cells can co-immunoprecipitate and form a physical complex *in vitro*, suggesting that these two pathways might be one common pathway during midline crossing. Moreover, recombinant Slit2 protein was found to bind to the surface of COS cells transfected with Robo1 and NP2 with binding affinities similar to that of Slit2 and Robo1 ($K_d \sim 1-2$ nM) (Zou, et al. unpublished).

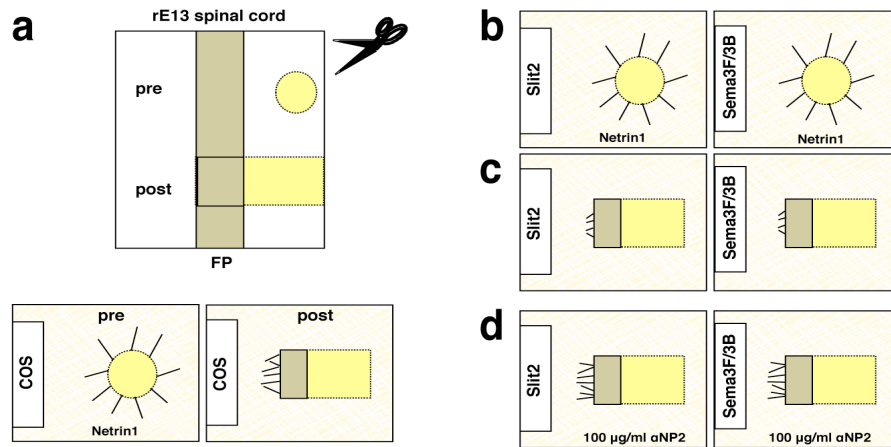


Figure 2.2 Schematic diagram of explant assays depicting responses of precrossing and postcrossing axons to chemorepellents Slit2 and Sema3F.

(a) Schematic of precrossing and postcrossing commissural axon explant assays dissected from open book spinal cord preparations. Precrossing axons grow radially into the collagen gel matrix in the presence of Netrin1, whereas postcrossing axons extend away from the floor plate into the collagen gel matrix. (b) Precrossing explants cocultured in the presence of COS cell aggregates expressing Slit2 or Sema3F do not respond to midline chemorepellent activities. (c) In contrast, postcrossing axons grown in the presence of Slit2 or Sema3F aggregates are strongly inhibited and failed to extend their axons after floor plate crossing. (c) postcrossing axons cultured in the presence of anti-Neuropilin2 antibody are no longer repelled by COS cells transfected with Slit2 or Sema3F. Schematics after Zou, et al., 2000 (b, c), (d) unpublished.

Together these data implies that NP2 might be a common receptor for mediating Slit2 and Semaphorin repulsion. Given that Slit2 can directly bind to NP2 *in vitro* and NP2 is always expressed in commissural axons during their trajectory across the ventral midline it is possible that Slit/NP2 along with Semaphorins/NP2 mediate expulsion of commissural axons at the floor plate. It is also likely that Slit2 binding to NP2 might enhance Slit signal recognition for the Slit2/Robo pathway

possibly through NP2 interactions with the Robo1 receptor as previously found (Zou, et al. unpublished).

In this chapter we present preliminary data indicating Slit2 and Semaphorin3 pathway activation in precrossing explants (**Fig. 2.5**). Our results show a convergence of Slit and Semaphorin3 signals that could lead to strong repulsive effects at the floor plate midline, where commissural axons need to be expelled abruptly and change their trajectory from a D-V to an A-P direction. We propose that Sema3F and Slit2 function synergistically to repel axons possibly through the NP2 receptor activating growth cone sensitivity to repulsive cues at the floor plate. Because NP2 proteins are binding receptors to Semaphorins, whereas PlexinA3 are required for Semaphorin3 signal transduction, we tested whether the Slit2/NP2 interaction required PlexinA3. Our preliminary findings indicate that recombinant Slit2 might also bind cell surface COS expressing PlexinA3 and this interaction could be required for Slit2/NP2 mediated repulsion (**Fig. 2.6**). Future experiments to expand these studies will be proposed in the discussion section of this chapter.

Another receptor for Slits, the Rig-1 receptor, was recently found to be highly expressed before midline crossing and during crossing, but downregulated after midline crossing with a high similarity to TAG-1 expression only present in precrossing axons (Sabatier, et al., 2004). This protein expression pattern is the opposite of those of Robo1 and Robo2, which are highly upregulated in commissural axons after crossing. Rig-1 was shown to prevent Slit activity prior midline crossing and in its absence commissural axons were completely unable to cross the midline as a

result of being prematurely responsive to Slits (Sabatier, et al., 2004). Rlg-1 mRNA levels were shown to significantly overlap with Robo1 and not Robo2 in the dorsal spinal cord where commissural neurons are born and although a large number of commissural axons express only Robo1, in its absence only a mild loss of repulsion was observed, which further suggests the presence of another receptor for sensing Slit repulsion (Long, et al., 2004).

Even though, removal of the NP2 receptor in the Rlg-1 mutant failed to rescue midline crossing, axons from Rlg-1 mutant explants prematurely responsive to Slits, were not inhibited by Sema3F repulsive activity (Sabatier, et al., 2004), suggesting that the guidance systems that regulate initial floor plate crossing might be different from the repulsive signals triggered upon axonal contact with floor plate cells. Importantly, Semaphorin repulsion does not seem to be affected by Rlg-1/Slit signals and despite the redundant repulsive functions of Slits and Semaphorins at the floor plate, little is known about how Semaphorin signals are regulated. We sought to investigate floor plate derived molecules that could prematurely activate repulsive Semaphorin3 signals based on spinal cord mRNA and protein expression patterns. In this chapter, we present preliminary findings showing direct regulation of Sema3F signals by the actions of Sonic Hedgehog (Shh) activities of the floor plate that exclude the regulation of Slit signals (**Fig. 2.7**). In **Chapter 3**, we present in depth analysis of the signaling mechanisms underlying Shh mediated regulation of Sema3F and Sema3B activities during commissural axon midline crossing.

Results

To investigate commissural axon responses to guidance systems prior or after floor plate crossing at the ventral midline, explant assays can be used to recapitulate commissural axon behaviors *in vitro* (**Fig. 2.3**) (Serafini, et al., 1994; Zou, et al., 2000). Here, we studied the axonal behaviors of explants generated from Rat E13 spinal cords prior floor plate crossing. Open book preparations were dissected out to generate explants composed of commissural progenitors from the dorsal domains of the spinal cord, known as precrossing explants (**Fig. 2.3a**). As previously described, precrossing explants were cultured in a three-dimensional two-layer collagen matrix gel for 16 hours prior harvesting and later fixed and stained for the cell adhesion molecule, TAG-1 (Serafini, et al., 1994). For our experiments, we modified this precrossing culture model so that we could introduce an uniform source of Netrin1 resuspended in the bottom collagen gel, while concomitantly exposing the explants to a COS cell aggregate expressing a protein of choice (**Fig. 2.3b**). Commissural axon behaviors were classified as no growth (**Fig. 2.3c**), diminished growth (**Fig. 2.3d**), normal growth (**Fig. 2.3e**), enhanced growth (**Fig. 2.3f**), inhibitory growth (**Fig. 2.3g**), or repulsion (**Fig. 2.3h**) as predictors of axonal behaviors. Explants displaying normal growth or repulsion were further analyzed based on the ratio of the total axonal length from the proximal (P) side of the COS cell clump divided by the total axonal length from the distal (D) side as depicted (P/D) (**Fig. 2.3b**), with a P/D ratio of 1 indicating normal growth and a P/D ratio $\ll 1$ indicating repulsion.

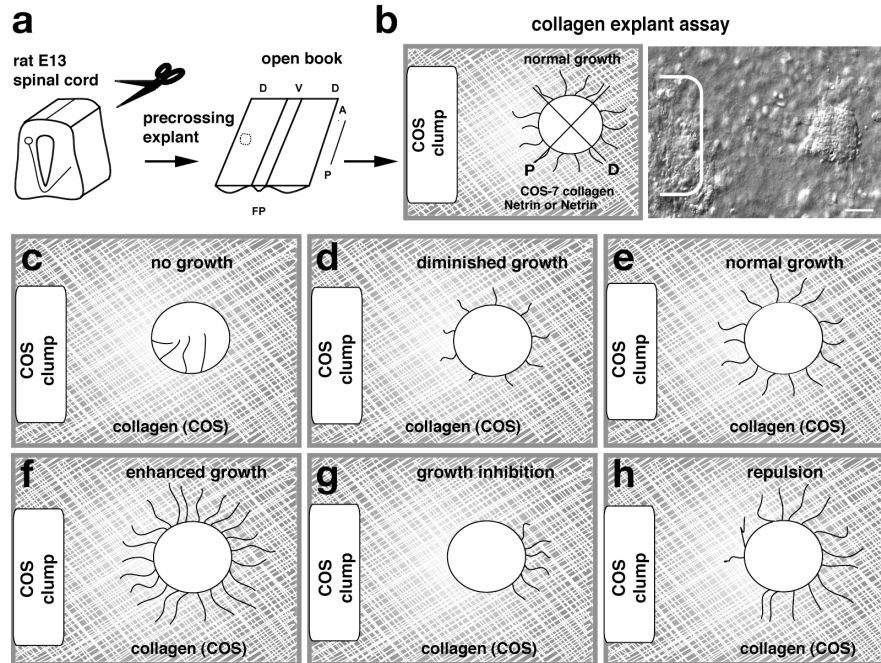


Figure 2.3 Precrossing explant assay analysis of commissural axon behaviors *in vitro*.

(a) Schematic diagram of a rat E13 spinal cord opened at the level of the roof plate and displayed as an open book preparation showing precrossing explants microdissected from dorsal domains of the spinal cord (Serafini, et al., 1994). (b) Schematic and differential interference contrast (DIC) microscope image of isolated explants cultured in a three-dimensional two layer collagen matrix gel, with the bottom gel containing a resuspension of transfected COS cells (e.g. Netrin1 or vector control), cocultured in the presence of a COS cell aggregate (COS clump) expressing a protein of choice. (c-h) Analysis of axonal growth was categorized according the axonal distribution around the explant circumference. Scale bar represents 100 μm.

To test the system we isolated precrossing explants and cultured them in different growth conditions (**Fig. 2.4**). As described above, in our modified precrossing explant assay, we cultured explants on a collagen gel bed containing transfected COS cells in the presence of a COS cell aggregate placed on one side of the explant (**Fig. 2.4a**). In our first control, we cultured precrossing explants in the presence of COS cells expressing a pCDNA vector control in both the bottom collagen

layer and in the COS cell aggregate (**Fig. 2.4b**). As expected, precrossing explants cultured under these conditions failed to grow in the absence of Netrin1 (Serafini, et al., 1994) (**Fig. 2.4b**). Furthermore, precrossing explants cultured on a collagen bed containing vector expressing cells in the presence of a Netrin1 cell aggregate showed preferential growth towards the proximal side of the cell aggregate (**Fig. 2.4c**). In contrast, explants cultured on a collagen bed containing Netrin1 expressing cells in the presence of a vector control cell aggregate grew in a radial fashion indicating equal axonal responses from both the proximal (P) and distal (D) side of the explant (**Fig. 2.4d**). To test the effects of floor plate derived chemorepellents, Slit2 and Sema3F, precrossing explants were cultured on a collagen bed containing Netrin1 expressing cells in the presence of cell aggregates expressing Sema3F (**Fig. 2.4e**) or Slit2 (**Fig. 2.4f**). Explants cocultured with Sema3F aggregates displayed an enhanced axonal growth, whereas explants cultured in the presence of a Slit2 cell aggregates had less growth than the growth observed in the presence of Netrin1 alone. Importantly, precrossing explants did not present repulsive growth in the presence of either Sema3F or Slit2 cell aggregates, consistent with the notion that the switch of responsiveness to repulsive signals are only triggered upon floor plate crossing (**Fig. 2.2c**) (Zou, et al., 2000).

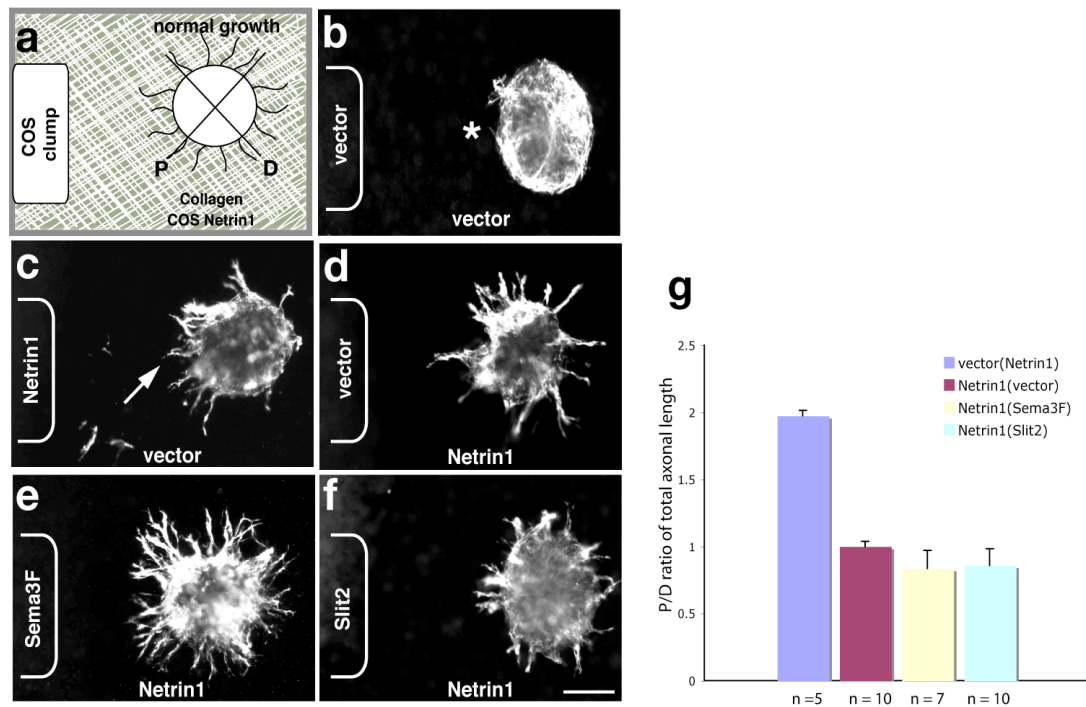


Figure 2.4 Precrossing explants respond to Netrin1 attractive signals, but ignore the chemorepellent signals of Slit2 and Semaphorin3F.

(a) Schematic diagram of precrossing commissural explants (b-c) As previously described, precrossing commissural explants need Netrin1 to grow *in vitro*. (b) In the absence of Netrin1, precrossing axons fail to grow (b) In contrast, explants exposed to a localized source of Netrin1 display directional attraction to the Netrin1 cell aggregate. (d) Precrossing explants grow radially on a collagen bed containing Netrin1 expressing COS cells in response to an uniform concentration gradient of the attractant. (e-f) Despite the known chemorepellent functions of Sema3F and Slit2, precrossing axons display radial outgrowth when exposed to a localized source of repulsive Sema3F or Slit2 signals. (e) Note however, the increase outgrowth of precrossing axons in the presence of Sema3F, but not in the presence of Slit2 (f). (f) Axonal growth is diminished in explants exposed to Slit2 expressing cell aggregates. Explants are stained with TAG-1 antibody. (g) P/D ratio of total axonal length of precrossing explants. Graph shows mean average $\pm$ SEM error bars from three sets of experiments and *n* indicates the total number of explants quantified. A P/D ratio $\gg 1$ represents attraction and a P/D ratio of $\ll 1$ represents repulsion. Scale bar represents 100 μm .

Given that precrossing axons do not respond to the chemorepellents activities of Semaphorins and Slits prior floor plate crossing, we sought to explore the biological

significance of the convergence of Semaphorin and Slit pathways (**Fig. 2.2**) (Zou, et al., unpublished). First, we tested whether Slit2 could turn Sema3F responsiveness. Based on the spinal cord expression patterns of Slit2 and Sema3F mRNA transcripts (**Fig. 2.1**), we hypothesized that Slit2 might switch on Sema3F signals after midline crossing. To test this, we cultured precrossing explants in a uniform gradient of both Netrin1 and Slit2 and compared P/D ratios of axonal growth in response to Sema3F cell aggregates and found that precrossing explants displayed significant attenuation of growth, which made it difficult to assess repulsion (**Fig. 2.5a**). Moreover, precrossing explants cultured in the presence of a uniform source of Netrin1 and Sema3F exposed to a Slit2 cell aggregate displayed signs of increased defasciculation and loss of axon rigidity (**Fig. 2.5b**) when compared to axons grown in control conditions (**Fig. 2.4**).

While neither Slit2 nor Sema3F could activate repulsion signals of each other, precrossing commissural axons were repelled in the presence of cell aggregates expressing both, Slit2 and Sema3F proteins (50:50), indicating that together they can synergize to repel axons (**Fig. 2.5c**). These data suggest that the effects of Slits and Semaphorins combined could exert greater repulsion activity than either Slits or Semaphorins alone and can functionally interact to repel commissural axons. We hypothesized that this mechanism of interactive signaling pathways could act as a layer of regulation to control growth cone sensitivity prior to reaching the midline, given that Slits are mainly present at the ventral midline floor plate and in motor columns at the base of the floor plate, while Semaphorins are not only present in the floor plate (Sema3B), but also abundant throughout the spinal cord (Sema3F) (**Fig.**

2.1). While commissural axons do not respond to secreted Slits and Semaphorins before midline crossing, during crossing together Slits and Semaphorins through the NP2 receptor might switch on this repulsion *in vivo* necessary to increase the strength of repulsive signals at the floor plate.

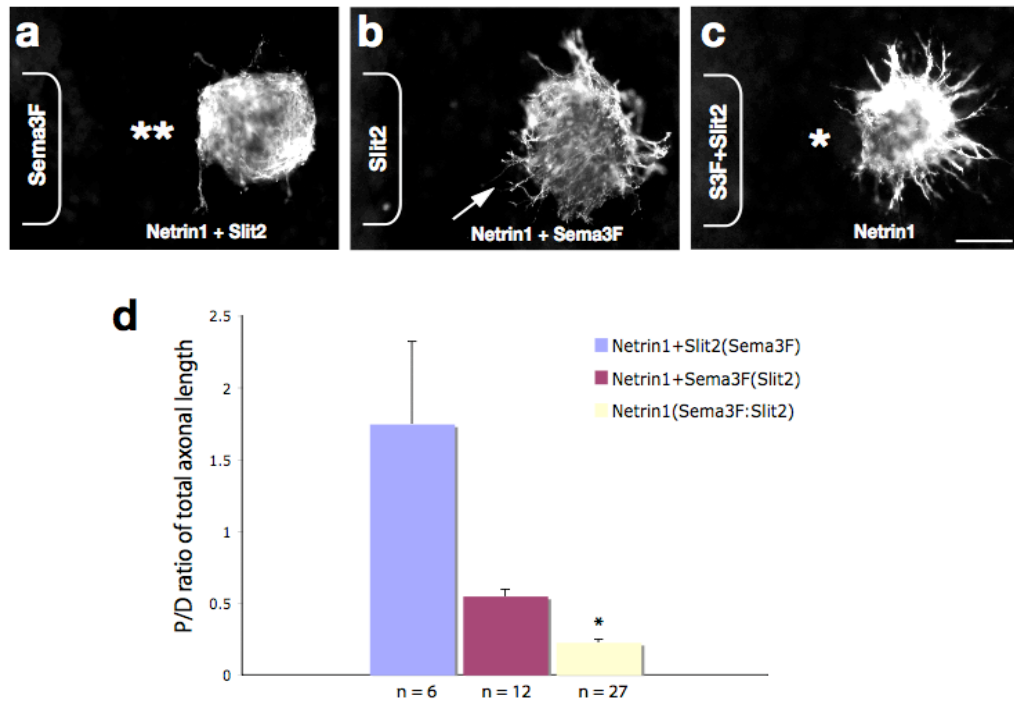


Figure 2.5 Slit2 and Sema3F signals together activate repulsion of precrossing commissural axons.

(a) Slit2 signals emanating from the Netrin1 collagen gel bed greatly diminished precrossing axon growth (double asterisks) in the presence of a localized source of Sema3F (S3F). (b) Netrin1 and Sema3F signals expressed uniformly in the bottom collagen gel failed to induce Slit2 repulsion in precrossing axons (arrow), but instead appeared wavy and defasciculated. (c) In contrast, precrossing axons were strongly repelled (asterisk) by the combinatorial effects of Slit2 and Sema3F activities emanating from a COS cell aggregate, suggesting a synergism between the two pathways. Explants are stained with TAG-1 antibody. (d) P/D ratio of total axonal length of precrossing explants. Graph shows mean average $\pm$ SEM error bars from three sets of experiments and n indicates the total number of explants quantified. * $P < 0.05$ (Student's t -test). P/D ratios $\ll 1$ indicate repulsion. Scale bar represents 100 μm

The data described above suggest the integration of Semaphorin3/Neuropilin2 signaling pathways with that of Slit/Robo pathways at the vertebrate ventral midline. Our results show that Slit2 and Sema3F together synergize to induce a robust repulsion of precrossing commissural axons *in vitro*.

Previously, NP2, the Semaphorin binding receptor of the Neuropilin/Plexin complex, was found to be required for both Sema3F and Slit2 induced repulsion of postcrossing commissural axons (**Fig. 2.2d**). In addition, *in vitro* cell binding assays showed that Slit2 could directly bind to NP2 receptors expressed on the cell surface of COS cells (Zou, et al., unpublished). To further test this, we transfected COS7 cells with NP2, PlexinA3 and Robo1 constructs and incubated these cells with recombinant Slit2 and Sema3F proteins fused with an alkaline phosphatase (AP) tag (**Fig. 2.6a**). The recombinant proteins were transfected in HEK293T cells and conditioned for three days in a reduced-serum medium, later concentrated in a Centrprep30 column. The concentration of the recombinant proteins obtained were determined based on the AP substrate reaction of NBT and BCIP, which yield a purple precipitate that can be quantified using a phosphate colorimetric assay of 405nm readings. Using the same NBT and BCIP color detection, cell binding was detected when Slit2 recombinant protein was incubated with cells transfected with Robo1 (**Fig. 2.6f**), NP2 (**Fig. 2.6i**), PlexinA3 (**Fig. 2.6l**) and a combination of NP2/PA3 (**Fig. 2.6o**). As controls, Slit2 did not bind to cells expressing vector only (**Fig. 2.6c**). Sema3F is known to bind to NP2/PA3 receptor complexes (Chen, et al., 1997; Zou, et al., 2000), used in this assay as positive controls. Recombinant Sema3F-AP protein was found to bind to cells

expressing NP2 (**Fig. 2.6j**) and NP2/PA3 (**Fig. 2.6p**), but did not bind to PlexinA3 only expressing cells (**Fig. 2.6m**) or to the Slit receptor, Robo1 (**Fig. 2.6g**). As a control, Sema3F-AP did not bind to COS cells expressing vector only (**Fig. 2.6d**). Together this data show that Slit2-AP proteins interact with NP2 and PA3 receptors transiently expressed in COS cells, an interaction that might be necessary for the activation of repulsive pathways *in vivo*.

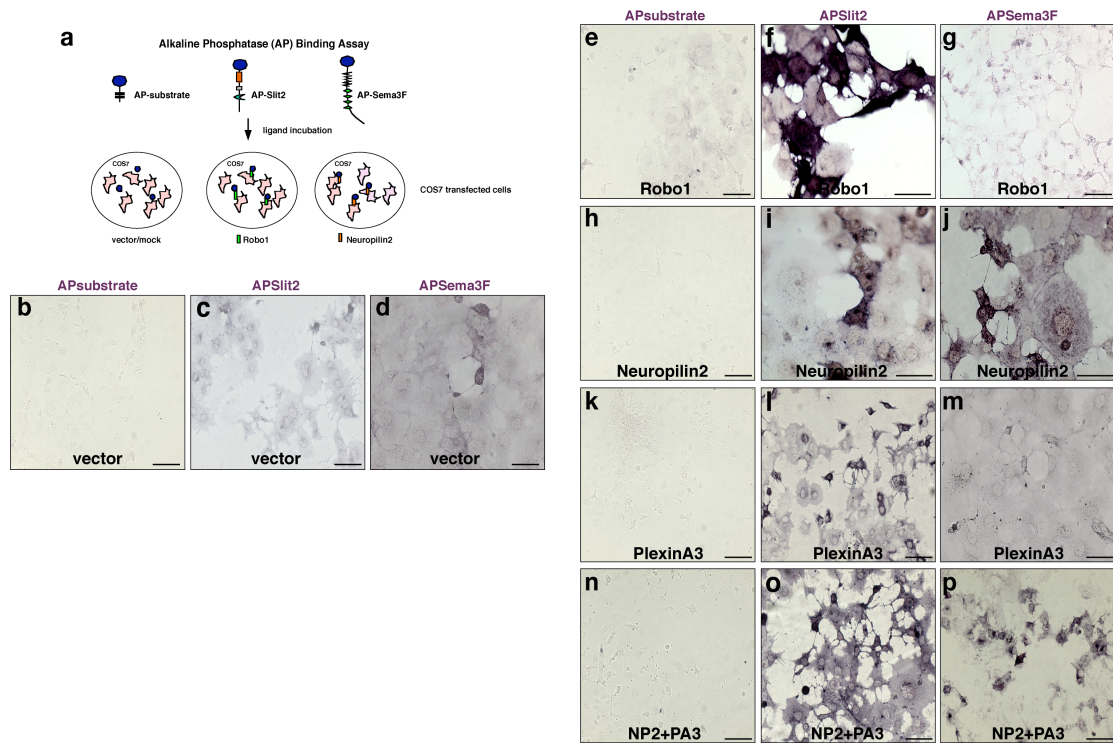


Figure 2.6 Slit2 binds to the Semaphorin3 receptors Neuropilin2 and PlexinA3 *in vitro*.

(a) Schematic diagram depicting the alkaline phosphatase (AP) cell binding assay using AP fusion proteins for Slit2, Sema3F and an AP substrate negative control. COS cells were transfected with an empty vector control, Robo1, Neuropilin2 (NP2), PlexinA3 or both NP2 and PlexinA3 and later incubated with AP fusion proteins for histological examination. (b-d) AP fusion proteins incubated with vector control cells showed no significant binding. (e-g) Robo1 expressing COS cells showed specific binding to AP-Slit2, but not to AP-Sema3F or the AP-substrate control. (h-j) COS cells expressing the NP2 receptor showed increased binding to both AP-Sema3F and AP-Slit2 recombinant proteins. (k-m) PlexinA3 expressing cells did not appear to have significant binding affinity to AP-Sema3F, but AP activity was observed in PlexinA3 cells incubated with Slit2. (n-p) Cotransfection of NP2 and PlexinA3 did not change the binding affinities of AP-Slit2 and AP-Sema3F to NP2. Scale bars represent 50 μm .

Furthermore, given that commissural axons do not respond to either Slits or Semaphorin3 proteins prior midline crossing, we hypothesized that a floor plate derived molecule could be regulating the repulsive activities of these signals acting

upstream of the pathways. To test this we screened candidate proteins that were expressed by floor plate cells during the time of commissural axon crossing that could induce premature repulsion in our modified precrossing explant assay (**Fig. 2.3**). In these experiments, explants were cultured in the three-dimensional two-layer collagen matrix gel previously described, containing Netrin1 expressing cells and candidate floor plate derived molecules, which were uniformly resuspended in the bottom collagen gel, while concomitantly exposing the explants to cell aggregates expressing a vector control, Slit2 or Sema3F proteins (**Fig. 2.7a**). The floor plate derived molecules tested included, Wnt4 (**Fig. 2.7b**), Wnt7b (**Fig. 2.7c**) (Lyuksyutova, et al., 2003) and Sonic Hedgehog (Shh) proteins (**Fig. 2.7d**) (Jessell, 2000). Precrossing explants grown in the presence of Netrin1 and Wnt4 expressing COS cells exposed to cell aggregates expressing a vector control failed to grow in culture (**Fig. 2.7f**) compared to control explants grown in the presence of Netrin1 alone (**Fig. 2.7e**). Precrossing explants grown in the presence of Netrin1 and Wnt7b next to a vector control cell aggregate appeared defasciculated and acquired a wavy appearance with less orderly growth, which made their effects on Sema3F and Slit2 signals difficult to determine (**Fig. 2.7g**). Our third molecule of choice was Shh, a protein also expressed at the floor plate during commissural axon crossing and in the adjacent notochord (**Fig. 2.7d**). Precrossing explants grown next to a vector control cell aggregate displayed normal radial growth (**Fig. 2.7h**) similar to explants grown in Netrin1 only expressing cells (**Fig. 2.7e**). Moreover, when precrossing explants were cultured next to a cell aggregate expressing Slit2 in the presence of Netrin1 and Shh, axons appeared

short and stubby and displayed poor growth resembling explants grown in the presence of Netrin1 and Slit2 next to a cell aggregate expressing Sema3F (**Fig. 2.5a**). Because of the reduction of axonal growth in these explants, the total axon length P/D ratio was not effective in determining induced repulsion. In contrast, precrossing explants grown on COS cells expressing Netrin1 and Shh, next to a cell aggregate expressing Sema3F, displayed robust repulsive growth on the proximal side of the explant (**Fig. 2.7j**). This finding prompted us to further investigate the effects of Shh on Semaphorin3 signals, results presented in **Chapter3**.

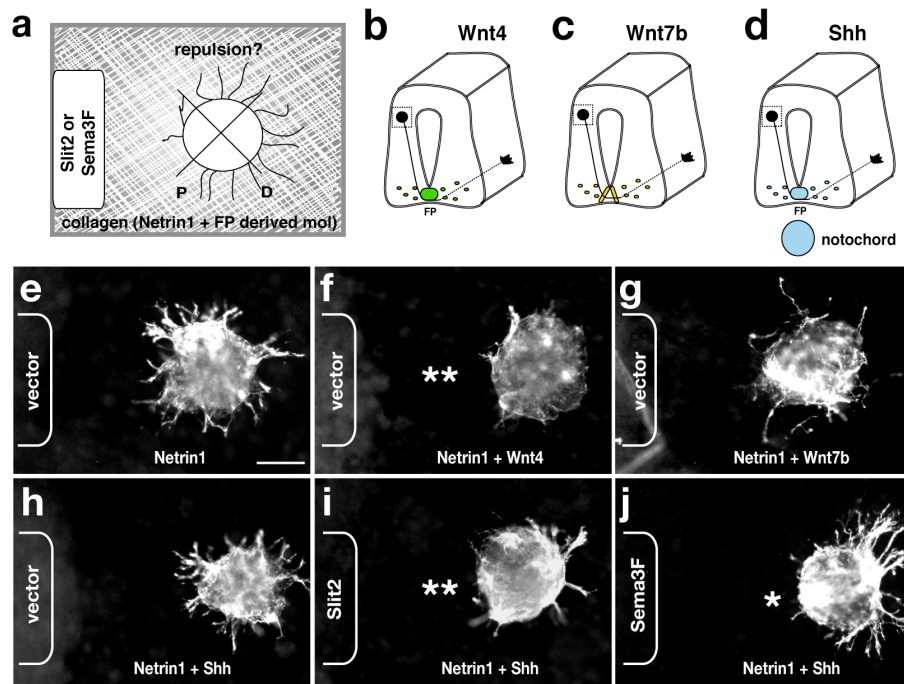


Figure 2.7 Sonic Hedgehog induces Semaphorin3F repulsion in precrossing commissural axons.

(a) Schematic depicting the floor plate protein screen assay. Precrossing spinal cord explants were cultured in the presence of Netrin1 and floor plate derived molecules next to a COS cell aggregate expressing vector control, Slit2 or Sema3F for 16 hours in a collagen gel matrix. (b-d) Based on floor plate expression during commissural axon crossing, Wnt4 (b), Wnt7b (c), and Shh (d) were analyzed as candidate molecules for regulating Slit and Semaphorin repulsive signals. (e) Control explants cultured in the presence of Netrin1 and a vector control cell aggregate displayed normal radial growth. (f) In the presence of Wnt4, however, precrossing axons failed to grow when exposed to a vector expressing cell aggregate. (g) Explants grown in the presence of Wnt7b expressing cells, showed increased defasciculation and less orderly axonal growth, which made it difficult to determine repulsion. (h) Explants cultured in the presence of both Shh and Netrin1 expressing cells presented radial growth when exposed to COS cell aggregates expressing vector controls. (i) Explants grown next to a Slit2 cell aggregate in the presence of Netrin1 and Shh did not show significant repulsive behaviors, but instead displayed reduced axonal growth. (j) Precrossing explants cocultured with COS cell aggregates expressing Sema3F in the presence of Netrin1 and Shh showed a robust premature repulsion to the chemorepellent properties of Sema3F. Explants are stained with TAG-1 antibody. Scale bar represents 100 μm .

Discussion

Commissural axons provide a useful model system for studying axonal navigation through intermediate targets, such as the floor plate. Commissural axons are first attracted to the chemoattractive properties of the floor plate, but become repelled during crossing. This ensures proper axonal transition from one side of the spinal cord to the other and is how commissures are formed in development (Dickson and Gilestro, 2006; Dickson, 2002; Chisholm and Tessier-Lavigne, 1999).

The growth cone of a navigating axon is composed of a myriad of receptors located on the surface membrane regulated by intrinsic mechanisms and extrinsic axon guidance systems. Successful navigation of the ventral midline requires precise and specific responses to guidance cues present in the developing embryo. Axonal responses are also dependent on the interactions with other axon guidance pathways and the coincident activation of other guidance signals. This raises the fundamental question of how growth cones integrate multiple guidance pathways and translate these signals into navigational behaviors. Our results imply that Slit and Semaphorin3 signals emanating from the floor plate and ventral regions of the developing spinal cord might work synergistically to repel commissural axons as they cross the ventral midline. While numerous guidance systems are present in the same growth cones, some can dominate over the other activating or inactivating their activities. For instance, at the ventral midline there is evidence that Netrin1 signals through the receptor DCC can be inhibited at the receptor level by direct binding of the Slit receptor, Robo to DCC in *Xenopus* spinal cord neurons. Slit/Robo signaling induces

silencing of Netrin1 attraction, which in turn activates Slit repulsive signals and prevents axons from stalling at the floor plate (Stein and Tessier-Lavigne, 2001). In addition, other guidance pathways can synergize to mediate an attractive or repulsive axonal response. For example, Shh and Netrin1 chemoattractive properties have been shown to cooperate to guide commissural axons towards the floor plate ventral midline *in vitro* and appears to be necessary for guidance to the floor plate by these axons *in vivo* (Charron, et al., 2003).

Slit/Robo and Semaphorin3/NP2 pathways synergize in precrossing commissural axons

Our studies suggest a crosstalk of Slit and Semaphorin3 signaling pathways consistent with previous findings (**Fig. 2.2d**) (Zou, et al., unpublished). This prompted us to test whether Slit/Robo and Semaphorin/NP2 pathways could dominate one over the other or activate each other, given that both pathways have been well characterized as ventral midline chemorepellent signals (Zou, et al., 2000; Long, et al., 2004). Our *in vitro* precrossing explant analysis suggest that while neither Slit2 nor Sema3F appear to directly function upstream of one another, together these pathways can promote a robust premature repulsion of precrossing commissural axons (**Fig. 2.5**). In addition, both Slit2 and Sema3F appear to have individual effects on precrossing axons, with Slit2 signals promoting shorter axons, whereas Sema3F signals stimulate growth of precrossing axons and in the presence of Slit2, these axons appear defasciculated and wavy in nature. In contrast cell aggregates coexpressing Slit2 and Sema3F can induce a robust premature repulsion of precrossing commissural

axons. The findings that Slit2 can also bind to the Semaphorin3 receptor, NP2, further corroborates the notion that while precrossing axons do not express the receptors for Slits, Robo1 and Robo2 during crossing, their expulsion from the midline might be regulated by the interaction of Slits and Semaphorin3 pathways through the NP2 receptor. Our preliminary findings also suggest that Slit2 binds to cells expressing PlexinA3 the signal transducer of the Semaphorin3 pathway. Our *in vitro* analysis shows that Slit and Semaphorin pathways functionally interact to mediate repulsion potentially through the NP2/PA3 signaling cascade. Future experiments should focus on determining whether Plexins are functionally required for Slit2 mediated repulsion. We have cloned a dominant-negative PlexinA3 construct, which lacks the cytoplasmic domain, but still contains the ectodomains and transmembrane domains of the receptor, into a pCIG2-IRES GFP vector. This vector has been consistently used in the lab for *ex utero* electroporations of spinal cord commissural neurons, which can be readily visualized under the expression of the green fluorescence protein (GFP) using confocal microscopy. Precrossing and postcrossing assays of dominant negative PA3 electroporated explants can be set up to determine whether the interaction of Slit and NP2 is mediated through PlexinA3 signals. Furthermore, Slit and Semaphorin3 pathways together might converge at common downstream signaling pathways, such as the RhoA family of small GTPase regulators (RhoA, Cdc42 and Rac), which are known to connect multiple axon guidance systems to cytoskeletal rearrangements to mediate axonal steering changes. The underlying molecular mechanisms of the convergence of these two chemorepellent pathways could increase repulsion by

removing attraction (e.g. decreasing active Cdc42), while promoting repulsion (e.g. increasing active RhoA) (Lowery and Van Vactor, 2009).

Sonic Hedgehog induces Semaphorin3 repulsion in precrossing commissural axons

As mentioned above, the intricateness of commissural axon guidance systems relies in the formation of multi-molecular complexes and crosstalks between guidance receptors, key transmembrane components and intracellular signaling molecules that will determine the outcome of the axonal response. The correct integration of these signals is necessary to induce the proper axonal responses despite the fact that numerous signals will be activated in the developing growth cone at any given time and place. Consequently, signal transduction can be interpreted by axons in the context of other relevant signals simultaneously present in their environment. We know from previous studies that commissural axons do not respond to either Slits or Semaphorin3 proteins prior midline crossing, we hypothesized that a floor plate derived molecule could be regulating repulsive activities upstream of these signals.

The floor plate ventral midline is a major organizer for axon wiring and an important intermediate target. Commissural axons change their trajectory after midline crossing by dynamically remodeling growth cone responsiveness to guidance cues. The floor plate is also known for secreting morphogen molecules of the Shh and Wnt family of proteins responsible for early patterning of the spinal cord (Jessell, 2000; Bovolenta, 2005; Zou and Lyuksyutova, 2007). In addition, morphogen gradients have been shown to regulate axonal pathfinding possibly by providing

growth cones with graded positional information (Charron, et al., 2003; Lyuksyutova, et al., 2003; Bovolenta, 2005). Given their expression in the floor plate, they are interesting candidates for regulators of midline chemorepellents, which prompted us to test whether they could activate chemorepellent Slit and Semaphorin3 signals. Using a novel *in vitro* assay, it was previously shown that Wnt protein activity was necessary for the anterior guidance of postcrossing commissural axons. Wnt4 and Wnt7b presented high mRNA transcript levels at the floor plate and were shown to affect postcrossing commissural axon growth (Lyuksyutova, et al., 2003). In addition, commissural axon guidance after floor plate crossing was severely disrupted in mice lacking the Wnt receptor Frizzled3 (Lyuksyutova, et al., 2003). Here, we tested if Wnt activity could mediate the switch of repulsive functions at the floor plate ventral midline, in particular whether it could prematurely turn on Slit or Sema3F function in precrossing commissural axons and found that in our assay, Wnt4 and Wnt7b proteins have effects on normal axonal growth and fasciculation, which made it difficult to test for premature repulsion in precrossing commissural axons.

The role of morphogens in development is known to rely heavily on signal strength, gradient stability and protein concentration, so it is not surprising that responses to the Wnt family of proteins could affect commissural axon growth. Another interesting molecule highly expressed at the ventral midline is the morphogen, Sonic hedgehog (Shh). Shh was previously shown to function as an axonal chemoattractant that can mimic the Netrin1 independent chemoattractant properties of the floor plate *in vitro* and conditional inactivation of the Shh receptor

Smoothened, resulted in defective commissural axon trajectories in the ventral spinal cord *in vivo* with no apparent changes in spinal cord patterning (Charron, et al., 2003).

Interestingly, Shh was also shown in chick embryos to direct the anteroposterior guidance of postcrossing commissural axons (Bourikas, et al., 2005). In this study, Shh was reported to guide postcrossing commissural axons anteriorly on the longitudinal axis of the contralateral side of the developing spinal cord. Silencing of Shh activity by RNAi resulted in some axons turning randomly on the A-P axis of the developing chick embryo. Intriguingly, this effect appeared to function independent of the canonical receptors for Shh, Patched or Smoothened and instead was proposed to be mediated through, Hip1, a Shh-binding membrane glycoprotein presumably expressed during floor plate crossing (Bourikas, et al., 2005). Hip1, like PTC1 is a general transcriptional target of Shh and binds to Shh with similar affinities to PTC1 (Chuang and McMahon, 1999). Moreover, Hip1 has been shown to attenuate Shh signals providing a negative regulatory feedback loop and unlike PTC1, Hip1 appears to inhibit Shh by physically sequestering Shh at the cell surface (Jeong and McMahon, 2004).

The molecular mechanisms underlying changes in commissural axon responsiveness are not fully understood. Here, we sought to investigate whether Shh could regulate the chemorepellent activities of Slit and Semaphorin3 in precrossing axons. As previously shown, Shh did not have a significant effect on precrossing commissural axon growth in the presence of Netrin1 alone (**Fig. 2.7h**) (Charron, et al., 2003). Furthermore, in the presence of a cell aggregate expressing Slit2, commissural

axons failed to grow sufficient enough for us to determine repulsion. In contrast, commissural axons exposed to cell aggregates expressing Sema3F in the presence of both Netrin1 and Shh displayed a robust premature repulsion, suggesting that Shh could as a regulator of Semaphorin3 signals emanating from the spinal cord ventral midline. The dual effects of Shh signals seen in previous precrossing and postcrossing explant studies described above might represent differences in regulation of axon guidance systems in the growth cone depending on the its location along concentration gradients. In cell patterning, Shh functions as a gradient signal to dictate cell fate, so it is conceivable that growth cones might also be guided through this gradient to interpret positional information. At the level of the floor plate, Shh signals are the highest compared to other regions of the spinal cord, which include Shh signals emanating from the adjacent notochord. This drastic change in concentration might induce intrinsic growth cone changes necessary to activate Semaphorin3 signals *in vivo*.

The regulation of Slit/Robo repulsive signals at the floor plate ventral midline has been extensively studied in both invertebrate and vertebrate systems (Dickson and Gilestro, 2006, Long, et al., 2004), but less is known about the mechanisms regulating Semaphorin3 repulsive signals. We sought to investigate the molecular mechanisms underlying Sema3F responsiveness by Shh signals in commissural axons. These studies will be presented in **Chapter 3**.

Further studies of morphogenic function in axon guidance can help understand the regulation of intracellular guidance mechanisms that subsequently transduce

axonal navigation events. In particular, morphogen signals are likely to modulate axonal sensitivity to guidance cues by regulating properties of membrane receptors and cytoplasmic signal components. In the future, it will be important to study morphogenic effects on local transduction mechanisms affecting growth cone morphology and axonal behaviors and also whether these functions are mediated through the canonical morphogenetic transcriptional signaling pathways or additional independent pathways.

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CHAPTER 3

Sonic hedgehog modulates the response of spinal cord commissural axons to class 3 Semaphorin signals during midline crossing

Introduction

Pathfinding axons undergo dynamic molecular remodeling to change responses to guidance cues as they search the developing nervous system for final targets. During midline crossing, spinal cord commissural axons acquire responsiveness to class 3 Semaphorins and Slits, which regulate their exit from the floor plate and restrict their trajectory into a longitudinal pathway after crossing (Zou, et al., 2000). We found that Sonic Hedgehog (Shh) can turn on class 3 Semaphorin mediated repulsion in pre-crossing commissural axons in culture. Blocking Shh function in commissural axons by treating open book spinal cord cultures with the function blocking antibody against Shh, 5E1, or by expressing a mutant form of Patched1 (PTC1) insensitive to Shh, Ptc1^{Δloop2} or a shRNA construct targeting Smoothened (Smo) resulted in axon guidance defects at the midline, including stalling, knotting, recrossing, anterior-posterior randomization and overshooting after crossing, reminiscence of Neuropilin2 (NP2), mutant phenotypes. In addition, altering Protein kinase A (PKA) activity levels in precrossing commissural axons diminished Shh mediated Semaphorin repulsion and caused profound midline stalling and overshooting/wandering of postcrossing axons in open book spinal cord cultures,

suggesting that Shh regulates responses to Semaphorin in part by regulating the ratio of cyclic nucleotides. These results suggest that Shh may regulate signaling of other axon guidance molecules by modulating second messenger levels.

NP2 mutant embryos showed severe midline guidance defects including stalling in the midline, overshooting to contralateral ventral spinal cord and randomized projection along the anterior-posterior (Zou, et al., 2000). These phenotypes are consistent with the proposed function of these chemorepellents to ensure proper midline exit and channel postcrossing commissural axons to turn into an anterior-posterior (longitudinal) trajectory on the contralateral side. Nonetheless, the molecular mechanisms underlying the switch in the responsiveness to multiple guidance systems, particularly the extrinsic signals that trigger these changes are still largely unknown. Here, we studied how commissural axons gain responsiveness to class 3 Semaphorin signaling during midline crossing. We found that Shh can activate Semaphorin repulsion in precrossing commissural axons and showed that the receptors PTC1 and Smo are both necessary for proper commissural axon guidance. We propose that Shh sensitizes growth cone responsiveness to Class 3 Semaphorins by down regulating the cAMP/PKA pathway, which suggests a novel role for Shh signals in regulating the responses of other guidance cues.

Results

Shh turns on Semaphorin repulsion in precrossing commissural axons

To further understand the molecular changes developing axons undergo at key intermediate targets, we sought to investigate the mechanisms that could activate Semaphorin repulsion in commissural axons during spinal cord midline pathfinding. To do this, we modified the classical precrossing collagen explant assay so that we could test the effects of various diffusible guidance molecules expressed during midline crossing on Semaphorin3 repulsion (**Fig. 3.1a-b**). The precrossing assay consists of an *in vitro* culture of spinal cord dorsal explants grown between two layers of collagen gel polymerized one on top of the other. In the top collagen layer, explants of dorsal commissural neurons from E13 rat embryos (equivalent to E11.5 mouse embryos) are embedded next to COS cell aggregates expressing Sema3B or Sema3F. The bottom collagen layer contains dispersed COS cells expressing Netrin1 alone or Netrin1 plus a secreted molecule from the floor plate (**Fig. 3.1b**, gray scratched pattern). We have used similar approaches to introduce secreted Frizzled-related proteins (sFRPs) in open book spinal cord explant cultures to test the effect of sFRPs on the anterior turning of the commissural axons (Lyuksyutova, et al., 2003). In our assay, explants cultured in the presence of Netrin1 alone grew radially around their circumference and did not respond to Sema3B or Sema3F expressing COS cell aggregates (**Fig. 3.1d,e**) similar to the vector only cell aggregate control (**Fig. 3.1c**). In contrast, surprisingly, precrossing commissural axons grown on top of collagen gel containing Netrin1 and Shh expressing cells were repelled by Sema3B and Sema3F

expressing cell aggregates (**Fig. 3.1g, h**), but not repelled by aggregates expressing the vector control (**Fig. 3.1f**). Our results indicate that the repulsive activities of Sema3B and Sema3F could be activated by Shh in precrossing commissural axons in our assay. Because Shh is highly abundant in the floor plate, surrounding tissue, and notochord, Shh is an excellent candidate for a midline switch signal.

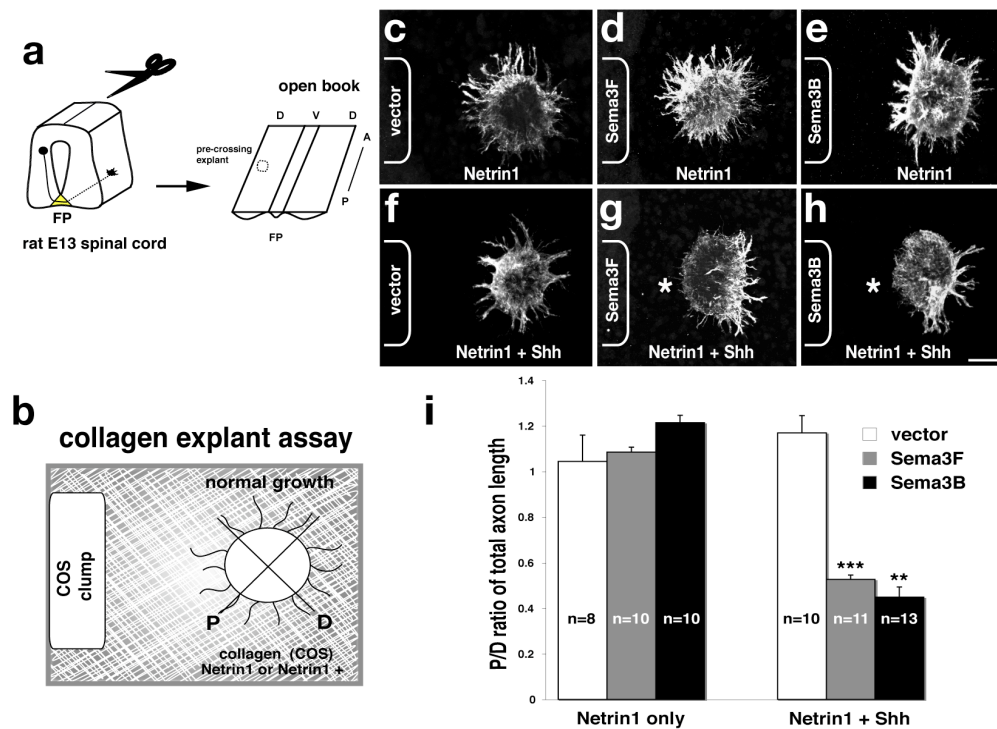


Figure 3.1 Shh induces Semaphorin responsiveness in precrossing commissural axons.

(a) A schematic of rat E13 embryo microdissections of precrossing explants from the dorsal domains of the developing spinal cord. (b) Diagram showing the precrossing collagen explant assay used to assess Semaphorin repulsion. Dorsal spinal cord pre-crossing explants are grown on a collagen gel bed containing a resuspension of COS cells secreting Netrin1 only or both Netrin1 and Shh and co-cultured next to a COS cell aggregate expressing a control vector, pCDNA (c and f), Sema3F (d and g) or Sema3B (e and h) constructs. The axon outgrowth of precrossing explants appear symmetrical in control co-cultures (c and f) and in explants grown in the presence of Netrin1 only cells and Semaphorin3 expressing cell aggregates (d and e). (g and h) In the presence of Shh expressing cells, commissural axons are repelled by Sema3F and Sema3B expressing cell aggregates (asterisks indicate reduced growth due to repulsion), whereas commissural axons are normally not repelled by Semaphorins in the absence of Shh (d and e). (i) Quantification of the total axon bundle length P/D ratio. Graph shows mean average $\pm$ SEM error bars from three sets of experiments and n indicates the total number of explants quantified. ** $P < 0.01$ and *** $P < 0.0025$ (Student's t -test). Scale bar represents 100 μ m.

Blocking Shh activity causes misguidance of commissural axons during midline crossing

To determine the role of Shh in regulating midline pathfinding, we analyzed the trajectory of commissural axons in rat E13 open book spinal cord preparations treated with the function-blocking antibody for Shh, 5E1. The projections of these commissural axons were visualized by injections of the lipophilic dye, DiI, at the dorsal margin of the spinal cord (**Fig. 3.2g**). In open book explants, commissural axons can recapitulate *in vivo* pathfinding and project from the spinal cord dorsal margin across the floor plate and then anteriorly on the contralateral side of the spinal cord (Zou, et al., 2000; Lyuksyutova, et al., 2003; Wolf, et al., 2008). Our results showed that Shh antibody, 5E1, treated open book explants displayed a cohort of axons that were unable to project towards the floor plate and instead extended along the anterior-posterior direction close to the ipsilateral border of the floor plate (**Fig. 3.2b-e**, arrowheads) when compared to untreated controls (**Fig. 3.2a**), consistent with the role of Shh as chemoattractant for precrossing commissural axons (Charron, et al., 2003). Whereas, the majority of commissural axons treated with 5E1, stalled and knotted within the midline (**Fig. 3.2c, d**, double headed arrows) and at the contralateral edge of the floor plate (**Fig. 3.2b-f**, double short arrows). In addition, a cohort of postcrossing axons wandered and misprojected on the contralateral side of the spinal cord (**Fig. 3.2f**, double long arrows) and a few axons misguided back into the floor plate (**Fig. 3.2d**, single arrow). Commissural axons make a sharp anteriorly after midline crossing and project into the ventral funiculus alongside the floor plate or fan out to grow laterally in a diagonal direction (**Fig. 3.2i**) (Lyuksyutova, et al., 2003).

After 5E1 open book treatments, postcrossing axons deviate from that organized trajectory and overshoot in the contralateral side and in the posterior direction, suggesting that they are ignoring a repulsive cue from the lateral direction (**Fig. 3.2j, k**). These phenotypes are similar to those seen in mice deficient for NP2, which encodes a key receptor component for Class 3 Semaphorins, Sema3B and Sema3F (Zou, et al., 2000).

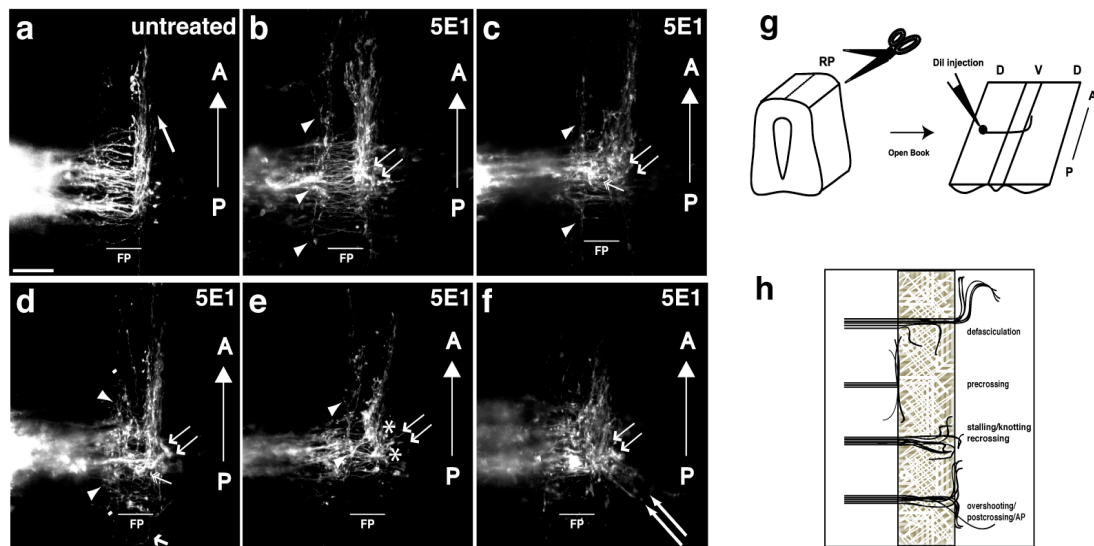


Figure 3.2 Shh function is required for normal midline pathfinding of commissural axons.

Confocal images of DiI injections labeling commissural axons in open book explants as seen in the schematic in **g**. (**a-f**) DiI injections showing commissural axon pathfinding **during** midline crossing. (**a**) Untreated open book injection shows the majority of axons crossing the floor plate (FP) and turning anteriorly (single arrow). (**b-f**) in the presence of the function-blocking Shh antibody, 5E1, a cohort of commissural axons fail to enter the FP (arrowheads in **b-e**), while others stall and form knots within the FP (double head arrow in **c** and **d**). Many axons fail to exit the FP (short double arrows **b-f**) and instead stall at the contralateral side of the FP forming distinct knots (asterisks in **e**). A few postcrossing axons loop back and recross the FP (single short arrow in **d**) and other postcrossing axons overshoot or wander into the contralateral side of the spinal cord instead of turning anteriorly (double long arrows in **f**) reminiscent of A-P axonal misguidance. (**j**) Schematic diagram of an open book explant showing a normal DiI injection crossing the midline and turning anteriorly. (**h**) Summary of commissural axon pathfinding errors in anti-Shh treated spinal cord explants.

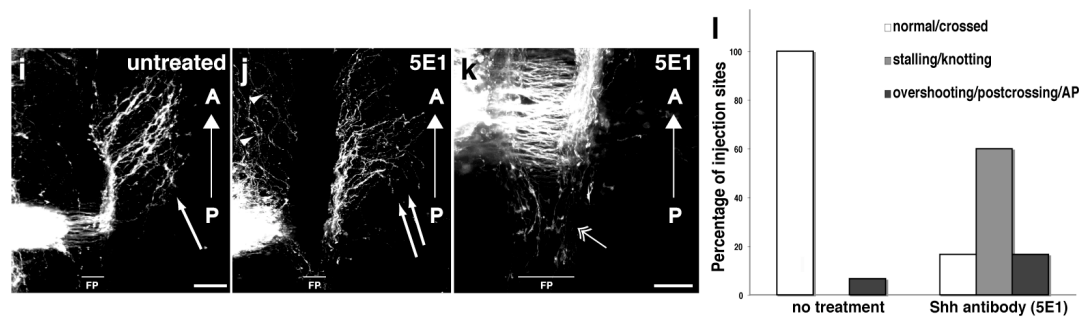


Figure 3.2 Shh function is required for normal midline pathfinding of commissural axons, continuation.

(i-k) DiI images showing commissural axon pathfinding treated **after** midline crossing (postcrossing axons). (i) DiI injection of an untreated spinal cord explant showing anterior turning of a stereotypical commissural axon trajectory after midline crossing projecting into the ventral and lateral funiculus alongside the FP (long single arrow). (j-k) DiI injection of open book explants treated with the function-blocking antibody for Shh, 5E1 after midline crossing displaying defasciculation errors on the contralateral side of the spinal cord. Postcrossing axons fan out from their fasciculated tracts and fall posteriorly (double long errors in j), while the majority of axons have already crossed the midline during the anti-Shh antibody treatment, a cohort of young axons are affected before crossing and fail to enter the FP (arrowheads in j) and a few others wandered within the FP (doubleheaded arrow in k). (l) Quantification of midline pathfinding behaviors in treated DiI injections as seen in a-f. n = number of DiI injections examined. Scale bar represents 50 μ m (a and k) and 100 μ m (i).

Perturbing Shh signaling through Patched1/Smoothed signaling causes commissural axon guidance defects at the ventral midline

To further examine how Shh may regulate repulsion during midline pathfinding, we tested the function of the Shh receptor, PTC1 by *ex utero* electroporation in open book spinal cord cultures (Fig. 3.3a) (Wolf, et al., 2008). Here, we generated a deleted form of PTC1, Ptc1^{Δloop2}, which lacks the Shh-binding domain, rendering it constitutively active in its ability to inhibit Smo. This construct was shown to downregulate the Shh pathway down to <10% of its potential activity acting as a dominant negative mutant protein (Taipale, et al., 2002). Ptc1^{Δloop2} was

cloned into the IRES-PCIG2 vector driven by a β -actin promoter to allow expression in commissural neurons and visualization of the green fluorescence protein (**Fig. 3.3b**). PTC1 expression was found in commissural neurons along the axon shaft and developing growth cone (**Fig. 3.3c**). Rat E13 embryos were electroporated *ex utero* and spinal cords dissected out as open book preparations. After three days in culture, pCIG2-IRES-GFP expressing commissural axons cross the ventral midline and turn anteriorly in control open book electroporated explants (**Fig. 3.3d**). In contrast, expression of $Ptc1^{\Delta loop2}$ resulted in severe commissural axon pathfinding defects during floor plate crossing (**Fig. 3.3e-h**). The number of axons reaching and entering the midline were significantly reduced, consistent with the notion that Shh acts as a chemoattractant for precrossing axons (**Fig. 3.3e-h**, arrowheads) (Charron, et al., 2003). In addition, the majority of commissural axons crossing the floor plate appeared misguided, with many axons looping back and recrossing the midline, an axonal behavior seen in *Drosophila* roundabout mutants at the central nerve cord midline (**Fig. 3.3f-h**, double headed arrows) (Dickson and Gilestro, 2006). A significant number of axons failed to make an anterior turn after midline crossing and instead, overshoot on the contralateral side making gradual turns randomly along the A-P axis (arrows in **Fig. 3.3e, f, h**), suggesting that they failed to respond to a repulsive cue from the lateral direction. These phenotypes are similar to those seen in NP2 mutants (Zou, et al., 2000).

To confirm that PTC1 is expressed in commissural axons at the stage when commissural axons are pathfinding at the midline, we performed

immunohistochemistry of rat E13 commissural neurons (**Fig. 3.3c**) and mouse E11.5 embryos (**Fig. 3.3k-n**). Here, we found that PTC1 is highly expressed in the commissural axon segment at the floor plate (**Fig. 3.3k-n**). The protein expression pattern is consistent with the *in situ* data in chick embryos (PTC1 transcript was found in DRGs at equivalent stages) (Marigo, et al., Development; 1996; Guan, et al., 2008). It is also known that PTC1 is a direct target of Shh transcriptional activity and Shh is highly expressed at the ventral midline and adjacent notochord (Marigo, et al., Nature, 1996; Jessell, 2000).

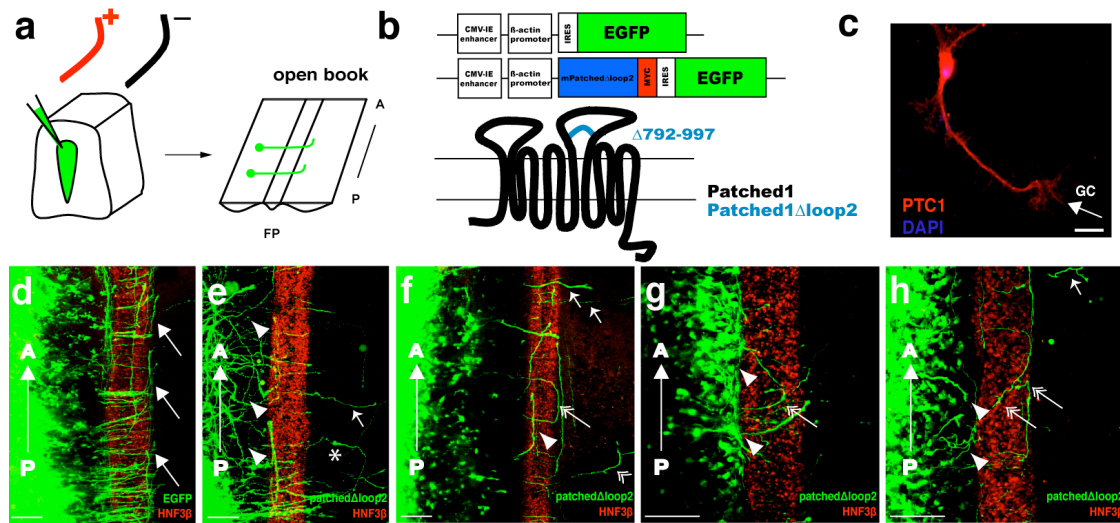


Figure 3.3 The Shh receptor Patched1 is required for normal midline crossing of commissural axons.

(a) Schematic diagram showing ex utero electroporations of rE13 embryos. (b) Constructs expressing a EGFP vector control and a Shh insensitive form of PTC1 that constitutively inhibits Smo signaling, Patched1 Δ loop2 (Ptc1 Δ loop2). The Ptc1 Δ loop2 construct is driven by a β -actin promoter conferring constitutive neuronal expression under the IRES translational system. (c) PTC1 (red) and DAPI staining of a rat E13 spinal cord neuron. PTC1 stains the plasma membrane throughout the axon and growth cone, DAPI stains the nucleus. (d) Normal midline pathfinding of commissural axons electroporated with EGFP. Axons enter the midline, cross the floor plate (FP) and turn anteriorly soon after crossing (arrows). (e-h) Commissural axons expressing Ptc1 Δ loop2 are misguided at the floor plate. The number of axons reaching the midline are significantly reduced, some axons appear misoriented and unable to enter the midline (arrowheads in e-h). Many axons loopback and recross the midline (double head arrows in f-h). A significant number of axons overshoot and wander into the contralateral ventral spinal cord (short arrows in e, f and h) and loop back (asterisk in e). Some of the overshoot axons do not turn anteriorly immediately after midline crossing but rather turn gradually at more lateral positions (double arrowhead in f).

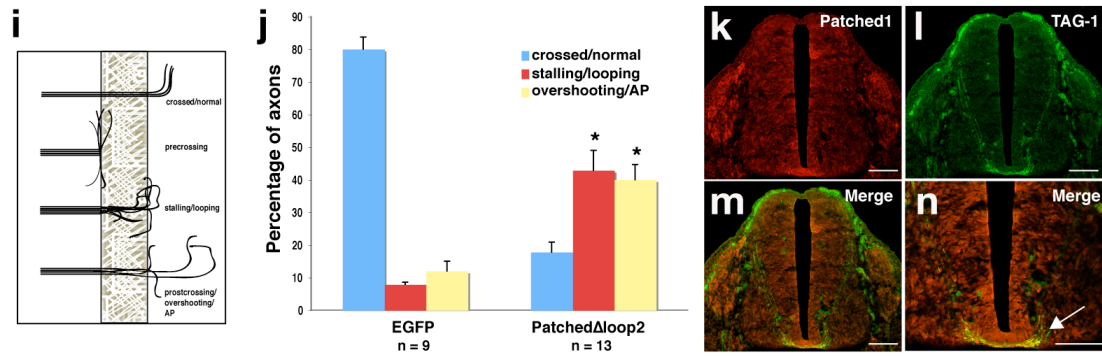


Figure 3.3 The Shh receptor Patched1 is required for normal midline crossing of commissural axons, continuation.

(i) Summary of misguidance behaviors observed in $Ptc1^{\Delta loop2}$ electroporated spinal cord explants. (j) Quantification of commissural midline pathfinding behaviors in EGFP and $Ptc1^{\Delta loop2}$ expressing axons. The graph shows the percentage of axons displaying normal and misguided behaviors in electroporated spinal cords. The mean $\pm$ SEM error bars are from three sets of experiments and n indicates the total number of explants quantified. * $P < 0.05$ (Student's t -test). (k-n) Patched-1 is highly enriched in commissural axons during midline crossing (arrow in n). Yellow in m and n indicate the costaining of Patched1 and the commissural axon marker TAG-1. Scale bars represent 50 μ m (d-f), 100 μ m (g and h), and 100 μ m (k-n).

Smo is broadly expressed in the spinal cord (Fig. 4.1) and in particular, Smo protein is present in developing commissural axon growth cones (Fig. 3.4b). To complimentary test the role of Smo during midline pathfinding, we obtained an shRNA construct targeting Smo (Fig. 3.4a) and electroporated rE13 embryos as described in Fig. 3.3a. We found that knocking down Smo caused severe midline guidance defects. Compared to dominant-negative PTC1, $Ptc1^{\Delta loop2}$, many axons turned backwards within the floor plate or at the contralateral border of the floor plate (Fig. 3.4d-f, arrowheads). A cohort of axons wandered after floor plate crossing on

the contralateral side of the spinal cord gradually turning anteriorly instead of turning immediately after crossing (**Fig. 3.4d,e**, asterisks).

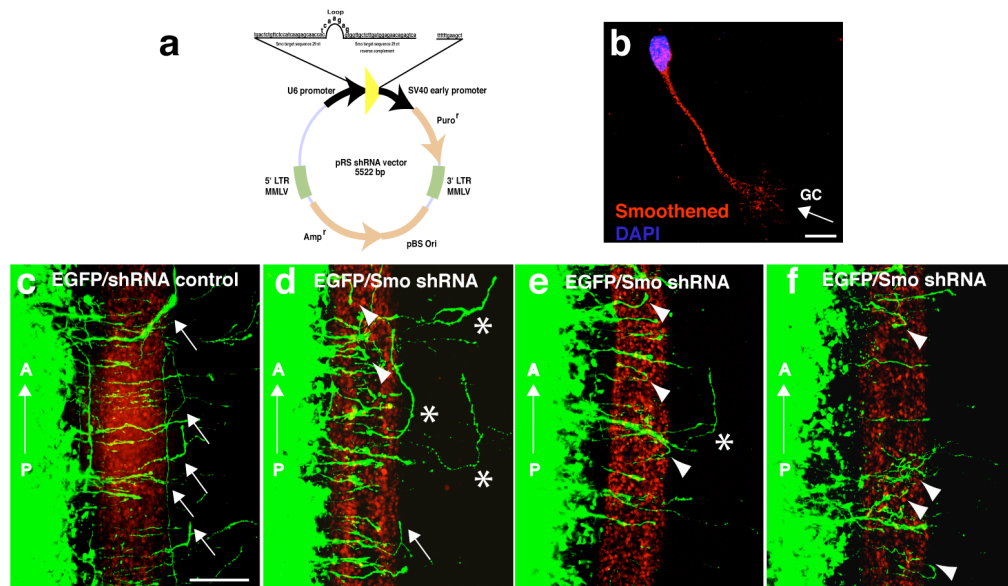


Figure 3.4 Smoothed is required for proper guidance of commissural axons during midline crossing.

(a) Schematic diagram depicting the pRS shRNA expression vector driving Smo shRNA under a U6 promoter. The shRNA expression cassette contains a 29 nt sequence targeting the Smoothed (Smo) protein. (b) Smo (red) and DAPI staining of a rat E13 spinal cord neuron. Smo is detected in the cell body, in the axon, and in the growth cone, DAPI stains the nucleus. (c-f) Open book spinal cord cultures of rat E13 embryos co-electroporated with (c) EGFP and a random shRNA vector or (d-f) EGFP and the Smo shRNA expression vector. Open book explants are stained for GFP (green) and the floor plate marker HNF-3 β (red). (c) Axons in open book explants of embryos electroporated with a random shRNA vector follow the stereotypic trajectory of commissural axons, enter the midline, cross and turn anteriorly on the contralateral side of the spinal cord in close contact with the floor plate (FP) border (arrows in c). (d-f) In contrast, the majority of electroporated axons with the Smo shRNA construct wander and fail to exit the FP and instead loop back into the midline (arrowheads in d-f). Post-crossing axons on the contralateral side overshoot away from the FP and do not turn anteriorly immediately after midline crossing but rather turn gradually at more lateral positions (asterisks in e and f). A few axons project anteriorly close to the contralateral floor plate border (arrow in d).

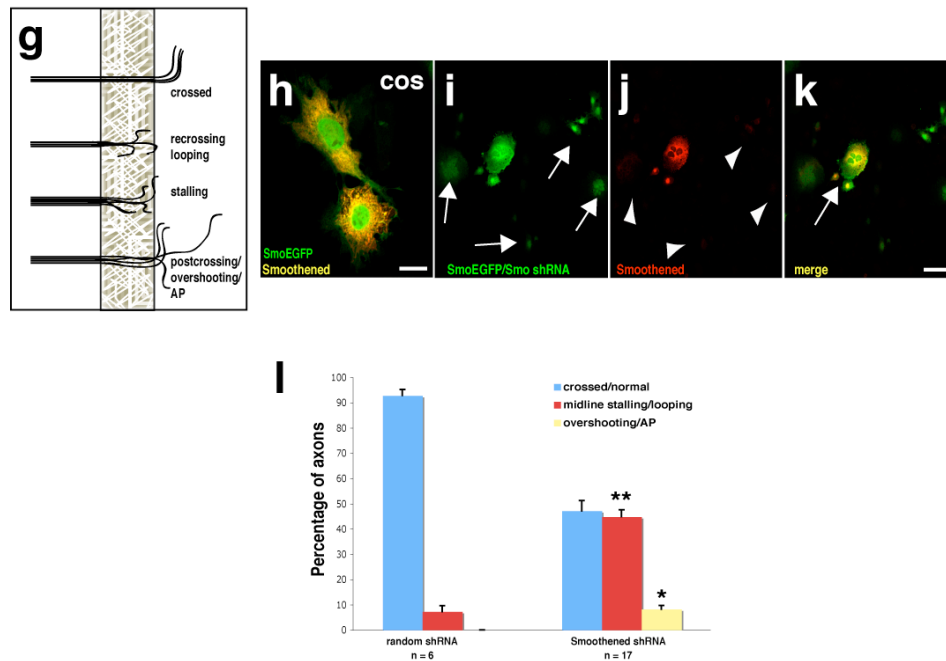


Figure 3.4 Smoothed is required for proper guidance of commissural axons during midline crossing, continuation.

(g) Schematic showing a summary of misguidance behaviors observed in Smo ShRNA electroporated embryos. (h-k) COS cells transiently transfected with (h) a Smo-IRES-GFP construct or cotransfected with (i-k) a Smo-IRES-GFP and Smo shRNA constructs. (h) High-magnification image of GFP and Smo immunofluorescence (red) showing levels of the Smo-IRES-GFP expression construct. (i-k) Knockdown of Smo staining (arrowheads) in Smo-IRES-GFP /Smo shRNA expressing cells compared to the level of IRES-GFP (arrows in i). A few cells cotransfected with Smo shRNA remain positive for Smo and GFP staining (arrow in k). (l) Quantification of open book explants coelectroporated with EGFP and a random shRNA or Smo shRNA constructs. The graph shows the percentage of axons displaying normal and misguided behaviors in electroporated spinal cords during midline pathfinding. SEM error bars are from three sets of experiments and *n* indicates the total number of explants quantified. ** $P < 0.01$, * $P < 0.05$ (Student's *t*-test). Scale bars represent 10 μm (b), 100 μm (c-f), 50 μm (h), and 100 μm (i-k).

Shh reduces cAMP/PKA activity and this reduction is necessary for Semaphorin repulsion

Cyclic nucleotides have been shown to regulate the function of guidance cues, such as Netrin1 and Semaphorins and can switch axonal responses from attraction to

repulsion and *vice versa* depending on the ratios of cAMP to cGMP. Overall, higher cAMP and lower cGMP associate with attraction and lower cAMP favors repulsion, differentially regulating actin cytoskeletal dynamics of growth cone motility (Song, et al., 1998). In addition, Protein Kinase A (PKA) activity has been shown to negatively regulate Semaphorin-PlexinA-mediated repulsion *in vivo* by PKA association with the A-kinase anchoring protein (AKAP), Nervy, and the PlexA receptor in the *Drosophila* central nerve cord (Terman and Kolodkin, 2004). We hypothesized that growth cone cAMP levels may be down regulated by floor plate signals, such as Shh, causing axons to become sensitive to Semaphorin3 chemorepulsive activity. To test this, we first examined whether appropriate PKA activity was necessary for correct midline guidance. In open book explant cultures commissural axons were treated with a PKA inhibitor KT5720 (**Fig. 4.2**) or the adenylyl cyclase agonist, Forskolin and analyzed commissural axon pathfinding by DiI tracing as shown in **Fig. 3.2j**. We found that downregulating PKA with the PKA inhibitor, KT5720 prevented a number of axons (~50%) from entering the floor plate (**Fig. 4.2, b, c**, single arrowheads), whereas elevating cAMP levels with Forskolin caused profound misguidance of commissural axons during midline crossing (**Fig. 3.5b-e**). Open book explants treated with Forskolin displayed a number of stalled axons at the midline (**Fig. 3.5c, d**, double arrows), forming knots at the contralateral edge of the floor plate (**Fig. 3.5b, c**, asterisks), whereas others displayed wandering behaviors after midline crossing, overshooting (**Fig. 3.5d**, single long arrow) or turning randomly in the A-P direction (**Fig. 3.5e**, single long arrow). In some injections, a few of these wandering axons

looped back and reentered the floor plate (**Fig. 3.5d**, short single arrow). Our results showed that, Forskolin treatments caused stalling of commissural axons still in the floor plate and A-P misguidance defects in postcrossing axons. These axonal behaviors are again very similar to those seen in NP2 ^{-/-} embryos (Zou, et al., 2000) and in 5E1-treated open book explants (**Fig. 3.2**) and suggest that Forskolin addition may interfere with the normal switching of repulsive responsiveness needed for midline pathfinding.

To further test this hypothesis, we analyzed precrossing explants (**Fig. 3.1a, b, Fig. 3.5j**) and found that Forskolin diminished Shh-induced Semaphorin repulsion in this assay (**Fig. 3.5k, l**). In addition, we tested biochemically, whether PKA activity could be regulated in commissural neurons exposed to recombinant Shh-N protein. Dissociated spinal cord commissural neurons were serum starved overnight and treated with 2.5 µg/ml (150 nM) of Shh-N recombinant protein 1 hour prior lysate collection. Forskolin (25 µM), the Smo agonist Purmorphamine (5 µM) and the PKA inhibitor, KT5720 (25 µM) were added to the commissural neuron culture as controls 15 minutes before lysis. Commissural neuronal lysates were processed for phosphorylated PKA levels with an antibody that specifically recognizes an activated form of the catalytic domain of PKA phosphorylated at the Threonine residue, 197. Western analysis showed a significant reduction of activated PKA after Shh-N recombinant protein treatments when compared to the lysate controls (**Fig. 3.5m**) suggesting that Shh might cause a reduction of PKA activity in the growth cones of commissural axons once they enter the floor plate allowing repulsion to take place. In

addition, phospho CREB levels were found to be regulated by Shh proteins in Western immunoblots (**Fig. 4.3e**). Together, these data implies that Shh could regulate growth cone sensitivity to Semaphorins by modulating cAMP levels.

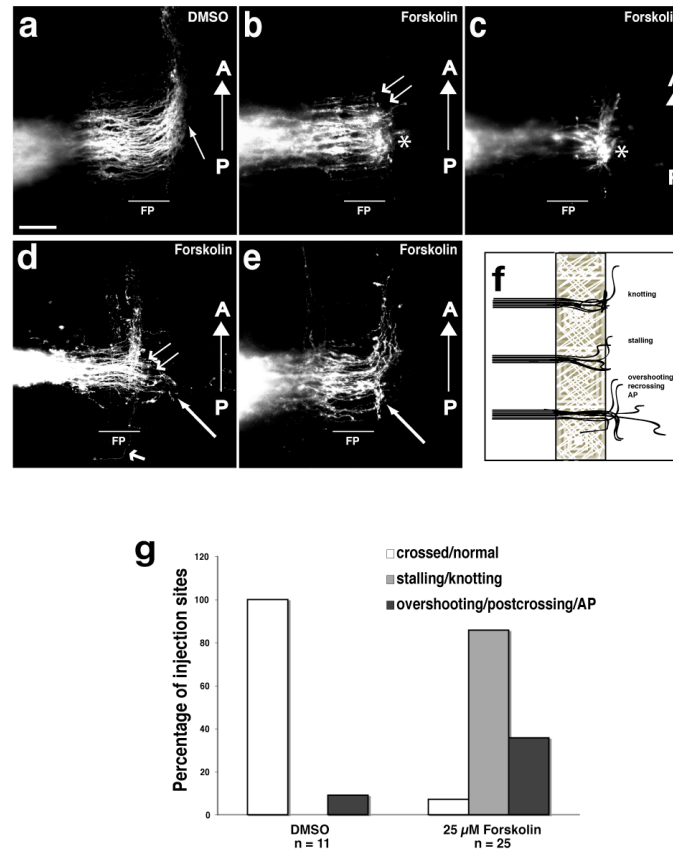


Figure 3.5 Shh modulating the activity of the cAMP/PKA pathway as a regulator of Semaphorin3 repulsive signals.

(a-e) Commissural axon projections during midline crossing revealed by DiI injections of open book treated explants. (a) Normal trajectory of commissural axons in a vehicle control (DMSO) treated spinal cord explant turning anteriorly after midline crossing. (b-e) Abnormal midline pathfinding of commissural axons in the presence of the adenylyl cyclase activator, Forskolin. The majority of axons in Forskolin treated spinal cord explants stall at the contralateral side of the floor plate (FP) (double arrows in b and d) with prominent knotting (asterisks in b and c). In some cases, wandering axons reenter the FP (single short arrow in d) while others overshoot to the contralateral side of the FP without turning (long arrow in d). (e) A number of injected axons projected randomly in the A-P direction (long arrow). (f) Summary of commissural axon misguidance errors observed in Forskolin treated open book explants. (g) Quantifications of midline pathfinding behaviors seen by DiI labeling in vehicle and Forskolin treated open book explants. Scale bar represents 50 μ m.

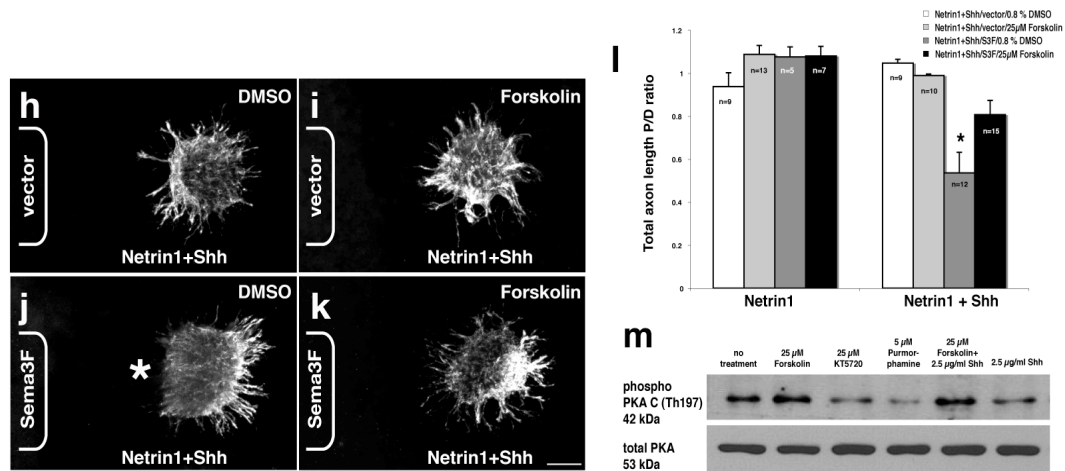


Figure 3.5 Shh modulating the activity of the cAMP/PKA pathway as a regulator of Semaphorin3 repulsive signals, continuation.

(h-k) Confocal images of spinal cord precrossing commissural explants in DMSO treated controls or Forskolin. (h and i) Symmetric outgrowth of precrossing commissural axons in the presence of Netrin1 and Shh expressing cells cultured in media containing vehicle control (DMSO) or Forskolin. (j) Shh induced Semaphorin3 repulsion of precrossing commissural axons is maintained in DMSO treated explants (asterisk indicates repulsion), but repulsion significantly diminishes in precrossing explants cultured in Forskolin containing media (k). (l) P/D ratios of the total axon bundle length in DMSO and Forskolin treated pre-crossing commissural explants. Graph shows mean average $\pm$ SEM error bars from three sets of experiments and n indicates the total number of explants quantified. * $P < 0.05$ (Student's *t*-test). (m) Levels of activated phospho PKA and of total PKA as seen by immunoblots of rE13 dorsal spinal cord primary cultures. The PKA inhibitor KT5720, N-Shh recombinant protein and the Smoothed agonist Purmorphamine reduced the level of activated PKA in dorsal spinal cord lysates. Scale bar represents 50 μ m (a) and 100 μ m (k).

Discussion

The midline is a major organizer for axon wiring and an important intermediate target. Axons change the direction of their trajectory after midline crossing by dynamic regulation of growth cone responsiveness to guidance cues. The extrinsic and intrinsic mechanisms of this switch of responsiveness are still not well known. Our results suggest that Shh might act as an extrinsic midline switch for

activating Semaphorin3 repulsion of commissural axons. This study implies that morphogens, such as Shh, might directly modulate growth cone signaling of other guidance cues and orchestrate growth cone remodeling at intermediate targets.

Shh binds to PTC1 and signals through Smo by suppressing the inhibition of PTC1 on Smo signals (Taipale, et al., 2002). Shh has been proposed to collaborate with Netrin1 as chemoattractants for vertebrate commissural axons binding to the Shh receptor Boc (Okada, et al., 2006). In addition, Shh is proposed to act as a chemorepellent for commissural axons in chick embryos by binding to the Shh binding receptor, Hip1 (Bourikas, et al., 2005). Shh signaling can have multiple functions in axon guidance depending on the receptor level expression and distribution of the Shh protein, a role consistent with its morphogenetic properties (Jessell, et al., 2000). In the retina, Shh was proposed to have a dual role on retinal ganglion cell growth depending on its concentration, low concentrations of Shh served as positive factors for growth, whereas high concentrations of Shh attenuated axonal growth (Kolpac, et al., 2005). In the developing spinal cord, Shh expression is highest at the ventral midline emanating from floor plate cells and the adjacent notochord (Jessell, 2000). As Shh diffuses throughout the spinal cord it appears to act as a positive regulator of attraction by the time commissural axons begin to travel ventrally and as a negative regulator at high concentrations as commissural axons begin to reach the floor plate. In this study, the Shh receptor, PTC1 was found to be highly expressed at the ventral midline, consistent with the notion that PTC1 is a direct transcriptional

target gene of Shh signaling (Marigo, et al., Nature, 2006) suggesting that high levels of PTC1 expression might be needed for Shh induction of repulsive signals.

Here, we found that both PTC1 and Smo are required for midline pathfinding. The guidance defects cause by constitutively active PTC1 or Smo shRNA knockdown, suggest a role for Shh signals in regulating repulsive responses.

Next, we hypothesize that growth cone cAMP levels might be regulated by floor plate signals causing axons to become sensitive to the Slit and Semaphorin3 chemorepellent activities. In *Drosophila*, the Semaphorin receptor Plexin can be regulated by cyclic nucleotides. Nervy, an A-kinase anchoring protein (AKAP), Nervy, was shown to link PKA to Plexin, which counteracted Semaphorin-directed repulsion (Terman and Kolodkin, 2004). In addition, the 7 transmembrane Frizzled-like structure of the signaling receptor for Shh, Smo, suggests that it could utilize heterotrimeric GTP-binding regulatory proteins acting as a G-protein coupled receptor (GPCR) (Dessaud, McMahon and Briscoe, 2008; Odgen, et al., 2008). Smo signaling as a true GPCR has been contentious for some time, however increasing studies continue to demonstrate the ability of Smo signals to activate $G\alpha_i$ pathways (DeCamp, et al., 2000; Chen, et al., 2004; Meloni, et al., 2006; Riobo, et al., 2006; Odgen, et al., 2008). Of particular interest, Shh has been shown to be a negative regulator of growth cone movement by decreasing cAMP levels in mouse retinal ganglion cell axons (Bovolenta, 2005; Sanchez-Camacho and Bovolenta, 2008).

Cyclic nucleotides have been extensively reported to convert the actions of guidance cues from attraction to repulsion (Ming, et al., 1997; Song, et al., 1998;

Hopker, et al., 1999; Nishiyama, et al., 2003). For example, the attraction of *Xenopus* spinal cord neurons can be converted to repulsion by inhibiting cAMP or PKA (Song, et al., 1998) or by modulating cAMP/cGMP levels (Nishiyama, et al., 2003). *In vivo*, PKA has been shown to regulate the guidance of olfactory sensory neurons through the olfactory placode boundary, an intermediate target in zebrafish embryos (Yoshida, et al., 2002) and in *Drosophila*, Sema1-a mediated repulsive guidance in motor axons is decreased with increased Nerve-PKA expression (Terman and Kolodkin, 2004). Therefore, we propose that Shh activation of Smo-G α_i pathways Semaphorin3 signals to repel commissural axons. Downregulation of PKA and pCREB levels through Shh activity might facilitate a microenvironment at the floor plate where repulsive signals can be quickly activated to expel commissural axons. Future studies will focus on how Shh regulates PKA activity and enhances growth cone Semaphorin3 signals and other downstream effectors involved in modulating repulsive signals.

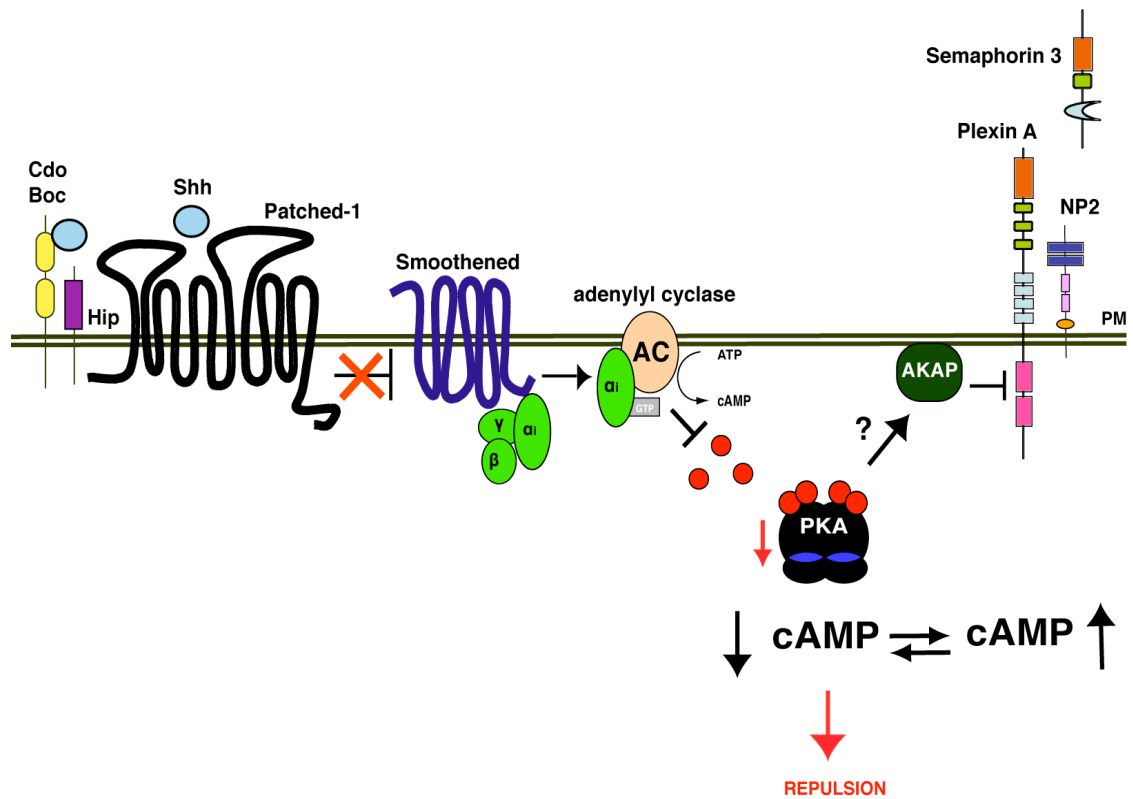


Figure 3.6 A model for the role of Shh signals in modulating the sensitivity of axons to guidance cues.

Schematic diagram depicting Shh signals regulating second messenger pathways through Smo $G\alpha_i$ signals. Smo G protein signals inhibit adenylyl cyclase (AC) activity, which in turn downregulate cAMP and PKA intracellular levels. The intracellular ratios of cyclic nucleotides have been shown to switch axonal responses to guidance molecules (Guan, et al., 2008). As in the *Drosophila* counterpart, downregulation of cAMP/PKA signaling could affect the function of PKA effector molecules involved in silencing Semaphorin signals (e.g. A-kinase anchoring proteins (AKAPs)) (Terman and Kolodkin, 2004).

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CHAPTER 4

General Principles of Axon Guidance at the Floor Plate Ventral Midline

Introduction

The collection of data presented in this dissertation aspires to contribute to the understanding of spinal cord commissural axon pathfinding. Although the key molecular cues and cognate receptors orchestrating commissural axon guidance across the developing spinal cord have been identified, the precise intracellular signaling cascades that control and direct commissural axonal growth remained to be fully understood. The pathfinding of commissural axons in the developing spinal cord has been extensively characterized and is not only one of the best understood models in axon guidance, but has proven to be a powerful tool for the analysis of growth cone dynamics.

We sought to dissect out the molecular mechanisms underlying floor plate crossing at the ventral midline of the spinal cord, in particular the mechanisms mediating the turning on growth cone responses to the chemorepellent cues secreted by floor plate cells. This dissertation focuses on the fundamental question of how commissural axons change their initial attraction to the floor plate to repulsion necessary to drive the growth cones across and beyond the ventral midline to the next segment of the trajectory.

Discussion

What are the molecular mechanisms underlying the change in commissural axon responsiveness to chemorepellent signals? The body of knowledge presented here discusses tantalizing scenarios where simple orchestration of attraction and repulsion events cannot account for the complex molecular mechanisms that developing axons must undergo in order to successfully navigate through intrinsic intermediate targets, such as the floor plate. The navigating axon generates a repertoire of dynamic behaviors in response to the simultaneous presence of environmental molecular guidance cues by differentially regulating extracellular and intracellular signaling networks (Chilton, 2006; Dickson and Gilestro, 2006; Song and Poo, 2001).

In our initial studies presented in **Chapter 2**, we confirmed a functional interaction between the known chemorepellents pathways of the spinal cord, Slit/Robo and Semaphorin3/NP2 signals. Neuropilin2 (NP2) receptors, the binding moieties for Semaphorin3F and Semaphorin3B were found to be required for Slit2 mediated repulsion in postcrossing commissural explants (**Fig. 2.2d**), which prompted us to rethink the receptor-ligand system role in the chemorepellent pathways of the ventral midline. Based on previous analysis of the NP2 mutant mice, where commissural axons displayed severely affected phenotypes during floor plate crossing, lingering, stalling and losing their directionality (Zou, et al., 2000) and analysis of the Slit receptors, Robo1 and Robo2, where axons displayed stalling phenotypes, but far less penetrant than the Slit triple and NP2 knockout phenotypes (Long, et al., 2004), it

seemed that the Slit/Robo receptor complex system failed to compensate for the loss of Semaphorin/NP2 signals. Together, these results suggested the possibility that NP2 could be involved in mediated Slit repulsion *in vivo*, which incited us to study the relevance of the interactions of these pathways in commissural axonal behaviors. We sought to investigate whether Slit and Semaphorin3 proteins could be hierarchical organized, so that one could dominate over the other, or whether binding to NP2 by Slit was a necessary step to activate Semaphorin3 signals. While neither Slit2 nor Sema3F were able to activate other in precrossing explant assays (**Fig. 2.5a, b**), together Slit and Sema3F induced repulsion in precrossing commissural axons (**Fig. 2.5c**). Further analysis of the biological significance of this interaction should focus on the characterization of Slit2/Np2 interaction with Semaphorin3 repulsive signals. In particular, whether Slit/Robo and Sema/NP2/PlexinA3 signals converge as one pathway to activate specific intracellular repulsive signaling cascade required to expel commissural axons from the floor plate.

As mentioned in **Chapter 2**, Slit and Semaphorin3 pathways together might converge at common downstream signaling pathways, such as the RhoA family of small GTPase regulators (RhoA, Cdc42 and Rac), which are known to connect multiple axon guidance systems to cytoskeletal rearrangements to regulate axonal steering. Convergence of these two chemorepellent pathways might increase repulsion by removing attraction (e.g. decreasing active Cdc42), while promoting repulsion (e.g. increasing active RhoA) (Lowery and Van Vactor, 2009).

Furthermore, using precrossing commissural axon and spinal cord open book assays we have obtained evidence that Shh regulates commissural axon responses to Semaphorin3 signals (**Chapters 2 and 3**). In addition, knockdown of the Shh binding receptor, PTC1 and the Shh signal transducer receptor, Smo, coincidentally disrupted proper guidance of commissural neurons during floor plate guidance, further confirming a role of Shh signals in axon guidance. We also found that PTC1 and Smo are expressed in dissociated cultures of commissural spinal cord neurons. In addition, PTC1 appears to be upregulated at the spinal cord ventral midline at the time commissural axons begin to cross the midline, implying a role for PTC1 in regulating midline crossing of commissural axons (**Fig. 4.1, Fig. 3.3 and Fig. 3.4**).

PTC1 upregulation as a direct target of Shh transcriptional activity might modulate the concentration and duration of the Shh signal and consequently influence axonal responses. This upregulation may be important for adjusting Shh pathway sensitivity in axons crossing the ventral midline, where axons are progressively exposed to increasing Shh levels from both floor plate cells and the adjacent notochord (Jessell, 2000; Dessaud, McMahon and Briscoe, 2008). In commissural neuronal cultures, PTC1 staining was detected at the plasma membrane of axonal growth cones and in ventral regions of mouse E11.5 spinal cords (**Fig. 3.3**). In contrast, Smo was detected in intracellular vesicles of spinal cord neurons (**Fig. 3.4**) and enriched in regions where commissural axons join the ventral and lateral funiculus on the contralateral side of the spinal cord (**Fig. 4.1**).

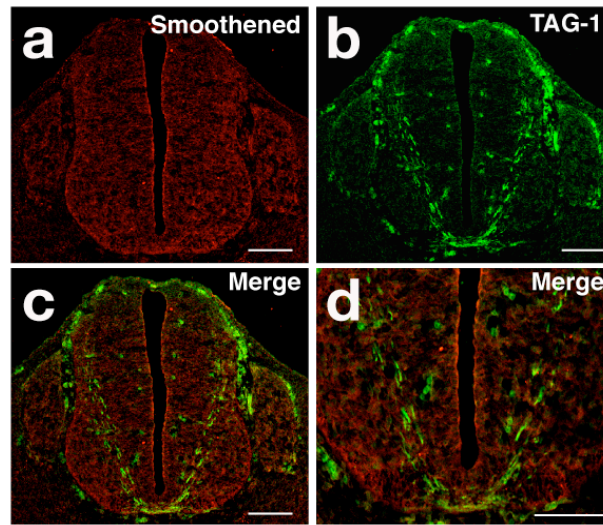


Figure 4.1 Smoothened staining is enriched in the subventricular zone and ventral regions of the developing spinal cord.

(a) Smoothened staining in the mouse E11.5 developing spinal cord is higher in ventral and subventricular spinal cord regions. (b) TAG-1 staining of precrossing commissural axons showing their circumferential migration towards the floor plate. (c-d) Merge of Smo and TAG-1 staining shows no significant overlap, zoomed in (d). TAG-1 is green (Cy2), Smoothened is red (Cy3). Scale bars represent 100 μm (a-d)

Our results imply that the activation of Semaphorin3 signals by Shh could be induced through the modulation of cAMP/PKA activities via Smo dependent activation of G-protein inhibitory signals ($G_{\alpha i}$) (**Fig. 3.5**). cAMP is a major second messenger cyclic nucleotide known to be an important intracellular signal for switching axon guidance responses in the growth cone, with elevated levels of cAMP mediating attraction and decreasing levels mediating repulsion (Song, et al., 1997; Song, Ming and Poo, 1997; Song, et al., 1998; Hopker, et al., 1999; Han, et al., 2007). cAMP signals are key regulators of the multifunctional holoenzyme PKA, known to phosphorylate a number of effector proteins for different signaling cascades.

In *Xenopus*, growth cones turn away from Netrin1 and brain derived neurotrophic factor (BDNF) when cAMP and PKA levels are low, but are attracted when levels are high (Ming et al., 1997; Song, Ming and Poo, 1997; Song, et al., 1998). In rat spinal cord commissural explants, PKA was shown to regulate the sensitivity of chemoattraction to Netrin1, but did not switch between attraction and repulsion when treated with the PKA inhibitor, KT5720, an ATP analog that competitively inhibits PKA (Moore and Kennedy, 2006). Inhibiting PKA with the same inhibitor, KT5720, in open book rat E13 spinal cord culture showed that PKA inhibition did alter commissural axon trajectory across the floor plate although not completely. Although in most injections, the majority of axons crossed the ventral midline and turned correctly, a number of axons (~40-50%) stalled at the floor plate border before crossing (**Fig. 4.2**). This is consistent with previous observations showing that PKA activation recruits the Netrin1 receptor DCC and enhances axon chemoattraction to Netrin1 (Bouchard, et al., 2008) but does not switch between chemoattraction and chemorepulsion (Moore and Kennedy, 2006).

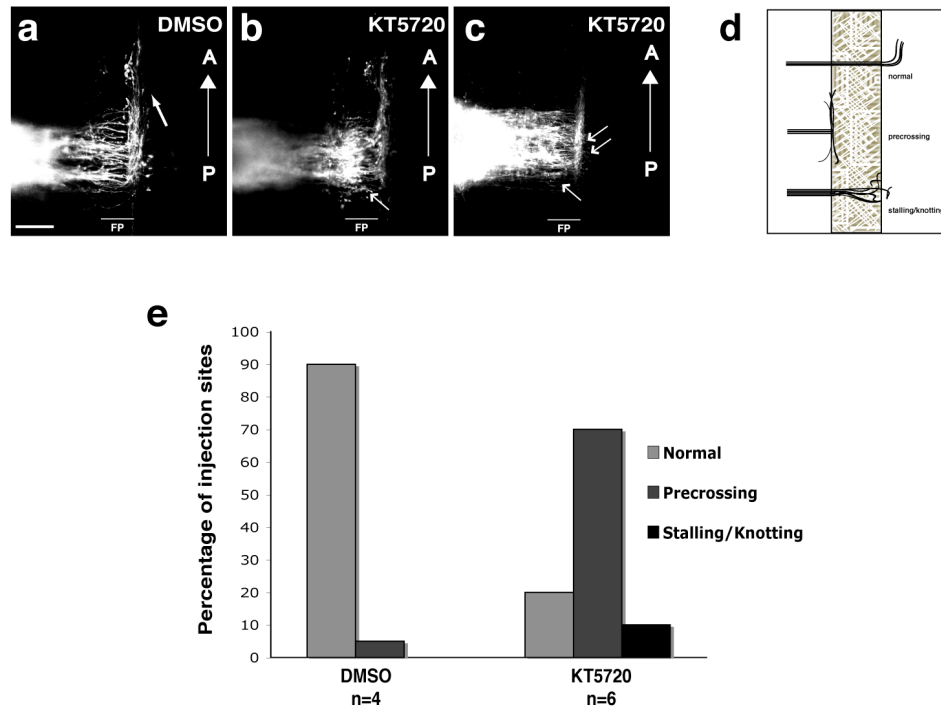


Figure 4.2 The PKA inhibitor, KT5720, decreases commissural axon attraction to the floor plate.

(a) Normal trajectory of commissural axons turning anteriorly after midline crossing in vehicle (DMSO) treated rat E13 spinal cord open book explant. (b-c) Open book explants cultured in the presence of the PKA inhibitor, KT5720 show a decrease attraction stalling before entering the floor plate (single arrows). A few axons stall on the contralateral side of the spinal cord after floor plate crossing (double arrows in c). (d) Schematic showing a summary of axonal behaviors in open book explants treated with the PKA inhibitor, KT5720. (e) Quantifications of midline pathfinding behaviors seen by DiI labeling in vehicle and Forskolin treated open book explants, with most of the quantified injections displaying errors entering the floor plate. Scale bar represents 50 μm .

Consistent with the knowledge that altering cAMP/PKA activities within nerve cells can dramatically change axonal responses to axonal guidance cues (Ming et al., 1997; Song, Ming and Poo, 1997; Song, et al., 1998), our results showed that increases of cAMP/PKA activity in commissural axons by Forskolin treatments resulted in loss

of repulsion of commissural axons from the floor plate (**Fig. 3.5**), whereas decreasing PKA activity prevented a cohort of axons from entering the floor plate (**Fig. 4.2**).

Here, we showed that Shh plays a pivotal role regulating Semaphorin3 signals in commissural axon pathfinding at the floor plate of the ventral midline and provide evidence that this modulation might involve Smo induction of G α i signals (**Fig. 3.5m**). Shh is a multifaceted protein that governs a wide variety of functions in the developing embryo and in adult tissue homeostasis. The hallmark of Shh signaling is the ability to act over extended regions to control the differentiation of different cell types as a function of its concentration. During ventral spinal cord patterning, Shh forms a ventral to dorsal gradient with different concentration specifying different ventral neural progenitors. Less clear is how these gradients are generated and maintained in development and how nerve cells transduce the different concentrations of Shh to elicit a specific developmental outcome (Jiang and Hui, 2008).

The strength, duration and amplitude of Shh signals play a crucial role in shaping and fine tuning the biological outcome in response to Shh activities. In addition, Shh activation of gene transcription leads to the upregulation of negative regulators of the pathway as a self-correcting mechanism to control signal strength (Jessell, 2000). Intriguingly, genes regulated by high levels of Shh required longer exposure time and are expressed at later stages of development. The adaptation of Shh signals at higher levels of Shh is proposed to be influenced by Shh-mediated upregulation of PTC1 (Dessaud, McMahon, Briscoe, 2008), which can result in two tentative scenarios, the attenuation of Shh signaling intensity by sequestering Shh

from the cell surface or the increase of Shh-PTC1 complexes thus increasing the ratio of free Smo and consequently Shh signal transduction.

The reiterative use of morphogenetic signaling in axon guidance adds a new layer of complexity to these pathways. The high expression of Shh in the ventral spinal cord makes it an ideal candidate for mediating floor plate crossing. It has been shown that Shh can act as a chemoattractant of commissural neurons through Smo signals interacting with the Shh binding protein, Boc (Charron, et al., 2003; Okada, et al., 2006; Yam et al., 2009). Boc belongs to the Ihog/Cdo family of immunoglobulin/fibronectin repeat containing proteins and are thought to positively regulate Shh signaling by enhancing binding to PTC1 increasing cell recognition (Jiang and Hui, 2008).

Conversely, in chick, Shh repels postcrossing commissural axons anteriorly along the longitudinal axis. Interestingly, this activity is not mediated through PTC1 or Smo and instead was argued to be mediated by the Shh binding protein, Hip1 (Bourikas, et al., 2005). Hip1 has been shown to act as a negative regulator of Hh by competing with PTC1 for binding creating a negative feedback loop to restrict Hh signals. In addition, Hip1 was shown to be a direct target of Shh transcriptional signals, which further enhances this effect (Jiang and Hui, 2008). The role of Shh in chick commissural axon guidance, remains unclear, especially how Shh chemorepellent signals can regulate axonal pathfinding in the absence of PTC1 and Smo activities.

Our findings suggest a neuromodulatory role of Shh signals in guiding commissural axons across the ventral midline. The high expression of Shh activity at the floor plate and the different thresholds of Shh required for signaling, beg the question of whether Shh studies in axon guidance lack a proper appreciation of the kinetics of the Shh pathway.

For instance, unlike other signals, Shh binding to PTC1 does not activate the pathway, but instead relieves the inhibition of Smo, the signal transducer. PTC1 is a twelve-transmembrane receptor homologous to a bacterial RND proton driven transporter and is thought to inhibit Smo through the translocation of small molecule intermediates across the plasma membrane (Taipale, et al., 2002). On the other hand, Smo topographically resembles seven transmembrane G protein couple receptors signaling through heterotrimeric G proteins, but the role of G proteins in Smo signals has been contentious for some time. Nonetheless, increasing evidence in *Drosophila* (Ogden, et al., 2008) and mammalian systems (Sánchez-Camacho and Bovolenta, 2008) continue to show the requirement of inhibitory G protein activation for Smo-mediated signal transduction (DeCamp, et al., 2000; Chen, et al., 2004; Meloni, et al., 2006; Riobo, et al., 2006; Odgen, et al., 2008).

The observed coupling of Smo to G α i could be part of a mechanism used to reduce PKA activity by decreasing the levels of cAMP. In axon guidance, it has been shown that manipulation of intracellular cyclic nucleotides can overwrite the effects of other extracellular guidance cues on axonal growth (Han, et al., 2007). As mentioned above, inhibition of PKA results in the conversion of Netrin1-dependent attraction to

repulsion (Song and Poo, 2001). Similarly, Semaphorin3 chemorepellent activities can be converted to attraction in a turning assay by altering the intracellular levels of cyclic nucleotides (Nishiyama, et al., 2003; Song, et al., 1998). For Netrin1 attraction, the adenosine receptor, A2bR is speculated to bind to Netrin1 and play a role in Netrin1 mediated cAMP production and commissural axon outgrowth (Corset, et al., 2000). Furthermore, the chemokine stromal cell-derived factor 1 (SDF-1) signal through the GPCR receptor, CXCR4 was shown to lead to the reduced chemorepellent activity of growth cones to Slits and Semaphorins by modulating cAMP and Rho levels (Chalasani, et al., 2003).

In this dissertation, we showed that Shh might positively modulate Semaphorin3 signals by downregulating levels of activated PKA (**Fig. 3.5m**) and levels of activated cAMP response element-binding protein, pCREB (**Fig. 4.3**). These results are consistent with the role of Shh in controlling growth and navigation of mouse RGC axons at the developing optic chiasm (Sánchez-Camacho and Bovolenta, 2008).

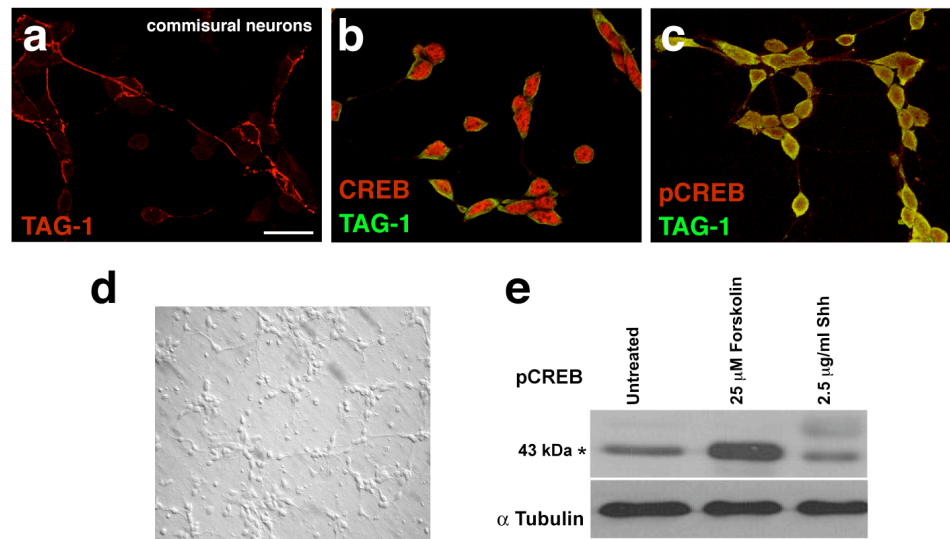


Figure 4.3 Shh modulates pCREB levels in commissural neurons.

(a) Progenitors of rat e13 commissural neuron culture are positive for TAG-1 (Cy3), the cAMP response binding protein, CREB (b) and its activated form, pCREB (c). (d) Commissural neuron culture for Western blot analysis. (e) Western blot analysis of commissural neuron lysates detecting pCREB. Commissural neurons were incubated with Forskolin or recombinant Shh-N protein. Forskolin treatments induce an increase in pCREB levels, whereas Shh-N treatments resulted in a decrease of pCREB levels compared to controls. Scale bar represents 100 μm .

The perturbation of Shh signals in commissural neurons was consistent with the inability of axons to respond to a chemorepellent signals during floor plate crossing, in addition, Shh was found to directly influence precrossing commissural axon responses to Semaphorin3 proteins *in vitro* (**Chapter 3**). Furthermore, preliminary analysis of postcrossing explants cocultured in the presence of COS cell aggregates expressing Shh displayed repulsion like behaviors compared to control explants, further suggesting a role for Shh signals in repulsion (**Fig. 4.4**).

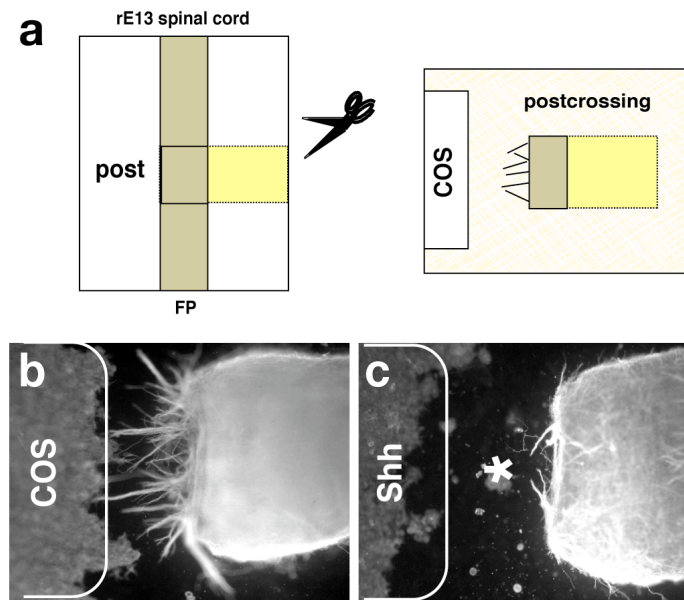


Figure 4.4 Postcrossing commissural axons are repelled by Shh expressing cell aggregates.

(a) Explant assay of rat E13 commissural postcrossing axons cocultured in the presence of a COS cell aggregate. (b-c) Postcrossing commissural explants stained for F-actin by phalloidin. (b) Postcrossing axons extend towards a cell aggregate expressing a vector control (c) Postcrossing explants cocultured in the presence of Shh expressing cell aggregates show repulsion of postcrossing commissural axons.

Shh dependent regulation of cyclic nucleotide levels during commissural axon pathfinding is an attractive mechanism by which Shh signals can modulate Semaphorin3 chemorepellent activities. In vertebrates, seemingly convincing data shows that SemaphorinA repulsion is increased by inhibiting PKA and diminished by increasing cAMP (Song, et al., 1998). Intriguingly, in *Drosophila*, a direct molecular link between cyclic nucleotides and Semaphorin signals was found to be a member of the family of A-kinase anchoring proteins (AKAPs), which were shown to regulate Sema1a-PlexinA repulsive axon guidance by bringing PKA in close proximity to

PlexinA, antagonizing its repulsive activity (Terman and Kolodkin, 2004). A *Drosophila* homologue of mammalian myeloid translocation genes (MTGs), Nervy, was found to act as an A-Kinase anchoring protein (AKAP) antagonizing PlexA signaling. AKAPs are scaffolding proteins coupled to cAMP-PKA known to coordinate cAMP dependent events by compartmentalizing PKA and other enzymes to discrete focal points of subcellular activity and signal transduction. However, MTGs, such as Nervy do not function at the plasma membrane and instead are largely localized to the nucleus where they mediate complex protein interactions involving transcriptional corepressors, histone modifying enzymes, and DNA binding transcription factors leading to increased levels of transcriptional repression. In mammals, MTG8 and MTG16 were found to bind to the regulatory subunit of type II PKA (PKA RII α) functioning as anchoring proteins in the Golgi of lymphocytes (Ice, et al., 2005).

Future studies should focus on the characterization of the precise subcellular distribution of mammalian MTG proteins in developing growth cones and their role in vertebrate Semaphorin3 repulsive signals. Based on the spatial localization of type II PKA in the filopodia of *Xenopus* growth cones, PKA-AKAP complexes are likely to be involved in the regulation of F-actin cytoskeleton dynamics (Han et al., 2007). The conserved family of Ena/VASP actin regulatory proteins are direct targets of cyclic nucleotide activated kinases (e.g. PKA) and are perfectly positioned at the tip of filopodia to relay information to the cytoskeleton (Drees and Gertler, 2008).

Better understanding of the growth cone's receptor composition upon integration of Shh and Semaphorin3 signals is crucial in determining how growth cones adjust their sensitivity to the environment during midline guidance decisions. Alternatively, the presence of Shh can desensitize the attractive responses to Netrin1 and sensitize the growth cone to Semaphorin3B signals emanating from the floor plate allowing axons to collapse at the ventral midline while still maintaining their motility, which in turn could mediate gradual adaptation changes to signals through an intracellular period of signal transduction enabling them to respond to new extracellular environments after floor plate crossing (Mann and Rougon, 2007; Dessaud, McMahon, Briscoe, 2008). Consequently, Semaphorin3F repulsive signals are also sensitized in the growth cone possibly to squeeze commissural axons into longitudinal tracts guided anteriorly on the contralateral side of the spinal cord by Wnt4 attractive signals (Lyuksyutova, et al., 2003).

Transcription of Shh signaling targets requires activity of the Gli family of zinc finger transcription factors. Upon Smo activation the Shh transcriptional signal is induced by the balance of activities between activator and repressor forms of the Gli proteins. In vertebrates, Gli3 proteins function mainly as repressors while Gli2 act as activators. Gli1 is a direct transcriptional target of Shh signals upregulated to positively regulate Gli2 transcriptional activities. In addition, Gli proteins are regulated by multiple molecular mechanisms including phosphorylation by kinases, PKA, GSK3 β and CKI, which mediate the targeting of Gli proteins for proteosomal degradation.

Activation of Gli proteins induces the differentiation of ventral progenitor domains in the developing spinal cord through the simultaneous repression of Class I homeodomain transcription factors (e.g. Pax6, pax7, Irx3) and the induction of Class II homeodomain transcription factors (e.g. Nkx2.2, Nkx6.1, olig2, Dbx1, Dbx2) (Jessell, 2000). Shh transcriptional signals in mammals are regulated by the Sufu protein, a major inhibitor of the translocation of Gli proteins into the nucleus (Jiang and Hui, 2008). Loss of Sufu in mammals leads to the ectopic activation of Shh pathways, similar to loss of PTC1. To date, it is not known whether the role of Shh in axon guidance requires Gli dependent transcriptional activation. Mouse Gli2 mutants lack floor plate cells and present abnormal guidance of commissural axons in the ventral regions of the spinal cord (Matise, et al., 1999). Although, this study suggested a role for Gli2 proteins in regulating ventral midline guidance, the direct function of Gli2 proteins on commissural axons can be difficult to determine in the absence of a floor plate.

To further address the role of Gli proteins in commissural axon guidance, we treated rat E13 open book preparations with the Gli1/Gli2 antagonist, GANT61 (**Fig. 4.5**) (Lauth, et al., 2007). DiI injections of explants treated with GANT61, showed commissural axon misguidance after floor plate crossing with a cohort of axons displaying stalling and knotting behaviors within the floor plate. Misguided axons were reminiscent of the Gli2^{-/-} phenotype, suggesting that Gli protein activity might be necessary to direct commissural axon guidance during midline crossing and on the contralateral side of the spinal cord.

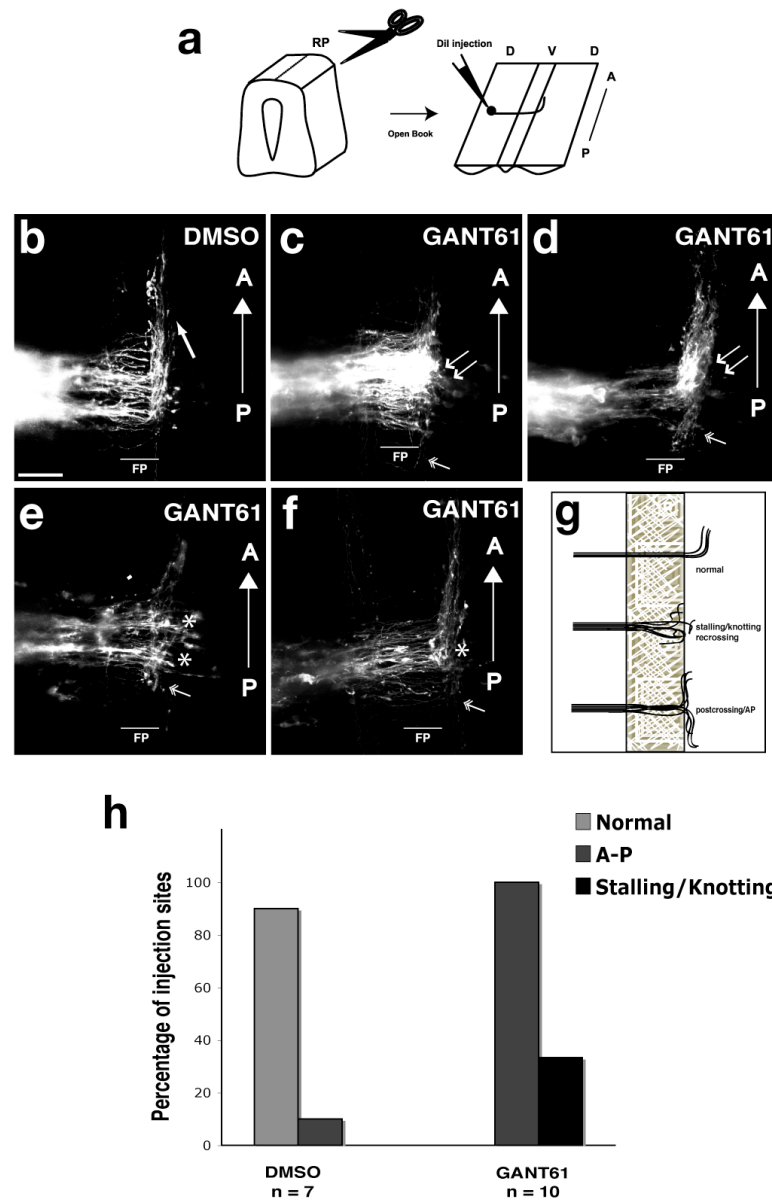


Figure 4.5 Commissural axon misguidance defects in open book explants treated with the Gli1/Gli2 inhibitor, GANT61.

(a) DiI injections of spinal cord open book preparations. (b) Normal projections of commissural axons treated with vehicle control (DMSO). (c-f) DiI injections of open book cultures treated with the Gli1/Gli2 antagonist, GANT61 (25 μ M) display abnormal projections, knotting/stalling (c), randomized turns on the A-P axis with stalling (d) or knotting (e) and misguided caudal projections (f). (g) Diagram of the summary of misguidance behaviors observed in GANT61 treated open book explants. (h) Quantification of injection sites displaying normal, stalling/knotting, or random A-P behaviors. Scale bar represents 100 μ m.

Furthermore, Wnt4 was found expressed at the floor plate in an anterior-high-posterior-low gradient necessary to attract commissural axons to rostral spinal cord regions. Postcrossing commissural axons in mutants for the Wnt receptor Frizzled3 were shown to project randomly in rostrocaudal regions of the spinal cord (Lyuksyutova, et al., 2003).

Shh and Wnt signaling pathways simultaneously regulate the development of the neural tube (Jessell, 2000; Ulloa and Briscoe, 2007). It has been shown that negative regulators of the Shh pathway, Sufu and Gli3 can significantly reduce Wnt activity in the dorsal neural tube by altering β -catenin protein levels, important effectors of Wnt intracellular signals (Ulloa and Briscoe, 2007). Together these data raise the question of how activities of Shh and Wnt signals are integrated in axon guidance. Future studies should address whether transcriptional activation by Gli and β -catenin pathways play a role in commissural axon pathfinding.

Recently, Wnt-Frizzled mediated attraction of commissural axons was shown to require phosphatidylinositol-3-kinase (PI3K) activity downstream of heterotrimeric G-protein signals. In open book electroporated explants, inappropriate activation of the PI3K catalytic subunit, p110 γ was sufficient to induce premature A-P turning of commissural axons (Wolf, et al., 2008). Our preliminary data from overexpressing p110 γ in precrossing commissural axons showed diminished axonal growth and increased growth cone collapse in the presence of Netrin1 (**Fig. 4.6c**) compared to GFP expressing vector controls (**Fig. 4.6b**). Furthermore, commissural neurons treated with recombinant Shh proteins showed increased levels of an active form of

the PI3K effector, Akt (**Fig. 4.6d**). Akt (PKB) is a serine/threonine kinase downstream of PI3K signals shown to positively regulate Shh activity by controlling PKA-mediated Gli inactivation (Riobo, et al., 2006). In addition, activation of Gli-dependent transcription was found to require the activity of G α i proteins through PI3K signals (Kanda, et al., 2003). Together these findings imply a crosstalk of PI3K and Akt pathways downstream of Shh and Wnt signals, but their direct role in commissural axon guidance remains to be fully determined.

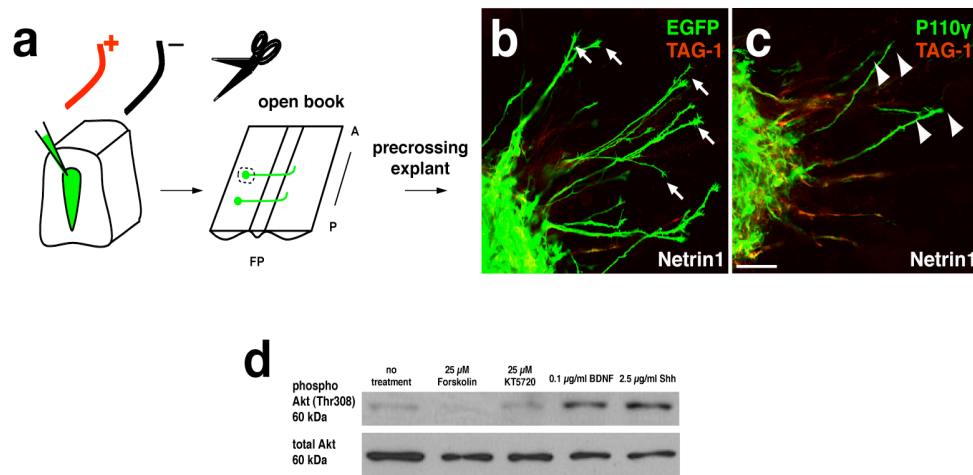


Figure 4.6 Phosphoinositide 3-kinase and Akt signals in commissural neurons.

(a-c) *ex utero* electroporations of precrossing explants. (b) Rat E13 embryos were electroporated with an EGFP construct or with an EGFP fusion protein expressing the catalytic subunit of the phosphatidylinositol-3-Kinase, p110 γ (c) (Wolf, et al., 2008) and cultured in the presence of Netrin1 as previously described (**Fig. 2.3**). Explants are costained for GFP (green) and the precrossing commissural marker TAG-1 (red). (b) Growth cones of EGFP control expressing axons (arrowheads). (c) Precrossing axons expressing p110 γ show decreased growth and increased growth cone collapse. (d) Western blot analysis of phosphorylated Akt (Thr308) and total Akt levels in rat E13 commissural neurons. Commissural neuronal lysates treated with BDNF or Shh-N recombinant proteins show increased pAKT levels. Scale bar represent 50 μ m.

Conclusions

Although numerous studies have shed new light into the mechanisms of axonal pathfinding, it is evident that further understanding of complex guidance signaling systems is required to fully grasp the biological outcomes involved in neuronal connectivity. A number of studies in invertebrates and vertebrates highlight the importance of the integration of axon guidance signals as key determinants in translating extracellular information into a biological response. Axon guidance in development is fundamental for the proper wiring of neural circuits and in the search for therapeutic targets of spinal cord injury and disease.

The findings of this dissertation envisage molecular mechanisms underlying the integration of guidance pathways in the development of neural circuits. In particular, the regulation of growth cone responses to repulsive guidance cues during ventral midline pathfinding (**Fig. 4.7**). Overall, the challenge in understanding neural circuit formation lies amidst the integration of molecular signals involved in general patterns of axonal connectivity. Advances in molecular biology have recently generated new classes of fluorescence probes for live cell imaging to study protein activity using techniques such as, Fluorescence Resonance Energy Transfer (FRET), developed to measure several intracellular signals, cyclic nucleotides, small G proteins, and balances between protein kinase and phosphatase activities (Giepmans, et al., 2006). Single cell imaging of commissural neurons in culture combined with new genetic information of axon guidance molecules can certainly improve our

attempts to dissect out the molecular mechanisms underlying the formation of neural circuits in development and axonal regeneration.

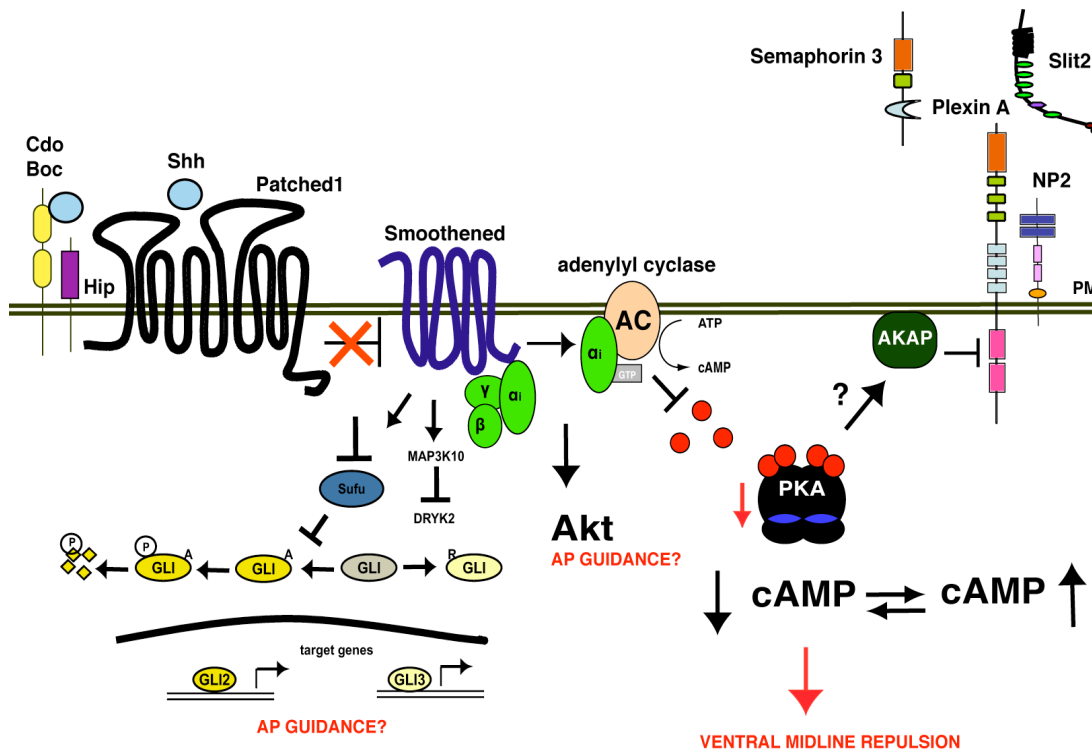


Figure 4.7 Molecular mechanisms of axon guidance at the floor plate ventral midline.

A schematic diagram of proposed Shh signaling in axon guidance. Shh signals through Smo dependent G protein activity regulate intracellular pathways involved in axon guidance systems. In addition, Shh transcriptional activity might induce upregulation of other axon guidance molecular components through Gli dependent mechanisms.

Axonal Regeneration

The increasingly complex mammalian nervous system when injured lacks the ability to regenerate new nervous tissue (Harel and Strittmatter, 2006). Even though progress has been made in overcoming the obstacles after mammalian CNS injury (e.g. glia scar formation), direct recovery of severed axons and functional circuits remains a challenge (Danek, 2006). This limited regenerative ability could perhaps be in itself challenged with a more faithful recapitulation of developmental signals. Most of the axon guidance obstacles present in the adult CNS are marked by an increase of axonal sensitivity to chemorepellents and the induced secretion of growth inhibitory myelin derived proteins (Nicolou, et al., 2006). Even though, axon guidance cues continue to be expressed in the adult and injured nervous system, the downregulation of attractive cues and overall changes in receptor distributions after development changes the mature spinal cord environment (Harel and Strittmatter, 2006). Nonetheless, it is possible that in order to reestablish earlier plasticity properties in damaged axons is necessary to at least in part reintroduce a favorable developmental environment that could change the intrinsic signals of mature neurons into more flexible alternatives suitable for regeneration. cAMP levels known to positively modulate neuronal responses to attractive guidance cues decreases with neuronal age, but can be artificially increased in injured adult fibers to promote some nerve regeneration after injury and degeneration (Nicolou, et al., 2006).

Thus, induction of axonal growth in the injured CNS is possible in certain cellular contexts, but the key approach lies in the successful recapitulation of the

intrinsic state of the developing neuron and the permissive growth-promoting environment of the embryo. Not only understanding how spinal cord axons behave after injury, but also dissecting out the molecular mechanisms of axon guidance pathways in development are crucial in generating models and therapies for the amelioration of spinal cord injury and disease.

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MATERIALS AND METHODS

Animals

Embryos from staged Sprague Dawley pregnant rats (Charles River) were collected at rat embryonic day 13 (E13). CD-1 mice (The Jackson Laboratory) were staged *in house* and embryos collected at mouse E11.5. Animals were handled according to The University of California, San Diego Institutional Animal Care and Use Committee.

Expression constructs

Netrin1, Slit2, Robo1, Semaphorin3F, Neuropilin2, PlexinA3 and Shh Δ CT (N-Shh) pcDNATM 3.1 vector expression constructs were a kind gift from M. Tessier-Lavigne (UCSF, Genentech). The Semaphorin3B expression construct was a generous gift from V. Castellani (Universite Claude Bernard) and subcloned into a pcDNATM 3.1 vector (Invitrogen) for COS cell expression. The Full-length p110 γ -N-terminal EGFP fusion construct was cloned in the lab as described previously (Wolf, et al., 2008).

Full length PlexinA3 (PA3) was cloned by PCR using the PlexinA3 pcDNATM 3.1 vector plasmid as a template (from M. Tessier-Lavigne) with the forward primer 5'ACTCAGGGCGCGGGGCCATGCCCTCTGTCTGCCTCCTC-3' and reverse primer 5'CTGAGTGGCGCGCCCTAATTCAGATCTTCTTCAGAAATAAGTTTTTGTTC

GCTGTCGGTGGACACGAGGCTGAT-3'. A Myc-tag epitope was added to the C-terminus before the stop codon to generate PA3-Myc. The construct was inserted in frame at the *AscI* insertion site of the pCIG2-IRES-GFP vector (from F. Polleux).

Dominant negative PlexinA3 (dnPA3) was cloned from the PA3 expression vector plasmid as described above with the cytoplasmic domain deleted using the forward primer 5'ACTCAGGGCGCGGGGCCATGCCCTCTGTCTGCCTCCTC-3' and reverse primer

5'CTGAGTGGCGCGCCCTAATTCAGATCTTCTTCAGAAATAAGTTTTTGTTCCTTGCGCTTGTACGCCACCAGCAC-3'. A Myc-tag epitope was added to the C-terminus to generate dnPA3-Myc. The construct was inserted in frame at the *AscI* insertion site of the pCIG2-IRES-GFP vector as described.

Patched1 (GenBank accession number U46155) was amplified from an embryonic day 11.5 whole embryo mouse cDNA library using the forward primer 5'ATGGCCTCGGCTGGTAACGCCGCCGGGGCC-3' and the reverse primer 5'GTTGGAGCTGCTCCCCACGGCCTCTCCTC -3'. Deletion of the second extracellular loop to block Shh signals was obtained by polymerase chain reaction deletion of amino acids 768-1026 as previously described (Marigo, et al., 1996) and blunt-end ligated with a modified *PmeI* linker site (GTTTcAACc). The coding sequence at the join is GLDLTDIVP/*VST*/LVCAVFLN, where *VST* codes for the modified *PmeI* linker site. A Myc-tag epitope was added to the C-terminus to generate PTC1^{Δloop2}Myc. The resulting PTC1^{Δloop2} Myc construct was inserted in frame between the *AscI* and *XmaI* sites of a pCIG2-IRES-GFP vector (from F.

Polleux, University of North Carolina, Chapel Hill) driven under the control of a CMV enhancer and chicken β -actin promoter (modified to include a unique *AscI* insertion site) (**Fig. 3.3b, e-h**).

Smoothed (GenBank accession number BC048091) was amplified from a E11.5 whole embryo mouse cDNA library using the forward primer 5'ATGGCCGCTGGCCGCCCCGTGCGTGGGCCC-3' and reverse 5'GAAGTCTGAGTCTGCATCCAAGATCTC AGC-3'. A Myc-tag epitope was added to the C-terminus to generate Smo-Myc. The resulting construct was inserted in frame at the *AscI* insertion site of the pCIG2-IRES-GFP vector as described above. A synthesized 29 nucleotide short hairpin RNA (shRNA) construct against Smoothed (Smo) was purchased in a pRS plasmid under U6 promoter coding for Smo sequence 5'-TGACTCTGTTCTCCATCAAGAGCAACCAC-3' (Origene) (**Fig. 3.4a, c-f**).

Immunohistochemistry

Rat E13 spinal cord precrossing commissural explants were fixed at 37°C with 4% PFA, blocked in 5% PDT (1xPBS, 5% normal donkey serum, 1% BSA, 0.1% Triton X-100) and processed for TAG-1 immunostaining (1:50; 4D7, Developmental Studies Hybridoma Bank) overnight, followed by secondary staining with Cy3 donkey anti-mouse IgM antibody (1:300; 715-165-140, Jackson ImmunoResearch) (**Fig. 2.4b-f; Fig. 2.5a-c; Fig. 2.7e-j; Fig. 3.1c-h; Fig. 3.5h-k**). Rat E13 spinal cord postcrossing explants were fixed as described above and stained for F-actin with Texas Red ®-x phalloidin from Molecular Probes (1:40; T7471, Invitrogen) (**Fig. 4.4b, c**).

Rat E13 electroporated open book spinal cultures explants (**Fig. 3.3d-h; Fig. 3.4c-f**) were fixed in the collagen gel matrix at 37°C with 4% PFA overnight and blocked with 5% PDT as previously described (Wolf, et al., 2008). Electroporated explants (**Fig. 4.6b,c**) were processed overnight at 4 °C with anti-GFP antibody (1:10000; A11122, Invitrogen) and the floor plate marker antibody, HNF-3 β (1:50; 4C7, Developmental Studies Hybridoma Bank) or costained with TAG-1 (1:50 mouse Ab). Next, open book explants were washed in 1% PDT and processed overnight with Alexa Fluor® 488 donkey anti-rabbit IgG (1:1500; A21206, invitrogen) and Cy3 donkey anti-mouse IgG (1:300; 715-166-150, Jackson ImmunoResearch) for open books or Alexa Fluor® 488 donkey anti-rabbit IgG and Cy3 anti-mouse IgM (1:300; 715-165-140, Jackson ImmunoResearch) for electroporated precrossing explants. Finally, explants were mounted in Fluoromount G (Fisher) between two glass coverslips for microscopic analysis.

E11.5 mouse embryos were harvested from CD-1 pregnant mice and fixed at 4°C in 4% PFA with rotation. Fixed embryos were washed with 1xPBS and equilibrated overnight with 30% Sucrose followed by OCT embedding. OCT blocks were frozen in a CO₂/ethanol bath and kept at -80°C for cryostat sectioning. Cryostat sections (20 μ m) were processed on Superfrost Plus slides (Fisher), air dried, and blocked in 5% PDT for 1 hour followed by 4°C overnight incubation with the primary antibodies TAG-1 (1:50) (**Fig. 1.2c**), TAG-1 and Patched1 (1:100; rabbit mAb53715, Abcam) (**Fig. 3.3k-n**) or TAG-1 (1:50, mouse Ab) and Smoothed (1:100; rabbit mAb60016, Abcam) (**Fig. 4.1a-d**). Slides were washed the next day three times with

1% PDT and incubated for 1hr at room temperature (RT), with secondary antibodies, Cy2 donkey anti-mouse IgM (1:300, 715-226-020, Jackson ImmunoResearch) for TAG-1 and Cy3 donkey anti-rabbit IgG (1:300, 715-166-150, Jackson ImmunoResearch) for Patched1 or Smoothened. The slides were subsequently washed in 1% PDT before mounted in Fluoromount G (Fisher) for microscopic analysis.

Dissociated commissural neuron cultures from rat E13 embryos were fixed at 37°C with 4% PFA for 20 minutes as described (Wolf, et al., 2008). After fixation, the commissural cultures were blocked for 1 hr in 1% PDT and stained for DAPI, Patched1 (1:100; rabbit mAb53715, Abcam) (**Fig. 3.3c**) or Smoothened (1:100; rabbit mAb60016, Abcam) overnight (**Fig. 3.4b**); TAG-1 (1:50, mouse Ab), CREB (1:50; Cell Signaling, rabbit mAb # 9197) or phospho-CREB (1:50; Cell Signaling, rabbit mAb # 9198) (**Fig. 4.3b, c**). This was followed the next day with secondary antibody incubation with Cy2 donkey anti-mouse IgM (1:300, 715-226-020, Jackson ImmunoResearch) for TAG-1 and Cy3 donkey anti-rabbit IgG (1:300, 715-166-150, Jackson ImmunoResearch) for CREB and phospho-CREB, 2 hrs before mounting for microscopy.

COS cells plated on glass coverslips for immunohistochemistry, were cotransfected with shRNA-Smo and pCIG2-Smo-IRES-GFP constructs at a 1:3 ratio respectively using FuGENE 6 (Roche) and cultured for 48 hours before being processed for GFP and Smoothened immunostaining as described above (**Fig. 3.4h-k**).

Precrossing commissural explant assay

Precrossing assays to study repulsion were carried out as described (Serafini, et al., 1994; Zou, et al., 2000). Rat E13 spinal cords were isolated by microdissection and dorsal explants from the caudal most region of the spinal cord were collected. Explants (~100-150 μ M width) were cultured on a collagen bed containing a resuspension of COS cells transiently transfected with FuGENE 6 to express pcDNATM 3.1 empty vector control (**Fig. 2.4b, c**), Netrin1 (**Fig. 2.4d-f; Fig. 2.5c; Fig. 2.7e; Fig. 3.1c-e**); Netrin1 and Slit2 (**Fig. 2.5a**), Netrin1 and Semaphorin3F (**Fig. 2.5b**), Netrin1 and Wnt4 (**Fig. 2.7f**), Netrin1 and Wnt7b (**Fig. 2.7g**), Netrin1 and Shh-N (**Fig. 2.7h-j; Fig. 3.1f-h; Fig. 3.5h-k**). In addition, explants were cocultured next to COS cell aggregates expressing pcDNATM 3.1 (empty vector control) (**Fig. 2.4b, d; Fig. 2.7e-h; Fig. 3.1c, f; Fig. 3.5h, i**), Netrin1 (**Fig. 2.4c**), Slit2 (**Fig. 2.4f; Fig. 2.5b; Fig. 2.7i**), Sema3F (**Fig. 2.4e; Fig. 2.5a; Fig. 2.7j; Fig. 3.1d, g; Fig. 3.5j, k**), Sema3F and Slit2 (**Fig. 2.5c**) or Sema3B (**Fig. 3.1e, h**) constructs. Precrossing explant cultures were incubated at 37°C with 5% CO₂ for 16 hours in spinal cord dorsal medium before fixation as previously described (Serafini, et al., 1994).

For compound treatment experiments (**Fig. 3.5**), the culture explant medium was changed into a Forskolin (25 μ M; F3917, Sigma) (**Fig. 3.5i, k**) or vehicle control, dimethyl sulfoxide (0.8% DMSO, D2438, Sigma) (**Fig. 3.5h, j**) containing medium 2 hours after explants were embedded in the collagen gel and cultured a total of 16-18 hrs before fixation. For quantification the total length of axon bundle was measured in the proximal (P) and distal (D) quadrants as explained above (**Fig. 2.3 and Fig. 2.4**).

The P/D ratio is a measure of attractive or repulsive activity, with a ratio of >1 indicating attraction and <1 indicating repulsion.

Postcrossing commissural explant assay

Preliminary postcrossing explant assays were carried out as described (Zou, et al., 2000). Spinal cord open book preparations were dissected out as described above and a strip of around 300 μM width was isolated with the floor plate attached using a sharp tungsten needle (Schematic diagram, **Fig. 2.2a, c, d; Fig. 4.4**). Postcrossing explants were cultured in the three-dimensional two-layer collagen matrix gel conformation described for precrossing explant cultures above and exposed to COS cell aggregates expressing either a pCDNA3.1 vector control (**Fig. 4.4b**) or cell aggregates expressing Shh-N (**Fig. 4.4c**). Explants were incubated at 37°C with 5% CO_2 for 18 hours prior harvesting and treated for immunohistochemistry as described above.

Alkaline phosphatase binding assay

To generate alkaline phosphatase (AP) fusion recombinant proteins, HEK293T cells were transfected using FuGENE6 with an AP-substrate control (AP-ss), AP-Slit2 and AP-Sema3F fusion constructs (from M. Tessier-Lavigne) and cultured for two days (**Fig. 2.6a**). On the third day, the media was changed to a low serum medium (1x), Opti-MEM (Invitrogen) to condition for recombinant AP-Slit2 and AP-Sema3F proteins on day 6. The recombinant protein containing media was collected and the

supernatant harvested at 5000 rpm for 5 minutes to clear residual cell debris. 1M HEPES was added to the collected supernatant for a final concentration of 20mM HEPES, pH7. The collected supernatant was concentrated using a Centriprep 30 (Amicon) column. The final concentration of the AP fusion proteins and the AP substrate control was determined by AP activity compared to alkaline phosphatase standards as described previously (Takahashi, et al., 1997).

pCDNA3.1 vector control, Robo1, Neuropilin2, and PlexinA3 constructs were transfected in COS cells using FuGENE 6 in 24 well plates and cultured for two days. Transfected cells were rinsed three times for 10 minutes in Hank's balanced salt saline (HBS) + 0.5 mg/ml BSA/0.1%NaN₃/20mM HEPES, pH7 and heparin (2 µg/ml) (HBS+). The AP binding of collected AP fusion proteins to transfected COS cells was carried out in unfixed cells for 2 hrs at room temperature. COS cells were washed in HBS+ solution five times and fixed for 1 minute in a 60% acetone/3%PFA/20mM HEPES solution, washed again in HBS+ solution and heated to 65 °C to inactivate endogenous AP activity. AP activity was detected by deposition of the insoluble reaction with nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl phosphate (BCIP). Reactions were terminated when the blue product was visible, rinsed in Tris/EDTA and mounted using fluoromount G. The intensity of AP signals was captured using a cooled-snap camera CCD camera as seen in **Fig. 2.6b-p**.

Open book spinal cord explants treatments and DiI injections

Open book preparations of rat E13 spinal cords were isolated as previously described (Lyuksyutova, et al., 2003, Wolf, et al., 2008). At 2-4 hrs of culture, open book explants were treated with vehicle control (0.8% DMSO, D2438, Sigma) (**Fig. 3.5a; Fig. 4.2a; Fig. 4.5a**), Shh blocking antibody (5E1; mouse, IgG1, 100 ng/ml, DSHB) (**Fig. 3.2b-f, j, k**), Forskolin (25 μ M; F3917, Sigma) (**Fig. 3.5b-e**), PKA inhibitor, KT5720 (25 μ M KT5720; EI-199, Biomol) (**Fig. 4.2b,c**) and Glit1/2 inhibitor GANT61 (GANT61, from R.Toftgård, Karolinska Institute, Sweden) (**Fig. 4.5c-f**).

Open Book explants were incubated at 37°C with 5% CO₂ for an additional 12-14 hrs. After culture, open books were fixed at 37°C with 4% PFA, washed in RT 1xPBS and injected with the lipophilic dye 1, 1'-Dilinoleyl-3,3,3'-tetramethylindocarbocyanine perchlorate (DiI, D282; 1 mg/ml, Molecular Probes) via iontophoresis (~7 volts) into the dorsal region where commissural neuron cell bodies reside. The DiI was allowed to diffuse for 1 day at 37°C to enable visualization of the complete commissural axon trajectory before mounting in Fluoromount-G between two coverslips for confocal microscopy analysis. Injection sites were scored based on their overall behavior at the ventral midline and presented as a percentage of all injected sites from at least three experiments.

Rat spinal cord *ex utero* electroporations

PTC1^{Δloop2} Myc-IRES-GFP or pCIG2-IRES-GFP (1 μg/μl) constructs were electroporated *ex utero* in Rat E13 embryos with three 100 ms pulses of 25V at 1s intervals using a BTX#ECM 830 electroporator directed at the progenitor domains of the dorsal spinal cord as described (**Fig. 3.3a, b**) (Wolf, et al., 2008). Spinal cords were microdissected immediately after electroporation and cultured as open book explants in a collagen gel matrix at 37 °C, 5% CO₂ for 3 days in fresh media before processing for fixation (**Fig. 3.3d-h**).

shRNA constructs against Smo (Origene) were coelectroporated in rat E13 embryos with the EGFP-N2 vector (Clontech) modified to include the chick β-actin promoter to allow GFP in commissural neurons. Using the same electroporation parameters as above at a 1:3 GFP vector to shRNA ratio, open book spinal cords were cultured for 3 days fixed and processed for immunohistochemistry (**Fig. 3.4**). For quantification the total number of crossed axons and misguided axons were counted and presented as the percentage of the total number of electroporated axons from at least three independent experiments.

Full-length p110 γ-N-terminal EGFP fusion constructs (**Fig. 4.6c**) described previously (Wolf, et al., 2008) or EGFP-N2 vector control constructs (**Fig. 4.6b**) were electroporated in rat E13 embryos as described above. Spinal cords were microdissected and the progenitor domains of the dorsal regions were dissected out as precrossing commissural explants (**Fig. 4.6a**). Precrossing explants were cultured in the three-dimensional two-layer collagen gel assay in the presence of Netrin1

expressing cells resuspended uniformly in the bottom collagen gel layer. Explants were cultured at 37°C with 5% CO₂ for 40 hrs, harvested, fixed, and processed for immunohistochemistry as described above.

Dissociated commissural neuronal culture and Western immunoblotting

Spinal cords were isolated from rat E13 embryos and commissural neurons were dissociated as previously described (Wolf, et al., 2008). In brief, embryos were treated with Dispase I (0.25 mg/ml; D4818, Sigma) for 5 minutes prior isolation of spinal cords. Followed by collection of spinal cord dorsal domains by microdissection and incubation with Trypsin (1 mg/ml; T47-99, Sigma) for 10-12 min in a 37 °C water bath. The tissue was then triturated with flamed glass pipettes and a single cell suspension was plated on poly-D-lysine (PDL; 20 µg/ml) and laminin (10 µg/ml) coated coverslips or 12 well plates (Corning). More than 90% cells in these cultures express TAG-1, a marker for spinal cord commissural neurons (**Fig. 4.3a**). 10⁵ dissociated cells were plated on PDL/laminin coated coverslips and cultured for 24 hrs before processed for immunohistochemistry as described above (**Fig. 3.3c; Fig. 3.4b; Fig. 4.3a-c**). For Western blot analysis, 10⁶ dissociated cells were plated on PDL/laminin coated 12-well tissue culture plates and cultured initially for 24 hrs in dorsal explant media (replenished daily) then changed to a low serum medium overnight prior to treatments (**Fig. 4.3d**). Commissural dissociated cells were subsequently treated with 25 µM Forskolin, 25 µM KT5720 (PKA inhibitor), 5 µM Purmorphamine (Smo agonist, Cat # 540220, CALBIOCHEM), 0.1 µg/ml

recombinant human Brain-derived neurotrophic factor (BDNF; 248-BD, R&D) or 2.5 µg/ml recombinant mouse Sonic Hedgehog amino-terminal peptide (125 nM Shh-N; 461-SH, R&D) for 15 minutes (**Fig. 4.6d**) or 2.5 µg/ml recombinant mouse Shh-N for 1 hr (**Fig. 3.5m**) and lysed in a buffer of 20mM HEPES, 1% Triton X-100, 150 mM NaCl, 2 mM CaCl₂, 2mM MgCl₂, 50µg/ml BSA, 10% glycerol, 1mM EDTA, 1mM EGTA containing a cocktail mix of protease and phosphatase inhibitors (Sigma). Samples were centrifuged and the supernatants without insoluble proteins were collected for protein concentration determination (Bio-Rad Protein Assay; 500-0006). Cell lysates were separated by SDS-PAGE gel electrophoresis in 1xSDS loading buffer after DNA disruption by sonication, transferred onto a nitrocellulose membrane, blocked with 5% w/vBSA in TBST and probed for relative amounts of phosphorylated levels of PKA C (-α, -β, -γ; MW 42kDa) at Thr197 (1:1000; rabbit mAb 4781, Cell Signaling), total PKA levels of regulatory subunit PKA_{R11β}, MW 53kDa (1:1000; mouse mAb 610625, BD Biosciences) (**Fig. 3.5m**), phosphorylated levels of CREB at Ser133, MW 43kDa (1:1000; rabbit mAb 9198, Cell Signaling), and total CREB (48H2), MW 43kDa (1:1000; Rabbit mAb 9197, Cell Signaling); loading control α-tubulin levels, MW 50kDa (mouse IgG, 1:1000) (**Fig. 4.3e**); Phosphorylated levels of AKT at Thr308, MW 60 kDa (1:1000; Rabbit mAb 9275, Cell signaling) or total AKT levels (1:1000; Rabbit mAb 9272, Cell signaling) (**Fig. 4.6d**). The blots were developed using the ECLTM (enhanced chemiluminescence) protein detection system (Armstrong) for horseradish peroxidase conjugated

secondary antibodies; anti-rabbit for phospho-PKA C, phospho-CREB, total CREB, phospho-AKT, total AKT, and anti- mouse for PKA_{R11β} and α -tubulin.

Microscopy

1024*1024 fluorescent photographs were taken using a Zeiss LSM510 confocal laser-scanning microscope with identical acquisition parameters for each experiment. Electroporated spinal cord images were at least 12 Z-slices of a 100 μ m thick Z stack.

Statistical analysis

Statistical analysis of experiments were performed using unpaired student's two tail *t* test of data analyzed from at least 3 independent experiments using ImageJ, Excel and SigmaPlot. Results are expressed as the mean average $\pm$ SEM. $P \leq 0.05$ was considered significant and was denoted by *, $P \leq 0.05$, **, $P \leq 0.01$, ***, $P \leq 0.0025$.

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