Title
Building the visual pathway

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Publication Date
2002

Peer reviewed|Thesis/dissertation

Building the Visual Pathway: The Role of Sema5A in Retinal Axon Guidance and Optic Nerve Formation

by

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DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biomedical Scienes

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO

Dedication

This dissertation is dedicated to my parents, Stuart and Leslie, who have been patiently sending me to school for over 20 years and counting…

Preface

I am most thankful for the guidance and mentoring of my graduate advisor, David W. Sretavan. Dave's hands-on approach, passion for teaching, and tireless efforts over the years made this work possible.

I am grateful to members of the Sretavan laboratory for their experimental contributions, patient teaching, and daily company. In addition, I am indebted to my thesis committee members, Todd Margolis and John Rubenstein for their advice and generous time commitment over this course of study. I am also thankful for the financial support of the National Institute of Health Medical Scientist Training Program and of the That Man May See Foundation.

Finally, I am grateful for the support I have enjoyed from my family and friends, who provided the perfect balance of encouragement and distractions.

The first two chapters of this dissertation have been submitted for publication in the following journals. Chapter 1 was submitted to *The British Journal of Ophthalmology*, and Chapter 2 to the journal *Development*.

Building the Visual Pathway: The Role of Sema5A in Retinal Axon Guidance and Optic Nerve Formation

Stephen F. Oster

Proper pathfinding of axons to their correct targets is fundamental to nervous system development. This pathfinding process is orchestrated by axon guidance molecules that can act either as attractants and support axon growth, or as inhibitory signals steering axons away from incorrect destinations. In the visual system, the axons of retinal ganglion cells are directed by a series of guidance molecules along the developing visual pathway. The earliest retinal axon guidance decisions include exiting the retina through the optic disc and traversing the length of the developing optic nerve. These tasks are known to involve the guidance cues Netrin-1, L1, and laminin. Netrin-1 is expressed by neuroepithelial cells surrounding the optic disc and along the optic nerve, and is necessary for retinal axon exit through the optic disc. Laminin and L1 are growth substrates available to retinal axons throughout the early pathfinding process, and both have been reported to modulate axon responses to other guidance molecules. Here we identified, through a mRNA expression screen, the presence of transmembrane Sema5A at the optic disc, and along the optic nerve, at times when retinal axons were passing through these regions. Given that semaphorins are a large, and generally inhibitory, family of axon guidance molecules, we tested how Sema5A influenced retinal axons, and whether this response was modulated by other guidance signals encountered along the retinal pathway. In both collapse assays and neurite outgrowth assays, our results

showed that Sema5A invariantly inhibited retinal axons in the context of Netrin-1, L1, or laminin signaling. This suggested that Sema5A inhibited these same axons in vivo as they traveled through the optic disc and along the optic nerve. A polyclonal antibody confirmed that Sema5A was distributed in a ring-like pattern surrounding axons in the retinal pathway. In addition, antibody perturbation of Sema5A function led to optic nerve defasciculation. Thus, these studies show that Sema5A acts invariantly on retinal axons as they progress through pathfinding tasks directed by other guidance molecules, and that the use of inhibitory sheaths is a mechanism for maintaining nerve integrity during central nervous system development.

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Chapter 1

Connecting the Eye to the Brain: The Molecular Basis of Ganglion Cell Axon Guidance

Prologue

This chapter introduces the principles of axon guidance, and some of the families of axon guidance molecules. It reviews pathfinding in the developing visual system, and highlights the relevance of retinal axon guidance to human visual developmental disorders and to retinal axon regeneration.

David Sretavan and I wrote this chapter as a review of axon guidance in the visual system. It has been submitted to *The British Journal of Ophthalmology*.

Connecting the Eye to the Brain: The Molecular Basis of Ganglion Cell Axon Guidance

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Abstract

In the last several years, a great deal has been learned about the molecular basis through which specific neural pathways in the visual system are established during embryonic development. This review provides a framework for understanding the principles of retinal ganglion cell axon guidance, and introduces some of the families of axon guidance molecules involved. In addition, the potential relevance of retinal axon guidance to human visual developmental disorders, and to retinal axon regeneration, is discussed.

Introduction

Wiring the human brain is one of nature's greatest feats, and one of its most daunting tasks. Neurons numbering in the billions must be specifically connected to one another to assemble functioning neural circuits. The basic framework of neuronal connectivity is built during fetal development in a process called axon pathfinding. During pathfinding, the axons of developing neurons navigate long distances along specific pathways to reach their appropriate targets. The characterization of the molecules in the developing brain environment that guide axons, as well as the receptors and signaling cascades through which guidance molecules exert their influence, form the central areas of investigation in the field of axon guidance.

One of the best-studied models of axon guidance is the developing retinal ganglion cell (RGC) and its axon. Recent work has begun to shed light on the molecular mechanisms that govern RGC axon guidance during optic nerve development, the formation of the optic chiasm, and the establishment of retinotopic maps in visual targets such as the superior colliculus. Our goal in this review is to discuss a number of the pathfinding tasks that developing RGC axons must accomplish. In doing so, we will provide examples of how guidance molecules steer RGC axons to illustrate fundamental principles of axon pathfinding. In addition, we will highlight aspects of RGC axon guidance that have potential relevance to visual developmental disorders, and discuss how

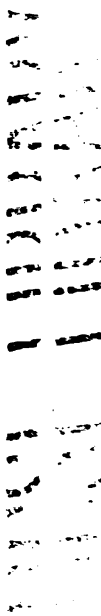
lessons of axon guidance may be applied towards a better understanding of axon regeneration in the visual system.

The axon growth cone as an exploratory apparatus

During pathfinding, axons elaborate specialized structures at their tips call growth cones through which they sense and respond to the environment (see figure 1). The growth cone is a fan-shaped motile structure with finger-like filopodia, and is constructed of actin filaments extending from a central microtubule core [1]. As a growth cone extends in the embryonic environment, receptor molecules on its surface interact with guidance molecules displayed in the extracellular matrix or on the surfaces of surrounding cells. Upon activation of these receptors by guidance molecules, intracellular signaling cascades are triggered which eventually feed into pathways altering the assembly of cytoskeletal components such as actin and tubulin. Signaling cascades causing a net addition of cytoskeletal components is thought to lead to growth cone advance, while net disassembly may lead to axon retraction. Asymmetric signaling on one side of the growth cone is thought to lead to turning and change in the direction of growth [1-3]. In the last several years, as researchers identified significant numbers of guidance molecules and begin to understand how particular combinations are used for specific pathfinding tasks, the rough outlines of how the visual system is assembled is beginning to emerge.

Basic building blocks - growth cone attraction versus repulsion

Amongst the earliest axon guidance molecules identified were extracellular matrix molecules such as laminin and fibronectin that promote axon growth. Analysis of the protein domain structure of these and other subsequently identified families of guidance molecules showed that guidance molecules in general all contain a number of common domain motifs such as immunoglobin-like repeats, EGF repeats, and fibronectin type III domains. Each family of guidance molecules is however, also defined by its own distinctive domain (see reviews [4, 5]). In addition to guidance molecules that promote axon outgrowth, an important contribution to our understanding of axon pathfinding was the discovery that a substantial number of guidance proteins control axons by inhibiting their ability to extend. Given that the nervous system is able to both encourage and inhibit axon growth, it would seem that one simple strategy for axon guidance is to use arrays of growth promoting and inhibitory guidance cues to steer developing axons along specific pathways to their targets. Indeed, as illustrated in the following examples, there are instances of RGC axon guidance that seem to reflect this strategy.



Growth promotion and axon fasciculation

The first major pathfinding task for a newly born RGC is to extend an axon towards the optic nerve head. During development, ganglion cells are born in a central to peripheral gradient such that the oldest RGCs are closest to the optic disc and the younger RGCs are in more peripheral retina. Newly formed RGC axons are in contact with axons of older RGCs and travel along, or fasciculate with, these neighboring axons to reach the optic nerve head. This fasciculation appears to be due to growth promoting

molecules such as L1 on the RGC axons themselves. L1 is a member of the immunoglobulin family of cell adhesion molecules [6], and functions in a homophilic manner. Homophilic binding means that a L1 molecule on a given axon binds a L1 molecule on an adjacent axon. It is thought that such L1 homophilic interactions encourage retinal axons to grow in bundles, or fascicles, within the retina on their way to the optic disc. This model is supported by the finding that experimental blockade of L1 function, or the function of related Ig guidance molecules, causes RGC axons to wander in the retina instead of growing directly to the optic disc [7, 8]. Thus RGC axon pathfinding to the optic disc appears to involve the ability of retinal growth cones to follow a trail of attractive axon guidance molecules.

Attraction from a distance

Along with guidance molecules such as LI that are displayed on surfaces of cells or other axons, the nervous system also uses secreted proteins to attract axons. These secreted attractants add a degree of versatility to pathfinding because they can diffuse away from their source to influence growth cones at a distance. Netrin-1 was the first secreted attractant identified in vertebrates, and was initially characterized as an important guidance molecule during development of the spinal commissural axons [9-11]. The floorplate, which forms the ventral midline of the developing spinal cord, secretes Netrins that can diffuse away to form a gradient. The axons of commissural neurons in dorsal spinal cord find their way ventrally to the midline by following this increasing Netrin gradient. In the visual system, several lines of evidence have shown that Netrin-1

plays a critical role in RGC axon exit from the retina into the developing optic nerve [12]. First, Netrin-1 protein is expressed by neuroepithelial cells at the developing optic nerve head which are in close contact with RGC axons. Second, RGC axons in vitro respond to Netrin-1 as an attractive guidance molecule. Lastly, mice with a targeted deletion of the Netrin-1 gene exhibit pathfinding errors at the optic disc where RGC axons fail to exit into the optic nerve and instead grow inappropriately into the other side of the retina (see figure 2). As a result of this aberrant pathfinding, these mice exhibit optic nerve hypoplasia [12].

Using inhibitory guidance in the retina

The discussion thus far has focused on guidance attraction either as a result of RGC growth cones contacting cell or axon surfaces displaying growth promoting cues or responding to diffusible molecules secreted at a distance. However, RGC axon pathfinding in the retina also uses inhibitory guidance molecules. After RGCs are born, what ensures that their axons head correctly towards the optic nerve head instead of growing towards peripheral retina? Although an explanation involving intrinsic RGC properties that properly orient initial axon outgrowth is possible, studies in rodents have provided evidence for an active inhibitory mechanism preventing RGC axons from heading incorrectly towards peripheral retina. This mechanism appears to involve a ring of chondroitin sulfate proteoglycan in the peripheral retina adjacent to the youngest RGCs (see figure 3). Chondroitin sulfate is found in the extracellular matrix, and has been shown to inhibit RGC axon growth in vitro [13, 14]. Enzymatic breakdown of

chondroitin sulfate during retinal development resulted in RGC axons with abnormal trajectories in peripheral retina [13]. These findings suggest that inhibition helps send RGC axons in the correct direction towards the optic disc at the very start of their journey.

Inhibitory guardrails during optic chiasm development

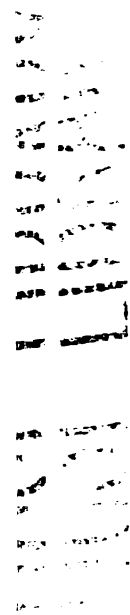
Growth cone inhibition is also important once RGC axons have left the retina. The Slit proteins are a family of inhibitory axon guidance molecules that were originally discovered to play a guidance role in the *Drosophilia* midline and longitudinal tracts [15, 16]. In mammalian development, Slits have been implicated in olfactory system guidance, neural migration, and axon branching ([17-19] for a Slit review see [20]). Recent work has demonstrated that Slit proteins govern where RGC axons form the optic chiasm on the ventral surface of the developing hypothalamus [21]. Slits are known to inhibit RGC axons in vitro [22, 23, 24], and are normally expressed in regions both anterior and posterior to the developing optic chiasm. In mice deficient for Slits, some RGC axons form a secondary optic chiasm anterior to the normally situated optic chiasm [21]. The region into which the chiasm was shifted normally expresses Slits, suggesting that Slit inhibition forms a barricade to properly channel RGC axons at the chiasm (see figure 3). However, since not all RGC axons are affected by the Slit deficiency, other guidance cues must also play a part in governing RGC axon pathways at the developing optic chiasm.

Growth cone repulsion at a distance

Just as growth promoting molecules exist as cell-attached and secreted proteins, inhibitory guidance molecules can also exist as cell-attached or diffusible repellents. The first diffusible inhibitory guidance molecule identified belonged to the Semaphorin family (for Semaphorin reviews see [25, 26]). Now called Sema3A, this protein was identified by its ability to "collapse" growth cones in vitro [27]. Collapse has become a hallmark of in vitro inhibition, and occurs when axons treated with an inhibitory molecule undergo actin depolymerization, lose their filopodia and fan-shaped structure leaving just a thin tip [27]. A role for Semaphorins in RGC axon guidance has yet to be clearly established. However, there is recent evidence that Sema3A collapses RGC axons from the frog Xenopus laevis in vitro [28], although it is not known where Sema3A is expressed in the developing visual system. Studies have also identified the presence of a transmembrane Semaphorin (Sema VI) in the mammalian retina [29]. However, since Semaphorins have diverse roles in development including control of neural migration [30], it is not clear whether the Semaphorin VI influences RGC axon guidance or other aspects of retinal development. While the role of Semaphorins in the development of central retinal pathways remains to be determined, it is of note that Sema3A is expressed in developing cranial tissues. Mice deficient for Sema3A, exhibit cranial nerve axon guidance errors resulting in abnormal innervation patterns of the facial and trigeminal nerves [31].

Mapping through gradients

The previous examples have illustrated how the visual system uses individual guidance molecules to provide simple forms of growth cone attraction or inhibition. It is clear however that more sophisticated strategies such as graded inhibition are also employed. The use of gradients of guidance molecules is best illustrated by the formation of the retinotopic map in the superior colliculus. The characteristic feature of the retinotopic map is that axons of neighboring RGCs project to neighboring sites in CNS targets to faithfully re-create a retinal map of visual space. The molecular mechanism of mapping represents an intriguing problem since the targeting of each axon must be coded by slightly different information than its neighbors for proper alignment. Rather than assigning a distinct guidance receptor to each axon, and an individual guidance molecule to each target cell, the nervous system uses gradient mapping to solve the problem.



Early insight came when researchers discovered that given a choice in vitro, RGC axons from the chick temporal retina avoided growing on cell membranes harvested from the posterior optic tectum (avian homolog of the superior colliculus), and that this avoidance was due to a repellent molecule present in posterior tectal membranes [32]. The repellent turned out to be one of a large group of protein ligands now collectively called the Ephrins (for a general Eph/Ephrin review see [33]). Subsequent work in both chick and mice revealed that specific Ephrins are expressed in an anterior-posterior gradient in the colliculus, while within the retina, a nasal-temporal gradient of the corresponding Eph receptors are expressed by RGCs (reviewed in [34]). Due to the inhibitory action of the Ephrins, axons from temporal retina carrying a relatively large number of Eph-receptors avoid posterior colliculus where ephrins are highly expressed.

Axons from progressively more nasal retina on the other hand, carry fewer Eph-receptors are thus capable of mapping to more posterior sites in the target. The graded expression of Eph receptors on different RGC axons and gradients of inhibitory guidance molecules, assists the visual system in generating a retinotopic mapping in the colliculus. Additional work has suggested that Eph-Ephrin gradients may also contribute to RGC topographic target recognition in other visual targets such as the lateral geniculate nucleus [35]. The use of inhibitory gradients to sort RGC axons enormously amplifies the amount of information carried by a small set of guidance molecule-receptor pairs.

Bifunctionality and reverse signaling

So far in this review, the discussion has categorized guidance molecules into those that promote axon outgrowth and those that exert inhibitory effects on growth cones. It is now known that this distinction is not always correct, and that, at times, the same axon guidance molecule can act as either an attractant or a repellent. Bifunctionality not only offers increased flexibility during axon guidance, but also maximizes the amount of guidance information that a single molecule can provide. Evidence in mammals for the ability of a single guidance molecule to both attract and repel axons has come from studies showing that Netrin-1, known to promote growth from spinal axons and RGCs, actually repels the axons of trochlear motor neurons [36]. These experiments corroborated earlier evidence from invertebrates that a single Netrin source could attract some neurons and repel others [37]. Bifunctional guidance information blurs the distinction between

attractive and repulsive guidance as the function of a given guidance molecule depends on the axon population being affected.

Another example of the nervous system maximizing guidance information resulting from ligand / receptor interactions can be found in the Eph/Ephrin family of guidance molecules. It is known that Ephrins normally bind to Eph proteins that serve as receptors in RGC axon mapping in the superior colliculus. However, a number of studies have also shown that Eph proteins can additionally act as ligands and trigger signaling events in cells expressing Ephrins. First described in biochemical studies [38], this reverse flow of information from Eph through Ephrin molecules has been shown to play an important role in RGC axon navigation within the retina to the optic disc. Although Ig family molecules such as L1 mediate retinal axon fasiculation during growth towards the optic nerve head, L1 by itself is not enough. The visual system also exposes retinal axons approaching the optic nerve head to an inhibitory gradient of EphB proteins in order to maintain tight axon fasiculation [39, 40] (see figure 2). Elimination of EphB proteins, which in this case are functioning as guidance molecules, results in retinal axon defasciculation with the affected retinal axons bypassing the optic nerve head exit point [39, 40].

Integration at the growth cone

As expression patterns of more guidance molecules in the developing brain were characterized, it became clear that the nervous system does not simply use a serial non-

overlapping sequence of guidance molecules to steer axons. Instead, growth promoting molecules are often found in regions overlapping with inhibitory guidance molecules. The simplest way for a growth cone to combine information presented simultaneously by multiple guidance cues is to sum all the guidance forces. This model predicts that growth cones are influenced by the net effect of the total attractive and repulsive forces. Some evidence for a summation model comes from genetic studies in *Drosophilia.* Experimental alterations in the expression levels of attractive Netrins versus inhibitory Semaphorins in individual muscle cells resulted in predictable behavior of the innervating motor axon growth cone. For example, ramping up Semaphorin mediated inhibition in the muscles, which repelled motor neuron axons, could be counterbalanced by also raising the levels of Netrin mediated attraction in the muscles [41]. In this system, it appears that motor axons find their appropriate muscle targets based on a proper balance of attraction and repulsion.

Modulation of responses

In addition to the summation model described above, evidence suggests that the nervous system can also combine guidance information in a less predictable fashion. For example, RGC axons from the frog *Xenopus* interpret Netrin-1 to be an attractive guidance molecule, and they will grow towards a Netrin-1 source in vitro [42]. However, if RGC axons are also simultaneously exposed to laminin, a molecule that normally promotes growth, an inhibitory response is triggered [43]. This surprising finding demonstrates that a growth promoting substrate and a diffusible attractant can combine into an

inhibitory force. In *Xenopus*, like in the mouse retina, the diffusible attractant Netrin-1 is present at the optic nerve head, and likely also governs retinal axon growth into the optic nerve. Given that laminin is present in the region of the mammalian optic nerve head, the results suggest that in normal development, as RGC axons simultaneously encounter both laminin and Netrin-1, inhibition drives axons away from the laminin rich retinal surface through the optic nerve head. In this situation, the growth cone's response to Netrin is switched by the presence or absence of laminin. This mode of action allows the nervous system to modulate the effects of a guidance molecule on an axon by adding or subtracting a second cue.

Cell signaling and switching between attraction and inhibition

One mechanism that could explain how growth cones exhibit varying responses to the same guidance molecule under different circumstances involves cyclic-nucleotide mediated intracellular signaling. Experiments have indicated that attraction and repulsion is determined downstream of the guidance receptor, and in fact a single axon can be made to grow towards or away from a guidance molecule source by manipulating only the cyclic nucleotides in the growth cone [2, 3, 42]. This finding made very clear that the attractive or inhibitory effects of guidance molecules and their receptors are not inherent to the proteins at the cell surface, but instead are a result of multiple interacting intracellular signaling cascades. These interacting signaling cascades present a mechanism for the integration of multiple guidance messages, and could explain how a growth cone changes its response to a given guidance molecule at different points in a pathway. A

better understanding of the intracellular events controlled by various ligand-receptor pairs will provide insight into how guidance molecules combine to steer growth cones in vivo.

Receptor silencing and hierarchy of guidance response

An important question in axon guidance is how axons that have grown into an attractive area can ultimately leave and continue on the next segment of their pathfinding journey. While several possibilities exist, there is evidence that exposure to a new guidance molecule can silence the effects of guidance forces that the growth cone was following previously. This silencing idea represents a novel model for how axons integrate multiple guidance forces. As described earlier in this review, a Netrin-1 gradient produced by the floorplate attracts spinal commissural axons to the ventral midline. Interestingly, the floorplate also produces inhibitory Slits. It appears that commissural axons approaching the floorplate are attracted by Netrins and ignore the Slit protein. However, once these same axons cross the ventral midline at the floorplate, they are no loner affected by Netrin-1 and instead are repelled by Slits. The underlying mechanism is that the Slit receptor itself, which only becomes available after midline contact, mediates not only the Slit-based repulsion but also binds directly to, and silences, the Netrin receptor [44]. This scenario allows the axons to be drawn towards the Netrin-rich floorplate, and then repelled by slit from the floorplate so the axons do not linger at or re-cross the midline [45]. Using receptor silencing in this fashion, the nervous system can potentially control which of many available guidance factors influence the growth cone at different points along an axon's journey. Currently it is not known whether receptor

silencing also works to mediate RGC axon pathfinding. However, one segment of the retinal pathways where this mechanism may come into play is the optic nerve, where both Netrin and Slit are known to be present. A form of receptor silencing may be required for RGC axons to leave Netrin attraction in the optic nerve and enter the chiasm midline region known to lack Netrin [12].

Axon guidance in the human visual system

Almost all of the studies discussed above were performed in the developing visual systems of lower vertebrates or rodents. Naturally, one question is how much of what we have learned about retinal axon guidance from these species can be applied towards understanding how the human visual system is assembled. Some insight can be obtained by considering the fact that the molecular basis of growth cone guidance is highly conserved throughout evolution. For example, homologs of many of the axon guidance molecules discussed here, such as Netrins, Slit, and Semaphorins, have been identified in invertebrates such as the fruit fly Drosophilia and the nematode *C elegans,* where they also participate in axon pathfinding during neural development [15, 37, 46-48]. Human homologs of Netrins, Slits, Semaphorins, and Ephrins have also been identified [46, 49], but as of yet, little is known about of their patterns of expression or function during human development. However, it seems highly likely that these same gene families, and similar axon guidance principles, contribute to patterning the human visual system.

Developmental Disorders

It is of note that the elimination of specific axon guidance molecules during development of the mouse nervous system have resulted in phenotypes that bear a striking resemblance to CNS abnormalities found in some human syndromes. Several examples come from axon guidance studies within the retina. For instance, the loss of Netrin-1 at the optic disc causes RGC axons to fail to exit the retina, and leads to Optic Nerve Hypoplasia [12]. Optic Nerve Hypoplasia is an important cause of childhood visual disability, and a number of potential etiologies have been proposed including abnormal RGC neurogenesis, or inappropriate RGC cell death due to defects in target derived trophic support. The examination of Netrin-1 function in mouse visual development suggests that an additional potential mechanism is defects in genes encoding retinal axon guidance molecules. In addition to defects in optic nerve formation, the lack of Netrin-1 function during development also results in abnormalities in other parts of the CNS. These include agenesis of the corpus callosum, and a number of cell migration and axon guidance defects in the hypothalamus [50]. In humans, the triad of Optic Nerve Hypoplasia, abnormal corpus callosum formation, and pituitary dysfunction is found in the syndrome Septo-Optic Dysplasia [51].

Mice lacking Eph B guidance proteins also exhibit retinal axon guidance defects. RGC axons in these mice stray away from normally tight fascicles and miss the optic disc exit point [40]. Of note, these guidance defects are found specifically in axons originating from the dorsal, or superior, part of the retina [40]. In humans, a similar restriction of an anatomical defect to superior retina is present in patients with Superior Segmental Nerve Hypoplasia patients [52]. One speculation is that the pathogenesis of Superior

Segmental Optic Nerve Hypoplasia shares similarities with the developmental mechanisms controlling expression of axon guidance molecules in the dorsal-ventral retinal axis.

Regeneration in the visual system

The remarkable capacity for growth, and specific wiring, during development is not present in the adult visual system. Adult retinal axons, after damage through disease or trauma, are incapable of regenerating and reestablishing functional circuitry. As a consequence, injury to the visual system, like damage elsewhere to the adult CNS, results in permanent functional impairment. Given the debilitating effects of CNS axon injury, substantial effort has been undertaken to identify the reasons for the failure of axon regeneration in the injured brain. Studies show that adult CNS neurons, including RGCs, retain the ability to regrow, but appear to be prevented in doing so by inhibitory proteins in their environment. Identified inhibitory proteins include CNS myelin proteins such as Nogo (reviewed in [53]) and myelin associated glycoprotein (MAG) [54], as well as extracellular matrix molecules like Tenascin [55]. Much like inhibitory axon guidance molecules, several of these proteins trigger growth cone collapse. This observation suggests that results from studies of the molecular basis of axonal pathfinding during development may to some degree be applicable towards regeneration therapies in the clinical setting. Further weight is lent to this argument by the recent findings that axon guidance molecules important during development re-appear in the setting of CNS injury. For instance, the inhibitory axon guidance molecule Sema3A is found in scar tissues

following spinal cord injury, and is hypothesized to play a role in preventing CNS axon regeneration across the site of injury ([56, 57] reviewed in [58]). In the visual system, members of the Ephrin family which mediate the development of the retinocollicular map continue to be expressed in the adult superior colliculus after optic tract injury [59]. This finding raises the prospect that sufficient molecular information may be present in the adult to reconstitute a functional retinal map in CNS targets. An implication of these findings is that it would be useful to examine whether the failure of adult retinal axon regeneration is in part due to the re-expression of inhibitory axon guidance molecules during the injury response. Important questions to be addressed include the cellular origin of these inhibitory molecules, whether these inhibitory molecules affect adult retinal axons, and whether strategies effective in changing embryonic retinal axon responses to specific guidance molecules will also work in adult axons. To answer these questions, a systematic characterization of the pathfinding ability of adult regenerating retinal axons will be required. With this information in hand, it may be possible in the future to formulate regeneration strategies based on an understanding of adult retinal axon pathfinding at the molecular level.

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Figure and table legends:

Table 1 Heading:"Partial Listing of Molecules Contributing to RGC Axon Guidance"

Figure 1 The growth cone. Extracellular guidance molecules bind surface receptors on the growth cone. In turn, these receptors activate signaling cascades that ultimately influence growth cone cytoskeletal components controlling morphology and motility. Signaling cascades are known to affect the actin cytoskeleton. It is not clear whether there are also direct influences on growth cone microtubule assembly during axon guidance.

Figure 2 Axon pathfinding to the optic disc. (A) A crystal of DiI placed in the periphery of a flat-mounted retina labels RGC axons projecting towards the optic disc (dashed circle). (B) In a wild-type retina, RGC axons travel directly to the optic disc and exit the retina. (C) In EphB2, EphB3 double null mutant retinas, axon fascicles split off from the main group, fail to exit at the optic disc, and grow aberrantly into the opposite side of the retina. (D) In Netrin deficient retinas, many axons at the optic disc splay out into surrounding retina instead of passing through the disc and into the optic nerve. All scale bars are 100 mm.

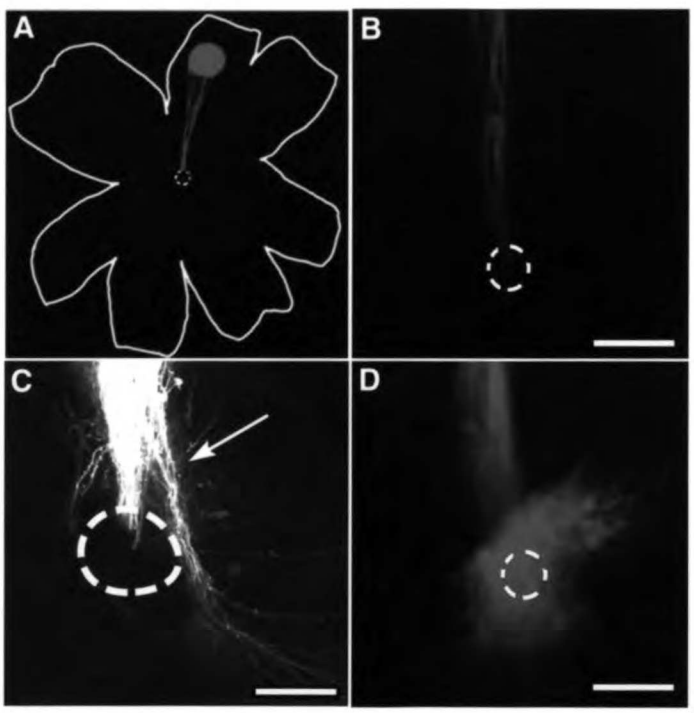
Figure 3 Mechanisms of RGC axon guidance in the vertebrate visual system. In the retina, axons are repelled from the periphery by chondroitin sulfate. En route to the optic disc, RGC axons fasciculate due to L1 expression and an Eph inhibitory gradient. At the optic disc, RGC axons exit into the optic nerve using a mechanism based on Netrin

mediated attraction. Within the optic nerves, RGC axons are kept within the pathway possibly by inhibitory Slit protein. Slits also contribute to positioning the optic chiasm by creating zones of inhibition. Finally with the superior colliculus, RGC axons map to specific target positions by responding to gradients of inhibitory Ephrin molecules. Axons from temporal retina, which express high levels of Eph receptors, map to anterior colliculus, which express low levels of the inhibitory Ephrin ligand.

[Permissions have been requested to reprint figure 2 panels A, B, D from Deiner et al 1997 (reference number 12) and figure 2 panel C from Birgbauer et al 2001 (reference 39)]

Guidance Family	Mode of Action	Reported Effect on RGC Axons	Location in Developing Visual System	Remarks	Known Receptors	Selected References
Netrins	Secreted	Attractive	Optic Disc Optic Nerve	Homology to Laminin	DCC, UNC5	12
Semaphorins	Secreted and Membrane Bound	Inhibitory?	Retina?	Large Family (>20 Members) Semaphorin Domain	Neuropilins Plexins	28, 29
Slits	Secreted	Inhibitory	Retina Optic Nerve Optic Chiasm Optic Tract	Form Boundaries for RGC Axons	Robos	20, 21, 22
Ephrins	Secreted & Membrane Bound	Inhibitory	Retina Superior Colliculus	Two subclasses: Ephrin-A and Ephrin-B Form gradients in retina and tectum	EphA Receptors EphB Receptors	34, 35
EphB Proteins	Membrane Bound	Inhibitory	Retina	"Reverse" Signalling Through Ephrins	Ephrin B Proteins	39, 40
L1	Membrane Bound	Attractive	Expressed on RGC Axons	Ig Superfamily member Maintains RGC Axon Fasiculation	Homophilic Binding	7, 8, 60
Laminin	Extracellular Matrix	Growth Promoting	Retina	Commonly used as Substrate for RGC axon growth In Vitro	Integrins	61, 62
Tenascin	Extracellular Matrix	Inhibitory	Optic Nerve	Expression Begins After Embryogenesis	Unknown	55
Chondroitin Sulfate	Extracellular Matrix	Inhibitory	Retina	Inhibitory Ring in Outer Retina	Unknown	13, 14

Guidance molecule
Guidance molecule
Actin
Signalling Molecules
Microtubules


A
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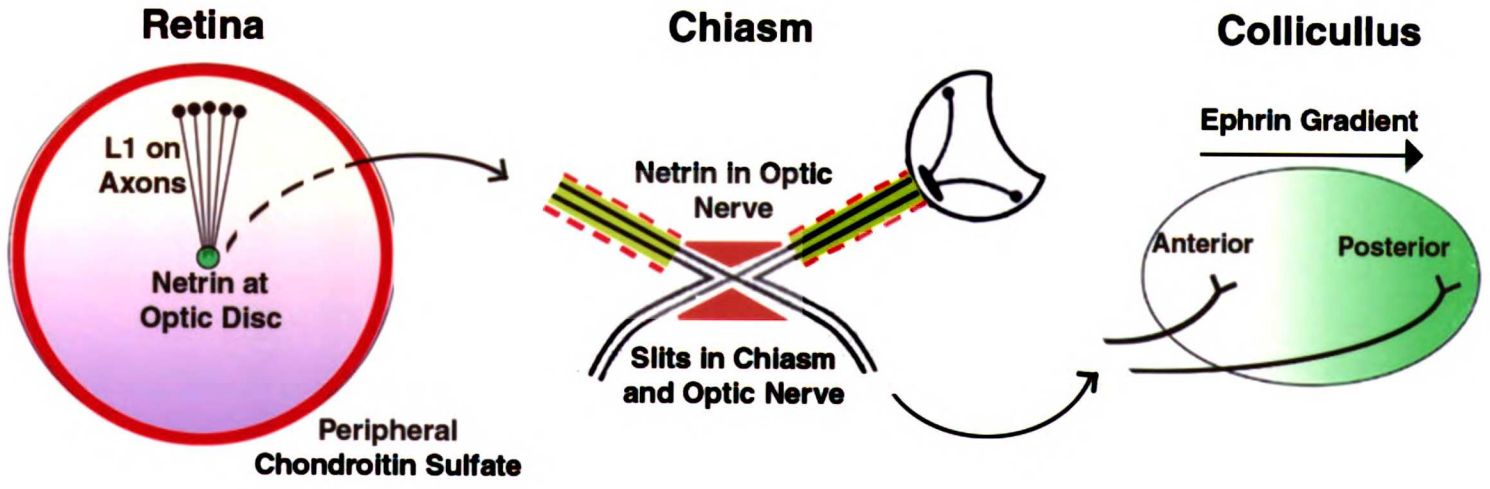
Retina
L1 on
Axons
Netrin at
Optic Disc
Peripheral
Chondroitin Sulfate
Chiasm
Netrin in Optic
Nerve
Slits in Chiasm
and Optic Nerve
Colliculus
Ephrin Gradient
Anterior
Posterior

Chapter 2

Invariant Sema5A Inhibition Serves An Ensheathing Function During Optic Nerve Development

Prologue

The studies in this chapter represent our characterization of Sema5A function in the developing visual pathway. They cover gene expression, Sema5A protein localization, in vitro effects of Sema5A on retinal axons, and finally in vivo studies using antibody mediated perturbation of Sema5A function.

F. He performed the work shown in Table 1. M. Bodeker performed many of the in situ hybridizations seen in Figure 1, and he was fundamental to making the polyclonal antibody described. D. Sretavan supervised this entire project, and contributed experimentally to the studies shown in Figure 6. This chapter has been submitted to the journal *Development*.

Invariant Sema5A Inhibition Serves An Ensheathing Function During Optic Nerve Development

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ABSTRACT

Retinal axon pathfinding from the retina into the optic nerve involves the axon guidance molecules L1, laminin, and Netrin-1, each of which governs axon behavior at specific regions along the retinal pathway. In identifying additional molecules regulating this process, we found that transmembrane Semaphorin5A mRNA and protein was specifically expressed in neuroepithelial cells surrounding retinal axons of the embryonic mouse optic disc and along the optic nerve. Given that growth cone responses to a specific guidance molecule can be altered by co-exposure to a second guidance cue, we examined whether retinal axon responses to Sema5A were modulated by other guidance signals axons encountered along the retinal pathway. In growth cone collapse and neurite outgrowth assays, Sema5A triggered an invariant inhibitory response in the context of L1, laminin, or Netrin-1 signaling, suggesting that Sema5A likely inhibited retinal axons throughout their course at the optic disc and nerve. Antibody-perturbation studies in living embryo preparations showed that blockade of Sema5A function led to retinal axons straying out of the optic nerve bundle, indicating that Sema5A normally helped ensheath the retinal pathway. Thus, CNS nerve development appears to require inhibitory sheaths to maintain integrity. Furthermore, this function is accomplished using molecules such as Sema5A that exhibit conserved inhibitory responses in the presence of co-impinging signals from multiple families of guidance molecules.

Introduction

Proper wiring of axons to correct targets is essential for development of the nervous system. This process is orchestrated by axon guidance molecules that act either as attractants and support growth, or as inhibitory signals to steer axons away from incorrect pathways and targets. The semaphorins are the largest family of inhibitory axon guidance molecules, consisting of seven distinct classes, and are defined by a characteristic 500 amino acid semaphorin domain near the N-terminus. Invertebrate semaphorins include classes I and II, while vertebrate semaphorins consist of classes III-VII and encompass over 15 members (Raper 2000). Previous work in vertebrates has focused on Class III semaphorins. These secreted guidance molecules contribute to development of cranial nerves, cortical architecture, and dorsal root ganglia sensory axon pathways (Messersmith et al. 1995; Taniguchi et al. 1997; Polleux et al. 1998). However, the majority of vertebrate semaphorins, such as class IV, V, and VI, are transmembrane molecules, and their function in vivo is not yet well understood.

While semaphorins are best known for their inhibitory activity during development, a number of reports have provided evidence for the opposite function. Studies in grasshopper have shown that transmembrane Sema-1a functions as an attractive guidance molecule in different regions of the limb bud (Wong et al. 1999). In the mammalian cortex, Sema3A has been reported to inhibit cortical axon growth but attract dendritic growth from the same cell population (Polleux et al. 2000). Similarly,

growth cones can switch their response to a given guidance molecule from attraction to repulsion, and vice versa, depending on signaling from a second guidance molecule (Hopker et al. 1999; Song and Poo 1999). In vertebrates, L1, a guidance molecule of the Ig superfamily, has been reported to switch growth cone response to secreted Sema3A from inhibition to attraction (Castellani et al. 2000). It is not clear whether a similar type of response switching occurs for transmembrane semaphorins. If so, axon responses to transmembrane semaphorins might continually change along a neural pathway as growth cones sequentially encounter signals from a variety of axon guidance molecules.

Amongst the vertebrate semaphorins, Sema5A and Sema5B have an unusual pairing of protein domains. The extracellular domain of these two semaphorins contains seven thrombospondin (TSP) type-1 repeats in addition to the sema domain (Adams et al. 1996). Given that thrombospondin type-1 repeats have been reported to promote neurite outgrowth and neuronal adhesion (Adams and Tucker 2000), the pairing of a typically inhibitory sema domain with TSP repeats poses an intriguing question of class V semaphorin function during neuronal development.

In the visual system, the optic nerve consists of the axons of retinal ganglion cells (RGCs), and conveys retinal information to CNS targets. During embryonic development, the optic nerve is formed as RGC axons extend from their cell bodies centrally towards the optic disc, pass through the optic disc, and travel down the optic stalk towards the brain. Some of the axon guidance molecules contributing to this process have been identified. These include L1, which is expressed by retinal axons, and is

involved in axon fasiculation in the retina (Brittis et al. 1995). L1 is also highly expressed by retinal axons traversing the optic nerve, chiasm, and optic tract, suggesting that a large number of retinal axon pathfinding tasks operate in the context of L1 signaling. A second retinal axon guidance molecule is Netrin-1, which is expressed by the group of neuroepithelial cells that form a collar surrounding the optic disc. Netrin-1 is essential for promoting retinal axon growth through the disc into the optic nerve (Deiner et al. 1997). In approximately the same region, retinal axons are also thought to interact with laminin-1, which cooperates with Netrin-1 to help steer retinal axons into the optic nerve (Hopker et al. 1999). Given that L1, Netrin-1, and laminin-1 are all involved in retinal axon exit at the optic disc leading to optic nerve formation, these three guidance molecules can potentially modulate how retinal growth cones respond to additional guidance cues in the retinal pathway.

To identify additional guidance molecules mediating optic nerve development, we examined expression patterns of known guidance molecules including semaphorins. Of eight semaphorins studied, we found that Sema5A was specifically expressed at the optic disc, and along the optic nerve, in a pattern suggestive of a role in retinal axon pathfinding. Functional assays revealed that Sema5A consistently triggered an inhibitory response in embryonic retinal growth cones when tested in the presence of signaling from other relevant guidance molecules. Antibody mediated disruption of Sema5A function resulted in retinal axon defasiculation, and axons straying away from the optic nerve. Our results indicated that transmembrane Sema5A acts as a continually present inhibitory

sheath encasing the retinal pathway as RGC axons carry out multiple pathfinding tasks involving different sets of axon guidance molecules.

Materials and Methods

Recombinant proteins and cell lines

Three recombinant Sema5A-Fc fusion proteins were produced. 1) full-length Sema5A extracellular domain (ECD-Fc), 2) Sema5A semaphorin domain (Sema-Fc), and 3) the seven thrombospondin repeats of Sema5A (TSP-Fc). cDNAs encoding each region were cloned into mammalian expression vectors pEx.Fc (Exelixis) or pSectag (Invitrogen), in frame with the human IgG-Fcγ domain. HEK 293 cell lines secreting each recombinant protein were grown in Optimem (Gibco) for 5 days, and the supernatants harvested. Fc-tagged proteins were isolated by protein-A chromatography (Amersham Pharmacia), and protein concentrations determined by coommassie blue staining and comparison with bovine serum albumin (BSA) standards. Protein purity was verified using silver staining. Fc control protein was prepared as described previously (Birgbauer et al. 2001).

The cDNA encoding the extracellular domain of human L1 (aa 1-1120) was cloned into pEX.Fc, and recombinant L1-Fc protein was isolated and characterized as described above for Sema5A proteins.

Full length Sema5A cDNA was cloned into the pEX-myc vector, and a stably transfected HEK 293 cell line was isolated. Immunoblots using membrane preparations from transfected cells showed a single band of the expected size (140kD) by anti-myc staining, and by staining using an anti-Sema5A antibody (see below).

In situ hybridization

E12-E18 wild-type (C57/B6) mouse embryo brains were fixed in 4% paraformaldehyde for 60 minutes, and 10um cryostat sections cut for in situ hybridization as previously described (Birgbauer et al. 2000). Digoxigen-labeled sense and anti-sense RNA probes for Sema3A, 3C, 3E, Sema4A, 4C, Sema5A, 5B, and Sema6A were 250-400 bases in length, and taken from the 3' end of these genes. An additional Sema5A in situ probe (bp 201-2065) and Sema5B probe (bp 337-2497) were also used. These probes spanned the Sema-domains and TSP repeats of both molecules.

Embryonic tissue culture

Retinal tissue was obtained from E14 mouse embryos harvested from anesthetized timed pregnant C57/B6 mice (detection of vaginal plug was counted as day 0). Laminin (Gibco) was coated onto polylysine covered dishes at 5 ug/ml for collapse assays, and at 2.5 ug/ml for Netrin-1 dependent neurite outgrowth assays (see below). To use L1 as a substratum, polylysine covered dishes were coated with anti-human Fc antibody (5

ug/ml, Jackson Immunochemicals). Anti-human Fc coated dishes were then blocked with 0.25% BSA, and L1-Fc (5 ug/ml) was applied. All coatings were performed at 37°C for 2 hours. L1-Fc coated dishes were PBS washed three times, used for explant cultures. Explants were maintained at 37°C, 5% CO2 in F12 medium and N2 supplement (F12/N2 medium) (Gibco).

Retina-optic nerve preparations

The retinal eye cup, and a length of optic nerve were removed from E14 mouse embryos. Three quarters of the retina was cut away, leaving a retinal wedge and the attached optic nerve stump (see Fig. 4A). Retina-optic nerve preparations were cultured overnight on L1 coated dishes, and held in place by a flap of fine wire mesh (#203025-A, Small Parts Inc.) attached to the culture dish by Sylgard (Dow Chemical).

Growth cone collapse assays

Collapse assays were performed in 8-well coverglass chamberslides (Fisher), ECD-Fc, Sema-Fc, TSR-Fc, or Fc were diluted in F12/N2 medium, and added to wells containing retinal explants at final concentrations of 2.5 nM-100 nM. Anti-human Fc antibody (Jackson Immunochemical) was added at 7.5 ug/ml to multimerize the Fc-tagged recombinant proteins. After exposure to reagents at 37°C in 5% CO_2 for 30 minutes, retinal explants were fixed with 4% paraformaldehyde. Growth cones were

stained using Texas-Red Phalloidin (Molecular Probes), and scored for collapsed or expanded morphology.

Time-lapse microscopy

35 mm coverslip dishes (MakTek) containing retinal explants or retina-nerve preparations were overlayed with pre-warmed mineral oil (Sigma) and maintained at 37°C on a microscope stage incubator with CO_2 influx. Time-lapse images of growth cones were captured at 1 minute intervals with a CCD camera (PXL2, Photometrics) using Hoffman optics and Deltavision image acquisition software (API). Baseline growth was recorded for 20-45 minutes before reagent application. Recombinant proteins and anti-Fc antibody were diluted to a volume of 100 uL, and then added by gel-loading pipette to cultures underneath the mineral oil to achieve a recombinant protein concentration of 12.5 nM. Following reagent application, growth cone behavior was recorded for an additional 45-80 minutes.

Neurite outgrowth assays

Supernatant from Netrin-1 expressing HEK 293 cells was concentrated using centriprep-10 concentrator columns (Amicon). Netrin-1 concentration was determined by coommassie blue staining and comparison with BSA standards. In Netrin-1 dependent outgrowth assays, laminin was used at 2.5 ug/ml (threshold for reliable

outgrowth was 4 ug/ml) and Netrin-1 (100 ng/ml) was added to the culture medium. Anti-DCC monoclonal antibody (Oncogene) was used at 1:150 dilution.

Co-culture assays of retinal explants with either Sema5A-myc 293 cells or parental 293 cells were performed in laminin-coated 35 mm coverslip dishes (MakTek). 293 cells were grown to either 20% or 40% confluence. Retinal explants obtained from GFP-transgenic E14 mouse embryos (Jackson Lab) were cultured overnight on top of the laminin and 293 cells in F12/N2 medium with Netrin-1 supplementation. Total neurite outgrowth was determined after fixation in 4% paraformaldehyde and tracing the fluorescent neurites on a video monitor. After conversion to digital images, total neurite length was quantified using Adobe Photoshop.

Antibody production and characterization

A rabbit polyclonal antibody was raised against bacterially expressed Sema5A without the thrombospondin-type 1 repeats (aa 1-544 fused with 939-974). Coding sequence was inserted into pTrcHis 2B (Invitrogen), in frame with a C-terminal myc epitope and a poly-histidine (His_6) tag. This recombinant protein was produced in E. coli (strain BL21(DE3)pLysS) after IPTG induction for three hours at 30°C. Bacteria were then lysed, and recombinant protein affinity purified on a NiNTA agarose column (Pharmacia). Antigen was injected into rabbits by Covance Inc. (Berkeley). Anti-Sema5A rabbit immune and pre-immune serum were precipitated using ammonium sulfate, and dialyzed against F12 medium. Fab fragments were made using papain

digestion, and separated from Fc fragments and uncut antibodies by protein A chromatography (Pierce).

Anti-Sema5A was used at 1:400 in Western blots to demonstrate immunoreactivity against recombinant ECD-Fc. Live 293 cells expressing Sema5A-myc were stained for 1 hour at 37°C with anti-Sema5A (1:100) in culture medium. After 3 washes with pre-warmed culture medium, they were fixed in 4% paraformaldehyde, and visualized using a Cy3 donkey anti-rabbit antibody (Jackson Immunochemicals).

Retinal immunostaining

Live E14 retinas were isolated along with a short segment of optic nerve, and incubated with anti-Sema5A antibody (1:100) in F12/N2 medium for 2-16 hours at 37°C. After three washes with warm culture medium, retinas were incubated Cy2 donkey anti-rabbit antibody (1:500) Immunochemicals) for another 2-16 hours. Retinas were then washed three times, fixed in 4% paraformaldehyde for 1 minute, and whole-mounted. Control retinas were processed in the same manner using pre-immune serum. The pattern of immunoreactivity was analyzed using a confocal microscope (Pascal LSM, Zeiss).

Sema5A function blocking experiments

For optic nerve function blocking experiments, live tissue preparations containing the retinas, optic nerves, and the optic chiasm were dissected from E14 embryos as

described (Sretavan and Reichardt 1993). Preparations were cultured for 8-10 hours at 37°C in the presence of either anti-Sema5A or pre-immune serum (1:100) in F12/N2 medium. Tissue preparations were then transferred to a second culture dish containing 2 mls of antbody-free F12/N2 medium, cultured overnight, and fixed with 4% paraformaldehyde for at least 24 hours. Retinas were removed, and a DiI crystal placed at the optic disc using a glass micropipette. After overnight incubation in 4% paraformaldehyde at 37°C, optic nerves were examined using rhodamine optics on a fluorescent microscope (Microphot-SA, Nikon) or a confocal microscope (Pascal LSM, Zeiss).

For intra-retinal function blocking experiments, gestational day 14 pregnant mice were anesthetized with xylazine/ketamine mixture, and a mid-abdominal incision made to expose the uterine sacs. A small incision was made through the uterine wall to reveal one eye of an embryo. Micropipettes loaded with Fab fragments (1 μg/ml in F12 medium) were used for intraocular injections, and an estimated 2-4 uL of antibody was delivered into each eye. Up to four embryos were injected in each animal. Uterine incisions were closed with 8-0 sutures, and the abdominal wall and overlying skin closed with 4-0 sutures. After a further 24 hours of in utero development, injected embryos were harvested and fixed in 4% paraformaldehyde for 24 hours. A DiI crystal was placed in the peripheral retina of treated eyes, and after 6-8 hours at 37°C, retinas were wholemounted, and visualized using standard fluorescence optics.

Results

Sema5A mRNA expression in the developing retinal pathway

To better understand the molecular basis of retinal ganglion cell axon guidance and optic nerve development, we carried out in situ hybridization analysis to identify axon guidance molecules expressed in the developing retinal pathway. Included were probes corresponding to eight different members of the semaphorin gene family, covering both secreted and transmembrane semaphorins (Table 1).

A number of semaphorins were found in the developing mouse retina during the period of active RGC neurogenesis and axon outgrowth through the optic disc into the optic nerve (Table 1). The mRNAs corresponding to Sema3A, Sema3C, Sema3E, and Sema6A were found in the retinal ganglion cell layer. However, only mRNA corresponding to Sema5A, a class V transmembrane semaphorin, was detected at the developing optic disc and optic nerve. Sema5A mRNA was expressed by the optic disc neuroepithelial cells that as a group surrounded the RGC axons exiting from the retina (Fig. 1A,B). Previous work has shown that this population of neuroepithelial cells expresses Netrin-1, which plays a major role in guiding axons into the optic nerve (Deiner et al. 1997). Sema5A mRNA was also found in neuroepithelial cells that line the embryonic optic stalk connecting the retina to the ventral diencephalon (Fig. 1A,E). Expression was however absent at the midline region of the optic chiasm. Sema5A mRNA was present at the optic disc and optic stalk from E12-E16, during the period of

active retinal axon growth through these areas, but was absent from these regions by E18 (data not shown). Sema5A mRNA expression was also observed in the lamina propria under the olfactory epithelium (Fig. 1G). Of note, mRNA for Sema5B, the other known vertebrate Class V semaphorin, was not detected in the developing retinal pathway (Fig. 1D). The pattern of Sema5A mRNA expression placed this molecule in regions traversed by embryonic retinal axons, and suggested a role in retinal axon guidance.

The presence of Sema5A mRNA in the optic nerve and at the optic disc, a major exit point for retinal axons, was at odds with the fact that vertebrate semaphorins are generally considered inhibitory axon guidance molecules. Given that growth cone responses have been reported to switch from inhibition to attraction in the context of signaling from a second guidance molecule (Hopker et al. 1999; Castellani et al. 2000), Sema5A function in the context of retinal pathway development needed to be determined. Since axon pathfinding through the optic disc into the optic nerve is known to involve L1, Netrin-1, and laminin-1, we tested retinal axon responsiveness to Sema5A in the context of signaling from each of these molecules.

Sema5A collapses retinal growth cones on laminin

A secreted recombinant Sema5A protein was generated consisting of the extracellular portion of Sema5A, encompassing both its semaphorin and thrombospondin domains, fused to the human IgG Fc domain (ECD-Fc) (Fig. 2A,B). Addition of 12.5 nM ECD-Fc, oligomerized with 7.5 ug/ml anti-Fc antibody, resulted in the collapse of 68% of

RGC growth cones cultured on laminin within 30 minutes (Fig. 2C). Increases in ECD-Fc concentration, or duration of exposure, did not further increase the response rate (Fig. 2C and data not shown). Oligomerization was required for maximal response, and was used in all further assays. In control experiments, axons treated with oligomerized human Fc protein responded at a rate of 12% ($n=89$, $p<0.001$) (Fig. 2C).

The ability of Sema5A to inhibit retinal axons when expressed as a transmembrane molecule on cell surfaces was also tested. To do so, we measured RGC axon outgrowth in the presence of stably transfected cells expressing full-length Sema5A. Retinal explants placed on top of untransfected 293 cells growing at 20% confluence on a laminin substratum resulted in a mean total neurite length of 5.3 mm per explant ($n=25$ explants). In the presence of Sema5A-myc transfected cells at the same confluence, the mean total length was reduced by 30% to 3.7 mm per explant ($n=25$ explants, $p<0.001$) (Fig. 2D,E,F). An increase in the density of 293 cells seeded onto the laminin-coated dishes to 40% confluence resulted in a mean total neurite length of 2.1 mm per explant for untransfected cells ($n=9$ explants), while Sema5A-myc transfected cells resulted in a mean total neurite length of only 1.3 mm per explant ($n=9$ explants, $p=0.011$) (Fig. 2F). Thus, the results indicated that retinal axons extending on laminin were inhibited by Sema5A presented either as a soluble protein fragment in collapse assays, or as a cell surface protein in neurite outgrowth studies.

Sema5A inhibition is maintained on a L1 substratum

The extracellular fragment of human L1 was used as a substratum for retinal axons in Sema5A growth cone collapse assays. After a 30 minute exposure to 12.5 nM ECD-Fc, 74% of growth cones extending on L1 exhibited a collapsed morphology (n=148). By comparison, Fc treatment alone resulted in the collapse of only 5% of retinal growth cones (n=178, $p<0.001$) (Fig. 3B). Time lapse microscopy confirmed that ECD-Fc treated growth cones extending on L1 (n=30) underwent a loss of filopodia and lamellopdial structures characteristic of growth cone collapse. Of note, unlike typical descriptions of growth cone collapse in which there is quick contraction from an open to a collapsed morphology, growth cones extending on L1 displayed an intermediate stage resulting from the shrinkage of the growth cone into a branched structure (Fig. 3A). This branched structure subsequently progressed to the fully collapsed, stick-like morphology.

Activities of the semaphorin and TSP domains

Class V semaphorins are unique in that they contain both a sema domain and seven thrombospondin type-1 repeats in their extracellular domain. To investigate the possible function of each sub-domain, recombinant proteins consisting of the semaphorin domain alone fused to an Fc tag (Sema-Fc), or the thrombospondin repeats fused to an an Fc tag (TSP-Fc), were purified (Fig. 1A,B) and used in collapse assays. The ability of the sema domain alone to trigger growth cone collapse appeared to be reduced compared to

the intact extracellular domain. 12.5 nM Sema-Fc led to only 13% growth cone collapse (n=93), compared to the 74% collapse response observed when 12.5 nM of the entire extracellular fragment was used (Fig. 3C). An increase of Sema-Fc to 100 nM resulted in a 62% collapse rate (n=103), suggesting that the sema domain alone was approximately 8-10 fold less potent in inhibitory potential (Fig. 3C).

Application of TSP-Fc up to 100 nM resulted in a collapse rate of only12% (n=146), which was not different to the 10% response rate seen after application of recombinant Fc control protein (n=52) (Fig. 3C). TSP-Fc was also tested as a growth substratum at concentrations up to 20 mg/ml, but failed to support axon outgrowth from embryonic retinal explants. This data indicated that the inhibitory activity of transmembrane Sema5A laid with its sema domain, as has been described for secreted Sema3A (Koppel et al. 1997). Moreover, the full inhibitory potential required activity supplied by other regions in the extracellular domain.

Sema5A function in the presence of Netrin-1

The mRNA expression pattern for Sema5A was remarkably similar to that of Netrin-1. To determine whether exposure to Netrin-1 modified retinal growth cone responses to Sema5A, we carried out Sema5A collapse assays in the presence of soluble Netrin-1, using retinal axons extending on either L1 or laminin.

Retinal explants were cultured overnight on an L1 substratum in medium containing 100 ng/ml Netrin-1, a concentration previously shown to effectively promote RGC axon outgrowth in collagen gels (Deiner et al. 1997). ECD-Fc was then added to a final concentration of 12.5 nM for 30 minutes, and the resulting growth cone morphology was analyzed. Sema5A in the presence of Netrin-1 resulted in collapse of 74% of RGC growth cones (n=252) similar to the response rate of 74% following ECD-Fc treatment without Netrin-1 in the culture medium (Fig. 3D). Addition of Fc alone in the presence of Netrin-1 resulted in the collapse of 11% of growth cones (n=116, $p<0.001$) (Fig. 3D).

In a second set of experiments, we examined the effects of ECD-Fc on retinal neurites that were more directly dependent on Netrin-1 for outgrowth. Retinal explants were cultured on a dish coated with 2.5 ug/ml laminin, a sub-optimal level for reliable axon outgrowth. However, the addition of 100 ng/ml Netrin-1 to these cultures elicited reproducible retinal axon outgrowth. This Netrin-1 dependence was demonstrated by the fact that addition of an antibody blocking the function of the Netrin receptor DCC attenuated this increased neurite outgrowth (Fig. 3E). In this Netrin-1 dependent outgrowth assay, ECD-Fc collapsed 70% of RGC growth cones (n=135) compared to only 24% in Fc treated controls (n=77, $p<0.001$)(Fig. 3F). Together, this data indicated that Netrin-1 was not capable of modulating the inhibitory effects of Sema5A on RGC axons. Thus, Sema5A appeared to maintain its inhibitory function as RGC axons encountered the reitnal pathfinding molecules L1, laminin, or Netrin-1.

Sema5A inhibition during navigation in the optic nerve

One feature of Sema5A mRNA expression was its presence along the entire course of the developing optic nerve. Given that exposure to guidance molecules at specific pathfinding regions can silence receptors and eliminate the ability of a growth cone to respond to a different guidance molecule (Zou et al. 2000; Stein and Tessier-Lavigne 2001), we investigated whether RGC axons after passing through the optic disc were altered in their responsiveness to Sema5A.

Retina-optic nerve preparations consisting of a portion of retina connected to the optic nerve were cultured on L1 (Fig. 4A). Retinal axons that had grown through the optic disc region readily extended from the cut end of the optic nerve (Fig. 4B) and their responsiveness to Sema5A was recorded using time-lapse microscopy. Axons growing out of the optic nerve were sensitive to Sema5A, with 57% of growth cones collapsing in response to ECD-Fc (n=30), compared to only 12% of Fc treated grwoth cones (n=24, p=0.002) (Fig. 4C). By timelapse analysis, there was no significant difference in the response rates between ECD-Fc treated axons growing from retinal explants or retina-optic nerve preparations (60% versus 57%) (Fig. 4C). These results suggested that post-optic disc, retinal axons remained sensitive to Sema5A during their course along the optic nerve.

Localization of Sema5A protein in retinal pathway

The results thus far indicated that Sema5A inhibited RGC axons, and that this inhibition was maintained despite co-impinging signals from several relevant guidance molecules. To understand how this inhibition contributed to retinal pathway development, we examined Sema5A protein localization using a polyclonal antibody. This antibody recognized recombinant ECD-Fc on western blots, and a single band of the appropriate size for Sema5A (135 kD) from embryonic retinal membrane preparations (Fig. 5A). Furthermore, the antibody also resulted in membrane staining of living cells transfected with Sema5A-myc, but not of untransfected cells (Fig. 5B and data not shown).

Retinal immunostaining using this anti-Sema5A antibody required the use of unfixed tissues. In E14 mouse retinas, immunoreactivity was detected as a ring at the perimeter of the optic disc, encircling RGC axons passing through the optic disc (Fig. 5C,D). Immunostaining was absent from the central region of the optic disc that contained retinal axons and the neuroepithelial cell processes known to display Netrin-1 protein on their surfaces (Deiner et al. 1997). This pattern of protein localization, together with the inhibitory activity of Sema5A on retinal axons, raised the possibility that Sema5A prevented RGC axons from straying away from the optic disc and the optic nerve.

Perturbation of Sema5A function results in axon guidance errors

To test the role of Sema5A in development of the retinal pathway, anti-Sema5A antibody, or Fab preparations, were applied intraocularly to embryos in utero and to living embryonic tissue preparations containing the optic nerve. In control experiments, anti-Sema5A antibody (1:100) was effective in blocking Sema5A mediated growth cone collapse in vitro. In the presence of anti-Sema5A antibody, growth cone collapse rates after ECD-Fc exposure dropped from 74% (n=148) (Fig. 3A) to 32% (n=121, $P<0.001$) (Fig. 6A). Pre-immune serum did not curb Sema5A mediated growth cone collapse, and resulted in a 70% response rate (n=95) (Fig. 6A).

Horizontal slice preparations of embryonic mouse brains, including the retinas and the optic nerves, were cultured in either anti-Sema5A antibody (1:100) or pre-immune serum (1:100) for 8-10 hours, and then grown overnight. In anti-Sema5A treated optic nerves (n=26), 35% exhibited pathfinding errors compared with no occurrence of errors among optic nerves incubated in pre-immune serum (n=22, p=0.007)(Fig. 6B). The guidance errors ranged from mild cases in which a small number of axons were observed straying from the main optic nerve bundle (Fig. 6D) to more severely affected cases in which fascicles of axons were observed veering off from their normal path (Fig. 6E,F).

Anti-Sema5A Fab fragments injected into eyes of E14 mouse embryos in utero led to retinal axon guidance errors in 4 of 18 treated retinas. The phenotypes were mild, and consisted of stray axons leaving the optic disc region and projecting aberrantly for short distances within the retina itself (Fig. 6G,H). In sum, disruption of Sema5A function resulted in axon straying from the main retinal pathway, and this effect was more prominent in the optic nerve than at the optic disc.

Discussion

In the present study, we found that while a number of semaphorins were expressed within the embryonic mouse retina, only Sema5A was specifically expressed at the optic disc and along the optic nerve during active retinal axon outgrowth. In growth cone collapse and neurite outgrowth assays, Sema5A triggered inhibitory responses in embryonic retinal axons. Furthermore, this inhibition was maintained when exposure to Sema5A was systematically paired with signaling mediated by L1, Netrin-1, or laminin; three guidance molecules that govern axon pathfinding in the region of the optic disc and optic nerve. This suggested that retinal axons continually responded to Sema5A inhibition as they progressed through the developing retinal pathway. Immunostaining in live tissues using an anti-Sema5A antibody confirmed mRNA in situ hybridization results, and showed that Sema5A protein was present as a ring surrounding retinal axons in the visual pathway. Antibody perturbation studies resulted in retinal axons straying

from the optic disc and optic nerve defasciculation, supporting the notion that Sema5A acts as an inhibitory sheath ensuring proper development of the optic nerve.

Sema 5A inhibition and L1, laminin, Netrin-1 signaling

A major pathfinding task for retinal axons is exiting the retina by growing through the optic disc into the optic nerve. This guidance event is mediated by Netrin-1 (Deiner et al. 1997), and occurs in the presence of L1 (Deiner et al. 1997), and laminin (Hopker et al. 1999). Given that Sema5A was also present at the optic disc and nerve, we examined how combinations of L1, Netrin-1, and laminin affected the ability of retinal axons to respond to Sema5A. The results showed that Sema5A clearly inhibited retinal growth cones in the presence of all guidance molecule combinations tested including laminin or L1 alone, and pairings of Netrin-1 with L1 or laminin. Thus, retinal growth cones interpreted Sema5A as an inhibitory molecule while receiving co-impinging signals from members of multiple families of axon guidance molecules.

Neurite outgrowth in the presence of laminin and Netrin-1

The assay testing retinal axon responses to Sema5A under the influence of both Netrin-1 and laminin involved culturing retinal explants on a laminin level that by itself was too low to produce reliable axon outgrowth, but which resulted in consistent neurite extension after the addition of soluble Netrin-1. The dependence of this assay on both laminin and Netrin-1 was demonstrated by the fact that Netrin-1 supplementation alone

without the laminin substratum supported no retinal axon outgrowth, and the outgrowth induced by the laminin/Netrin-1 combination was decreased by an antibody against the Netrin receptor DCC (Fig. 2E). The ability of this laminin/Netrin-1 combination to augment neurite outgrowth was somewhat unexpected given a previous study reporting that in the presence of laminin-1, Xenopus retinal growth cone response to Netrin-1 was switched from attraction to repulsion (Hopker et al. 1999). One explanation for this difference is the micropipette delivery of a Netrin-1 point source to Xenopus axons, while Netrin-1 was bath applied in the present study and did not provide neurites with a Netrin-1 gradient to respond to. A second possibility is that the substratum laminin level in the present study by itself did not reliably support retinal axon growth in vitro. In contrast, Xenopus growth cones were first grown on effective concentrations of laminin-1, and then tested with Netrin-1 gradients. If this second explanation is correct, then whether switching occurs in vivo may depend critically on the precise concentration of particular axon guidance molecules to specific growth cones.

Effect of L1 on responses to Sema5A

Several aspects of Sema5A action on retinal axons such as effective concentration, localization of inhibition to the sema domain, and increased effectiveness after oligomerization, were consistent with previous studies of semaphorin function (Koppel et al. 1997; Koppel and Raper 1998; Xu et al. 2000). One difference, however, was the regulation of responses to semaphorins by the Ig superfamily guidance molecule L1. Cortical axons, like retinal axons, express abundant L1 on their surfaces in vivo, and

likely carry out numerous pathfinding tasks in the presence of L1 signaling. In a study in which cortical axons were exposed to Sema3A in the presence of L1-Fc, growth cones did not collapse, but instead maintained an open morphology (Castellani et al. 2000), suggesting that growth cones responding to L1 signaling were freed of Sema3A mediated inhibition. This was not the case for Sema5A, as retinal growth cones responded to Sema5A inhibition in the presence of L1. Furthermore, concentrations of Sema5A required to trigger growth cone collapse were similar for retinal axons grown on laminin or L1. These results raise the possibility that the growth cone signaling cascades triggered by Sema3A and Sema5A are different, and cross-talk with L1 signaling exists in the case of Sema3A but not Sema5A.

Function of Thrombospondin type-1 repeats

A unique aspect of class V semaphorins is the seven thrombospondin type-1 repeats in their extracellular domains. While TSP repeats in other molecules have been shown to promote axon growth and to mediate neural attachment (Adams and Tucker 2000), the present results showed that TSP repeats combined with a semaphorin domain clearly resulted in a molecule with overall inhibitory activity for retinal neurite outgrowth. In addition, the TSP repeat domain by itself did not trigger growth cone collapse, nor did it support retinal axon outgrowth. Given that the intact Sema5A extracellular domain, which included the TSP region, was a more potent inhibitor of retinal axons than the sema domain alone, it is possible that TSP repeats contribute to ligand-receptor binding, a known function for the non-sema domains of other

semaphorins (Feiner et al. 1997). However, given that TSP repeats have also been implicated in protein binding (Adams and Tucker 2000), an additional possibility is that Sema5A associates with other proteins through the TSP repeats and these protein complexes represent an as yet unrecognized aspect of Sema5A function.

Localization of Sema5A

In situ hybridization and immunostaining showed that Sema5A was expressed by the group of optic disc neuroepithelial cells that surround the retinal axon bundle exiting the retina. These same cells are known to extend Netrin-1 bearing processes radially towards the center of the optic disc (Deiner et al. 1997). Although expressed by the same cell population, Sema5A protein was localized mostly to the cell bodies of the neuroepithelial cells arrayed at the periphery of the optic disc, and Sema5A protein was not detected on the Netrin-1 bearing processes. This suggested that Sema5A may be specifically excluded from neuroepithelial cell processes, possibly through anchoring of transmembrane Sema5A via its cytoplasmic domain.

An inhibitory sheath for optic nerve development

Blockade of Sema5A function resulted in retinal axon defasciculation from the main axon bundle of the optic nerve. Although some cases showed sizable retinal axon bundles leaving the optic nerve, other preparations had only a few stray axons, and in many anti-Sema5A treated nerves, no errors were noted. This variation in occurrence

and severity of pathfinding errors could be due to the use of a function-blocking antibody over a relatively short time period; a necessary limitation to maintain the health of tissue preparations. A second possibility is the presence of other inhibitory molecules such as Slit proteins in the developing optic nerve (Niclou et al. 2000) serving a similar ensheathing function.

In principle, axon defasciculation from the optic nerve could be interpreted as a loss of growth promoting/fasciculation activity rather than a decrease of axon inhibition. However, given the in vitro evidence that Sema5A inhibited retinal axon outgrowth and caused growth cone collapse, combined with the evidence that Sema5A mRNA and protein were found surrounding the optic nerve bundle, we favor the model that Sema5A served as an inhibitory sheath. Furthermore, since new retinal growth cones entering the optic nerve generally extend along the external surface of the axon bundle (Reese et al. 1991; Williams et al. 1991), a Sema5A sheath places this inhibitory molecule in a direct position to contain the growth of newly arriving axons.

Maintaining Inhibition during Pathfinding

Previous studies have shown that receptor signaling cascades activated by guidance molecules can interact and modulate growth cone responses. These interactions set up a logic system in which a growth cone's response to multiple cues presented simultaneously is greater than just the sum of the individual guidance forces, and creates increased functionality given a limited repertoire of guidance molecules.

During pathfinding however, there are instances when axons grow over long distances, and it may be useful to maintain a constant responsiveness to a single guidance molecule while accomplishing sequential pathfinding tasks involving various others. In such cases, the nervous system could utilize molecules such as Sema5A that appear to trigger a conserved response in growth cones in the face of co-impinging signals from multiple families of guidance molecules.

Acknowledgements

We would like to thank Y. Luo and L. Suh for providing reagents. S.F.O. was supported by the National Institutes of Health Medical Scientists Training Program and a That Man May See Research Award. This research was supported by the NIH and the That Man May See Foundation.

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Figure Legends:

Figure 1: Expression of Sema5A mRNA. (A) Sema5A in situ hybridization in the E14 mouse retinal pathway. Sema5A was expressed at the optic disc and along the developing optic nerve to the ventral diencephalon. The boxed areas are shown at higher magnification in panels B and E. (B) Sema5A mRNA expression at the optic disc. Hybridization signal using antisense probe was present in neuroepithelial cells that flank the exiting retinal axons (*). As a group, these Sema5A positive neuroepithelial cells formed a collar around the retinal axon bundle. (C) Hybridization at the optic disc using the Sema5A sense probe. (D)Hybridization at the optic disc using the Sema5B probe showed no signal. (E) Sema5A mRNA expression in the developing optic nerve. Note the presence of hybridization signal in neuroepithelial cells flanking the central core containing retinal axons. (F) Hybridization in the optic nerve using the Sema5A sense probe. (G) Sema5A mRNA expression in the lamina propria (LP) under the olfactory epithelium (OE). Scale bars, (A) 150 μm; (B-F) 25 μm; (G) 75 μm.

Figure 2: Recombinant Sema5A proteins inhibit RGC axons on laminin substratum. (A) Domain structure of Sema5A, and diagrams of three Sema5A extracellular domain constructs. (B) Silver staining of the three Sema5A recombinant proteins (ECD-Fc, sema-Fc, TSP-Fc). (C) The percent of growth cones on laminin substratum exhibiting collapse with increasing amounts of oligomerized ECD-Fc. (D, E) Composite images showing the pattern and density of axon outgrowth for 25 explants grown on a laminin substratum in the presence of Sema5A-myc expressing 293 cells (D), or parental 293

cells (E), at 20% confluence. (F) Mean total neurite outgrowth per explant on a laminin substratum in the presence of HEK 293 cells transfected with full length Sema5A-Myc or parental cells. At both 20% and 40% confluence, explants grown in the presence of cells transfected with Sema5A-Myc exhibited less neurite outgrowth. Scale bars, (D,E) 250 μm.

Figure 3: Growth cone responses to Sema5A in the presence of L1 and Netrin-1. (A) Timelapse sequence showing a retinal growth cone extending on an L1 substratum and exhibiting collapse in response to application of oligomerized ECD-Fc. (ECD-Fc was applied at t=0). Numbers at top left refer to minutes elapsed. During collapse, axons on L1 tended to show an intermediate stage characterized by the shrinkage of the growth cone into a branched structure (t=20). Growth cones eventually progressed to a fully collapsed morphology (t=40). (B) The percent of growth cones on L1 substratum exhibiting collapse with increasing amounts of oligomerized ECD-Fc. (C) The percent of growth cones on L1 substratum exhibiting collapse with increasing amounts of oligomerized sema-Fc or TSP-Fc. (D) The percent of growth cones extending on laminin in the presence of Netrin-1 responding to oligomerized ECD-Fc. The presence of Netrin-1 did not alter the ability of ECD-Fc to mediate growth cone collapse. (E) Assay of Netrin-1 dependent outgrowth. Sub-optimal levels of laminin resulted in little axon outgrowth (column 1). 100 ng/ml of Netrin-1 by itself was not able to support outgrowth (column 2). The combination of sub-optimal laminin levels and 100 ng/ml Netrin-1 resulted in robust outgrowth (column 3). This Netrin-1 dependent outgrowth was reduced by the addition of antibody against the Netrin receptor DCC (column 4). (F)

Axons dependent on Netrin-1 for outgrowth remained responsive to ECD-Fc mediated growth cone collapse. Scale bar, (A) 10 μm.

Figure 4: Sema5A inhibition of post-optic disc retinal axons. (A) Diagram of the Retina-Optic Nerve preparation. The boxed region from a tissue preparation is shown in B. (B) Retinal axons and growth cones extending from the cut optic nerve of a Retina-Optic Nerve preparation and stained with Texas-Red Phalloidin. (C) The response rate of growth cones from Retina-Optic Nerve preparations compared to standard retinal explants. Growth cone responses to oligomerized ECD-Fc were observed with time-lapse microscopy. Scale bar, (B) 50 μm.

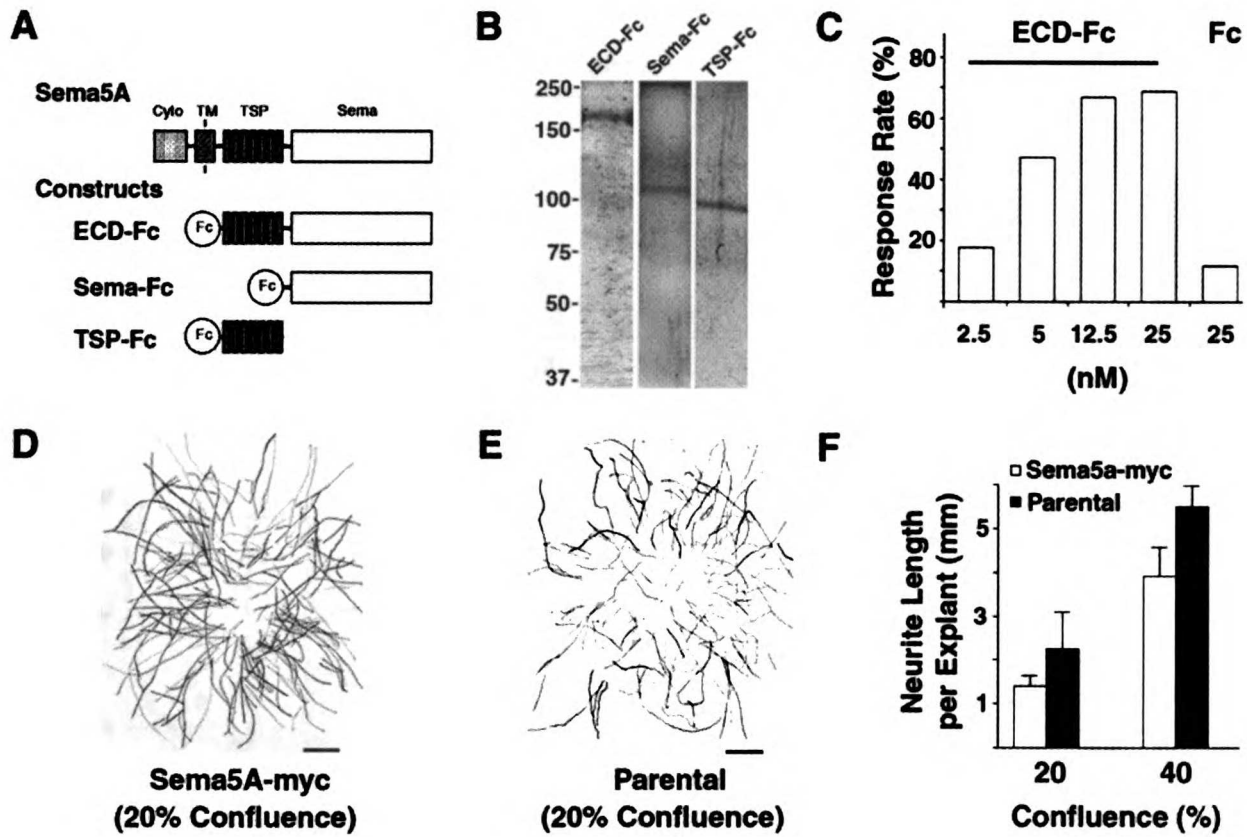
Figure 5: Anti-Sema5A antibody characterization and Sema5A localization at the optic disc. (A) In immunoblots, the Sema5A antibody recognized ECD-Fc protein (lane 1), Sema5A-myc from transfected 293 cells (lane 2), and yielded a band of the expected size for Sema5A (135kd) from E14 retina (lane 3). (B) Anti-Sema5A staining of live Sema5A-myc expressing 293 cells. Top row shows Sema5A-myc expressing cells stained with Anti-Sema5A, and the bottom row shows Sema5A-myc cells similarly treated with pre-immune serum. (C) Brightfield image of the optic disc. Arrowheads point to retinal pigment epithelium cells, and (*) marks the center of the optic disc. (D) Same optic disc as in (C). Sema5A immunoreactivity was localized to the peripheral rim of the optic disc, and (*) marks the center of the optic disc. Image compiled from optical sections through a depth of 25 μm at the optic disc. Scale bars (B) 25 μm; (C-D) 50 μm.

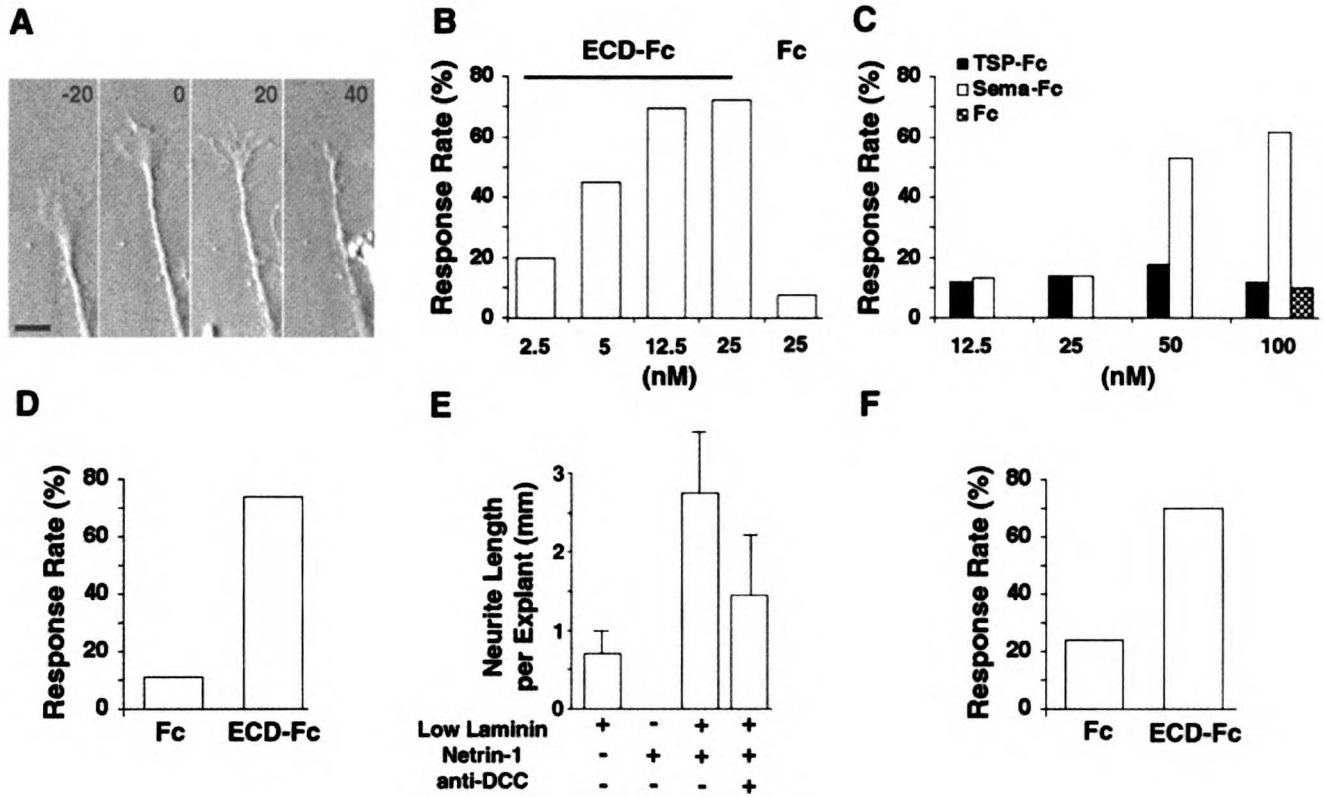
Figure 6: Sema5A function blockade and RGC axon pathfinding errors. (A) ECD-Fc mediated retinal growth cone collapse in the presence of anti-Sema5A antibody or pre-immune serum. (B) The incidence of axon guidance errors in optic nerves treated with anti-Sema5A antibody. (C) A normal, DiI labeled E14 optic nerve showing retinal axons contained within the retinal pathway. The retina and optic disc are towards the left. (D-F) Examples of axon guidance errors in anti-Sema5A treated optic nerves. The retina is towards the left. (D) A mild phenotype consisting of a pair of short axons tipped with growth cones (arrowheads) straying from the optic nerve bundle. (E) A more severe error showing a bundle of axons (arrowhead) veering away from the main portion of the optic nerve. Of note, some aberrantly projecting axons grew in parallel to the optic nerve (arrow). (F) A large fascicle of axons near the optic disc (arrowhead) splitting away from the main optic nerve bundle, and terminating in a knot-like structure. (G and H) Examples of stray RGC axons within the retina (arrowheads). Unlike normal axons, stray axons failed to grow completely through the optic disc, and extended inappropriately within the retina. Scale bars, (C-H) 50 μm.

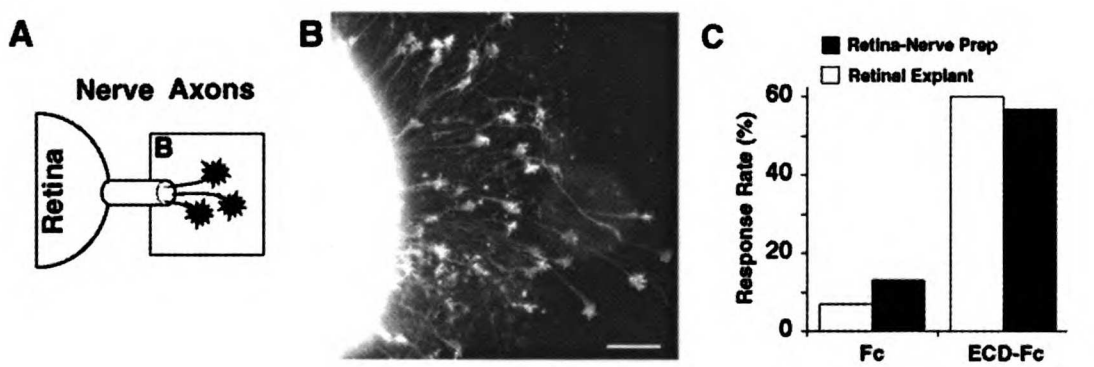
Table 1: Semaphorin mRNA Expression in E13-E15 Mouse Retina and Optic Nerve

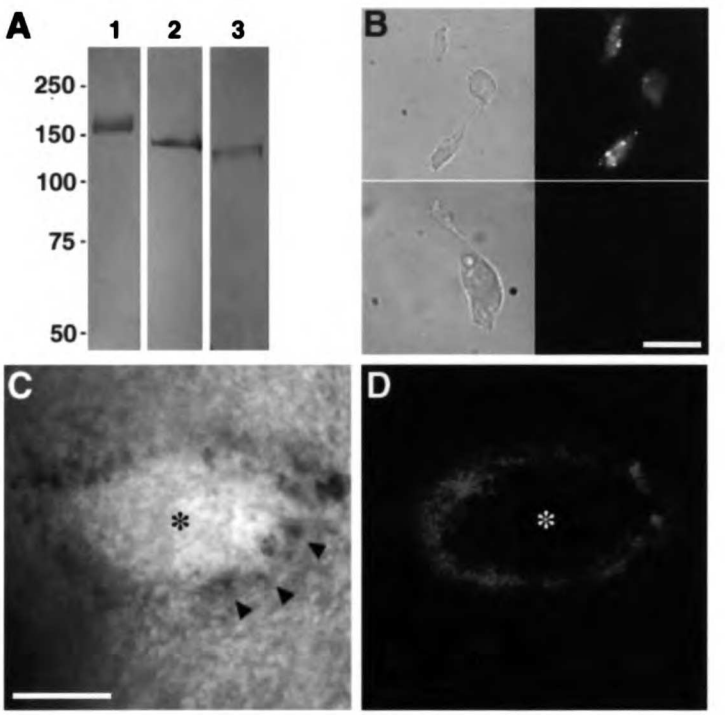
Semaphorin	RGCs	Optic Disc/Nerve
Secreted:		
Sema3A	++	-
Sema3C	++	-
Sema3E	++	-
Transmembrane:		
Sema4A	+	-
Sema4C	-	-
Sema5A	-	+++
Sema5B	+	-
Sema6A	+++	-

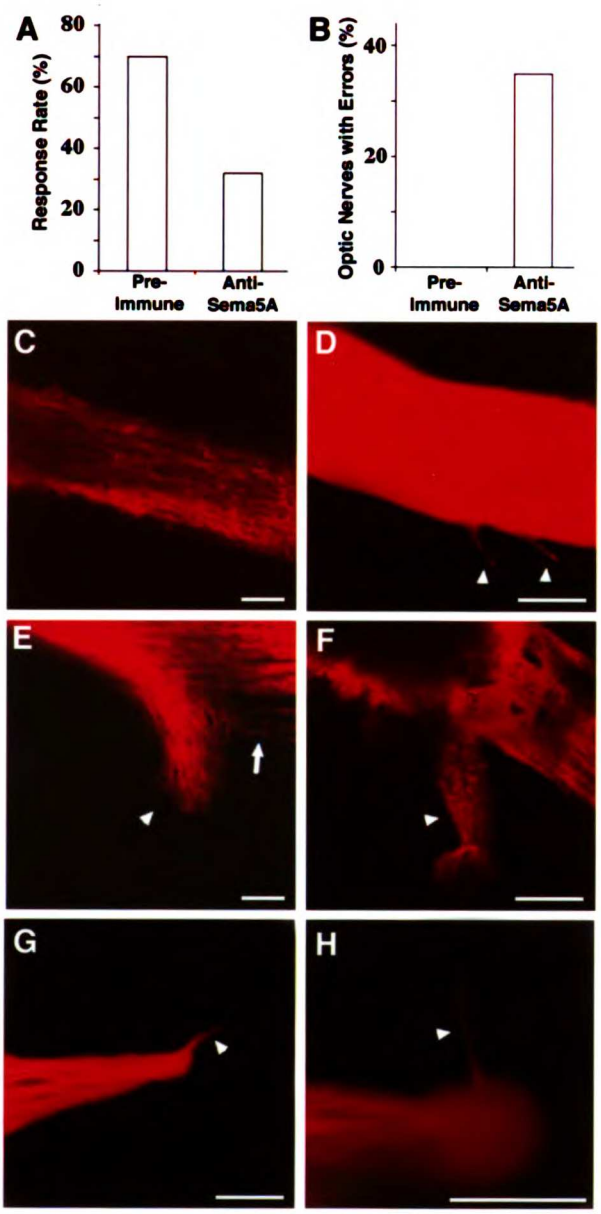
A
B
E
B
E
F
C
G
D
LP
OE


A
Sema5A
Cyto
TM
TSP
Sema
Constructs
ECD-Fc
Fc
Sema-Fc
TSP-Fc
B
ECD-Fc
Sema-Fc
TSP-Fc
250
150
100
75
50
37
C
ECD-Fc
Fc
Response Rate (%)
80
60
40
20
2.5
5
12.5
25
25
(nM)
D
Sema5A-myc
(20% Confluence)
E
Parental
(20% Confluence)
F
Sema5a-myc
Parental
Neurite Length
per Explant (mm)
5
3
1
20
40
Confluence (%)


A
-20
0
20
40
B
ECD-Fc
Fc
Response Rate (%)
0
20
40
60
80
2.5
5
12.5
25
25
(nM)
C
TSP-Fc
Sema-Fc
Fc
Response Rate (%)
12.5
25
50
100
(nM)
D
Response Rate (%)
Fc
ECD-Fc
E
Neurite Length per Explant (mm)
0
1
2
3
Low Laminin + - + +
Netrin-1 - + + +
anti-DCC - - - +
F
Response Rate (%)
Fc
ECD-Fc


A
Nerve Axons
Retina
B
B
C
Retina-Nerve Prep
Retinal Explant
Response Rate (%)
60
40
20
0
Fc
ECD-Fc


A
1
2
3
250
150
100
75
50
B
C
D


A
Response Rate (%)
80
60
40
20
0
Pre-Immune
Anti-Sema5A
B
Optic Nerves with Errors (%)
40
20
0
Pre-Immune
Anti-Sema5A
C
D
E
F
G
H

Chapter 3

Discussion

Discussion

The original goal of these studies was to identify additional molecules contributing to retinal pathfinding at the optic disc, and to investigate how they operate in combination with guidance molecules known to direct RGC axons in the region. Ultimately, the purpose of such experiments is to better understand how the many molecular cues contributing to a pathfinding decision cooperate to control axonal growth and neural development. The optic disc and optic nerve are an ideal system to study such a complicated process. RGC pathfinding decisions and axon routes are well described, there is a good fund of knowledge about retinal guidance, and the RGC axon is amenable to in vitro culture as well as in vivo manipulation. By taking advantage of this system, we gained some insight into vertebrate transmembrane semaphorin function, and into how the nervous system uses guidance molecules to build nerves.

Transmembrane semaphorins in vertebrates are a poorly understood group of molecules. There is evidence that Sema6A inhibits sympathetic axons, and that high concentrations of a class IV semaphorin collapse RGC axons (Encinas et al., 1999; Xu et al., 2000). Similarly, our results indicate that Sema5A inhibits retinal axons, however, our study also places Sema5A inhibition in a functionally relevant context. We showed that Sema5A surrounds the retinal axon bundle, and ensheaths the developing optic nerve to prevent defasciculation.

There have been previous reports relating semaphorins to cranial nerve fasciculation. Mice deficient for the secreted Sema3A exhibit defasciculation and

widening of the trigeminal, facial, glossopharyngeal, and vagus nerves (Taniguchi et al., 1997). However, Sema3A expression in these mice did not follow along these cranial nerves, but instead appeared more widely expressed in areas surrounding the nerve trajectories. This may reflect an interesting difference in mechanism of action between secreted and transmembrane semaphorins. It would not make sense to ensheath a nerve with a secreted semaphorin, as the inhibitor would diffuse into the growing nerve bundle and halt axon growth. However, transmembrane semaphorins can be anchored to cells lining the perimeter of the developing nerve, and not interfere with proper axon extension. This is the pattern of protein localization we saw for Sema5A in the optic nerve, and reflects an advantage of stationary guidance molecules.

Finally, our results provide some insight into how guidance molecules are used to link individual axon decisions together into roadmap for targeting. A long pathfinding journey, like the one undertaken by retinal axons, can be broken down into a series of discrete tasks such as exiting through the optic disc, travelling the optic nerve, and choosing to cross the midline at the optic chiasm. Often these individual decisions are controlled by a set of guidance cues whose interactions are tailored to the task at hand. For example, a combination of Netrin-1 activity and proper laminin-1 substrate positioning is necessary to drive axons into the optic disc (Hopker et al., 1999), and a complicated series of events involving Netrins, Slits, and their respective receptors control spinal commissural axon crossing at the ventral midline (Stein and Tessier-Lavigne, 2001). Furthermore, in each of these cases, the extending axon's response to the guidance cues presented at the choice point changes as the growth cone integrates

multiple signals, and this change is part of driving axons past the decision point and onto the next leg of the journey. However, Sema5A function shows that the nervous system also deploys guidance molecules to control an axon population over a longer series of events than a single choice point. Furthermore, cues such as Sema5A can affect axons in a consistent manner despite co-impinging signaling from the molecules directing the individual tasks at hand. This use of invariant guidance information is an interesting technique for linking a series of discreet choice points into a more complete set of pathfinding information.

Future Directions

There are a number of unresolved issues surrounding Sema5A function, and a number of possible directions for future efforts. One question remaining is the function of Sema5A's thrombospondin repeats. Their ability to potentiate the collapsing abilities of Sema5A's extracellular domain, and the known capacity of similar repeats to mediate protein-protein interactions (Adams and Tucker, 2000), strongly suggest that the repeats are involved in Sema5A receptor binding. This raises another interesting question, namely what the Sema5A receptor expressed by RGC axons is. There are a number of known semaphorin receptors. These are split into two families, the neuropilins and the plexins (Yu and Kolodkin, 1999), but none have been reported to bind to Sema5A. Furthermore, Neuropilin-1 and Neuropilin-2 deficient mice show no optic nerve phenotype (Chen et al., 2000; Giger et al., 2000; Kitsukawa et al., 1997), suggesting that these molecules are not the Sema5A receptor. Neuropilin-1 was identified through

expression cloning (He and Tessier-Lavigne, 1997), and a similar strategy might reveal the Sema5A receptor.

Despite our efforts using antibody-mediated Sema5A function blockade, the majority of treated retinas and optic nerves displayed no pathfinding errors. Furthermore, the retinal errors seen were mild, especially compared to the obvious optic disc phenotypes resulting from Netrin-1 loss of function (Deiner et al., 1997). These observations lead to two interesting experimental possibilities. The first is to create mice deficient for Sema5A, and upon examination of their optic discs and optic nerves we would expect a more serious lost axon and defasciculation phenotype. The second series of experiments is to combine Sema5A loss of function (antibody blockade or a genetic loss of function) with deletions for other axon guidance molecules acting on RGCs at relevant places in the visual pathway. For example, Slit-2 is expressed along the developing optic nerve, and is known to inhibit retinal axons in vitro (Niclou et al., 2000). These results suggest that, like Sema5A, it might also contribute to maintaining optic nerve fasciculation. Antibody blockade of Sema5A function in a Slit-2 deficient mouse might lead to far greater defasciculation along the optic nerve than either manipulation alone, and would demonstrate how these guidance molecules cooperate in vivo. Similar experiments using other guidance cues relevant to retinal pathfinding could also reveal how Sema5A function complements these molecules.

Finally, it would be interesting to examine Sema5A expression patterns in the adult visual pathway before and after optic nerve injury. One goal of studying embryonic

pathfinding is to understand the molecules controlling axon growth so that this knowledge might ultimately be applied to axon re-growth. Class III semaphorins are well described in the developing mouse spinal cord, and mounting evidence indicates that these axon growth inhibitors are re-expressed in the spinal cord after injury (Pasterkamp and Verhaagen, 2001). Possibly a similar process occurs along the optic nerve, and Sema5A re-expression after optic nerve injury could contribute to the difficulty of adult retinal axon re-growth in these circumstances.

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