Title
Molecular studies of axonal chemorepulsion

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Publication Date
1997

Peer reviewed|Thesis/dissertation

Molecular Studies of Axonal Chemorepulsion

by

Eduardo David Leonardo

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Cell Biology

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

To my family, past, present, and future.

Acknowledgments

Graduate school has been a long journey, often over difficult and varied terrain. Now I find myself at an intermediate target, ready to alter course and embark on a new direction. The journey has been made easier, more exciting, and more fun by wonderful colleagues, friends, and family whose support has been invaluable.

I first want to thank my advisor Marc Tessier-Lavigne for his example, guidance, and support. His tireless dedication, the clarity and precision of his scientific thinking, and his remarkable ability to recognize fundamental issues in the most ordinary appearing results, are inspirational.

I also want to thank my thesis committee; Cori Bargmann, Bill Mobley, and Lou Reichardt; for their encouragement, support and insight over the years. With each meeting they helped me to alter course just enough to pursue my project most effectively.

Over the last five years, the lab has been a second home where I have worked, debated, played, and grown with an incredible group of people. For the past three years, I have worked closely with Lindsay Hinck, initially expression cloning for netrin receptors, later working with the UNC-5 homologues in the seemingly endless search for their function. Our collaboration has been a contrast in personality and style from which we learned to strike a balance between my cautious apprehension and her optimistic energy. I have really valued Lindsay as a partner with whom to share every detail of this project, the struggles, the disappointments (of which there have been many) and the successes.

I am greatly indebted to many wonderful people in the lab, especially: Chen-Ming Fan for his unique and effective style of imparting lessons in technical wizardry with a dose of humility; Tim Kennedy for his late night advice and comic relief; Esther Stoeckli whose unbounded enthusiasm for science is truly marvelous; José de la Torre for his advice on food, wine and music, his active participation in any debate, for keeping up my Spanish, and for his friendship; Christine Mirzayan for her appreciation of good music, for

always being there when there is an issue to be discussed, and her infectious smile and energy; Michael Galko for teaching me to dissect, always providing an opposing viewpoint, and for his example of dedication; Ellen Kuwana for all the early sequencing in this project, her organization, many great dinners and her friendship; Kevin Wang for his energy in the final phase of the UNC5H4 project; Katja Brose for her invaluable editing skills; Jen Zallen, an MTL lab wanna-be, for her critical assessment of my work and her unique perspective on life. Thank you to everyone on the fourteenth floor: this community has been an incredible source of support, late-night company, scientific perspectives, and random thoughts on the world.

During the past five years I have had the opportunity to work on several wonderful collaborative projects. I especially appreciated the opportunity to work with Alex Kolodkin and Liz Messersmith, with whom I toiled on the semaphorin project presented in chapter two.

Throughout this graduate school process, I have been sustained by the friends I have made here. Hundreds, perhaps thousands, of hours of intense discussion with Adrian Erlebacher have been instrumental in honing my critical thinking skills. Luke Hoffman has been a friend since before the beginning. He has kept me going with bike rides ("only 15 minutes..."), his consistently positive outlook, and his unfailing friendship. To my friends and classmates, thank you.

Finally, I wanted to give my thanks to my parents for instilling the confidence to pursue this career and for their love and support for all these years, and to my wife, Elizabeth, who has made each day of this journey special and who, I am thankful, will continue on with me.

ADVISOR'S STATEMENT

Regarding previous publications and material with multiple authors.

Chapter 2 has been published (Messersmith, E. K. et al (1995) Neuron *14*, 949-59), and is reproduced with permission from Cell Press (see page vii). The in situ hybridization studies in that paper were performed by Elizabeth Messersmith. All of the in vitro studies were performed as a complete collaboration between David, Elizabeth, and Alex Kolodkin, with the three authors making similar contributions.

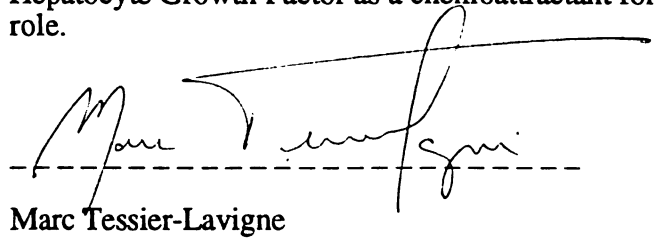
Chapter 3 has been published (Leonardo et al. (1997) Nature *386*, 833-8). It is reproduced with permission from Nature (see page viii). David worked collaboratively with Lindsay Hinck and Masayuki Masu to isolate cDNAs encoding two vertebrate homologues of UNC-5. He collaborated with Lindsay Hinck in characterizing the sites of expression of the mRNAs for these two proteins, as well as the binding properties of the proteins and of a third UNC-5 homologue cloned by Susan Ackerman. This work was a collaboration between David and Linsday, who both participated in all the experiments, with David focusing more on the binding studies and Lindsay more on the expression studies.

Chapter 4 is unpublished. David cloned cDNAs encoding UNC5H4 and performed the binding studies. The in situ hybridization studies were performed by Lindsay Hinck and Kevin Wang.

Chapter 5 is unpublished. David performed all of the work reported in this chapter (the two-hybrid screen).

Appendix II has been previously published (Serafini, T. et al. (1996) Cell *87*, 1001-14). It is reproduced with permission from Cell Press (see page ix). David's contribution to this study was the cloning of the mouse netrin-1 cDNA, which was then used by the other authors in the analysis of netrin-1 function in vivo.

Appendix III has been previously published (Ebens et al. (1996) Neuron *17*, 1157-72). It is reproduced with permission from Cell Press (see page viii). The study reported in that paper was initiated by David, who sought to identify the cellular interactions between motor axons and surrounding tissues, and discovered an attactant effect of sclerotome on these axons. The other authors built on this discovery, identifying Hepatocyte Growth Factor as a chemoattractant for motor axons, and defining its in vivo role.



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Messersmith, E.K., Leonardo, E.D., Shatz, C.J., Tessier-Lavigne, M., Goodman, C.S., and Kolodkin, A.L. (1995) Semaphorin III can function as a selective chemorepellent to pattern sensory projections in the spinal cord. Neuron, 14(5):949-59.

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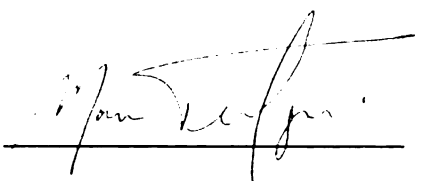
Molecular Studies of Axonal Chemorepulsion

by

E. David Leonardo



Marc Tessier-Lavigne

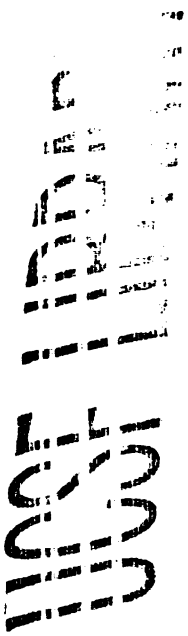
Chair, Thesis Committee

Abstract

The ability of neurons to establish connections with their targets is critical to the development and proper functioning of the nervous system. In order to achieve this, neurons extend axons, tipped by growth cones, to navigate through the environment in search of cues that will direct them to their appropriate targets. This process of directed outgrowth to a target, called axon guidance, results in remarkably stereotyped trajectories, from which few axons deviate. The specificity and accuracy of this process is due, in part, to the use of multiple distinct cues in combination. These cues can be target or non-target derived, positive or negative, and function at long range or short range. The work presented in this thesis focuses on several aspects of the mechanisms of action of long range negative cues, chemorepellents.

In chapter two, in an in vitro approach, we use a three dimensional collagen gel assay to demonstrate that sema III can act selectively as a chemorepellent for specific subpopulations of sensory neurons. We demonstrate that the effect is truly chemorepulsion and not simply inhibition, as axons can extend parallel to sema III gradients but will not grow up them. Chapters three and four describe the use a candidate gene approach to identifying netrin-1 receptors. Netrin-1 is a bifunctional molecule that can act both as a chemoattractant and a chemorepellent for distinct populations of neurons. We

describe the identification of a family of vertebrate UNC-5 proteins, demonstrate that members of the family can bind netrin, and that they are candidates for mediating repellent functions of netrin. Finally, chapter five describes the use of a yeast two-hybrid strategy to identify candidate molecules that interact with UNC-5 family proteins and are candidates for mediating their intracellular signals. Taken together, these studies provide some of the first investigations of the biological function of chemorepellents, the receptors for chemorepellents, and the intracellular messengers that ultimately effect the changes in the cytoskeleton that mediate chemorepulsive responses.



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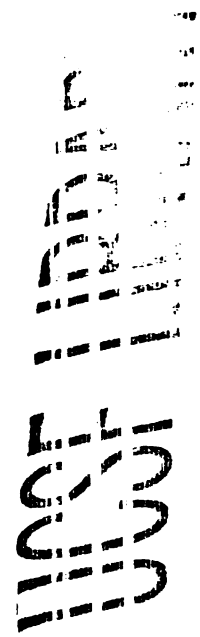
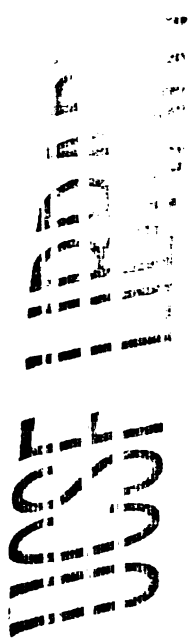


Chapter 1

Introduction

Axon Guidance

The function of the nervous system depends critically on its ability to wire itself properly during the course of development. The establishment of connections occurs in two phases, an initial activity independent phase, which guides axons to the vicinity of their targets, and a second activity dependent phase, in which fine tuning and refinement of contacts between axons and targets occurs (Reviewed in Goodman and Shatz, 1993; Shatz and Katz, 1996). This discussion will focus on the first phase of initial outgrowth towards the target, a process called axon guidance. This process is remarkably accurate, with the vast majority of axons following stereotyped trajectories to their appropriate destination without error.



Axons are guided to their targets by the growth cone, a specialized motile sensory apparatus, located at their distal end. The growth cone is a dynamic structure that senses cues present in the environment. It is now known that these cues can be of two types, attractive (or permissive) and repellent (or inhibitory). Additionally, these cues can be local (substrate bound) or long range (diffusible). While these categories provide a useful conceptual framework, clear distinctions between categories at the molecular level may be difficult to make (reviewed in Goodman, 1996; Tessier-Lavigne and Goodman 1996). For example, the netrins, a family of guidance cues, can function as diffusible chemoattractants but can also repel certain classes of axons, presumably due to the different cell surface receptors expressed on the axons (reviewed in Hedgecock et al., 1997). In addition, although netrins can act at a distance as diffusible cues, they also interact with components of the extracellular matrix (Serafini et al., 1994) and in some contexts appear to act as local cues (Diner et al., 1997). Similarly, the semaphorins, a family of repellent guidance cues, comprise both secreted members which can act at a distance and transmembrane members which act locally (reviewed in Kolodkin, 1997). It is therefore possible that, at least in some instances, the distinction between permissive/attractive cues, as well as the distinction between inhibitory/repellent cues,

may be a contextual difference rather than a mechanistic one. This thesis will focus on the role of chemorepulsion as a mechanism of axon guidance, the molecular nature of these repulsive cues, and the possible intracellular mechanisms by which they may effect their response.

Chemorepulsion

Support for the idea that the growth cone guidance is influenced by negative cues is relatively recent. Early examples of these cues suggested that guidance mechanisms act locally as they required contact between the growing axon and "inhibitor tissue." For example, it was discovered in chicks that sympathetic axons of the peripheral nervous system avoid retinal axons of the central nervous system and vice versa (Kapfhammer et al., 1986; Kapfhammer and Raper, 1987a). These encounters result in the retraction of lamellopodia and filopodia in a process known as growth cone collapse. Numerous examples of contact mediated collapse have since been described (Kapfhammer & Raper 1987b; Raper & Kapfhammer, 1990; Cox et al., 1990; Raper & Grunewald, 1990; Bandtlow et al., 1990). Another early avoidance assay, the membrane stripe assay, required neurons to choose between membrane carpets made from different tissues, revealing activities that made axons avoid membranes that were otherwise permissive (Walter et al., 1987). These assays were performed on two dimensional substrates and required direct contact between the growing axon and inhibitory cells or axons.

The breakthrough for long range repulsion was the use of three dimensional collagen gels which stabilize molecular gradients. These gels were used successfully to develop chemotropic assays and have played a critical role in the demonstration of chemorepulsion. Initially, the idea that axons could be repelled at a distance was met with skepticism. An early study by Ebendal first hinted at chemorepulsion (Ebendal, 1982), however, it wasn't until 1993 with a study by Adrian Pini that the concept of chemorepulsion was established. He was able to demonstrate that embryonic axons from

the developing rat olfactory bulb are specifically repelled by the septum (Pini, 1993). Reports of other chemorepulsive events followed, and the first chemorepellent molecules were identified (Fitzgerald et al., 1993; Messersmith et al, 1995; Colamarino, 1995, Guthrie and Pini, 1995; Tamada et al., 1995). The principle emerged that non-target tissue in the vicinity of axonal pathways could establish exclusion zones for axons by secreting diffusible repellent molecules. Target tissue (attractive cues) and non-target tissue (repellent cues) gained equal stature as providers of long range guidance cues.

Molecular basis of chemorepulsion

The molecular nature of some of these chemorepellent cues is now known, with much of the evidence centering around two families of guidance molecules, the netrins and the semaphorins (reviewed in Varela-Echavarria and Guthrie, 1997). The netrins were identified due to their ability to promote outgrowth of commissural axons from rat embryonic dorsal spinal cord explants into collagen gels (Serafini et al., 1994). Cloning of netrin-1 led to the remarkable discovery that the netrins are vertebrate homologues of the *C. elegans* protein UNC-6, as they share 50% sequence identity (Serafini et al., 1994). It was immediately apparent that the conservation of molecular structure extends to function, as both netrin-1 in vertebrates, and UNC-6 in worms, guide circumferential migrations of axons that extend in a ventral direction, presumably due to chemoattraction (Serafini et al., 1994; Kennedy et al., 1994; Serafini et al., 1996). The analogy was further extended by the demonstration that netrin-1 can mediate chemorepulsion of trochlear motor axons in vitro (Colamarino and Tessier-Lavigne, 1995), much like motor axons in *C. elegans* that are also guided by UNC-6 (Hedgecock et al., 1990), presumably also through repulsion (Wadsworth et al., 1996). This repulsive action of netrin-1 in vertebrates has since been described for a number of other axon populations (Tamada et al., 1995; Varela-Echavarria et al., 1997).

Insights into the mechanism of netrin-mediated repulsion has come from genetic studies in *C. elegans*. In addition to UNC-6, the guidance of motor axons in *C. elegans* is

critically dependent on the product of the *unc-5* gene. UNC-5 is a transmembrane receptor that is required for UNC-6 dependent cell and axon migrations away from the ventral midline (Leung-Hagesteijn et al., 1992). Chapter 3 describes the identification of a family of vertebrate UNC-5 proteins that are expressed in the developing nervous system, and demonstrates that they are netrin binding proteins, providing the first evidence for a direct biochemical interaction between netrins and UNC-5 proteins. Independently, Sue Ackerman has demonstrated that a mutation in a vertebrate UNC-5 (*Unc5h3*^{rcm}) causes defects in cell migrations (Ackerman et al., 1997). Taken together, the evidence suggests that in vertebrates, as well as in nematodes, UNC-5 family members can function as receptors that mediate the function of netrin.

A second family of guidance cues, the semaphorins, comprise approximately twenty-eight members including both secreted and transmembrane forms (Kolodkin, 1997). They are characterized by an approximately 500 amino acid semaphorin domain which contains 15 conserved cysteine residues and many blocks of non-repeating conserved residues (Kolodkin et al., 1992; Kolodkin et al., 1993; Luo et al., 1993). The first semaphorin, grasshopper Sema I (G-Sema I, formerly Fasciclin IV), was identified in a monoclonal screen for molecules expressed in a restricted spatial and temporal fashion that suggested a role in axon guidance. Two independent approaches led to the identification of vertebrate semaphorins. In one approach, a phylogenetic walk was used to clone a number of related genes in Tribolium (T-sema I), *Drosophila* (D-sema I, D-sema II), and Human (H-sema III) (Kolodkin et al., 1993). Independently, a chick semaphorin, collapsin-1 (the species homologue of Sema III), was purified from embryonic chick brains as an activity capable of collapsing growth cones (Luo et al., 1993).

Insights into the function of the semaphorins have been obtained by complementary studies in vertebrates. Chapter two describes experiments that implicate semaphorin III (collapsin-1) as a chemorepellent for subpopulations of sensory neurons.

Previous studies had demonstrated that the ventral spinal cord secretes a molecule that inhibits the outgrowth of sensory neurites from dorsal root ganglia (DRG) in culture (Fitzgerald et al., 1993). This thesis extends these studies by demonstrating that Sema III is expressed at the appropriate time and place, and is capable of mimicking the inhibitory effect of the ventral spinal cord. Moreover, the repulsion is selective for a specific subset of sensory neurons that normally do not invade the ventral spinal cord. Chapter three presents these results in the context of a model whereby repulsion by Sema III plays a role in patterning the projection of sensory neurons into the spinal cord. This study provided the first evidence for a physiological role for semaphorins in vertebrates. Studies by other authors have since confirmed and extended these studies. Raper and colleagues have demonstrated that in the chick, antibodies to collapsin-1 will block the inhibitory effect of ventral spinal cord on sensory DRG axons (Shepherd et al., 1997). Furthermore, they find that all DRG sensory axons appear to respond to the semaphorin chemorepellent at early embryonic stages, and that NT-3 dependent neurites, which invade the ventral spinal cord, selectively lose their sensitivity to the cue as they begin to penetrate the spinal cord (Shepherd et al., 1997). Thus, the evidence now suggests that SemaIII may establish a waiting period that initially prevents axons from entering the spinal cord, and that later selective loss of responsiveness allows one population of neurons (NT-3 dependent) to enter the ventral spinal cord, while restricting others (NGF dependent) to the dorsal spinal cord. Finally, evidence from analysis of Sema III deficient mice suggest that Sema III does indeed play a role in patterning these projections (Behar et al., 1996).

Signaling in Chemorepulsion

The recent identification of receptors for the netrin and semaphorin families of guidance cues sets the stage for an analysis of the signaling pathways they activate. The combined evidence of studies in *C. elegans* and vertebrates implicates the DCC/UNC-40

and UNC-5 families in netrin mediated signaling and provides a solid foothold for this area of investigation (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Hamelin et al., 1993; Chan et al., 1996; Kolodziej et al., 1996 (evidence for DCC-like protein in *Drosophila*); Keino-Masu et al., 1996; Ackerman et al., 1997; Leonardo et al., 1997). Similarly, the recent identification of neuropilins as a semaphorin receptors now provides an opening into the study of semaphorin signaling as well (Chen et al., 1997; He and Tessier-Lavigne, 1997; Kolodkin et al., 1997). Interestingly, all of the candidate chemorepellent receptors for axon guidance identified to date have no homology to known catalytic domains, and there is little about their structure or sequence that hints at their mechanism of signaling. This is quite unlike other chemosensory systems such as chemoattraction in leukocytes and *Dictyostelium*. In these instances the receptors that mediate these events appear to be G-protein coupled seven transmembrane receptors (reviewed in Devreotes and Zigmond, 1988). Therefore, one must postulate that chemorepellent receptors perform their function by specifically bringing together other cellular components that are themselves capable of transmitting the signal. Chapter 5 describes preliminary results from a yeast two-hybrid screen that represent a first attempt to identify molecules in the UNC-5 signaling pathway. We have identified numerous candidate interactors, including phosphatases and proteins regulated by intracellular calcium. Here, we will examine what is currently known about possible mechanisms of growth cone signaling and in particular how they might apply to the cases of netrin and semaphorin signaling.

Signal transduction

The ability of chemorepellents to affect the direction of a growing axon ultimately requires regulation of the actin cytoskeleton. There is extensive evidence that points to actin as a determinant of axon motility and directional growth (Reviewed by Smith, 1988, Cooper, 1991). However, it is unclear how binding of chemorepellents to their receptors

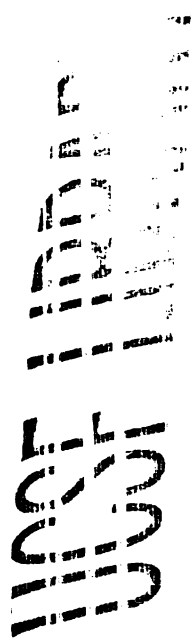
at the cell surface results in the rapid and specific reorganization of the actin cytoskeleton. We know that actin exists in the cell in a highly dynamic, regulated state oscillating between filamentous F-actin and monomeric G-actin (Theriot and Mitchison, 1992). Directional growth cone motility can be envisioned as the selective accumulation of F-actin at sites of new growth, and similarly the selective decrease of F-actin in regions that retract or cease to grow (Smith, 1988; Fan et al., 1993; Lin and Forscher, 1995). In the case of chemorepulsion, where a decrease in F-actin would be expected, the decrease can result in a variety of ways. A decrease in F-actin could result from an activation of actin severing/destabilizing proteins, thus shifting the balance toward G-actin. Alternatively, in a highly dynamic environment, simply decreasing the rate of F-actin formation may be sufficient to shift the equilibrium in favor of G-actin. The signaling pathways known to affect axon motility and actin dynamics are varied, and several are candidate pathways for transducing a repellent signal from the cell surface receptor to the actin cytoskeleton.

Calcium

It is clear that fluctuations in the concentration of intracellular calcium can have numerous effects on growth cones and axon outgrowth. Changes in calcium concentration have been implicated in such processes as axon outgrowth, axons turning toward chemoattractants (Zheng et al., 1996), arrest of outgrowth (Lipton and Kater, 1989), and growth cone collapse (Bandtlow et al., 1993). How changes in calcium concentrations can result in these seemingly distinct behaviors is less clear. It is likely that the effects of calcium are highly dependent on the specific parameters of the spatial and temporal change in its concentration, and on the responsive machinery in the growth cone.

The effects of calcium in the growth cone are likely to be mediated at least in some cases by the Ca++ sensitive protein calmodulin. Studies in *Drosophila* have

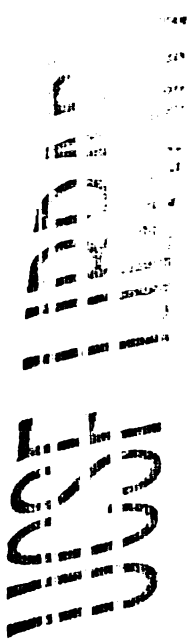
demonstrated that calmodulin is required for certain axon guidance events, as transgenic flies expressing a calmodulin antagonist show defects in axon targeting and fasciculation (VanBerkum and Goodman, 1995). However, the evidence for calcium as a mediator of axonal repulsion is mixed. Studies using the growth cone collapse assay have demonstrated that calcium is required for collapse in some but not all cases: specifically, intracellular calcium stores are required for the collapse evoked by CNS myelin (Bandtlow et al., 1993), but not that evoked by Sema III/Collapsin-1 (Ivins et al., 1991). Another study has shown that turning in response to localized encounters with collapsin occurs without changes in intracellular calcium (Fan and Raper, 1995). It is therefore possible that Sema III/Collapsin does not require calcium to mediate its effects. Whether the same is true for netrin-1 mediated repulsion is unknown (although appears likely, e.g. Ming et al., 1997). Interestingly, as discussed in chapter five, a two hybrid screen with the C-terminus of UNC5H1 has identified a number of Ca++/Calmodulin dependent effectors as candidates for mediating an UNC-5 signal.



Kinases and Phosphatases

The evidence implicating receptor tyrosine kinases (RTK's) and receptor tyrosine phosphatases (RTP's) in axon guidance is growing daily. For instance, recent work on *Drosophila derailed*, EPH RTK's, as well as RTP's like DLAR, has implicated RTK's/RTP's in a variety of axon guidance events (Callahan, 1995; Desai et al., 1996; Krueger et al., 1996; Reviewed in Stoker, 1996 and Orioli and Klein, 1997). Similarly, there is also now much evidence that non-receptor tyrosine kinases and phosphatases modulate the cytoskeleton and are involved in axon guidance (Maness et al., 1988; Elkins et al., 1990; Ignelzi et al., 1994; Clemens et al., 1996). Tyrosine phosphorylation at the growth cone appears to be tightly regulated (Wu and Goldberg, 1993), and numerous kinases and phosphatases are highly enriched in growth cones (Bixby and Jhabvala, 1993).

Recent data also suggests a role for protein kinase A in mediating attractive and repulsive axon guidance responses. Poo and colleagues have demonstrated that axons can turn towards point sources of BDNF and that this response can be converted into a repulsion by inhibitors of protein kinase A (Song et al., 1997). This result suggests that attraction and repulsion are mechanistically related and that modification of a single signaling pathway can transform one to another. This PKA "switch" also appears to operate in the case of netrin (Ming et al., 1997), perhaps hinting at the messengers responsible for translating netrin signals into responses at the cellular level. Highlighting the complexity and possible interactions that exist between signaling cascades is the observation that the protein kinase A mediated attraction to BDNF and netrin is, in addition, dependent on extracellular calcium (Ming et al., 1997; Song et al., 1997).



Rho GTPases

The rho family of small GTPases has received considerable attention as regulators of cytoskeletal dynamics. Experiments conducted primarily in non-neuronal cells have demonstrated that members of this family can have very specific and dramatic effects on the actin cytoskeleton (Reviewed in Hall, 1994). Specifically, injections of activated forms of rac1, rho and Cdc42 into fibroblasts result in membrane ruffling and lamellopodia formation, stress fiber formation, and filopodial formation respectively. More recently, experiments in *Drosophila* have more directly addressed the role of these protein in neurons. Overexpression of dominant negative or constitutive forms of Rac1 in sensory neurons led primarily to axon termination defects while expression of similarly altered Cdc42 led to more general defects in both axons and dendrites (Luo et al, 1994). Other studies in PC12 cells implicate rho in mediating growth cone retraction in response to lysophophatidic acid (LPA) and thrombin (reviewed in Luo et at., 1996). These experiments and others show that the effects of rho family GTPases can be highly specific (Zipkin and Kenyon, 1997).

While it is accepted that small GTPases are important regulators of the cytoskeleton, surprisingly little is known about their activators and effectors (Tapon and Hall, 1997). In particular, what has been missing is a link between an axon guidance molecule and activation of one of these small GTPases. An elegant study by Jin and Strittmatter seems a step in this direction. They introduced constitutively active and dominant negative forms of rac1 into DRG neurons and showed that these mutant rac1 proteins specifically alter the neuronal response to Sema III/Collapsin-1 (Jin and Stritmatter, 1997). No such effect is seen with similar mutant forms of Cdc42. This suggests that rac1, but not related molecules like Cdc42, may mediate Sema III induced growth cone collapse. Similar, experiments may have potential for shedding light on the mechanisms of action of netrin, both in cases of chemoattraction and chemorepulsion.

Future Directions

The past few years has seen a dramatic explosion of new information on axon guidance. The work presented in this thesis highlights some of the important progress that has been made, including the identification of the first chemorepellent (Chapter 2), and the identification of a family of vertebrate UNC-5 like receptors that may mediate the repellent effects of netrin (Chapter 3,4). Despite considerable progress, numerous challenges remain. One remaining mystery is understanding how the growth cone integrates all the signals in its environment to execute a single coordinated response. The identification of protein kinase A as "switch" that converts attraction into repulsion may be an important step in this direction. The candidate proteins isolated in the two hybrid screen presented in chapter 5 are a first step toward linking axon guidance molecules and their receptors at the cell surface with the intracellular messengers that ultimately lead to the remodelling of the cytoskeleton and the behavior we observe as repulsion.

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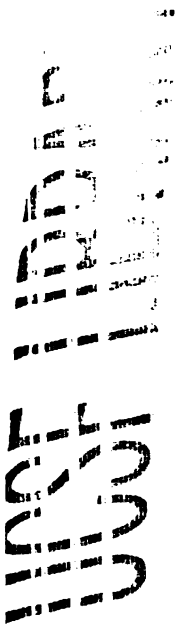
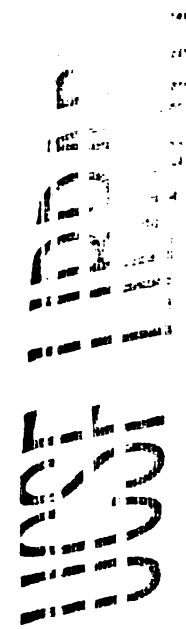


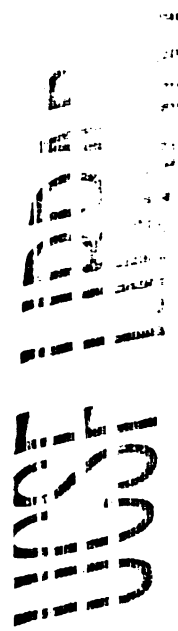
Chapter 2

Semaphorin III Can Function as a Selective Chemorepellent to Pattern Sensory Projections in the Spinal Cord



Summary

Distinct classes of primary sensory neurons in dorsal root ganglia subserve different sensory modalities, terminate in different dorsoventral locations in the spinal cord, and display different neurotrophin response profiles. Large diameter muscle afferents that terminate in the ventral spinal cord are NT3-responsive, whereas small diameter afferents subserving pain and temperature are NGF-responsive and terminate in the dorsal spinal cord. Previous in vitro studies showed that the developing ventral spinal cord secretes a diffusible factor that inhibits the growth of sensory axons. Here we show that this factor repels NGF-responsive axons but has little effect on NT3-responsive axons. We also provide evidence implicating semaphorin III/collapsin, a diffusible guidance molecule expressed by ventral spinal cord cells, in mediating this effect. These results suggest that semaphorin III functions to pattern sensory projections by selectively repelling axons that normally terminate dorsally.



Introduction

Mammalian dorsal root ganglia (DRG) comprise several functionally distinct classes of primary sensory neurons mediating different types of somatosensory information (Brown, 1981; Willis and Coggeshall, 1991). Different classes have characteristic physiological properties and axonal diameters, and project axons and axon collaterals to second order neurons in the spinal cord and medulla. The pattern of projections within the spinal cord is highly stereotyped, with the axons entering the dorsal horn from the dorsal funiculus and extending ventrally to laminar termination sites that are characteristic for each class. For example, small diameter afferents involved in thermoreception and nociception terminate in the dorsal-most laminae (I and II), larger diameter afferents involved in detection of non-noxious stimuli terminate in deeper laminae (III - IV) in the dorsal horn, and the large group Ia muscle spindle afferents which mediate the monosynaptic stretch reflex terminate on motoneurons in the ventral spinal cord (Rexed,

1952; Eccles et al., 1957; Burke, 1977, Christensen and Perl, 1970, Light and Perl 1979). During development, these sensory axons reach the dorsal funiculus several days before sending collaterals into the spinal cord. In the rat, the larger diameter afferents then project to intermediate and ventral termination sites starting at embryonic days 14 -15 (E14-15), and small diameter afferents project to laminae I and II starting at E16 (Windle and Baxter, 1936; Smith, 1983; Altman and Bayer, 1984; Snider et al., 1992; Zhang et al., 1994; I. Silos-Santiago and W. Snider, personal communication).

The mechanisms that direct the projections of DRG axons to different dorsoventral termination sites in the spinal cord are largely unknown. Permissive and attractive guidance cues are known to direct axonal projections in a variety of systems (reviewed by Goodman and Shatz, 1993; Tessier-Lavigne, 1994), and it is possible that different laminae are sources of attractive factors for distinct classes of axons. For example, neurotrophin NT3 is expressed by motoneurons and has been proposed as a candidate for attracting Ia afferents to the ventral horn (discussed by Zhang et al., 1994).

In addition, axons can be guided by both short- and long-range inhibitory or repulsive cues (e.g., Kapfhammer and Raper, 1987; Walter et al., 1987; Bandtlow et al., 1990; Davies et al., 1990; Raper and Grunewald, 1990; Moorman and Hume, 1990; Pini, 1993; Nose et al., 1994; Matthes et al., submitted; Colamarino and Tessier-Lavigne, submitted). Previous studies have implicated inhibitory mechanisms in shaping axonal projections into the spinal cord. Peterson and Crain (1982) showed that axons from cultured DRG selectively failed to invade explants of ventral spinal cord in long-term culture. More recently, Fitzgerald et al. (1993) showed directly that the ventral spinal cord from E14-15 rat embryos secretes a diffusible factor that has an inhibitory effect on the growth of axons from DRG at a distance in vitro. In both sets of experiments, the inhibitory effect of the ventral spinal cord was demonstrated on axons that grew out of the DRG in response to nerve growth factor (NGF). These NGF-responsive axons correspond to the small diameter axons that terminate in superficial laminae (I and II) of the dorsal

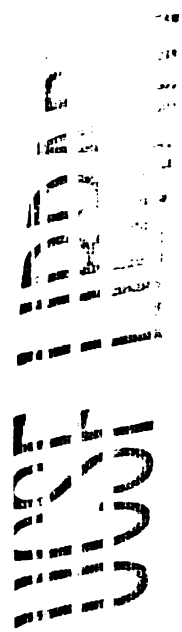
horn (Ruit et al., 1992; Mu et al., 1993; and see below). The effect of the ventral spinal cord factor on other classes of axons that are not NGF-responsive and which terminate more ventrally was not determined. It therefore remained unclear from this study whether the likely role of the factor was to delay ingrowth of all afferents into the spinal cord until the appropriate developmental stage, or alternatively, to prevent selectively the small diameter axons from ever approaching the ventral spinal cord. In addition, the molecular identity of the factor was not determined.

One family of molecules that comprises candidates for inhibitory guidance cues is the semaphorins (Kolodkin et al., 1992; Luo et al., 1993; Kolodkin et al., 1993). The first member of the family, semaphorin I (sema I, formerly fasciclin IV), a transmembrane semaphorin in insects, has been implicated in guiding pioneer axons in the grasshopper limb bud (Kolodkin et al., 1992). Other members of the semaphorin family were subsequently identified by sequence similarity in Drosophila (D-sema I and D-sema II) and in human (H-sema III). Independently, a member of this family, collapsin, was identified in chicken on the basis of its ability to cause collapse of growth cones of DRG neurons in culture (Luo et al., 1993). Although there appear to be many distinct members of the semaphorin family in vertebrates (see Results), collapsin and H-sema III appear to be species homologues. Unlike sema I, sema II and sema III/collapsin lack transmembrane domains and are likely to be secreted and to act as diffusible factors. Recent evidence has shown that sema II can function in vivo as a chemorepellent for motor axons in Drosophila (Matthes et al., submitted). The fact that sema III/collapsin causes sensory growth cone collapse in vitro suggests that it too may function as a chemorepellent, in this case for vertebrate sensory axons.

To determine the role of sema III/collapsin in directing the central projections of axons from DRG neurons, we have focused on the development of these projections in rodents. We isolated a murine homologue of this molecule (hereafter referred to as sema III), and examined its expression pattern in relation to primary sensory afferent projections

in mouse and rat. High levels of *semaIII* mRNA expression were detected in the ventral spinal cord at appropriate developmental stages, and COS cells secreting sema III were found to mimic the inhibitory effect of the ventral spinal cord on axons extending in response to NGF. These results suggest that sema III contributes to mediating the long-range inhibitory effect described by Fitzgerald et al. (1993).

In addition, we have further extended these observations by examining whether sema III has effects on other classes of DRG neurons. For this, we took advantage of the fact that NT3 but not NGF evokes outgrowth in vitro of muscle afferents that terminate in the ventral spinal cord (Hory-Lee et al., 1993; see also Davies, 1986; Hohn et al., 1990, and Discussion). In contrast to their effects on NGF-responsive axons, we have found that cells secreting sema III do not affect the growth of axons from explants grown in NT3. Together, our results suggest that sema III secreted by ventral spinal cord cells contributes to patterning sensory projections in the spinal cord by creating an exclusion zone for axons that terminate dorsally, without preventing the ingrowth of axons that terminate ventrally.



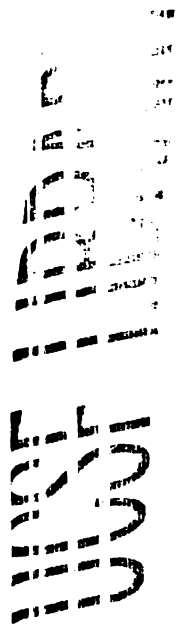
Results

The inhibitory effect of ventral spinal cord explants is selective for NGF-responsive axons

The inhibitory effect of ventral spinal cord cells was previously demonstrated by culturing E14 rat lumbar DRG at a distance from explants of E14 rat ventral spinal cord in three dimensional collagen gels in medium containing NGF. Outgrowth from the DRG was strongly inhibited on the side of the explants, but was unaffected by explants of dorsal spinal cord (Fitzgerald et al., 1993). NGF is expected to evoke axon outgrowth only from thermoreceptive and nociceptive neurons, which project to laminae I and II in the dorsal spinal cord (Ruit et al., 1992; Zhang et al., 1994; Snider, 1994). To determine whether the ventral spinal cord can inhibit outgrowth of sensory axons that project to other laminae, we

focused on axons that are responsive to the neurotrophin NT3. These include Ia muscle spindle afferents which project to the ventral spinal cord, and perhaps also some axons projecting to other laminae (see Discussion) but not laminae I and II.

We first sought to replicate the observations of Fitzgerald et al. (1993) by coculturing E14 rat DRG at distances of 150 - 950 mm from target explants of E14 rat dorsal or ventral spinal cord in collagen gels, in the presence of NGF. In agreement with these authors, we found that the pattern of outgrowth from DRG cultured alone or with dorsal spinal cord was radially symmetric (data not shown), whereas outgrowth from DRG cultured with ventral spinal cord explants was reduced on the side of the explant and directed away from it (Figure 1A). Quantification of the effect by comparing the extent of neurite outgrowth towards the ventral spinal cord explant (Figure 1C, proximal quadrant) to that away from the explant (Figure 1C, distal quadrant) showed that the inhibition was highly significant ($p < 0.0001$, Table 1).



Although in some cases the DRG axons in the proximal quadrant appeared to contact some axons from the ventral spinal cord explant (e.g. Figure 1A), in most cases the inhibition was observed in the absence of such contact (data not shown), indicating, as previously described by Fitzgerald et al. (1993), that the effect is mediated by a diffusible factor secreted by ventral spinal cord cells. This is further supported by the observation that the inhibitory effect decreased roughly linearly with increasing distance between the DRG and the ventral spinal cord explant (Figure 2). This observation suggests that the ventral spinal cord secretes limiting amounts of the factor, and that its concentration within the collagen matrix decreases with distance. A clear inhibitory effect was detected at considerable distances, as the extent of neurite outgrowth from the proximal side of each DRG was shorter than that from its distal side for all cultures in which the explants were separated by less than 800 mm (Figure 2 and data not shown). The in vivo distance at E14 between the ventral spinal cord and the dorsal funiculus (where the NGF-responsive axons are located) is about 200 mm.

We next examined the effects of ventral spinal cord explants on neurites extending from DRG cultured with NT3. In all experiments, DRG were taken exclusively from brachial (forelimb) regions which contain large numbers of NT3-responsive neurons (DRG at non-limb levels have a much smaller cohort of such neurons; Hory-Lee et al., 1993). In contrast to their effect on axons growing out of DRG in the presence of NGF, ventral spinal cord explants had no apparent effect on the radial profile of axons growing out of DRG in the presence of NT3 (Figure 1B and Table 1). Dorsal spinal cord explants also did not affect axons growing out in the presence of NT3 (data not shown). Although no difference was observed in the lengths of neurites extending towards or away from the ventral spinal cord explants, in a few cocultures the density of neurites extending towards the explant appeared less than the density of those extending away (data not shown), raising the possibility that the NT3-responsive population is heterogeneous and that the ventral spinal cord affects a subpopulation of these axons. In most cocultures no such difference was apparent. Thus, most if not all NT3-responsive axons that grow out of DRG in these cultures appear unaffected by the inhibitory factor secreted by ventral spinal cord explants.

Sema III is expressed in the ventral spinal cord during the period of sensory axon ingrowth

Because collapsin/sema III can cause collapse of growth cones from DRG neurons in vitro (Luo et al., 1993), we next examined whether it was expressed in the developing spinal cord in a manner consistent with it having a role in influencing sensory axon projections. To determine the expression of sema III in the spinal cord of rodents, we isolated a cDNA comprising the murine sema III coding sequence (except the first 318 bp; see Experimental Procedures). The predicted product is 95% and 90% identical to the human sema III and chick collapsin sequences, respectively (see Experimental Procedures). Figure 3 shows the alignment of portions of the ~500 amino acid semaphorin domains (Kolodkin et al., 1993) of these mouse and human sema III. In addition, a search of the dbest database (see

Experimental Procedures) identified eleven human expressed sequence tags that appear to code for at least four (including sema III) and as many as nine human proteins with semaphorin domains. The relation of these sequences to sema III and previously identified insect semaphorins is also shown in Figure 3.

In an initial set of experiments, we used a coding region probe to examine the expression of *semaIII* mRNA by in situ hybridization in transverse sections of spinal cord from mouse embryos. Expression was detected very transiently in the roof plate region of the neural tube at early stages of neural tube formation (E9.5 in the mouse), but was then not detectable in the spinal cord until E11.5. From that time on, transcripts were detected in the ventral spinal cord until E18, the latest time examined (data not shown). The level of expression was high between E12.5 and E14.5, and decreased steadily thereafter (data not shown).

Because the timing of sensory afferent ingrowth has been studied in most detail in the rat, we also examined the expression of *semaIII* mRNA in rat embryos. Similar expression patterns were observed in rat and mouse embryos of comparable developmental stages. At E14 in the rat (which corresponds to E12.5 in the mouse), a high level of expression was detected in the ventral spinal cord, excluding the floor plate region (Figure 4A,B). The expression was not uniform but was highest in the ventricular zone, and next highest in the developing motor columns; expression also appeared to extend into the dorsal horn for a short distance (Figure 4A). A similar pattern was observed at all axial levels of the spinal cord that were examined at this stage (data not shown). The level of expression subsequently decreased without change in the pattern of expression; significant though lower expression was still detected at E18 (Figure 4C, D), when muscle afferents have reached the ventral horn and after NGF-responsive axons have started to invade laminae I and II (see Discussion). Interestingly, Fitzgerald et al. (1993) reported that the ventral spinal cord inhibitory activity was still present but at lower levels in E18 embryos.

Thus, the pattern and level of *semaIII* mRNA expression is consistent with sema III mediating the inhibitory activity.

COS cells secreting sema III selectively repel NGF-responsive axons

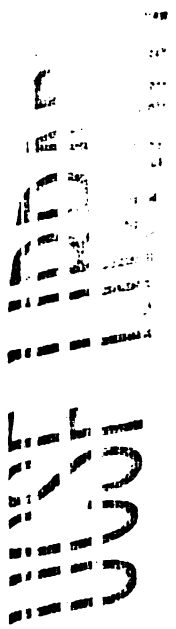
Previous studies have established that collapsin/sema III causes collapse of DRG axons when applied acutely in culture (Luo et al., 1993), but they have not determined its effects on DRG axons in chronic cocultures like those with ventral spinal cord (Figure 1A), nor have they determined which classes of DRG neurons are affected. To test directly whether sema III can contribute to the inhibitory effects of the ventral spinal cord, we cultured DRG together with aggregates of COS cells that had been transfected with a human sema III expression construct, and which were shown by immunoblotting to secrete sema III protein (see Experimental Procedures).

We first examined the effect of cells secreting sema III on NGF-responsive axons. When DRG explants were cultured in collagen gels in medium containing NGF, COS cells secreting sema III dramatically reduced the amount of outgrowth on the proximal side of the DRG (i.e. in the proximal quadrant) without effect on the axons in the distal quadrant (Figure 5B and Table 1). Control COS cells had no apparent effect on the axons (Figure 5A and Table 1). Contrary to what was observed with ventral spinal cord, the degree of inhibition by COS cells secreting sema III was similar whatever the distance separating the cells from the explants (in the range 200 - 950 mm; data not shown), suggesting that the amount of sema III that reaches the DRG in these experiments is far above threshold for the inhibitory effect.

These results suggest that sema III contributes to the inhibitory effect of ventral spinal cord. To attempt to correlate further the sites of expression of sema III with the ventral spinal cord inhibitory activity, we took advantage of the observation that sema III transcripts were not detected in floor plate cells (Figure 3). Microdissected floor plate from E14 embryos had no effect on NGF-responsive axons (data not shown; similar results have

been obtained by G. Kwiat and M. Fitzgerald, 1993, Soc. Neurosci. Abstract), indicating that the inhibitory factor is found only in the portion of the ventral spinal cord that expresses *semaIII*.

In contrast to their effect on NGF-responsive axons, COS cells secreting sema III had no apparent effect on the outgrowth of NT3-responsive axons from the DRG (Figure 5D and Table 1). The density of axons projecting towards the COS cells also appeared in all cases to be unaffected (data not shown). The absence of an inhibitory effect of the COS cells on the NT3-responsive axons was further suggested by the observation that axons that reached the COS cells could actually invade the aggregates (see e.g. Figure 5D). Control COS cells, like sema III-expressing cells, had no effect on NT3-responsive axons in this assay (Figure 5C and Table 1). Thus, sema III functions as a selective diffusible repellent of NGF-responsive axons without apparent effect on NT3-responsive axons.



Discussion

When the axons of different classes of primary sensory neurons enter the spinal cord during development, they project ventrally from the dorsal funiculus to different laminar termination sites along the dorsoventral axis that are appropriate for their specific modalities. Such a pattern of specific projections might in principle be controlled by differential attractive signals, repulsive signals, or a balance of both. Little is known, however, about the identity and function of guidance molecules that direct this patterning. Here we provide evidence that one of these molecules is semaphorin III/collapsin. We show that sema III is a selective chemorepellent of a specific set of sensory axons that terminate dorsally, and suggest that it functions in vivo to help prevent those axons from projecting ventrally (Figure 6).

Semaphorin III is a Ventral Spinal Cord-Derived Repellent for NGF-Responsive Sensory Axons

Fitzgerald et al. (1993) showed that ventral spinal cord explants from E14 rats secrete a diffusible factor that has an inhibitory action on sensory axons that grow out in response to NGF. Our results implicate sema III in mediating this effect. We found that *semaIII* transcripts are present in the ventral spinal cord at E14 in rats, and that COS cells secreting sema III can mimic the inhibitory effect of ventral spinal cord explants. The detailed spatial and temporal pattern of *semaIII* mRNA expression is consistent with sema III being the inhibitor. Within the ventral spinal cord, neither the inhibitory activity nor *semaIII* transcripts are expressed by floor plate cells. Moreover, the levels of *semaIII* transcripts expressed by ventral spinal cord cells decreases by E18, consistent with the decrease in inhibitory activity at that age reported by Fitzgerald et al. (1993). Thus, sema III is likely to mediate in whole or in part the activity in ventral spinal cord explants. In our assay, COS cells secreting sema III were more effective than ventral spinal cord explants; we presume that this simply reflects a higher level of secretion of sema III by the COS cells.

The factor secreted by ventral spinal cord explants was previously described as "inhibitory", but our results appear more consistent with this factor (and sema III) being a chemorepellent rather than an outright blocker of outgrowth of NGF-responsive axons. Extensive outgrowth was observed on the side of the DRG explants facing ventral spinal cord explants or COS cells secreting sema III, but these axons extended parallel to target, appearing to have turned away from it (Figures 1A and 5B). This effect was particularly pronounced with COS cells secreting sema III, which could prevent axons from projecting towards them at a distance of over 900 μm, but which did not appear to have affected the length of axons that were growing parallel to the target even when as close as 200-400 mm (Figure 5B and data not shown). Thus, a source of sema III does not block the growth of axons in its vicinity provided the axons are growing away from the source.

The cellular events involved in repulsion of these axons by sema III have not been determined. Collapsin (the sema III homologue in chick) causes rapid collapse of growth cones of chick DRG axons when added acutely to these axons (Luo et al., 1993); the recombinant human sema III used in our studies has similar effects (unpublished data). However, when growth cones are exposed to a localized but non-diffusible source of collapsing signal (in the form of chick brain extract enriched in collapsin that is immobilized on beads), these growth cones often turn away from the source without collapsing following filopodial contact with the source (Fan and Raper, 1995). It is possible that growth cone collapse only occurs when many filopodia are exposed simultaneously and rapidly to a step change in concentration of the factor, such as is achieved in the collapse assay. Whether collapse occurred in our experiments where axons were chronically exposed to a presumed gradient of the factor, and indeed whether collapse in response to sema III occurs in vivo, remain to be determined.

A Role for Semaphorin III in Patterning Sensory Axon Projections in the Spinal Cord

Distinct classes of sensory axons project to different termination sites in the spinal cord, and our results indicate that sema III affects only a subset of these axons. In the rat, the first axons project from the DRG to the developing dorsal funiculus two days before entering the gray matter at E14-15 (Windle and Baxter, 1936; Altman and Bayer, 1984; a similar "waiting period" is observed other species, e.g. Knyihar et al., 1978; Lee et al., 1988; Mendelson et al., 1992). The axons to enter into the spinal cord at this time include the Ia afferents which project to the ventral horn (Kudo and Yamada, 1987; Ziskind-Conhaim, 1990). This early projecting population also likely includes the afferents from the low threshold mechanoreceptors which terminate in intermediate regions of the dorsal horn in laminae III and IV (Kudo and Yamada, 1987; Snider, 1992; Zhang et al., 1994). Small diameter afferents involved in pain and temperature perception, which terminate in the dorsal-most laminae (I and II), only enter the gray matter later, starting at E16 (Snider

et al., 1992; Zhang et al., 1994; I. Silos-Santiago, personal communication). These small diameter DRG neurons have later birthdates than the larger ones (Altman and Bayer, 1984), which may in part explain their delayed entry into the spinal cord.

We have found that sema III repels all NGF-responsive axons in our assay without apparent effect on NT3-responsive axons. The NGF-responsive axons studied here are almost certainly the small diameter afferents that terminate in laminae I and II (Ruit et al., 1992, Zhang et al., 1994). The NT3-responsive axons are likely to include the Ia afferents that project to the ventral spinal cord (Hory-Lee et al., 1993). These axons also likely include other large diameter afferents that relay information to the cerebellum (reviewed in Snider, 1994). At lumbar and thoracic regions, these afferents send collaterals to a specialized nucleus in the thoracic spinal cord called Clarke's column, positioned roughly midway along the dorsoventral axis. At the brachial levels used in our studies, these afferents project to a nucleus in the brainstem (the lateral cuneate nucleus) not the spinal cord. Interestingly, although sema III never appeared to affect the NT3-responsive axons, ventral spinal cord explants occasionally caused an apparent reduction in the density of this axonal population on the side of the explants, without effect on the length of those present (see Results). One possibility is that an inhibitory factor distinct from sema III is produced by the ventral spinal cord that affects a subpopulation of NT3-responsive axons, perhaps those normally destined for Clarke's column or the lateral cuneate nucleus. Finally, our studies have not addressed whether sema III plays a role in directing the projections of afferents from the low-threshold mechanoreceptors, which terminate in laminae III and IV. Culture conditions that can selectively elicit the outgrowth of these axons have not been determined, and molecular genetic studies suggest that they may even be neurotrophin-independent at this stage of development (reviewed in Snider 1994b; see also Zhang et al., 1994). It is therefore unclear from our experiments whether sema III affects this class of axons.

Our results suggest that sema III secreted by ventral spinal cord cells patterns sensory projections by restricting the ventral growth of NGF-responsive axons that terminate in laminae I and II without effect on axons that terminate in the ventral spinal cord. However, at least three different models for the role of sema III are compatible with our data (see also Fitzgerald et al., 1993). First, if sema III diffuses throughout the dorsal spinal cord, it may function as a "waiting cue" preventing the ingrowth of NGF-responsive axons into laminae I and II until E16-17. The down-regulation in expression of *semaIII* mRNA (and presumably also sema III protein) would therefore be key in allowing these axons to enter. Second, sema III protein may not normally reach the NGF-responsive axons in laminae I and II, and may only be present more ventrally to help redirect any of these axons that have mistakenly wandered beyond those laminae. Third, sema III may be present in a gradient throughout the dorsal spinal cord that does not actually block ingrowth into the gray matter but which does inhibit the progression of the NGF-responsive axons further ventral than laminae I and II. In model three, sema III is responsible for making the axons stop in laminae I and II, whereas in models one and two, a separate cue is required for that purpose. Zhang et al. (1994) recently showed that injections of NGF in utero around E14 - E16 cause the axons that normally terminate in laminae I and II to extend further into the dorsal horn. The axons could reach and even cross the midline, but never projected into the ventral spinal cord. These results appear to favor the first two models over the third. Similar conclusions were recently drawn by Sharma et al. (1994) from studies of sensory projections into spinal cord explants in vitro.

Whatever the precise role of sema III, several features of the projections of these axons indicate that other cues must also function with sema III to guide them. The axons that project to the ventral spinal cord or laminae III and IV enter the spinal cord medially, while axons that project to laminae I and II enter laterally; the afferents from the low-threshold mechanoreceptors initially overshoot laminae III and IV, then turn upward in a characteristic "flame-shaped appearance" (Ramon y Cajal, 1909, Scheibel and Scheibel,

1968). Specific cues must exist that constrain axons to these trajectories. It is also possible that the ventral spinal cord is the source of chemoattractant for the Ia afferents (discussed in Zhang et al., 1994).

Semaphorins: A Growing Family of Axon Guidance Molecules

Semaphorin III/collapsin (Luo et al., 1993; Kolodkin et al., 1993) is a member of the semaphorin family, which comprises cell surface and secreted growth cone guidance molecules that are conserved from insects to humans (Kolodkin et al., 1993). Semaphorins are ~750 amino acids in length (including signal sequence), and are defined by a conserved ~500 amino acid extracellular semaphorin domain containing 14-16 cysteines, many blocks of conserved residues, and no obvious repeats (Kolodkin et al., 1993). The transmembrane semaphorins have an additional ~80 amino acid stretch, a transmembrane domain, and a 80-110 amino acid cytoplasmic domain. The secreted semaphorins have an additional ~20 amino acid stretch, a single immunoglobulin domain, and a 70-120 amino acid C-terminal region. A search of the dbest database has revealed the existence of a large family of semaphorins in humans (Figure 3). In addition, two semaphorins are encoded in viral genomes (Kolodkin et al., 1993).

All available evidence thus far suggests that different members of the semaphorin family function as either cell surface or secreted chemorepellents or inhibitors of axon pathfinding, branching, or targeting. Although there are similarities in how they function, there are also some interesting differences, and these differences may reflect either real differences in function or differences in the assays used (Kolodkin et al., 1992; Luo et al, 1993; Fan and Raper, 1995; Matthes et al., 1995; results presented here).

The first identified member of the family was semaphorin I (formerly fasciclin IV; Kolodkin et al., 1992), a transmembrane semaphorin in insects. Antibody-blocking experiments in the grasshopper limb bud have shown that expression of sema I by a stripe of epithelial cells prevents Ti1 axons that encounter it from defasciculating and branching.

However, sema I is not an absolute inhibitor of growth, since the Til growth cones grow on the sema I-expressing cells (see discussion in Kolodkin et al., 1993).

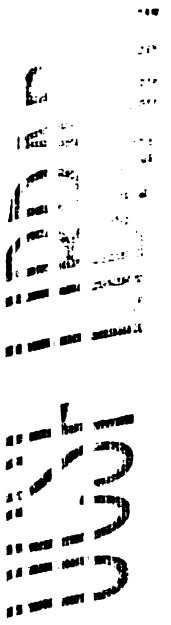
Semaphorin II, a secreted semaphorin in Drosophila (Kolodkin et al., 1993), is transiently expressed in the embryo by a subset of neurons in the central nervous system, and by a single large muscle during motoneuron outgrowth and synapse formation. To test the in vivo function of sema II, transgenic Drosophila were created which generate ectopic sema II expression by muscles that normally do not express it. The results show that in some cases sema II can function to repel axons. In the case of a single identified motoneuron (RP3), however, sema II inhibits the formation of its synaptic terminal arborizations without actually repelling it (Matthes et al., submitted).

Thus, in the developing organism, semaphorins appear capable of inhibiting branching (sema I in grasshopper), influencing steering decisions (sema I in grasshopper and sema III in mammals), preventing axons from entering certain target regions (sema II in Drosophila and perhaps sema III in mammals), or inhibiting the formation of synaptic terminal arborizations (sema II in Drosophila). The full range of effects of different or even individual semaphorin family members on developing axons remain to be determined. In particular, given the evidence that some guidance cues can have both repulsive and attractive effects (discussed in Goodman, 1994, Colamarino and Tessier-Lavigne, submitted), it will be important to determine whether any semaphorins function as attractants. Nothing is yet known about the identity of the receptors for semaphorin family members.

Emerging Diversity of Repulsive Guidance Molecules

It is not yet known how many semaphorins are encoded in the genome of any one species: there are at least two in Drosophila (Kolodkin et al., 1993) and four in human (Kolodkin et al., 1993; and EST analysis in Figure 3), but this is probably an incomplete representation of either genome's total number. Moreover, the semaphorins represent just

one family of repulsive or inhibitory guidance molecules. A number of other molecules have been identified which can function in a repulsive or inhibitory fashion, including the cell surface proteins connectin (Nose et al., 1994) and myelin-associated glycoprotein (MAG; McKerracher et al, 1994; Mukhopadhyay et al., 1994), the diffusible protein netrin-1 (Colamarino and Tessier-Lavigne, submitted), the related extracellular matrix proteins tenascin and janusin/restrictin/J1-160-180 (Faissner and Kruse, 1990; Pesheva et al., 1993), and proteoglycans (see Snow et al., 1990). What is emerging is a picture of a great diversity of molecules that can repel or inhibit various aspects of axon growth, pathfinding, and targeting. The literature contains numerous cases of repulsion (e.g., Kapfhammer and Raper, 1987; Walter et al., 1987; Bandtlow et al., 1990; Davies et al., 1990; Raper and Grunewald, 1990; Moorman and Hume, 1990; Pini, 1993). It remains to be determined which of these events are mediated by members of known families of repellents or inhibitors, and whether other as yet unknown families also contribute to inhibitory and repellent interactions in the developing nervous system.



Experimental Procedures

Isolation and Sequence Analysis of Mouse *semaIII*

DNA cloning techniques were performed as described by Sambrook et al. (1982) unless otherwise stated. Mouse *semaIII* (*M-semaIII*) sequences were isolated by PCR, using as a template cDNA made to poly(A)+ RNA isolated from whole mouse E14 brain. First, oligonucleotides (LSTH5, LSTH3; see below) derived from the *H-semaIII* sequence (Kolodkin et al., 1993) were used to amplify a 1300 bp fragment. This fragment was cloned into the Sma I site of pBluescript (Stratagene) generating pMSemaIIIA (which was used for RNA in situ analysis). An oligonucleotide (M3A) derived from this 1300 bp fragment was used in conjunction with an oligonucleotide (M4B) derived from a more 3' portion of the *H-semaIII* coding sequence (see below). This reaction generated a 970 bp fragment which was directly cloned into pCR II (Invitrogen). Both cloned PCR fragments were sequenced on both strands using an ALF DNA Sequencer and Autoread reagents (Pharmacia LKB). The composite *M-semaIII* sequence derived from these two PCR fragments encodes a protein sequence that is 666 amino acids in length, 95% identical at the amino acid level to H-sema III starting from amino acid 106 of H-sema III to the end of the coding sequence. The *M-semaIII* sequence is incomplete at the 5' end, missing ~59 amino acids at the start of the semaphorin domain, and ~47 amino acids at the amino-terminus of the protein. The high degree of similarity between available *M-semaIII* and *H-semaIII* coding sequences identifies the encoded protein M-sema III as a homologue of H-sema III and chick collapsin (Luo et al., 1993). The sequence of *M-semaIII* can be obtained via Genbank accession # L40484. Sequences described in Figure 3 were obtained by searching the dbest database (Boguski et al., 1993, and Adams et al., 1991) with previously identified semaphorin sequences. The PCR cycling conditions were: 35 cycles of 96°C, 30 sec.; 55°C, 1 min.; 72°C, 3 min. Oligonucleotide sequences were: LSTH5-GGAAAAGACATCCCTGAAAGAA corresponding to the *H-semaIII* DNA sequence

encoding amino acids 107-113; LSTH3--CTGGGAATGACACGAACCCTA (encoding H-sema III amino acids 532-538); M3A-ATGTTCATCGGAACAGAT (encoding M-sema III amino acids MFIGTDV); and M4B-GACACTCCTGGGTGCCCTCTCAAA (encoding H-sema III amino acids 764-771).

Production of Recombinant Human semaphorin III

A COS cell expression vector pCOS(LB)-BMN-myc was generated by modifying pMLP (which contains an SV40 ori, an E1A enhancer/MLP/tripartite leader, polylinker sequences, and DHFR and SV40 poly A+ sequences; a gift from Lisa Brunet), through the addition in the polylinker of a sequence encoding a peptide from c-myc (EEQKLISEEDLLRKRREQLKHKLE) recognized by monoclonal antibody 9E10 (Evan et al., 1985). Three unique restriction sites were also inserted upstream of the c-myc epitope sequence to allow open reading frames (ORFs) to be cloned in frame 5' to the c-myc epitope. Primers derived from the *H-semaIII* sequence were used to amplify the *H-semaIII* ORF by PCR using Vent Polymerase (New England Biolabs), and the resulting amplification product was cloned into pCOS(LB)-BMN-myc to yield pCOS(*H-semaIII-myc*). COS cells were transfected with pCOS(*H-SemaIII-myc*) using LipofectAMINE (Gibco BRL). COS cell aggregates were prepared by the hanging drop method as previously described (Kennedy et al., 1994). Western analysis using 9E10 demonstrated the presence of secreted myc-tagged *H-semaIII* in the medium conditioned by transfected COS cells.

RNA In Situ Hybridization

Rat embryos (E14) were obtained from timed pregnant rats (vaginal plug equivalent to E=0, Simonsen Laboratories). Embryos were removed into L15 medium, embedded in OCT compound (Tissue Tech), flash frozen in liquid nitrogen and stored at -80°C. Cryostat sections (25μm) were collected on Superfrost Plus slides (Fisher). ^{35}S-labelled sense and

antisense riboprobes corresponding to the 1300 base pair coding region sequence of M-SemaIII were generated with T3 and T7 RNA polymerase using pMSemaIIIA. Probes were base-hydrolyzed to an average size of 200 base pairs. For prehybridization, slides were brought to room temperature and fixed in 4% paraformaldehyde (PFA) in 0.1M phosphate buffered saline (PBS) for 20 min, rinsed four times (5 min each) in PBS and once in depc-treated water prior to dehydration through a graded ethanol series (50, 80, 95%). Sections were digested with 1 mg/ml proteinase K for 20 minutes, refixed in 4% paraformaldehyde/0.1M PBS, washed in PBS, washed in depc-treated water, dehydrated in ethanol, treated with 0.1M TEA and acetic anhydride, washed in depc-treated water and dehydrated with ethanol. Sections were incubated with hybridization buffer containing $1x10^6$ counts/section of either sense or antisense riboprobe, incubated overnight at 55°C in a humidified chamber, washed twice (30 min each) in 2x SSC with 10mM b-mercaptoethanol (bME) at room temperature, treated with 50µg/ml RNaseA in 2x SSC, 1 mM EDTA for 40 min, washed twice with 50% formamide in 2x SSC, 1 mM EDTA and 10mM bME at 55°C (1 hr each), washed in 0.2xSSC at 55°C and then with 0.1xSSC for 30 min at 55°C. Slides were dehydrated through an ethanol series, exposed to x-ray film and processed for autoradiography as described (Luskin and Shatz, 1985).

Explant Co-Cultures

Spinal cord tissue and dorsal root ganglia from the brachial region were dissected from rat embryos (E14) obtained from timed pregnant rats, and embedded in collagen gels as described (Tessier-Lavigne et al., 1988). Co-cultures consisted of DRG with either spinal cord (dorsal or ventral), floor plate, or COS cells [mock-transfected or transfected with pCOS(*H-semaIII-myc*)]. Explants of the ventral spinal containing the floor plate were oriented with the floor plate side pointing away from the DRG, thus exposing the dorsal edge of the explant to the DRG. Explants were cultured for 36 hr in F12/N3 medium as described (Tessier-Lavigne et. al. 1988) except that the medium contained only 0.5% heat-

inactivated horse serum and was supplemented with either 50 ng/ml 2.5S NGF (Bioproducts for Science Inc.) or 50 ng/ml NT3 (a generous gift from David Shelton, Genentech, Inc.).

Immunohistochemistry

After culture, explants were fixed with 4% PFA in PBS for ~2 hours at room temperature. Immunostaining with a neurofilament specific antibody (NF-M ; Lee et al., 1987; 1:1000) and an HRP conjugated secondary antibody (Boehringer-Mannheim; 1:400) was performed essentially as described (Kennedy et. al. 1994) except that antibody incubations and washes were in PBS/1% heat inactivated goat serum/0.1% Triton X-100. Explants were mounted in Aquamount (Lerner Laboratories) for analysis of neurite outgrowth.

Quantification of Neurite Outgrowth

DRG explants cultured in the presence of NGF or NT3 displayed a radial pattern of neurite outgrowth. For quantification, the region of neurite growth was divided into four quadrants as diagrammed in Figure 1C. Neurite outgrowth into the collagen gel was measured from the outer border of DRG to the outer perimeter of the bulk of neurofilament stained neurites (thus measuring the perimeter at its region of highest density of staining). In instances where the perimeter was not entirely symmetric within a quadrant, the perimeter border was defined as the distance between the minimal and maximal radii. Statistical analysis was performed using Microsoft Excel 4.0.

Acknowledgments

Correspondence should be addressed to Marc Tessier-Lavigne. We thank Sophia Colamarino for critical comments on the manuscript, Virginia Lee for the generous gift of NF-M and David Shelton for the generous gift of NT3, and Jay Rehm, Krystyna Kilomanski, Caroline Esmurdoc, and Larry Nelson for technical assistance. We thank

William Snider, Doug Wright,and colleagues for communication of results prior to publication. E.D.L. was supported by NIH grant GM07618 through the UCSF MSTP program. A.L.K. was supported by NIH grant HD21294 (to C.S.G.). E.K.M. was supported by the March of Dimes Birth Defects Foundation (to C.J.S.) and by Fight for Sight National Society to Prevent Blindness Postdoctoral Fellowship (PD93007). M.T.-L. was supported by the Lucille P. Markey Charitable Trust. C.J.S. and C.S.G. are Investigators and M.T.-L. is an Assistant Investigator with the Howard Hughes Medical Institute.

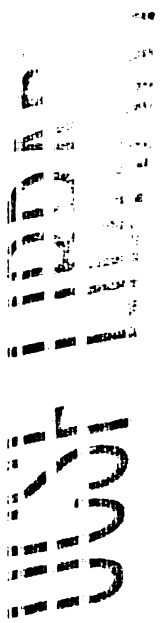


Figure 1. The embryonic ventral spinal cord has a long-range inhibitory effect on NGF-responsive but not NT3-responsive sensory axons.

E14 rat DRG from brachial (forelimb) levels (outlined with dots) were cultured for 40 h at a distance from ventral spinal cord explants (on right hand side of figure) in collagen gels in medium containing 50 ng/ml NGF (**A**) or 50 ng/ml NT3 (**B**). Ventral spinal cord explants were positioned with floor plate (fp) to the right. Cultures were stained with the anti-neurofilament antibody NF-M. Neurites growing from DRG cultured with NGF but not NT3 were redirected away from the ventral spinal cord explant. (**C**) Schematic representation of the method used to quantify neurite outgrowth. The field surrounding the DRG was divided into four quadrants, and the distance from the explant to the neurite front was measured in the proximal quadrant and the distal quadrant as described in Experimental Procedures. Scale bar, 200 μm.

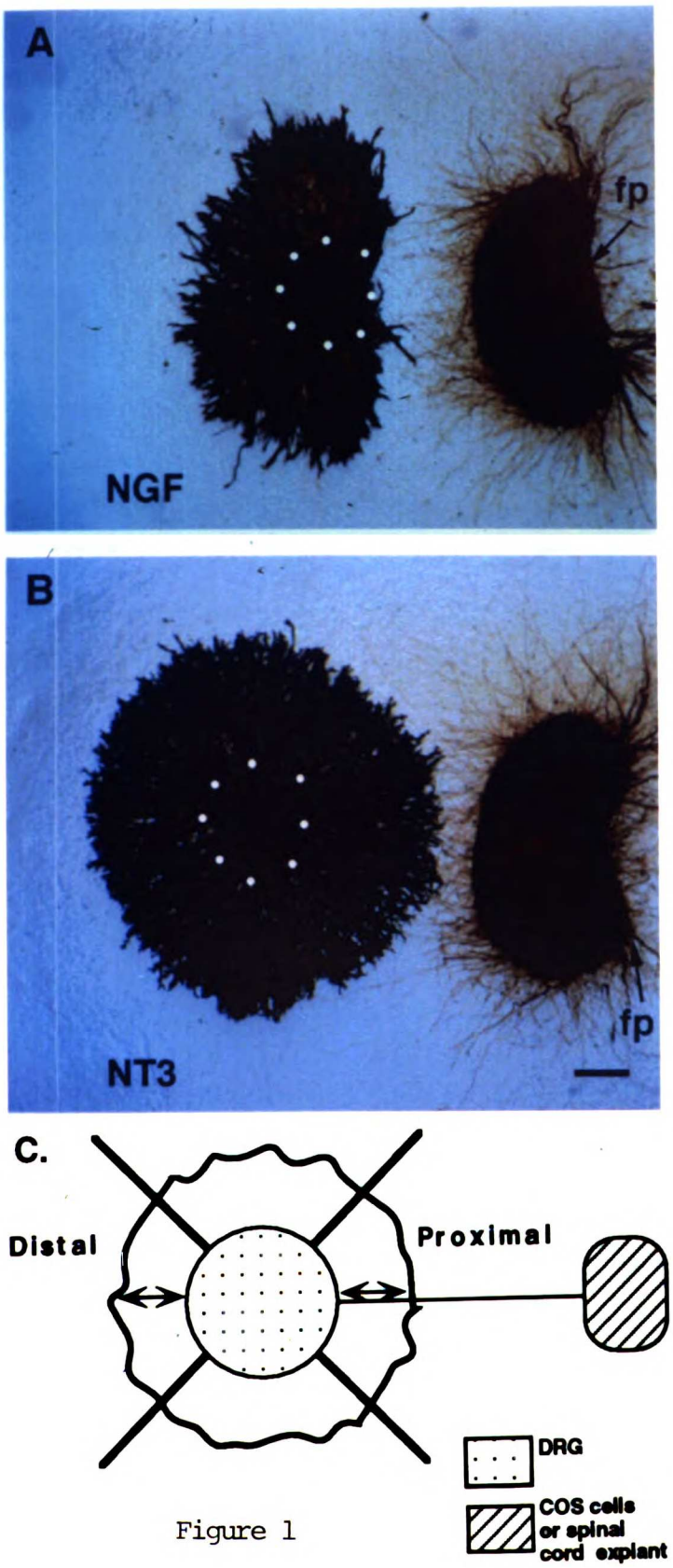

A
fp
NGF
B
fp
NT3
C.
Distal
Proximal
DRG
COS cells
or spinal
cord explant

Figure 1

Figure 2. The degree of inhibition of NGF-responsive neurites by ventral spinal cord explants decreases with distance.

DRG were cultured with ventral spinal cord explants in medium containing 50 ng/ml NGF as shown in Figure 1A. Length of outgrowth in the proximal quadrant (squares) and the distal quadrant (triangles) (quantified as shown in Figure 1C) was plotted as a function of the distance between the proximal edges of the ventral spinal cord explant and the DRG. A strong correlation was observed between distance separating the explants and the length of the outgrowth from the proximal quadrant (line fitted by linear regression, $r = 0.84$). Only a very modest correlation was found for the distance separating the explants and the length of the outgrowth in the distal (control) quadrant ($r = 0.54$)

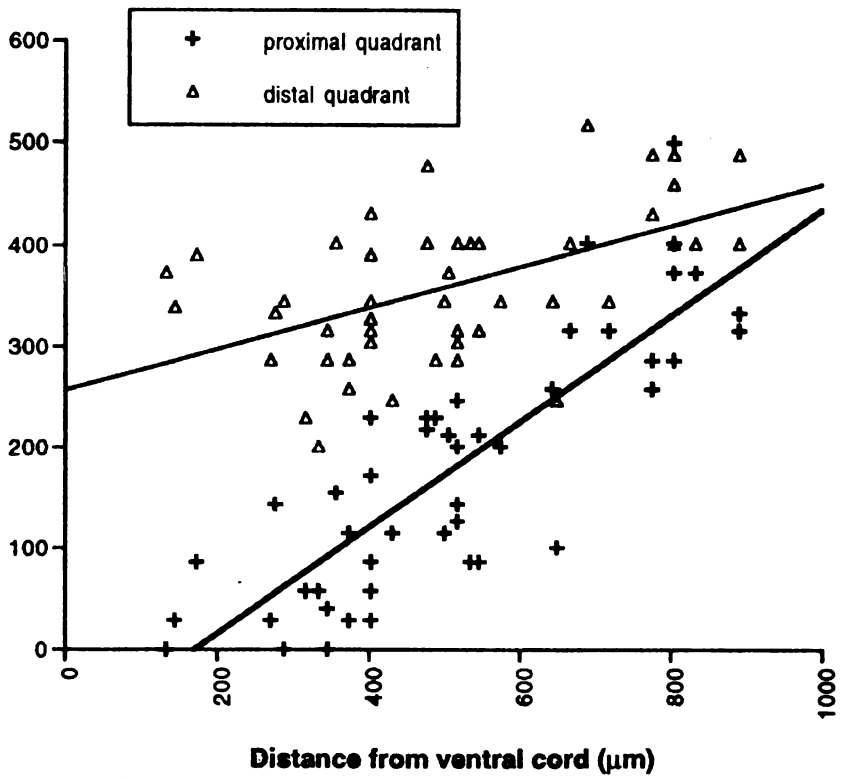
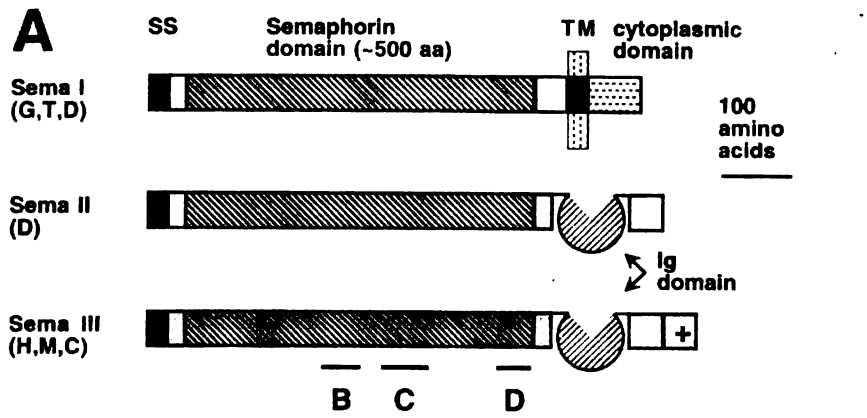

proximal quadrant
distal quadrant
600
500
400
300
200
100
0
0
200
400
600
800
1000
Distance from ventral cord (μm)

Figure 2

Figure 3. Comparison of the murine semaphorin III sequence with the sequences of other insect and vertebrate semaphorins.

(A) Diagram of structures of sema I [a transmembrane semaphorin identified in three insect species: grasshopper (G), Tribolium (T) and Drosophila (D)], sema II (a secreted semaphorin identified in Drosophila), and sema III (a secreted semaphorin previously identified in human and chick, and reported here in mouse). All proteins have a signal sequence (SS, filled area) and share a ~500 amino acid (aa) conserved semaphorin domain (hatched). The secreted proteins have a single immunoglobulin domain (Ig). In addition, sema III has a basic domain at its extreme C terminus (+). Lines over "B", "C" and "D" indicate the regions of the semaphorin domain whose sequences are compared in (B), (C) and (D) below. **(B - D)** Comparison of the amino acid sequences of the three insect sema I proteins (G-, T- and D-SI), the Drosophila sema II (SII) protein, the human sema III protein (SIII), and sequences encoded in eight human expressed sequence tags (EST1-8) from the dbest database (see Experimental Procedures). Where the murine sema III sequence diverges from the human, the residue in the murine sequence is shown immediately above the corresponding residue in the human sequence. Some of the EST sequences contain putative frameshifts and are adjusted accordingly. The EST sequences appear to code for at least three and as many as eight distinct human members of the semaphorin family in addition to sema III. Boxed letters are all residues found in the ESTs that are identical to the corresponding residue in at least one of the previously known semaphorin proteins. Note that the EST sequences appear to fall into different subclasses. EST7 is most closely related to the three insect sema I sequences. EST1-5 are most closely related to sema III. EST6 and EST8 are most closely related to each other, and show hallmarks of both sema I and sema III. The sequence shown for each EST is only a portion of the sequence available in the database. In addition to the eight ESTs shown here, dbest also contains a portion of the sema III sequence. Accession numbers: EST1, T48905 and T49107; EST2, Z21993;



B

```
G-SI  AIYSRVARVCKHDKGGPHQF-GDRWTSFLKSRLNCSVPGD-YPFYF---NEIQ 282
T-SI  VIYSRVARVCKDDKGGPHQ-SRDRWTSFLKARLNCSIPGE-YPFYF---DEIQ 280
D-SI  AIYSRVARVCKNDKGGPHRF-RNRWTSFLKSRLNCSIPGD-YPFYF---NEIQ 276

SII   AVYSRIARVCKKDVGGKNLL-RHNWATYLKARLNCSISGE-FPFYI---NEIG 329

SIII  ATHARIGQICKNDFGG-HRSLVNKWTTFLKARLICSVPGP-NGIDTHH-DELQ 309

EST2                                LKGRIVCSVPGE-DGIETHH-DEIG
EST3                        RVWAMSQWTTFLKARL
EST7  AFFPEVAGVLKNDMGGSQRVWEKQWTSFLKARLNCSVPGRLSHFYH---NILG
```

C

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G-SI  GAFSRKSILESFD-GPFKEQETMNSNWLAVPSLKVPEPRPGQCVNDSR---------TLFL 367
T-SI  GADQMADILRVFE-GSFKHQETINSNWLPVPQNLVPEPRPGQCVRDSR---------ILPD 365
D-SI  GAFALQDIADTFE-GQFKEQTGINSNWLPVNNAKVPDPRPGSCHNDSR---------ALFL 361

SII   GSFHINEIQAAFN-GKFKEQSSSSNSAWLPVLNSRVPEPRPGTCVNDTS---------NLPD 408
                  L

SIII  GTSMSDVRRVFL-GPYAHRDGPNYQWVP-YQGRVPYPRPGTC--PSKTFGGFDSTKDLFL 397

EST2  GVYSMADIRMVFN-GPFAHKEGPNYQWMP-SQGRVPYPRPGTCPGGNLHGXSMKSTKQYPG
EST4     RPQDIRTVLN-GPFRELKHDCNRGLPVVDNDVPQPRPGECITNNMKLRHFGSSLSLPD
EST5  GEYQLKEVQRVFE-GPYKEYHGEPQKWDR-YTDXVPSPRPGSCINNWHRXHGYTSSLELPN
```

D

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G-SI  DVWVSDDEILAEKLHRCGSDKITNCRECVSLQDPYCAWDNVELKC 521
T-SI  VVWVGKDEIRLANLNHCASS-K-TRCKDCVELQDPHCAWDAKQNLC 511
D-SI  SIIVTDSQVVAECLHRCHNDKITSCSFCVALQDPYCAWDKIAGKC 525

SII   LYIGTDHRIKQIDLAMG-NRRYDNCFRCLV--RDPYCGWDKEANTC 550
                                              S

SIII  LYIGSTAGVAQLPLHPCDIYG-KACAECCLARDPYCAWD--GSAC 542

EST1  LTQASRTG-GQIALHRCAAHX-RVCIECCLARDPYCAWD  GVAC
EST6  LFAGSRSQLVQLPVADCMKY--RSCADCVLARXPYCVWSVNTSRC
EST8  LLVGSRTEVTQVNTTNCGRLQ-QSCSECILAQDPVCAWSFRLDEC
```

Figure 3

Figure 4. Expression of *semaIII* mRNA in the developing rat ventral spinal cord. **(A, B)** Dark field (A) and bright field (B) views of a transverse section through the spinal cord of an E14 rat embryo hybridized with an antisense *semaIII* riboprobe, showing high levels of *semaIII* transcripts in the ventral half of the spinal cord. Asterisk (*) indicates intense hybridization in the ventricular zone. No expression was detected in the floor plate (fp). Hybridization was also observed in a stripe of cells just lateral to the DRG. Hybridization was not observed with the corresponding sense probe, except in the surface ectoderm; counts of silver grains overlying the surface ectoderm at E18 failed to reveal any difference in the intensity of labelling between sense and antisense, suggesting that the apparent ectodermal hybridization is artifactual (data not shown). Double headed arrow indicates section orientation: D, dorsal; V, ventral. **(C, D)** Dark field (C) and bright field (D) views of a transverse section through the spinal cord of an E18 rat embryo processed in the same way as the section shown in (A). The pattern of expression of *semaIII* transcripts is similar to that observed at E14, but the level of expression is lower. Scale bar, 100 μm.

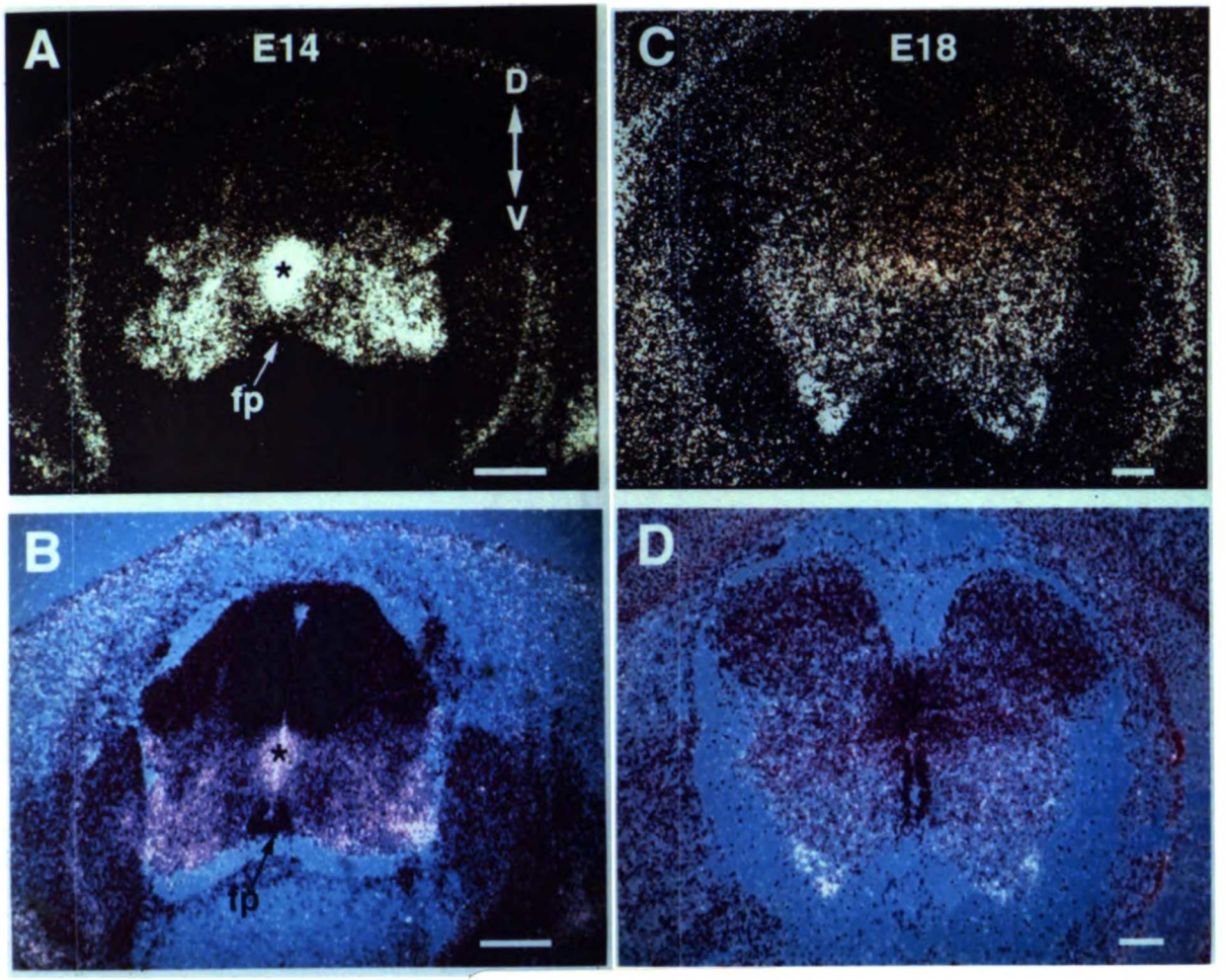


Figure 4

Figure 5. COS cells secreting sema III inhibit NGF-responsive but not NT3-responsive sensory axons.

E14 rat DRG from brachial (forelimb) levels (outlined with dots) were cultured for 40 h at a distance from aggregates of control COS cells (-) **(A, C)** or COS cells secreting recombinant human sema III (+) **(B, D)** in medium containing 50 ng/ml NGF **(A, B)** or 50 ng/ml NT3 **(C, D)**. Cultures were stained with the anti-neurofilament antibody NF-M. Outgrowth from DRG cultured with NGF but not NT3 is repelled by COS cells secreting sema III. Scale bar, 200 μm.

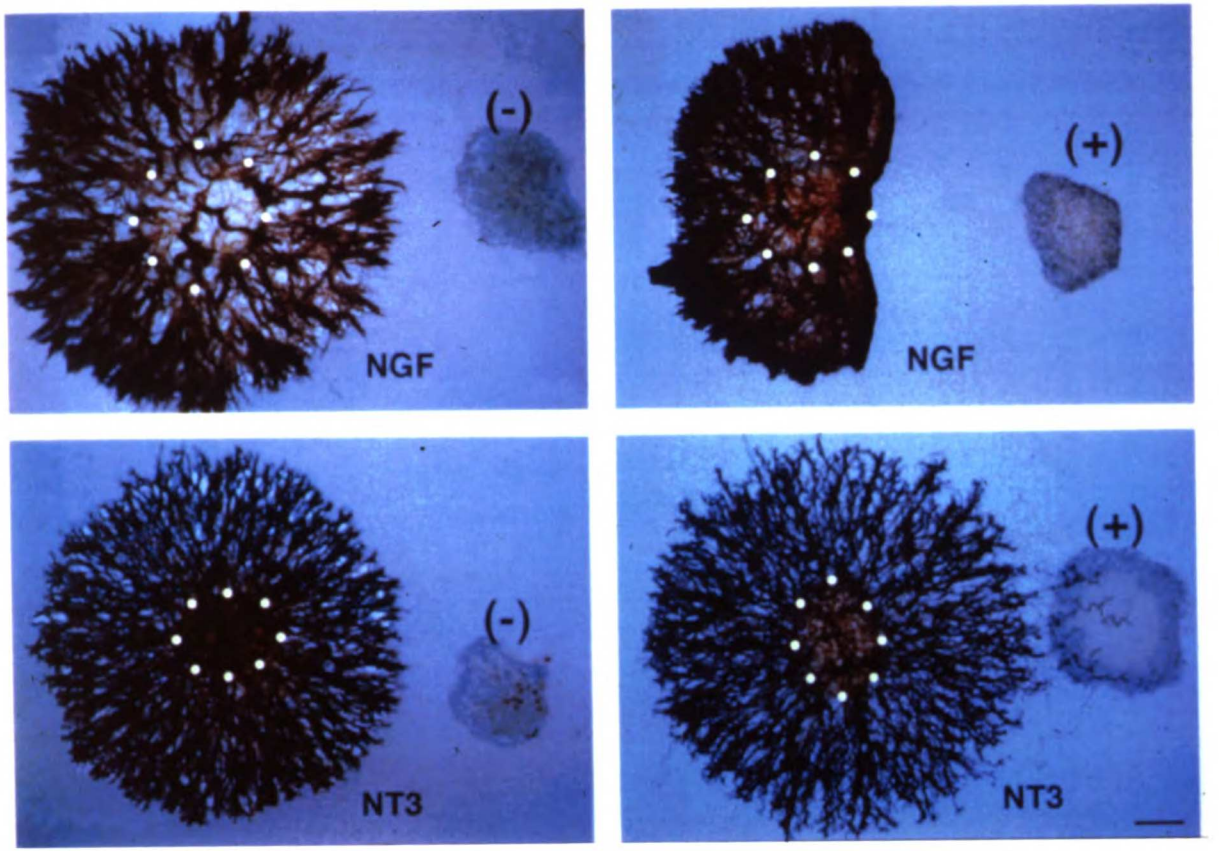
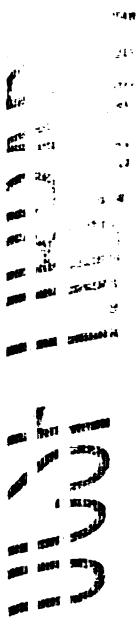


Figure 5

Figure 6. Trajectory of NGF-responsive axons and NT3-responsive Ia afferents in relation to *semaIII* expression in the embryonic rat spinal cord.

Group Ia afferents (NT3-responsive) (open circle) enter the spinal cord from the medial aspect of the dorsal funiculus starting around E14. NT3-responsive axons also include large diameter afferents that relay information to the cerebellum via Clarke's column (which is located at intermediate levels along the dorsoventral axis in the thoracic and high lumbar spinal cord) or the lateral cuneate nucleus in the medulla (not shown, see text). Axons from NGF-responsive neurons (solid circle) project into the spinal cord starting around E16, and target the dorsal-most laminae I and II. *semaIII* transcripts are detected in the ventral spinal cord during this period. It is proposed that sema III protein emanating from the ventral spinal cord contributes to targeting the NGF-responsive axons to laminae I and II. An additional class of afferents is those from low threshold mechanoreceptors (stippled circle) which enter the spinal cord medially, overshoot their targets and then turn upward to terminate in laminae III and IV. The neurotrophin dependence of these cells is uncertain; our experiments do not address the role if any of sema III in directing their projection. Diagram adapted from Snider (1994). See text for details.



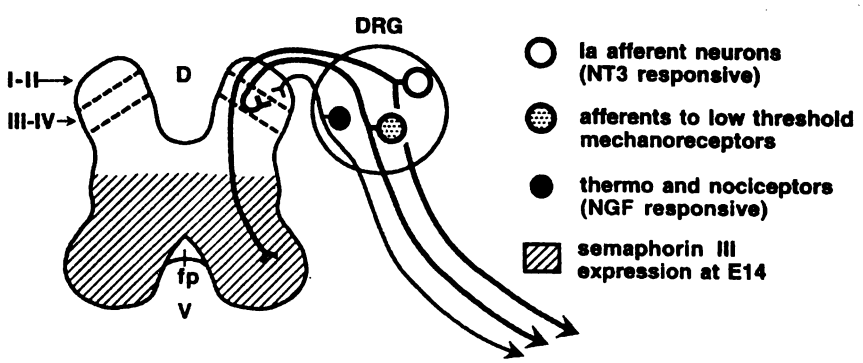

DRG
I-II
III-IV
D
V
fp
V
Ia afferent neurons
(NT3 responsive)
afferents to low threshold
mechanoreceptors
thermo and nociceptors
(NGF responsive)
semaphorin III
expression at E14

Figure 6

Table 1. Outgrowth ratios of DRG neurons when co-cultured with E14 ventral spinal cord explants or with COS cell aggregates.

Culture condition	Mean ratio of quadrant (1:3) ± standard error.	Mean ratio of quadrant (2:4) ± standard error.	Sample size n=	t-test: two sample assuming unequal variance. p-value=
Ventral cord co-cultures:				
NGF 50 ng/ml	.47 ± .08	1.01 ± .04	47	<.0001
NT3 50 ng/ml	.92 ± .03	1.02 ± .05	50	n.s.
Mock transfected COS cell co-cultures:				
NGF 50ng/ml	1.03 ± .02	1.06 ± .02	21	n.s.
NT3 50 ng/ml	.99 ± .02	.98 ± .01	22	n.s.
Sema III transfected COS cell co-cultures				
NGF 50 ng/ml	.07 ± .01	1.01 ± .23	24	<.0001
NT3 50 ng/ml	.91 ± .02	.98 ± .02	24	n.s.

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Chapter 3

Vertebrate homologues of *C. elegans* UNC-5 are candidate netrin receptors

In the developing nervous system, migrating cells and axons are guided to their targets by cues in the extracellular environment. The netrins are a family of phylogenetically-conserved guidance cues that can function as diffusible attractants and repellents for different classes of cells and axons [1-10]. Recent studies in vertebrates, insects and nematodes have implicated members of the DCC subfamily of the immunoglobulin (Ig) superfamily as receptors involved in migrations toward netrin sources [6, 11-13, 14]. The mechanisms that direct migrations away from netrin sources (presumed repulsions) are less well understood. In Caenorhabditis elegans, the transmembrane protein UNC-5 [15] has been implicated in these responses, as loss of unc-5 function causes defects in these migrations [16, 17], and ectopic expression of unc-5 in some neurons can redirect their axons away from a netrin source [18]. Whether UNC-5 is a netrin receptor or simply an accessory to such a receptor has not, however, been defined. We report the identification of two vertebrate homologues of UNC-5 which, with UNC-5 and the product of the mouse rostral cerebellar malformation gene (rcm) [19], define a novel subfamily of the Ig superfamily, and whose mRNAs show prominent expression in various classes of differentiating neurons. We provide evidence that these two UNC-5 homologues, as well as RCM, are netrin-binding proteins, supporting the hypothesis that UNC-5 and its relatives are netrin receptors.

cDNAs encoding two rat homologues of UNC-5, termed UNC5H1 and UNC5H2, were isolated from an E18 rat brain cDNA library (see Methods). The predicted proteins show sequence similarity with UNC-5 over their entire lengths (Fig. 1a), but are more similar to one another (52% identity) than to UNC-5 (28% identity in each case). Like UNC-5 [15], both possess two predicted Ig-like domains and two predicted thrombospondin type-1 repeats in their extracellular domains, a predicted membrane spanning region, and a large intracellular domain (Fig. 1b). The UNC5H proteins also each possess a signal sequence which, curiously, is lacking in UNC-5 [15]. The predicted topology of the

UNC5H proteins in cell membranes (Fig. 1b) was verified using recombinant versions of the proteins expressed in transfected cells and antibodies directed against the extracellular and intracellular domains (see methods). The cytoplasmic domains of the two UNC5H proteins do not contain obvious signaling motifs, but do possess a small region of homology to Zona Occludens-1 (ZO-1) (Fig. 1c), a protein that localizes to adherens junctions and is implicated in junction formation[20, 21]. ZO-1 contains PDZ-domains[20, 21], structures implicated in protein clustering[22], but the region of homology with UNC-5 homologues corresponds to a unique sequence at the carboxy terminus of ZO-1 (Fig. 1c). The homology between ZO-1 and C. elegans UNC-5 is less pronounced (and is not detected by computer BLAST search), but is nonetheless apparent when all four sequences are aligned (Fig. 1c). This homology is also apparent in the newly identified UNC-5 homologue RCM[19]. RCM and UNC5H2 are more similar to one another (66% identity) than is either to UNC5H1 (55% identity between RCM and UNC5H1).

To determine whether UNC5H1 and UNC5H2 are candidates for receptors involved in neuronal migration or axon guidance, we first examined their sites of expression by RNA in situ hybridization in rat embryos. *Unc5h1* transcripts are detected at early stages of neural tube development in the ventral spinal cord (Fig. 2a-c). At embryonic day 11 (E11), when motoneurons are beginning to differentiate in that region[23], transcripts are present throughout the ventral spinal cord, excluding the midline floor plate region, but are most intense in the ventricular zone and at the lateral edges (Fig. 2a and data not shown). At E12, expression is observed in the motor columns and is becoming excluded from the ventricular zone, and also extends more dorsally into a region encompassing the cell bodies of some commissural and association neurons (Fig. 2b). This more dorsal expression appears transient, as expression by E13 is confined to postmitotic cells in the ventral spinal cord, apparently including the motoneurons (Fig. 2c). *Unc5h2* transcripts are not detected at significant levels in the spinal cord until E14, when they are found in

the roof plate region (Fig. 2d). They are, however, detected in developing sensory ganglia that flank the spinal cord, at low levels at E12 (data not shown), and at higher levels by E14 (Fig. 2d). The expression of these two genes is thus observed in regions where differentiating neurons are undergoing axonogenesis, consistent with a possible role in this process.

Expression of these genes is also observed at higher axial levels of the nervous system, as well as in non-neural structures. At E13, *Unc5h1* is expressed in the basal plate (ventral neural tube) in the hindbrain and midbrain, in the developing hypothalamus and thalamus, and in the pallidum (Fig. 3a). *Unc5h2* expression at this stage is detected in the nervous system in the dorsal aspect of the developing optic cup and in restricted regions of the midbrain and caudal diencephalon (Fig. 3b and data not shown). By E16, *Unc5h1* mRNA is also detected at high levels in the entorhinal cortex and at lower levels throughout the cortex (data not shown). *Unc5h2* is also detected at this stage at low levels in the cortex, and at high levels in hypertrophic chondrocytes (data not shown). Expression of the two homologues persists postnatally, with, at postnatal day 10 (P10), continued expression of both at low levels throughout the cortex, expression of both in distinct patterns in the septal area, and high level expression of *Unc5h1* in the developing hippocampus and entorhinal cortex (Fig. 3c, d). In addition, a prominent site of postnatal expression of both genes is in the cerebellum (Fig. 3c-f). Both are expressed in the inner granule cell layer (Figs. 3e, f), and *Unc5h2* is in addition expressed in the inner aspect of the external germinal layer (Fig. 3f), where granule cell precursors differentiate prior to migrating to their final destination in the inner granule cell layer[24, 25].

Thus, the expression patterns of the two *Unc5h* genes are suggestive of potential roles in cell or axon migration. It is particularly striking that *Unc5h2* expression overlaps with *rcm* in granule cells that are about to undergo a migration that is defective in *rcm*

mutant mice[19]. The co-expression of these related proteins might account for the fact that only a subset of *rcm* expressing cerebellar cells are affected in *rcm* null mutants[19].

To test whether UNC-5 homologues can be involved in mediating responses to netrins we tested whether netrin-1 can bind cells expressing these proteins. Transfected human embryonic kidney 293 cells expressing UNC5H1, UNC5H2, or RCM showed significant binding of netrin-1 protein above background (Fig. 4a-d, and data not shown), as is also observed for transfected cells expressing the netrin receptors DCC and neogenin, but not for transfected cells expressing TAG-1 or L1, two other members of the Ig superfamily[13]. In these experiments, binding was performed in the presence of soluble heparin, which eliminates non-specific binding of netrin-1 to the cells[13] but does not evidently prevent binding to the UNC5 homologues. Similar specific binding was observed when cells were incubated with netrin-1 without added heparin and only subsequently washed with heparin-containing medium (data not shown), indicating that heparin is not required for the binding interaction. To verify further, in the case of UNC5H2, that exogenously added heparin is not required for the interaction, we generated a soluble protein comprising the extracellular domain of UNC5H2 fused to the constant region (Fc) of a human immunogloblin molecule. This UNC5H2-Fc fusion protein bound transfected 293 cells expressing netrin-1 (some of which remains associated with the surface of these cells[3, 10]) in the absence of added heparin (Fig. 4e) but did not show binding to non-transfected cells (Fig. 4f), nor to cells expressing UNC5H2 itself, DCC, or neogenin (data not shown). The UNC5H2-Fc fusion also did not bind transfected cells expressing F-spondin, an adhesive extracellular matrix protein made by floor plate cells[26], or Semaphorin III, a chemorepellent for sensory axons at the stages that *Unc5h2* is expressed in sensory ganglia[27](data not shown). Both of these proteins, like netrin-1, are secreted but partition between cell surfaces and the soluble fraction[26, 28]. Thus, the interaction between netrin-1 and UNC5H2 appears specific, and

does not require heparin nor reflect a generalized interaction with proteins that associate non-specifically with cell surfaces.

The affinity of UNC-5 homologues for netrin-1 was estimated in equilibrium binding experiments using netrin(VI•V)-Fc, a fusion of the amino terminal two-thirds of netrin-1 to the constant portion of human IgG[13]. This netrin-1 derivative is bioactive but, unlike netrin-1, does not aggregate at high concentrations, and it binds DCC with a Kd comparable to that of full length netrin-1[13]. Specific binding of netrin(VI•V)-Fc to each of the three UNC5 homologues showed saturation and the binding curves were fitted to the Hill equation (Fig. 5a-c), yielding Kd values of 19 ± 0.8 nM, 3.4 ± 1.0 nM, and 6.9 ± 1.8 nM for UNC5H1, UNC5H2 and RCM respectively. These values are comparable to the Kd for the DCC-netrin(VI•V-Fc) interaction (~5 nM), and are consistent with the effective dose for the axon outgrowth promoting effects of netrin-1[2, 13].

The phenotype of *rcm* mutant mice implicates RCM in cell migration[19], but establishing the involvement of UNC5H1 and UNC5H2 in cell migration and axon guidance will require perturbing their functions in vivo. In the meantime, however, our results are at least consistent with such an involvement, as these homologues are expressed by some populations of cells that are undergoing migrations or extending axons. For example, *Unc5h1* is expressed by spinal motoneurons, whose axons are repelled in vitro by floor plate cells[29], and whose outgrowth in vitro can be suppressed by netrin-1[30]. It is also expressed in the region of trochlear motoneurons, which can be repelled by netrin-1[4].

Our evidence that vertebrate UNC5H proteins bind netrin-1 provides direct support for the idea that members of this new subfamily of the Ig superfamily are netrin receptors. This idea was first proposed for C. elegans UNC-5, based on the findings that *unc-5* is required cell-autonomously for dorsal migrations that require the function of the netrin

UNC-6[15], and that ectopic expression of *unc-5* in neurons that normally project longitudinally or ventrally can steer their axons dorsally[18]. Although consistent with the possibility that UNC-5 is an UNC-6 receptor, these results are also consistent with a role for UNC-5 in modifying the function of a distinct UNC-6 receptor. A modifier function was made more plausible by evidence that the DCC homologue UNC-40, a putative UNC-6 receptor involved in ventral migrations[11], is expressed by axons that project dorsally and is required for those projections[11, 16, 17], suggesting that UNC-5 might function by switching an attractive netrin receptor (UNC-40) into a repulsive netrin receptor. Although such a switching role is still possible, our results suggest that UNC-5 might itself also function directly as a netrin receptor. A model in which UNC-40 and UNC-5 can form a receptor complex but UNC-5 can also function alone in transducing the UNC-6 netrin signal would provide an explanation for the observation that loss of *unc-40* function results in a much less severe phenotype for dorsal migrations than do either loss of *unc-5* or loss of *unc-6* function[17, 18]. It is noteworthy in this context that *Unc5h1* and *Unc5h2* are expressed in regions of the vertebrate nervous system where the *unc-40* homologues *DCC* and *neogenin* are expressed (e.g., dorsal and ventral spinal cord, dorsal retina, dorsal root ganglia, and cerebellum (refs. 13 and 31 and data not shown). Thus, a model in which UNC-5 and UNC-40 form a receptor complex might also be applicable to vertebrates.

Recent studies have demonstrated a remarkable phylogenetic conservation in function of netrin proteins in guiding axons towards a source of netrin at the midline of the nervous systems of nematodes, flies and vertebrates[1, 7, 8, 9], as well as a conserved role for members of the DCC subfamily of the Ig superfamily in mediating the axonal responses that underlie those guidance events[11, 12, 13]. The identification of vertebrate homologues of UNC-5, and the evidence that they are netrin-binding proteins, raises the

possibility that the signaling mechanisms through which netrins elicit repulsive responses are also conserved.

Methods

Isolation of rat UNC-5 homologues, and *in situ* hybridization. A search of the human expressed sequence tag (EST) databases revealed a small sequence (Genbank accession number R11880) with distant similarity to the carboxy-terminal portion of UNC-5. The corresponding cDNA fragment, amplified by polymerase chain reaction from an embryonic human brain cDNA library (Stratagene), was used to screen the library, resulting in the isolation of a 3.8 kB cDNA clone comprising all but the first 440 nt of the coding region of the human homologue of UNC5H1. Non-overlapping probes from this cDNA were used to screen an E18 rat brain library (gift of S. Nakanishi), leading to the isolation of seven partial and one full length UNC5H1 cDNA and one full length UNC5H2 cDNA. Additional screens of E13 rat dorsal and ventral spinal cord libraries resulted in isolation of a second full length UNC5H2 cDNA as well as a nearly full length UNC5H1 cDNA. Sequencing was performed on a Licor (L4000) automated sequencer as well as by ^{33}P cycle sequencing. Genbank accession numbers are U87305 and U87306 for rUNC5H1 and rUNC5H2 respectively. RNA *in situ* hybridization was performed as described [13].

Antibodies, expression constructs and immunohistochemistry. Rabbit polyclonal antisera were raised to a peptide corresponding to a sequence (YLRKNFEQEPLAKE) in the extracellular domain of UNC5H2 that is almost completely conserved in UNC5H1 (one amino acid substitution), and to peptides corresponding to unique sequences in the

cytoplasmic domains of UNC5H1 (GEPSPDSWSLRLKKQ) and UNC5H2 (EARQQDDGDLNSLASA). Antisera were affinity-purified on their respective peptides (Quality Controlled Biochemicals). cDNAs for the various constructs were subcloned into the expression vectors pMT21 (Genetics Institute) and pCEP4 (Invitrogen) (for UNC5H1 and UNC5H2), or pRc/CMV (Invitrogen) (for RCM), and transiently transfected into 293 cells using lipofectamine. The antiserum to the extracellular peptide can detect UNC5H1 and UNC5H2 proteins expressed in transfected cells without cell permeabilization, whereas the antisera directed against the cytoplasmic domain peptides detected their respective proteins after cell permeabilization (data not shown). Netrin-1 protein was produced, purified, used and visualized in binding assays as described [13], except that a monoclonal antibody (9E10)[32] directed to a C-terminal myc-epitope tag was used to detect recombinant netrin-1. Quantitative binding with netrin (VI•V)-Fc on 293T cells transiently transfected with UNC5H1, UNC5H2 or RCM was performed essentially as described [13], except that after incubation with ligand, cells were washed once rapidly with 600 µl PBS and then fixed with 100% methanol followed by 4% paraformaldehyde. Cells were then washed once in PBS prior to proceeding with the secondary antibody incubation. The full length UNC5H1 protein appears to be toxic for transfected 293 cells (data not shown), so binding experiments used a truncated UNC5H1 protein lacking a portion of the cytoplasmic domain (truncation at amino acid 494), which was not toxic. A 293-EBNA cell line stably expressing the UNC5H2-Fc fusion was derived and maintained as described[10, 13]. The fusion protein was purified from serum-free medium conditioned for seven days by affinity chromatography on protein A agarose. The 293 cell line expressing netrin-1 has been described [13]. Binding of the UNC5H2-Fc fusion to this line was visualized using a Cy3-conjugated secondary antibody (Jackson Immunoresearch) directed against human Fc.

Acknowledgements

We thank A. Faynboym for purification of netrin proteins, C. Mirzayan for the 293 cell line expressing netrin-1, D. Shehu for sequencing, V. Head for assistance with in situ hybridization and S. Nakanishi for the brain cDNA library. Supported by grants to M.T.-L. from the International Spinal Research Trust, the American Paralysis Association, and the Howard Hughes Medical Institute, and to S.L.A. from the National Institutes of Health. E.D.L. was supported by a UCSF Chancellor's Fellowship, L.H. by a fellowship from the Jane Coffin Childs Memorial Fund, and K.K.-M. by the National Defense Medical College, Japan. M.M. is an Associate and M. T.-L. an Assistant Investigator of the Howard Hughes Medical Institute.

Figure 1. Structures of UNC5H1 and UNC5H2. *a*, Sequences of the two rat UNC5H proteins compared to that of *C. elegans* UNC5. Signal peptide sequences in the UNC5H proteins are indicated by a black bar, amino acid identities are indicated by black boxes and similarities by shaded boxes, extents of the the two Ig-like domains (Ig-1 and Ig-2) and the two Tsp type 1 domains (Tsp-1 and Tsp-2) are indicated by arrows, membrane-spanning region is indicated by asterisks, and region of homology with ZO-1 is bounded by ^ symbols. *b*, Predicted topology of UNC5 and its homologues, indicating the predicted structural domains, ZO-1 homology region, and percent sequence identity. *c*, Alignment of sequences of UNC5, UNC5H proteins, and ZO-1, in their regions of homology. Shading as in *a*.

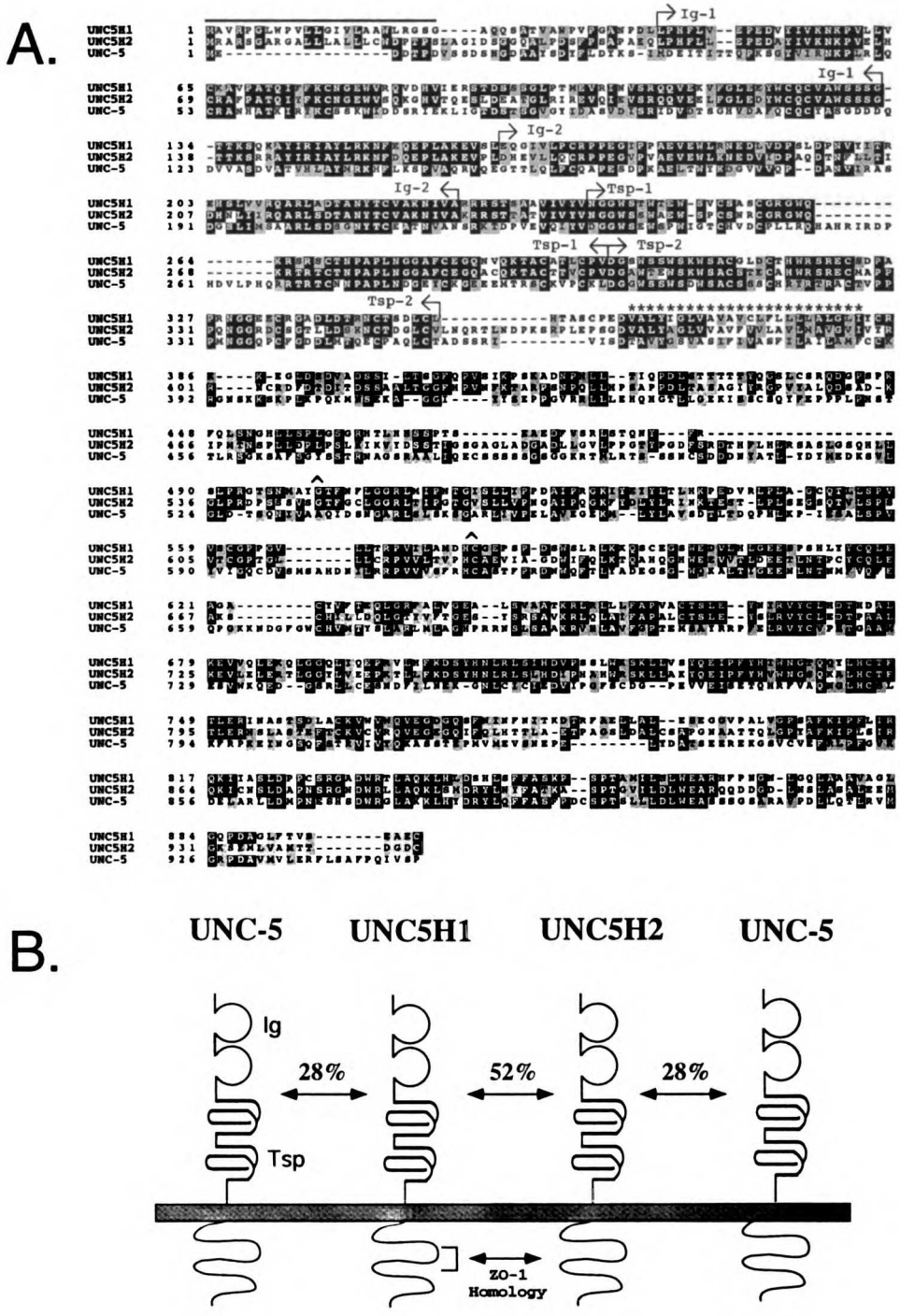

A.
Ig-1
Ig-2
Tsp-1
Tsp-2
B.
UNC-5
UNC5H1
UNC5H2
UNC-5
Ig
Tsp
28%
52%
28%
ZO-1
Homology
C.

Figure 1

Figure 2. *a-c,* Expression of *Unc5h1* mRNA in tranverse sections through the developing rat spinal cord at E11 (*a*), E12 (*b*), and E13 (*c*). Note that at E12, expression is observed in the motor column (m), but also in more medially positioned cells in the ventral spinal cord (vertical arrowhead) as well as more dorsally (horizontal arrowhead). *d*, expression of *Unc5h2* in developing sensory ganglia and roof plate of the spinal cord in an E14 rat embryo. Abbreviations: d, dorsal root ganglia, f, floor plate; m, motor column, r, roof plate, v, ventricular zone. Scale bar shown in *d* is 30, 100, 130, and 320 μm in *a*, *b*, *c*, and *d*, respectively.

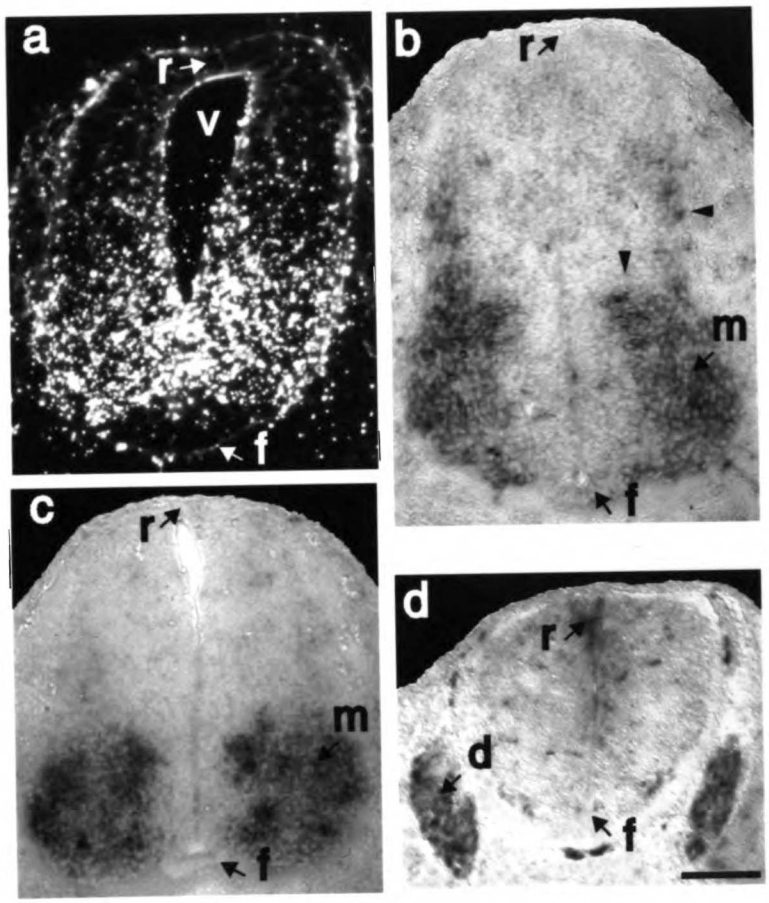


Figure 2

Figure 3. Other sites of expression of *Unc5h1* (*a*, *c*, *e*) and *Unc5h2* (*b*, *d*, *f*). *a*, *b*, Expression at E13, visualized in a parasagittal section through the head (*a*) or in an embryo whole-mount (*b*). Asterisk in *b* indicates expression at the midbrain hindbrain junction. *c*, *d*, Semi-adjacent horizontal sections of P10 brain. Asterisks indicate expression in cerebral cortex. *e*, *f*, Semi-adjacent parasagittal sections through folia in the cerebellum. Abbreviations: l, limb; b, basal plate; c, cerebellum; d, caudal diencephalon; do, dorsal aspect of the optic cup; dg, dendate gyrus; e_i, inner aspect of the external germinal layer; g, inner granule cell layer; h, hypothalamus; m, ventral aspect of midbrain; n, nasal pits; p, pallidum; s, septal region. Scale bar shown in *f* is 500 μm in *a*, 6.6mm in *b*, 160 μm in *c* and *d*, and 200 μm in *e* and *f*.

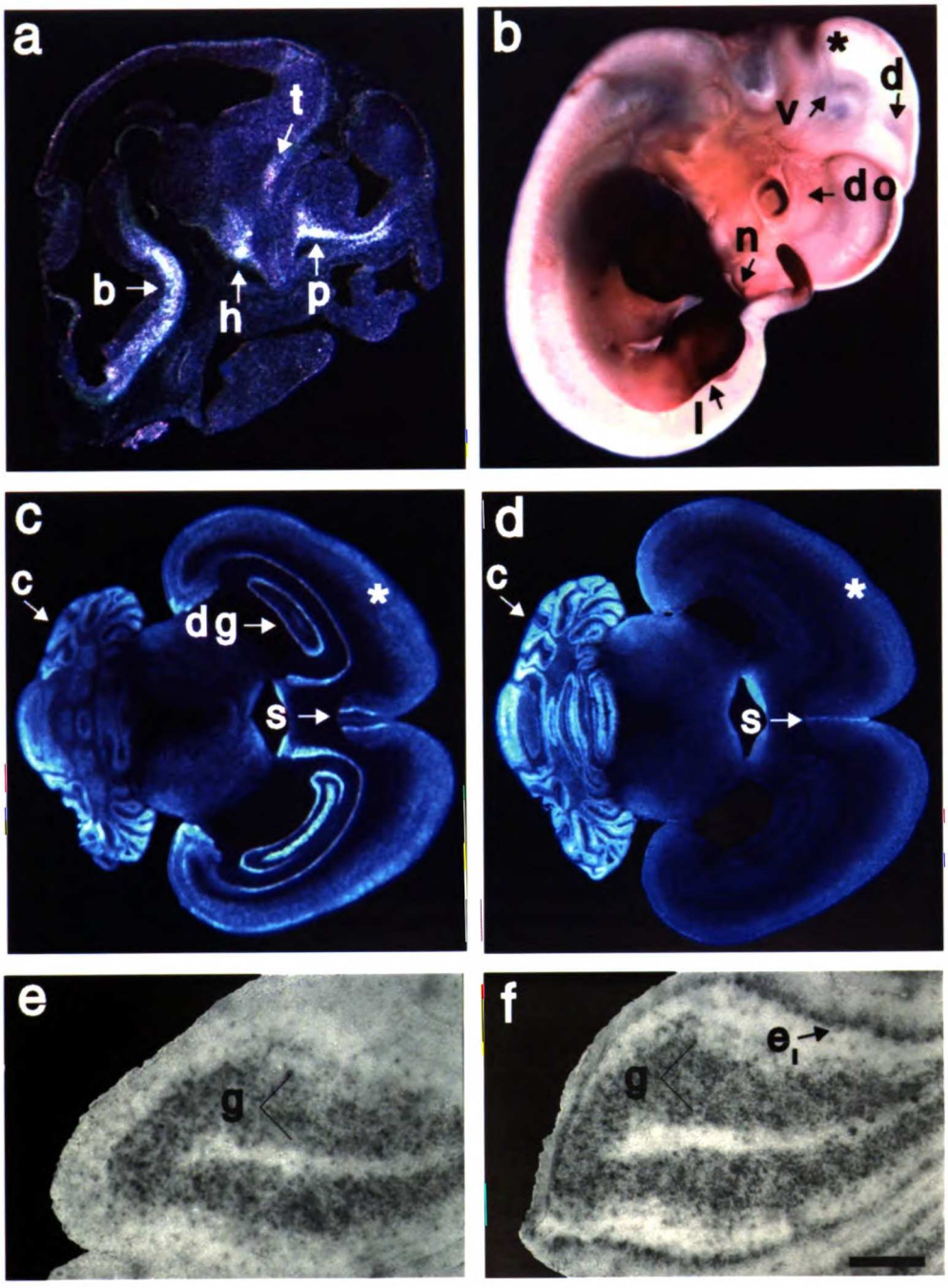


Figure 3

Figure 4. Interactions of netrin-1 and UNC5H proteins, as demonstrated in experiments in which purified netrin-1 (*a-d*) is incubated with cells expressing candidate receptors or in which a soluble UNC5H2-ectodomain-Fc fusion protein is incubated with cells expressing netrin-1 (*e,f*). Panels *a-f* show fluorescence photomicrographs. *a-c*, Binding of netrin-1, detected with antibody 9E10 against a C-terminal myc-epitope on the protein, to 293T cells expressing UNC5H1 (*a*), UNC5H2 (*b*), or RCM (*c*). *d*, In control experiments, netrin-1 did not show significant binding to 293T cells expressing the Ig superfamily member L1. Note that in these transient transfections, only ~10% of cells express the constructs. *e,f*, An UNC5H2-ectodomain-Fc fusion protein binds 293 cells stably expressing netrin-1 (*e*) but not the parental 293 cell line (*f*). Note that all cells express netrin-1 on their surface in this case. Scale bar shown is 66 μm.

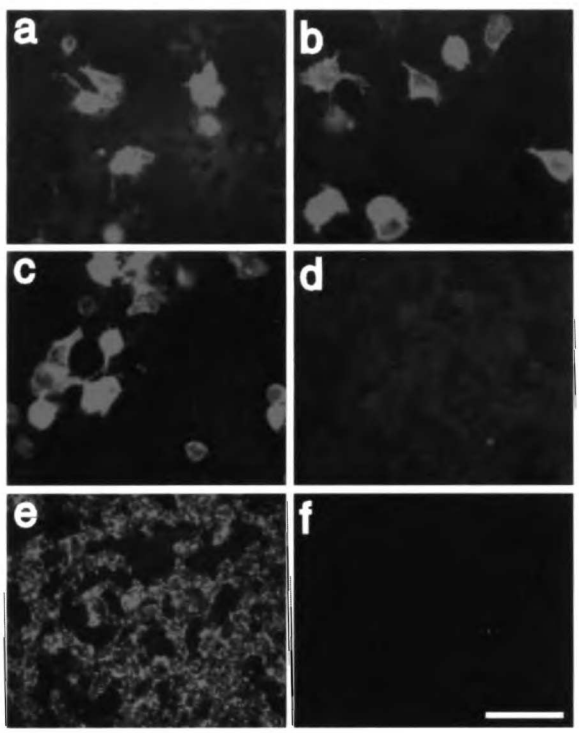
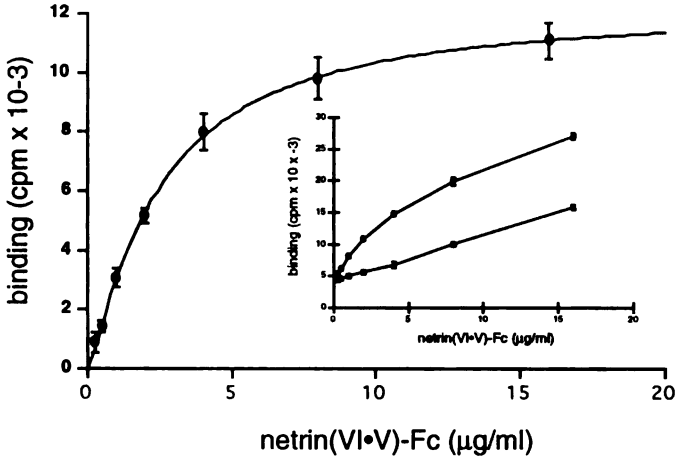


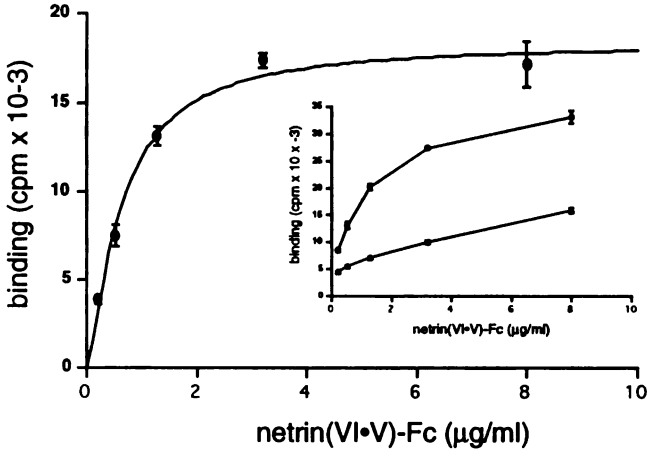
Figure 4

Figure 5. *a-c,* Equilibrium binding of the netrin (VI•V)-Fc fusion protein to UNC5H1 (*a*), UNC5H2 (*b*), and RCM (*c*). Binding of netrin (VI•V)-Fc was determined by measuring the radioactivity associated with the cells after subsequent incubation with radiolabeled anti-human IgG antibody. In each panel, the inset shows raw data (closed circles: total binding to receptor expressing cells; closed triangles: total binding to mock transfected cells). Specific binding curves were fitted using the Hill equation (larger scale graph in each panel). Kd values for the interaction of netrin (VI•V)-Fc with UNC5H1, UNC5H2 and RCM were 3.04 ± 0.13 μg/ml, 0.54 ± 0.17 μg/ml and 1.14 ± 0.29 μg/ml, respectively (1 μg/ml corresponds to 6.25 nM). Bars indicate SEM for triplicates. Results are from one representative of two (UNC5H2) or three (UNC5H1, RCM) experiments performed.

A.


binding (cpm x 10-3)
netrin(VI•V)-Fc (µg/ml)

B.


binding (cpm x 10-3)
netrin(VI•V)-Fc (µg/ml)

C.

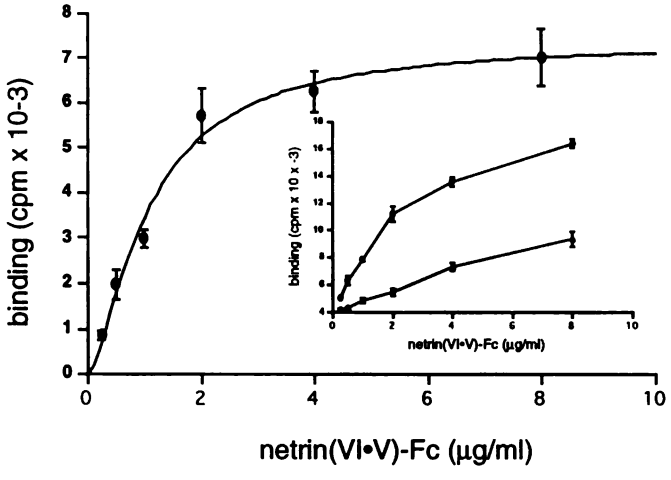

binding (cpm x 10-3)
netrin(VI•V)-Fc (µg/ml)

Figure 5

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Chapter 4

Identification of a novel alternatively spliced UNC5 homologue that does appear to bind netrin-1

Introduction

The netrin family of guidance cues has been remarkably conserved in both structure and function (Ishii et al., 1992; Serafini et al., 1994; Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995; Wadsworth et al., 1996; Mitchell et al., 1996; Harris et al., 1996; Serafini et al., 1996). As discussed previously, netrin-1 is a bifunctional guidance cue that can function as a chemoattractant for some neurons, such as commissural neurons, and as a chemorepellent for others, such as trochlear motor neurons and branchiomotor neurons (Colamarino and Tessier-Lavigne., 1995; Tucker et al., 1996). This bifunctionality is also apparent in *C. elegans*, where the netrin UNC-6 is responsible for guiding circumferential migrations both the toward and away from an UNC-6 source (Hedgecock et al., 1990).

More recently, it has become clear that the cellular receptors responsible for mediating the effects of netrin-1 are also conserved. In *C. elegans*, genetic evidence suggests that UNC-40 is an UNC-6 receptor for ventrally directed migrations (Hedgecock et al., 1990; Chan et al., 1996). Likewise, DCC, a vertebrate homologue of UNC-40 binds netrin-1 and mediates the netrin-evoked outgrowth of commissural axons into collagen gels (Keino-Masu et al., 1996). Mice deficient for DCC have defects in commissural axon guidance, and the phenotype of these mice is virually identical to that of netrin-1 deficient mice (Fazeli et al., 1997). By contrast, the dorsally directed migrations in *C. elegans* require UNC-5, with a minor role for UNC-40, suggesting that UNC-5 like proteins, possibly together with DCC like proteins, mediate repellent effects of netrins. (Hedgecock et al., 1990; Leung-Hagesteijn, 1992; Hamelin et al., 1993). In keeping with this suggestion, the vertebrate homologues UNC5H1, UNC5H2, and UNC5H3 (formerly RCM) bind netrin-1 and are thus candidates to mediate repulsive cues in vertebrate systems (Ackerman et al., 1997; Leonardo et al., 1997).

Although the parallels between *C. elegans* and vertebrates are striking, there is an added level of complexity in the vertebrate systems. To date, only single *unc-5*, *unc-6*, and *unc-40* genes have been identified in *C. elegans*. This stands in stark contrast to the vertebrate system where there are at least two netrins, two DCC like molecules and four UNC-5-like molecules (Serafini et al., 1994; Vielmetter et al., 1994; Keino-Masu et al., 1996; Leonardo et al., 1997). This molecular redundancy raises the possibility that different members of these families may have evolved distinct functions in addition to those that are paralleled in their nematode counterparts.

Here, we report the identification of a novel vertebrate homologue of UNC5, designated UNC5H4. The mRNA for UNC5H4 is alternatively spliced and encodes at least two distinct proteins. We show that UNC5H4, like other vertebrate UNC-5 proteins, is expressed in a spatially and temporally regulated manner, both within and outside of the nervous system. In addition, we provide evidence that unlike other vertebrate UNC-5 homologues, UNC5H4 does not bind netrin-1, raising the possibility of an additional ligand. Finally, as an initial step towards identifying non-netrin ligands for UNC5H4, we demonstrate potential sites of ligand expression using an AP-in situ binding technique.

Results

Identification of a novel vertebrate UNC-5

We have previously described the identification of a family of UNC-5 vertebrate homologues (See chapter 2). Sequences in the human EST database suggested that the family may contain at least one additional member. Using probes derived from these sequences we isolated complementary cDNAs encoding a novel vertebrate homologue of UNC-5 (designated UNC5H4) in rat and mouse (see methods). The rat UNC5H4 gene encodes a predicted transmembrane protein with all the hallmarks of an UNC5 homologue (Fig. 1A). In addition, UNC5H4 appears to be alternatively spliced resulting in cDNAs that encode either a full length UNC5H4 or a splice variant (UNC5H4-tsp) that

is missing the first thrombospondin type-1 repeat but is otherwise identical to the full length form (Fig. 1A, B). PCR analysis suggests that both splice variants are expressed at approximately equal levels in the embryonic day 13 rat brain (data not shown). Alternative splicing has not been reported for the three previously identified UNC-5 homologues, and no function has yet been ascribed the Tsp-1 domain.

The full length UNC5H4 shows 42%, 49% and 45% identity to rat UNC5H1, UNC5H2 and mouse UNC5H3 respectively as well as 21% identity to *C. elegans* UNC-5. While sequence similarity can be detected throughout the entire protein, it is most striking in the extracellular domains (Fig. 1C). This finding is consistent with homologies amongst other UNC-5 family members, which also share homology mainly in the extracellular domain.

UNC5H4 is expressed in the developing spinal cord and cerebellum.

In order to further characterize this new gene, we examined the pattern of mRNA expression, with particular attention to the spinal cord and cerebellum where other family members are known to be expressed (Ackerman et al, 1997; Leonardo et al, 1997). UNC5H4 transcripts are detected in the developing spinal cord at embryonic day 13, when expression can be detected at low levels throughout the spinal cord with highest levels seen in the lateral motor column, consistent with expression in subpopulations of motoneurons (Fig 2A). In addition, strong expression is seen in the lateral aspect of the dorsal spinal cord, perhaps corresponding to expression in commissural cell bodies. At embryonic day 14, transcripts are detected in the ventral spinal cord as well as in the intermediate zone of the spinal cord, excluding both the ventricular zone and the lateral edge (Fig 2B).

Notable sites of high embryonic expression outside of the nervous system include the branchial arches, precursors to the major craniofacial structures (Fig. 3 A-C). Expression progresses from the posterior margin of the hyoid process and the posterior margin of the third branchial arch, to the anterior half of the mandibular process, to the

epibranchial groove of the first branchial arch (between the mandibular and maxillary processes).

Postnatal expression in the developing cerebellum can be seen in Purkinje cells at postnatal day 5 (P5) (Fig. 4A). Low level expression can also be detected in the outer aspect of the external germinal layer. At P10, expression persists in Purkinje cells and in the outer aspect of the external germinal layer but has dramatically expanded to include the internal granule cell layer (Fig. 4B). The expression in the internal granule cell layer is of interest because, to date, all known homologues of UNC-5 are expressed in this layer (Ackerman et al., 1997; Leonardo et al., 1997). In addition, *Unc5h3*^{rcm} mutants show cell autonomous defects in these cells. Interestingly, the expressivity of this granule cell defect is quite low (Ackerman et al., 1997). One possibility for the low expressivity may be the overlapping expression and function of other UNC-5 family members.

UNC5H4 does not bind netrin-1:

We have previously demonstrated that UNC5H1, UNC5H2, and UNC5H3 bind netrin-1 protein with a Kd in the 1-10 nM range (Leonardo et al., 1997). In order to perform similar experiments for UNC5H4 we first confirmed expression of the protein in transfected cells using a polyclonal antibody that cross-reacts with the extracellular domain of UNC5H4. Expression on the surface of cells is detected for UNC5H4 but not for UNC5H4-tsp (Fig 5 A, B and data not shown). Since the only difference between the two clones is the alternatively spliced tsp-1 domain, the reason for this failure to detect expression of UNC5H4-tsp is unclear. One possibility is that in the context of 293T cells, the shorter spliced form cannot fold properly.

In order to determine whether cells expressing full length UNC5H4 bind netrin, we performed netrin binding experiments using 293T cells that were either transiently transfected to express UNC5H4, UNC5H4-tsp, or mock transfected controls (Fig. 5D, E). As a positive control for binding, we used 293T cells expressing UNC5H2 (Fig. 5F).

Under conditions where netrin binding can be detected on cells expressing UNC5H2, no binding is seen for UNC5H4 (Fig. 5E). Similar results were obtained using netrin-2: netrin-2 binds UNC5H2 transfected cells, but fails to bind cells transfected with UNC5H4. These results suggest that netrins do not interact with UNC5H4 in the same manner as they do with the other UNC5H proteins. However, we cannot rule out that UNC5H4 is misfolded or that the netrin-UNC5H4 interaction is too weak for detection in this assay.

Other ligands?

The surprising result that UNC5H4 does not appear to bind netrin-1 raises the possibility that there may be additional, non-netrin, ligands for members of the vertebrate UNC-5 family. One hypothesis is that UNC5H4 binds no ligand and that its intracellular domain acts as a dominant negative, competing for UNC5H binding proteins. Another hypothesis is that UNC5H4 has evolved a ligand distinct from netrin-1. In order to investigate these possibilities, we constructed a chimeric protein that consists of the extracellular domain of UNC5H4 fused to alkaline phosphatase (H4-AP). This reagent was used as a probe to examine sites of receptor-binding in situ (Cheng and Flanagan, 1994). In these experiments, whole embryos are incubated with a fusion protein consisting of UNC5H4 ectodomain coupled to alkaline phosphatase and then incubated with alkaline phosphatase substrate to reveal receptor binding sites. Using this technique, we revealed receptor binding sites in the developing facial structures of an E13 embryo (Fig. 3D). Interestingly, the observed binding sites are near regions where mRNA expression of the receptor is observed by in situ hybridization (Fig. 3C). The pattern is specific to the UNC5H4 fusion protein, as it was not detected using either a UNC5H2 fusion protein or alkaline phosphatase alone (data not shown). The control UNC5H2 protein, but not the UNC5H4 protein, can bind to netrin expressing cells in culture. In addition to failure of UNC5H4-AP fusion protein to bind netrin expressing cells in

culture, netrin-1 does not appear to be expressed in this region. Taken together, this data suggests that UNC5H4-AP may be binding to a ligand distinct from netrin-1.

Discussion

We have described the identification of a novel member of the vertebrate UNC-5 family. Like the other known vertebrate UNC-5 homologues, UNC5H4 mRNA is expressed in a temporally and spatially restricted fashion both within and outside of the developing nervous system. A novel feature of UNC5H4 is the alternative splicing of the first thrombospondin type-1 repeat. Although the functional implication of this splicing is unknown, it is possible that this domain plays a regulatory role by altering receptor specificity. To begin to address this possibility, it will first be essential to show that both isoforms are expressed on the cell surface since, at least in 293T cells, the tsp-1 deleted form does not appear to be appropriately directed to the plasma membrane.

Previously described UNC-5 family members bind netrin-1(see chapter 3), and might thus be predicted to act as receptors that mediate repulsive guidance by netrin cues. While direct evidence for such a repellent function for UNC-5 homologues is still lacking, the analysis of UNC5H3^{rcm} mutant mice suggests that UNC5H3 may mediate cell migration of granule cells and their precursors (Ackerman et al., 1997). This finding is consistent with the role of UNC-5 in mediating cell migrations in *C. elegans*. Furthermore, given that UNC5H3 binds netrin-1 tightly, there is at least strong circumstantial evidence to suggest that UNC5H3 may function as a receptor or a component of a receptor for netrin-1. Future analysis of mice mutant for UNC5H1 and UNC5H2 should address the functions of these molecules in the development of the nervous system. It will be particularly interesting to determine whether UNC5H1 and UNC5H2 , as proposed for UNC5H4 may also have netrin-independent functions.

The absence of an interaction between UNC5H4 expressing cells and soluble netrin-1 suggests additional roles for UNC-5's in vertebrates, or at least that UNC5H4 may have evolved a distinct function. As mentioned previously, our assay cannot

completely rule out the possibility that this receptor interacts with netrin-1. It does suggest however that any hypothesized interaction would have to be of low affinity (Kd>10nM). One possibility is that UNC5H4 may be a receptor for an as yet unidentified netrin, or an altogether unrelated ligand.

Support for the novel ligand hypothesis is provided by the RAP-in situ data. Specific staining is observed in the vicinity of the branchial arches, the region of highest UNC5H4 expression early in development. Furthermore, the specificity is clear, as RAP in situ probes for UNC5H1, UNC5H2 and UNC5H3 fail to detect any such pattern. In principle, this result provides a means for identifying the UNC5H4 binding protein. The UNC5H4 receptor-AP fusion could be used to screen an expression library made from the embryonic tissue that has been revealed to contain binding sites. There are numerous examples of other Ig family members binding multiple ligands, both by distinct and shared domains. For example F3/F11 is known to interact with tenascin and Nr-CAM using different domains (Brummendorf et al., 1993). In fact, the existence of UNC-40 phenotypes that are not UNC-6 dependent, as well as the fact that axon guidance of commissural neurons appears to be more severely disrupted in DCC mutant mice than in netrin-1 mutants suggest that these receptor(s) for netrin mediated attraction may have additional ligands (Hedgecock et al., 1990; Chan et al., 1996; Fazeli et al., 1997). Our identification of a non-netrin binding UNC-5 homologue suggests that there may be other ligands for the repellent receptor family as well.

A final feature of the UNC-5 family that has emerged from our studies is the remarkable conservation of the extracellular domains (Ackerman et al., 1997; Leonardo et al., 1997 and Fig. 1C). By contrast, the intracellular domain is much less conserved. In fact, the approximately 500 amino acid cytoplasmic domain contains only two recognizable motifs: ZO-1 and "death domains" (Hofmann and Tschopp, 1995; Ackerman et al., 1997; Leonardo et al., 1997). While there is one additional short stretch of homology, high level of divergence exists throughout the rest of the molecule.

Although the functional significance of these similarities and differences are not known, it is possible that certain functions of the UNC-5 proteins have been conserved and may map to regions of conserved sequence. Likewise, non-conserved regions may have evolved to allow for specific differentiation of function between the UNC-5 family members. One possible example of this is a functional difference between UNC5H1 and UNC5H2. While transient transfection of 293T cells with UNC5H2 results in high levels of expression, transfection with full length UNC5H1 results in cell death and little or no surface expression (Leonardo et al., 1997 and data not shown). Since expression of UNC5H1 in 293T cells is only possible when the cytoplasmic domain is truncated, differences in the C-terminal domains of UNC5H1 and UNC5H2 (possibly the death domain) may account for differences observed between UNC5H1 and UNC5H2. The inability of UNC5H4 to bind netrin-1 may be yet another example of functional differentiation between the vertebrate UNC-5's.

Methods:

Identification of a novel vertebrate UNC-5 homologue.

A search of the EST database using sequences from UNC5H1 and UNC5H2 revealed a 181 bp human sequence that shares homology with the Ig-2 domain of the previously identified rat clones. The EST, (D38687), was identified by an exon trapping method specific for human chromosome 8. Although the sequence showed highest homology to the vertebrate UNC-5's, additional sequence was required to determine if the homology extended to other domains. Two antisense primers 8-2 (CAC AAC AGT GGC CGA CAG GC (LSATVV)) and 8-1 (CTC TTA GCC ACG ATG TTG GC (ANIVAKR)) were used sequentially in combination with a standard T3 primer to amplify fragments from a human embryonic brain library (Stratagene) using the polymerase chain reaction (PCR). Analysis of the products revealed a smear with primer 8-2 which resolved into several bands in the 200-600 base pair range after a nested reaction with primer 8-1. The major bands from the nested product were subcloned into

pBluescript SK- (Stratagene) and the products sequenced. One of the products, 3-3T3, contained the sequence from the EST as well as an additional 390 bp of sequence that included sequence homologous to domain Ig-1 of the vertebrate UNC-5's. This clone was used to screen 1.1 million clones of a mouse adult brain cDNA library (Gift of George Minowada). Three positive clones were identified. The longest one (10-1) was characterized. This clone contained a 5 Kb insert which encoded a novel UNC5 homologue designated mouse UNC5H4. This clone contained all the predicted UNC-5 structural domains and was homologous throughout the coding region. The clone was incomplete however as it was missing the 5' end, predicted to be at least 180 base pairs upstream of the end of the clone. Identification of a full length clone required screening approximately 750,000 clones from an embryonic day 13 rat spinal cord library. Three positive clones were identified (12, 70, 94) and the two longest (approx. 7kb insert) which appeared similar were characterized. Clones were sequenced using a Licor L4000 sequencer or an ABI sequencer.

PCR for alternative splicing

Two primers H4SPL5 (AAG AGT AGG AAA GCT TCC GTG CGC A) and H4SPL3 (TTT ATC TAG AAT GCA GAG ACC ATC C) corresponding to sequences just after Ig-1 and TSP-2 respectively. These primers should amplify a 460 bp predicted fragment for clones lacking Tsp-1 and a 628 bp fragment for clones containing Tsp-1. PCR was performed using cDNA that was reverse transcribed using random hexamers from E13 rat brain mRNA. The reaction products were run out on an agarose gel, subcloned into pBluescript SK- (Stratagene) and sequenced.

Generation of expression constructs

Clone 12-1 contains the 5' ATG as well as the 3' stop codon. This clone encodes a protein that contains two Ig domains and one thrombospondin domain in its extracellular

domain. Clone 94-8 is an alternatively spliced clone that contains both thrombospondin type-1 repeats, but is truncated at is 5' end. Clone 12-1 and 94-8 are identical in all other respects. In order to construct a full length expression construct for clone 94-8, a 400 bp EcoRI/HindIII fragment from clone 12-1 was used to replace the EcoRI/HindIII of clone 94-8. This results in a clone that contains a 5' ATG from clone 12-1 and the remaining coding region from clone 94-8. Both constructs are in the expression vector pMT21 (Genetics Institute).

Binding assays and immunohistochemistry

Binding assays and transfections were performed essentially as described (Leonardo et al., 1997). UNC5H4 protein was visualized on the surface of transfected cells with a mouse polyclonal antibody raised against the extracellular domain of UNC5H1 that cross-reacts with UNC5H4. The bound antibody was visualized with a Cy-3 conjugated goat anti-mouse secondary antibody (Jackson Immunoresearch).

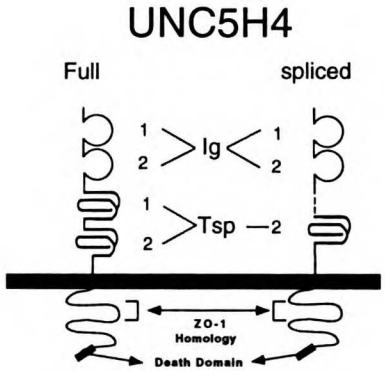
In situ hybridization

In situ hybridization was performed as previously described (Leonardo et al., 1997).

Figure 1

Structure of UNC5H4. **A**. Predicted topology of UNC5H4 , indicating the predicted structural domains which include 2 immunoglobulin like domains (Ig-1 and Ig-2) and two Tsp type-1 domains (Tsp-1 and Tsp-2) in the full length form and one Tsp type-1 domain (Tsp-1) in the alternatively spliced form (UNC5H4-tsp). Also indicated are the cytoplasmic ZO-1 homology region, and the C-terminal death domain. **B.** Alignment of UNC5H4 to UNC5H4-tsp illustrating the precise boundary of the alternatively spliced domain. **C.** Sequences of the four known UNC-5 homologues compared with that of *C. elegans* UNC-5. The signal peptide sequences are indicated by a black bar, amino-acid identities by black boxes, and similarities by shaded boxes; extents of the two immunoglobulin-like domains (Ig-1 and Ig-2) and the two Tsp type-1 domains (Tsp-1 and Tsp-2) are indicated by arrow; the membrane-spanning region is indicated by asterisks; the region of homology with ZO-1 is bounded by ^ symbols, and the death domain is indicated with #.

A.


UNC5H4
Full
spliced
Ig
Tsp
ZO-1
Homology
Death Domain

B.


Tsp-1
Tsp-1
Tsp-2
Tsp-2

C.

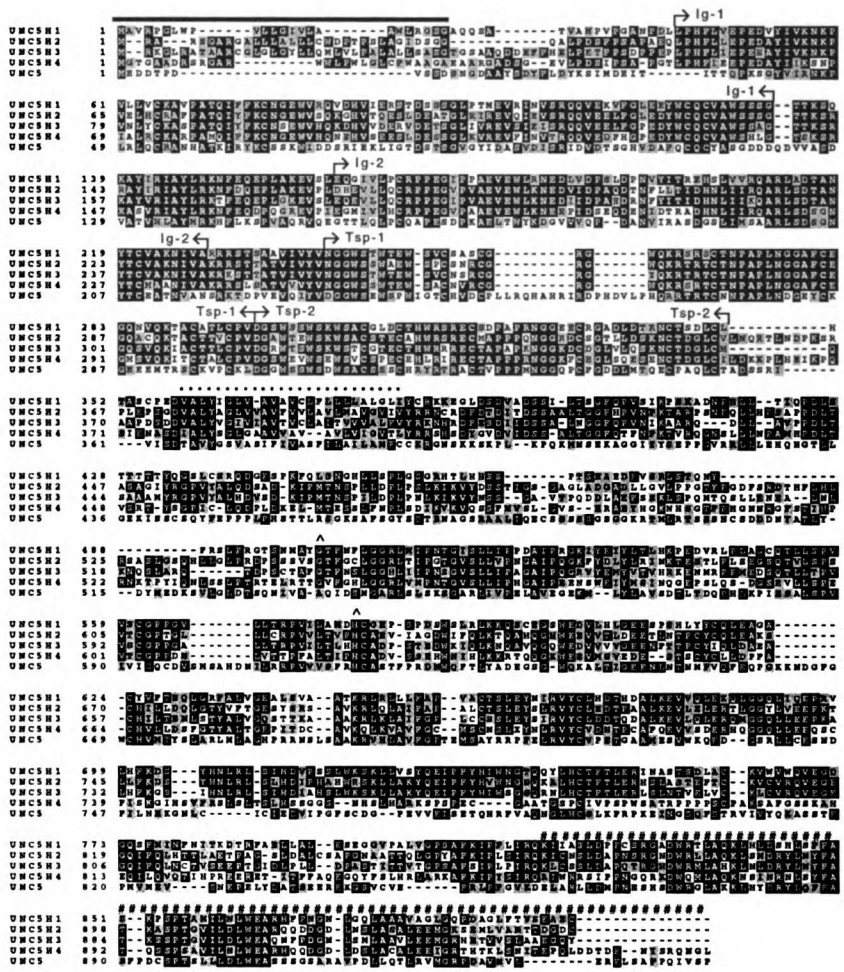

Ig-1
Ig-1
Ig-2
Ig-2
Tsp-1
Tsp-1
Tsp-2
Tsp-2

Figure 1

Figure 2

Expression of *Unc5h4* mRNA in transverse sections through the developing rat spinal cord at E12 (**A**), and E14 (**B**) Note that at E12, expression is observed in the motor columns (m), but is also seen more dorsally along the lateral aspect of the spinal cord, in a pattern that suggests expression in differentiated cells but not proliferating cells. This expression may also encompass the cell bodies of commissural neurons (c). At E14, expression can be seen throughout the ventral spinal cord including motor neurons (m), and extending dorsally at an imtermediate position between the lateral edge and the ventricular zone of the spinal cord.

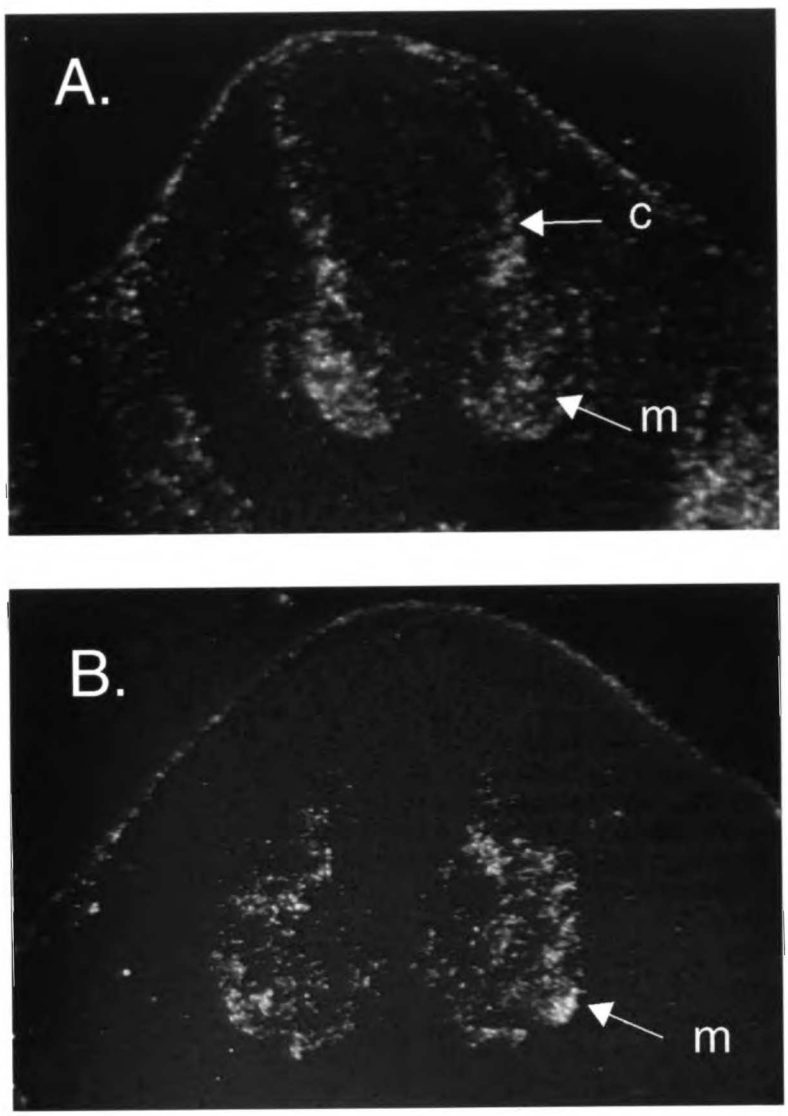


Figure 2

Figure 3

Other sites of *Unc5h4* mRNA in the embryo (**A**, **B**, **C**) and RAP in situ with UNC5H4 ectodomain probe (**D**). **A**. Expression at embryonic day 12 as viewed by whole mount in situ hybridization. Note expression at the posterior margin of the hyoid process (arrow). Signal in the otic vesicle is likely to be non-specific trapping (*). **B**. By E13 expression has moved to the anterior half of the mandibular arch (ma). **C**. At E14 more restricted expression is visible in the epibranchial groove (eg). **D**. At E14, binding sites for the UNC5H4 ectodomain are detected superficially in a striped pattern laterally on the developing facial structures (+).

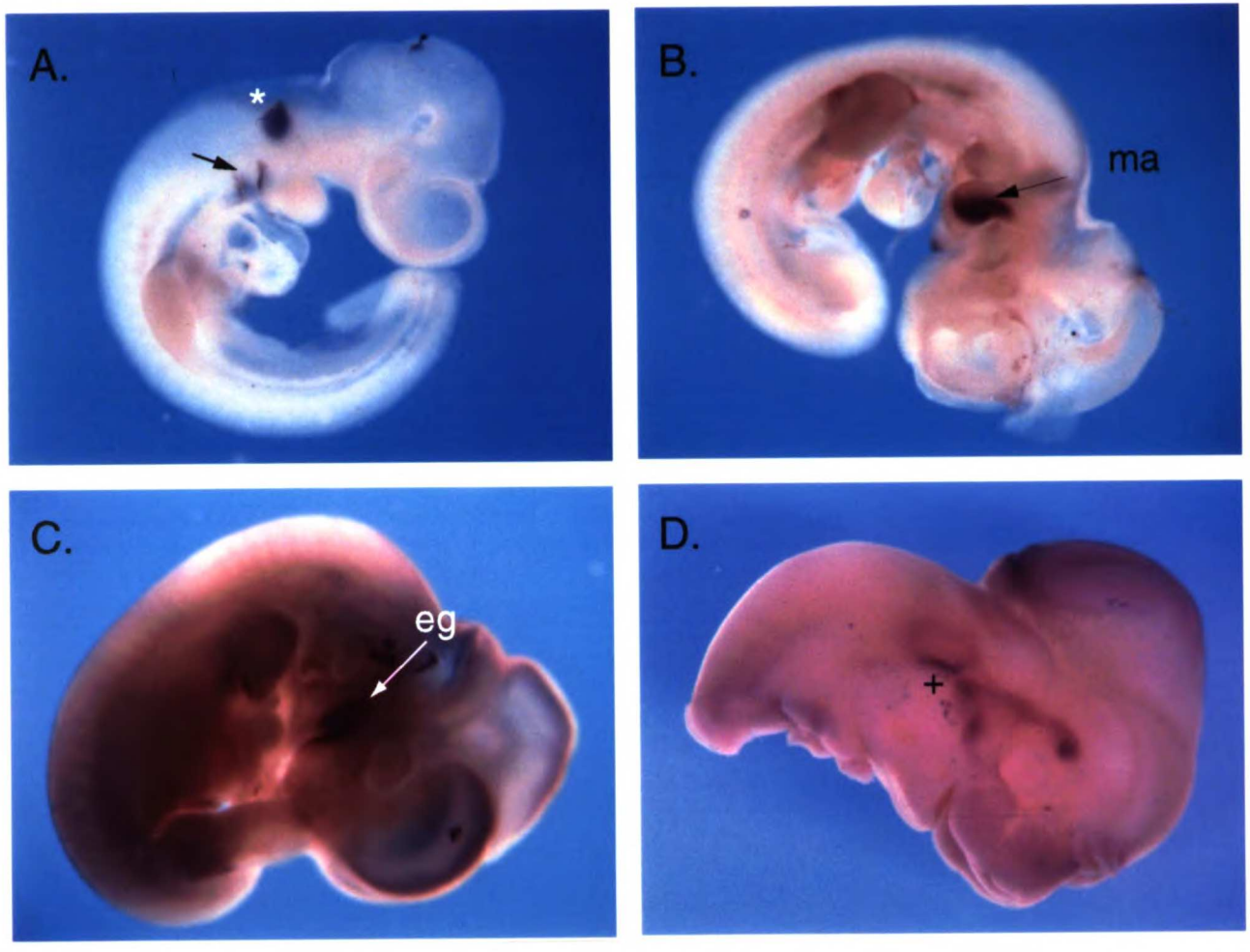
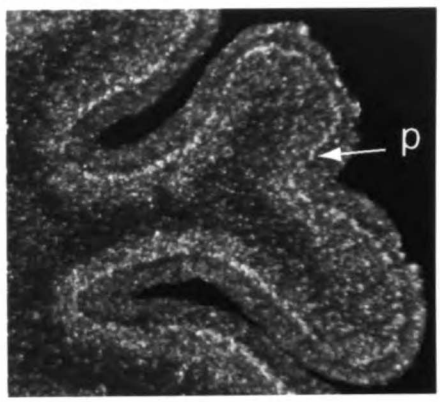


Figure 3

Figure 4

Postnatal expression of *Unc5h4* in the developing cerebellum. **A**. At P5 prominent expression can be seen in the Purkinje cell layer (p). **B**. At P10 expression has expanded to include the external germinal layer (e) and the internal granule cell layer (i).

A.



B.

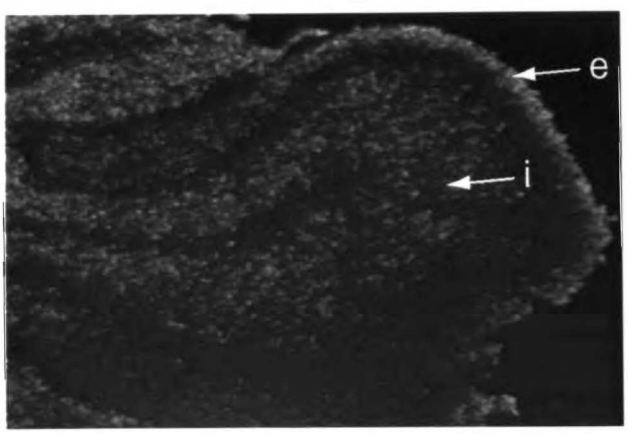


Figure 4

Figure 5

Expression of alternatively spliced forms of UNC5H4 (**A**,**B**) or full length UNC5H2 (**C**) on the surface of transfected 293T as visualized by immunohistochemistry with an antibody that recognizes the extracellular domain. **A**. UNC5H4-tsp **B**. UNC5H4. Interactions of netrin-1 and UNC5H4 , as demonstrated by experiments in which purified netrin-1 is incubated with mock transfected cells (**D**), UNC5H4 transfected cells (**E**) and UNC5H2 transfected as a positive control. Netrin-1 binding is visualized in red. Note that cells expressing UNC5H2 but not cells expressing UNC5H4 bind to netrin-1.

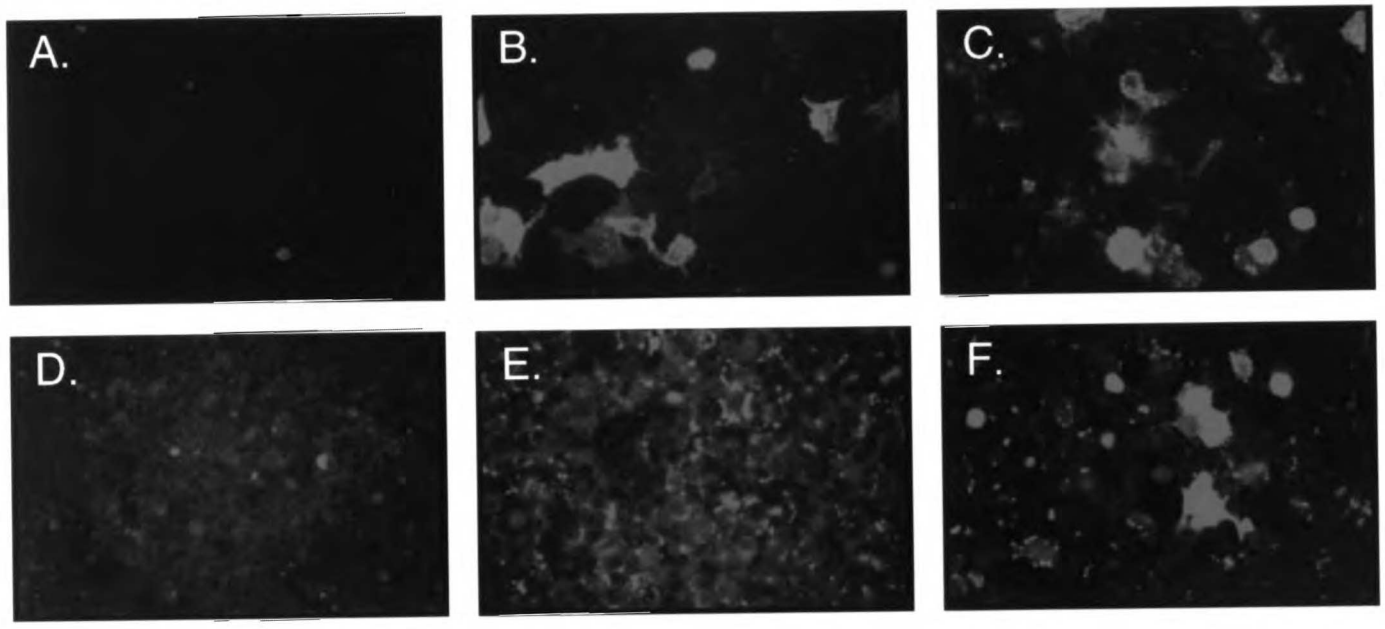


Figure 5

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Chapter 5

Identification of Candidate Effectors of UNC-5 Signaling: 2 Hybrid Screen for Interactions with UNC5H1 Death Domain

Introduction:

UNC-5 and its vertebrate homologs are candidate receptors for mediating the repellent functions of netrins. In *C. elegans*, strong genetic evidence from both loss of function and gain of function studies implicate UNC-5 as a repellent receptor for UNC-6 (Hedgecock et al., 1990; Hamelin et al., 1993). While there has been no direct demonstration in vertebrates of such a repulsive function for the UNC-5 homologues, there is ample circumstantial evidence to suggest a role (Ackerman et al., 1997; Leonardo et al., 1997). Firstly, the strong conservation of structure and function between netrin-1 and UNC-6 and their attractive receptors DCC and UNC-40 suggest that a similar functional conservation between *C. elegans* UNC-5 and UNC-5 homologues in vertebrates may exist. Second, as discussed in chapter 3, UNC5H1, UNC5H2 and UNC5H3 bind tightly to netrin-1. Finally, very preliminary ectopic expression studies with UNC-5 homologues in Xenopus suggest that UNC5H proteins are capable of confering netrin mediated repulsion, perhaps providing the direct link between netrin and UNC5H function that has been so elusive (Kyon-Soo Hong and Mu-Ming Poo, personal communication).

To date, the clearest function for vertebrate UNC-5 homologues has been described in the cerebellum. Netrin-1, all of the UNC-5 homologues, as well as DCC and neogenin are expressed in the cerebellum. Furthermore, analysis of *Unc5h3*^{rcm} mutant mice suggest a role for UNC5H3 in mediating migrations of cerebellar granule cells or their precursors. The mechanism by which UNC-5 proteins might mediate their effects is not known. One possibility is that UNC-5 proteins might directly transduce a signal in response to netrin-1, or other as yet unidentified ligands. Another possibility, discussed in chapter 3, is that UNC-5 proteins may act as accessory receptors, modulating the function of DCC/UNC-40 (or another as yet undiscovered signaling partner) in response to netrin-1.

Whether UNC-5 proteins signal directly or indirectly, they are likely to do so by interacting with proteins through their cytoplasmic domain. The large ~ 550 amino acid domain that is present in all family members contains no known catalytic domain and has only two regions, conserved in all UNC-5 family members, that show homology to other proteins (Leung-Hagesteijn, 1992; Ackerman et al., 1997; Leonardo et al., 1997). One, designated the ZO-1 homology domain (see chapter 3) is an approximately 90 amino acid homology motif which corresponds to the C-terminal-most portion of ZO-1.

The other homology region, also approximately 90 amino acids in length, shares homology to the death domain motif found in Fas, the tumor necrosis factor receptor, as well as in other proteins thought to play a role in cell death (reviewed by Hofmann and Tschopp, 1995; Boldin et al., 1995, Chinnaiyan et al., 1995). While this motif has generally been found in proteins associated with apoptosis, this motif may actually represent a more general protein-protein interaction domain and thus may function in signaling events unrelated to programmed cell death (Feinstein et al., 1995; Hofmann and Tschopp, 1995).

Given the strong conservation of these two motifs in all UNC-5 family members, we reasoned that these protein domains might be important for UNC-5 function. Therefore, as a first step toward understanding how UNC-5 proteins transduce signals, we performed a yeast two hybrid screen to identify proteins that interact with the C-terminal death domain of UNC5H1.

Screen for proteins that interact with the UNC5H1 death domain

The last 95 amino acids of UNC5H1 including the death domain homology region were used to screen a postnatal day 10 rat cerebellum library in a yeast two-hybrid screen. We screened 6.9 million yeast transformants which represent 1.7 million independent original clones. Clones were initially screened for interaction with the UNC5H1 death domain by their ability to promote growth on leucine deficient medium, leading to the identification of 581 positive colonies (See Fig. 1). These clones were re-streaked on

Xgal containing plates in a more stringent test for interactions, based on the ability of the clone to induce lacZ expression and turn the colonies blue. This secondary screen yielded 84 positive clones. A subset (43) of these clones were further characterized by a combination of sequencing and re-screening to eliminate clones that interact with control baits. The re-screening procedure resulted in the elimination of all but 23 clones which were further characterized.

Identity of interacting proteins

The candidate clones were further characterized based on three criteria. One important criterion is their representation in the pool of positive clones. First, clones were prioritized based on their representation in the pool of positive clones: two interacting proteins, designated DDIR1 and DDIR2 (Death domain interacting Protein #) were isolated multiple times in this screen, four isolates in the case of the former and two isolates for the latter. All remaining candidates were isolated only once and are listed in Table 1. Secondly, promising candidates were screened by sequence analysis. Clearly, only proteins with known functions can be assessed in this manner (For example, SNRNP's were eliminated by this method). In the future, *in situ* hybridization will be used to identify interacting proteins that are co-expressed with UNC5H1. Interactors that meet these three initial criteria will be subjected to more rigorous biochemical tests to determine if the interactions identified in the two-hybrid screen are indeed physiologically relevant.

Based on sequence information, the identified clones fall into a number of broad categories. These include phosphatases, calcium binding proteins, and proteins containing known protein-protein interaction domains. Among previously identified proteins, the phosphatases include the protein tyrosine phosphatase delta (clone 3-22), the regulatory domain of PP2-A, as well as the Ca++/calmodulin sensitive serine-threonine phosphatase calcineurin (clone 1-17). A number of novel proteins with identifiable domains were also found. These include a protein with homology to NCS-1 (neuronal

calcium sensor-1), an ankryin-repeat containing protein (clone 2-41), a PDZ domain-containing protein (DDIR2) and several novel in the database (eg. DDIR1. See table 1). Other clones of interest include the rat homologue of Drosophila Flightless-I, a gelsolin like protein (Claudianos and Campbell, 1995).

While the physiological relevance of these interactions awaits further experimentation, these UNC5H1 interacting proteins point to a number of potential signal transduction mechanisms that might act to transduce an UNC5H1 signal.

Discussion

The two hybrid screen strategy described here was based on the assumption that the cytoplasmic portion of UNC5H1 is required for its function and that its C-terminal death domain may help to mediate protein-protein interactions. As discussed earlier, death domains were initially identified in proteins involved in signaling pathways leading to programmed cell death (Reviewed by Hofmann and Tshopp, 1995). However, there is also evidence to suggest that these "death domains" mediate protein-protein interactions in other contexts. For example, the *Drosophila* proteins TUBE and PELLE contain death domains, interact with each other, and are involved in the Toll-Dorsal signal transduction pathway. (reviewed in Belvin and Anderson, 1996). The validity of the assumption that at least some aspects of UNC5H signal transduction occurs via its death domain awaits the results of structure/function studies which are currently in progress.

The screen for interactors identified a number of candidates with reported activities in other intracellular signaling pathways. For example, calcineurin, a calcium/calmodulin-dependent protein phosphatase, is associated with the cytoskeleton and has been found to be particularly enriched in the growth cones of elongating neurons (Ferreira et al., 1993). In addition, in vitro experiments using chick DRG neurons show that local inactivation of calcineurin results in specific, localized filopodial and lamellopodial retraction and lateral growth away from the site of inactivation (Chang et al., 1995). These results suggest that inhibition of calcineurin phosphatase activity can

result in growth cone changes that are reminiscent of a repellent response. In one model, local inactivation of calcineurin by UNC5H1 in response to netrin could cause axons to turn away from the netrin source. Interestingly, in addition to a possible role in growth cone guidance, calcineurin has been found to mediate signals resulting in programmed cell death (Shibasaki and McKeon, 1995). This is particularly intriguing since UNC5H1 expression in heterologous cells can result in cell death (See chapter 3).

We have also also identified receptor tyrosine phosphatase delta as a candidate interactor for the C-terminal domain of UNC5H1. RPTP-δ is a member of the RPTP subfamily that include DLAR, a *Drosophila* RPTP that has been directly implicated in axon guidance (Brady-Kalnay and Tonks, 1995). RPTP-δ is developmentally regulated and is highly expressed in the developing rat nervous system (Sommer et al., 1997). In addition to DLAR, phosphatase activity has been shown to be involved in axon guidance in a number of systems. For example, mutants in DPTP69D have defects in axon defasciculation (Desai et al., 1996). While the intracellular substrates for most of these phosphatases are largely unknown, there is abundant data to suggest that a balance between tyrosine kinase and phosphatase activites may modulate axon behavior via their interactions with the actin cytoskeleton (Stoker, 1996; Brady -Kalnay and Tonks, 1995). The intracellular domain of at least one phosphatase has been suggested to transduce its signal to the cytoskeleton via its interaction with β-catenin (Kypta and Reichardt, 1996). The extracellular ligands for most of these phosphatases are unknown and it is unclear to what degree, if any, the activity or compartmentalization of these phosphatases might be regulated by ligand binding. One model is that interactions with transmembrane receptors, such as UNC-5, may help to co-localize the phosphatases and their substrates and in this way impart specificity to the signaling process.

In addition to known proteins, a number of novel candidate interactors containing previously identified motifs were found. Firstly, we identified a novel interactor that contains a PDZ domain. PDZ domain containing proteins are thought to function as

scaffolds that assemble and localize multimeric signaling complexes to highly specialized sub-membranous regions (Ponting et al., 1997). For example, the inaD protein in *Drosophila* contains five PDZ domains and serves as a scaffold for proteins involved in the phototransduction cascade, bringing together light activated ion channels with such effectors as phospholipase C-β and protein kinase C (Tsunoda et al., 1997).

We also identified a novel interacting protein that contains an ankyrin repeat motif. Ankyrin repeat motifs in other proteins appear to mediate protein-protein interactions (Michaely and Bennett, 1993; Bork, 1993). Consistent with this biochemical interaction between UNC5H1 and an ankryin-repeat containing protein, genetic experiments in *C. elegans* have identified the *C. elegans unc-44* ankryin related gene as a suppressor of ectopic UNC-5 redirection of axon outgrowth (Chan et al., 1996; Otsuka et al., 1995). One intriguing possibility is that the ankyrin repeat containing interactor identified by our screen is an *unc-44* homologue.

The candidates identified by this screen suggest several adaptor proteins and signaling pathways that may be involved in UNC5H mediated signal transduction, including mediators of calcium signaling and regulators of phosphorylation. While the identified interactors offer tantalizing clues as to how an UNC5H mediated signal might be transduced, establishing the physiological relevance of the interactions will certainly require further testing aside from two-hybrid analysis. Initially, co-immunoprecipitation experiments can be used to confirm a direct interaction. In addition, UNC5H1 mediated repulsion of Xenopus spinal cord neurons may provide a manipulable system where the function of these candidates can be assessed (Kyon-Soo and Mu-Ming Poo, personal communication). Furthermore, a systematic structure/function analysis of the cytoplasmic domain of UNC-5 proteins, including, in particular, deletions of the death domain will be required to established the relevance of these death domain interactions in UNC-5 signaling. Likewise, future two-hybrid screens using either the ZO-1 domain or other cytoplasmic elements may reveal other candidate downstream signaling

components. This information will provide a first glimpse into the mechanism of action of the UNC-5 family of receptors, and in turn, may offer critical insight into the signaling pathways in the growth cone that mediate a repulsive response. Together with results from similar experiments with the attractive netrin receptor, DCC, we may gain insight into whether attraction and repulsion are in fact mechanistically similar or different.

Material and Methods

Library and bait plasmid construction

Cerebellar tissue was removed from postnatal day 10 rats and snap frozen in liquid nitrogen. RNA from 600 mg of this tissue was isolated using RNASOL yielding 1.55 mg of total RNA. The mRNA fraction was purified using Oligotex beads(Qiagen) yielding 107 ug mRNA. This mRNA was then used to construct a random primed directional library. The library was constructed using the Stratagene cDNA synthesis kit except that the primer RNDLIB (GAGAGAGAGAACTAGTCTCGAGNNNNNNNNNNNNNN) was substituted for the primer that is included with the kit. The cDNA was size fractionated to eliminate clones < 500bp, and 100 ng of the resulting cDNA was ligated into vector pJG4-5. The ligation was then transformed into E. Coli and amplified, resulting in a 3.4 X10^6 primary transformants. The average insert sized was determined to be approximately 750 base pairs. The bait plasmid was constructed by inserting a 285 base pair PCR fragment encoding the last 95 amino acids of UNC5H1 into the vector pEG202. The resulting plasmid (pEG202-DD1) encodes a fusion protein that contains the death domain homology region of UNC5H1.

Library Screen

Yeast strain EGY48 was transformed with pEG202-DD1, pJK103. The resulting transformants were then transformed with the P10 cerebellar library resulting in 9X10^6 primary transformants. This represents 93.2% coverage of the original plasmid library. The transformants were scraped off plates and frozen in glycerol at -80^{o}C. An aliquot was thawed, and 6.9 X10^6 transformants were screened. This represents 53.5% coverage of the original yeast transformants and represents 49.9% coverage of the primary library. The screen therefore represents 1.7 X10^6 independent clones screened.

Sequencing of clones

Clones were either sequenced directly on an ABI automated sequencer, or the inserts subcloned into pBluescript prior to sequencing.

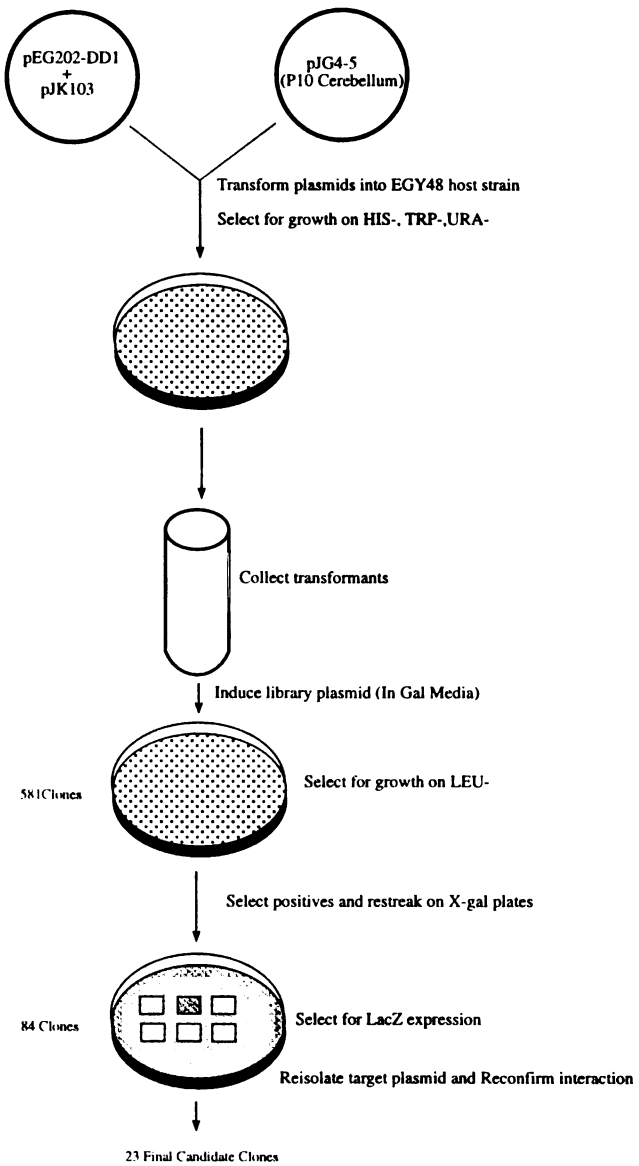
In Situ's

In Situ's were performed as previously described (Leonardo et al., 1997)

Figure 1.

Schematic of two hybrid screen for UNC5H1 death domain interactors (**A**) and illustration of selection strategy (**B**). **A.** First, EGY48 host strain is tranformed with the pJK103 LacZ reporter plasmid and the death domain bait (pEG 202-DD1), as well as the prey plasmid PJG4-5 containing the P10 cerebellum library. These transformants are selected for on the appropriate media and collected into a single pool. These transformants are then induced with galactose to express the prey protein. Interaction between the prey is detected first by the ability to confer growth on LEU- media, and by the ability to activate the LacZ reporter system. Positives clones are isolated and retested to ensure that the selected phenotype is plamid mediated. **B.** Selection is achieved by using reporter genes that are contain LexA binding sites in their promoters. The bait is a fusion protein that contains the LexA DNA binding domain fused to the UNC5H1 death domain. The fusion protein bound to the LEXA sites is transcriptionally inactive on its own. The prey construct is a fusion of the library protein to the Gal4 activation domain. Interaction between bait and prey brings the Gal4 activation domain to the promoter of the reporter, activating its transcription. The readout is growth on LEU- and blue colonies on galactose containing plates.

A.


pEG202-DD1
+
pJK103
pJG4-5
(P10 Cerebellum)
Transform plasmids into EGY48 host strain
Select for growth on HIS-, TRP-,URA-
Collect transformants
Induce library plasmid (In Gal Media)
581Clones
Select for growth on LEU-
Select positives and restreak on X-gal plates
84 Clones
Select for LacZ expression
Reisolate target plasmid and Reconfirm interaction
23 Final Candidate Clones

B.

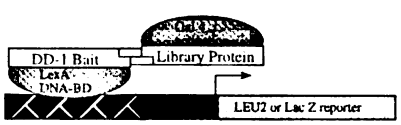

DD-1 Bait
Library Protein
LexA
DNA-BD
LEU2 or Lac Z reporter

Figure 1

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Chapter 6

Summary and Conclusions

The concept of chemorepulsion was a late arrival to the field of axon guidance, providing a balanced view of how axons are guided to their targets. The prominent roles of both positive and negative cues, acting locally and at a distance is today almost dogma. The work presented in this thesis illustrates some of the early progress in the field of chemorepulsion, and a summary of the major conclusions from each chapter is presented below.

We have used an in vitro approach to demonstrate that sema III can act as a chemorepellent for subpopulations of sensory DRG axons, and that sema III likely accounts for the ability of the ventral spinal cord to repel these DRG axons in culture. This work introduces the concept of chemorepellents as molecular sieves, selectively allowing the passage of some populations of axons, while restricting the advance of others. As described in chapter two, sema III expressed in the ventral spinal cord may create an exclusion zone by selectively repelling small diameter afferents, restricting them to the dorsal spinal cord. The concept of exclusion zones has been further developed with the demonstration of "surround repulsion", in which multiple sources of repellent cues are sufficient to channel axons into specific pathways, even in the absence of other positive cues (Keynes et al., 1997). In addition, a physiological role for exclusion zones has been confirmed by phenotypic analysis of sema III mutant mice (Taniguchi et al., 1997). For example, they observe that in wild-type mice, the peripheral nerves and their distal branches specifically avoid regions of sema III expression, creating regions devoid of axons and their branches. This is not the case in mutant embryos, where exuberant growth and branching is observed inappropriately in regions that would normally express sema III (Taniguchi et al., 1997).

Whether sema III establishes exclusion zones for the central projections of sensory neurons, as it seems to for peripheral nerves, is less clear. DiI labelling of central sensory axon projections in E15.5 sema III mutant embryos reveals nearly normal trajectories (Taniguchi et al., 1997). By contrast, analysis of central projections in sema

III mutants as visualized by CGRP demonstrates that small diameter sensory afferents overshoot their targets in the dorsal spinal cord, growing into regions where sema III is normally expressed (Behar et al., 1996). The reason for the different phenotypes observed is unclear. However, it is possible that the CGRP positive neurons represent only the small subset of small diameter afferents that are affected and which are thus obscured in the DiI labelling. It is also possible that the distinct phenotypes are the result of distinct genetic backgrounds, as suggested by the fact that mutants in a C57BL/6 background survive and can breed (Taniguchi et al., 1997), while mutants in a 129 background are perinatal lethals (Behar et al., 1996). Further analysis will be required to determine the contribution of sema III in patterning the central projections of DRG afferents.

Netrin-1 in vertebrates, and UNC-6 in *C. elegans* guide axons both toward and away from the ventral midline. The receptors (DCC/UNC-40) that mediate the attractive function of netrins have been conserved (Keino-Masu et al., 1996). We have identified a family of vertebrate UNC-5 homologues and demonstrate that UNC5H1, UNC5H2, and UNC5H3 bind netrin-1, providing the first evidence of a biochemical interaction between netrins and UNC-5 proteins. In addition, we demonstrate the vertebrate UNC-5 proteins are prominently expressed in the developing nervous system during axon outgrowth. These data suggest that the receptors that mediate repulsion have also been conserved.

In addition, we describe a new member of the UNC5H family that does not appear to bind netrin-1, raising the possibility that this protein may have evolved a distinct function, perhaps acting as a dominant negative with no ligand, or alternatively, having acquired an additional ligand. Our data are consistent with the latter possibility as non-netrin receptor binding sites can be detected near areas of receptor mRNA expression. The functional role of this and the other UNC5H proteins awaits the results of knockout studies in mice. It will be particularly interesting to compare the phenotypes of the

different mutant mice, looking for evidence of distinct functions, perhaps even netrin independent functions as we have proposed for UNC5H4.

We have also identified proteins that interact with the C-terminus of UNC5H1, including proteins predicted to be sensitive to changes in intracellular calcium, as well as receptor tyrosine phosphatases. These experiments are a first step in understanding the mechanisms by which candidate receptors relay repellent information to the cytoskeleton and affect growth cone behavior. One promising step in this direction is the recent evidence that modulation in the levels of protein kinase A (PKA) activity can change an attractive response to netrin (mediated by DCC) into a repellent one, in a calcium dependent manner (Ming et al., 1997). We have previously discussed evidence to suggest that DCC-like and UNC-5 like proteins interact (see chapter 3), perhaps cooperating in the generation of a repellent signal. One attractive hypothesis consistent with the data is that DCC-like proteins transmit "the signal" and that UNC-5 signaling alters the signal by modulating levels of PKA activity. How UNC-5 proteins would produce this modulation is unclear. Two conserved motifs in the UNC-5 cytoplasmic domain, the ZO-1 domain and the death domain, are likely to play a role, perhaps through interacting proteins such as those identified in chapter 5. Specific targeting of these domains in structure/function studies will firmly establish their roles in UNC-5 signaling.

Finally, recent experiments in collaboration with Mu-Ming Poo's lab suggest that ectopic expression of UNC-5 proteins in Xenopus spinal neurons, normally attracted to netrin in a DCC dependent fashion, results in repulsion (Kyon-Soo Hong and Mu-Ming Poo, personal communication). This assay may provide the missing functional link between vertebrate UNC-5 homologues and netrin-1. In addition, this assay may allow further analysis of the cell biology of chemorepulsion, and may facilitate the analysis of candidate signaling molecules identified by other means.

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Appendix I

Identification of Netrin-Binding Proteins

Introduction:

The screen presented below was initiated prior to the identification of vertebrate UNC-5 homologues described in chapters 3 and 4, and before the molecular identity of UNC-40 was known. In addition to the candidate gene approach, we sought netrin receptors using an unbiased approach based on netrin-1 binding. We designed an expression cloning strategy to identify netrin-1 binding proteins with the expectation that they would be candidates for mediating netrin function in vertebrates.

Results:

Characterization of the netrin-1 ligand

The ability to detect netrin binding proteins from COS cell expression cloning requires a suitable signal to noise ratio in order to avoid the identification of false positive clones. Preliminary studies suggested that netrin-1 was unsuitable as a ligand for these studies due to its high level of background binding to COS cells. In addition, netrin-1 is expressed at relatively low levels in transfected cells, and the availability of netrin ligand would be limiting for the screen. In an attempt to identify a more suitable ligand, we generated several netrin-1 deletion mutants as well as netrin-fusion proteins. These mutant proteins were screened on the basis of two criteria. First, mutant proteins were assayed for their ability to promote outgrowth of commissural neurons from embryonic day 13 rat dorsal spinal cord explants. Second, the mutant constructs were tested for levels of protein expression when transfected in COS cells. We determined that a fusion protein that comprised domains VI and V of netrin-1 fused at its C-terminus to the constant (Fc) region of human IgG met our criteria for activity and expression levels (Keino-Masu et al., 1996). In addition, we noticed that background binding of the VIV-Fc fusion protein, though lower than that of netrin-1, was further reduced by including heparin in the binding solution (Keino-Masu et al., 1996). The addition of this amount of heparin did not inhibit the ability of netrin-1 to promote commissural axon outgrowth from dorsal spinal cord explants. This VIV-Fc fusion protein, which retained netrin

bioactivity and was easily expressed in COS cells, was therefore chosen as the ligand for screening the expression library.

Screen of Embryonic Day 13 Dorsal Spinal Cord Library

In order to identify physiologically relevant netrin-1 binding proteins, we chose to screen an embryonic day 13 rat dorsal spinal cord expression library. At this age, commissural neurons located in the dorsal spinal cord respond to netrin-1 and must therefore express netrin-1 receptors. The library was divided into pools of approximately 5000 clones, and each pool was transfected into COS cells in a 35 mm dish. Netrin-1 binding was assessed by a ligand overlay assay (Keino-Masu et al., 1996, Leonardo et al., 1997). VIV-Fc ligand was first incubated with transfected cells. The cells were then washed and fixed. Cells were then incubated with a anti-human-Fc antibody conjugated to alkaline phosphatase (AP). Residual VIV-Fc binding was revealed by exposing cells to the AP substrate BCIP/NBT which is converted into a blue precipitate by alkaline phosphatase activity. Individual pools were examined under dissecting microscopes to identify transfected cells that had turned blue in this procedure, indicating that they were expressing netrin-binding proteins. Approximately 200 pools were screened in this manner.

Expression of glucosaminyl N-deacetylase/N sulfotransferase (GNDNS) confers netrin-binding to COS cells

Pool 34 was identified as containing a candidate netrin binding protein. Several rounds of sib selection yielded a single clone with a 3.7 kb cDNA insert (clone 43). This clone conferred visible netrin binding above background on the surface of transfected cells(data not shown). No such binding was observed in untransfected cells, and binding above background levels was not observed if the Fc domain alone was used as ligand. These results suggest that netrin domain VI or domain V was responsible for the binding observed in clone 43 transfected cells. Sequence analysis revealed the clone to be a glucosaminyl N-deacetylase/N sulfotransferase (GNDNS). This type II membrane

protein has been previously identified as an enzyme involved in the heparin biosynthesis pathway (Orellana et al., 1994).

Other isolates

In the initial screen, numerous additional pools were identified as containing candidate netrin-binding proteins. Subsequent rounds of sib selection eliminated most of these pools as false positives from the initial screen. These pools either failed to reproduce positive clones, or were found to contain clones that bound specifically to the Fc portion of the VIV-Fc fusion protein. The high rate of false positives seemed to result from an inadequate signal to noise ratio ratio in the screen. Nevertheless, at least one additional promising pool was identified (pool 175), and sib selection was initiated.

At this time, we received information that UNC-40 was a *C. elegans* homologue of vertebrate DCC (J. Culotti, personal communication). In order determine whether DCC might be the netrin-binding protein in pool 175, we performed southern blot analysis on DNA isolated from the pool using a DCC cDNA as a probe. This southern blot showed a large molecular weight hybridizing band. The cross-hybridizing clone was isolated from the library, and sequencing identified it as a full length rat DCC clone. Transfection of this clone into COS cells conferred specific netrin (VIV-Fc) binding (data not shown and Keino-Masu et al., 1996).

Discussion

We have used an expression cloning strategy to identify netrin-binding proteins that are candidate receptors. We demonstrate that a cDNA for a glucosaminyl N-deacetylase/N sulfotransferase (GNDNS) will confer netrin-1 binding on the surface of transfected COS cells. This result is consistent with two possibilities. Either GNDNS binds directly to netrin, or alternatively, GNDNS may modify the surface of transfected cells in order to increase their affinity for netrin. We have no direct evidence for either possibility, although the latter might appear more plausible.

GNDNS is a type II membrane protein that has been implicated in the biosynthesis of heparin, specifically in the sulfation of sugar chains (Orellana et al., 1994). Expression of GNDNS in human 293 kidney cells increases the level of sulfation of heparan sulfate proteoglycans (Cheung et al., 1996). Interestingly, netrin binds tightly to the extracellular matrix and to cell membranes, presumably due to interactions with heparan sulfate proteoglycans, and was purified on the basis of its tight interactions with heparin (Serafini et al., 1994). Non-specific netrin interactions with cell surfaces can be eliminated with increasing concentrations of heparin in the media without disrupting the specific interactions seen with receptors such a DCC and UNC-5 homologues (Keino-Masu et al., 1996; Leonardo et al., 1997). In addition, heparin is more effective at releasing "non-specific" cell surface bound netrin than is heparan sulfate (Christine Mirzayan, unpublished observations). Together, these results suggest the possibility that by increasing the sulfation of endogenous heparan-sulfate proteoglycans in transfected cells, the GNDNS may increase these cells' affinity for netrin above that of non-GNDNS expressing cells. In this context, it would be interesting to test whether higher concentrations of heparin could compete away the increase in binding in the GNDNS expressing cells. Similarly, one could compare background levels of netrin binding on COS cells that are deficient in sulfation versus netrin binding on COS cells that have high levels of sulfation. If GNDNS can regulate "non specific" binding of netrin-1 to cell surfaces, it is conceivable that such a mechanism may operate to modulate the partitioning of netrin-1 protein between soluble and cell bound forms. This in turn could be envisioned as a mechanism for fine tuning the range of netrin action away from a source of production.

Materials and Methods

Library Construction

Rat embryonic day 13 embryos were dissected and their spinal cords removed, as described (Serafini et al., 1994). The spinal cords were bisected into dorsal and ventral halves and snap frozen in liquid nitrogen. mRNA was prepared from the dorsal spinal cord tissue and was converted to cDNA using the Stratagene cDNA synthesis kit. The cDNA was then size fractionated. Pools of cDNA that contained the largest transcripts were then ligated into the vector pMT21 (Genetics Institute). The result was a directional cDNA library with an average insert size of 2.5 kb. The library was transformed into E. Coli and plated in pools of approximately 5000 clones. DNA was prepared from each individual pool and stored separately. Greater than 200 pools representing approximately 1 million clones were generated in this way.

Preparation of netrin (VIV-Fc ligand)

A cDNA encoding the VIV-Fc fusion protein was transiently transfected into COS cells using lipofectamine. Twelve hours after initial transfection, the medium of the cells was changed to Optimem (Gibco). The medium was conditioned for 72 hours. The supernatents were then collected, spun at 1000g to remove debris, filtered with a .45 um filter, and supplemented with .05% azide.

Library Screen

Approximately 1ug of DNA from each of the library pools was transfected (with lipofectamine) into COS cells in a single well of a 6 well tissue culture dish (35 mm per well, Corning). Twelve hours after transfection the medium was changed to Optimem (Gibco) and the cells were allowed to grow for 30 hours. The cells were then rinsed with PBS, and incubated with binding solution (VIV-Fc supernatent as described above, 1% heat inactivated goat serum (HINGS), and .25ug/ml heparin) for 90 minutes. Cells were

then quickly washed with 3 changes of PBS +1% HINGS and quickly fixed by serial dehydration into methanol, then rehydrated into PBS and rinsed with PBS + 1% HINGS. The rehydrated cells were then incubated with a secondary antibody solution (goat anti-Human IgG coupled to alkaline phosphatase, 1:1000 (Gibco), 1% HINGS in PBS) for 30 minutes. Cells were rinsed three times and incubated with the alkaline phosphatase substrate BCIP/NBT (Gibco). The reaction was monitored using dissecting microscopes and was stopped after approximately 15 minutes, or when background staining became apparent. Each dish was then scanned with the dissecting microscope searching for individual cells that were stained purple by the alkaline phosphatase reaction product, indicating that they had bound VIV-Fc.

Positive pools were identified as those which contained significantly more purple cells than mock transfected pools. DNA from candidate positive pools was reintroduced into bacteria, and replated into smaller pools. DNA from the subpools was then retransfected into COS cells and the screening process repeated. After several rounds of selection, single clones responsible for VIV-Fc binding could be identified.

Sequencing

Sequencing was performed as described (Serafini et al., 1994).

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Appendix II

Preface

Motor axons grow out of the ventral spinal cord in a stereotyped and precise fashion. Axons of motoneurons extend from the spinal cord into the anterior half of each sclerotome, actively avoiding posterior sclerotome, resulting in a segmented pattern of motor nerves (Keynes and Stern, 1984; Oakley and Tosney, 1993). In addition, motor axons from each segment exit the spinal cord in a single ventral root. Early in their trajectory the epaxial motor axons, a subset of motor axons which will innervate the dorsal muscle mass, make a sharp turn dorsally, abandoning the major ventral root and proceeding to the dermomyotome. Ablation studies in chick embryos have suggested that the dermomyotome secretes a chemoattractant for epaxial motor axons (Tosney, 1987). These and other experiments with sclerotome and dermomyotome suggested that several discrete signals emanating from surrounding tissue patterned the early projections of motor axons (Stern et al., 1986; Oakley and Tosney, 1989, Soc. Neurosci., abstract) . In an attempt to understand and identify these cues, I decided to take an in vitro approach, using a three dimensional collagen gel assay to examine the effect of neighboring tissues on the outgrowth of motor axons. The initial goals were to identify a chemoattractant activity from dermomyotome as well as to identify a chemorepellent activity from posterior sclerotome. To my surprise, I was unable to reconstitute either of these activities, instead discovering a powerful outgrowth promoting effect of sclerotome for motor axons. I performed the initial characterization of the outgrowth promoting activity, including initial experiments to suggest that it might function as a chemoattractant, as well as the initial characterization of the tissue specificity of the effect. My initial results were followed up by Allen Ebens and Katja Brose, together with some critical input from Nira Pollack, who very elegantly demonstrated that the in vitro effect of sclerotome on motor axons was due to Hepatocyte Growth Factor (HGF). Furthermore, they demonstrated that HGF was likely to act as a chemoattractant as well as a trophic factor for motor axons in vivo. The combined results of this work are presented in the appendix that follows.

Hepatocyte Growth Factor/Scatter Factor Is an Axonal Chemoattractant and a Neurotrophic Factor for Spinal Motor Neurons

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Summary

In the embryonic nervous system, developing axons can be guided to their targets by diffusible factors secreted by their intermediate and final cellular targets. To date only one family of chemoattractants for developing axons has been identified. Grafting and ablation experiments in fish, amphibians, and birds have suggested that spinal motor axons are guided to their targets in the limb in part by a succession of chemoattractants made by the sclerotome and by the limb mesenchyme, two intermediate targets that these axons encounter en route to their target muscles. Here we identify the limb mesenchyme–derived chemoattractant as hepatocyte growth factor/scatter factor (HGF/SF), a diffusible ligand for the c-Met receptor tyrosine kinase, and we also implicate HGF/SF at later stages as a muscle-derived survival factor for motoneurons. These results indicate that, in addition to functioning as a mitogen, a motogen, and a morphogen in nonneural systems, HGF/SF can function as a guidance and survival factor in the developing nervous system.

Introduction

Developing axons are guided to their targets in the nervous system by molecular guidance cues in their local environment. One mechanism that contributes to accurate axon guidance is chemoattraction, the guidance of axons by gradients of diffusible factors secreted by the intermediate and final cellular targets of these axons. Despite the considerable evidence for the operation of chemoattractant mechanisms in axon guidance, to date only one small family of molecules, the netrins, that function as chemoattractants for developing axons has been identified (reviewed by Tessier-Lavigne and Goodman, 1996). In vertebrates, embryological and tissue culture experiments have provided evidence for the operation of chemoattractant mechanisms in the guidance of many different classes of axons (reviewed by Tessier-Lavigne, 1992), raising the question of what other molecules function as chemoattractants. Are other chemoattractants related to the netrins in any way? Is chemoattraction a unified mechanism of guidance, with all chemoattractants acting via similar types of receptors and signal transduction mechanisms? Or are there different kinds of chemoattraction specialized for different types of guidance events?

To identify other chemoattractants and address these questions, we have focused on the development of the projections of spinal motor axons to peripheral targets in vertebrates, because previous studies have provided evidence for the existence of chemoattractants that guide these axons over different portions of their trajectory (for review, see Tosney, 1991; Eisen, 1994). Spinal motor neurons are born in the ventral portion of the spinal cord and project axons out toward target muscle cells. Different targets, such as the axial and limb musculatures, are innervated by distinct subpopulations of motoneurons whose cell bodies are located in distinct dorsoventral and rostrocaudal positions within the ventral spinal cord (see, e.g., Tsuchida, 1994). All spinal motor axons project ventrolaterally to exit the spinal cord, then migrate through the sclerotome, precursor of the vertebral column and ribs. Motor axons in each segment all project via the anterior portion of the sclerotome, owing to contact-repulsion by posterior sclerotome cells (Keynes and Stern, 1984; Oakley and Tosney, 1993), thus giving rise to the segmented pattern of ventral roots. Some evidence has also been provided that anterior sclerotome cells might secrete a chemoattractant for motor axons that could contribute to this segmentation (Stern, et al, 1986; Oakley and Tosney, 1989, Soc. Neurosci., abstract; Hotary and Tosney, 1996; see Discussion).

Motor pathways diverge within the sclerotome, presumably in response to both local and long-range guidance cues from tissues along their paths. For instance, epaxial motoneurons send their axons dorsally toward the dermomyotome, precursor of the axial musculature. Ablation studies in chick embryos suggest that the epaxial motor axons are attracted by a chemoattractant secreted by dermomyotome cells (Tosney, 1987); similarly, evidence has been provided that Xenopus laevis myotomal cells can attract axons from neural tube explants in culture (McCaig, 1986). Other motor axons avoid the dermomyotome, projecting ventrolaterally through the sclerotome. At limb levels, these axons converge at the base of the limb before projecting to their muscle targets in the limb along dorsal or ventral paths (Tosney and Landmesser, 1985). Grafting studies in a variety of species have suggested that spinal motor axons can be attracted by a limb-derived chemoattractant (Detwiler, 1934; Hamburger, 1939; Okamoto and Kuwada, 1991b). Furthermore, in tadpole, limb mesenchyme has been shown to enhance neurite outgrowth

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from spinal cord explants in culture (Pollack and Liebig, 1977). Collectively, these experiments suggest that the limb may secrete cues that can attract and guide motor axons at a distance. In addition, the ability of chick motor axons displaced by limb shifts or spinal cord reversals to project long distances through regions not normally followed by nerves has suggested the existence of muscle-derived attractants for motor axons (Lance-Jones and Landmesser, 1981).

Hepatocyte growth factor/scatter factor (HGF/SF) is a multifunctional growth factor which stimulates the proliferation, motility, or branching morphogenesis of various cell types (reviewed by Rosen et al., 1994; Jeffers et al., 1996). HGF/SF was originally identified as a mitogen for hepatocytes (hence, hepatocyte growth factor) (Nakamura et al., 1989) and a motogen (motility stimulator) for epithelial cells (hence, scatter factor) (Stoker et al., 1987). These diverse actions of HGF/SF all appear to be mediated by the c-Met receptor tyrosine kinase (Bottaro et al., 1991; Naldini et al., 1991; Weidner et al., 1993). HGF/SF is secreted as an inactive precursor of 728 amino acids, which can be cleaved extracellularly to yield an active two-chain form, composed of 69-kDa α and 34-kDa β chains (reviewed by Rosen et al., 1994; Jeffers et al., 1996). Mice with null mutations in the *Hgf/Sf* and *c-Met* loci have indistinguishable phenotypes (Schmidt et al., 1995; Uehara et al., 1995; Bladt et al., 1995). Mutant embryos die in utero starting at embryonic day 14 (E14) from a failure in liver and placental proliferation (a reflection of the mitogenic effects of HGF/SF); in addition, these embryos have defects in the migration of myoblasts to the limb, diaphragm, and tongue (a reflection of the motogenic effects of HGF/SF). Both *Hgf/Sf* and *c-Met* are expressed in the nervous system (see Discussion), and possible roles in nervous system development have been suggested on the basis of the ability of HGF/SF to promote neural induction (Stern et al., 1990; Streit et al., 1995), stimulate Schwann cell proliferation (Krasnoselsky et al., 1994), promote axon outgrowth from P19 embryonal carcinoma cells (Yang and Park, 1993; Yamagata et al., 1995), and increase c-Fos expression in septal neurons (Jung et al., 1994).

In this study, we have sought to identify chemoattractants for developing spinal motor axons. Our approach has been to search for chemoattractant effects of pathway-derived tissues by using in vitro coculture assays and to use such assays to identify candidate chemoattractants. This approach led to us to identify HGF/SF as a limb mesenchyme–derived chemoattractant for motor axons and also to implicate it as a survival factor for motoneurons.

Results

Identification of Pathway-Derived Activities Affecting Motor Axons In Vitro

To characterize diffusible cues that might participate in motor axon patterning, we cultured explants of ventral spinal cord (ventral explants) containing motoneurons together with explants of tissues that form the pathway for their axons in vivo. Tissues were dissected from E11 rat embryos and cultured for 40 hr in three-dimensional collagen matrices. At the time of dissection, motoneurons are the predominant or only neuronal population in the ventral neural tube and are in the final stages of differentiation and early axonogenesis; over the next 40 hr in vivo, they would normally project axons out the neural tube and innervate peripheral target tissues (Altman and Bayer, 1984; data not shown). Extensive axon growth was observed in ventral explants cultured alone, as assessed by immunolabeling of axons with antibodies to neurofilament (Figure 1A)). These axons were identified as motor axons by expression of p75, the low affinity NGF receptor, and peripherin, an intermediate filament protein (data not shown), two markers of motor axons at these stages (Yan and Johnson, 1988; Gorham et al., 1990).

Although motor axons extended in ventral explants cultured alone, most axons did not enter the surrounding collagen matrix, instead forming a swirling mass over the surface of the explant (Figure 1A). In contrast, when ventral explants were cultured at a distance of ~100–400 μm from explants of either forelimb mesenchyme or sclerotome, tissues that the axons normally invade, most axons left the explant to invade the collagen matrix (Figures 1B and 1C). The anterior and posterior portions of the sclerotome were equally effective in promoting outgrowth of motor axons (data not shown). The pathway-derived tissues consistently caused more profuse and longer outgrowth from the side of the ventral explant closest to the target (Figures 1B and 1C; data not shown), apparently reflecting a chemoattractant effect of the tissues (discussed below).

The effect of pathway-derived tissues was specific, since motor axon outgrowth in this assay was not elicited by explants of other neighboring non-pathway-derived tissues (Table 1). In particular, although previous experiments have suggested the existence of a dermomyotome-derived chemoattractant (Tosney, 1987), we have been unable to detect its activity (Figure 1D; Table 1). Thus, these results provide evidence for the existence of directional invasion–promoting activities from rat forelimb mesenchyme and sclerotome.

HGF/SF Possesses Invasion-Promoting Activity for Motor Axons

We next examined whether the activity of pathway-derived tissues could be recovered in soluble form. Medium conditioned by exposure to sclerotome tissue was as effective as pathway-derived tissues in causing invasion of the collagen matrix by motor axons, although the outgrowth was radial rather than asymmetric (Figure 1E). The activity in conditioned medium was stable to freeze–thaw, was lost by heating (60°C, 10 min) or treatment with protease, and was retained on an ultrafiltration membrane with a molecular mass cut-off of 500 kDa. In search of a more abundant source of activity, several cell lines of mesodermal origin were screened (Table 1). C3H10T1/2 cells (10T1/2 cells) (Reznikoff et al., 1973) were found to secrete a similar activity (Figure 1F), which was also heat and protease sensitive and retained on a 500 kDa cut-off ultrafiltration membrane. When fractionated by heparin-affinity chromatography, the activity eluted in a single peak centered at 1 M NaCl (Figure 2A). Fractionation of this eluate by gel-filtration

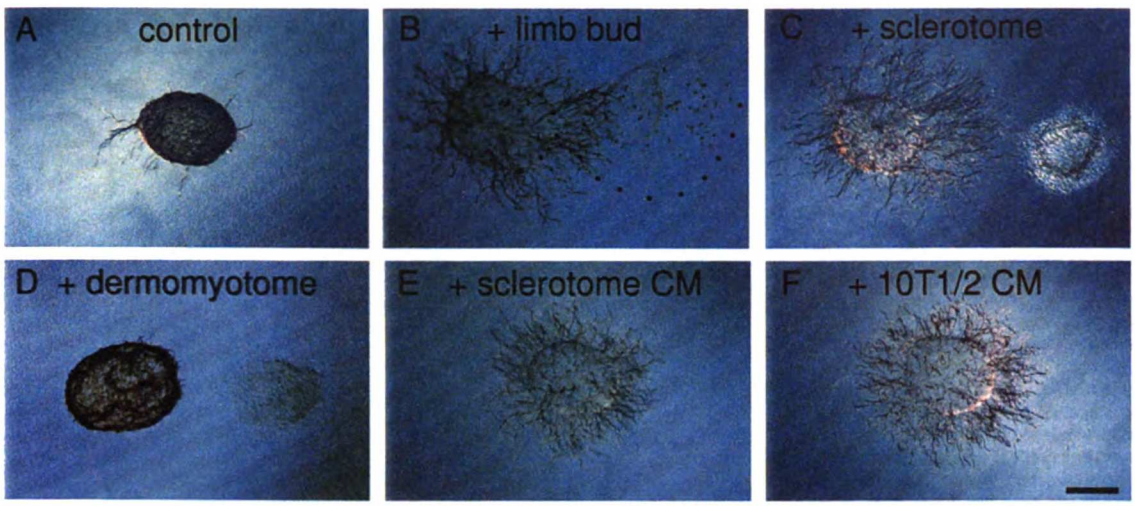

Figure 1. Pathway-Derived Tissues and Conditioned Media Promote Invasion of Collagen Gels by Spinal Motor Axons

E11 rat ventral spinal cord explants were cultured alone (A), with indicated tissues from E11 rat embryos (B–D), or with indicated conditioned media (E, F) for 40 hr in three-dimensional collagen matrices, then stained with anti-neurofilament antibodies to visualize axons.

(A) In ventral explants cultured alone, motor axons extend but are confined primarily to the body of the explant and rarely invade the collagen matrix.

(B, C) Both mesenchyme derived from the forelimb bud (B) and sclerotome tissue (C) promote invasion of the collagen matrix by motor axons with longer and more profuse outgrowth on the side facing the target. Note that the body of the ventral explant is relatively free of staining, indicating that most or all motor axons leave the explant. Axons seen within the sclerotome explant in (C) likely extend from neurons derived from neural crest cells in the explants; they were not always observed, and their presence or absence did not correlate with outgrowth promoting activity (which was always observed; see Figure 5).

(D) Dermomyotome does not promote outgrowth of motor axons.

(E, F) Medium conditioned by sclerotome explants (E) or 10T1/2 cells (F) promotes a radial invasion of the collagen matrix by motor axons. Scale bar, 100 μm.

chromatography on Sephacryl 200 indicated an apparent molecular mass of ~50–100 kDa (data not shown), suggesting that the original activity in 10T1/2 cell–conditioned medium was a high molecular mass complex that dissociated during chromatography.

These initial results prompted us to test heparin-binding factors in this molecular mass range for activity. Recombinant human HGF/SF was found to possess a similar invasion-promoting activity (Figure 2B; Table 1). Maximal activity was observed at concentrations of 5–10 ng/ml, with inhibition at higher concentrations (Figure 2C) (a characteristic of factors whose effects require receptor dimerization [e.g., Wells, 1996]). As with other heparin-binding factors, HGF/SF activity was potentiated by addition of soluble heparin (Figures 2B and 2C). Western blotting with antibodies to HGF/SF indicated that 10T1/2 cells produce HGF/SF, which cofractionates precisely with the invasion-promoting activity (Figure 2A) and which likely accounts for all the invasion-promoting activity of 10T1/2 cell–conditioned medium (as assessed by quantitative Western blotting; data not shown). Other factors we tested (including other heparin-binding factors and factors that influence the differentiation or survival of motoneurons or the outgrowth of motor axons) did not possess invasion-promoting activity (Table 1).

Table 1. Tissues and Factors Tested for Motor Axon Invasion–Promoting Activity

Tissues from E11 Rat Embryos	Survival/Growth/ Differentiation Factors		ECM Molecules/Chemoattractants/ Soluble CAMs	Cell Lines
Dorsal spinal cord	BDNF	EGF	fibronectin	C12
Dermomyotome	CNTF	bFGF[a]	laminin-1	C518
Lateral plate	GDNF	**HGF/SF**	laminin-2	
Notochord	LIF	MSP	netrin-1	**C3H10T1/2**
Forelimb ectoderm	NT-3	SHH	soluble L1–Fc fusion	
Forelimb mesoderm	NT-4/5	TGFβ1	soluble NCAM–Fc fusion	
Sclerotome			tenascin	
			thrombospondin	

E11 ventral spinal cord explants were cultured in collagen gels with the indicated tissues or factors (at the concentrations indicated in the Experimental Procedures). Conditions under which motor axon outgrowth was stimulated are underlined and in boldface type.

[a] bFGF also caused a small degree of axon outgrowth, but this outgrowth was morphologically distinct from that seen under all other culture conditions (axon bundles were short, thin, and spindly) and was associated with an increase in cell proliferation within the explants (data not shown).

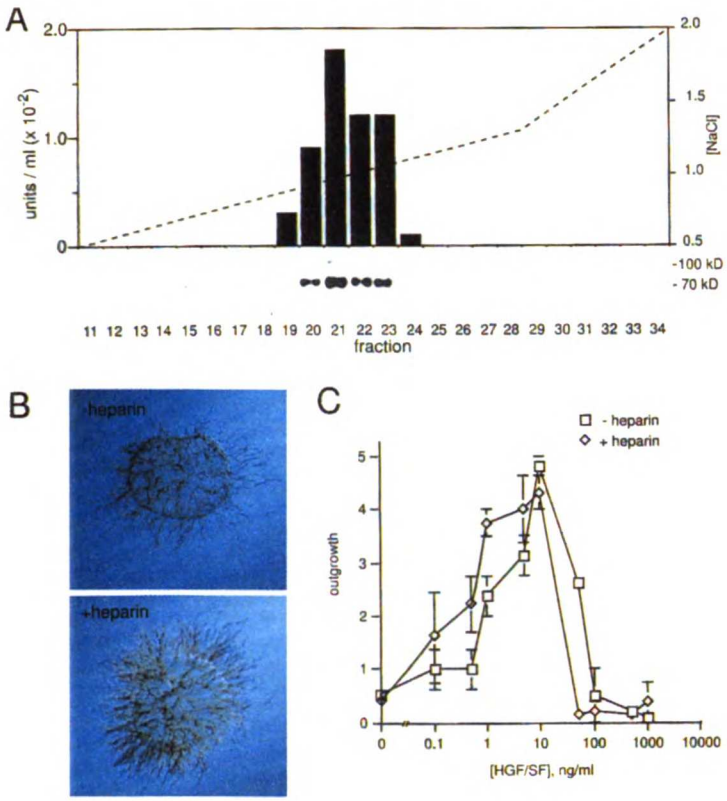

Figure 2. HGF/SF Possesses Motor Axon Invasion–Promoting Activity

(A) Cofractionation of invasion-promoting activity and HGF/SF in medium conditioned by 10T1/2 cells during heparin-affinity chromatography. Conditioned medium bound to a heparin column was eluted with a 0.5–1.5 M NaCl gradient followed by a step to 2 M NaCl; salt concentration of 500 μl fractions was estimated from conductivity measurements on 1:100 dilutions of the fractions (broken line). Motor axon invasion–promoting activity (thick vertical bars; see Experimental Procedures for definition of a unit of activity) was observed in a subset of the fractions centered at 1 M NaCl and cofractioned with HGF/SF detected in the fractions by Western blotting (gel in [A]). The antiserum detects the α chain (~70 kDa) of HGF/SF; the faint upper band likely corresponds to a small amount of uncleaved HGF/SF precursor (~100 kDa) in the medium.

(B) Outgrowth elicited by 5 ng/ml recombinant human HGF/SF is potentiated by addition of 50 ng/ml heparin.

(C) Dose–response curve for the invasion-promoting activity of HGF/SF, in the absence (squares) or presence (diamonds) of 50 ng/ml heparin. Outgrowth was scored blindly by two observers using a 0–5 scale (see Figure 9). Points indicate means ± SEM for eight ventral explants in two separate experiments at each concentration.

HGF/SF Possesses Chemoattractant Activity for Motor Axons

The asymmetric growth of axons toward pathway-derived tissues was suggestive of a chemoattractant effect. However, since outgrowth was observed from all sides of the explant (see, e.g., Figures 1B and 1C), the asymmetry could also in principle be caused by a dose-dependent effect of an invasion-promoting activity that does not actually direct axon growth (see Lumsden, 1988, for discussion). To distinguish these possibilities, we performed tandem coculture experiments (Charlwood et al., 1972; Lumsden and Davies, 1983), in which two ventral explants, proximal and distal, were exposed in tandem to a source (Figure 3)). Outgrowth from the near side of the distal explant can be more profuse than outgrowth from the far side of the proximal explant only if the activity is chemoattractant (Figures 3A and 3B).

To present HGF/SF as a point source, we generated a stable cell line secreting recombinant rat HGF/SF. Aggregates of these cells elicited outgrowth from ventral explants that was qualitatively indistinguishable from outgrowth in response to either limb mesenchyme or sclerotome and that could be completely blocked with an antibody directed against rodent HGF/SF (data not shown). In tandem cocultures with limb mesenchyme, sclerotome, or HGF/SF-secreting cell aggregates, the amount of outgrowth from the near side of the distal explant was greater than outgrowth from the far side of the proximal explant, even though the more distal explant was farther from the source (Figures 3C–3E, and legend), demonstrating that the tissues and cells are producing a chemoattractant effect. It should be noted that we did not observe a major reorientation of axons toward the source, as might have been expected for a chemoattractant. This may be explained by fact that the axons grew in bundles (fascicles) in the collagen, and axons that can reorient growth when unfasciculated can have difficulty reorienting within collagen matrices when fasciculated (e.g., compare Heffner et al., 1990, with Placzek et al., 1990, and Pini, 1993).

HGF/SF Is a Candidate for the Limb-Derived Chemoattractant

We next examined whether the sites and timing of expression of HGF/SF and its receptor, c-Met, are consistent with a role in motor axon guidance in vivo. These expression studies were performed in mouse because of the availability of relevant transgenic strains (see below). Early stages of rat and mouse development are similar, except that the mouse develops more rapidly. Thus, E11 in the rat, the developmental stage at which we dissect ventral explants, corresponds to E9.5 in the mouse. At this stage, when motoneurons are in late stages of differentiation or have recently initiated axonogenesis (Figure 4A), c-*Met* mRNA is not yet detectable in motor neurons (Figure 4B), and *Hgf/Sf* mRNA is expressed at high levels in the proximal forelimb but is not detectable in the sclerotome (Figure 4C). A day later (E10.5 in the mouse or E12 in the rat), when motor axons have reached the

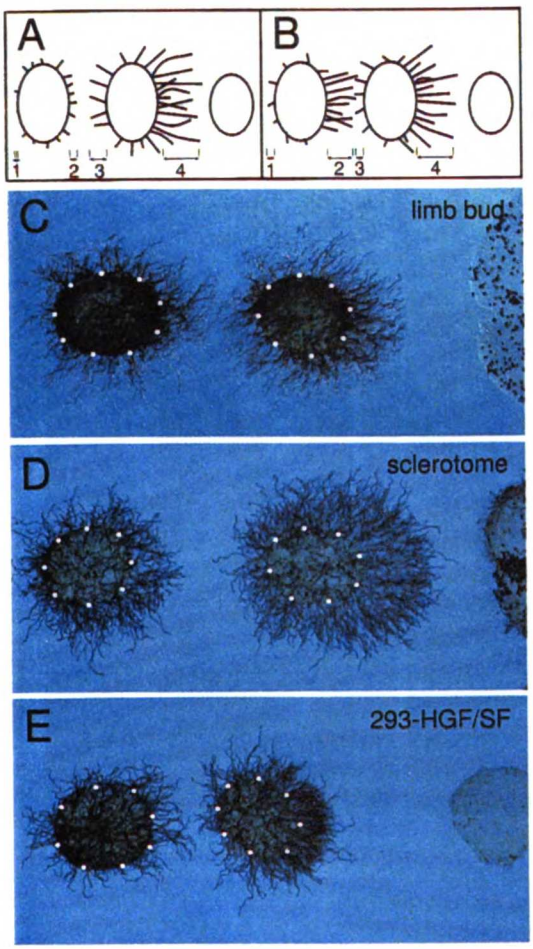

Figure 3. Pathway-Derived Tissues and HGF/SF Have Chemoattractant Activity for Motor Axons

Chemoattractant activity of tissues and cells was assessed by use of a tandem coculture paradigm in which two ventral explants, proximal and distal, are exposed in tandem to a source of activity.

(A) If the activity promotes outgrowth in a concentration-dependent but direction-independent manner, outgrowth from the near side of the distal explant should be less profuse than outgrowth from the far side of the proximal explant (i.e., distance 2 < distance 3 in the diagram).

(B) If the source has chemoattractant activity, then outgrowth from the near side of the distal explant can be more profuse than outgrowth from the far side of the proximal explant, even though the distal explant is farther from the source (i.e., distance 2 > distance 3).

(C–E) Coculture of two ventral explants in tandem with forelimb mesenchyme (C), sclerotome tissue (D), or an aggregate of 293 cells secreting HGF/SF (E). In all cases, the amount of outgrowth from the near side of the distal explant was greater than outgrowth from the far side of the proximal explant. To quantify this effect, we measured the lengths of the five longest axon bundles in regions 2 and 3 (compare Lumsden and Davies, 1983); for each coculture, the average length of the five axons in region 2 was greater than that in region 3. The mean values (± SE) of these averages in regions 2 and 3 were 220 ± 9.2 μm and 163 ± 13 μm (n = 10) for limb mesenchyme; 249 ± 9 μm and 167 ± 9 μm (n = 12) for sclerotome; and 215 μ 1 μ and 148 ± 4 μm (n = 6) for HGF/SF-secreting cells. In each case, the mean length in region 2 was significantly greater than that in region 3 ($p < 0.0005$, pairwise one-tailed Student's t test). Thus, the limb and sclerotome-derived activities, as well as HGF/SF, are chemoattractants for motor axons.

Scale bar, 100 μm.

base of the forelimb (Figure 4D), c-*Met* mRNA is expressed in a subset of motoneurons at the forelimb level (Figure 4E), and *Hgf/Sf* mRNA is expressed in the dorsal and ventral muscle masses within the forelimb (Figure 4F). At E11.5 in the mouse (E13 in the rat), when motor axons have initiated complicated branching patterns within the limb (Figure 4G), c-*Met* mRNA expression persists in a subset of motoneurons (Figure 4H), and *Hgf/Sf* mRNA persists in the developing muscle masses (Figure 4I). These results parallel results obtained on *Hgf/Sf* and c-*Met* expression in the chicken (Théry et al., 1995). The expression of *Hgf/Sf* in the limb at E9.5 and E10.5 appears to be within mesenchymal cells rather than myoblasts, since it is also observed in limbs from embryos homozygous for a mutation in c-*Met* (data not shown), in which the myoblasts fail to invade the limb (Bladt et al., 1995).

Thus, expression data are consistent with the possibility that HGF/SF, acting via c-Met, is responsible for the limb-derived activity and plays a role in motor axon progression, since *Hgf/Sf* mRNA is expressed in the limb, and motoneurons express c-*Met* mRNA prior to the stage of limb invasion by these axons. The evidence does not, however, support a role for HGF/SF in mediating invasion of the sclerotome, since *Hgf/Sf* mRNA is never detected in the sclerotome and since motoneurons do not express detectable c-*Met* mRNA at the time when they are growing through the sclerotome.

Direct evidence that motor axons are not initially responsive to HGF/SF was obtained by exposing E11 rat ventral explants to HGF/SF at different times in culture. When ventral explants were cultured for 24 hr in the absence of factor, then exposed to HGF/SF, axon outgrowth at 40 hr was indistinguishable from that observed when ventral explants were cultured for the entire 40 hr in the presence of HGF/SF (data not shown). In contrast, when ventral explants were cultured for 24 hr in HGF/SF, then washed extensively and cultured further in its absence, no outgrowth above background was observed at 40 hr (data not shown). These results suggest that for the first ~24 hr after explanting, the axons are not responsive to HGF/SF and that they become responsive thereafter. This timing fits with the expression of c-*Met*, which is first detected at E10.5 in the mouse, equivalent to E12 in the rat, i.e., 24 hr after the developmental stage at which ventral explants are isolated.

It is important to note that not all motoneurons express c-*Met*. Examination of serial sections from E10.5 and E11.5 embryos shows that c-*Met* mRNA is expressed by subsets of motoneurons in both the medial and lateral halves of the motor column (insets to Figures 4E and 4H; data not shown), which send axons to the dorsal and ventral muscle masses in the limb, respectively. The pattern of expression does not correlate in obvious ways with the initial pathway choices made by the axons of these neurons (Tsuchida et al., 1994).

HGF/SF Mediates the Limb-Derived Activity for Motor Axons

To test whether HGF/SF mediates the limb-derived activity, we made use of a neutralizing antiserum directed against murine HGF/SF (see Experimental Procedures)

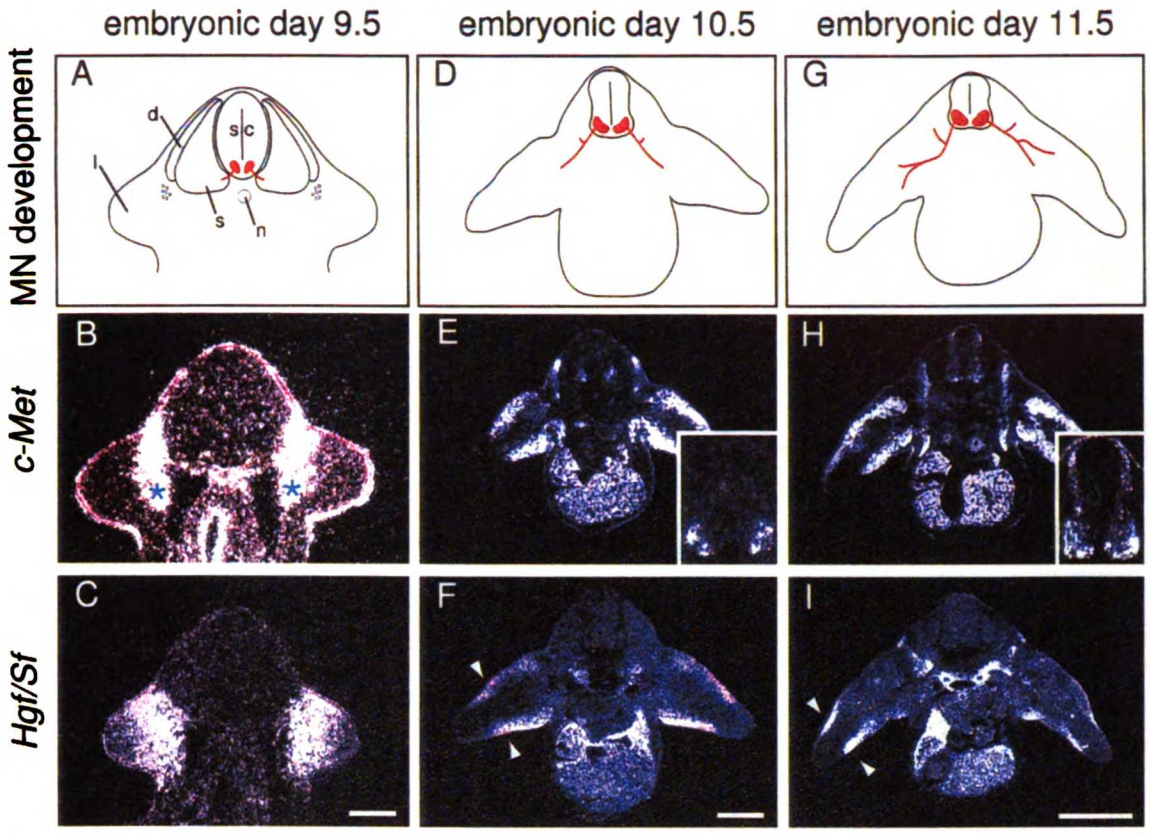

Figure 4. Expression of c-*Met* mRNA and *Hgf/Sf* mRNA in Relation to the Development of Motor Axon Projections in the Mouse

Diagrams of progression of motor axon outgrowth in mouse embryos (A, D, G) determined by DiI labeling (data not shown), compared with transverse sections at the forelimb level hybridized to a *c-Met* probe (B, E, H) or an *Hgf/Sf* probe (C, F, I).

(A–C) E9.5. Motor axons (red in [A]) have started to exit the neural tube to invade the sclerotome (s). Also diagrammed are the spinal cord (sc), dermomyotome (d), limb (l), and notochord (n). At this age, *c-Met* expression (B) is not observed in the spinal cord, although it is prominent in the lateral tip of the dermomyotome and in myoblasts that have delaminated and started to migrate into the forelimb (asterisk). *Hgf/Sf* mRNA expression (C) is prominent in the proximal forelimb buds but not in sclerotome.

(D-F) E10.5. Motor axons have reached the base of the limb, and rudiments of the epaxial branches (projecting to the dermomyotome) have formed (D). At this age, *c-Met* mRNA is detected in a subset of motor neurons in the ventral spinal cord ([E]; higher power view provided in inset), as well as in myoblasts in the dorsal and ventral muscle masses in the forelimb, and in other peripheral structures. *Hgf/Sf* mRNA is detected in dorsal and ventral forelimb (arrows) and in viscera but not in sclerotome (F).

(G–I) E11.5. Motor axons have started to invade the forelimb and to elaborate a complex branching pattern (G). At this age, *c-Met* mRNA expression continues to be detected in subsets of motoneurons ([H]; higher power view provided in inset); expression is also observed in cells in the dorsal spinal cord, as well as in the dorsal and ventral muscle masses and other peripheral structures. *Hgf/Sf* mRNA expression continues in the dorsal and ventral aspects of the forelimb (arrows) and viscera, as well as the pelvic girdle, but is still absent from sclerotome (I).

Scale bar, 300 μm (A–C); 600 μm (D–F); 1000 μm (G–I); 1300 μm (inset in [E]); and 2000 μm (inset in [H]).

and of mice carrying inactivating mutations in the *Hgf/Sf* or c-*Met* genes (Schmidt et al., 1995; Bladt et al., 1995). Forelimb mesenchyme from wild-type E9.5 mouse embryos was as effective as that from E11 rat embryos in promoting outgrowth of motor axons from E11 rat ventral explants (Figure 5A). In contrast, forelimb mesenchyme derived from littermates homozygous for the mutant *Hgf/Sf* allele did not elicit outgrowth above that observed with ventral explants cultured alone (Figure 5B; statistical analysis indicates the absence of any detectable activity [Figures 5D and 5E]). This result suggested that HGF/SF is responsible for the limb-derived activity. However, since HGF/SF is required for migration of muscle precursor cells into the limb (Bladt et al., 1995), it seemed possible that muscle precursors secrete a factor distinct from HGF/SF that mediates the limb-derived activity, whose absence in limbs from *Hgf/Sf*$^{-/-}$ animals was a secondary consequence of the absence of these cells. This possibility was, however, ruled out by the finding that limb explants from embryos homozygous for the mutation in the c-*Met* gene, which also lack muscle precursors (Bladt et al., 1995), elicited outgrowth indistinguishable from that elicited by wild-type limbs (Figure 5C).

The suggestion that HGF/SF is responsible for the invasion-promoting activity of limb mesenchyme was further supported by the finding that the anti-HGF/SF antiserum (which binds rodent but not human HGF/SF)

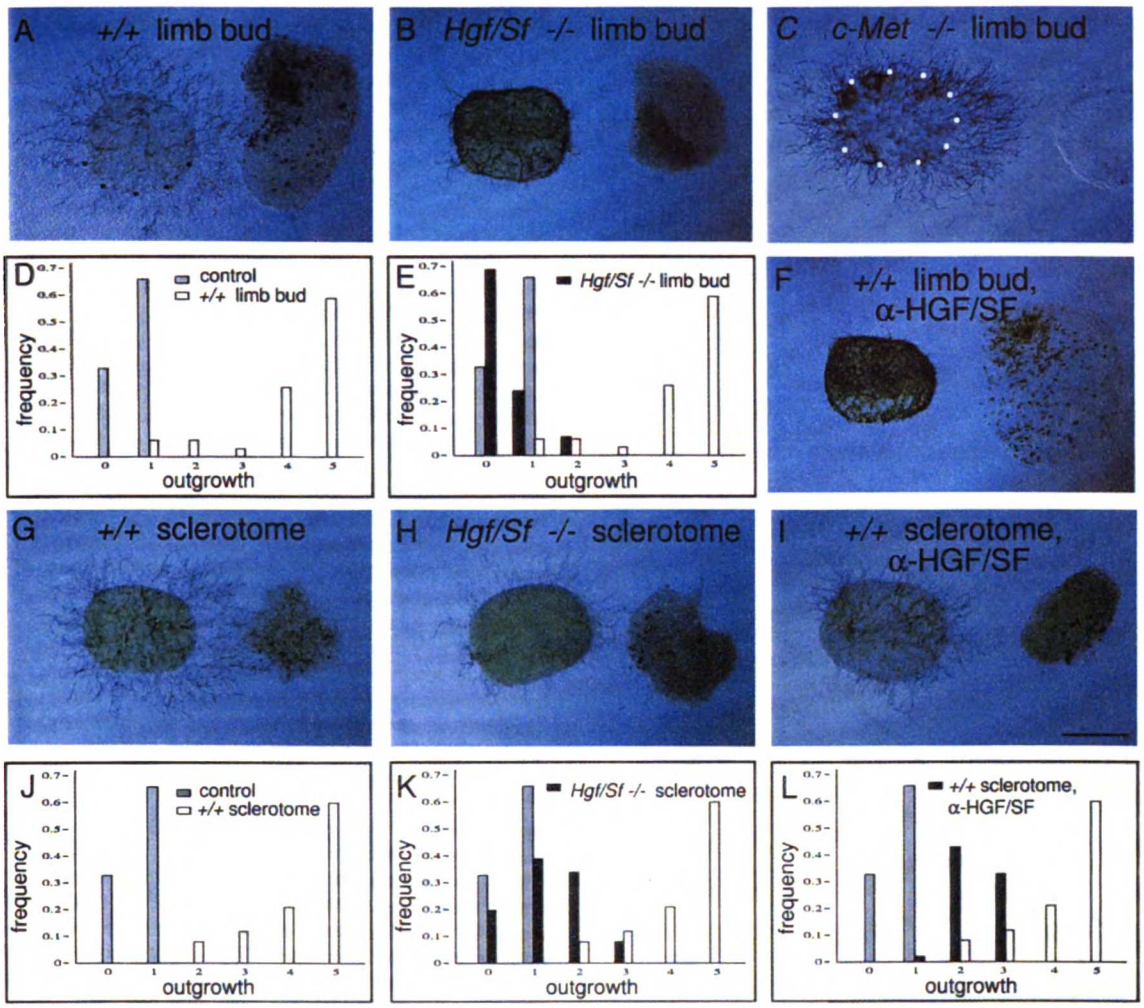

Figure 5. HGF/SF Accounts for the Entire Limb-Derived Activity but Only for Part of the Sclerotome-Derived Activity Observed In Vitro

Test of the involvement of HGF/SF in mediating the limb-derived (A–F) and the sclerotome-derived (G–L) activities for motor axons. In all panels, outgrowth of motor axons from E11 rat ventral spinal cord explants was examined after 40 hr in culture in collagen matrices in the presence of tissues derived from wild-type (A, G, F, I), *Hgf/Sf*$^{-/-}$ (B, H), or c-*Met*$^{-/-}$ (C) E9.5 mouse embryos. In (F) and (I), tissues from wild-type embryos were cultured in the presence of 20 μg/ml of a neutralizing antibody directed against HGF/SF. Outgrowth was scored blindly on the 0–5 scale of Figure 9 (see Experimental Procedures) by two independent observers, and the responses under different culture conditions were quantified in histograms (D, E, J, K, L) (data in histograms show the cumulative results for four forelimb mesenchyme and four sclerotome explants obtained from each of 27 *Hgf/Sf*$^{-/-}$ embryos and 16 wild-type littermates). Explants shown in (A)–(C) and (F)–(I) were representative of the average response for each condition.

(A–C) Profuse motor axon outgrowth is elicited by forelimb mesenchyme from wild-type (A) and c-*Met*$^{-/-}$ (C) but not from *Hgf/Sf*$^{-/-}$ (B) E9.5 embryos (similar responses observed for four explants from each of five *Met*$^{-/-}$ embryos).

(D) Histograms of the frequency of occurrence of different outgrowth responses (0–5 scale of Figure 9) observed for ventral explants cultured alone (negative control, gray bars) and with wild-type forelimb mesenchyme (positive control, open bars).

(E) Histograms comparing responses elicited by forelimb mesenchyme from *Hgf/Sf*$^{-/-}$ embryos (closed bars) to responses under the negative and positive control conditions plotted in (D). Outgrowth elicited by *Hgf/Sf*$^{-/-}$ forelimb mesenchyme was significantly reduced compared with that elicited by wild-type forelimb mesenchyme ($p < 0.001$, Mann–Whitney U test) and was not statistically different from that observed from ventral explants cultured alone ($p = 0.9$).

(F) The anti-HGF/SF antibody (αHGF/SF) completely blocks the invasion-promoting activity of wild-type E9.5 mouse forelimb mesenchyme (A).

(G, H) Motor axon outgrowth elicited by sclerotome derived from *Hgf/Sf*$^{-/-}$ E9.5 embryos (H) is partly reduced compared with that elicited by sclerotome from wild-type E9.5 embryos (G) but not completely eliminated.

(I) Similarly, the anti-HGF/SF antibody blocks part but not all of the invasion-promoting activity of wild-type E9.5 mouse sclerotome tissue (G).

(J) Histograms of the frequency of occurrence of different outgrowth responses (0–5 scale of Figure 9) observed for ventral explants cultured alone (negative control, gray bars) and with wild-type sclerotome (positive control, open bars).

(K, L) Histograms comparing the responses elicited by sclerotome from *Hgf/Sf*$^{-/-}$ embryos (closed bars in [K]) or by wild-type sclerotome when cultured with anti-HGF/SF (closed bars in [L]) to the responses under the negative and positive control conditions plotted in (J). Outgrowth elicited by *Hgf/Sf*$^{-/-}$ sclerotome (K) or by wild-type sclerotome when cultured with anti-HGF/SF (L) was reduced compared with that elicited by wild-type sclerotome in the absence of antibody ($p < 0.001$ in both cases, Mann–Whitney U test) but was still significantly greater than that observed from ventral explants cultured alone ($p < 0.001$ in both cases).

Scale bar in (I), 100 μm.

completely blocked the outgrowth-promoting effect of E9.5 mouse forelimb mesenchyme when used at 10–60 μg/ml (Figure 5F; data not shown). This blocking effect did not appear to reflect a nonspecific or toxic effect, since the antibody did not affect commissural axon outgrowth from E11 rat dorsal spinal cord explants in response to 500 ng/ml netrin-1 or sensory axon outgrowth from E14 rat dorsal root ganglia in response to 10 ng/ml nerve growth factor (NGF), nor did it block outgrowth elicited from ventral explants by recombinant human HGF/SF (data not shown).

If HGF/SF is indeed responsible for the limb-derived activity, then motor axons from c-*Met*$^{-/-}$ embryos should be unresponsive to both HGF/SF and limb mesenchyme. We have attempted to test this prediction but unfortunately found that neural tissue (whether wild-type or mutant) isolated from the strain of mice carrying the mutant c-*Met* allele does not exhibit good axon outgrowth under our culture conditions.

We next examined whether HGF/SF contributes to the activity of sclerotome tissue. This was not expected, since *Hgf/Sf* mRNA is not detected in sclerotome in vivo. To our surprise, we found that outgrowth elicited by *Hgf/Sf*$^{-/-}$ sclerotome was significantly reduced, but not eliminated, compared with that from wild-type sclerotome (Figures 5G and 5H). Quantification of the effect (Figures 5J and 5K) showed that the outgrowth elicited by *Hgf/Sf*$^{-/-}$ sclerotome is significantly greater than that observed from ventral explants cultured alone. Similarly, the neutralizing antiserum directed against HGF/SF reduced, but did not eliminate, outgrowth (Figures 5I and 5L).

These results indicate that part of the sclerotome-derived activity is due to a factor(s) distinct from HGF/SF. They also indicate, however, that part of the activity is due to HGF/SF, a paradoxical result, since sclerotome does not express detectable levels of *Hgf/Sf* mRNA in vivo. The paradox was, however, resolved by the finding that expression of *Hgf/Sf* mRNA by sclerotome is induced over 500-fold when the sclerotome tissue is explanted and cultured for 40 hr (Figure 6).

Defects in Limb Motor Axon Branching in *Hgf/Sf*$^{-/-}$ Mice

We next examined whether HGF/SF is required for normal guidance of motor axons to their targets in the limb by tracing the trajectories of motor axons in *Hgf/Sf*$^{-/-}$ embryos every day between E9.5 and E13.5. We did not expect a misrouting of all motor axons, since not all motoneurons at limb levels express c-*Met* mRNA (Figures 4E and 4H).

In *Hgf/Sf*$^{-/-}$ mice, motor axons first emerged from the spinal cord at E9.5 and reached the base of the limb by E10.5 without apparent defects or delays (data not shown). At E11.5, subtle but consistent defects in the pattern of motor axon branching within the limb became apparent (data not shown), presaging defects seen at E12.5 in a subset of motor axon branches (Figure 7). Two branches (labeled 1 and 7 in Figure 7) show reduced or delayed branching of axon terminal arbors on the nascent muscle target. A prominent branch in the dorsal anterior limb (branch 2) is consistently missing. A prominent branch in the dorsal limb (branch 3) bifurcates abnormally. Finally, a smaller branch in the ventral limb

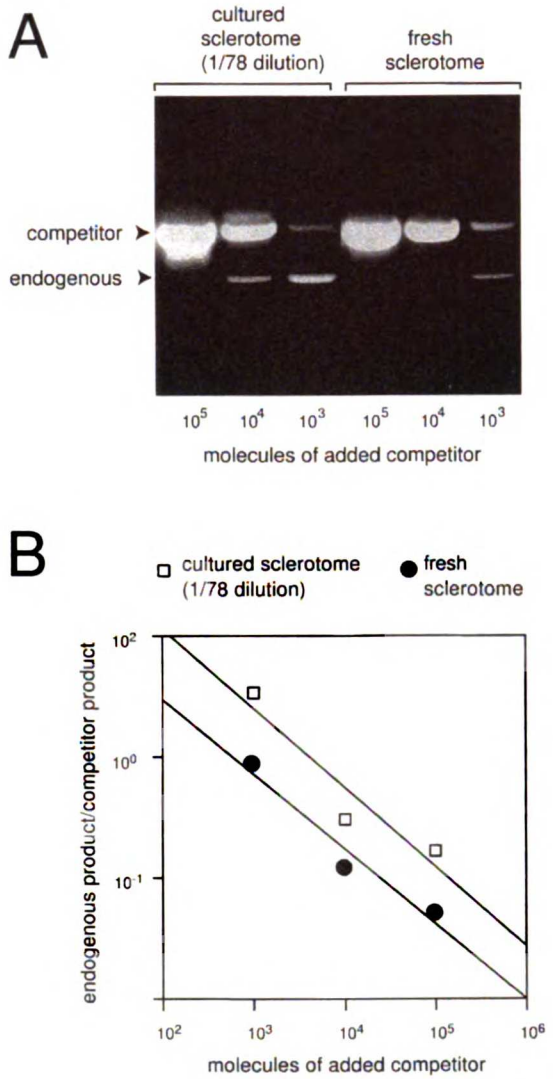

Figure 6. Induction of *Hgf/Sf* Expression in Cultured Sclerotome

Sclerotome was isolated from E9.5 mouse embryos and either frozen immediately or cultured for 40 hr. The number of *Hgf/Sf* transcripts in fresh or cultured sclerotome was estimated by PCR amplification in the presence of serial 10-fold dilutions of exogenously added competitor and ^{32}P-labeled nucleotides.

(A) PCR amplification products from cDNA obtained from fresh or cultured sclerotome (the latter cDNA was diluted 78-fold after normalization, prior to use). Products from added competitor or endogenous *Hgf/Sf* transcripts are indicated. The number of molecules of added competitor is shown below each lane.

(B) Plot of the ratio of endogenous to competitor *Hgf/Sf* product (obtained as the ratio of the intensities of the bands in each lane in [A]) as a function of the number of molecules of added competitor. The initial number of *Hgf/Sf* cDNA molecules in the reaction is equal to the number of molecules of added competitor required to obtain a ratio of 1. The plot indicates a ~7-fold higher initial number of *Hgf/Sf* cDNA molecules in samples from cultured sclerotome than from fresh sclerotome; since the former sample was diluted 78-fold prior to the experiment, the actual induction of *Hgf/Sf* was 540-fold in cultured sclerotome compared with fresh sclerotome. An independent experiment yielded an estimate of 560-fold induction.

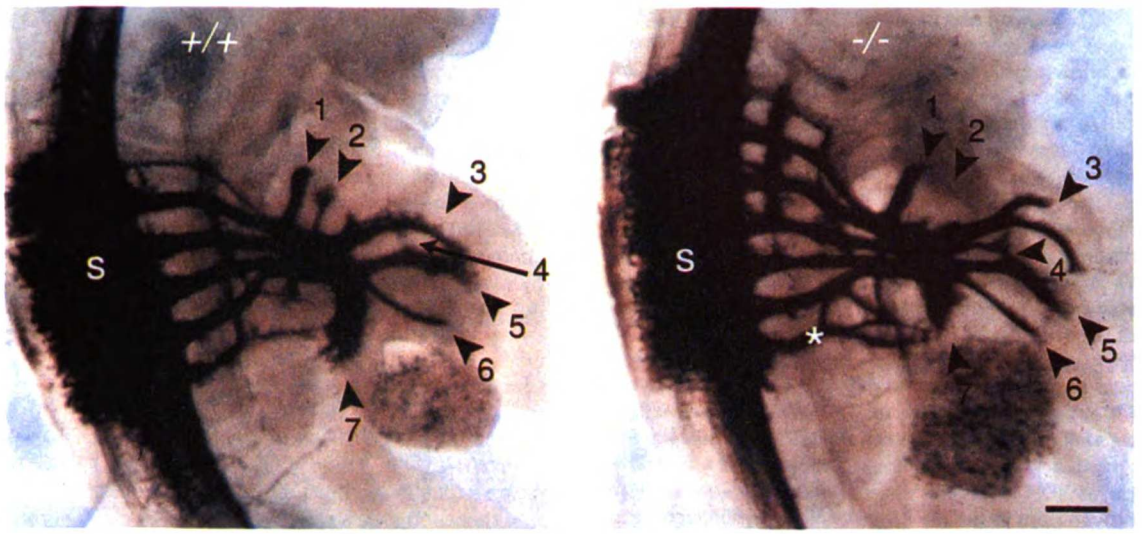

Figure 7. Pattern of Motor Axon Projections in Wild-Type and *Hgf/Sf*$^{-/-}$ Embryos at E12.5

Motor axons at the level of the forelimb in E12.5 wild-type and *Hgf/Sf*$^{-/-}$ embryos were labeled with DiI, which was then photoconverted to a visible reaction product. Branches within the limb were assigned arbitrary numbers between 1 and 7. Branch 1 lies quite proximal in the forming shoulder region, branches 2 and 3 innervate dorsal regions of the limb, and branches 4 to 7 innervate ventral regions of the limb. In the mutant, branches 1 and 7 were intact but had considerably reduced volume (the micrograph shown here failed to capture the reduction for branch 1, which was evident when directly observed under the microscope). These defects may result from a failure of terminal arborization in the muscleless limb (see Discussion). Branch 2 was altogether missing. Branch 3 in mutants bifurcated, whereas in controls it showed only a light defasciculation along the length of the branch. Branch 4 lies directly under branch 3 in wild-type embryos; in mutants, this branch consistently appeared in a more medial position with a flared termination. Branches 5 and 6 were not obviously affected in the mutant, at this level of analysis. The asterisk indicates axons that were labeled to varying extents depending on precise placement of the DiI crystals; no significant difference in these axons was observed between wild-type and mutant embryos, when all embryos were considered together (data not shown). Epaxial motor projections appeared unaffected in the mutants (data not shown). Ten wild-type and six mutants were examined at this age, and within each group the projections were identical.

Scale bar, 190 μm.

(branch 4) is misdirected to a more medial position and terminates in an abnormal flared ending. These defects appear to be fully penetrant, as they were observed in all six of the E12.5 mutant HGF/SF embryos examined. Although a detailed analysis of defects in c-*Met*$^{-/-}$ embryos was not performed, we did examine projections at E11.5 in these embryos. Branching defects in E11.5 c-*Met*$^{-/-}$ limbs were identical to those seen in E11.5 *Hgf/Sf*$^{-/-}$ limbs, with the exception that the defect of branch 2 was observed in only 4 of 6 embryos examined (data not shown).

HGF/SF Promotes the Survival of Spinal Motor Neurons

Once their axons reach muscle cells, motoneurons become dependent for their survival on a muscle-derived trophic factor(s). Competition for a limiting supply of the trophic factor(s) results in death of about half of all motoneurons (reviewed by Oppenheim, 1996), which, in the lumbar spinal cord in mice, occurs between E13 and E18 (Lance-Jones, 1982). At these stages, a subset of motoneurons still expresses c-*Met* mRNA (data not shown), and muscle cells now express *Hgf/Sf* mRNA (Sonnenberg et al., 1993). To determine whether HGF/SF can function as a survival factor for motoneurons in cell culture, motoneurons were isolated from E15 rat embryos, purified to greater than 80% homogeneity by panning, and cultured in defined medium without added factor, with HGF/SF, or with several other factors that promote motoneuron survival. Motoneurons cultured without added factor died rapidly, but HGF/SF was able to rescue many of these neurons during the 3-day culture period (Figures 8A and 8B) in a dose-dependent manner (Figure 8C) and was as effective as basic fibroblast growth factor (bFGF) and glial cell line–derived neurotrophic factor (GDNF), the most potent of the other factors tested (Figure 8D).

Discussion

The guidance of spinal motor axons to their target muscles has been proposed to be directed in part by chemotropic factors secreted by cells that form the intermediate or final targets for these axons. We have identified HGF/SF as a limb-derived chemoattractant for the axons of motoneurons, which can function to direct the growth of these axons and also to promote the survival of these neurons at later stages of their development. These results add HGF/SF to the roster of candidate guidance cues for developing axons, of which the netrins were the only previously known chemoattractants. They also extend the range of functions of HGF/SF, which had previously been implicated in regulating cell proliferation, motility, and morphogenesis but had not been shown to affect the growth of developing axons or neuronal survival.

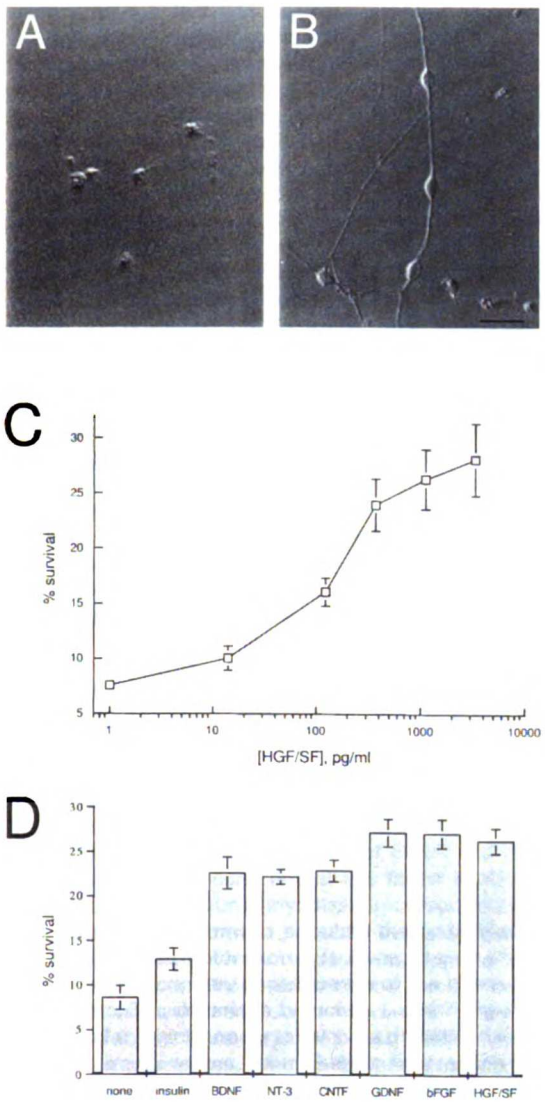

Figure 8. HGF/SF Promotes the Survival of Embryonic Motor Neurons

(A, B) Phase-contrast micrographs of motoneurons from E15 rat spinal cord cultured at low density on laminin-2 (merosin) for 72 hr in the absence (A) or presence (B) of 10 ng/ml HGF/SF. Scale bar, 75 μm.

(C) Dose-dependence of the survival-promoting effect of HGF/SF on motoneurons. Survival was assessed at 72 hr by mitochondrial uptake of MTT dye (see Experimental Procedures). Values shown are normalized to survival at 1 hr and represent means ± SD (all experiments done in triplicate in each experiment, for three separate experiments).

(D) HGF/SF is at least as potent as six other trophic factors in promoting the survival of motoneurons. Survival assessed and quantified as in (C). All factors were tested at 10 ng/ml.

HGF/SF Is a Limb Mesenchyme–Derived Chemoattractant

In vivo studies in amphibians, fish, and birds have shown that limb buds (or, in the case of fish, fin buds) grafted to ectopic locations can attract motor axons (Detwiler, 1934; Hamburger, 1939; Okamoto and Kuwada, 1991b). Similarly, in vitro studies using tadpoles have shown that limb mesenchyme can promote outgrowth of the axons of some spinal cord neurons (presumably motor neurons) (Pollack and Liebig, 1977). These findings have suggested that motor axons may be attracted to the base of the limb or into the limb by a factor secreted by the limb mesenchyme.

Similarly, we have found that in rodents the limb mesenchyme can promote the invasion of a collagen matrix by motor axons and attract these axons in vitro. We have also identified HGF/SF as the factor that mediates these activities in rodents. Since c-Met is currently the only known receptor for HGF/SF (Naldini et al., 1991; Bottaro et al., 1991; Weidner et al., 1993) and since motoneurons express c-*Met* mRNA, we presume that the actions of HGF/SF on motor axons are mediated by c-Met. If so, we would predict that only a subset of motor axons is sensitive to the actions of HGF/SF and limb mesenchyme, since only a subset of motoneurons expresses c-*Met* mRNA. This prediction might seem at odds with the observation in vitro that most axons appear to leave ventral explants in response to HGF/SF and limb mesenchyme (see Figures 1 and 2). It is possible, however, that only axons expressing c-Met do in fact respond directly to these factors and that other axons then grow out by fasciculating with the responders. Alternatively, since we have not yet been able to test directly whether loss of c-Met function abolishes motor axon responses to HGF/SF and limb mesenchyme (see Results), we cannot exclude the formal possibility that some motor axons (perhaps those that do not express c-Met) express an as yet unidentified receptor for HGF/SF.

What is the function of HGF/SF in motor axon guidance? Previous grafting and in vitro studies have been interpreted to suggest that a limb-derived chemoattractant contributes to directing motor axons to or into the limb. The sites and timing of expression of *Hgf/Sf* and c-*Met*, as well as our in vitro data, are certainly consistent with such a role; yet we have not observed obvious delays or defects in the projection of motor axons to the base of the limb or in their initial projection into the limb at E9.5 and E10.5 in *Hgf/Sf*$^{-/-}$ embryos. There are several ways in which the in vivo loss of function and the in vitro data might be reconciled. First, since our dye-tracing experiments enabled us only to examine all motor axons in bulk, we cannot exclude the presence of more subtle defects in initial projections in *Hgf/Sf*$^{-/-}$ embryos. In particular, axons that express c-Met might exhibit more severe defects that were obscured by the other axons; this possibility cannot be addressed at present, owing to the absence of reagents to visualize the c-Met-expressing axons selectively. If this explanation proves correct, we would conclude that different factors are responsible for guiding the axons that express c-Met and those that do not. An alternative explanation would be the existence of multiple redundant guidance systems for all motor axons in their initial projections to or into the limb in rodents, such that loss of any one system does not markedly impair any projections. The results of limb-bud ablation studies in amphibians and birds, and the analysis of a limbless mutant

in chick, have, in fact, been largely interpreted as showing that limb-derived factors, although capable of attracting motor axons, are not absolutely required to direct the axons to the base of the limb or into the limb (Detwiler, 1934; Hamburger, 1934; Piatt, 1956; Hollyday and Hamburger, 1976; Oppenheim et al., 1978; Ferguson, 1983; Lanser and Fallon, 1984), consistent with the presence of multiple redundant guidance systems. Other experiments have, however, suggested more stringent requirements for limb-derived cues. In the Medaka fish, motor nerves innervating the pectoral fin converge from five segmental levels to the base of the fin. If a fin bud is ablated, the fin nerves fail to converge, a defect also observed in a finless mutant (Okamoto and Kuwada, 1991a, 1991b), consistent with the presence of a fin-derived chemoattractant (perhaps HGF/SF) that is essential for directing motor nerves into the fin. Similarly, some evidence for misrouting following limb ablations has been provided in chick embryos (Tosney and Landmesser, 1984).

Although the inital pattern of projections in *Hgf/Sf*$^{-/-}$ embryos appeared normal, subtle defects were consistently observed in the formation of the stereotyped pattern of motor nerve branches within the forelimbs of the mutants, beginning around E11. Three branches were reduced or missing, one large branch showed an abnormal bifurcation, and one small branch was consistently mispositioned. Although these results are consistent with a guidance role for HGF/SF, several factors make it difficult to pinpoint what this role might be. The first is that our studies have focused on mRNA expression patterns, and it is not known how the defects that are observed relate to the expression of c-Met and HGF/SF proteins. The second is that this factor is also required for the migration of myoblast precursor cells from the paraxial mesoderm to populate the limb (Bladt et al., 1995), so that motor axon defects could arise at least in part as a secondary consequence of the absence of myoblasts. The defects in branches 1 and 7 (Figure 7) in particular, which appear to involve a defect in elaboration of terminal arbors, seem likely to have arisen from an absence of target muscles, since qualitatively similar defects in branching have been observed in chick embryos in which limb myoblast precursors had been eliminated by either X-irradiating or extirpating the somitic mesoderm (Lewis et al., 1981; Phelan and Hollyday, 1990). This interpretation is complicated even further, however, by the fact that myoblasts themselves begin to express *Hgf/Sf* after they reach their destinations (Sonnenberg et al., 1993). Thus, even if the defects in some branches result from the absence of muscle cells, they could still arise specifically from the absence of HGF/SF secreted by the muscle cells. There is, indeed, evidence for a muscle-derived chemoattractant for motor axons in chick embryos (Lance-Jones and Landmesser, 1981), which could be HGF/SF.

A Role for Chemoattractants in Guiding Axons into the Sclerotome and to Dermomyotome?

Sclerotome cells also secrete an invasion-promoting and chemoattractant activity for motor axons in our assay, and part of this activity is attributable to HGF/SF. Since *Hgf/Sf* mRNA is not detected in sclerotome in vivo, this must presumably reflect an up-regulation of HGF/SF expression by sclerotome cells in vitro. We have indeed obtained evidence for a dramatic induction of *Hgf/Sf* mRNA expression. This induction may reflect a nonphysiological response (e.g., an induction by a serum component). Alternatively, since the paraxial mesoderm is highly plastic at the stages we are studying and its differentiation in vivo is tightly controlled by a number of patterning molecules secreted by many surrounding tissues (see, e.g., Pourquié et al., 1996, and references therein), it is possible that the induction of *Hgf/Sf* expression reflects the existence of a signal that is present in vivo, but absent in our cultures, that normally actively represses *Hgf/Sf* expression by these cells.

Although some of the activity in sclerotome cells is due to this in vitro induction, we also demonstrated the existence of a second invasion-promoting activity, distinct from HGF/SF, secreted by sclerotome cells in culture. It is possible that this activity contributes to directing motor axon projections into the sclerotome in vivo, supporting suggestions obtained from experiments done in chick embryos (Stern et al., 1986; Oakley and Tosney, 1989, Soc. Neurosci., abstract; Hotary and Tosney, 1996). Identification of the factor(s) that mediates this effect will be required to establish its role.

Dermomyotome ablation studies in vivo have provided evidence for the existence of a chemoattractant from dermomyotome cells that attracts epaxial motor axons (Tosney, 1987). Surprisingly, we have not found evidence for such an attractant in our assay. It is possible that our culture conditions do not support the proper differentiation of epaxial motoneurons or extension of their axons, or that dermomyotome cells fail to express the attractant when explanted in culture.

HGF/SF Is a Trophic Factor for Motoneurons

Muscle cells in the limb, which are initially dependent on HGF/SF for migration into the limb, subsequently themselves express HGF/SF (Sonnenberg et al., 1993). We have shown that HFG/SF is a motoneuron survival factor as potent as other survival factors described to date (Figure 8). Similar results have been obtained by V. Wong and G. Yancopoulos (personal communication). Thus, HGF/SF is a good candidate for an endogenous muscle-derived trophic factor for motoneurons involved in the competitive interactions that regulate motoneuron cell death. The list of such candidates is, however, ever increasing (reviewed by Oppenheim, 1996), raising the question of what the specific function of HGF/SF might be. One possibility is that different subpopulations of motoneurons respond principally to different trophic factors; the fact that only a subpopulation of motoneurons expresses c-*Met* fits with this hypothesis, as does our observation that the effects of HGF/SF are additive with those of brain-derived neurotrophic factor (BDNF), ciliary neurotrophic factor (CNTF), or bFGF (when tested pairwise; unpublished data). One advantage of such a scheme is that it could be used to ensure that competitive interactions occur only among motoneurons within

each subpopulation. Testing the function of HGF/SF in regulating motoneuron cell death is, however, made difficult both by the fact that the *Hgf/Sf*$^{-/-}$ and c-*Met*$^{-/-}$ embryos die around E13.5–E14.5, and by the absence of limb muscle in such embryos.

Parallels between Neural and Nonneural Functions of HGF/SF?

The ability of HGF/SF to promote invasion of a collagen matrix by motor axons in vitro is reminiscent of its ability to stimulate the invasiveness of transformed cells (reviewed by Jeffers et al., 1996). Similarly, while the function of HGF/SF as an axonal chemoattractant is unprecedented, the guidance role discussed here is reminiscent of its motogenic effects on epithelial or mesodermal cells. An intriguing parallel can in fact be drawn between the migration of motor axons and the migration of muscle precursors to the limb. Limb muscle precursors arise from the ventrolateral aspect of the dermomyotome and express c-*Met* beginning approximately at E9.5, until E15 (Sonnenberg et al., 1993). In *Hgf/Sf*$^{-/-}$ or c-*Met*$^{-/-}$ mice, these precursors fail to migrate into the limb (Bladt et al., 1995), whereas in transgenic mice in which *Hgf/Sf* is expressed ectopically under the control of the metallothionein promoter, ectopic muscle formation is observed (Takayama et al., 1996), suggesting that muscle precursors are attracted by HGF/SF derived from nonmuscle tissue in the limb. The apparent guidance effects of HGF/SF on muscle precursors thus parallel the guidance effects on motor axons postulated here. The HGF/SF–c-Met ligand–receptor pair therefore appears to provide a simple and unified signaling system used to attract both the axons of motoneurons as well as their ultimate target to the same locale.

Other Functions for HGF/SF and c-Met Family Members in Axon Guidance and Neuronal Survival?

Our studies have focused on the potential roles of HGF/SF and c-Met in motor axon guidance and survival, but HGF/SF and c-Met are expressed in other parts of the nervous system as well. It is intriguing that a prominent site of c-Met expression is in basal forebrain neurons, which, like motoneurons, are also cholinergic (Jung et al., 1994). It is possible that HGF/SF, acting via c-Met, also has chemoattractant, invasion-promoting, or survival promoting effects (or combinations of these) on these neurons, or indeed on any other neuronal class that expresses c-Met. In addition, c-Met is the prototype for a large family of presumed receptors for secreted factors, including c-Sea, Ron, and members of the Sex subfamily, some of which also signal via tyrosine kinases, and some of which are also expressed in the nervous system (Ronsin et al., 1993; Huff et al., 1996; Wang et al., 1994, 1995; Théry et al., 1995; Maestrini et al., 1996). While the ligands for most of these receptors remain to be identified, macrophage stimulating protein (MSP; also known as HGF-like), has been established as a ligand for Ron (Wang et al., 1994). It is possible that MSP or other as yet unidentified ligands for c-Met family receptors have guidance or trophic functions in other parts of the nervous system.

Axon Guidance Mediated by Receptor Tyrosine Kinases

The demonstration of a role for HGF/SF and its receptor in the guidance of developing motor axons opens the way to understanding signal transduction mechanisms involved in axonal chemoattraction. While little is known about how signaling through guidance receptors is translated into changes in the actin cytoskeleton, recent studies have led to the suggestion that tyrosine phosphorylation plays an important role in axon guidance (reviewed by Tessier-Lavigne and Goodman, 1996). In particular, our findings implicating HGF/SF in motor axon guidance parallel recent reports supporting a role for FGF and neurotrophins (NGF and neurotrophin 3 [NT3]), also acting via receptor tyrosine kinases, in axon extension and target invasion (reviewed by Tessier-Lavigne and Goodman, 1996).

Although the signal transduction machinery that links receptor tyrosine kinases to cytoskeletal rearrangements is unknown, it is interesting to note that the human SH2/SH3 adaptor protein Nck, whose Drosophila homolog, Dreadlocks (DOCK), is required for guidance of photoreceptor cell axons (Garrity et al., 1996), has been shown to bind c-Met in vitro (Kochar and Iyer, 1996). It is therefore an intriguing possibility that Nck/Dock plays a role in translating signaling at guidance receptors into a reorganization of the cytoskeleton required for growth cone steering.

Superficially, axon guidance by receptor tyrosine kinases and their ligands might appear mechanistically distinct from that by other known axonal chemoattractants, the netrins, and their receptors, which appear to be members of the immunoglobulin superfamily (reviewed by Tessier-Lavigne and Goodman, 1996). However, there is considerable evidence that immunoglobulin superfamily members may signal by pathways involving other receptor or cytoplasmic tyrosine kinases (reviewed by Tessier-Lavigne and Goodman, 1996). The identification of c-Met as an axonal chemoattractant receptor not only provides a tool for dissecting the downstream components of receptor tyrosine kinase signal transduction in axon guidance but, with the identification of netrin receptors, also provides a framework for determining whether signal transduction in axonal chemoattraction converges on a unified group of effectors in the growth cone.

Experimental Procedures

Explant Cultures

Neural tube, somitic mesoderm, and limb explants from E11 rat embryos (E0 denotes day of vaginal plug for rats) or E9.5 mouse embryos (E0.5 denotes day of vaginal plug for mice) were obtained after enzymatic treatment and embedded in collagen gels as described (Serafini et al., 1994; Fan and Tessier-Lavigne, 1994) and cultured for 40 hr in 25% F12, 70% Optimem containing Glutamax (GIBCO), 5% fetal calf serum, 40 mM glucose, and 1% penicillin–streptomycin. Cultures using mouse tissues were supplemented with 5% conditioned medium from a CHO cell line secreting recombinant leukemia inhibitory factor (LIF), to enhance survival (this did not affect other aspects of the assays; data not shown). For antibody blocking experiments, purified IgG fraction of a sheep anti-serum against murine HGF/SF (which cross-reacts with rat but not human HGF/SF; gift of E. Gherardi; used at 10–60 μg/ml) was added once at the beginning of the incubation period.

Generation of Sclerotome-Conditioned Medium

Somites (200) were pooled in L15 medium with 5% heat-inactivated horse serum (HIHS), transferred to culture medium, and carefully triturated to separate dermomyotome from sclerotome. Dermomyotomes were decanted and the sclerotome suspension transferred to one well of a 96-well plate and cultured for 40 hr. Supernatant was harvested, centrifuged briefly to remove debris, and snap-frozen in liquid nitrogen. Medium conditioned by 4000 somites was pooled, generating a total of 2 U (see below) of activity (corresponding to 20 ng of HGF/SF).

Factors Tested for Invasion-Promoting Activity

The following factors were tested for invasion-promoting activity on ventral spinal cord explants in a series of 10-fold dilutions from 1 μg/ml to 1 ng/ml: HGF/SF (gift of R. Schwall); BDNF, NT3, NT4/5, CNTF, and GDNF (gifts of D. Shelton and A. Rosenthal), epidermal growth factor (EGF) (Collaborative), bFGF (FGF-2) (gift of C.M. Fan), LIF (GIBCO BRL), human MSP (HGF-like) (gift of E.J Leonard), netrin-1 (gift of A. Faynboym), Sonic Hedgehog (SHH) (gift of P. Beachy), transformalin growth factor β1 (TGFβ1) (gift of R. Derynck), NGF (Bioproducts for Science, Incorporated), tenascin-C (GIBCO BRL), thrombospondin (GIBCO BRL), fibronectin (Collaborative), laminin-1 (EHS-laminin) (Collaborative), laminin-2 (merosin) (Gibco/BRL). COS-7 cells transiently transfected with expression vectors that direct expression of soluble L1 and neural cell adhesion molecule (NCAM) ectodomain–Fc fusions (gifts of F. Walsh and P. Doherty) were tested as cell aggregates cocultured with ventral explants. C3H10T1/2 (Resnickoff et al 1973; American Type Culture Collection), C12, and C58 (gifts of G. Stewler) cell lines were tested as aggregates in coculture with ventral explants and also used to condition medium.

Immunohistochemistry

Explant cultures were fixed with 4% paraformaldehyde (PFA) in PBS for 1 hr at room temperature. Staining with a neurofilament-specific antibody (RMO270) (gift of V. Lee), anti-p75 (gift of L. Reichardt), or anti-peripherin (Chemicon), and a horseradish peroxidase (HRP)–conjugated secondary antibody (Amersham) was performed as described (Colamarino and Tessier-Lavigne, 1995), except that antibody incubations and washes were in PBS, 1% heat-inactivated goat serum, 0.1% Triton X-100. In situ hybridization was carried out as described (Fan and Tessier-Lavigne, 1994), with probes prepared as described by Sonnenberg et al. (1993).

Tracing of Motor Axon Projections with DiI

Embryos were fixed, crystals of DiI were inserted in the motor column from the lumenal side of the neural tube, embryos were incubated at 37 C for 7–10 days to allow the dye to diffuse down axons, the dye was photoconverted to a visible diaminobenzidine (DAB) precipitate (Sretavan, 1990), and embryos were dehydrated in methanol and cleared in 1:1 benzyl alcohol/benzyl benzoate (details available on request). The following numbers of *Hgf/Sf* embryos (and at least a similar number of stage-matched wild-type littermates) were examined: four E9.5, four E10.5, five E11.5, six E12.5, and three E13.5 embyos. Six E11.5 c-*Met* embryos were also examined.

Protein Chemistry

Conditioned medium was prepared from 10T1/2 cells by growth to confluence in DME-H21 (10% FCS, glutamine and antibiotics). Cells were washed twice with PBS and cultured 7 days in Optimem containing glutamax (GIBCO) and antibiotics. The conditioned medium was harvested and cleared by low speed centrifugation.

Protease sensitivity was assessed by incubating 10T1/2 cell-conditioned medium or sclerotome-conditioned medium with trypsin–agarose beads (Sigma) and in separate experiments with proteinase K conjugated to AffiGel resin (Bio-Rad) (details available on request). In all cases, exposure to trypsin or proteinase K abolished the activity, whereas exposure to BSA-conjugated control beads or resin did not. Protease sensitivity of sclerotome-conditioned medium was assessed by incubating 200 μl of culture medium conditioned by exposure to 200 dissected sclerotomes with 50 μl of trypsin–acrylic or BSA beads.

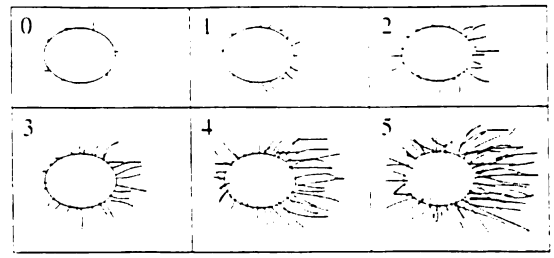

Figure 9. Scale Used to Score the Degree of Motor Axon Outgrowth

Motor axon outgrowth from E11 rat ventral spinal cord explants was scored by two independent observers using the 0–5 scale shown here.

For heparin affinity chromatography, 10T1/2 cell-conditioned medium was adjusted to 500 mM NaCl and 25 mM Tris and centrifuged at 100,000 × g for 1 hr. The supernatant was applied to a 1 ml heparin Hi-Trap column (Pharmacia) and eluted with a 10 ml gradient (0.5–1.5 M NaCl) followed by a step to 2 M NaCl. Fractions (0.5 ml) were collected, 0.3 ml of which was dialyzed overnight against F12 medium in a BRL microdialyzer and assayed in a 3-fold dilution series on ventral explants for invasion-promoting activity. For quantification of activity, a unit was defined as the amount of material that, in 1 ml, gives outgrowth with a score of 4 on the scale of Figure 9; in the case of HGF/SF, one unit is 10 ng. The remainder of the fractions were TCA-precipitated and subjected to Western blotting with a sheep anti-human HGF/SF antiserum (gift of R. Schwall). Bound primary antibody was visualized with an anti-sheep HRP conjugate (BMB) and ECL (Amersham).

Generation of a Cell Line Secreting Rat HGF/SF

A rat *Hgf/Sf* cDNA was obtained by reverse transcription–polymerase chain reaction (RT-PCR) using mRNA derived from E11 rat forelimb and the following primers: ACAGACTCGTTTGAGAC CATC and TCTCAATGGGCAACGAGAATG. The amplifed product was cloned into the pCEP4 expression vector (Invitrogen) and transfected into 293 cells to generate a stable cell line, as described by Shirasaki et al. (1996). HGF/SF secretion was detected by Western blotting (see above) and by bioactivity (Figure 3E).

Generation and Typing of Mutant Embryos

Heterozygous mice carrying inactivating mutations in either the *Hgf/Sf* or the c-*Met* locus were mated, and embryos were harvested at E9.5–E13.5. After removal of tissues required for particular experiments (Figure 5), the remaining tissues were used to genotype each embryo by PCR as described (Schmidt et al., 1995; Bladt et al., 1995).

Quantitative PCR

Quantitative PCR was carried out according to the methods of Gililand et al. (1990). In brief, sclerotomes were isolated from E11 rat embryos and frozen immediately (fresh), or cultured 40 hr in a collagen gel under standard assay conditions (cultured). Total RNA was prepared by using Ultraspec (Biotecx), and oligo-(dT)-primed cDNA prepared as described (Fan and Tessier-Lavigne, 1994). cDNA preparations were normalized for RNA recovery and cDNA synthesis efficiency by competitive amplification of β-*actin* (primers AGTA CAACCTCCTTGCAGCTC and TCACAATGCCAGTGGTACGAC, amplification product of 509 nt). Relative *Hgf/Sf* transcript content was determined in parallel experiments by competitive amplification of *Hgf/Sf* (primers ATCAGACACCACACCGGCACAAAT and GAAA TAGGGCAATAATCCCAAGGAA, product of 610 nt). Competitive amplifications involved using a constant amount of cDNA against a log dilution series of an appropriate competitor template constructed to contain the β-*actin* or *Hgf/Sf* primer sequences and to yield larger products (779 nt for the β-*actin* competition template, 890 nt for the *Hgf/Sf* competition template). Amplifications involved 30 cycles of 95 C (30 sec), 63 C (1 min), 72 C (1 min). Reaction products were

separated on 1.5% agarose gels, dried, and quantitated with a Molecular Dynamics Phosphorimager.

Motoneuron Survival Assay

Spinal cords from E15 rats spinal cords were dissected, dissociated, and spun through a BSA cushion, then a metrizamide cushion, as described (Camu and Henderson, 1992). Cells were panned by incubation for 12 hr at 4 C on Petri dishes previously coated with 40 μg/ml goat anti-mouse IgG (Jackson Immunoresearch) followed by anti-p75 supernatant (mAb 192, gift of E. Shooter); adherent cells were recovered by trypsin digestion. Oligodendrocytes and microglia were removed by panning the cells on dishes coated with an anti-galactocerebroside antibody (RmAb; Ranscht et al., 1982). More than 80% of the nonadherent cells recovered were motoneurons as determined by islet-1 immunostaining. Approximately 3000 purified motor neurons per well were cultured in 96-well plates coated with laminin-2 (merosin) (GIBCO) in 100 μl of serum-free medium containing L-15, 0.63 mg/ml sodium bicarbonate, 0.1 mg/ml BSA, 30 nM selenium, 0.1 mM putrescine, 0.1 mg/ml transferrin, 1 mM pyruvate, 1 mM glutamine, penicillin/streptomycin, and the trophic factors at 10 ng/ml (in these experiments, bFGF was obtained from Preprotech, human BDNF and CNTF from Regeneron, and GDNF from Synergen). Survival rates were normalized to the percentage of surviving cells at 1 hr after plating. Cell survival was assessed at 76 hr by mitochondrial uptake of MTT dye (Sigma) (Mosmann, 1983), added to culture media at a final concentration of 0.56 mg/ml for 1 hr at 37°C.

Acknowledgments

We are greatly indebted to N. Pollock, a rotation student, who first observed activity in 10T1/2 cell conditioned medium following a suggestion by C.-M. Fan. We also wish to thank R. Schwall and his colleagues at Genentech for providing numerous HGF/SF agonists and antagonists, as well as helpful suggestions; E. Gherardi for generously providing the neutralizing antiserum to HGF/SF; G. Yancopoulos for communicating results prior to publicaton; G. Strewler for cells lines; M. Galko for expert advice on biochemistry and cell culture; V. Head for assistance with in situ hybridization; J. Mak and M. Embry for assistance with breeding and typing of mice; and P. Beachy, R. Derynck, P. Doherty, C. M. Fan, V. Lee, E. J. Leonard, D. Shelton, E. Shooter, A. Rosenthal, and F. Walsh for factors, plasmids, and antibodies. A. E. was supported by postdoctoral fellowships from the Howard Hughes Medical Institute and the American Cancer Society, K. B. by a predoctoral fellowship from the National Science Foundation, and E. D. L. by a University of California, San Francisco Chancellor's Fellowship. Supported by grants from the March of Dimes Birth Defects Foundation to B. A. B., from the European Union (Biotechnology and Human Capital and Mobility Programs) to C. B., and from the Howard Hughes Medical Institute to M. T.-L.; M. T.-L. is an Assistant Investigator of the Howard Hughes Medical Institute.

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Received October 22, 1996; revised November 12, 1996.

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Appendix III

Preface

The following appendix describes the identification of the mouse *netrin-1* gene, characterization of its expression pattern in the developing embryo, as well as the generation and characterization of netrin-1 deficient mice. My contribution to this work is the identification and cloning of the mouse netrin-1 gene, including preliminary characterization of its expression pattern. The mouse *netrin-1* gene was identified using degenerate PCR as described in the materials and methods section. At the time this work was initiated, partial cDNA clones for chick *netrin-1* existed, and the homology with *unc-6* was evident.

Netrin-1 Is Required for Commissural Axon Guidance in the Developing Vertebrate Nervous System

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Summary

During nervous system development, spinal commissural axons project toward floor plate cells and trochlear motor axons extend away from these cells. Netrin-1, a diffusible protein made by floor plate cells, can attract spinal commissural axons and repel trochlear axons in vitro, but its role in vivo is unknown. Netrin-1 deficient mice exhibit defects in spinal commissural axon projections that are consistent with netrin-1 guiding these axons. Defects in several forebrain commissures are also observed, suggesting additional guidance roles for netrin-1. Trochlear axon projections are largely normal, predicting the existence of additional cues for these axons, and evidence is provided for a distinct trochlear axon chemorepellent produced by floor plate cells. These results establish netrin-1 as a guidance cue that likely collaborates with other diffusible cues to guide axons in vivo.

Introduction

Embryological experiments in both vertebrates and invertebrates have provided evidence that developing axons are guided to their targets in the nervous system by the combined actions of attractive and repulsive guidance cues, but the identity of these cues and their precise contributions to axon guidance are only now being elucidated (reviewed in Tessier-Lavigne and Goodman, 1996). One family of putative guidance cues for developing axons are the netrins, large (~70–80 kDa), soluble proteins that show homology in their amino termini to portions of the extracellular matrix molecule laminin and that have been implicated in axon guidance through distinct and complementary lines of evidence in worms, flies, and vertebrates (reviewed in Culotti and Kolodkin, 1996; Harris et al., 1996; Mitchell et al., 1996; see also Discussion).

In the nematode C. elegans, the netrin family member UNC-6 was implicated in axon guidance through loss-of-function studies that demonstrated that UNC-6 is required for accurate circumferential projections of axons in both a dorsal and a ventral direction (Hedgecock et al., 1990; Ishii et al., 1992). The finding that the UNC-6 gene product is concentrated in the ventral portion of the nematode during the period of axon guidance has suggested a model in which UNC-6 functions to attract ventrally directed axons and to repel dorsally directed axons (Wadsworth et al., 1996). However, the fact that a substantial fraction of circumferential projections are normal even in homozygous null mutants for *unc-6* indicates the existence of other factors that work with UNC-6 to direct these projections (Hedgecock et al., 1990).

Netrin proteins have been implicated in directing circumferential axonal projections in vertebrates as well. Commissural axons pioneer a circumferential trajectory to the floor plate at the ventral midline of the neural tube during embryogenesis (reviewed in Colamarino and Tessier-Lavigne, 1995a). Floor plate cells secrete a diffusible factor that can promote the outgrowth of commissural axons from explants of dorsal spinal cord into collagen matrices in vitro and reorient these axons within the neural epithelium (Tessier-Lavigne et al., 1988; Placzek et al., 1990). Two netrin proteins were purified from embryonic chick brain on the basis of their ability to mimic the outgrowth-promoting effect of floor plate cells (Serafini et al., 1994), and recombinant versions of these proteins are capable of reorienting commissural axon growth in vitro (Kennedy et al., 1994). In chickens, *netrin-1* is expressed by floor plate cells and is therefore a candidate for mediating the outgrowth-promoting and reorienting activities of these cells, whereas *netrin-2* is expressed in the ventral two thirds of the spinal cord (Kennedy et al., 1994). These results have suggested a model in which a gradient of netrin protein contributes to directing the growth of commissural axons toward the ventral midline (Kennedy et al., 1994), and further evidence has suggested that this mechanism operates in the hindbrain as well (Shirasaki et al., 1995). The floor plate and netrin-1 were also found to be capable of repelling in vitro a group of axons, trochlear motor axons, that grow away from the floor plate in vivo (Colamarino and Tessier-Lavigne, 1995b), suggesting that a gradient of netrin protein contributes to directing the growth of some axons away from the ventral midline as well.

Studies in vertebrates, however, have not yet determined whether and how netrin proteins contribute to guiding different classes of axons during development in vivo. Does the ability of netrin-1 to attract commissural axons in vitro reflect a role for netrin-1 in attracting these axons to the ventral midline in vivo? Is its ability to repel trochlear motor axons important for setting the trajectory of those axons? To begin to address these questions, we have taken advantage of the results of

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a gene-trapping study (Skarnes et al., 1995) that has provided the means to isolate a loss-of-function allele of the murine *netrin-1* gene. Here we report an analysis of neural morphology and axonal trajectories in mice homozygous for this mutation. Our results indicate important roles for netrin-1 in vivo in establishing the projections not only of commissural axons, but of other axonal classes as well.

Results

Netrin-1 Is Expressed beyond the Floor Plate in the Developing Mouse Spinal Cord

To analyze netrin-1 function in the mouse, we isolated cDNA clones comprising the entire coding region of murine *netrin-1*. The predicted sequence of the mature murine netrin-1 protein is 89% identical to that of chick netrin-1, and 69% identical to that of chick netrin-2 (data not shown). Northern analysis using both coding region and 3′-UTR probes revealed a major 6.0 kb hybridizing species present in mouse brain (data not shown). In situ hybridization analysis demonstrated that, at the beginning of the period of commissural axon growth to the floor plate in the mouse spinal cord (E9.5; see Colamarino and Tessier-Lavigne, 1995a), the *netrin-1* gene is expressed not only at high levels in the floor plate but also in roughly the ventral two thirds of the spinal cord (Figure 1A). Expression is maintained at high levels in the floor plate region through the period of commissural axon growth to the floor plate (E10.5 and E11.5) (Figures 1B and 1C). Expression beyond the floor plate is also maintained during this period, albeit in a region progressively more restricted to the ventricular zone. This pattern of murine *netrin-1* expression resembles a composite of the patterns observed for chick *netrin-1* and *netrin-2* expression (Kennedy et al., 1994). Only one other *netrin* gene has so far been identified in mouse, but this gene is not expressed in the spinal cord at these ages (H. W. and M. T.-L., data not shown).

Generation of a Hypomorphic Allele of the Murine *Netrin-1* Gene

A previous report described the use of a β-galactosidase-encoding gene trap vector (pGT1.8TM) in embryonic stem (ES) cells to selectively recover mutations in genes encoding proteins with signal sequences by virtue of creating fusions of amino terminal portions of these proteins with an exogenous transmembrane domain (Skarnes et al., 1995). That report also described the generation of an ES cell line (ST514) in which the murine *netrin-1* gene had been so mutated, but this allele could not be transmitted through the germ line. However, a second ES cell line (ST629) yielded chimeric mice whose β-galactosidase activity pattern matched that of chimeric mice generated using ST514, and this new mutant allele was transmitted through the germ line. The integration event was found to be an insertion into an intron of the *netrin-1* gene (see Experimental Procedures). The translation product of the mutant transcript (6.8 kb by Northern analysis of brain RNA) is predicted to be a transmembrane protein comprising (1) an extracellular portion consisting of domain VI and the first EGF-like repeat of domain V of netrin-1 (Serafini et al., 1994) together with a short fragment derived from the splice acceptor, (2) a transmembrane domain encoded by the vector (from the CD4 protein), and (3) a cytoplasmic domain consisting of β-geo (β-galactosidase fused to neomycin phosphotransferase). Immunohistochemical analysis suggests that this fusion protein is found intracellularly (data not shown; Figures 3D and 3E), consistent with the localization of such pGT1.8TM-generated fusion proteins in ES cells to a subcellular compartment rather than the cell surface (Skarnes et al., 1995).

A multiplex reverse transcription–polymerase chain reaction (RT–PCR) method was used to analyze relative levels of wild-type and fusion transcripts in spinal cord or caudal hindbrain (Figure 1D), where *netrin-1* is expressed at high levels from at least E9.5 to P0 (Figures 1A–1C, data not shown). Southern analysis of genomic DNA confirmed that the assignment obtained by RT–PCR accurately reflects zygosity (data not shown). In these RT–PCR experiments, a product apparently corresponding to wild-type *netrin-1* mRNA was detected in animals homozygous for the mutant allele (Figure 1D). Furthermore, in the homozygotes a low level of *netrin-1* mRNA containing exons downstream of the inserted DNA was detected by RNase protection (Figure 1E) and by in situ hybridization at the floor plate (Figures 1F–1H). The simplest explanation for these results is a splicing of the pre-mRNA transcribed from the mutant alleles around the newly inserted exon. Thus, the mutant allele is likely to be severely hypomorphic rather than a complete null.

Homozygotes are born, but apparently do not suckle (milk is never found in their stomachs) and die within a few days. Blind trials indicate that a homozygote can be faithfully selected from among littermates by scoring for the lack of ingested milk and for two behavioral phenotypes: (1) the almost complete inability to move its forelimbs independently of one another (so that it "paddles") and (2) the inability, when placed on its back, to turn its upper body to one side, so that it bends at its midsection when attempting a righting response.

Severe Defects in Spinal Commissural Axon Trajectories in Netrin-1 Mutants

To determine how a reduction in *netrin-1* function affects spinal commissural axon growth, we examined the developing spinal cords of mutant embryos at E10.5 and E11.5. The overall morphology of the spinal cord at E11.5 was relatively normal (data not shown), with the exception that the ventral commissure of the spinal cord was reduced greatly in mutant animals when compared to wild-type and heterozygote animals at this age (Figure 2).

To determine the cause of this reduction, the trajectories of commissural axons in the spinal cord were examined by immunohistochemistry using an antibody to TAG-1, a marker of commissural axons as they project to the floor plate (Dodd et al., 1988). In wild-type embryos, commissural axons project ventrally near the edge of the spinal cord until they reach the level of the developing motor column. A few of the earliest projecting axons at E9.5–E10 continue to grow near the edge all

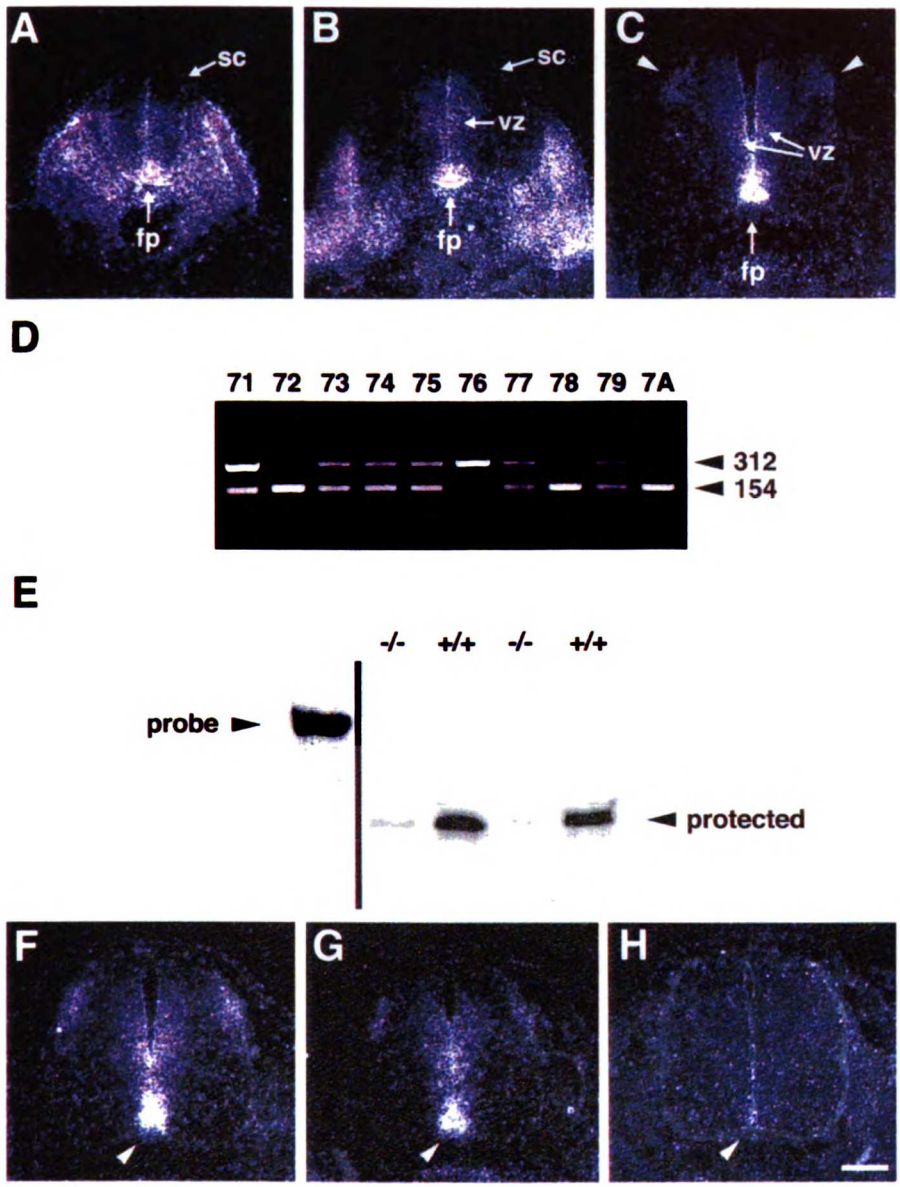

Figure 1. Expression of *Netrin-1* in Wild-Type and Mutant Mouse Embryos

(A–C) Transverse sections of embryos at brachial level were subjected to hybridization with a ^{35}S-labeled ***netrin-1*** antisense riboprobe. (A) Embryonic day 9.5 (E9.5). The domain of *netrin-1* expression at this age includes both the floor plate (fp) and the ventral two thirds of the spinal cord (sc), as well as somites flanking the neural tube. (B) E10.5. The expression of *netrin-1* within the spinal cord becomes more restricted to the ventricular zone (vz). Expression is also observed in the lateral portion of the paraxial mesoderm. (C) E11.5. Another, more dorsal and dynamic pattern of *netrin-1* expression is seen at this stage in a dorsolateral region of the spinal cord that is contacted by ingrowing dorsal root ganglion (DRG) sensory axons (arrowheads) (see also [F]).

(D) Multiplex RT–PCR analysis of a litter of P0 mice from a heterozygote × heterozygote mating. The 5′ primer corresponded to wild-type *netrin-1* sequences 5′ to the site of insertion of the secretory gene trap construct. Two 3′ primers were used: one corresponding to wild-type *netrin-1* sequences 3′ to the insertion site, the other corresponding to sequences within the gene trap construct. The upper band (312 bp) is the product expected from the mutant *netrin-1* fusion transcript, while the lower band (154 bp) is the product expected from the wild-type transcript. Note that the animals fall into three classes: (1) wild-type product alone (mice 72, 78, and 7A), (2) roughly similar intensities of wild-type and mutant products (mice 73–75, 77, and 79), and (3) much higher ratio of mutant to wild-type products (mice 71 and 76).

(E) RNase protection performed on P0 brain RNA from wild-type and homozygous mutant animals using a 3′-UTR riboprobe. The positions of the undigested probe and protected fragments are indicated. Left lane is a control (no RNase digestion; 2 min exposure); reactions containing RNA from embryos of the indicated genotype were electrophoresed in the same gel in the lanes indicated (71 hr exposure). No protected fragments were observed when brain RNA was omitted (not shown).

(F–H) In situ hybridization patterns of 3′-UTR probe on transverse sections from the brachial regions of E11.5 wild-type (F), heterozygote (G), and mutant (H) littermates. Arrowhead indicates floor plate. Note the very low level of signal detectable in the mutant floor plate. Four embryos of each genotype were examined with results similar to those shown here.

Scale bar is 55 μm in (A), 110 μm in (B) and (C), and 120 μm in (F)–(H).

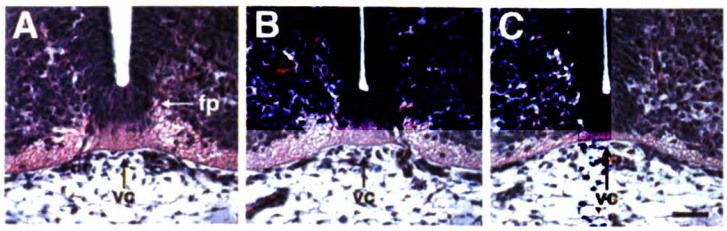

Figure 2. Reduction of the Ventral Commissure in Netrin-1 Deficient Animals

Compared to wild-type (A) and heterozygote (B) animals, homozygous mutant animals (C) have a greatly reduced ventral commissure (vc) underlying the floor plate (fp) of the spinal cord. This was true for all comparably sized spinal cords examined (n = 12 wild-type, 13 heterozygote, 14 homozygote). Scale bar is 30 μm.

the way to the floor plate, and many later-developing axons appear to grow along the path marked out by the earliest projecting axons, but as the motor columns increase in size due to the generation of motoneurons, many axons grow through the motor columns in a directed manner toward the ventral midline (Wentworth, 1985). These normal trajectories were observed in wild-type and heterozygous embryos (Figures 3 and 4, data not shown).

Embryos homozygous for the mutation, however, displayed profound disturbances in commissural axon trajectories. At E10.5, most commissural axons in the mutants appeared foreshortened compared to those in age-matched heterozygote and wild-type littermates, having extended only to the level of the developing motor column, and some axons can be seen apparently turning toward the ventricle (Figure 3D). By E11.5, severe disturbances in the growth of these axons were apparent (Figure 3B). Axons continue to extend but in aberrant directions, with some projecting medially toward the ventricle and others projecting in the ventral spinal cord near its lateral edge. A third, more numerous group progresses further ventromedially but is not as directed toward the floor plate as in the wild type: Instead of all being oriented at an angle toward the floor plate, the majority of axons project more vertically into the motor column, and only a few axons reach the floor plate. These errors were accompanied by a substantial reduction in the number of commissural axons that successfully invade the ventral spinal cord, which is most easily observed in sagittal (side) views of these projections (Figure 4).

These defects in commissural axon trajectories could also be observed in the pattern of axonal projections

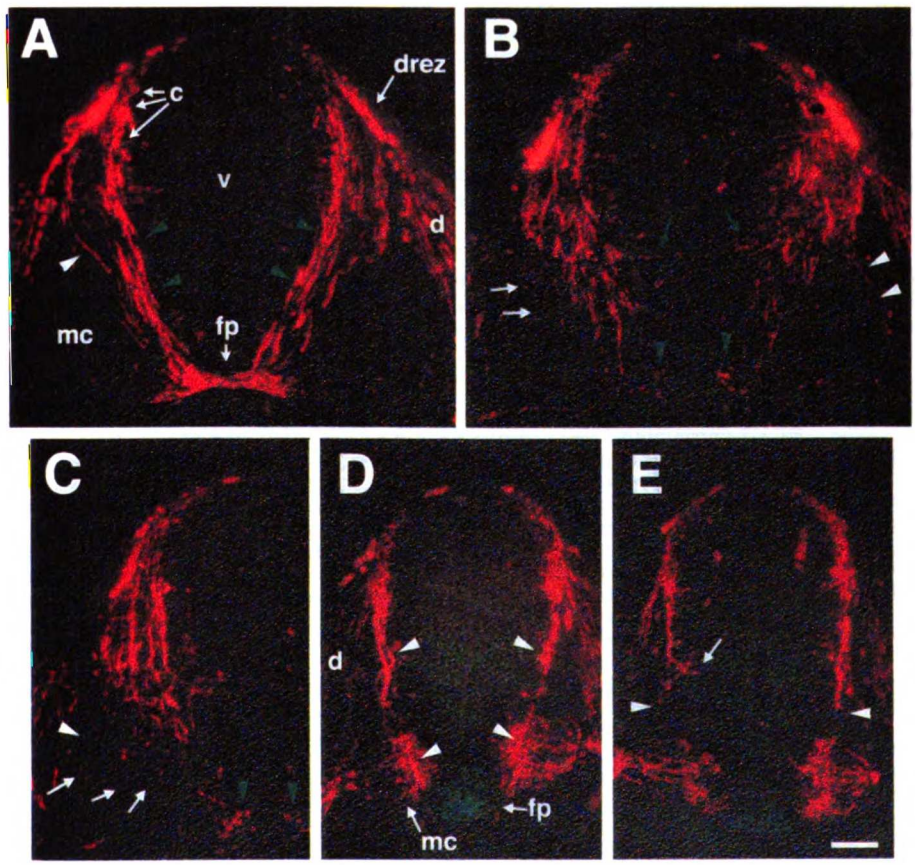

Table 1. In Vitro Activities of Floor Plate Explants from E11.5 Mouse Embryos

Activity	Genotype		
	Wild Type (+/+)	Heterozygote (+/−)	Mutant (−/−)
Commissural axon outgrowth[a]	7/7	6/6	0/6[b]
Commissural axon turning	8/8	19/19	5/5
Trochlear motoneuron repulsion	10/10	22/22	7/7

[a] Four floor plate explants surrounded four E13 rat dorsal spinal cord explants.
[b] Very low levels of outgrowth were observed in some cases.

seen in sections stained with an antibody to neurofilament protein, which labels all axons in these sections (Figure 5, data not shown). Other aspects of pattern formation that we examined, however, appeared normal. Motoneurons were found in their expected ventrolateral location and projected normally, as assessed using antibodies to TAG-1 and neurofilament (Figures 3C, 3D, and 5), and Islet-1 (a nuclear protein expressed in motoneurons [Ericson et al., 1992]; data not shown). Expression of the floor plate markers HNF3β (Ruiz i Altaba et al., 1993; Sasaki and Hogan, 1993) and *F-spondin* (Klar et al., 1993) also appeared normal (data not shown). Finally, the expression of the *netrin-1* gene itself in both the ventral midline region and the ventral ventricular zone appeared unchanged, as assessed by the pattern of expression of the β-galactosidase reporter construct (Figures 3C and 3D). It should be noted that the localization of this fusion protein reflects the cell bodies of the cells expressing the *netrin-1* gene (Figure 1B), not necessarily the localization of the wild-type netrin-1 protein.

Trochlear Motor Axons Trajectories Are Largely Normal

Netrin-1 also functions as a chemorepellent for trochlear motor axons in vitro (Colamarino and Tessier-Lavigne, 1995b). We therefore examined the trajectory of these axons in the *netrin-1* mutant embyros between E9.5 and E10.5, the period during which trochlear motoneurons first differentiate and extend their axons along a ventral-to-dorsal trajectory away from the floor plate in the mouse embryo (Fritzsch et al., 1995). Axons were visualized in embryo whole-mounts by labeling with antibodies to neurofilament, which labels both trochlear motor axons as well as other axons that project longitudinally in the hindbrain (Figure 6).

Trochlear motor axon trajectories were largely unaffected by loss of *netrin-1* function throughout the period E9.5–E11.5 (Figure 6, data not shown). A minor but consistent defect was the presence of a larger number of cell bodies of trochlear motoneurons in the floor plate region of the mutant embryos compared to wild-type or heterozygous littermates (arrows in Figure 6), suggesting that netrin-1 plays a role in the placement of the cell bodies of these neurons. It does not, however, appear to be required for the dorsally directed guidance of these axons.

Finally, another reproducible defect observed in this region is that the longitudinal axon tracts are thicker and fewer in number, and somewhat disorganized (Figure 6C). We do not know how to interpret this defect. Among other possibilities, netrin-1 might be a permissive factor for these axons, such that in its absence the growth of these axons on each other is favored over growth on nonaxonal surfaces.

Mutant Floor Plates Fail to Elicit Commissural Axon Outgrowth, but Do Cause Commissural Axon Turning and Trochlear Axon Repulsion In Vitro

The observations that some commissural axons reach the floor plate and that trochlear axon trajectories are largely normal in the homozygous mutants suggested

Figure 3. Commissural Axon Trajectories Are Aberrant in *Netrin-1* Homozygous Mutant Animals

(A–C) Transverse sections of wild-type ([A]; n = 8) and homozygous mutant ([B] and [C]; n = 7) E11.5 embryos at brachial level subjected to immunohistochemistry with antibody 4D7, which recognizes TAG-1, expressed on commissural neurons and their axons (c). In wild-type embryos (A), commissural axons grow in a directed fashion (green arrowheads) toward the floor plate (fp), where they cross the midline. Even axons that at first grow along the edge of the spinal cord turn to grow toward the floor plate (white arrowhead). Heterozygotes appeared similar to wild-type embryos (not shown). In contrast, in mutant embryos (B and C), commissural axon growth within the spinal cord is disorganized, and few commissural axons reach the floor plate. Many axons continue to grow ventromedially but are not as directed toward the floor plate and are instead oriented more vertically. Some axons continue to grow along the edge of the spinal cord around the motor column (white arrowheads), and some grow within the motor column (white arrows). Other axons appear to turn to grow toward the ventricle, even at dorsal levels (green arrows). Note in (B) and (C) a population of TAG-1$^+$ cells near the floor plate (green arrowheads) that are obscured by commissural axons in (A).

(D and E) Transverse sections of heterozygous ([D]; n = 7) and homozygous mutant ([E]; n = 8) E10.5 embryos at brachial level subjected to immunohistochemistry with antibody 4D7 (red). Directed growth of commissural axons through the motor column to the floor plate at this age (arrowhead) is observed in heterozygotes (and also wild type, not shown). (At this age, motor neurons also express TAG-1 [Dodd et al., 1988].). At this early age in the mutant, commissural axons already display defective trajectories; none have reached the floor plate (arrowheads), and some appear to have turned to grow toward the ventricle (arrows). In contrast, the *netrin-1* expression pattern (as revealed by the localization of the fusion protein using a polyclonal antiserum against β-galactosidase [green]), is similar in both heterozygotes and mutants (n = 4 in each case). Note that distribution of the fusion protein does not necessarily reflect the distribution of wild-type netrin-1 protein.

Scale bar is 75 μm (different embedding media used in [A]–[C] and [D] and [E]). v, ventricle; mc, motor column; d, dorsal root ganglion; drez, dorsal root entry zone.

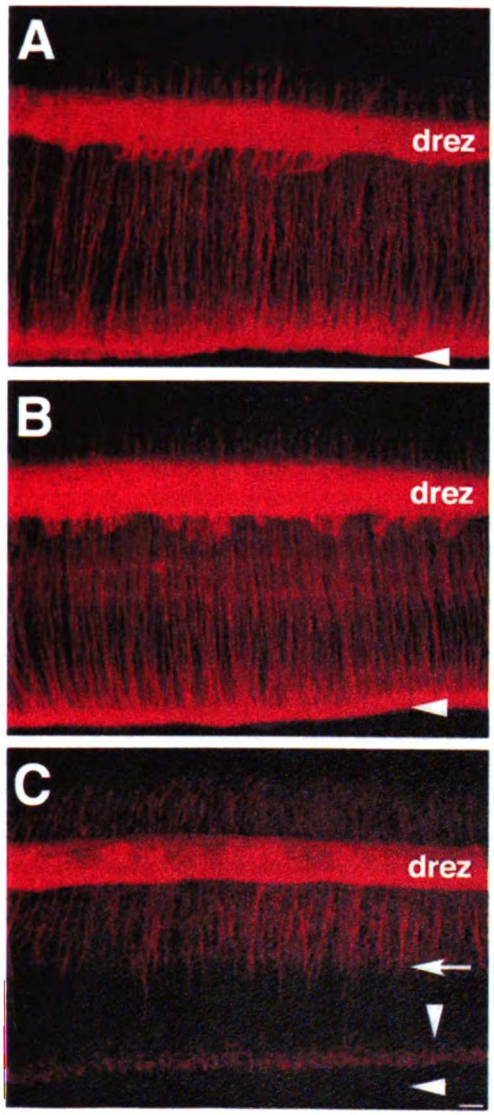

Figure 4. Few Commissural Axons Reach the Floor Plate in *Netrin-1* Homozygous Mutants

Spinal cords of wild-type ([A]; n = 9), heterozygote ([B]; n = 15), and homozygous mutant ([C]; n = 6) E11.5 embyros were fixed and subjected to whole-mount immunohistochemistry with antibody 4D7. Small pieces (~2 mm in length) were cut from the spinal cords just below forelimb level, and the roof plates and floor plates were cut to yield a hemicord that was mounted for viewing in the sagittal plane. In the mutant, although many commissural neurons are present dorsally, most axons fail to project further ventrally (arrow), and few reach the floor plate (horizontal arrowhead). The growth that is observed is disorganized when compared to that seen in wild-type or heterozygote littermates. Note the TAG-1$^+$ cells adjacent to the floor plate (vertical arrowhead) in the mutant whose presence is obscured in the wild type and heterozygote (see also Figures 3B and 3C). The roof plate is just off the top of the panel in each case. Scale bar is 50 μm; drez, dorsal root entry zone.

that other guidance cues might function with netrin-1 to guide these axons. One possibility we considered is that the floor plate might express additional diffusible attractants or repellents. Indeed, although previous studies have shown that floor plate cells and netrin-1 both possess several guidance activities in vitro (the ability to elicit commissural axon outgrowth from rat dorsal spinal cord explants into a collagen matrix, the ability to reorient the growth of commissural axons within rat dorsal spinal cord explants, and the ability to repel the growth of trochlear motor axons extending into a collagen matrix from explants of the ventral hindbrain–midbrain junction [HMJ] [Tessier-Lavigne et al., 1988; Placzek et al., 1990; Kennedy et al., 1994; Serafini et al., 1994; Colamarino and Tessier-Lavigne, 1995b]), these studies have not determined whether netrin-1 alone accounts for all of these activities when produced by floor plate cells.

We therefore examined whether floor plate cells from mutant embryos possessed the in vitro activities of wild-type floor plate cells, using explants of rat dorsal spinal cord or rat ventral HMJ as sources of responding axons. As compared to floor plates from wild-type or heterozygous embryos, floor plates from E11.5 mutant embryos were ineffective in promoting commissural axon outgrowth from E13 rat dorsal spinal cord explants, eliciting no outgrowth or outgrowth that was only slightly above background (Figures 7A–7C and Table 1). Thus, netrin-1 appears to account for most, or perhaps all, of the outgrowth-promoting activity of floor plate cells; the residual activity might be due to a small amount of wild-type netrin-1 made by mutant floor plate cells (Figure 1H). In contrast, floor plate explants from mutant embryos were as effective as those from wild-type embryos in eliciting turning of commissural axons (Figures 7D–7F and Table 1), raising the possibility that floor plate cells secrete another attractant for commissural neurons in addition to netrin-1 (see Discussion). Mutant floor plates were also able to repel the growth of trochlear motor axons at a distance (Figures 7G–7I and Table 1), indicating that floor plate cells secrete a repellent for trochlear motor axons distinct from netrin-1 (see Discussion).

Multiple Selective Defects in Brain Commissures and Pontine Nuclei in Netrin-1 Mutant Mice

We next examined whether netrin-1 was required for the development of the nervous system rostral to the spinal cord, where *netrin-1* expression can be demonstrated by in situ hybridization (see below). Serial sectioning of brains from late gestation (E18.5) mice of all three genotypes revealed striking and fully penetrant defects in brain development in the mutant animals (Figure 8 and Table 2).

Both the corpus callosum, which joins the left and right cerebral cortices, and the hippocampal commissure (ventral commissure of the fornix), which joins the left and right hippocampi, are completely absent in homozygous mutants (but are always present in wild-type and heterozygous littermates [Figures 8A–8C and Table 2]). What appear to be swirls of axons ("Probst bundles"; Probst, 1901) are present in the mutants instead of the commissures, and the fimbria are also malformed. The only obvious defect seen in heterozygotes is the presence of cell bodies within the hippocampal commissure that appear largely absent in wild-type animals. Another

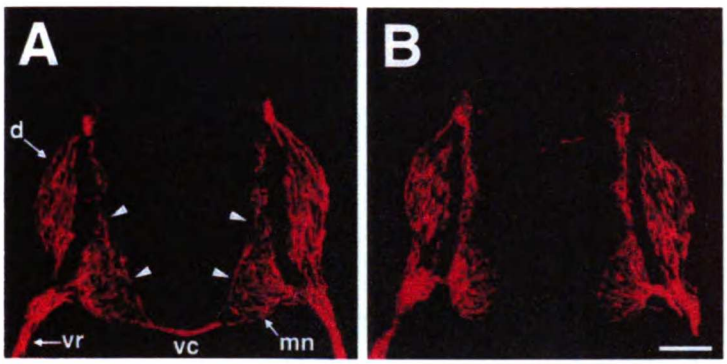

Figure 5. The Deficit in Commissural Axon Growth in Netrin-1 Deficient Embryos Is Selective

Transverse sections of E10.5 mutant embryos stained with a neurofilament antibody show that on E10.5, except for the lack of a ventrally directed commissural projection (arrowheads in [A]), spinal cords of mutant embryos ([B], six embryos examined) display apparently normal neuronal positioning and axonal trajectories as compared to heterozygote embryos of the same age ([A], seven embryos examined; wild type is similar, not shown). Motoneurons (mn) develop in their expected ventrolateral locations and project to form the ventral roots in both cases. Axons extend from sensory neurons in appropriately positioned dorsal root ganglia (d) both toward the spinal cord and to join the ventral roots (vr) in both cases. Scale bar is 120 μm.

major commissure of the brain, the more ventrally located anterior commissure, is also defective in the mutants (Figures 8D–8F and Table 2). The anterior commissure is composed of anterior and posterior limbs, which meet and contribute to a thick commissure at the midline. Both limbs of the anterior commissure are missing in homozygous mutants, with only a small vestige of crossing axons remaining at the midline (the severity of this defect was variable [Table 2]: in 3/8 embryos, there was a remnant of the commissure [data not shown], whereas in the remaining embryos few or no axons were seen crossing [Figure 8F]). Not all brain commissures are affected in the mutants, however. Two dorsal commissures, the habenular and posterior commissures, appear intact in the mutants (Figures 8G–8I and Table 2), and a new major commissure is found in the roof of the fourth ventricle at the junction of hindbrain and midbrain (Figures 8J–8L and Table 2). Mutant animals also completely lack pontine nuclei (Figures 8M–8O and Table 2). This defect may indicate a role for netrin-1 in cell migration rather than axon guidance (see Discussion).

In situ hybridization reveals that *netrin-1* is expressed at appropriate times and locations to play a guidance role for axons forming the corpus callosum, hippocampal commissure, and anterior commissure. In the case of the corpus callosum and hippocampal commissure, *netrin-1* is expressed along the path of the fimbria and in cells at the junction of the two cerebral hemispheres where the commissural fibers cross, before and during the period of formation of these commissures (Figures 9A–9D). *netrin-1* is also expressed at appropriate times at the midline more ventrally, where the axons of the anterior commissure cross (Figures 9E–9H). The *netrin-1*-expressing midline cells partially envelop these commissural axons (Figures 9F and 9H). Some of the axons forming the commissure also pass through or near other domains of *netrin-1* expression on their way to the midline.

Discussion

There is considerable evidence that axons can be guided in vitro by diffusible chemoattractants and chemorepellents, and in recent years progress has been made in identifying candidates for the molecules that mediate these effects (reviewed in Tessier-Lavigne and Goodman, 1996). However, the in vivo functions of diffusible guidance cues, including netrin-1, have not so far been defined in vertebrates. The generation of an insertional mutation in the murine *netrin-1* gene has made it possible to assess the function of netrin-1. Our findings provide evidence that netrin-1 guides axons during development of the vertebrate central nervous system, and also provide evidence for the existence of other diffusible cues that collaborate with netrin-1 to effect accurate guidance.

A Loss-of-Function Allele of *Netrin-1*

The *netrin-1* allele described here appears to be a loss-of-function allele. The major product of the allele is a chimeric transmembrane protein that retains some amino-terminal netrin-1 sequences in its extracellular

Table 2. Defects in Central Nervous System Rostral to Spinal Cord

Defect[a]	Genotype		
	Wildtype (+/+)	Heterozygote (+/−)	Mutant (−/−)
Corpus callosum and hippocampal commisure absent	0/9	0/12	11/11
Anterior commisure defective	0/9	0/12	8/8[b]
Posterior or habenular commisure absent	0/9	0/12	0/11
"Aberrant" commisure present	0/9	0/12	11/11
Pontine nuclei absent	0/9	0/12	8/8

[a] Defects were determined by collecting serial paraffin sections from E18.5 brains.
[b] Three of these had a remnant of the commisure at the midline; in the other five, the commissure was almost absent (see Figure 8F). See text for details.

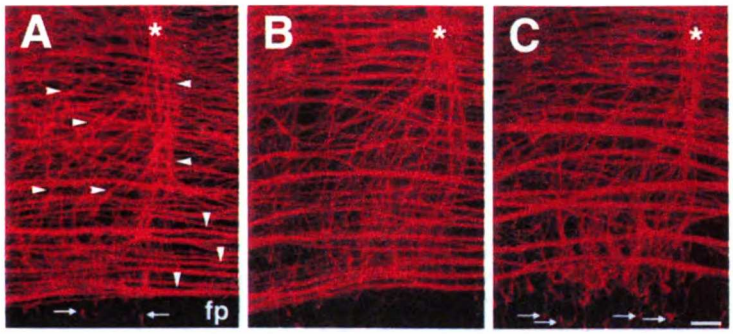

Figure 6. Trochlear Motoneurons Project Normally in *Netrin-1* Homozygous Mutant Mice

Sagittal (side) views of the region of the trochlear nucleus, in (A) wild-type, (B) heterozygote, and (C) homozygous mutant E10.5 mice visualized after whole-mount immunohistochemistry with an antibody recognizing neurofilament-M (NF-M). Dorsal is up; rostral, to the right. Trochlear axons (several are indicated by horizontal arrowheads in [A]) run toward the top of the figure and coalesce (asterisk) before exiting the neural tube. Their trajectory is partially obscured by bundles of axons coursing longitudinally (i.e., horizontally in the figure) (several are indicated by vertical arrowheads). Several trochlear neurons among those present are indicated (arrows). When compared to wild-type and heterozygote littermates, mutants show no obvious defects in trochlear axon trajectories (n = 12, 27, and 14 trochlear nuclei examined at E10.5, respectively). However, more trochlear neurons are present within the floor plate region in the mutants (compare arrows in [A] and [C]). In addition, longitudinal axon tracts are somewhat disorganized. Scale bar is 60 μm.

domain but that appears confined to an intracellular compartment within expressing cells (as observed here; see also Skarnes et al., 1995), where it would be inaccessible to developing axons. This intracellular targeting does not appear to have significant dominant effects (e.g., by interfering with secretion of other proteins), since the phenotypes we observe are fully recessive. (The one minor phenotype we observe in heterozygotes, the increased presence of cell bodies in the hippocampal commissure, could reflect a dosage sensitivity.) The mutant allele is likely to be hypomorphic rather than null, since some wild-type transcripts are produced from the allele, presumably by splicing over the inserted sequences. Only very low levels of wild-type transcript are produced, however, suggesting the presence of only small amounts of wild-type netrin-1 protein in homozygous mutants. This suggestion is supported by the observation that the outgrowth-promoting activity of floor plate cells is almost completely eliminated in homozygous mutants (Figure 7C).

Guidance of Spinal Commissural Axons by Netrin-1

Based on studies in chick embryos, it was hypothesized that a decreasing ventral-to-dorsal gradient of netrin-1 and netrin-2 protein might be present in the spinal cord and guide commissural axons to the floor plate (Kennedy et al., 1994; Serafini et al., 1994). In mouse, netrin-1 appears to have taken over any required netrin functions in the spinal cord, since the pattern of *netrin-1* expression in mouse resembles a summation of the *netrin-1* and *netrin-2* expression patterns in chick, and since the murine *netrin-2* gene is not expressed in the spinal cord at these stages (H. W. and M. T.-L., unpublished observations). Although the pattern of *netrin-1* mRNA expression is consistent with the presence of a gradient of netrin-1 protein, the actual distribution of the protein remains to be determined.

The misrouting of spinal commissural axons observed here in homozygous mutant embryos supports the guidance role postulated for netrin-1. The axons appear to project along a ventral trajectory in the dorsal spinal cord, but as they approach the motor column at the midpoint of dorsoventral axis they deviate from the normal trajectory toward the floor plate. Some axons make medial projections in the direction of the ventricular zone. Many others project ventrally rather than ventromedially and do not orient toward the floor plate, unlike the axons that progress into the ventral half of the spinal cord in wild-type embryos. As a result, many of these axons invade the motor column rather than skirting it, as occurs in wild-type embryos. The simplest interpretation of these errors is that the axons are confused and unable to locate the ventral midline in the absence of a strong directional cue provided by a (presumed) gradient of netrin-1 protein.

It is important to note that the errors are not absolute, as some axons appear to project along a roughly normal trajectory and reach the ventral midline. It is possible that the small amount of residual netrin-1 protein predicted to be present is sufficient to guide the axons. Alternatively, other cues might be present and, on their own, capable of effecting some guidance. This appears to be the case in C. elegans and Drosophila, where loss of function of *netrin* genes (*unc-6*, and *Netrin-A* and *Netrin-B*, respectively) only partially impairs ventrally directed projections (Hedgecock et al., 1990; Harris et al., 1996; Mitchell et al., 1996). What might these cues be? One possibility is that the dorsalmost (roof plate) region of the spinal cord secretes a repellent for commissural axons, directing the initial ventral trajectories seen in the absence of netrin-1 (J. Dodd, personal communication). Closer to the ventral half of the spinal cord, the netrin cue may become important, so that the axons in the mutants begin to show major deviations from normal trajectories only in the ventral portion of the spinal cord. In this model, netrin-1 is the sole ventral cue required for axons to find the ventral midline efficiently.

An additional, and not mutually exclusive, possibility suggested by the turning of commissural axons toward homozygous mutant floor plates in vitro (Figure 7F) is the existence of a second chemoattractant made by floor plate cells. If such a second chemoattractant is present, why then do any axons get misrouted? If the factor is secreted solely by floor plate cells, it is possible that its range of action in the ventral spinal cord is more limited than that of netrin-1, which is expressed not only

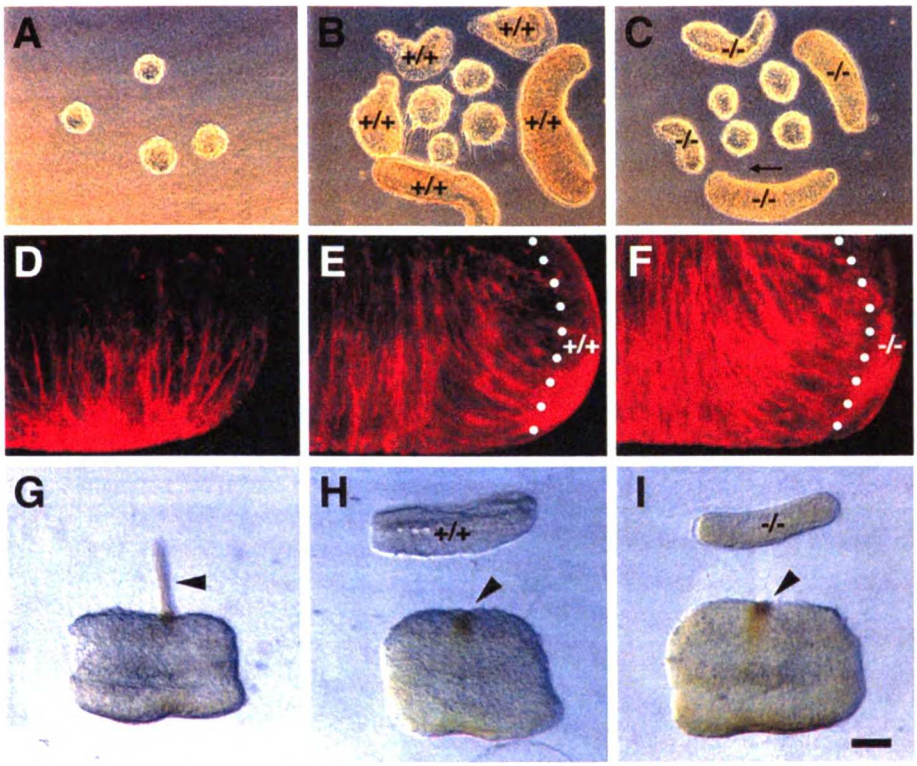

Figure 7. Floor Plate Tissue from Homozygous Mutants Lacks Commissural Axon Outgrowth Activity but Can Still Elicit Commissural Axon Turning and Repel the Growth of Trochlear Axons In Vitro

Commissural axon outgrowth assays (A–C), commissural axon turning assays (D–F), and trochlear motoneuron axon repulsion assays (G–I) performed without any floor plate present (A, D, and G), with floor plate from a wild-type E11.5 embryo present (B, E, and H), and with floor plate from a homozygous mutant E11.5 embryo present (C, F, and I). See Table 1 for quantitation, including testing of floor plates from heterozygotes.

(A–C) Each panel shows four explants of E13 rat dorsal spinal cord cultured for 16 hr in collagen gels (Serafini et al., 1994). Without floor plate (A), commissural axons do not grow out of E13 rat dorsal spinal cord explants into the surrounding collagen over 16 hr. Floor plate explants from a wild-type embryo (+/+) elicit robust outgrowth (B), whereas floor plate explants from a homozygous littermate (−/−) elicit very little outgrowth (arrows in [C]).

(D–F) E11 rat dorsal spinal cord explants were cultured for 40 hr in collagen gels, and commissural axons in the explants were visualized by whole-mount immunohistochemistry with antibodies to TAG-1 (Kennedy et al., 1994). Dorsal is up in each case. Commissural axons project along their stereotypical dorsal-to-ventral trajectory within an E11 rat dorsal spinal cord explant cultured alone (D), but reorient toward floor plate explants from both wild-type (E) and mutant (F) embryos (dots indicate border between dorsal and floor plate explants).

(G–I) Explants of ventral hindbrain–midbrain junction (HMJ) from E11 rat embryos were cultured for 40 hr in collagen gels and stained with antibody F84.1 to visualize trochlear motor axons (Colamarino et al., 1995). The trochlear axons (arrowheads) extend from these explants in the absence of floor plate (G), but are repelled by floor plate from both wild-type (H) and homozygous mutant (I) embryos.

Scale bar is 160 μm in (A)–(C), 80 μm in (D)–(F), and 180 μm in (G)–(I).

by floor plate but also by ventral spinal cord cells. In this model, diffusion gradients of chemoattractants emanating from floor plate cells would serve primarily to direct the last leg of commissural axon trajectories, and would be most effective when the spinal cord is small. The expression of *netrin-1* beyond the floor plate would become more important for guidance as the spinal cord grows, and the distance that commissural axons would have to project to reach the floor plate would increase. It should also be noted that this turning of axons toward mutant floor plate cells in vitro could reflect an indirect effect. The mutant mouse floor plate cells are expected to express Sonic hedgehog (Echelard et al., 1993), which can induce *netrin-1* expression in early chick intermediate neural plate explants in a contact-dependent manner (Tanabe et al., 1995). If Sonic hedgehog can also induce *netrin-1* in the developmentally older rat dorsal neural tube explants used here, then the mutant floor plates may have caused axons to turn in our experiments by inducing at short range a gradient of netrin-1 protein within the rat dorsal explants.

Another strong phenotype that requires explanation is the decrease in invasion of the ventral spinal cord by commissural axons in homozygous mutants as compared to wild-type animals. This could be a simple consequence of the absence of a directional netrin-1 cue. Growth cones are known to slow down their migration at important decision points, presumably the better to sample the guidance information in these regions (reviewed in Tessier-Lavigne and Goodman, 1996). It is therefore possible that the absence of a strong directional cue at the level of the developing motor column

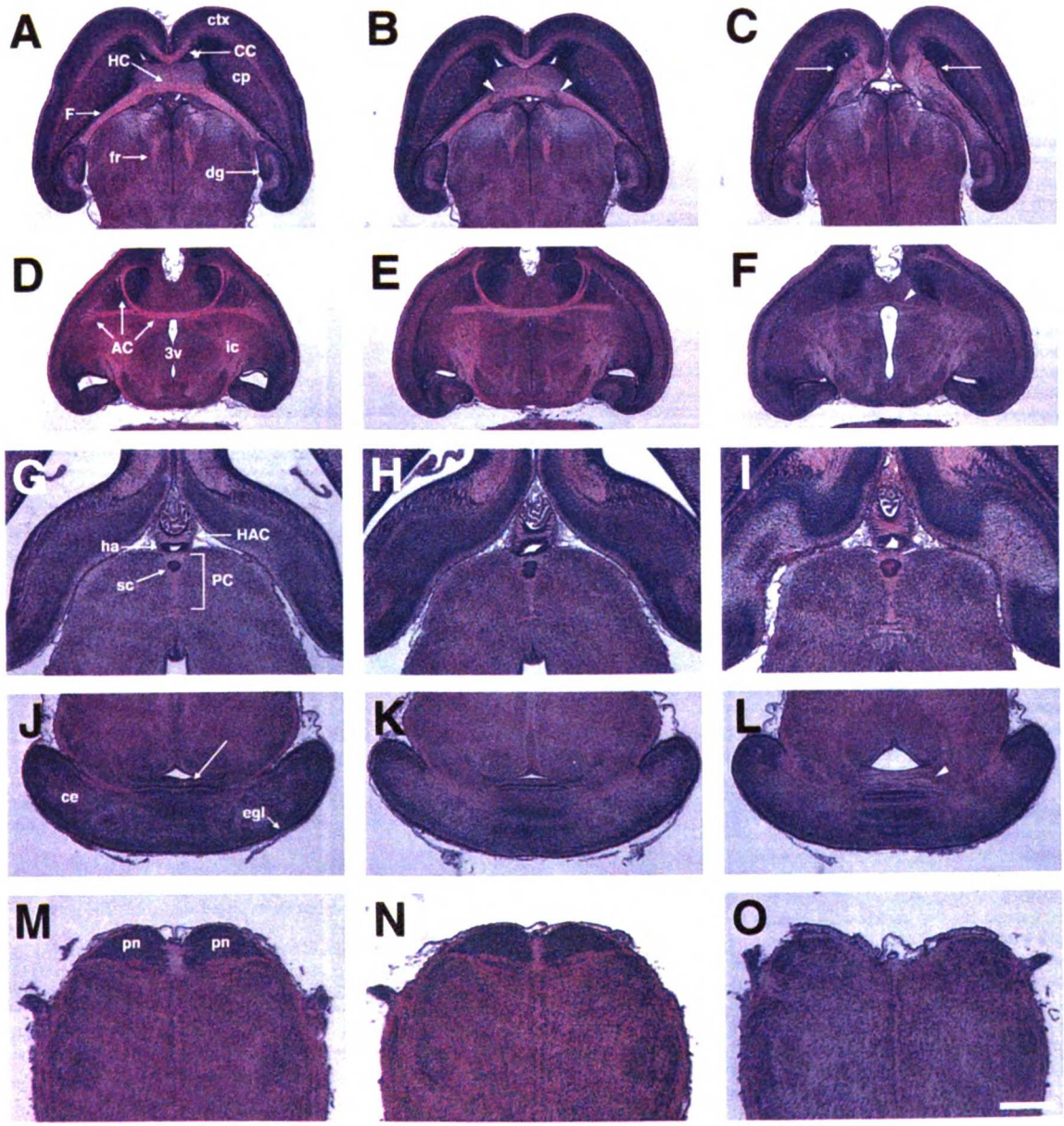

Figure 8. Homozygous Mutants Have Selective Brain Commissure Defects and Lack Pontine Nuclei

Horizontal sections (rostral toward the top in each case) from E18.5 brains of wild-type (A, D, G, J, and M), heterozygote (B, E, H, K, and N), and homozygous mutant (C, F, I, L, and O) embryos (stained with hematoxylin and eosin). Serial sections were examined for all embryos; see Table 2 for quantitation.

(A–C) Level of section displaying the corpus callosum (CC) and hippocampal commissure (ventral commissure of the fornix) (HC). Compared to wild-type and heterozygote littermates, the mutants have malformed fimbria (F) and lack any CC or HC. Instead, the mutants have Probst bundles (arrows) where the commissures should be. A larger number of cell bodies in the heterozygote as compared to wild-type animals (arrowheads) are found within the HC, the only defect seen so far in heterozygotes.

(D–F) Level of section displaying the anterior commissure (AC). Except for a few crossing axons (arrowhead in [F]), both anterior and posterior limbs of the AC are absent in mutant animals (note that in some embryos, a remnant of the commissure was seen at the midline: see text for details). (The full extent of the posterior limb of the AC, which appears lacking in this particular heterozygote section, is seen in adjacent sections.)

(G–I) Level of section displaying both the habenular commissure (HAC) and the posterior commissure (PC). Both are present in animals of all three genotypes.

(J–L) Level of section displaying the developing cerebellum (ce) and roof of the fourth ventricle at the junction of midbrain and hindbrain (anterior medullary velum) (arrow). In this region, mutant embyros have a large commissure (arrowhead) that is not present in wild type or heterozygotes.

(M–O) Level of section displaying hindbrain structures, including the pontine nuclei (pn). Pontine nuclei are completely absent in mutant animals.

Scale bar is 815 μm in (A)–(F), 385 μm in (G)–(I), and 510 μm in (J)–(O). ctx, cerebral cortex; cp, caudo-putamen; dg, dentate gyrus; fr, fasciculus retroflexus; ic, internal capsule; 3v, third ventricle; ha, habenula; sc, subcommissural organ; egl, external germinal layer.

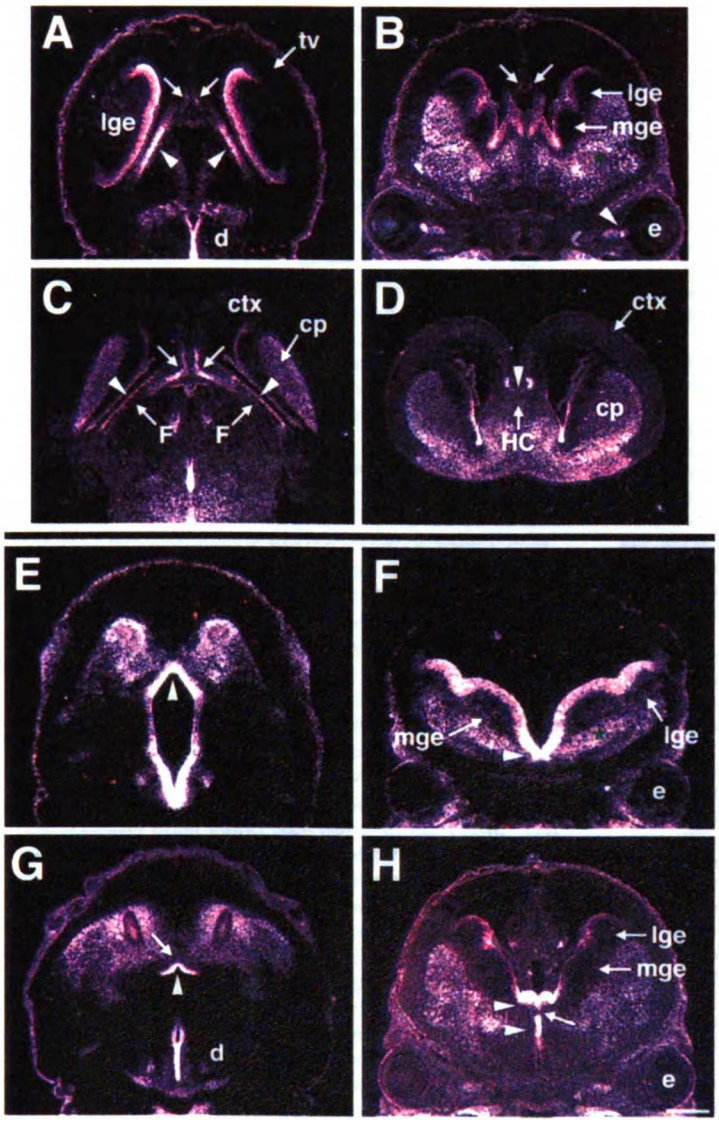

Figure 9. *Netrin-1* Is Expressed Appropriately to Play a Direct Role in the Formation of the Fimbria, Hippocampal Commissure, Corpus Callosum, and Anterior Commissure

Horizontal (A, C, E, and G; rostral is up) and coronal (B, D, F, and H; dorsal is up) sections of E12.5 (E and F), E14.5 (A, B, G, and H) embryos and E16.5 brains (C and D) were subjected to in situ hybridization with a ^{35}S-labeled *netrin-1* probe. Sections in (E) and (G) are more ventral than those in (A) and (C); sections in (F) and (H) are more anterior than those in (B) and (D).

(A and B) At E14.5, *netrin-1* is expressed in the forebrain in the medial walls of the telencephalic vesicles (tv) (arrowheads) and is expressed at the surfaces where the two vesicles meet more rostrally (arrows). Expression is prominent in the ventricular zones of the ganglionic eminences (medial [mge] and lateral [lge]) and in cells of the ventral forebrain that have migrated away from the ventricular surface (green asterisk). Expression is also observed in the diencephalon (d) and epidermis. Expression at the exit point of the retina and along the optic nerve is also present (arrowhead).

(C and D) At E16.5, the developing fimbria (F) lie along the region of *netrin-1* expression at the medial hemisphere walls (arrowheads). At their rostral end the axons lie between this region and the *netrin-1*-expressing region where the two hemispheres meet (arrows in [C]; note higher expression at this age). This region of expression continues across the midline (arrowhead in [D]) in cells that are contacted by axons that are crossing to the opposite hemisphere to form the hippocampal commissure (HC).

(E and F) At E12.5, *netrin-1* is expressed at the midline of the developing forebrain (arrowheads in [E] and [F]) just caudal to where anterior commissural axons will cross.

(G and H) At E14.5, some anterior commissural axons have already crossed, contacting the midline (arrow). In the coronal plane (H), the axon tract is seen to be partially enveloped by the *netrin-1* expressing midline cells (arrowheads).

Scale bar is 480 μm in (A), (B), (G), and (H), 600 μm in (C) and (D), and 375 μm in (E) and (F). Additional abbreviations: ctx, cerebral cortex; cp, caudo-putamen; e, eye.

forces the axons to search for alternate cues, slowing down or even halting their progression. An alternative interpretation is suggested by the fact that netrin-1 possesses outgrowth-promoting activity in vitro, enabling commissural axons to invade a collagen matrix that is otherwise an unfavorable environment for commissural axon growth (Serafini et al., 1994). It is therefore possible that the environment of the ventral spinal cord is relatively nonpermissive to commissural axon growth in the absence of netrin-1. In this model, netrin-1 would have the additional guidance role of a "permissive" factor, in addition to its role as an "instructive" chemoattractant.

Finally, it seems likely that other non–floor plate–derived cues contribute to shaping commissural axon trajectories. In both wild-type and mutant embryos, commissural axons remain largely confined to the lateral aspect of the dorsal spinal cord and, for the most part, do not approach the ventricular zone in the ventral spinal cord either, despite the presence there of *netrin-1* expression. It is possible that cells in the ventricular region present inhibitory cues that discourage commissural axon invasion even in the presence of netrin-1 protein, or that additional positive cues mark out the lateral aspect (discussed in Colamarino and Tessier-Lavigne, 1995a).

Netrin-1 Is Not Essential to Trochlear Motor Axon Guidance

Netrin-1 can repel trochlear motor axons in vitro (Colamarino and Tessier-Lavigne, 1995b), but loss of *netrin-1* function does not significantly alter the projections of

trochlear motor axons. A minor disorganization of the placement of the cell bodies of trochlear motor neurons is observed, but the axons seem otherwise to grow along a largely normal trajectory. Thus, other cues must be present that can guide the axons in the absence of netrin-1. One candidate is a second chemorepellent for trochlear motor axons, whose existence is indicated by our in vitro experiments demonstrating that floor plate explants from homozygous mutant embryos can repel rat trochlear motor axons in vitro. It is possible that this second chemoattractant is a semaphorin family member (Culotti and Kolodkin, 1996), since semaphorin III/D can also repel trochlear motor axons (unpublished data; A. Tucker et al., submitted). Recent evidence also indicates the existence of a floor plate–derived chemorepellent(s) distinct from both netrin-1 and semaphorin III (Hu and Rutishauser, 1996; R. Shirasaki et al., submitted).

A Role for Netrin-1 in Axon Guidance and Cell Migration in the Brain?

In addition to the defects in the spinal cord, severe defects were observed in the formation of several commissures in the brain: the corpus callosum, hippocampal commissure, and anterior commissure. The defects did not reflect a generalized defect in brain commissure formation, since some other commissures were unaffected. We do not know at present whether the defects reflect a direct role for netrin-1 in guiding any of the affected axonal classes. Loss of *netrin-1* function could conceivably influence the formation of these commissures indirectly, if netrin-1 is required, for example, for the establishment of the cellular substrates that the axons normally grow along (see Silver et al., 1993). The distribution of *netrin-1* mRNA along the paths of the axons that are affected and at the points where they cross the midline is, however, at least suggestive of a role for netrin-1 in guiding these axons.

We have likewise not determined how the absence of pontine nuclei arises in the homozygous mutant animals. These nuclei are formed by a long-range migration of postmitotic pontine precursor cells from the lateral recess of the IVth ventricle along a ventral, circumferential trajectory (Bourrat and Sotelo, 1990), not unlike that of commissural axons. It is therefore tempting to speculate that these cells are normally guided by netrin-1 and that the absence of the pontine nuclei in the mutant reflects a mismigration. This would then parallel the finding in C. elegans that UNC-6 is also involved in directing cell migrations (Hedgecock et al., 1990).

Thus, further studies are required to determine how netrin-1 influences the migrations of cells and growth cones in the developing brain. Nonetheless, our results raise the possibility that netrin-1 plays important roles in axon guidance not only in the relatively simple environment of the early neural tube, but also in the more complex environment of the developing brain, and that, as in C. elegans, netrins in vertebrates might function to guide not only developing axons, but migrating cells as well.

Experimental Procedures

Cloning of cDNAs Encoding Murine Netrin-1

PCR was performed on reverse-transcribed E14.5 mouse brain poly(A)$^+$ RNA. Primers were designed based upon sequence homologies between chick netrin-1 and C. elegans UNC-6. The degenerate sense primer sequence corresponded to the amino acid sequence WVPFEFY, and the degenerate antisense primer sequence to DCKHNTA. A probe derived from the amplified product was used to screen 1×10^6 clones of an E14.5 mouse telencephalon cDNA library, yielding a clone with a poly(A)-sequence at its 3′ end and all but 250 bp of the most 5′ coding sequence, which was used to isolate a second clone extending an additional 480 bp. These clones were used to construct a cDNA containing the entire coding region of murine *netrin-1* (pMNET1).

Northern Analysis

A major 6.0 kb band was observed using probes synthesized from domain VI–encoding sequences or 3′UTR sequences hybridized to E14.5 brain poly(A)$^+$ RNA or from the 5′ portion of the pMNET1 insert hybridized to P0 brain RNA. Hybridization of the 5′ pMNET1 probe to P0 heterozygote brain RNA also yielded a 6.8 kb species, at much lower abundance, which matched the band observed using a *lacZ* probe, and therefore likely represents the fusion transcript.

RNA In Situ Hybridization

A ^{35}S-labeled *netrin-1* antisense riboprobe was obtained from construct pMNET3UTRA$^-$, which includes most of the 3′-UTR but lacks the poly(A) tract. Embryos and brains were fixed and embedded into paraffin. Sections 8–10 μm in thickness were processed for in situ hybridization using a slightly modified version of a previously described protocol (Frohman et al., 1990) (details available from authors on request). *F-spondin* transcripts were detected using a probe generated from FP5 (gift of A. Klar).

Generation of Netrin-1 Mutant Mice

Embryonic stem cell line ST629, containing an insertion of the secretory gene trap vector pGT1.8TM within an intron of the *netrin-1* gene, was generated as previously described (Skarnes, et al., 1995). 5′-RACE on ES cell RNA was performed as previously described (Skarnes et al., 1995), and yielded 42 bp upstream of the splice site that perfectly matched *netrin-1* sequence (except for a single bp gap) at the end of domain V-1. A primer-extended cDNA library was constructed in pMT21 from P0 heterozygote brain poly(A)$^+$ RNA essentially as described (Serafini et al., 1994), using a primer for first-strand synthesis complementary to the *lacZ* sequence present 917 bp downstream from the expected splice junction. Clones (1×10^6) were screened with the *engrailed-2* splice acceptor probe (see below); 90 positives were scored, and of seven sequenced, five extended across the splice junction, and three of these perfectly matched *netrin-1* sequence at the same position as found by the 5′-RACE (the other two did not match any sequences in GenBank). Founder chimeric males, generated as described (Skarnes et al., 1995), were bred to CD1 females, and heterozygous progeny (as determined by Southern analysis using a *lacZ* probe) were mated to maintain the allele.

Southern Analysis, RT–PCR, RNase Protection

For Southern analysis, a probe specific for *engrailed-2* splice acceptor sequences was synthesized from the 500 bp fragment generated by digesting plasmid p1.8HX with BamHI and BglII. A probe specific for *lacZ* sequences was synthesized from an EcoRV fragment of pML62 (gift of Mark Lewandowski), and a probe specific for *neo* sequences was synthesized from an XhoI / SalI fragment of pMC1neoPolyA (Thomas and Capecchi, 1987). RT–PCR to genotype embryos was performed using RNA isolated from caudal portions of embryos containing neural tube (E9.5–E11.5) or from rostral spinal cord or caudal hindbrain of excised brains (E18.5–P0). Multiplex PCR (details available on request) was performed on reverse transcribed RNA using the following primers: forward (TGACTGTAGGCACAACA CGG; within netrin-1 domain V-1 sequence), reverse wild-type (CTCCATGTTGAATCTGCAGC; within netrin-1 domain V-2 sequence), and reverse mutant (GCCTTCCATCTCAACTCTCC; within CD4 sequence).

The ^{32}P-labeled antisense riboprobe for RNase protection analysis was transcribed from NotI-linearized pMNETXH by T_7 RNA polymerase. pMNETXH was generated by cloning a 300 bp XbaI/HindIII fragment of the insert of pMNET3UTRA$^-$ into pBluescript. RNase protection reactions were performed with 10 μg brain RNA and 1×10^5 cpm of probe using a commercially available kit (Clontech).

Explant Culture and In Vitro Assays

In vitro assays of floor plate-derived commissural axon outgrowth, commissural axon turning, and trochlear motoneuron repulsion activities were performed as previously described (Tessier-Lavigne et al., 1988; Placzek et al., 1990; Kennedy et al., 1994; Serafini, et al., 1994; Colamarino and Tessier-Lavigne, 1995b), except that glutamine was replaced by 2 mM GlutaMAX I.

Immunohistochemistry

Sections were blocked in PHT (1 × PBS/1% heat-inactivated goat serum/0.1% Triton X-100) and were incubated overnight with antibodies to TAG-1 (monoclonal 4D7 [Dodd et al., 1988] supernatant, diluted 1:100 in PHT), β-galactosidase (rabbit polyclonal, diluted 1:2000; Boehringer Mannheim), neurofilament (monoclonal 3A10 supernatant, used without dilution, Developmental Studies Hybridoma Bank), Islet-1 (monoclonal 4D26 supernatant, diluted 1:5; Developmental Studies Hybridoma Bank), and HNF3β (rabbit polyclonal, diluted 1:100; gift of Dr. Brigid Hogan). After several washes (10 min) at ambient temperature with PHT, sections were incubated with fluorescein (DTAF)- or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch) for 1 hr at 4°C and washed before mounting in Fluoromount-G (Fisher).

Whole-mount immunohistochemistry was performed on E11 rat dorsal spinal cord explants and E11.5 mouse spinal cords using antibody 4D7, on E11 rat ventral HMJ explants using antibody F84.1 (gift of W. Stallcup), and on E9.5–E11.5 mouse HMJ regions using an antibody to NF-M (gift of Dr. Virginia Lee), essentially as described (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995b), with the following modifications: PHT (0.1% Triton X-100) was used instead of PHTX, and immunohistochemistry with NF-M was performed in PBSMT (PBS with 2% nonfat milk and 0.1% Triton X-100) using 2-day incubations in antibody and 1- to 2-day washes at 4°C.

Acknowledgments

We thank D. Townley for assistance with 5′-RACE; B. Hogan, A. Klar, V. Lee, M. Lewandowski, W. Stallcup, and M. Yamamoto for antibodies and plasmids; J. Rubenstein and C.-M. Fan for libraries; C.-M. Fan and M. Bedolli for advice on in situ hybridization; L. Prentice for advice on histological procedures; and S. Catalano and M. Masu for discussions on forebrain development and anatomy. This work was supported by postdoctoral fellowships from the American Cancer Society (T. S. and H. W.), a Howard Hughes Medical Institute predoctoral fellowship (S. A. C.), a UCSF Chancellor's Fellowship (E. D. L.), and grants from the National Institutes of Health and the Human Frontiers Science Program (M. T.-L.). M. T.-L. is an Assistant Investigator and R. B. and International Scholar of the Howard Hughes Medical Institute.

Received July 30, 1996; revised October 17, 1996.

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GenBank Accession Number

The accession number for the murine *netrin-1* sequence described in this paper is U65418.

Notes Added in Proof

The paper cited as R. Shirasaki et al., submitted is now in press:

Shirasaki, R., Mirzayan, C., Tessier-Lavigne, M., and Murakami, F. (1996). Guidance of circumferentially growing axons by netrin-dependent and -independent floor plate chemotropism in the vertebrate brain. Neuron, in press.

In addition, papers describing axonal projections in mice deficient in two other diffusible guidance cues defined in vitro have recently appeared or are in press:

Behar, O., Golden, J.A., Mashimo, H., Schoen, F.J., and Fishman, M.C. (1996). Semaphorin III is needed for normal patterning and growth of nerves, bones, and heart. Nature *383*, 525–528.

Ebens, A., Brose, K., Leonardo, E.D., Hanson, M.G., Bladt, F., Birchmeier, C., Barres, B. and Tessier-Lavigne, M. (1996). Hepatocyte Growth Factor/Scatter Factor is an axonal chemoattractant and a neurotrophic factor for spinal motor neurons. Neuron, in press.