Title
Mechanisms of netrin-1 action in the formation of the retinal projection in the frog xenopus laevis

Author
Torre, José Ramón de la

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Mechanisms of Netrin-1 Action in the Formation of the Retinal Projection in the Frog Xenopuslaevis

by

José Ramón de la Torre, III

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

in the

GRADUATE DIVISION

of the

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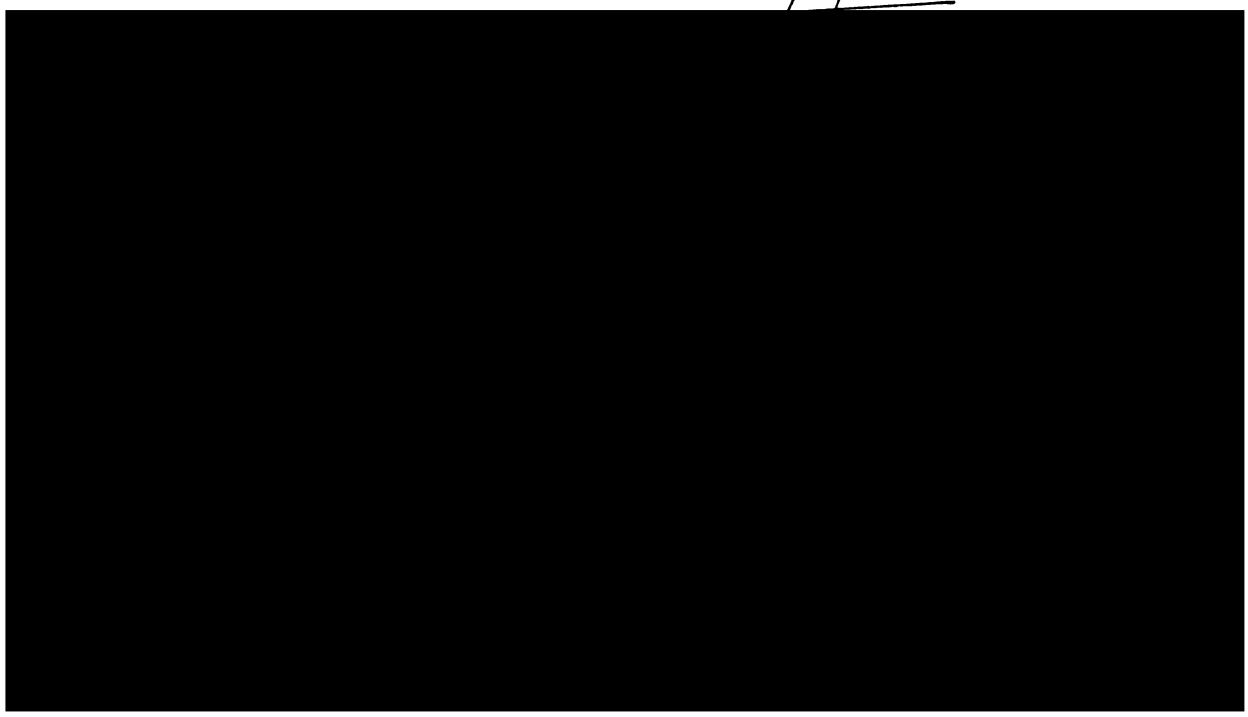
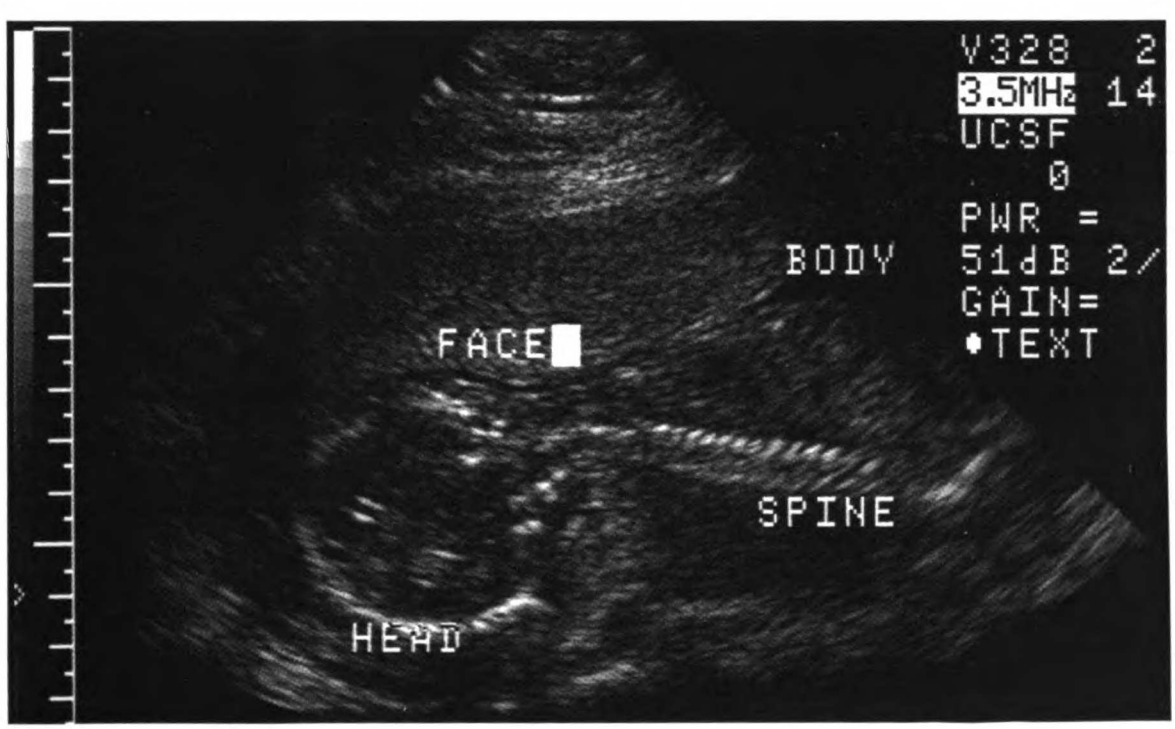


I dedicate this thesis

a mi pichoncita adorada,
bella sirena cuyo amor me guia por los mares,
y sin cual se undaria mi barca,

and to my family,
whose unconditional love and support
have made me who I am,
who pried open the entire world
to shape my life, my thoughts and my dreams,

et à l'être adoré(e)
qui pousse en ce moment dans le ventre de ma bien aimée,
et dont la présence remplira bientôt
mes jours et mon coeur de bonheur.



Tal vez no ser es ser sin que tú seas,
sin que vayas cortando el mediodía
como una flor azul, sin que camines
más tarde por la niebla y los ladrillos,

sin esa luz que llevas en la mano
que tal vez otros no verán dorada,
que tal vez nadie supo que crecía
como el origen rojo de la rosa,

sin que seas, en fin, sin que vinieras
brusca, incitante, a conocer mi vida,
ráfaga de rosal, trigo del viento,

y desde entonces soy porque tú eres,
y desde entonces eres, soy y somos
y por amor seré, serás, seremos.

Pablo Neruda, *Cien Sonetos de Amor.*

Acknowledgments

This thesis is the product of much more than seven and a half years of work -- it represents the synthesis of lessons learned over nearly three decades. It is equal parts science (air)', tears (water), blood (fire) and joy (earth?), thus encompassing all the elemental forces. It is also the result of my interaction with numerous friends, colleagues and collaborators without whom it could not have been done and to whom I much indebted.

I owe my love of science to my family. From an early age, they opened my eyes to the mysteries of the Earth, the night sky and the sea. I am forever grateful to my parents for their unquestioning love, for their courage in letting me find my own path through life while always supporting me, and for exposing me from the earliest of ages to the incredible diversity of our world. Likewise, I want to thank my sister Cristina, who, despite my efforts to open windows with her head, has always been a source of love and support for me. I am also eternally thankful to my grandfathers, Abuelo Tio, who took me to the Smithsonian every single weekend whenever we visited and who patiently waited while I memorized every last information in the Natural History and the Air & Space Museums, and Abuelo Pepe, who taught me all the botany I know.

Over the years, numerous people have guided me along the academic path toward this degree. Of the early years, one name stands out: Monsieur Couteau, a Lycée mathematics teacher whose sadistic tendencies, rationalized as a gallic form of "tough love", were the driving force behind the development of my critical thinking. No doubt he would credit himself for the completion of this Ph.D., and for that I thank him. Later role models were more compassionate. At U.C. Berkeley I had the good fortune to become associated with laboratory of Richard Harland, an event that changed the course of my life and continues to influence me to this day. Richard remains a close friend, an important role model and a template upon which I hope to pattern myself as a scientist and a person. Also

in the Harland lab I met my dear friend Ali Hemmati-Brivanlou. Ali is one of the few people I have met whose passions run as deeply and openly as my own. His generosity is legendary and his willingness to sacrifice everything for those he cares about have made him like a brother to me. In a very real sense, I owe this thesis to his kindness.

I would also like to thank my thesis advisor, Marc Tessier-Lavigne. Marc and I moved to UCSF at almost the same time and it has been interesting to watch us both grow within this close-knit community. I thank Marc for his continued enthusiasm and advice, for his generosity, and for his willingness to support scientific endeavors that carried me from one end of the country to another. I also thank him for bringing together an incredible group of colleagues whose insights and ideas were critical to my development as a scientist. These colleagues, many of whom became dear friends, provided a challenging forum for discussions ranging from the scientific to the political. In particular, I will most miss the core nucleus of coffee-drinking, salsa-listening philosophers: Dave "Sunshine" Leonardo, graduate student extraordinaire; Michael "Shi-Shi" Galko, fellow adventurer and punmaster; Christine "Too-Much" Mirzayan, whose passionate nature I expect to be subverted toward leading conservative think tanks in Washington; Katja Brose, whose training is finally complete; Bob "Ouch" O'Connor, a man with a serious grudge against mountains, hills and rocks, and a liability in any outdoor activity; Philipus Leigtonensis, a charming fellow despite his transient association to the Cardinal; and finally Fan, who although sorely missing is not in the least forgotten. In addition, I thank the other members of the Tessier-Lavigne lab, past and present, particularly Hao Wang, Lindsay Hinck, Allen Ebens and Tim Kennedy, for interesting discussions, insightful comments and fun times. Most importantly, I thank them all for putting up with my dubious brand of humor, my repetitive story-telling and my horrendous singing.

I would also like to thank my many collaborators, particularly those in New York and San Diego. I will never forget the nights spent at the Rockefeller University Faculty Club with the Hemmati-Brivanlou lab debating philosophy, politics and (occasionally)

science. In particular, I am grateful to Paul Wilson, Dan Weinstein, Anne Wilson, Pamela Hoodless, Eric "Lizard King" Honoré, Greg Cox, Giorgio Lagna, Akiko Hata, Chenbei Chang, Gerry Thomsen and Peg Bolce for making me feel at home so far from San Francisco. Likewise, I thank the members of the laboratories of Christine Holt and Bill Harris at UCSD for being such gracious hosts, particularly Elsa Cornel-Maliwat, Wes Chang and Veit Höpker.

In addition, I wish to thank my many friends here at UCSF and in San Francisco who have provided an extensive and entertaining network and made the past seven plus years enjoyable. Most of all, I am grateful for the friendship of Bill Wells, Kurt Zingler, Pratima Raghunathan, Tanya Awabdy, Geoff Parsons, Nicole Valtz, Abby Dernburg and Jeremy Minshull. Life would have been very dull without them. Likewise, I continue to be amazed at the fact that my childhood friends Marc Laurent and Glenn Berry are still here with me after over twenty five years, and I thank them for their love.

Finally, none of this would have been possible without the love and support of my wife, Lynn Connolly. Lynn and I came to UCSF together and we will leave it together as well. Throughout my tenure here, Lynn has been the single most important person to me, providing balance and perspective, as well as love and companionship. Lynn is my closest friend, my soul-mate, and it seems marvelously fitting that as I finish this dissertation, we also celebrate our marriage and the arrival of our first child. For all these years of patience, love and support I thank her.

Statement Regarding Previously Published Material with Multiple Authors

Chapters Two and Three have previously been published as:

> Kennedy, T. E., Serafini, T., de la Torre, J. R., and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. Cell *78*, 425-35.

and

> de la Torre, J. R., Höpker, V. H., Ming, G.-l., Poo, M.-m., Tessier-Lavigne, M., Hemmati-Brivanlou, A., and Holt, C. E. (1997). Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC. Neuron *19*, 1211-1224.

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My contribution to Chapter Two was the temporal and spatial analysis of *netrin-1* and *netrin-2* expression in the chick embryo, presented in Figures 2-1 and 2-2. This work was initiated by me following the identification of cDNA clones for *netrin-1* and *netrin-2* by Dr. Tito Serafini, Dr. Tim Kennedy and Christine Mirzayan in the laboratory. The majority of this work was done by me, although I was assisted by Dr. Kennedy, a postdoctoral fellow in our laboratory, in the weeks prior to submission of the manuscript.

The work described in Chapter Three was initiated as a collaboration between our laboratory and the laboratories of Dr. Ali Hemmati-Brivanlou, Dr. Christine E. Holt and Dr. Mu-ming Poo. I was responsible for initiating this project, and carried out most of the work myself on location at The Rockefeller University and the University of California, San Diego (UCSD). At UCSD, I was joined in the project by Dr. Veit H. Höpker, a postdoctoral fellow in the laboratory of Dr. Holt. Dr. Höpker was responsible for teaching me the techniques for the dissociated retinal cultures, and carried out the turning assays described in Figure 3-7 and Table 3-3.

Finally, the work presented in Chapter Four was also initiated by me in collaboration with the laboratory of Dr. Holt at UCSD. For this study, I was assisted by Elsa Cornel-Maliwat, a research assistant in the laboratory of Dr. Holt.

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JOSÉ R. DE LA TORRE, III
PROGRAM IN DEVELOPMENTAL BIOLOGY
DEPARTMENT OF ANATOMY
SAN FRANCISCO, CALIFORNIA 94143-0452

TELEPHONE: (415) 502-6508
FAX: (415) 476-3493
E-MAIL: jose@cgl.ucsf.edu

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PROGRAM IN DEVELOPMENTAL BIOLOGY
DEPARTMENT OF ANATOMY
SAN FRANCISCO, CALIFORNIA 94143-0452

TELEPHONE: (415) 476-3187
FAX: (415) 502-3927
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Mechanisms of Netrin-1 Action in the Formation of the Retinal Projection in the Frog *Xenopus laevis*

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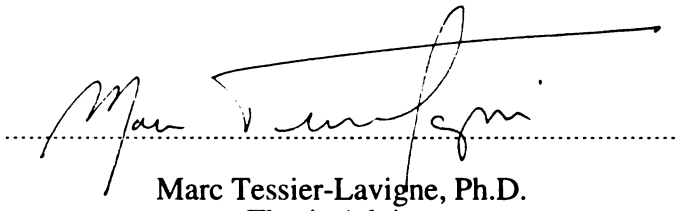

Marc Tessier-Lavigne, Ph.D.
Thesis Advisor
Chairman, Thesis Committee

Abstract:

A crucial step in the development of the nervous system is the establishment of appropriate connections between neurons and their targets. Floor plate cells at the ventral midline of the developing spinal cord have been shown to secrete a diffusible factor or factors capable of promoting and orienting the growth of spinal commissural axons *in vitro*. Two novel proteins, netrin-1 and netrin-2, isolated from embryonic chick brain are capable of mimicking the activities of the floor plate on commissural axons *in vitro*. We show that both *netrin-1* and *netrin-2* are expressed in the developing spinal cord during the period of commissural axon migration, *netrin-1* specifically in the floor plate. Furthermore, heterologous cells expressing recombinant netrin-1 or netrin-2 are capable of attracting commissural axons at a distance and causing re-orientation of commissural growth cones within dorsal spinal cord explants. These results suggest that the netrins, and netrin-1 in particular, may play a role in guiding commissural axons to the floor plate *in vivo*, a result supported by the analysis of netrin-1-deficient mice.

Recent experiments have shown that netrin-1 promotes the outgrowth of spinal commissural axons and retinal ganglion cell axons *in vitro* through a receptor mechanism involving the Deleted in Colorectal Cancer (DCC) protein. It was not known, however, whether netrin-1 alone could elicit growth cone turning or whether additional factors provided by the tissue explant are required. Further, it was not known whether the DCC receptor is involved in mediating the turning effect, as opposed to the outgrowth-promoting effect, of netrin-1. We show here that *Xenopus* retinal ganglion cell (RGC) growth cones

on a glass substrate orient rapidly towards a point source of netrin-1 in a cell free environment *in vitro*, an effect that is blocked by antibodies to DCC. Netrin-1-treated growth cones elaborate many long filopodia *in vitro* reminiscent of the complex growth cone morphology found at the optic nerve head, a site of netrin-1-rich expression and growth cone turning *in vivo*. These results demonstrate that netrin-1 can function alone to induce attractive turning and implicate DCC in mediating this response, and are consistent with a role for netrin-1 in guiding RGC axons at the optic nerve head *in vivo*.

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Chapter One

General Introduction

One of the key events in the evolution of animals was the formation of a nervous system. Fundamentally basic behaviors such as locomotion, reproduction, learning and feeding (as well as avoiding being eaten by others) rely on an animal's ability to perceive the world around it and respond accordingly. All developing nervous systems, whether as simple as the nerve net of cnidarians such as *Hydra*, or as complex as the more than 10^{12} nerve cells of the human brain, are therefore faced with a similar challenge: how to ensure the precise and reproducible establishment of the connections between each nerve cell (neuron) and its synaptic targets that are required for the proper functioning of the system. Studies on the formation of the nervous system in a variety of species have shown that this process occurs with remarkable precision and very few mistakes. How does the organism regulate the establishment of these connections? What cellular mechanisms and molecules are responsible for instructing developing neurons where to send their connections? In this thesis, I describe experiments that have begun addressing these issues. In particular, I will focus on a recently discovered family of proteins, the netrins, which appear to play an evolutionarily-conserved role in directing the trajectories of neuronal connections. I will describe experiments designed to elucidate the molecular mechanism of netrin action, and attempt to understand the role played by a member of the netrin family, netrin-1, in the formation of the visual system in the frog *Xenopus laevis*.

Basic Mechanisms of Axon Guidance

The formation of a nervous system can be divided into four separate stages. First, individual neurons form a growth cone which extends toward the target while remaining connected to the cell body by a thin cellular process known as an axon. These growth cones must then navigate the embryonic environment, sensing cues that guide them to their targets. Upon reaching the target, growth cones cease to migrate and form a synapse. In the final stages, these synapses are tested by the developing nervous system; appropriate connections are strengthened, while inappropriate ones are removed.

Several factors cooperate to facilitate the proper formation of the nervous system. First, developing neurons extend axons in successive waves during embryonic development. These first "pioneer" axons navigate smaller distances through a simpler environment to reach their targets, forming an initial network of axon tracts. Subsequent waves of axons use these tracts as a framework upon which to build additional complexity, emphasizing the importance that initial axons navigate correctly. Second, individual axons appear to simplify their task by breaking the migration to their final targets into a series of short trajectories (several hundred microns) between intermediate targets. The problem of axon guidance thus becomes a question of accurate detection of sequential guidance cues over short distances. Experimental evidence indicates that guidance cues can be separated into four basic categories based on their effect on navigating axons (reviewed in Tessier-Lavigne and Goodman, 1996): short-range and long-range mechanisms, each of which may act on the axon in an attractive or repellent manner.

Short-range non-diffusible guidance cues. Short-range cues are thought to be mediated by contact between the extending axons and sources of non-diffusible cell surface or extracellular matrix-bound (ECM) molecules. Contact repulsion has been described in the vertebrate central and peripheral nervous systems. For instance, axons from the nasal retina express surface-bound molecules that induce the collapse of temporal retinal growth cones (Raper and Grunewald, 1990). Likewise, the cell adhesion molecule (CAM) T-cadherin has been implicated in the avoidance of the caudal sclerotome by growing motor axons (Fredette et al., 1996). Instances of contact attraction can also be mediated by CAMs. In *Drosophila*, the CAM Fasciclin II (Fas II) is expressed by a variety of CNS axons in three longitudinal tracts (Grenningloh et al., 1991). Loss of Fas II function leads to pathfinding errors by these axons due to a failure to properly fasciculate. Conversely, increased levels of Fas II on these axons can cause the fusion of the three tracts (Lin et al., 1994). Likewise, experiments in chicken embryos have implicated CAMs such as L1 in the retina (Brittis and Silver, 1995) and axonin-1, Nr-CAM and Ng-CAM in the spinal cord

(Stoeckli and Landmesser, 1995), in the contact-mediated attraction of growing axons. Experimental evidence suggests that, *in vivo*, axons are often subjected to the coordinated action of both attractive and inhibitory cues. Thus, short-range cues may guide axons by simultaneously providing a positive or favorable substrate for growth (contact attraction), while inhibiting extension in inappropriate directions (contact repulsion) (discussed in Tessier-Lavigne and Goodman, 1996).

Long-range diffusible guidance cues. In addition to these substrate-bound non-diffusible cues, experiments have also provided evidence for the existence spatially graded distributions of molecules that can guide developing axons at a distance. Initially proposed by Ramón y Cajal (1892), this hypothesis was based on the guidance of motile non-neuronal cells in response to gradients of diffusible molecules (for review, see Devreotes and Zigmond, 1988). However, it is only within the last two decades, using *in vitro* co-culture assays to confront growing axons with potential sources of diffusible guidance cues, that direct evidence for the existence of such cues has become available. For instance, sensory axons of the mouse trigeminal ganglion appear to be attracted to their targets in the periphery by target-secreted diffusible attractants (Lumsden and Davies, 1983; Lumsden and Davies, 1986). Likewise, *in vitro* co-culture experiments showed that first spinal commissural axons, then commissural axons from all rostro-caudal levels, are attracted to an intermediate target, the floor plate, by a diffusible chemoattractant (Tessier-Lavigne et al., 1988; Placzek et al., 1990a; Shirasaki et al., 1996). Experiments also showed that these diffusible attractants need not necessarily act on the growth cone: a diffusible activity produced by the basilar pons can induce and attract collateral branches from corticospinal axons that had already grown past this target (Heffner et al., 1990). Moreover, these diffusible guidance cues are not limited to being attractive. The ventral spinal cord secretes an activity that repels sympathetic axons *in vitro* (Ebendal and Jacobson, 1977). Likewise, chemorepellent activities have been identified in the floor plate for certain populations of cranial motor neurons (Colamarino and Tessier-Lavigne, 1995;

Guthrie and Pini, 1995; Varela-Echavarria et al., 1997), and in the septum for developing olfactory bulb interneurons (Pini, 1993).

Finally, it is interesting to note that, as was described above for short-range cues, there is increasing evidence that both attractive and repulsive diffusible guidance cues act coordinately to regulate the direction of axon growth. As an example, *in vitro* co-culture experiments suggest that trochlear motor axons, which grow and exit the spinal cord dorsally partly in response to a chemorepellent in the floor plate (Colamarino and Tessier-Lavigne, 1995), may also be attracted to an activity expressed in the roof plate of the spinal cord (Colamarino, 1996). Furthermore, preliminary evidence suggests that the roof plate may also produce a chemorepellent for spinal commissural axons which may assist in driving their migration ventrally toward the floor plate (J. Dodd, personal communication). Thus, axon trajectories *in vivo* appear to reflect the coordinated actions of opposing forces.

Candidates for Diffusible Guidance Cues

In spite of the growing evidence in favor of diffusible guidance cues, only a handful of molecular candidates have been identified to date. Two new families of proteins, the semaphorins and the netrins, account for several of the chemotropic responses characterized at this time (Tessier-Lavigne and Goodman, 1996). Although *in vitro* experiments have shown that developing axons respond chemotactically to gradients of neurotrophins and neurotransmitters (Gundersen and Barrett, 1979; Lohof et al., 1992; Zheng et al., 1994; Song et al., 1997), the functional significance of these responses in the context of the developing embryo remains unclear. Recent experiments have also implicated hepatocyte growth factor/scatter factor (HGF/SF) as a potential chemotropic factor for developing vertebrate motor axons (Ebens et al., 1996). One final important point is the striking degree of evolutionary conservation encountered in these guidance mechanisms. Indeed, many of the lessons learned using invertebrates are applicable directly to axon guidance questions in vertebrates, and vice versa. Therefore, in presenting

the evidence in favor of these molecules as chemotropic factors, I will not make distinctions between species.

Neurotrophins and neurotransmitters. Nearly two decades ago, Gundersen and Barrett (1979) identified nerve growth factor (NGF) as the first molecule capable of eliciting a chemotropic response from sensory axons *in vitro*. Similar experiments involving the response of *Xenopus* spinal neurites to artificial gradients *in vitro* revealed the chemotactic activities of a number of other neurotrophins and neurotransmitters (e.g. BDNF, NT-3, acetylcholine, glutamate) (Lohof et al., 1992; Zheng et al., 1994; Song et al., 1997). *In vivo*, neurotrophins play an important role in branching and innervation at the target, as illustrated by the failure of sympathetic axons to innervate the pineal gland and external ear in NT-3-deficient mice (El Shamy et al., 1996). However, neither neurotrophins nor neurotransmitters appear to be involved in the long-range guidance of axons to their targets (discussed in Hoyle et al., 1993).

HGF/SF. Evidence has been provided for the involvement of chemotropic factors in the guidance of spinal motor axons to their peripheral targets (reviewed in Tosney, 1991; Eisen, 1994). Recent co-culture experiments have shown that the developing limb bud mesenchyme produces an activity capable of attracting growing motor axons at a distance *in vitro* (Ebens et al., 1996). HGF/SF, a motogen for epithelial cells (Stoker et al., 1987), has been implicated in this limb bud-derived chemotropic activity *in vitro*, although mice deficient in either HGF/SF or its receptor c-Met show only mild defects of limb motor axon guidance (Ebens et al., 1996).

Semaphorins. The semaphorins are a large phylogenetically-conserved family of diffusible and transmembrane proteins primarily associated with inhibition or repulsion of axon growth (Tessier-Lavigne and Goodman, 1996). Cell surface (transmembrane) semaphorins have been implicated in contact repulsion in insects (Kolodkin et al., 1992; Matthes et al., 1995). Diffusible members of the semaphorin family are effective chemorepellents for a variety of axons (Dodd and Schuchardt, 1995). Nevertheless, these

molecules exhibit a high degree of specificity in their inhibitory effects. For instance, Semaphorin III (Sema III) selectively repels NGF-responsive sensory axons *in vitro*, but not NT-3-responsive axons. Consequently, Sema III may play a role in establishing the patterns of sensory axon innervation of the spinal cord *in vivo* (Messersmith et al., 1995). In addition, Sema III is a potent inducer of sensory growth cone collapse *in vitro* (Luo et al., 1993), suggesting that its chemorepulsive activity may reflect a direct destabilizing action on the growth cone. Recently, a new family of molecules, characterized by the transmembrane protein neuropilin-1, was identified as components of the semaphorin receptor. The observation that neuropilin-1 can mediate both the growth cone collapse and chemorepellent activities of Sema III (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997) suggests that growth cone collapse and repulsion may be mechanistically linked.

Netrins. The netrins are diffusible proteins identified in vertebrates on the basis of their ability to stimulate outgrowth and reorient growth of spinal commissural axons *in vitro* (Kennedy et al., 1994; Serafini et al., 1994). Molecular analysis indicates that the netrins form an evolutionarily-conserved family of laminin-related proteins, including the *Caenorhabditis elegans* UNC-6 gene (Ishii et al., 1992) and homologues in *Drosophila* (Harris et al., 1996; Mitchell et al., 1996). In vertebrates, worms and flies, the netrins have been implicated in directing circumferential axon migrations, both as chemoattractants and chemorepellents, toward and away from the ventral midline of the CNS (see Culotti and Kolodkin, 1996). Recent studies have also implicated these molecules in axonal migrations in regions other than the midline (Deiner et al., 1997; Métin et al., 1997; Richards et al., 1997). Two families of transmembrane proteins have been shown to act as components of the netrin receptor machinery. These include homologues of the *C. elegans* genes UNC-40 and UNC-5 (reviewed in Guthrie, 1997; Kolodziej, 1997). In vertebrates, the UNC-40 homologue Deleted in Colorectal Cancer (DCC), is required for the response of commissural and retinal axons to the outgrowth-promoting effect of netrin-1 (Keino-Masu et al., 1996; Fazeli et al., 1997), and for the chemotactic response of retinal and

spinal neurites in gradients of pure netrin-1 protein *in vitro* (de la Torre et al., 1997; Ming et al., 1997), suggesting that the receptor mechanisms involved in the outgrowth and chemotropic responses may be the same.

Mechanism of Netrin-1 Action in the Vertebrate Nervous System

As described above, commissural neurons in the dorsal spinal cord extend axons circumferentially toward the ventral midline of the neural tube at least partly in response to netrin-1 secreted by floor plate cells (Tessier-Lavigne et al., 1988; Placzek et al., 1990a; Kennedy et al., 1994; Serafini et al., 1994; Shirasaki et al., 1995). In addition, netrin-1 has been implicated in the repulsion of a subset of cranial motor axons by the floor plate (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997). Similar attractive and repulsive activities have been described for netrin homologues in *C. elegans* and *Drosophila* (Hedgecock et al., 1990; Harris et al., 1996; Mitchell et al., 1996; Wadsworth et al., 1996). Furthermore, genetic and biochemical studies have suggested that these attractive and repulsive activities of the netrins may be mediated by separate domains of the netrin molecules and by different receptor mechanisms (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Wadsworth et al., 1996; Ackerman et al., 1997; Leonardo et al., 1997).

Recent studies in a variety of vertebrate species, including the analysis of axon trajectories in netrin-1-deficient mice, have also implicated netrins in numerous axon guidance events apart from the midline of the nervous system (Kennedy et al., 1994; Wang et al., 1994; Serafini et al., 1996; de la Torre et al., 1997; Deiner et al., 1997; Lauderdale et al., 1997; Livesey and Hunt, 1997; MacDonald et al., 1997; Métin et al., 1997; Richards et al., 1997; Strähle et al., 1997). However, in spite of the evidence that netrins, and particularly netrin-1, are required for axon guidance *in vivo*, the mechanism of netrin action on extending axons remains poorly understood. *In vitro*, commissural axons normally grow within spinal cord explants without invading the surrounding collagen matrix

(Tessier-Lavigne et al., 1988; Placzek et al., 1990a), suggesting that this environment is unfavorable for axon growth. The fact that these axons invade the collagen in the presence of netrins suggests that netrins have a "permissive" function that allows axons to overcome this inhibition (Serafini et al., 1994). Conversely, the fact that a point source of netrin-1 (such as an aggregate of heterologous cells expressing recombinant netrin protein) can reorient commissural axon growth within dorsal spinal cord explants (Kennedy et al., 1994) implies that the netrins also posses an "instructive" (chemotropic) activity that can guide axons at a distance. Recently, similar permissive and chemotropic activities have been demonstrated for netrin-1 on early cortical efferents *in vitro* (Métin et al., 1997; Richards et al., 1997), suggesting that these activities may be general properties of the netrins.

These observations raise several important questions concerning the role of netrin-1 *in vivo*. First, the chemotropic response of axons to netrin-1 has only been demonstrated within explants of neural tissue, raising the possibility that netrin-1 acts in coordination with accessory factors provided by the responding tissue to cause turning of axons. Second, studies into the receptor mechanisms involved in mediating the permissive and chemotropic responses of axons to netrin-1 have shown that the netrin-1 receptor DCC is required in commissural and retinal axons for the outgrowth-promoting activity of netrin-1 on those axons (Keino-Masu et al., 1996; de la Torre et al., 1997; Deiner et al., 1997). However, attempts to investigate the role of DCC in mediating the chemotropic response to netrin-1 using anti-DCC function blocking antibodies were inconclusive, perhaps due to poor penetration of the explants by the antibody (Keino-Masu et al., 1996).

In this thesis, I describe experiments designed to address these issues. In Chapter Two I present the initial characterization of the expression pattern and chemotropic activity of the first vertebrate netrins, chick netrin-1 and netrin-2. Beyond implicating the netrins in the guidance of spinal commissural axons, these studies, supplemented by the data presented in the Appendix, suggest that the netrins may be involved in a number of axon

guidance and cell migration events throughout the developing embryo. In Chapter Three, I focus on the role of netrin-1 and DCC in the guidance of retinal ganglion cell (RGC) axons in the frog *Xenopus laevis*, where we have studied the responses of individual retinal growth cones to netrin-1 protein in a simplified cell-free *in vitro* environment. Our finding that retinal neurites turn rapidly (within minutes) in a cell-free environment towards a source of purified netrin-1 protein demonstrates that these molecules can function as chemotropic cues acting directly on extending growth cones. Furthermore, these experiments show that the netrin receptor DCC is required for this chemotactic response, at least in this *in vitro* setting. Finally, in Chapter Four, I present preliminary evidence for two novel roles of netrin-1 in the guidance of developing axons. Again focusing on the development of the retino-tectal projection in *Xenopus*, we show that in the optic tract, netrin-1 appears to function as a substrate-bound, non-diffusible guidance cue for migrating retinal axons. Moreover, embryological manipulations that disrupt netrin-1 localization the optic tract suggest that netrin-1 may play a role in target recognition for retinal axons at the optic tectum. These studies raise many interesting questions regarding the functions of the netrins on developing axons, and may also help us gain insight into general principles that govern axon guidance in the embryo.

Chapter Two

Netrins are Diffusible Chemotropic Factors for Commissural Axons in the Embryonic Spinal Cord

Summary

The guidance of axons to their targets in the developing nervous system is believed to involve diffusible chemotropic factors secreted by target cells. Floor plate cells at the ventral midline of the spinal cord secrete a diffusible factor or factors that promotes the outgrowth of spinal commissural axons and attracts these axons *in vitro*. Two membrane-associated proteins isolated from embryonic brain, netrin-1 and netrin-2, possess commissural axon outgrowth-promoting activity. We show here that netrin-1 RNA is present in floor plate cells, whereas netrin-2 RNA is detected at lower levels in the ventral two thirds of the spinal cord, but not in the floor plate. Heterologous cells expressing recombinant netrin-1 or netrin-2 secrete diffusible forms of the proteins, and can mimic the long-range chemotropic activity of floor plate cells, reorienting commissural axons within the neural epithelium. These results show that netrin-1 is a chemotropic factor expressed by floor plate cells, and suggest that the two netrin proteins guide commissural axons in the developing spinal cord.

Introduction

The establishment of neuronal connections during embryogenesis involves the accurate guidance of axons to their targets. The possibility that axonal growth is oriented by graded spatial distributions of guidance molecules has long been entertained, in part due to evidence for the guidance of non-neural motile cells by both haptotactic mechanisms (which involve responses to gradients of substrate-bound factors) and chemotactic mechanisms (involving responses to gradients of soluble factors). The idea that axons might be guided by chemotropism, i.e., by gradients of factors diffusing from target cells, was first proposed by Ramón y Cajal over one hundred years ago (Ramón y Cajal, 1892). Experiments *in vitro*, in which developing axons were confronted with target tissues and were attracted by these tissues, have provided direct evidence for the existence of chemotropic factors secreted by intermediate or final targets in three different systems: the guidance of trigeminal sensory axons to their peripheral epithelial targets (Lumsden and Davies, 1983), of spinal commissural axons to the floor plate of the spinal cord (Tessier-Lavigne et al., 1988), and of cortical projection axons to subcortical targets (Heffner et al., 1990). Other experiments have provided evidence for the operation of chemotropic mechanisms in several other systems, including the projections of motor axons to various embryonic targets (reviewed in Tessier-Lavigne, 1992).

The chemotropic effect of floor plate cells on circumferentially-projecting spinal commissural axons was demonstrated in experiments showing that floor plate cells can promote the outgrowth of commissural axons into collagen gels and reorient these axons within explants of developing neural epithelium (Tessier-Lavigne et al., 1988; Placzek et al., 1990a). In the preceding paper, we described the isolation from embryonic brain of two structurally-related proteins, the netrins, that possess commissural axon outgrowth-promoting activity (Serafini et al., 1994). The netrins are homologues of UNC-6, a

protein involved in guiding circumferential axonal migrations in *Caenorhabditis elegans*(Hedgecock et al., 1990; Ishii et al., 1992).

This homology, together with the biological activity of the netrins, raised the possibility that, just as UNC-6 is involved in directing circumferential migrations in the nematode, so too the netrins might play a role in directing circumferential axonal migrations in the developing spinal cord, perhaps by mediating some of the long-range actions of floor plate cells on these axons. To examine these possibilities, however, it was necessary to address three issues. First, the netrins were purified from embryonic brain; are they also expressed in the spinal cord and, specifically, is either expressed by floor plate cells? Second, the netrins were purified as cell-associated proteins, yet floor plate cells secrete a diffusible outgrowth-promoting activity. Does either netrin exist in a diffusible form that would be a candidate for mediating the long-range outgrowth activity of floor plate cells? Third, the netrins were purified on the basis of their ability to promote commissural axon outgrowth. However, floor plate cells secrete not only an outgrowth activity but also a diffusible chemotropic activity that can reorient the growth of commissural axons. Do the netrins possess chemotropic activity in addition to their outgrowth activity?

To answer these questions, we first examined whether the netrins are expressed in the floor plate at the time of commissural axon extension towards the ventral midline. We show that both netrin-1 and netrin-2 transcripts are expressed in the developing spinal cord. Netrin-1 RNA is expressed selectively by floor plate cells at the time that commissural axons are growing to the floor plate, whereas netrin-2 RNA is expressed at lower levels in a restricted domain in the ventral two thirds of the spinal cord, but is excluded from the floor plate. Second, we examined whether the netrins can exist in diffusible form. COS cells expressing netrin genes secrete diffusible forms of the netrin proteins that can act as long-range outgrowth-promoting molecules. Finally, COS cells secreting diffusible recombinant netrins reorient commissural axons at a distance *in vitro*,

mimicking the chemotropic effect of the floor plate. These results implicate netrin-1 in mediating both the long-range outgrowth-promoting and the long-range chemotropic activities of floor plate cells, and suggest that the two netrins may guide commissural axons to the ventral midline of the spinal cord.

Results

Netrin-1 and *Netrin-2* Transcripts Are Expressed in the Developing Spinal Cord

Northern analysis was used to examine whether the netrin genes are expressed in the embryonic spinal cord. Expression of both genes in the spinal cord and brain was observed from embryonic day 6 (E6), the earliest stage at which these tissues were separately dissected, through to the adult, although the levels of expression varied with age (Figure 2-1). A single abundant *netrin-1* transcript of 4.9 kb, and three major *netrin-2* transcripts of 5.4, 3.6 and 3.2 kb were detected. In addition, expression of both genes was detected at various stages in several mesodermally- and endodermally-derived tissues (Figure 2-1), including heart, lung and ovary in the case of *netrin-1*, and heart, lung, gut, ovary, testes and spleen in the case of *netrin-2* (see also Figure 2-1 legend). Both *netrin-1* and *netrin-2* transcripts were also detected in RNA derived from whole embryos at Stage 15 (Hamburger and Hamilton, 1951) (E2.5, Figure 2-1), when commissural axons are beginning to extend towards the floor plate (Holley, 1982; Yaginuma et al., 1990).

To determine whether *netrin-1* RNA is expressed in floor plate during the period of commissural axon growth to the ventral midline, RNA *in situ* hybridization was performed on chick embryos at stages 10-11 (prior to commissural axon growth), stages 15-16 (at the onset of commissural axon growth), and stage 31 (after commissural axons have crossed the floor plate and initiated their projection to other axial levels). *Netrin-1* RNA was not detected in the neural plate prior to neural tube closure, but it was detected in the notochord (Figures 2-2A and 2-2B). After neural tube closure, however, *netrin-1* RNA was detected in the floor plate (Figure 2-2C). Throughout the period of commissural axon growth, *netrin-1* RNA continues to be expressed at high levels selectively by floor plate cells (Figures 2-2D - G). Since netrin-1 promotes commissural axon outgrowth (Serafini et al., 1994) this suggests that netrin-1 contributes to the commissural axon outgrowth-promoting activity of floor plate cells in the spinal cord.

However, floor plate cells are not restricted to the spinal cord and are also found more rostrally in the CNS in the hindbrain, midbrain and caudal diencephalon (Kingsbury, 1920; Puelles et al., 1987). Moreover, commissural axon outgrowth-promoting activity has been detected in ventral midline tissue taken from these axial levels (Placzek et al., 1990a; Placzek et al., 1993). Whole-mount RNA *in situ* hybridization revealed that, at stage 15, *netrin-1* RNA is expressed at high levels in the ventral midline of the CNS from the spinal cord into the caudal diencephalon (Figure 2-2E). In the spinal cord, hindbrain and midbrain, this expression is restricted to the floor plate. In the diencephalon, *netrin-1* RNA expression appears to broaden into the basal plate. Thus, netrin-1 may contribute to the commissural axon outgrowth-promoting activity observed in floor plate at all axial levels. *Netrin-1* RNA was also detected at this stage in the developing optic cup and optic stalk, and in thin stripes in the hindbrain that may correspond to rhombomere boundaries (Figure 2-2E and data not shown).

Netrin-2 RNA was also detected in the spinal cord before and throughout the period of commissural axon growth, but at much lower levels than *netrin-1* RNA (Figure 2-2H and 2-2I, and data not shown). Moreover, in contrast to the highly restricted spatial expression of *netrin-1* RNA within the spinal cord at all stages examined, at stage 15 *netrin-2* RNA was detected in a broad band throughout the ventral two-thirds of the spinal cord excepting the floor plate region (Figure 2-2H). Examination of neighboring sections from the same embryo suggested that the *netrin-2* RNA expression domain abuts the *netrin-1* RNA expression domain at stage 15 (data not shown). Between stage 15 and stage 31, *netrin-2* RNA expression remains in a band at apparently the same dorso-ventral level, but becomes progressively restricted to the ventricular zone (Figure 2-2I). The two netrin genes are also expressed in several non-neural structures at these early stages, including the notochord, paraxial mesoderm and branchial arches in the case of *netrin-1*, and the paraxial mesoderm and gastro-intestinal tract in the case of *netrin-2* (Figure 2-2 and data not shown; see Appendix and Figure 6-3).

Transfected COS Cells Secrete Diffusible Netrins

The expression of *netrin-1* RNA by floor plate cells suggested that netrin-1 contributes to the commissural axon outgrowth-promoting activity of the floor plate. However, the netrins were identified as cell-associated proteins (Serafini et al., 1994), yet floor plate cells express both cell-associated and diffusible forms of outgrowth activity, raising the question whether diffusible forms of the netrins also exist. We therefore examined whether COS cells transfected with a tagged netrin-1 or netrin-2 expression construct secreted diffusible forms of the proteins. Salt extracts of transfected COS cells contained recombinant netrins, as assessed by outgrowth activity (Serafini et al., 1994), and by immunoblotting (Figure 2-3, fourth and sixth lanes). Recombinant netrins were also detected by immunoblotting as soluble proteins in conditioned medium from the transfected cells (Figure 2-3, third and fifth lanes). The amount of soluble netrin-1 was at least 20% of the amount of salt extractable netrin-1, as assessed in dilution experiments (data not shown), whereas less soluble netrin-2 was found in the conditioned medium (~10% of the salt extractable netrin-2; data not shown). For each netrin, most of the immunoreactive species in the supernatant or the extract had a molecular weight comparable to that of the protein purified from brain (Serafini et al., 1994). However, minor species, which may have arisen by differential post-translational modification or proteolysis, were also observed (Figure 2-3).

The soluble recombinant netrins in conditioned medium promoted axon outgrowth from E13 rat dorsal spinal cord explants, although the total amount of activity present in the conditioned medium was less than that in the salt extract (data not shown), consistent with the lower amount of recombinant netrin protein observed in the medium compared to the extract. The outgrowth activity of the diffusible forms of the netrins was also detected by presenting aggregates of transfected COS cells directly to E11 rat dorsal spinal cord explants in collagen gels (Figure 2-4). Aggregates of transfected COS cells

expressing netrin-1 promoted the outgrowth of axons from dorsal spinal cord explants (10/10 explants) in the same manner as floor plate (Figures 2-4A and 2-4C). Control COS cells, transfected with vector alone, did not evoke outgrowth (6/6 explants, Figure 2-4B). Transfected COS cells expressing netrin-2 likewise evoked axon outgrowth (12/17 explants tested), albeit less than floor plate or COS cells expressing netrin-1 (Figure 2-4D). This weaker outgrowth in response to netrin-2-expressing cells may be explained by the fact that COS cells appear to secrete less netrin-2 than netrin-1 in diffusible form (see above). Netrin Synergizing Activity (NSA), a factor that potentiates the outgrowth activity of salt-extracted netrins (Serafini et al., 1994), also potentiated the outgrowth activity of the diffusible netrins (T. S., M.J. Galko, and M. T.-L., data not shown).

COS Cells Secreting Recombinant Netrins Mimic the Chemotropic Effect of Floor Plate Cells

Taken together, the patterns of expression of the netrins and their ability to function as diffusible outgrowth-promoting factors suggest that netrin-1 contributes to the commissural axon outgrowth-promoting activity of floor plate cells. However, floor plate cells also secrete a chemotropic activity, which can be revealed by the reorientation of commissural axons toward a floor plate explant *in vitro* (Tessier-Lavigne et al., 1988; Placzek et al., 1990a). This raises the question whether the netrins can also function as chemotropic factors.

Chemotropic activity can be demonstrated by culturing a floor plate explant adjacent to a dorsal spinal cord explant, parallel to the original dorso-ventral axis of the spinal cord as diagrammed in Figure 2-5A (Placzek et al., 1990a). In the absence of floor plate, commissural axons, visualized with an antibody to TAG-1 (Dodd et al., 1988), grow within the neural epithelium along a stereotyped dorso-ventral trajectory similar to the one they take *in vivo* (Figure 2-5C; see also Tessier-Lavigne et al., 1988; Placzek et

al., 1990a). In the presence of floor plate, the axons were deflected from this trajectory and turned towards the floor plate over a distance of 330 ± 40 μm (Figure 2-4B; see also Placzek et al., 1990a). To test the netrins for chemotropic activity, we examined whether aggregates of COS cells secreting recombinant netrins could also deflect commissural axons when positioned adjacent to a piece of dorsal spinal cord. Aggregates of transfected COS cells secreting netrin-1 deflected axons from their dorso-ventral trajectory over a distance of 260 ± 50 μm (Figure 2-5D and Table 2-1), slightly shorter than that observed with floor plate. Transfected COS cells secreting netrin-2 also deflected commissural axons (Figure 2-5E). However, the turning occurred on average over an even shorter distance (160 ± 50 μm; Table 2-1), consistent with the lower levels of outgrowth evoked by COS cells secreting netrin-2 (Figure 2-4D). Control aggregates of COS cells transfected with vector alone had no effect on commissural axon trajectories in these experiments (Figure 2-5C and Table 2-1). These results indicate that the netrins function as chemotropic factors for commissural axons, although we cannot exclude the formal possibility that the netrins induced expression of a distinct chemotropic factor by COS cells, or that additional factors may be involved.

Commissural axons that grew to the COS cells secreting either netrin-1 or netrin-2 were routinely observed to invade the COS cell aggregates (Figures 2-5D and 2-5E, and Table 2-1), whereas control aggregates of COS cells transfected with vector alone were not invaded by nearby commissural axons (Figure 2-5C and Table 2-1). Commissural axons that grew to floor plate explants remained close to the floor plate cells at the ventral-most edge of the explant, rather than invading the non-floor plate ventral spinal cord tissue in these explants (Figure 2-5B). One possible explanation for this observation is that additional mechanisms exist in the spinal cord that confine commissural axons to the floor plate region once they have reached it.

Discussion

The guidance of commissural axons to the ventral midline of the spinal cord has been proposed to be directed partly by a chemotropic factor secreted by floor plate cells. The netrins were purified on the basis of their ability to elicit commissural axon outgrowth (Serafini et al., 1994), an activity previously only described for a secreted factor from floor plate cells. We report here that during the period of commissural axon growth to the ventral midline, *netrin-1* is expressed by floor plate cells, and *netrin-2* is expressed at much lower levels in an adjacent domain in the ventral two-thirds of the spinal cord. Moreover, recombinant netrin-1 and netrin-2 expressed by COS cells can exist as diffusible proteins which possess both outgrowth-promoting and chemotropic activity for commissural axons. These findings strongly implicate netrin-1 in mediating the long-range actions of floor plate cells on commissural axons, and suggest that netrin-2 also contributes to the guidance of commissural axons.

Active Netrins Exist in Diffusible Form

The floor plate can act at a distance to promote commissural axon outgrowth (Tessier-Lavigne et al., 1988; Placzek et al., 1990a), indicating the existence of a diffusible activity, yet the outgrowth activity in floor plate homogenates is associated with the membrane fraction and is salt extractable (Serafini et al., 1994). Strikingly, recombinant netrins expressed in COS cells have the properties of the outgrowth activity in floor plate. The two recombinant proteins associate with COS cell surfaces but can be solubilized at high salt concentrations. In addition, a fraction of the netrins secreted by COS cells diffuse into the surrounding medium. As with the activity in floor plate, both cell-associated and diffusible recombinant netrins promote axon outgrowth from dorsal spinal cord explants. These observations raise the possibility that the range of diffusion of the netrins may be limited in the embryo by their avid binding to cell surface or ECM

components. It is also possible that netrin diffusibility is subject to regulation, for example by post-translational modification of the proteins or by modification of the substrates that bind netrins.

The Netrins Function as Chemotropic Factors

Since commissural axons turn toward a spatially-restricted source of diffusible netrin-1 or netrin-2, we infer that the netrins diffuse through the neural epithelium, forming gradients to which the axons respond with directed growth. However, it is not known how far the predicted gradients extend nor how steep they are. Interactions of the netrins with cell surfaces or the ECM could conceivably contribute to stabilizing and sharpening a gradient of netrins. It also remains to be determined whether netrin-1 and netrin-2 diffuse to similar extents. Although both proteins have similar specific activities when added in soluble form to spinal cord explants (Serafini et al., 1994), COS cells secreting netrin-2 are less potent sources of long-range outgrowth-promoting and chemotropic activity than those secreting netrin-1 (Figures 2-4 and 2-5), presumably because less netrin-2 diffuses away from the COS cells (Figure 2-3). This raises the possibility that netrin-2 acts more locally than netrin-1, in the vicinity of the neuroepithelial cells that produce it.

Roles of the Netrins in Commissural Axon Guidance

Our studies are consistent with netrin-1 functioning as a floor plate-derived chemotropic factor that contributes to guiding commissural axons to the floor plate, an intermediate target for these axons. *Netrin-1* transcripts are detected in floor plate cells from the earliest stages of floor plate development, well before the initiation of commissural axon growth around stage 15. Although we have not monitored production of netrin-1 protein by floor plate cells, it is reasonable to assume that it is secreted at these early stages. The observation that a restricted source of recombinant netrin-1 is almost as effective as the floor plate in reorienting commissural axons *in vitro* supports the hypothesis that netrin-1

produced by floor plate cells *in vivo* diffuses through the neural epithelium to reorient the growth of these axons towards the ventral midline of the developing spinal cord. It is unclear whether netrin-1 accounts for all of the outgrowth-promoting and chemotropic activity of the floor plate. It is possible that floor plate cells are also a source of the Netrin Synergizing Activity (NSA) (Serafini et al., 1994), which could function with netrin-1 to mediate the long-range activities of floor plate cells.

Netrin-2 transcripts are also expressed from early stages of spinal cord development, but at much lower levels than *netrin-1* transcripts. At the time that commissural axons are initiating growth towards the floor plate, *netrin-2* RNA is expressed in a broad domain adjacent to the floor plate (Figures 2-2 and 2-6). Thus, it is possible that, as commissural axons grow from dorsal regions to the ventral midline of the spinal cord, they encounter a continually increasing gradient of netrin protein. Commissural axons may be exposed to little or no netrin protein in the dorsal-most portion of the spinal cord. As they project ventrally, they are likely to be exposed to a low level of netrin-2 as they approach and enter the netrin-2 expression domain. Then, in the ventral region of the spinal cord, the axons may encounter an ever-increasing concentration of netrin protein due to high levels of netrin-1 that are presumed to be secreted by floor plate cells. Thus, the patterns of expression of the netrin genes suggest that the two proteins could together establish a long-range gradient of tropic activity, which directs commissural axon growth to the ventral midline of the spinal cord.

The Tropic Response of Commissural Axons : Chemotaxis or Haptotaxis?

The observation that the netrins can adhere to cell surfaces or ECM components (Serafini et al., 1994, and Figure 2-3) raises the question whether commissural axons detect the netrins in solution (a chemotactic response) or bound to cells or the ECM (a haptotactic response). The possibility that commissural axons respond to the netrins after they become substrate-bound cannot be excluded since axons are capable of responding to

smooth gradients of immobilized substrate-bound factors (Baier and Bonhoeffer, 1992). In the immune system, heparin-binding chemoattractants of the chemokine family have been shown to bind cell surfaces or the ECM and to be capable of functioning as haptotactic factors when immobilized (Rot, 1992; Tanaka et al., 1993; Wiedermann et al., 1993). If netrin-1 can function when substrate-bound, it is also possible that it mediates some of the contact-dependent guidance functions of floor plate cells on commissural axons (Jessell and Dodd, 1990), or indeed on any axons that navigate the midline by using short-range cues on floor plate cells. Conversely, it is possible that some known ECM or cell-surface modulators of axon growth also function as diffusible chemotropic factors under some circumstances.

The Netrins and UNC-6: A Functional Homology

The striking structural homology between the netrins and UNC-6 (Serafini et al., 1994) is further underscored by the possibility that these proteins have similar embryonic functions. Our studies have suggested that a gradient of netrin protein may direct circumferential axonal migrations in the spinal cord, but establishing the precise contribution of the netrins to guidance will require determining the extent to which perturbations of netrin function *in vivo* impair these migrations. Conversely, although *unc-6* mutants show defects in circumferential migration around the body wall of both mesodermal cells and axons (Hedgecock et al., 1990), the precise mechanism by which UNC-6 acts remains to be established. UNC-6 appears to be expressed selectively by cells in the ventral domain of the embryo (W. Wadsworth and E. Hedgecock, personal communication), suggesting the existence of an UNC-6 protein gradient. Moreover, the fact that the netrins can modulate the growth of axons in vertebrates supports the view that UNC-6 directly influences the migrations of cells and axons in the nematode. Thus, although in principle UNC-6 may participate only indirectly in guidance (discussed in Ishii et al., 1992), the simplest model is that UNC-6 is a guidance cue that is distributed

in a gradient with its high point at the ventral midline, to which cells and axons respond with directed migration (Ishii et al., 1992, W. Wadsworth and E. Hedgecock, personal communication).

Importantly, both dorsally and ventrally-directed migrations depend on UNC-6 activity (Hedgecock et al., 1990, and Figure 2-6). Thus, the UNC-6 protein may function as an attractant for cells that migrate ventrally and a repellent for cells that migrate dorsally. By analogy, it is possible that the netrins function as repellents of axons or cells that grow away from the floor plate. Furthermore, although mutations in *unc-6* disrupt circumferential migrations, for any given set of cells or axons, some but not all migrations are misdirected (Hedgecock et al., 1990). Thus, guidance cues other than those dependent on *unc-6* function must exist that also direct circumferential migrations. The existence of at least two vertebrate netrins raises the possibility that additional UNC-6/netrin-related proteins contribute to directing circumferential migrations in *C. elegans*.

Finally, studies of genes that interact with *unc-6* may provide insight into proteins that interact with the netrins, in particular netrin receptors. Mutations in the *unc-5* gene impair the dorsal migrations of cells and axons, whereas ventral migrations are unaffected (Hedgecock et al., 1990). *unc-5* acts cell-autonomously and encodes a predicted transmembrane protein with two immunoglobulin C2 domains, two thrombospondin type 1 domains and a cytoplasmic SH-3-like domain (Leung-Hagesteijn et al., 1992). Moreover, ectopic expression of *unc-5* in touch cells whose axons normally grow longitudinally or ventrally causes these axons to project dorsally (Hamelin et al., 1993). These dorsal migrations mediated by UNC-5 require the presence of UNC-6. Thus, UNC-5 is likely to be a receptor (or a component of a receptor) for the guidance molecule that directs dorsal migrations, presumably UNC-6. The high degree of sequence conservation between the netrins and UNC-6 may, therefore, make it worthwhile to examine predicted vertebrate homologues of UNC-5 as potential netrin receptors. Identification of the *C. elegans* gene encoding the axonal receptor that mediates

responses to the UNC-6-dependent ventral migratory cue (Hedgecock et al., 1990) may likewise provide further insight into the nature of netrin receptors.

Guidance of Other Cells and Axons by the Netrins

Netrin gene expression is not restricted to the developing spinal cord at the time of commissural axon growth, suggesting that other classes of axons or motile cells may be guided by the proteins. The expression of *netrin-1* in the floor plate at levels rostral to the spinal cord could contribute to guiding the many classes of axons that grow circumferentially or cross the midline in the hindbrain and midbrain; there are also populations of neuronal precursors at these levels whose cell bodies have been documented to translocate circumferentially, and whose migration has been proposed to be influenced by the floor plate (Bourrat and Sotelo, 1990a; Bourrat and Sotelo, 1990b). More generally, the expression of the *netrin* genes in the brain and spinal cord at late stages of development, and in mesodermal and endodermal derivatives, suggests a more widespread involvement of netrins in cell migrations and axon guidance during development, and possibly in cell migrations in the adult. It is likely that the netrins are secreted in diffusible form by several of the tissues where the genes are expressed, since two tissues where they are expressed – young (but not old) notochord and dermomyotome (Figure 2-2) – are already known to possess diffusible commissural axon outgrowth-promoting activity (Placzek et al., 1990a; Placzek et al., 1990b).

The idea that chemotropic cues guide axons in the developing nervous system was first proposed by (Ramón y Cajal, 1892), who also suggested specifically that spinal commissural axons are guided to the floor plate by such cues (Ramón y Cajal, 1909). The isolation of the netrins should make it possible to explore the extent to which commissural axon guidance to the floor plate is dependent on a chemotropic mechanism, and, more generally, to determine the contribution of the netrins to axon guidance throughout the vertebrate nervous system.

Experimental Procedures

Northern Analysis

For RNA isolation, tissues were dissected in L-15 medium from appropriately staged embryos or hatchlings, frozen in liquid nitrogen and stored at -80°C. Adult tissues were obtained from Pel-Freeze. Total embryo or tissue RNA was extracted using Ultraspec (BIOTECX Laboratories, Inc.) and a Polytron homogenizer (Brinkmann). To isolate poly(A)$^+$ RNA, total RNA was batch-bound to oligo-dT cellulose (Collaborative Research) in high salt and column eluted with 1 mM EDTA after an intermediate salt wash (Sambrook et al., 1990). Northern analysis was performed as described (Sambrook et al., 1990). For each tissue, 5 μg poly(A)$^+$ RNA was separated on a 1% formaldehyde agarose gel. Blots were sequentially probed with netrin-1, netrin-2 and GAPDH probes by exposing them damp and stripping them by boiling in 1 mM Tris pH 7.4 with 1% SDS. Probes were generated by incorporation of α^{32}P-dCTP during synthesis by PCR. The netrin-1 probe corresponded to domain C (143 bp) and 88 bp of 3' UTR sequences; the netrin-2 probe corresponded to 551 bp of 3' UTR sequences. The same patterns of transcripts detected with these probes were also detected with non-overlapping probes corresponding to coding sequences of netrin-1 and netrin-2. The control probe was generated by random priming a rat GAPDH cDNA (Fort et al., 1985).

RNA *in situ* hybridization

White leghorn chicken embryos (Feather Hill Farms, Petaluma, Ca.) were incubated at 38°C. Embryos were dissected from surrounding membranes in ice-cold L-15 medium, fixed overnight in 4% paraformaldehyde (PFA) in DEPC-treated PBS at 4°C, and washed once in DEPC-treated PBS at 4°C for 1 h. *In situ* hybridization was performed as follows.

For whole-mount *in situ* hybridization, embryos before stage 15 were dehydrated through a PBS/methanol series, stored in methanol at -20°C, and processed as described by Coutinho et al. (1992) with the following modifications: (1) the proteinase K (PK) treatment was reduced to 15 min. at 37°C, and embryos were subsequently refixed 30 minutes in 4% PFA/0.2% glutaraldehyde in PBS; (2) the antibody was pre-adsorbed to acetone embryo powder (Wilkinson and Nieto, 1993) in 10% heat-inactivated goat serum, 0.1% Triton-X100 in PBS. Embryos from which the CNS was to be excised were refixed for only 10 min. in 4% PFA after PK treatment; at the end of the entire whole-mount procedure, the CNS was teased out using tungsten needles. Prior to mounting, embryos were passed through 30%, 50% and 80% glycerol. For sectioning, embryos were washed in PBS, embedded in albumin/gelatin (20% sucrose, 30% egg albumin, 0.5% gelatin in PBS, filtered through cheese cloth, and gelled by addition of glutaraldehyde to 0.4%: M. Goulding, personal communication), and sectioned at 20-30 μm on a vibratome.

For *in situ* hybridization on sections, the brachial region of stage 15 and older embryos was fixed as described above, incubated overnight at 4°C in 30% sucrose in DEPC-treated PBS, transferred to OCT compound for 1-4 h at 4°C, and embedded in OCT at -70°C. 10μm cryostat sections were collected on Superfrost Plus slides (Fisher) and stored at -80°C. *In situ* hybridization using digoxygenin-labeled probes was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993). *In situ* hybridization using ^{33}P- or ^{35}S-labeled probes was performed as described (Frohman et al., 1990).

For both netrin-1 and netrin-2, several antisense riboprobes were prepared from the coding region and from the 3' untranslated region. For netrin-1, a probe corresponding to the first 900 bp of coding sequence was routinely used; similar results were obtained with a probe corresponding to domain C (455 bp). For netrin-2, a 2.1 kb probe comprising 1.1 kb of coding and 1.0 kb of 3' UTR sequences was routinely used; similar results were obtained with a 455 bp probe corresponding to 3' UTR sequences.

Fragments were subcloned into pBluescript (Stratagene), or amplified by PCR and subcloned into pCR (Invitrogen); plasmids were linearized with appropriate enzymes and transcribed with T7 or SP6 RNA polymerase, as appropriate. Probes were used at a final concentration of 200 ng/ml. Prior to whole-mount *in situ* hybridization, large (>900 bp) probes were base-hydrolyzed to an average probe size of ~200 bp.

Explant Culture

Dissection and culture of E11 (E0 = day of vaginal plug) rat spinal cord explants were performed as described (Tessier-Lavigne et al., 1988), except that explants were cultured in 25% F12, 70% OptiMEM I (Gibco/BRL), 5% heat-inactivated horse serum (HIHS), supplemented with 40 mM glucose, 2 mM L-glutamine, 100 µg/ml streptomycin sulfate and 100 U/ml penicillin G.

Expression of Netrins in COS Cells

Transfections of pMT21, pGNET1^{myc}, and pGNET2^{myc} (Serafini, et al., 1994) into COS-1 cells (under 20 passages) were performed using Lipofectamine (Gibco/BRL) as directed. Cells were seeded at ~2.5 X 10^5 cells per 35 mm well (in six well dishes) and were transfected 16-20 h later with 1 µg DNA using 6 µl Lipofectamine in a 1 ml volume; transfections were stopped after 5 h by adding an equal volume of medium containing 20% heat-inactivated fetal bovine serum (HIFBS). To produce clusters of transfected COS cells, 11-12 h after the beginning of transfection cell layers were trypsinized, washed twice with 10 ml DME H-21 with 10% HIFBS, and resuspended in DME with 1% HIFBS (0.25 ml per 35 mm well). Twenty microliter drops of the cell suspension were placed onto the lids of 35 mm dishes, which were inverted over dishes containing 2 ml of DME. These hanging drop cultures were incubated 14-16 h; aggregates were harvested into warm L15 medium containing 5% HIHS and trimmed with tungsten needles for use in explant cultures.

Analysis of Recombinant Protein Localization

Cell layers in 35 mm wells were washed 24 h after start of transfection with OptiMEM I supplemented with GlutaMAX I (Gibco/BRL), 100 μg/ml $CaCl_2$, and antibiotics, and were incubated in 1 ml of this medium for two days before harvesting medium. To prepare cell extracts, cell monolayers were incubated after removal of media with 0.5 ml of Extraction Buffer as described (Serafini et al., 1994). Media and extracts were centrifuged 5 min. in a microcentrifuge to remove cells and large debris, then at ~60,000 X g for 100 min. Two-fifths of the TCA-precipitated protein from media and extracts was subjected to Western analysis using a 1:200 dilution of a concentrated 9E10 culture supernatant (see Serafini et al., 1994).

Whole Mount Immunohistochemistry

After culturing, explants were fixed for 1.5 h at 4 °C with fresh 4% paraformaldehyde in PBS, and were routinely stored in PHTX (PBS / 1% heat-inactivated goat serum / 1% Triton X-100) for up to two months in the presence of 0.1% NaN_3. Collagen gels containing the explants were trimmed, incubated in PHTX with gentle agitation in 96-well plates for at least 24 h, incubated in 4D7 culture supernatant (Yamamoto et al., 1986) diluted 1:4 in PHTX for 12 h at 4 °C, washed four or five times for 2 h each in PHTX, incubated in affinity-purified Cy3-conjugated goat anti-mouse IgM antibody (Jackson ImmunoResearch, 1:700 dilution in PHTX) for 12 h at 4 °C, and washed as before prior to mounting with Fluoromount G (Fisher).

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Table 2-1: Commissural Axon Turning in Response to COS Cells Secreting Netrins

Cells apposed to side of E11 rat dorsal explant	Explants exhibiting axon turning (%)	Distance from apposed cells over which axons responded (μm ± s.e.m.)	COS cell aggregates invaded by axons (%)
E11 floor plate	14/14 (100%)	330 ± 40	na [a]
Control COS cell aggregates	1/12 (8%) [b]	40 [b]	0/12 (0%)
Netrin-1 expressing aggregates	16/16 (100%)	260 ± 50	15/16 (94%)
Netrin-2 expressing aggregates	19/20 (95%)	160 ± 50	19/20 (95%)

[a] Not applicable.

[b] In the one case where turning was apparently seen, the edge of the dorsal explant contacting the COS cells was uneven, perhaps reflecting a curling of one leaf of neural epithelium, which may explain the appearance of turning.

Figure 2-1: Northern Analysis of *Netrin-1* and *Netrin-2* Gene Expression in Poly $(A)^+$ RNA Prepared from Chick Embryos and Various Embryonic and Adult Tissues.

A single 4.9 kb *netrin-1* transcript, and three major *netrin-2* transcripts of 5.4, 3.6, and 3.2 kb are detected. A longer exposure of the Northern blot than was used for this Figure demonstrated low levels of expression of *netrin-1* in E10 and post-hatch gut, and in adult brain, heart, skeletal muscle and thymus (data not shown). A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to monitor RNA content in each lane. P, first day after hatching.

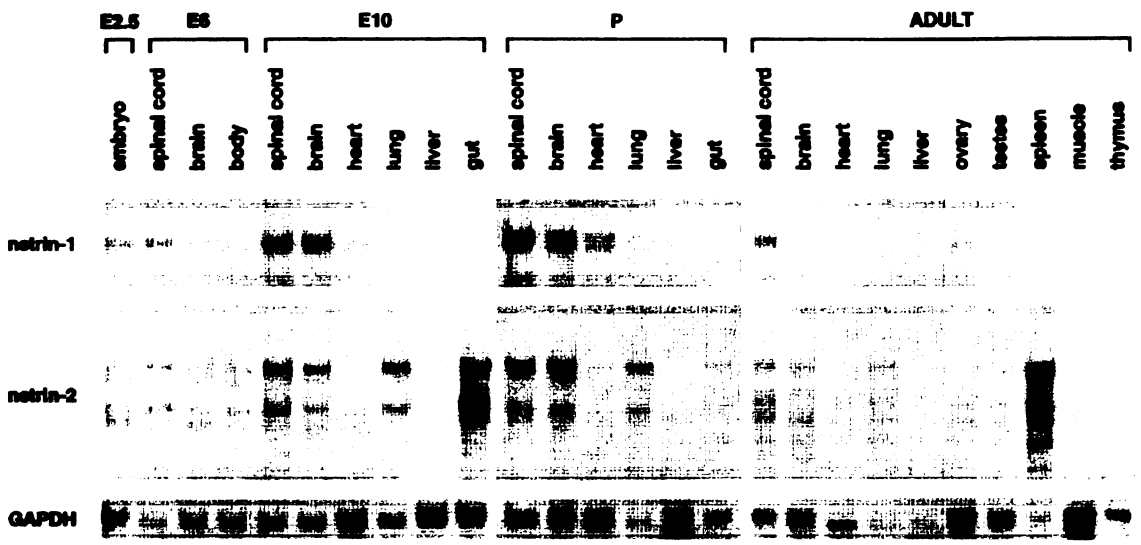

E2.5
E6
E10
P
ADULT
embryo
spinal cord
brain
body
spinal cord
brain
heart
lung
liver
gut
spinal cord
brain
heart
lung
liver
gut
spinal cord
brain
heart
lung
liver
ovary
testes
spleen
muscle
thymus
netrin-1
netrin-2
GAPDH

Figure 2-2: Expression of *Netrin-1* and *Netrin-2* Genes From Neurulation through the Period of Commissural Axon Growth to the Floor Plate.

Whole embryos (A-E) or transverse sections of embryos (F-I) were hybridized with antisense netrin-1 (A-G) or netrin-2 (H-I) RNA probes. Digoxygenin-labeled probes were used for all panels except (H), for which a ^{33}P-labeled probe was used.

(A) Dorsal view of a stage 11 embryo hybridized with a netrin-1 probe. Arrows indicate position of sections in panels (B) and (C).

(B) Transverse vibratome section through the neural fold region of the stage 11 embryo shown in panel (A). *Netrin-1* RNA is detected in the notochord and at low levels in the pre-somitic mesoderm.

(C) Transverse vibratome section through somite 3 of the stage 11 embryo shown in panel (A). *Netrin-1* RNA is detected in floor plate, notochord and somites.

(D) Transverse vibratome section through the spinal cord of a stage 15 embryo after hybridization with a netrin-1 probe. *Netrin-1* RNA is expressed at this stage in the floor plate and dermomyotome, but is no longer detected in notochord. Low levels of expression are detected in sclerotome.

(E) Lateral view of the isolated central nervous system of a stage 15 embryo showing expression of *netrin-1* transcripts in the floor plate at all axial levels. An arrow marks the rostral-most extent of expression in the caudal diencephalon.

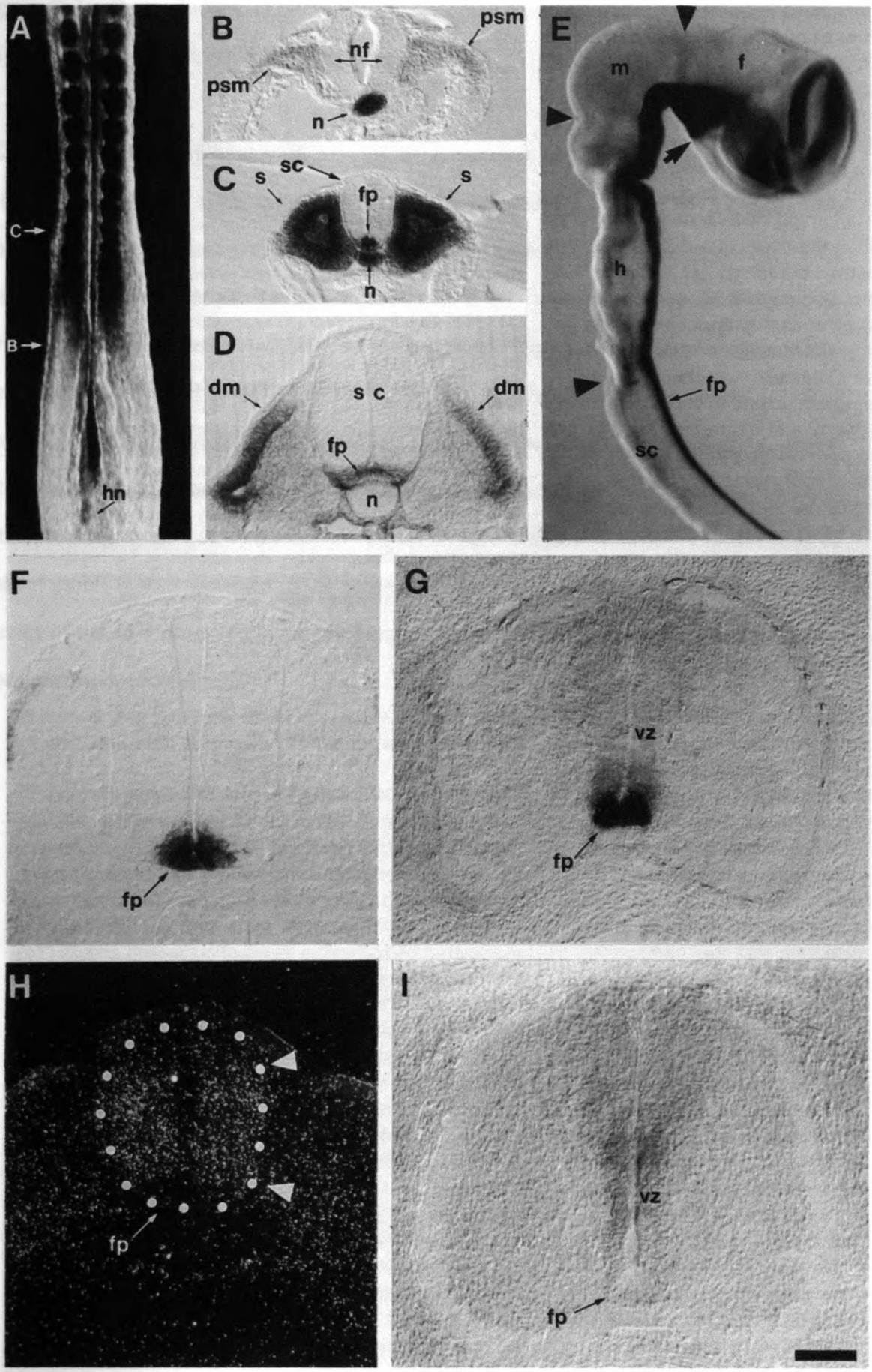
(F-G) Transverse sections through the spinal cord of a stage 15 embryo at a rostral level (F) and through the spinal cord of a stage 31 embryo (G), hybridized with an antisense netrin-1 riboprobe. Note the restricted expression of *netrin-1* RNA to the floor plate.

(H-I) Transverse sections though the caudal spinal cord of a stage 16 embryo (H) and through the spinal cord of a stage 31 embryo (I), hybridized with antisense netrin-2 riboprobes. A digoxygenin-labeled probe was used for the older embryo (I). Because of the low levels of expression in the younger embryo, a ^{33}P-labeled probe was used to

detect *netrin-2* transcripts in the younger embryo (H). The spinal cord is outlined with dots. *Netrin-2* RNA is detected in a broad band in the spinal cord at stage 15 (arrowheads in H), and becomes restricted to the ventricular zone by stage 31.

dm, dermomyotome; f, forebrain; fp, floor plate; h, hindbrain; hn, Hensen's node; m, midbrain; n, notochord; nf, neural folds; psm, pre-somitic mesoderm; s, somites; sc, spinal cord; vz, ventricular zone.

Scale bars are 240 μm (A), 90 μm (B - D), 250 μm (E), 90 μm (F-I).


A
C
B
hn
B
psm
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C
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vz
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fp

Figure 2-3: Recombinant Tagged Netrins Secreted by COS Cells Are Present in the Soluble as well as the Cell-Associated Fraction.

COS cells were transfected with expression constructs designed to yield myc-tagged netrin-1 ("net-1^{myc}", right two lanes), myc-tagged netrin-2 ("net-2^{myc}", middle two lanes), or no recombinant protein ("control", left two lanes). Conditioned medium ("M") and 1 M NaCl extract of the cell monolayers ("E") were subjected to high speed centrifugation to generate soluble protein fractions. Equivalent volumes were TCA-precipitated, subjected to SDS-PAGE (7.5% gel), and immunoblotted using the 9E10 monoclonal antibody to detect the expressed tagged netrins. In order to detect the tagged netrins in the conditioned medium ("M" lanes), it was necessary to overexpose the lanes corresponding to the salt extracts ("E" lanes). Under these conditions, minor bands of distinct mobility were detected in the netrin-1- and netrin-2-containing extracts. Multiple species of netrin-1 observed in the medium may arise from differential post-translational modification or proteolysis.

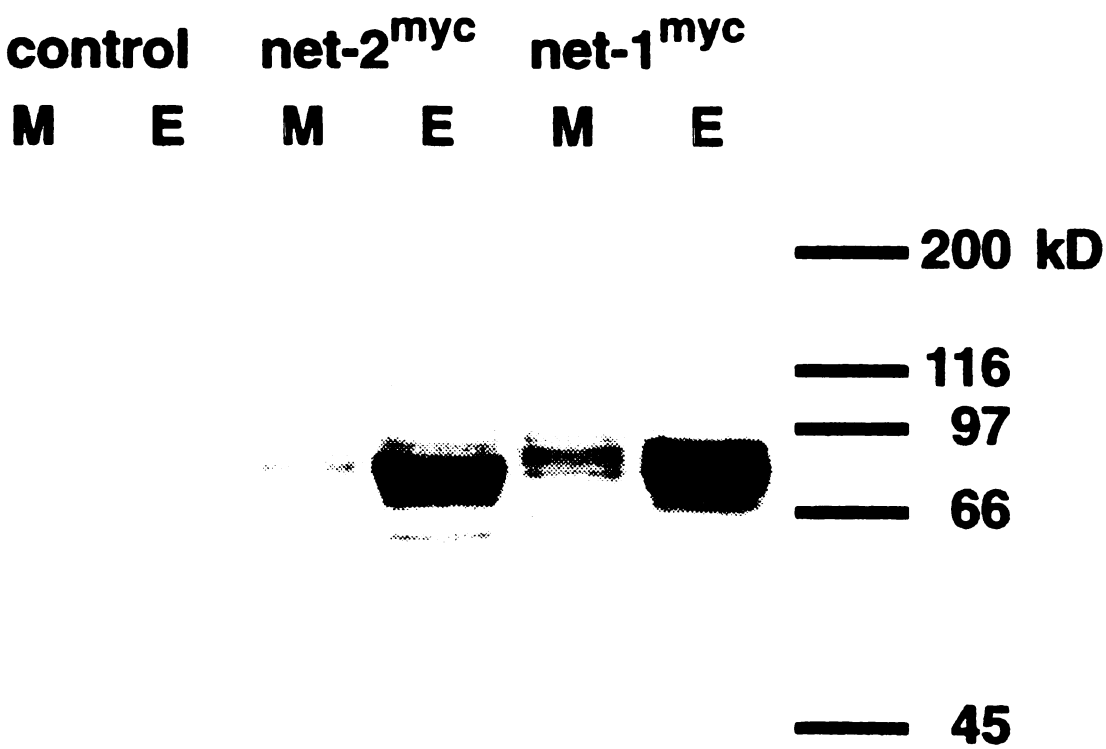

control
net-2myc
net-1myc
M
E
M
E
M
E
200 kD
116
97
66
45

Figure 2-4: Aggregated COS Cells Secreting Netrin-1 or Netrin-2 Elicit Commissural Axon Outgrowth from E11 Rat Dorsal Spinal Cord Explants at a Distance.

Outgrowth evoked from the cut ventral edge of E11 rat dorsal spinal cord explants by a floor plate explant (A), an aggregate of COS cells that had been transfected with vector alone (B), an aggregate of transfected COS cells secreting netrin-1 (C), or an aggregate of transfected COS cells secreting netrin-2 (D). In each case, the dorsal explant is the upper object in the photograph and is shown with its roof plate (dorsal surface) oriented toward the top of the photograph. Outgrowth was scored as positive if multiple axon bundles extended from the ventral edge of the dorsal explant towards the COS cell aggregate. Scale bars are 100 μm.

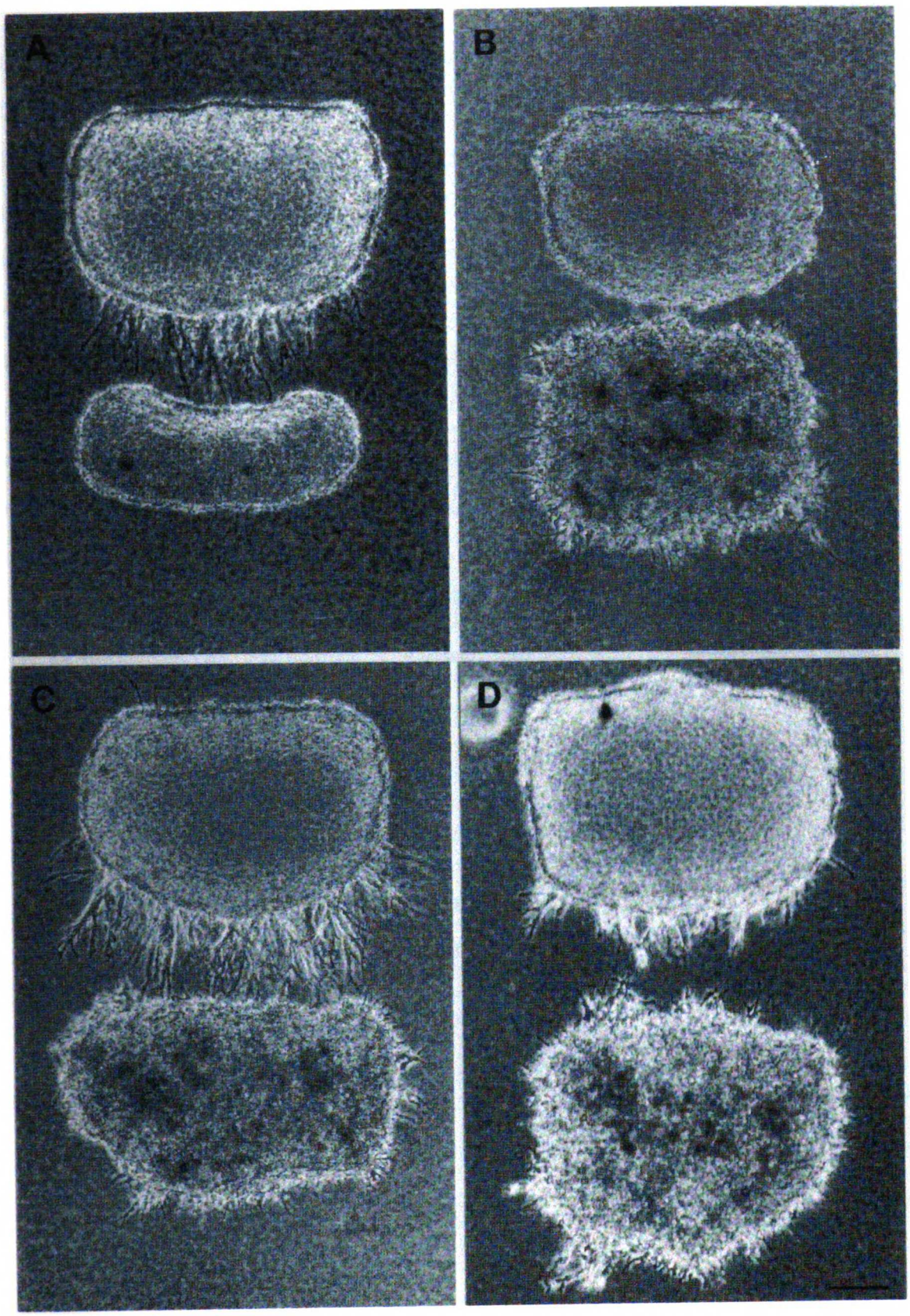

A
B
C
D

Figure 2-5: Netrin-1 and Netrin-2 Reorient Commissural Axon Growth.

(A) Diagram of the positioning of explants used to demonstrate the chemotropic effects of floor plate cells, in which a floor plate explant is positioned against the cut edge of an E11 rat dorsal spinal cord explant, along the dorso-ventral axis of the explant. When explants are co-cultured in this way, commissural axons turn towards the floor plate over a distance of several hundred micrometers (Placzek et al., 1990a).

(B-E) After 40 h of culture, explants were processed for whole-mount immunohistochemistry using the 4D7 monoclonal antibody (Yamamoto et al., 1986) and secondary immunofluorescence to visualize commissural axons. As previously reported (Placzek et al., 1990a), the floor plate caused commissural axons to turn within dorsal explants (B); axons reoriented growth within the range indicated by the arrowhead. Commissural axons did not turn when an aggregate of COS cells previously transfected with the expression vector alone was positioned alongside the dorsal spinal cord explant instead of floor plate (C), and instead grew along a stereotyped dorso-ventral trajectory, similar to the one they take in the dorsal spinal cord *in vivo* (Altman and Bayer, 1984; Dodd et al., 1988). However, when the aggregated COS cells secreted netrin-1 (D), reorientation of commissural axons was observed over almost the same distance as seen with floor plate (range indicated by arrowhead). Turning was also seen when the aggregated COS cells secreted netrin-2 (E), although the range of the effect (indicated by arrowhead) was less than that seen with netrin-1. In (D) and (E), axons invaded the netrin-expressing COS cells (arrows). The dotted line in (B-E) delineates the edge of the dorsal explant.

fp, floor plate; rp, roof plate

Scale bars are 50 μm.

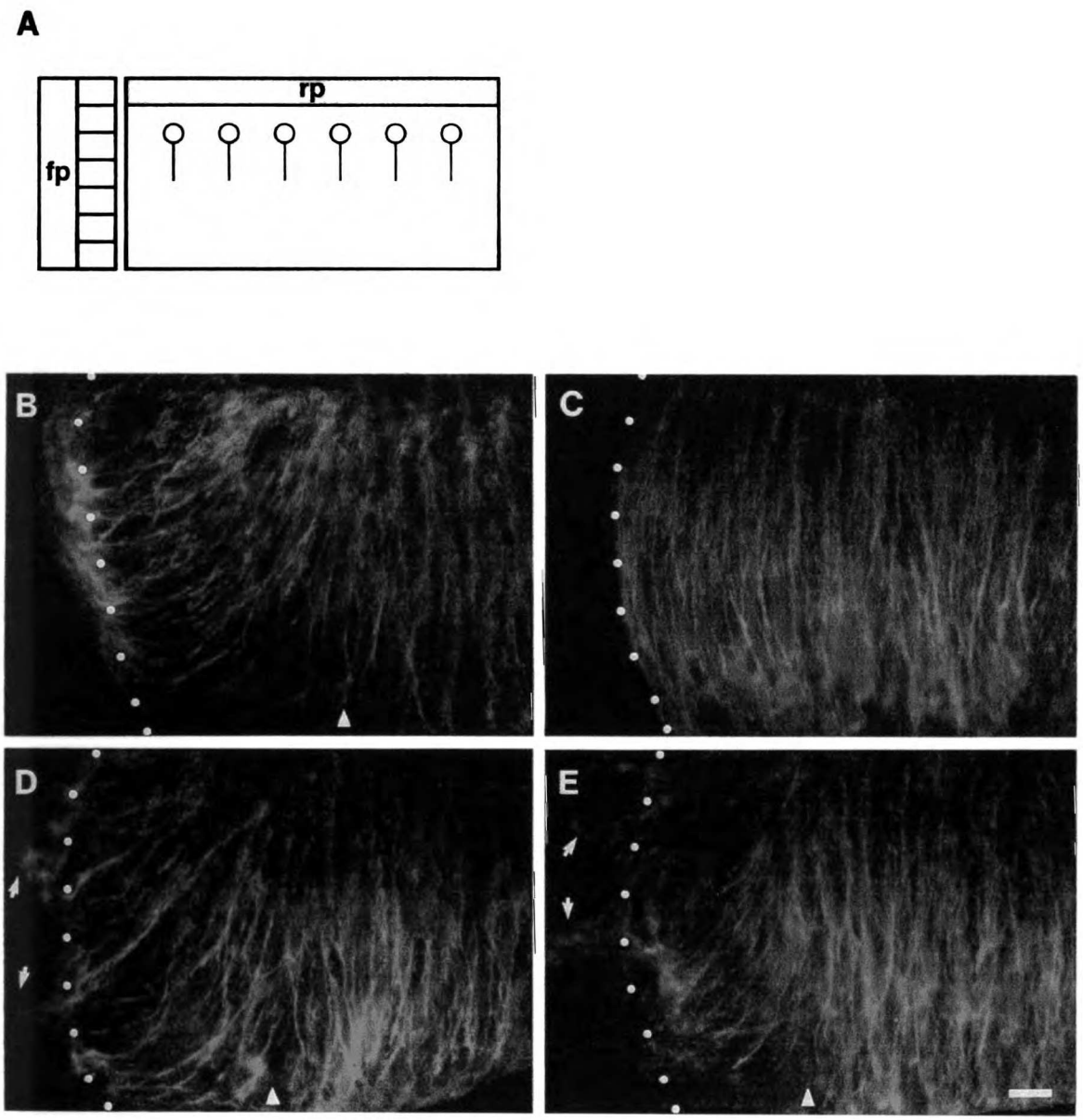
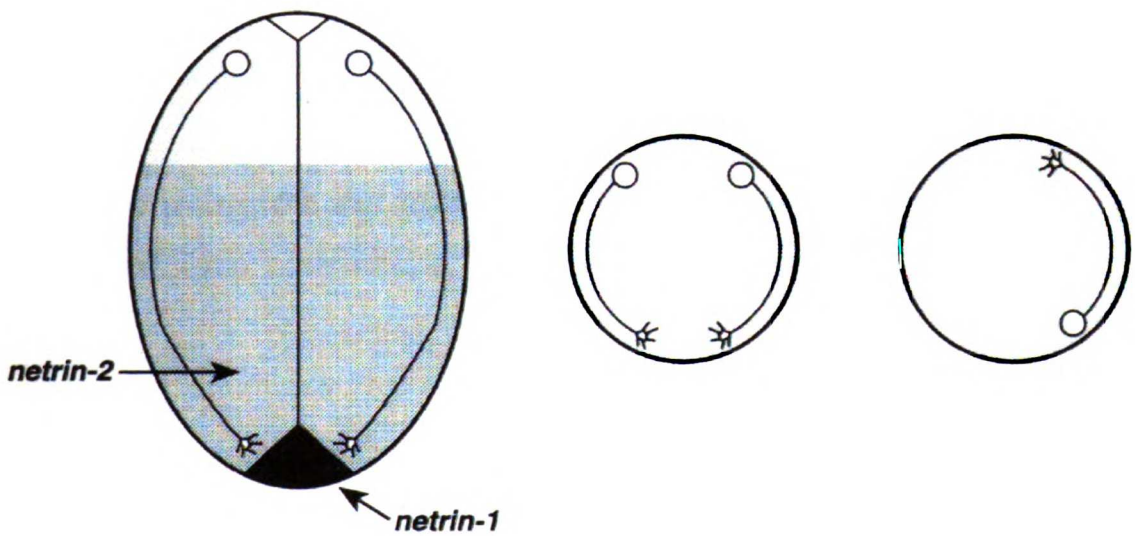

A
rp
fp
B
C
D
E

Figure 2-6: Similarities in the Growth of Vertebrate Commissural Axons and Axons That Are Misrouted in *unc-6* Mutants in *C. elegans*.

In the vertebrate spinal cord (left figure), the dorsally-located commissural neurons (which are sensory interneurons) extend axons ventrally near the lateral edge of the spinal cord, then turn and grow toward the ventral midline once they are within ~150 μm of the floor plate (Dodd et al., 1988). Similarly, those sensory neurons in *C. elegans* whose axon trajectories are affected in *unc*-6 mutant animals (central figure) have dorsally-located cell bodies and extend axons ventrally along the lateral edge of the nematode (in this case, the hypodermis) toward the ventral midline (Hedgecock et al., 1990). The other major class of axons in *C. elegans* whose guidance is affected in *unc*-6 mutant animals are motor neurons (right figure), which have ventrally-positioned cell bodies and extend axons dorsally along the hypodermis. The expression patterns of the two netrin genes, illustrated in the left figure, suggest that the two proteins may cooperate to establish a long-range tropic gradient of netrin protein with its high point at the ventral midline of the spinal cord. Since UNC-6 appears to be expressed in the ventral domain of the embryo (W. Wadsworth and E. Hedgecock, personal communication) it is possible that UNC-6 serves as a chemoattractant for ventrally-projecting axons in *C. elegans*. Furthermore, the expression pattern of UNC-6 suggests that it may act as a chemorepellent for dorsally-projecting axons (W. Wadsworth and E. Hedgecock, personal communication), raising the possibility that the netrins act as repellents for axons that grow away from the floor plate.


netrin-2
netrin-1

Chapter Three

Turning of retinal growth cones in a netrin-1 gradient mediated by the netrin receptor DCC

Summary

Netrin-1 promotes outgrowth of axons *in vitro* through the receptor Deleted in Colorectal Cancer (DCC) and elicits turning of axons within embryonic explants when presented as a point source. It is not known whether netrin-1 alone can elicit turning nor whether DCC mediates the turning response. We show that *Xenopus* retinal ganglion cell growth cones orient rapidly towards a pipette ejecting netrin-1, an effect blocked by antibodies to DCC. *In vitro*, netrin-1 induces a complex growth cone morphology reminiscent of that at the optic nerve head, a site of netrin-1 expression *in vivo*. These results demonstrate that netrin-1 can function alone to induce turning, implicate DCC in this response, and support the idea that netrin-1 contributes to steering axons out of the retina.

Introduction

Developing axons in the nervous system can be guided by diffusible factors secreted by target cells, a process known as chemoattraction (reviewed by Tessier-Lavigne and Goodman, 1996). One family of chemoattractants for developing axons are the netrins, laminin-related molecules that appear to have a phylogenetically conserved role in guidance of axons in vertebrates, *Caenorhabditis elegans*, and *Drosophila*. In rodents, commissural neurons, which are born dorsally in the spinal cord, extend axons circumferentially toward the floor plate at least partly in response to netrin-1 secreted by floor plate cells (Tessier-Lavigne et al., 1988; Placzek et al., 1990a; Placzek et al., 1990b; Kennedy et al., 1994; Serafini et al., 1994; Serafini et al., 1996). Similarly, the netrin homologue UNC-6 in *C. elegans* is required for the proper migration of axons and motile cells towards UNC-6-expressing midline cells (Hedgecock et al., 1990; McIntire et al., 1992; Wadsworth and Hedgecock, 1996), and in *Drosophila*, loss of function of Netrin-A and Netrin-B, normally expressed at the midline, results in misrouting of axons that normally project to the midline (Harris et al., 1996; Mitchell et al., 1996). In addition to their apparent roles in attraction, the netrins have been implicated as repellents of axons that migrate away from the midline in both *C. elegans* and vertebrates (Colamarino and Tessier-Lavigne, 1995; Wadsworth et al., 1996; Varela-Echavarria et al., 1997). Furthermore, genetic and biochemical evidence suggests that the attractive and repulsive actions of netrin proteins might involve different functional domains of the netrin molecules and may be mediated by distinct receptor mechanisms (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992; Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996; Wadsworth et al., 1996; Ackerman et al., 1997; Leonardo et al., 1997). In addition to this role in midline guidance, studies in a variety of vertebrates species have implicated netrins in guidance in many different regions of the nervous system apart from midline regions (Kennedy et al., 1994; Shirasaki et al., 1995; Serafini et al., 1996; Shirasaki et al., 1996; Wang et al., 1996; Lauderdale et al.,

1997; Livesey and Hunt, 1997; Métin et al., 1997; Richards et al., 1997; Strähle et al., 1997) including the guidance of RGC axons into the optic nerve head (Deiner et al., 1997).

Despite the demonstration that netrin proteins are required for a number of axon guidance events *in vivo*, the precise role played by netrins in these guidance events is only partially understood. The cellular aspects of the apparent attractive function of netrins have been explored in greatest depth in the context of spinal commissural axon growth to the ventral midline of the spinal cord. The netrins have two experimentally separable activities on spinal commissural axons *in vitro*. First, these proteins can promote outgrowth of commissural axons from explants of dorsal spinal cord into the otherwise unfavorable environment of a collagen matrix (Serafini et al., 1994), an apparent "permissive" action (see Discussion). Second, when presented from a point source (i.e., transfected cells secreting recombinant netrins), the netrins can cause spinal commissural axons to reorient growth within the tissue explants over a 40 hr period (Kennedy et al., 1994), mimicking the chemotropic or "turning" activity of floor plate cells (Tessier-Lavigne et al., 1988; Placzek et al., 1990a; Placzek et al., 1990b). Similar "permissive" and "chemotropic" activities have been demonstrated for the effects of netrin-1 on early cortical efferents (Métin et al., 1997; Richards et al., 1997), and may both be important for axon guidance *in vivo* (discussed in Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1996; Tessier-Lavigne and Goodman, 1996).

Several aspects of the chemotropic activity of netrins are poorly understood. First, chemotropic activity has only been demonstrated on axons growing within explants of neural tissue, not in a cell free environment (Kennedy et al., 1994; Métin et al., 1997), raising the possibility that the tissue explant provides accessory factors that are necessary for the turning effect. Second, the receptor mechanisms that mediate the turning response have not been defined. Previous studies have shown that the netrin receptor DCC expressed by commissural and RGC axons is required for the outgrowth-promoting activity of netrin-1 on those axons, since antibodies to DCC markedly reduce the amount of

axon outgrowth from dorsal spinal cord or retinal explants that is elicited by netrin-1 (Keino-Masu et al., 1996; Deiner et al., 1997). It has not, however, been resolved whether DCC also mediates the turning response of commissural axons towards sources of netrin-1 *in vitro*. In a previous study, function blocking antibodies to DCC failed to abolish turning of rat commissural axons within dorsal spinal cord explants towards cells secreting netrin-1 (Keino-Masu et al., 1996), suggesting that either DCC is not involved in this turning response or that these antibodies penetrated poorly into the explant.

To address these issues we have studied the responses of individual growth cones to netrin-1 protein *in vitro*, focusing on the role of netrin-1 and DCC in the guidance of retinal axons in *Xenopus*. Netrin-1 and DCC are expressed in the developing eye in a variety of vertebrate species (Kennedy et al., 1994; Pierceall et al., 1994; Keino-Masu et al., 1996; Deiner et al., 1997; Lauderdale et al., 1997; Livesey and Hunt, 1997; Strähle et al., 1997), and have been implicated in retinal axon guidance into the optic disc in rodents (Deiner et al., 1997). We report here the identification of a *Xenopus* homologue of netrin-1 and show that, as in rodents, netrin-1 is expressed in the optic nerve head (disc) and optic nerve, and DCC is expressed by RGC axons, consistent with their playing a role in *Xenopus* RGC axon guidance similar to that shown in mouse (Deiner et al., 1997). To examine at a cellular level how growth cones respond to netrin-1, we have taken advantage of an experimental system in which growth cones of embryonic *Xenopus* neurites are exposed to stable gradients of soluble chemoattractants established by a calibrated pulsatile release mechanism from a small glass capillary (Lohof et al., 1992; Zheng et al., 1994). This system has been used to investigate chemoattractant responses of *Xenopus* spinal neurites to a variety of different factors, including neurotransmitters and neurotrophic factors (Lohof et al., 1992; Zheng et al., 1994; Song et al., 1997). We find that netrin-1 increases the complexity of retinal growth cones *in vitro*. Furthermore, we show that retinal neurites turn rapidly towards a point source of netrin-1 in a cell-free environment *in vitro*, a response that is abolished by blocking DCC function. Our results suggest that

netrin-1 might be responsible for the morphological changes observed in RGC growth cones at the optic nerve head *in vivo* (Holt, 1989; Brittis and Silver, 1995; Mason and Wang, 1997). They also indicate that *Xenopus* retinal growth cones can be guided by a gradient of netrin-1 protein and implicate the netrin receptor DCC in mediating these responses.

Results

Expression of *Netrin-1* in the Developing *Xenopus* Nervous System

Potential *netrin* homologues in *Xenopus* were identified by a combination of low stringency library screening and degenerate Polymerase Chain Reaction (PCR) (see Experimental Procedures). The predicted amino acid sequence of the *Xenopus* netrin cDNA isolated in this way is most similar to that of netrin-1 proteins in chicken, mouse and zebrafish (Figure 3-1 and Table 3-1), suggesting that it is the netrin-1 orthologue in *Xenopus laevis*. Northern blot analysis detected a single transcript of approximately 2.3 kb from stage 28 mRNA (Figure 3-2A). Reverse Transcription-PCR (RT-PCR) indicated that *netrin-1* is present as a maternal transcript (Figure 3-2B; stage 2, lane 1) but disappears within the first few cell cycles (data not shown) and is largely absent at the mid-blastula transition (stage 9, lane 2). In contrast to chick embryos (Kennedy et al., 1994), *Xenopus netrin-1* transcripts do not appear to be expressed during gastrulation (stage 10.5, lane 3) and only begin to accumulate at stage 11 (lane 4), increasing in abundance throughout late gastrulation (stage 12, lane 5), neurulation (stages 13-20; lanes 6-13), and swimming tadpole stages (stages 33-40; lanes 16-17).

Whole-mount RNA *in situ* hybridization was used to localize *netrin-1* transcripts. *Netrin-1* transcripts can first be detected by *in situ* hybridization by stage 12 in two stripes on either side of the neural plate midline (Figure 3-3A, arrows). Two medio-lateral stripes are also visible in the anterior neural plate (Figure 3-3A, arrowheads), possibly corresponding to the expansion of netrin-1 expression to the lateral neural plate (Figures 3-3C - D, arrowheads) and later to the dorsal midline in the caudal diencephalon (Figures 3-3E - F, 3-3I and 3-4I, arrowheads). Similar patterns of *netrin-1* expression are also observed in other vertebrates (Kennedy et al., 1994; Serafini et al., 1996; Lauderdale et al., 1997; Strähle et al., 1997). Interestingly, expression in the developing axial mesoderm, particularly in the notochord, appears to be absent in *Xenopus* embryos (Figure 3-3G),

similar to zebrafish (Strähle et al., 1997) but contrary to what is observed in amniote embryos (Kennedy et al., 1994; Serafini et al., 1996). By stage 19, the strongest staining is clearly in the developing floor plate, although some staining is also detected in the cells immediately adjacent to the midline (Figures 3-3B and 3-3G). In the anterior neural plate, *netrin-1* mRNA is also present in the developing optic primordia (prospective ventral optic stalk and vesicle), as well as the presumptive lateral diencephalon (Figure 3-3C). At the onset of commissural axon outgrowth (stage 25; Roberts et al., 1988), *netrin-1* is strongly expressed by the cells of the floor plate (Figure 3-3D, 3-3F and 3-3H). This ventral domain of expression appears to be slightly wider than the morphological floor plate (Figure 3-3H), reminiscent of the expression pattern of *netrin-1* mRNA in chick and mouse embryos (Kennedy et al., 1994; Serafini et al., 1996). At later stages, *netrin-1* is expressed in the developing forebrain and midbrain (Figures 3-3I and 3-4I) in a pattern reminiscent of the early axon tracts of the developing brain (Taylor, 1991). Similar observations in zebrafish embryos (Lauderdale et al., 1997) suggest that netrin-1 may play a role in the formation of these axons tracts.

Netrin-1 and DCC are Expressed in the Developing Visual System

Netrin-1 transcripts are detected in the eye primordia by stage 19 (Figure 3-3C), well before the optic vesicles develop from the diencephalon. This expression becomes localized to the ventral-anterior aspect of the developing eye vesicle and the optic stalk (stage 25, Figure 3-3D). By stage 27, before the first RGCs begin to differentiate, *netrin-1* expression has become restricted to the ventral retina adjacent to the ventral choroid fissure and to the ventral optic stalk (Figures 3-4A - B). *Netrin-1* continues to be expressed in the ventral optic stalk (Figures 3-4B and 3-4F), where the first RGC axons grow and eventually form the optic nerve. High levels of netrin-1 transcripts are detected in the ventral optic nerve head (Figures 3-4D - E), where RGC axons make a sharp turn to exit the retina. Expression in the optic nerve persists throughout the initial period of RGC axon

extension out of the retina (stages 33-37, Figures 3-4E - F). *Netrin-1* is detected at the optic chiasm at early stages (Figure 3-4B), but not later (Figure 3-4F). Beyond the chiasm, *netrin-1* is expressed in the presumptive optic tract (Figures 3-3I and 3-4I - J), suggesting that netrin-1 may play a role in the guidance of RGC axons to their targets in the tectum.

Several candidate netrin receptors, including DCC, have been shown to be expressed in the developing visual systems of various species (Pierceall et al., 1994; Vielmetter et al., 1994; Keino-Masu et al., 1996; Leonardo et al., 1997; Livesey and Hunt, 1997). Using an antiserum raised against the cytoplasmic domain of *Xenopus* DCC (Pierceall et al., 1994), we found that several retinal cell types appear to express DCC. In particular, RGC axons appear to be labeled in the fiber layer, through the optic nerve head to the optic nerve, the chiasm, and the optic tectum (Figures 3-4G - H). DCC immunoreactivity is also detected on several other cell types in the outer and inner plexiform layers (OPL and IPL, respectively; Figure 3-4G), suggesting possible expression on the dendrites of RGCs or amacrine cells (OPL) or of horizontal cells (IPL). The findings that *netrin-1* is expressed in the optic disc and nerve and that its receptor DCC is expressed by RGC axons parallel similar observations in rodents (Deiner et al., 1997), raising the possibility that netrin-1 might affect *Xenopus* retinal axon growth as well.

Netrin-1 Stimulates *Xenopus* Retinal Neurite Outgrowth via DCC and Increases Growth Cone Complexity *In Vitro*

To determine whether retinal neurons in *Xenopus* respond to netrin-1, we first examined the effect of netrin-1 on retinal neurites growing in dissociated retinal cultures on various substrates. These mixed cultures are expected to contain both RGCs as well as other cell types. Netrin-1 stimulated neurite growth on laminin in a dose-dependent manner, with a peak increase of about two fold at 300 ng/ml and a reduction in outgrowth at higher concentrations (Figures 3-5A - C). This dose response curve is similar to that observed in other netrin-1-induced outgrowth assays such as spinal commissural axon and mouse

retinal ganglion axon outgrowth into a collagen matrix (Serafini et al., 1994; Deiner et al., 1997). The two-fold increase in outgrowth by 300 ng/ml netrin-1 is similar to the stimulation previously observed for Fibroblast Growth Factor 2 (FGF-2) on retinal neurites (Figure 3-5D; see also McFarlane et al., 1995). The stimulatory effect of netrin-1 was more pronounced when sub-optimal concentrations of laminin were used to coat the coverslip (0.5 μg/ml; data not shown). A similar two fold increase in response to 300 ng/ml netrin-1 was also observed on a fibronectin substrate (Figure 3-5A). Retinal neurite growth on poly-L-ornithine was poor, and was not significantly enhanced by netrin-1 (Figure 3-5A). Because outgrowth was greatest on laminin, that substrate was used in subsequent experiments, at the optimal concentration.

We next examined whether DCC is involved in the response of *Xenopus* retinal neurites to netrin-1. Addition of anti-DCC antibodies (2 μg/ml) to cultures grown with netrin-1 (300 ng/ml) fully blocked the increase in neurite length caused by netrin-1, whereas a control IgG (2 μg/ml) did not (Figure 3-5D). The anti-DCC antibody did not affect the stimulation of neurite outgrowth elicited by FGF-2 (Figure 3-5D), showing that the inhibition of netrin-1 effects by this antibody is specific.

These results implicate DCC in mediating the stimulation of *Xenopus* retinal neurite outgrowth *in vitro* by netrin-1. It should be noted, however, that it is in principle possible that the antibody is interfering not just with DCC function in retinal axons but also with that of an immunologically cross-reactive molecule expressed by these axons. The facts that the antibody is a monoclonal antibody and that in rodents it selectively binds DCC and not the only other known DCC relative (Neogenin) (Keino-Masu et al., 1996) argue against this possibility, but do not exclude it entirely.

Interestingly, netrin-1 added to cultures of retinal cells caused dramatic changes in growth cone morphology (Figure 3-6 and Table 3-2). After one day in culture, growth cones had a larger number of filopodia in cultures grown with netrin-1 when compared to controls, and there was also an increase in the total length of all filopodia per growth cone,

though no change in the total area of the growth cone (Table 3-2). Netrin-1 also induced changes in neurite morphology (Table 3-2). The frequency of neurite branching points per unit length of neurite was significantly increased by netrin-1, though no effect was observed on the number of neurite initiation sites per cell body (Table 3-2). Finally, time-lapse recording of growth cones demonstrated a 50% increase of growth cone speed in netrin-1 treated cultures compared to controls (Table 3-2). In agreement with this, the length of both the longest process and the total length of all processes per cell were significantly increased after 24 hours in netrin-1 supplemented cultures (Table 3-2).

Retinal Growth Cones Turn Towards a Point Source of Netrin-1

To test whether netrin-1 is capable of reorienting neurite growth in a cell-free environment *in vitro*, we examined the behavior of isolated growth cones extending from explants of stage 23/24 retina cultured on acid washed coverslips. The longest neurites in such cultures, which we studied exclusively, are those of RGCs (Harris et al., 1985; Sakaguchi et al., 1988; Sakaguchi et al., 1989). After 18 hours in culture, these growth cones were exposed to a gradient of netrin-1 protein established by repetitive pulsatile application of a concentrated solution (5 μg/ml) from a micropipette placed 100 μm from the growth cone at an angle of 45° from the direction of neurite extension. Previous studies have shown that stable gradients of proteins around 5%-10% over 10 μm can rapidly be generated by this method (Lohof et al., 1992; Zheng et al., 1994) and that the concentration of protein at the growth cone is approximately a thousand fold lower than that in the pipette (Lohof et al., 1992; Zheng et al., 1994), i.e. ~5 ng/ml. The shape of such a gradient is not strongly dependent on protein size since the diffusion coefficient is inversely proportional to the cubed root of the molecular weight (Lohof et al., 1992), and differs only by a factor of ~1.5 between Nerve Growth Factor (NGF) (~25 kD) and netrin-1 (~80 kD).

Retinal growth cones turned towards such a source of netrin-1 (Figure 3-7A-D and Table 3-3). Turning was evident as early as 15 minutes after the application of netrin-1

(Figure 3-7B) and was robust after one hour (Figure 3-7D). The average turning angle in the presence of netrin-1 was approximately 20°, compared to a turning angle close to 0° for control neurites (Table 3-3). Turning was not observed when the culture medium was supplemented with a function-blocking anti-netrin antibody (data not shown; T. E. Kennedy and M. Tessier-Lavigne, in preparation), nor when the micropipette contained a heat-treated netrin-1 solution (70°C, 30 minutes; 5 μg/ml). The rate of neurite extension was not significantly affected during the short period of exposure of the growth cone to netrin-1 (Table 3-3). To better illustrate this chemotactic response to netrin-1, the migration paths of growth cones were plotted during the one hour culture period and presented as composite drawings (Figures 3-7E - F). Under control conditions, growth cones did not show any bias in growth towards the micropipette (Table 3-3). In the presence of netrin-1, however, the vast majority of growth cones turned towards the micropipette (12 of 15; Table 3-3). In addition, repositioning the micropipette after one hour to a position 100 μm away from the growth cone and a 45° angle to the direction of growth caused the growth cone to turn once more (data not shown), suggesting that the receptor mechanisms in the growth cone are able to detect dynamic changes in the information cues provided by the extracellular environment.

To determine whether the observed turning response to netrin-1 is mediated through the netrin receptor DCC, we incubated the cultures with the anti-DCC antibody (2 μg/ml) for 30 minutes prior to application of netrin-1 (see Experimental Procedures). Turning of retinal axons toward the source of netrin-1 was abolished by addition of this antibody but not by addition of control rabbit IgG (2 μg/ml) (Table 3-3). Neither antibody significantly affected the rate of neurite elongation (Table 3-3). These data implicate the netrin receptor DCC in mediating the turning response of retinal growth cones in the presence of a netrin-1 gradient.

Discussion

Previous studies have suggested that gradients of the axon guidance molecule netrin-1 can orient the growth of extending axons *in vitro* and *in vivo* (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995; Shirasaki et al., 1995; Serafini et al., 1996; Shirasaki et al., 1996; Métin et al., 1997; Richards et al., 1997; Varela-Echavarria et al., 1997). In this study, we have examined the effects of netrin-1 on the growth of *Xenopus* retinal neurites *in vitro*. We provide direct evidence that a gradient of netrin-1 can act directly on the growth cone to cause turning of an extending neurite in a cell free environment. Furthermore, we show that antibodies to the netrin receptor DCC block this chemotropic response. These data, along with other previously published studies (Kennedy et al., 1994; Serafini et al., 1994; Fazeli et al., 1997), support the hypothesis that gradients of netrin-1 protein in the developing nervous system are interpreted by extending axons using a receptor mechanism that includes the DCC protein.

Chemotropic Action of Netrin-1 on Retinal Neurites in a Cell Free Environment

Netrin-1 and netrin-2 were originally isolated on the basis of their ability to promote the outgrowth of commissural axons into a collagen matrix (Serafini et al., 1994). Normally, these axons grow within the tissue explant but do not invade the collagen, suggesting that collagen may be an unfavorable substrate for axon extension (Tessier-Lavigne et al., 1988; Placzek et al., 1990a), and that netrin-1 has a "permissive" function, enabling the axons to overcome this inhibition (Serafini et al., 1994; Serafini et al., 1996). A similar "permissive" effect of netrin-1 has been demonstrated on RGC and cortical axons in collagen gels (Wang et al., 1996; Deiner et al., 1997; Métin et al., 1997; Richards et al., 1997). In addition, however, a point source of netrin protein provided by cells secreting netrins can cause turning of commissural axons within explants of dorsal spinal cord

(Kennedy et al., 1994) and of cortical axons within explants of cortex (Métin et al., 1997), providing evidence that netrins can exert a chemotropic action on growth cones. Significantly, netrins have not been found to be capable of reorienting axon growth once they have left tissue explants and entered collagen matrices. This negative result could be due to the extensive fasciculation of axons that is observed once axons enter the collagen (Serafini et al., 1994; Wang et al., 1996; Deiner et al., 1997; Métin et al., 1997; Richards et al., 1997), which may impair their ability to change direction. Alternatively, the chemotropic effect of netrin-1 observed within tissue explants might require the function of accessory factors provided by the tissue, and which are not present in the collagen matrix. A precedent for the operation of accessory factors in netrin function is provided by the observation that a strong outgrowth-promoting effect of netrin-1 on axons in explants of young (E11) (but not older [E13]) rat dorsal spinal cord requires the presence of the accessory factor Netrin Synergizing Activity (NSA) (Serafini et al., 1994). Finally, a more extreme hypothesis to account for the lack of turning in collagen gels is that netrin-1 itself has no chemotropic activity, but can induce cells within the tissue explants to secrete a distinct factor that causes the turning of axons. This possibility seems unlikely but could not previously be excluded, especially since the turning was studied over a long time course (40 hr) which could easily have allowed for secondary inductions.

Our demonstration that retinal neurites turn in a cell free environment towards a source of purified netrin-1 protein shows directly that a netrin protein can in fact function as a chemotropic factor and does not require accessory factors, at least in this *in vitro* setting. Several observations suggest that in our experiments growth cones are detecting a gradient of netrin-1 protein in solution (a chemotactic effect) rather than a gradient of netrin-1 protein adsorbed to the substrate (a haptotactic effect). First, growth cones turn rapidly (15 min.) towards the source. Second, a growth cone that has changed direction once can be made to turn again rapidly towards a repositioned pipette. Third, and perhaps most convincingly, the concentration of netrin-1 protein in solution at the growth cone is

expected to be about 5 ng/ml (see Results and Experimental Procedures), which is just above threshold for responses of axons to netrin proteins (Serafini et al., 1994; Deiner et al., 1997; Métin et al., 1997, and this study) and is in the range of the dissociation constant (Kd) for the DCC-netrin-1 interaction (Keino-Masu et al., 1996). It seems unlikely that enough netrin-1 protein could adsorb to the glass substrate in the vicinity of the growth cone in a fifteen minute period to cause a significant haptotactic response. Thus, netrin-1 is likely to have caused turning in our assay through a chemotactic mechanism. However, molecules such as NGF and laminin affect axon growth both as soluble and substrate-bound molecules (Gundersen and Barrett, 1979; Gundersen, 1985; Rivas et al., 1992), and it seems likely that substrate gradients of netrin-1, which could exist *in vivo*, will also be capable of causing a haptotactic response in the growth cone. Since netrins can associate non-specifically with cell membranes (Kennedy et al., 1994; Serafini et al., 1994), it has been suggested that netrin-1 may even act primarily through substrate-bound gradients of protein, rather than freely diffusing molecules, to guide extending axons *in vivo* (discussed in Kennedy et al., 1994; Serafini et al., 1996; Tessier-Lavigne and Goodman, 1996; Deiner et al., 1997; MacLennan et al., 1997). Our results do not address whether haptotactic or chemotactic mechanisms predominate *in vivo*, but merely demonstrate that retinal neurites are capable of responding chemotactically to a gradient of soluble netrin protein *in vitro*.

DCC Is Implicated in Mediating the Chemotropic Effect of Netrin-1 *in Vitro*

DCC binds netrin-1, and antibodies to DCC block the outgrowth-promoting activity of netrin-1 on spinal commissural and RGC axons in rodents in collagen gels (Keino-Masu et al., 1996; Deiner et al., 1997). These antibodies do not, however, block the chemotropic activity of netrin-1 on commissural axons within explants of dorsal spinal cord (Keino-Masu et al., 1996). These results have raised the question of whether the DCC receptor mediates both activities of netrin-1 or only the outgrowth-promoting activity. Attempts to address this question through analysis of DCC-deficient mice have so far been

inconclusive. Certainly, the finding that similar defects in projections of various axonal classes are observed in netrin-1-deficient and DCC-deficient mice has provided evidence that DCC is required for the action of netrin-1 *in vivo* (Deiner et al., 1997; Fazeli et al., 1997), but it is not known at present how much of the phenotypes is accounted for by loss of the permissive function of netrin-1 as opposed to loss of the chemotropic function of netrin-1 (discussed in Serafini et al., 1996; Tessier-Lavigne and Goodman, 1996).

Our finding that antibodies to DCC block the turning responses of growth cones to netrin-1 supports an involvement of DCC in mediating the chemotropic action of netrin-1, at least in this *in vitro* setting, and raises the possibility that the signal transduction mechanisms involved in mediating the outgrowth-promoting and chemotropic effects of netrin-1 might be similar or the same. Moreover, these results also suggest that the previous failure of the anti-DCC antibodies to block the turning response in tissue explants (Keino-Masu et al., 1996) may reflect poor penetration of the explant by the antibody. However, it remains possible that DCC or downstream signaling mediators function differently in retinal neurites and commissural axons, perhaps reflecting molecular differences in these two classes of axons.

Netrin-1 and the Guidance of RGC Axons at the Optic Nerve Head

During embryonic retinal development, RGCs born throughout the retina extend axons directly toward the optic nerve head (disc), where these axons make a sharp turn to exit the eye and migrate along the optic nerve to reach their targets in the brain. Several mechanisms have been proposed to explain the unidirectional migration of these axons within the retina to the optic nerve head (disc). Ramón y Cajal (1892) initially proposed that RGC axons are guided to the optic disc by long range diffusible cues within the retina. However, available evidence argues against the existence of a long range chemoattractant secreted by the optic nerve head (disc) and in favor of the operation of short-range cues that direct axons to the disc (Halfter and Deiss, 1984; Brittis and Silver, 1995; Halfter, 1996).

Evidence has been provided that these local cues involve intercellular spaces or channels formed by retinal glial endfeet (Silver and Sidman, 1980; Krayanek and Goldberg, 1981; Silver and Sapiro, 1981; Silver and Rutishauser, 1984), gradients of inhibitory proteoglycans (Brittis and Silver, 1995), and cues provided by other axons in the form of cell adhesion molecules (Brittis et al., 1995; Brittis and Silver, 1995).

Recent evidence has implicated netrin-1 in the guidance of RGC axons in the immediate vicinity of the optic nerve head (disc). First, in chicken, mouse, rat and fish embryos *netrin-1* is expressed in the developing optic nerve head and the optic nerve at the time of initial RGC axon extension (Kennedy et al., 1994; Serafini et al., 1996; Deiner et al., 1997; Lauderdale et al., 1997; Livesey and Hunt, 1997; Strähle et al., 1997). Second, several candidate netrin receptors are present in the developing retina, with DCC being expressed by RGCs (Pierceall et al., 1994; Vielmetter et al., 1994; Keino-Masu et al., 1996; Deiner et al., 1997; Leonardo et al., 1997; Livesey and Hunt, 1997). Third, netrin-1 can induce the outgrowth of mouse RGC axons from retinal explants *in vitro* (Wang et al., 1996; Deiner et al., 1997), a process which requires DCC function (Deiner et al., 1997). Finally, in netrin-1- and DCC-deficient mice, retinal axons extend normally to the vicinity of the optic disc but then are impaired in their ability to properly exit the eye though the optic disc (Deiner et al., 1997). Similar defects in RGC axon trajectories are observed in the zebrafish mutant No-isthmus (Noi), in which *netrin* gene expression in the optic nerve head and nerve is absent (MacDonald et al., 1997), although it has not yet been established whether loss of netrin-1 function in this mutant accounts for the defect.

Our results complement and extend these previous studies. As in other species, we find in *Xenopus* that *netrin-1* is expressed in the developing optic nerve head and nerve, that DCC is expressed by RGC axons (consistent with previous studies by Pierceall et al., 1994), that retinal neurites *in vitro* are responsive to netrin-1 protein, and that these responses require DCC function. These results suggest that netrin-1 in *Xenopus* might play a similar role in guiding RGC axons into the optic nerve to that demonstrated in

rodents. It is interesting to note in this context that studies in both amphibians and rodents have reported changes in the morphology of growth cones at the optic nerve head, where they appear more complex than those traveling within the retina (Holt, 1989; Brittis and Silver, 1995; Mason and Wang, 1997). Similar observations have also been made in rat spinal cord for commissural growth cones encountering the floor plate (Bovolenta and Dodd, 1990). Our finding that netrin-1 increases the complexity of retinal growth cones *in vitro* raises the possibility that the morphological changes observed *in vivo* are induced by netrin-1 in the proximity of the optic nerve head or the floor plate. Direct tests of this possibility will require examining growth cone complexity *in vivo* in the absence of netrin-1 or of a morphological floor plate. Interestingly, we find that both growth cone complexity and extension rate are increased by netrin-1 *in vitro*, whereas *in vivo* observations suggest a slowing at the optic nerve head (Holt, 1989; Brittis and Silver, 1995; Mason and Wang, 1997). This discrepancy may be due to additional signals present *in vivo* and lacking from our simplified culture conditions, or to the fact that our *in vitro* measurements were performed on neurites growing on laminin, an optimal substrate for neurite extension.

The precise role played by netrin-1 in guiding RGC axons at the optic nerve head has not been fully defined. As discussed in the context of spinal commissural axons above, it is possible that the region of the optic nerve head is inhibitory for axon growth, possibly to prevent other retinal axons from entering the optic nerve. Netrin-1 would then provide a permissive cue for RGC axons to overcome this inhibition, respond to other guidance cues, and exit the retina. An alternative hypothesis is that soluble or substrate-bound netrin-1 protein might be present in a short range gradient (a few cell diameters) that directly guides RGC axon growth towards the optic nerve head. Such a gradient might, for instance, direct the growth of pioneer RGC axons in the direct vicinity of the optic nerve head, with subsequent axons being guided into the optic nerve by selective fasciculation. Immunohistochemical studies in mouse have failed to demonstrate a long-range gradient of

netrin-1 immunoreactivity emanating from the optic disc, though the presence of a short-range gradient over a few cell diameters was not excluded (Deiner et al., 1997). In addition, while *netrin-1* transcripts and netrin-1 protein are localized tightly in the region of the optic disc in mouse (Deiner et al., 1997), *netrin-1* transcripts in *Xenopus* (this study) and zebrafish (Lauderdale et al., 1997) were localized not only to the optic nerve head, but also in the ventral retina (Figure 3-4). Further studies will determine whether a gradient of netrin-1 protein exists in the vicinity of the optic nerve head in the developing *Xenopus* retina.

The observation that some axons still manage to exit the retina in netrin-1- and DCC-deficient mice embryos (Deiner et al., 1997) suggests that netrin-1 is not the sole cue involved in the guidance of RGC axons into the optic nerve. Nonetheless, our finding that RGC axons can turn towards a source of netrin-1 at short range *in vitro* makes it of interest to examine whether the misrouting of RGC axons observed in netrin-1- and DCC-deficient mice reflects partly the failure of these axons to detect a short-range gradient of netrin protein in the immediate vicinity of the optic nerve head.

Experimental Procedures

Embryos

Xenopus laevis embryos were obtained by *in vitro* fertilization as previously described (Cornel and Holt, 1992). Embryos were raised in 10% Holtfreter's solution (Holtfreter, 1943) between 15°C and 20°C, and staged according to (Nieuwkoop and Faber, 1967).

Cloning of a *Xenopus* Netrin-1 Homologue

A radiolabeled chicken *netrin-1* probe was used to screen 10^6 plaques of a stage 28 head λZAPII *Xenopus* cDNA library at moderate stringency (42°C in 6X SSC, 50% formamide). Two identical clones were isolated and sequenced, and found to contain a partial open reading frame encoding a protein homologous to chicken *netrin-1*. The 5' end of the *netrin-1* open reading frame was cloned by PCR amplification using a degenerate oligonucleotide primer to the peptide sequence AQPDPC and two primers in the 5'-most region of the cDNA clone. Amplification products were cloned into pCRII and sequenced. The missing sequences at the 3' end of the *netrin-1* open reading frame were amplified and cloned by 3' RACE (Frohman and Martin, 1989).

Northern Blot Analysis

Polyadenylated RNA was isolated from stage 28 embryos using the FastTrak 2.0 mRNA isolation kit (Invitrogen). 4 μg of mRNA were electrophoresed on a denaturing formaldehyde gel, blotted onto Genescreen nylon membrane (DuPont) and crosslinked to the membrane using a Stratalinker (Stratagene). Blots were hybridized with a radiolabeled *netrin-1* probe using standard hybridization conditions (Sambrook et al., 1990) and exposed at -70°C using intensifying screens.

RT-PCR

RT-PCR was performed as described in (Wilson and Hemmati-Brivanlou, 1995). EF-1α primers were described in (Hemmati-Brivanlou and Melton, 1994). The following two primers were designed to detect *Xenopus netrin-1* transcripts: 5'-TATGACCAAAGCCATCACCC-3' and 5'-TGTCACAGTCTGCTGGTTCC-3'.

Whole-Mount *In Situ* Hybridization

Whole-mount *in situ* hybridization was performed as described in Hemmati-Brivanlou et al. (1990), with the modifications described in (Harland, 1991) using BM purple (Boehringer Mannheim) as substrate. A digoxigenin-labeled antisense riboprobe was generated by *in vitro* transcription of an EcoRI-linearized *Xenopus netrin-1* cDNA in pBluescript (Stratagene) using T7 RNA polymerase (Harland, 1991). Embryos were photographed in PBS or after clearing in 2:1 benzyl benzoate:benzyl alcohol. Following whole mount RNA *in situ* hybridization, some embryos were sectioned in paraffin. 10 μm serial sections were collected on Superfrost+ slides (Fisher), dewaxed in xylenes and mounted using Permount (Sigma). In addition some embryos were sectioned using a Vibratome (Oxford) as previously described (Kennedy et al., 1994).

Antibodies

Anti-DCC polyclonal antiserum 723 (provided by W. Pierceall, Yale University) is directed against the cytoplasmic domain of *Xenopus* DCC (Pierceall et al., 1994), and was used at 0.5 μg/ml in PBT + 5% goat serum. For DCC function blocking experiments, an anti-DCC mouse monoclonal (AF5, Oncogene Science) was used at 2 μg/ml; total IgG (2 μg/ml) was used as a control.

Immunocytochemistry

Xenopus embryos were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5, transferred to 30% sucrose, imbedded in OCT at -70°C,

sectioned (10 μm), and stained as described (Cornel and Holt, 1992). Prior to incubation with primary antibody, slides were boiled in a microwave for 10 minutes in 0.1 M sodium citrate, pH 8.0 to reveal masked epitopes (T.E. Kennedy and M. Tessier-Lavigne, in preparation).

Retinal Cell and Explant Cultures

Poly-L-ornithine-treated glass coverslips were coated with either laminin (10 μg/ml in Phosphate Buffered Saline [PBS]), fibronectin (10μg/ml in PBS) or washed with PBS for 30 minutes at room temperature in 35 mm petri dishes (Falcon). Following coating, coverslips were washed three times with PBS, once with 60% L-15 and placed in culture medium (60% L-15 medium plus 0.1% bovine serum albumin and 1% fungibact) for 30 minutes prior to plating the cells. Dissociated retinal cells from stage 24/26 *Xenopus* embryos prepared as described previously (Harris et al., 1985; Harris and Messersmith, 1992) were plated onto protein-coated glass coverslips in fresh culture medium with or without purified recombinant chick netrin-1 protein. Cultures were incubated for 24 hours at room temperature, fixed for 30 minutes in 0.5% glutaraldehyde in PBS, washed three times in PBS, and mounted on microscope slides with Aqua-Mount (Lerner Laboratories).

Retinal explants for turning assays were dissected as described (Harris et al., 1985), placed on chromic acid-washed glass coverslips, and cultured in culture medium (see above) for 18 hours at room temperature before exposure to gradients of netrin-1 protein (see below). The longest neurites in these cultures are those of RGCs (Harris et al., 1985; Sakaguchi et al., 1988; Sakaguchi et al., 1989). For antibody blocking experiments, cultures were pre-incubated with antibodies for one hour prior to netrin-1 application in small diameter polypropylene restriction rings to limit the volume of antibody solution required. Prior to netrin-1 application, restriction rings were removed and additional medium was added.

Production of Microscopic Netrin-1 Protein Gradients

Stable gradients of netrin-1 protein were formed as described (Lohof et al., 1992; Zheng et al., 1994; Song et al., 1997) by pulsatile ejection of a concentrated solution of purified recombinant netrin-1 protein (5 μg/ml) from a glass micropipette (tip opening of ~ 1 μm) placed 100 μm from the growth cone and at an angle of 45° to the direction of growth of the neurite (determined by the last 10 μm segment of the neurite prior to application of netrin-1). Stable concentration gradients of 5-10% over a width of 10 μm, the approximate width of a growth cone, can be established at a distance of 100 μm from the micropipette by this method; under these conditions, the estimated concentration of netrin-1 at the growth cones is ~ 5 ng/ml (~ 10^3 fold lower concentration than in the pipette) (Lohof et al., 1992; Zheng et al., 1994).

Measurement of Neurite Extension and Growth Cone Turning

Neurite lengths were measured by capturing phase-contrast images from a microscope (Axiophot, Zeiss) onto a computer (Power Macintosh, Apple) with a video camera (CCD-IRIS/RGB, Sony), and tracing the neurites using NIH Image (NIH, Bethesda). Statistical analysis was performed using GBSTAT software package. Elongation rates were measured as described (Chien et al., 1993). For turning assays, images were captured onto a computer (see above) at regular time intervals using a video camera attached to a phase-contrast inverted microscope (Nikon Diaphot). Path plots and turning angles could then be computed by analyzing the images with NIH Image software (NIH, Bethesda). The turning angle for each neurite was defined as the angle between the original direction of neurite growth and a line drawn between the original position of the growth cone and its position after one hour. Only growth cones with a net extension of >5 μm over the one hour period were analyzed.

Acknowledgments

We thank E. Cornel and D. Shehu for technical assistance; A. Faynboym, T.E. Kennedy and W. Pierceall for antibodies; L.E. Connolly, K. Brose, C.M. Mirzayan, P. Leighton, M.S. Deiner and P. Wilson for comments on the manuscript, and members of the Tessier-Lavigne, Hemmati-Brivanlou, Holt and Harris laboratories for helpful discussions. This work was supported by grants from the National Institutes of Health (MmP, NS22764; MTL, NS33713; AHB, HD32105; CEH, NS23780) and the Pew Foundation (CEH). MTL is an Investigator of the Howard Hughes Medical Institute. AHB is a Searle, McKnight and Merck scholar. The Genbank accession number for the *Xenopus netrin-1* sequence is AF033341.

Table 3-1: Percentage Amino Acid Identity Between *Xenopus* Netrin-1 and Other Netrin Homologues

	***Xenopus* netrin-1**			
	Overall	**Domain VI**	**Domain V**	**Domain C**
Chicken netrin-1	91%	91%	94%	86%
Mouse netrin-1	90%	88%	95%	89%
Zebrafish netrin-1a	91%	91%	96%	85%
Zebrafish netrin-1	85%	88%	95%	68%
Chicken netrin-2	72%	66%	90%	62%
Human netrin-2	59%	55%	78%	43%
Zebrafish netrin-2	[a]	[a]	95%	[a]
***C. elegans* UNC-6**	53%	52%	75%	33%
***Drosophila* netrin-A**	43%	46%	74%	18%
***Drosophila* netrin-B**	39%	36%	61%	24%

[a] partial sequence only

Table 3-1: Percentage of amino acid identity between *Xenopus* netrin-1 and chicken netrin-1 and netrin-2 (Serafini et al., 1994), mouse netrin-1 (Serafini et al., 1996), zebrafish netrin-1a (Lauderdale et al., 1997), netrin-1 (Strähle et al., 1997) and netrin-2 (MacDonald et al., 1997), human netrin-2 (Van Raay et al., 1997), *Drosophila* netrin-A and netrin-B (Harris et al., 1996; Mitchell et al., 1996), and *C. elegans* UNC-6 (Ishii et al., 1992). Overall amino acid identity is presented, as well as amino acid identity between the different protein domains (Ishii et al., 1992).

Table 3-2: Netrin-1 Causes Changes in Neurite and Growth Cone Morphology and Elongation Rate.

characteristic	condition	*n*	mean ± s.e.m.	p value
Neurite elongation rate	control	20	71.77 ± 9.87	
[μm/h]	netrin-1	20	108.40 ± 11.7	p = 0.025
Number of filopodia	control	100	5.41 ± 0.28	
per growth cone	netrin-1	100	6.64 ± 0.31	p = 0.004
Total filopodial length	control	100	53.83 ± 5.31	
per growth cone [μm]	netrin-1	100	72.00 ± 4.93	p = 0.0133
Growth cone area	control	100	78.11 ± 8.01	
[μm^2]	netrin-1	100	85.42 ± 4.46	p = 0.43
Longest neurite	control	200	138.16 ± 11.8	
per cell [μm]	netrin-1	151	218.43 ± 16.45	p = 0.0001
Total neurite length	control	200	228.37 ± 27.93	
per cell [μm]	netrin-1	151	357.40 ± 30.15	p = 0.002
Number of processes	control	200	1.325 ± 0.037	
arising from cell body	netrin-1	151	1.430 ± 0.051	p = 0.097
Number of branching	control	200	0.52 ± 0.09	
points per cell	netrin-1	150	1.30 ± 0.16	p < 0.0001
Number of branch	control	200	0.001795 ± 0.00034	
points per unit	netrin-1	151	0.003896 ± 0.00055	p = 0.0014
of neurite length				

Table 3-2: Summary of Changes in Growth Cone and Neurite Morphology Caused by Netrin-1.

Values shown represent the mean ± s.e.m.

Table 3-3: RGC Growth Cones Turn Toward a Point Source of Netrin-1.

protein in pipette	antibody in medium [a]	extension [μm/h] [b]	turning angle [deg.] [c]	turning response [%(*n*)] [d] -	0	+	*n*
none		20.7 ± 3.9	- 2.4 ± 3.7	37 (6)	44 (7)	19 (3)	16
netrin-1 (5 μg/ml)	none	19.2 ± 3.4	18.6 ± 6.7 *	19 (3)	0 (0)	81 (13)	16
	α-DCC Ab (1 μg/ml)	16.7 ± 2.2	- 1.3 ± 3.7	46 (6)	31 (4)	23 (3)	13
	r-IgG Ab (2 μg/ml)	20.4 ± 6.7	14.8 ± 5.0 *	8 (1)	25 (3)	67 (8)	12
netrin-1 (heat-inactivated; 5 μg/ml)	none	27.9 ± 4.4	- 1.9 ± 5.0	62 (8)	23 (3)	15 (2)	13

Table 3-3: Chemotropic Response of RGC Growth Cones to Netrin-1.

[a] Antibodies were pre-adsorbed at the indicated concentrations in a small volume to explants for 30 minutes prior to application of netrin-1, then diluted to ~ 0.2 μg/ml during the actual experiment (see Experimental Procedures).

[b] The rate of neurite extension was determined by measuring the trajectory of each neurite during the one hour period of netrin-1 application (Figures 3-7E - F). Values represent the mean ± s.e.m.

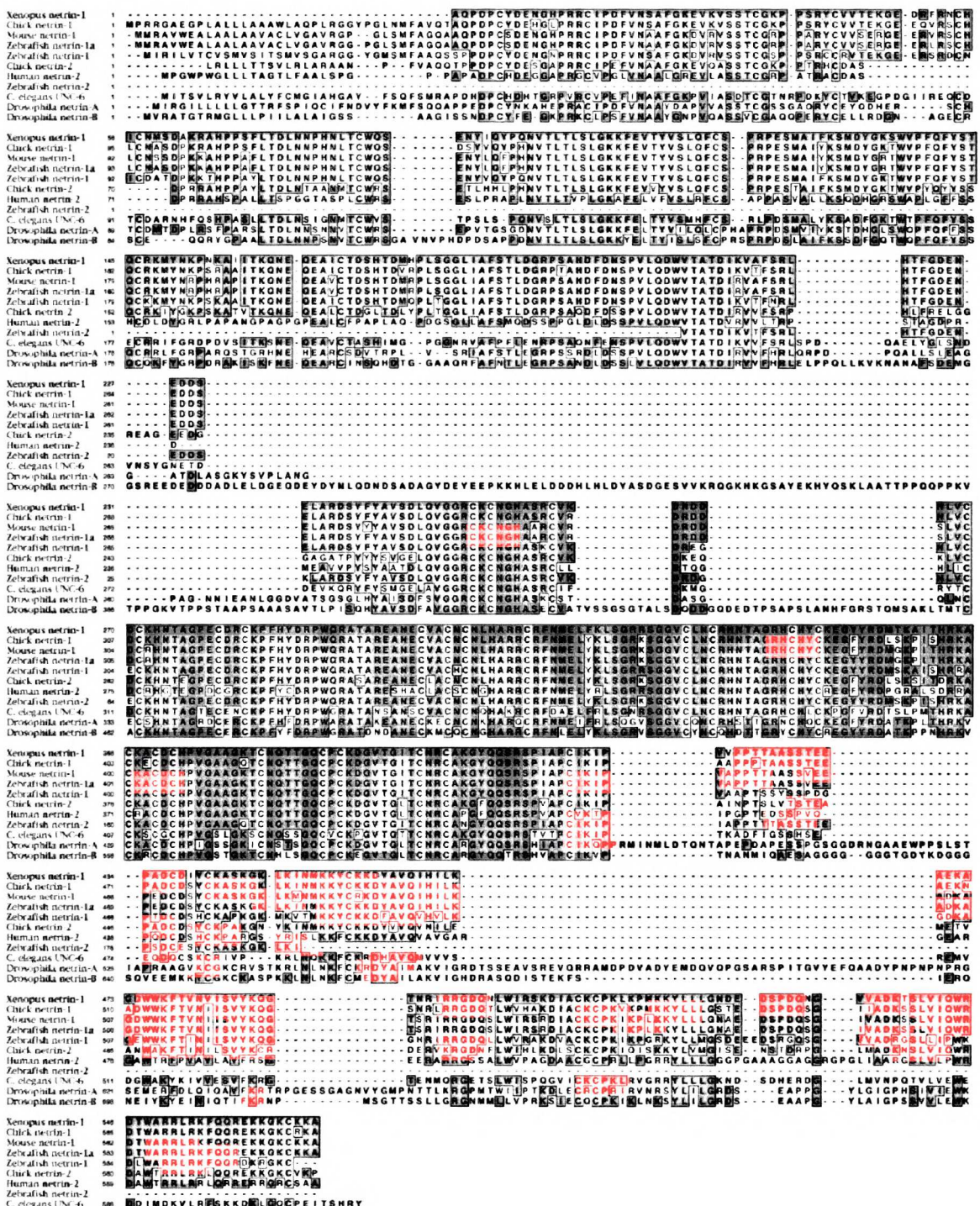
[c] Retinal growth cones were exposed to gradients of netrin-1 protein emanating from a micropipette, and turning angles were measured as described in Experimental Procedures.

[d] Turning angles superior to 5° were scored as positive (toward the gradient source; "+"), angles less than -5° were scored as negative (away from the gradient source; "-") and angles between -5° and 5° were scored as no turning ("0").

* $p < 0.05$ Student's t-test

Figure 3-1: Amino Acid Sequence Alignment of Netrin Proteins.

Alignment of the predicted amino acid sequence of *Xenopus* netrin-1 compared to that of chicken netrin-1 and netrin-2 (Serafini et al., 1994), mouse netrin-1 (Serafini et al., 1996), zebrafish netrin-1a (Lauderdale et al., 1997), netrin-1 (Strähle et al., 1997) and netrin-2 (MacDonald et al., 1997), human netrin-2 (Van Raay et al., 1997), *C. elegans* UNC-6 (Ishii et al., 1992), and *Drosophila* netrin-A and netrin-B (Harris et al., 1996; Mitchell et al., 1996). Overall amino acid identity is presented, as well as amino acid identity between the different protein domains (Ishii et al., 1992). Shaded amino acids are identical to the *Xenopus* netrin-1 sequence. Domain VI is indicated in blue, domain V in green and domain C in red (Ishii et al., 1992). Conserved cysteine residues are shown in yellow.


Xenopus netrin-1
Chick netrin-1
Mouse netrin-1
Zebrafish netrin-1a
Zebrafish netrin-1
Chick netrin-2
Human netrin-2
Zebrafish netrin-2
C. elegans UNC-6
Drosophila netrin-A
Drosophila netrin-B

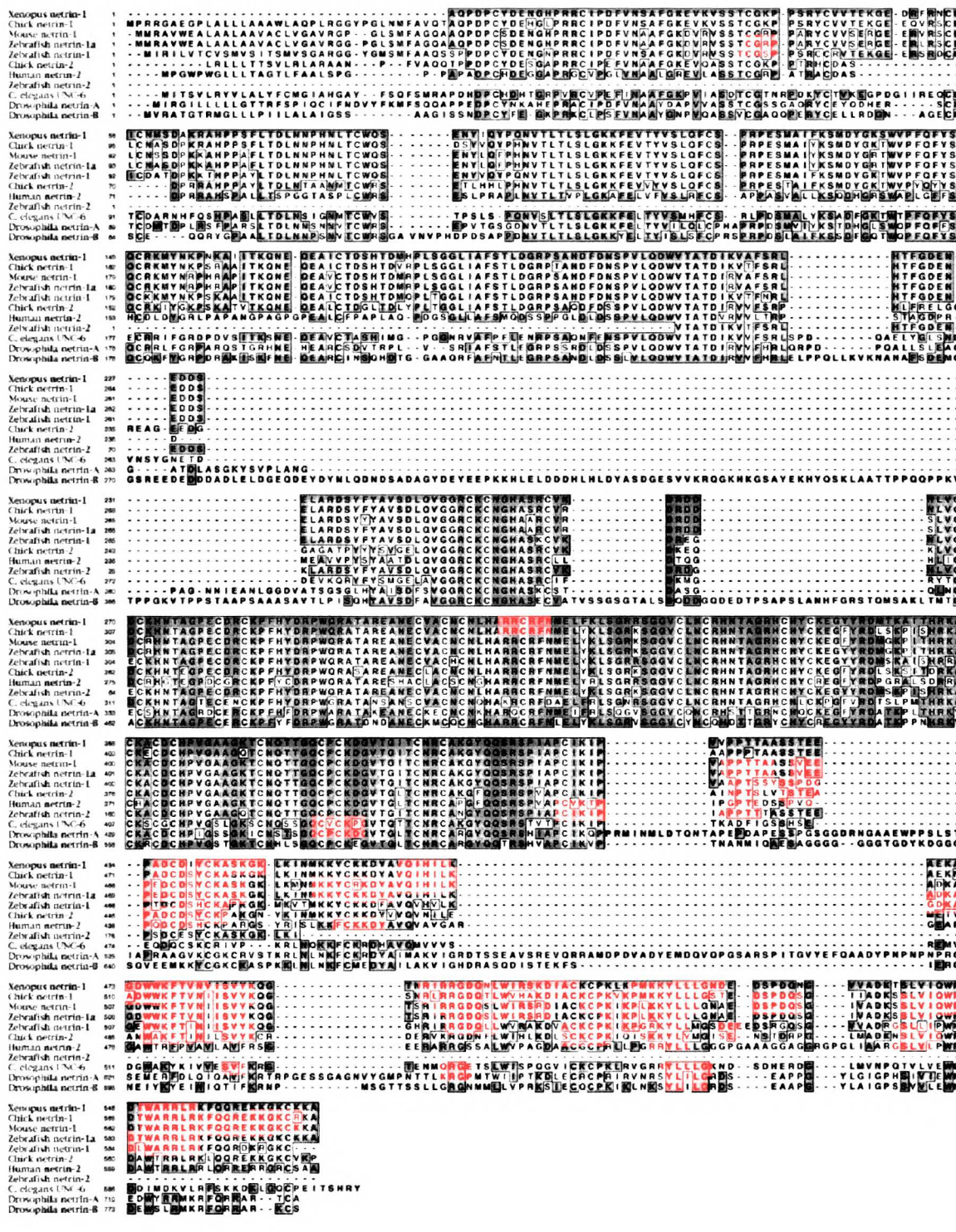

Xenopus netrin-1
Chick netrin-1
Mouse netrin-1
Zebrafish netrin-1a
Zebrafish netrin-1
Chick netrin-2
Human netrin-2
Zebrafish netrin-2
C. elegans UNC-6
Drosophila netrin-A
Drosophila netrin-B

Figure 3-2: Temporal Analysis of *Xenopus Netrin-1* Expression.

(A) Northern blot of stage 28 mRNA probed with a *Xenopus netrin-1* probe. A single transcript of approximately 2.3 kb is detected.

(B) RT-PCR analysis of *Xenopus netrin-1* expression during development. cDNA was made from total RNA of the appropriate stages using random hexamers (Wilson and Hemmati-Brivanlou, 1995) and used to amplify *Xenopus netrin-1* sequences. EF-1α primers (Hemmati-Brivanlou and Melton, 1994) were included as a control for cDNA synthesis and amplification. In lane 18 ("-R.T."), reverse transcriptase was omitted from the reaction as a control.

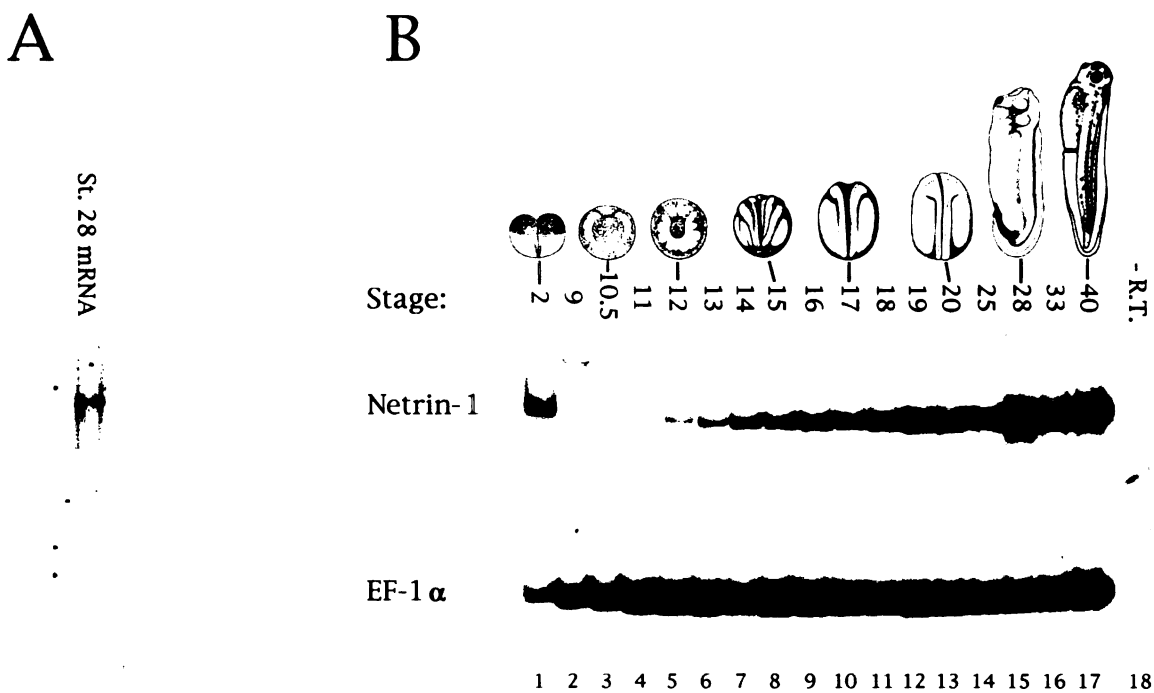

A
St. 28 mRNA
B
Stage:
2
9
10.5
11
12
13
14
15
16
17
18
19
20
25
28
33
40
- R.T.
Netrin-1
EF-1 α
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18

Figure 3-3: Localization of *Xenopus Netrin-1* Transcripts During *Xenopus* Development.

Xenopus netrin-1 transcripts were visualized by whole-mount RNA *in situ* hybridization using a digoxigenin-labeled *netrin-1* antisense riboprobe. In all panels except C, G and H, anterior is to the left. In panels C-I, dorsal is up.

(A) Dorsal view of a late gastrula embryo (stage 12). Arrows indicate two stripes running adjacent to the neural plate midline. Arrowheads show two medio-lateral stripes in the anterior neural plate.

(B) Dorsal view of a stage 19 embryo. Arrowheads indicate expression at the prospective midbrain/hindbrain junction. Arrows point to weak expression adjacent to the CNS midline.

(C) Anterior view of the embryo shown in (B). Arrowheads indicate expression in the prospective midbrain/hindbrain junction. Also, note expression in the prospective lateral diencephalon, presumptive optic vesicles and optic stalk.

(D) Head-on view of a stage 25 embryo. *Netrin-1* is expressed in ventral retina and forebrain, and in the floor plate at the ventral midline of the spinal cord.

(E) Lateral view of a tailbud stage embryo (stage 27). Note staining in the ventral retina, the somites and the branchial arches.

(F) Lateral view of another stage 27 embryo cleared in benzyl benzoate/benzyl alcohol to show expression in the neural tube. Notice staining in the ventral midline domain of the neural tube.

(G) Transverse section through the trunk region of the embryo shown in (B). Expression is highest at the midline of the neural plate, but lower levels of mRNA can also be detected lateral in the neural plate.

(H) Transverse vibratome section through the trunk region of a stage 28 embryo after whole mount RNA *in situ* hybridization with a *netrin-1* probe. Notice that *netrin-1* is expressed in a ventral midline domain slightly larger than the morphological floor plate.

(I) Lateral view of the isolated anterior CNS of a stage 28 embryo showing netrin-1 expression in the ventral midline at all axial levels. Arrowheads mark the boundaries between forebrain, midbrain, hindbrain and spinal cord.

b, branchial arches; e, eye; f, forebrain; fp, floor plate; h, hindbrain; m, midbrain; n, notochord; np, neural plate; ov, optic vesicle; pld, prospective lateral diencephalon; pov, presumptive optic vesicle and optic stalk; s, somite; sc, spinal cord.

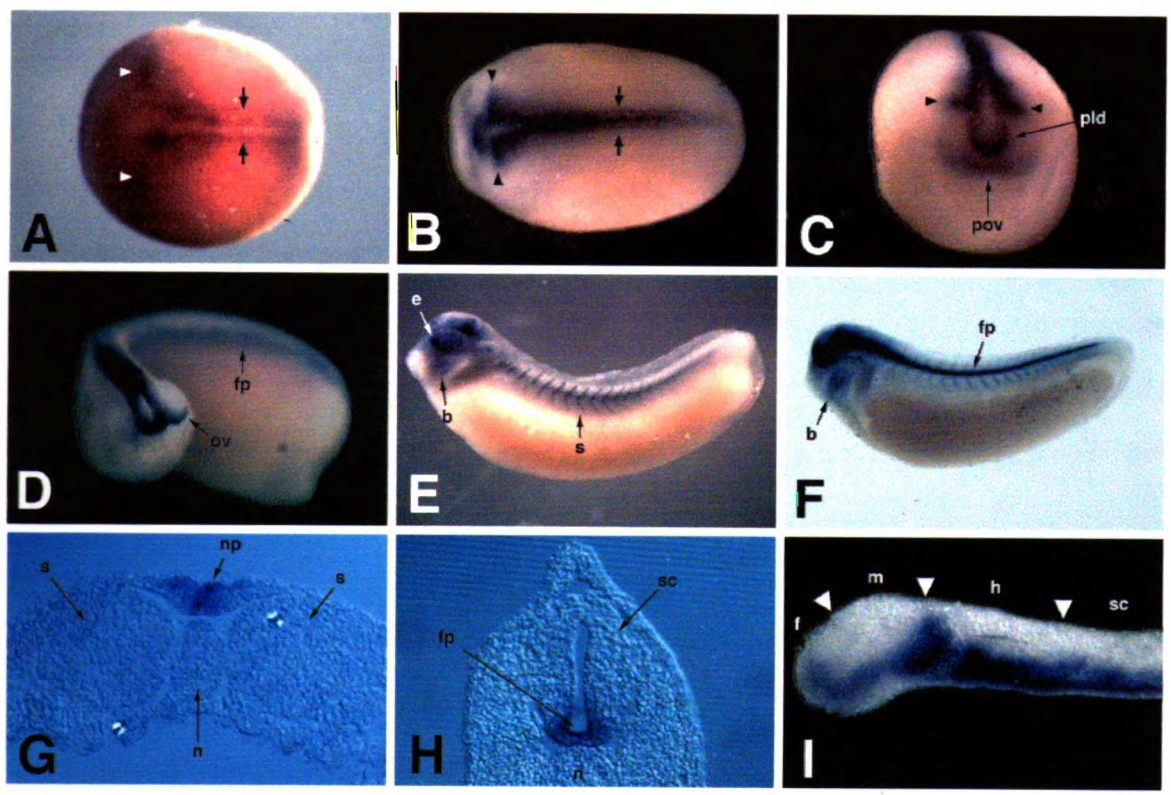

A
B
C
pld
pov
D
fp
ov
E
e
b
s
F
fp
b
G
np
s
s
n
H
sc
fp
n
I
m
h
sc
f

Figure 3-4: Expression of *Xenopus Netrin-1* and DCC in the Developing Visual System.

(A) Lateral view of the optic vesicle of a stage 27 embryos after whole mount RNA *in situ* hybridization with an antisense *netrin-1* riboprobe. Note expression adjacent to the choroid fissure, particularly on the nasal side. Anterior is to the left and dorsal is up.

(B) Transverse vibratome section through the optic vesicle, optic stalk and ventral diencephalon of a stage 27 embryo after whole mount RNA *in situ* hybridization with an antisense *netrin-1* riboprobe. *Netrin-1* expression is detected in the ventral optic vesicle and optic stalk, and laterally in the diencephalon.

(C-F) Transverse (C, D and F) and saggital (E) sections through the retina of a stage 40 embryo, hybridized with an antisense *netrin-1* riboprobe. Note expression ventrally in the nasal retina (C), in the optic nerve head (D) and in the ventral optic nerve (E and F).

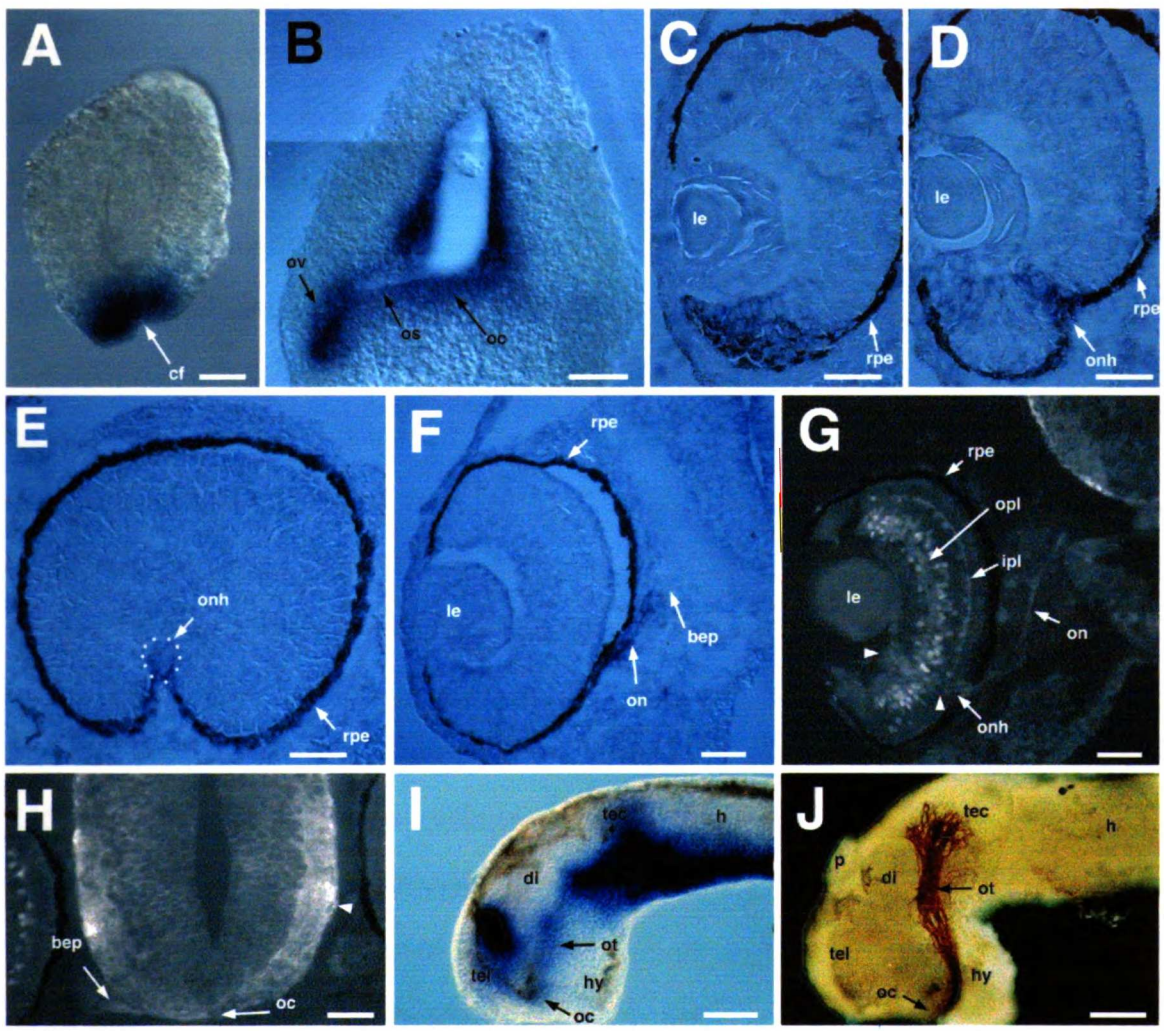
(G and H) Transverse sections through a stage 40 embryo showing staining with an anti-DCC antiserum. Note staining of RGC axons in the fiber layer, optic nerve head and optic nerve (G). Weaker staining is also observed in the inner and outer plexiform layers. (H) DCC staining of RGC axons at the optic chiasm. Arrowhead indicates RGC axons in the presumptive optic tract.

(I) Lateral view of the isolated anterior CNS of a stage 34 embryo after whole mount RNA *in situ* hybridization with an antisense *netrin-1* riboprobe. Note staining in the prospective optic tract, developing forebrain and midbrain.

(J) Lateral view of a whole-mounted stage 40 brain with a HRP-filled optic tract. Compare labeling of the optic tract with the *netrin-1* expression shown in (I).

bep, brain entry point of optic nerve; cf, choroid fissure; di, diencephalon; hy, hypothalamus; ipl, inner plexiform layer; le, lens; oc, optic chiasm; on, optic nerve; onh, optic nerve head; opl, outer plexiform layer; os, optic stalk; ot, optic tract; p, pineal; rpe, retinal pigmented epithelium; tec, optic tectum; tel, telencephalon.

The scale bars correspond to 50 μm (A-H) and 100 μm (I and J).


A
cf
B
ov
os
oc
C
le
rpe
D
le
rpe
onh
E
onh
rpe
F
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le
bep
on
G
rpe
opl
ipl
le
on
onh
H
bep
oc
I
tec
h
di
ot
tel
hy
oc
J
tec
h
p
di
ot
tel
hy
oc

Figure 3-5: Netrin-1 Promotion of Retinal Neurite Outgrowth *In Vitro* Requires the Netrin Receptor DCC.

Dissociated retinal cells were grown on various substrates. The length of the longest neurite of 30 randomly-selected neurons was measured and tabulated (see Experimental Procedures) for a minimum of three independent experiments with duplicate cultures for each condition. Neurite lengths are shown as the mean ± s.e.m. (in μm). Numbers in parenthesis represent the sample size (*n*) for each condition.

(A) Graph of the mean length of the longest neurite of dissociated retinal neurons grown on a variety of substrates in the presence or absence of recombinant netrin-1 protein (300 ng/ml). On both laminin- and fibronectin-coated coverslips (previously coated with poly-L-ornithine), netrin-1 induced a nearly two fold increase in retinal neurite length. No significant stimulation of outgrowth by netrin-1 was observed on poly-L-ornithine alone.

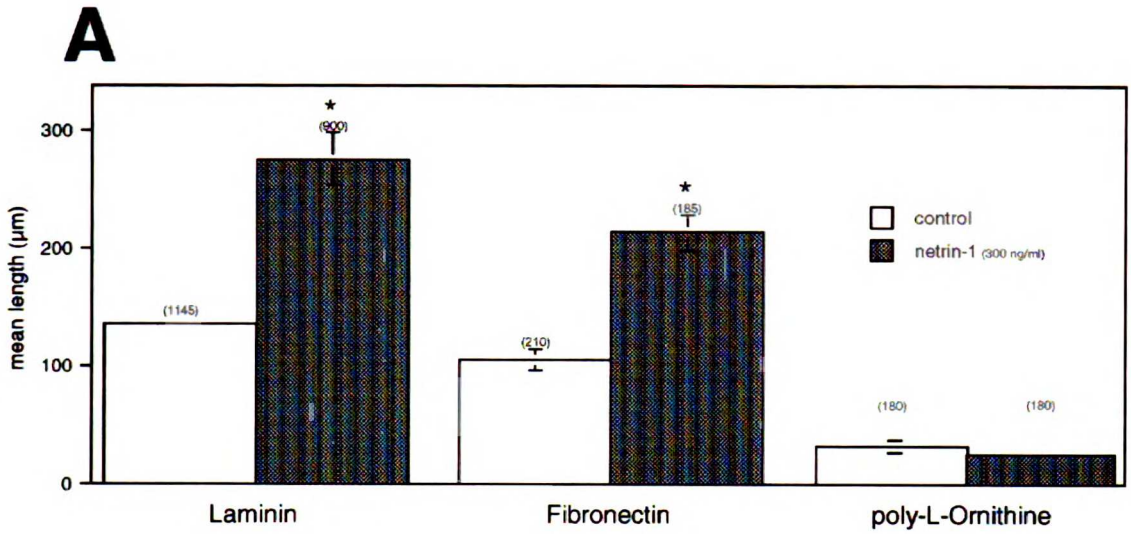
(B) Cumulative histogram of the length of the longest neurite of retinal neurons grown on laminin in the presence or absence of netrin-1. Dashed line represents control; solid represents cultures containing netrin-1 (300 ng/ml). Ordinate corresponds to the percentage of neurites in each condition that are longer than the indicated length (abscissa). The median neurite length for each population is indicated by the dashed blue lines (78 μm and 246 μm for control and netrin-1 cultures, respectively). Sample sizes were 692 and 625 for control and netrin-1, respectively.

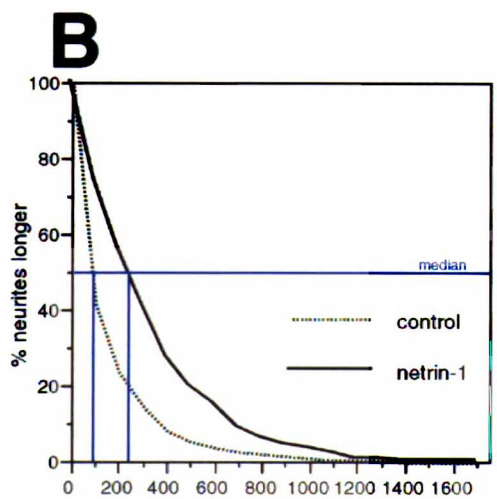
(C) Graph of retinal neurite length in response to different concentrations of soluble netrin-1. Abscissa indicates the concentration of netrin-1 protein in the medium (shown as a log scale), while ordinate shows mean length of longest neurite of each neuron. Retinal neurites responded to netrin-1 in a dose-dependent manner, with maximal response at 300 ng/ml netrin-1, and a decrease in response at higher concentrations.

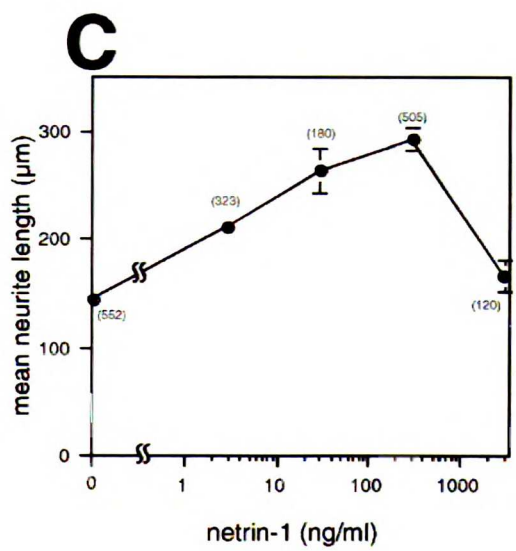
(D) Effect of antibodies to DCC on netrin-1-induced retinal neurite growth. Anti-DCC antibodies (2 μg/ml) abolished retinal neurite outgrowth in response to 300 ng/ml netrin-1

but not to 20 ng/ml FGF-2. Control antibodies (2 μg/ml) had no effect on netrin-1-induced outgrowth.

* $p < 0.05$, ANOVA and Bonferroni t-test


A
mean length (µm)
300
200
100
0
(900)
(185)
(1145)
(210)
(180)
(180)
control
netrin-1 (300 ng/ml)
Laminin
Fibronectin
poly-L-Ornithine


B
% neurites longer
100
80
60
40
20
0
median
control
netrin-1
0 200 400 600 800 1000 1200 1400 1600


C
mean neurite length (µm)
300
200
100
0
(505)
(180)
(323)
(552)
(120)
0 1 10 100 1000
netrin-1 (ng/ml)

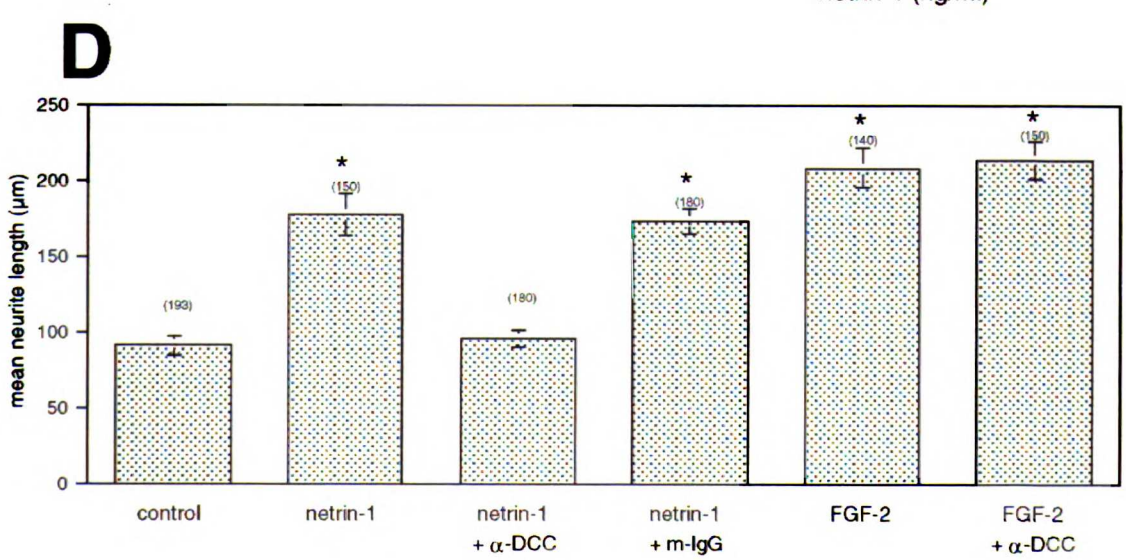

D
mean neurite length (µm)
250
200
150
100
50
0
(193)
(150)
(180)
(180)
(140)
(150)
control
netrin-1
netrin-1 + α-DCC
netrin-1 + m-IgG
FGF-2
FGF-2 + α-DCC

Figure 3-6: Netrin-1 Affects Neurite and Growth Cone Morphology *In Vitro*.

The morphology of retinal growth cones on a laminin substrate is more complex in presence of 300 ng/ml netrin-1 (B) than in its absence (A). Scale bar in (B), 20 μm.

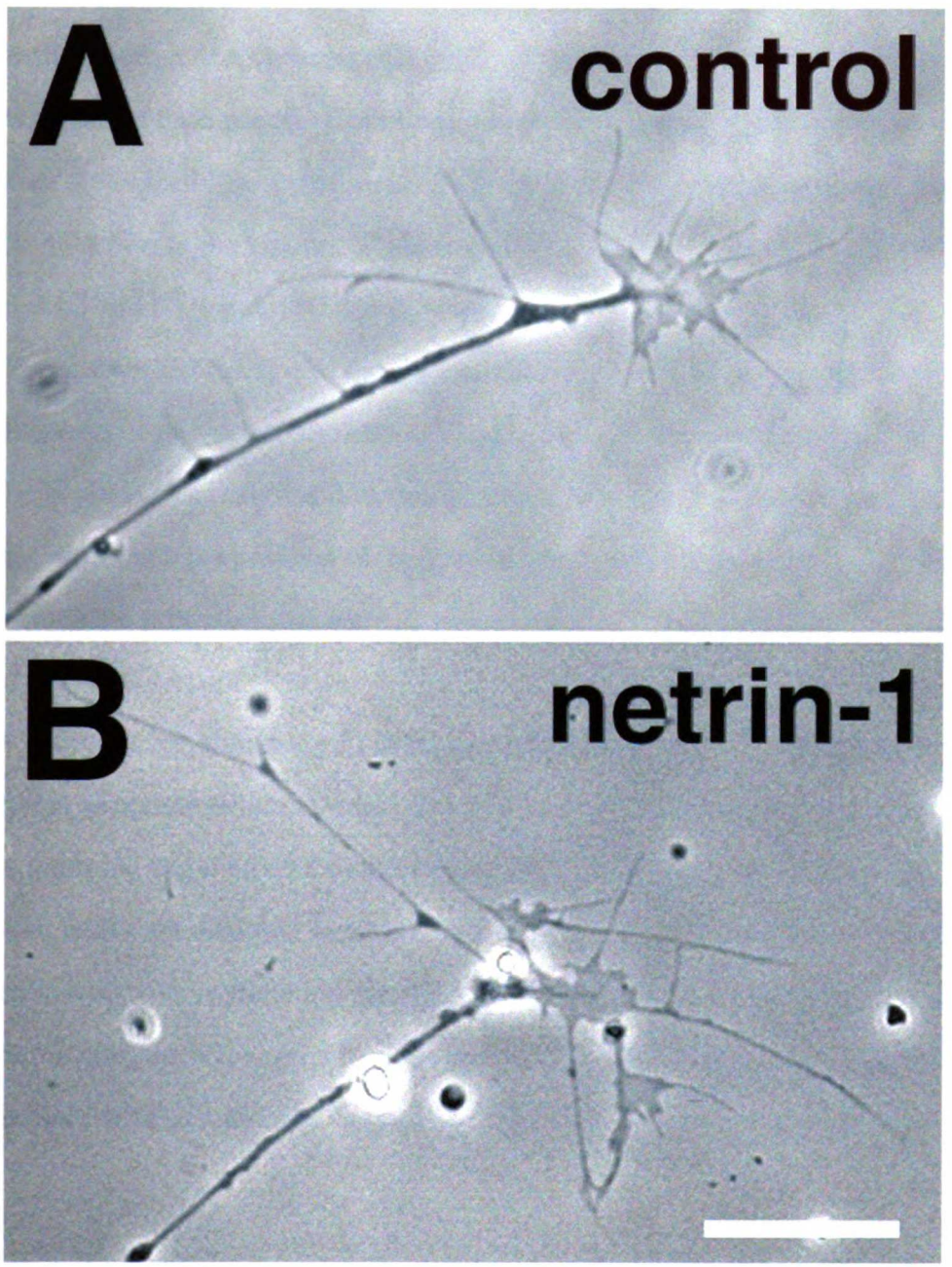
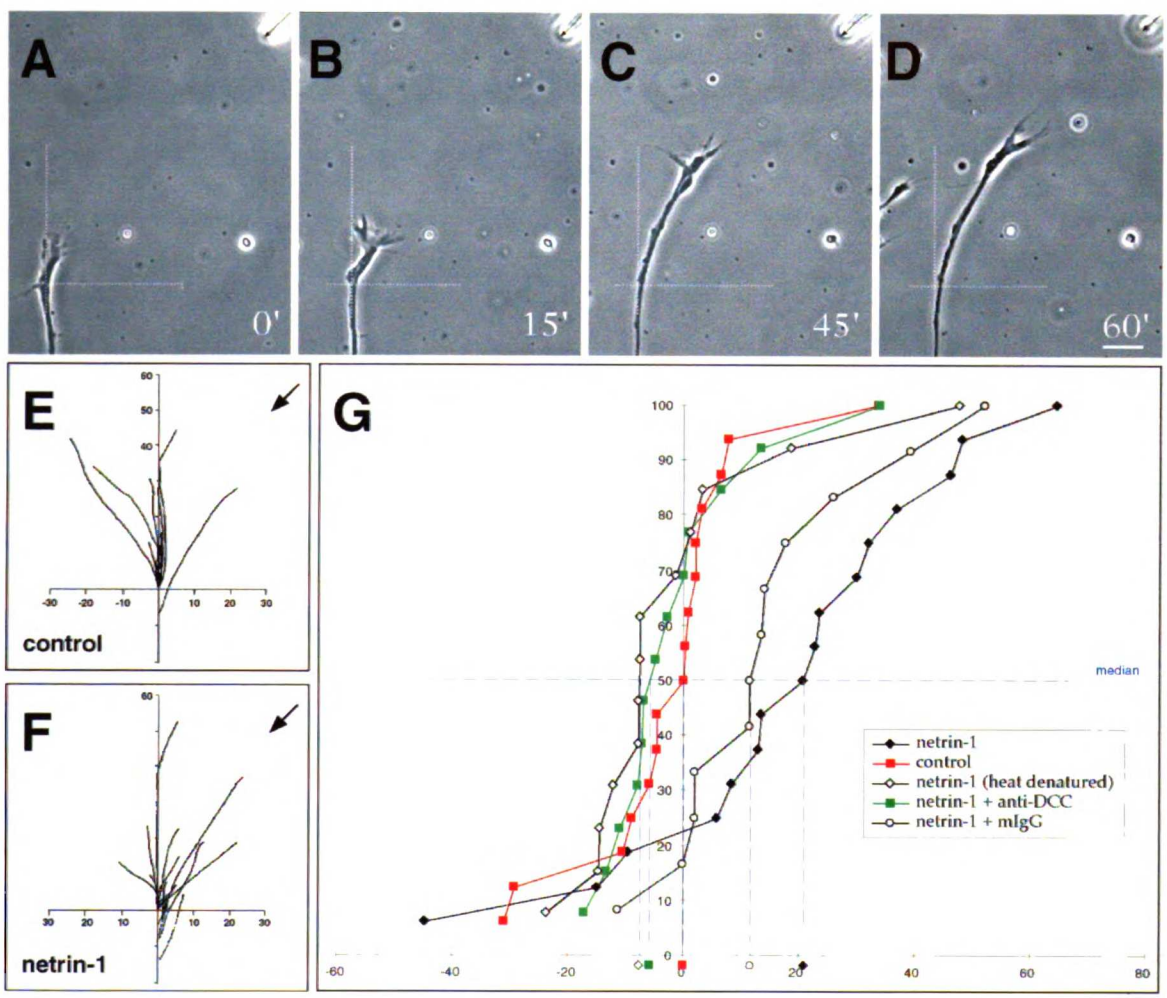

A
control
B
netrin-1

Figure 3-7: Turning Response of *Xenopus* Retinal Neurites in a Netrin-1 Gradient Requires DCC Function.

(A-D) Time-lapse series of a growth cone exposed to a gradient of netrin-1 created by pulsatile ejection of a concentrated protein solution from a micropipette (arrow in upper right corner of each panel). Coordinates in each panel indicate the position of the growth cone at the onset of the experiments (A). By 15 minutes (B), a clear reorientation can be observed towards the source. Complete reorientation and growth toward the source are seen at 45 and 60 minutes (C and D, respectively). Scale bar, 10 μm.

(E-F) Composite drawings of the migration paths of neurites in control conditions (E), and in the presence of a netrin-1 gradient (F) during a one hour period following the application of the gradient. The original direction of growth was aligned with the ordinate axis and the origin represents the position of the growth cone at the onset of the experiment. Dashed lines indicate processes that were displaced by growth cone turning. Tick marks on both axes represent 10 μm.

(G) Cumulative histogram of turning angles of neurites in netrin-1 gradients under different experimental conditions. The ordinate axis indicates the percentage of neurites with a turning angle inferior to the value shown on the abscissa. Under conditions where growth cones are attracted (with netrin-1 alone, or netrin-1 with a control antibody), the distribution curve is shifted to the right, with a median value significantly higher than 0°. Under conditions where growth cones are not attracted (control, heat-inactivated netrin-1, or netrin-1 with an anti-DCC antibody), the distribution curve centers close to 0°.


A
0'
B
15'
C
45'
D
60'
E
control
F
netrin-1
G
median
netrin-1
control
netrin-1 (heat denatured)
netrin-1 + anti-DCC
netrin-1 + mIgG

Chapter Four

Role of Netrin-1 in Retinal Axon Guidance in the Developing Optic Tract

Summary

Commissural and retinal axons can orient their growth *in vitro* toward a source of netrin-1 protein. Recent evidence suggests that the growth cones at the tips of these axons are turning in response to gradients of soluble netrin-1 protein. However, it remains unclear whether netrin-1 *in vivo* is freely diffusible or bound to cell surfaces or other extracellular matrix proteins. Over a large portion of the optical projection, retinal ganglion cell axons migrate over and remain restricted to tracts of netrin-1-expressing cells. We show here that disrupting the discrete localization of netrin-1 protein in the optic tract with exogenous netrin-1 protein leads to severe retinal axon pathfinding defects *in vivo*. Furthermore, we show that substrate-bound netrin-1 is capable of promoting retinal neurite outgrowth *in vitro*. These results suggest that netrin-1 can act as a positive, short-range guidance cue, potentially by mediating contact attraction in extending retinal axons.

Introduction

Axons growing in the developing nervous system must carefully select their pathway in order to reach and innervate their appropriate targets. To accomplish this task, growth cones, the sensory structures at the tip of axons, must be able to identify guidance cues in their environment and respond accordingly. A variety of cellular and molecular mechanisms have been proposed to explain the efficient and reproducible navigation of these neuronal processes through the developing embryonic environment and to their final targets. These mechanisms can be divided into four principal classes of guidance cues: short-range and long-range cues, which can exert either an attractive or repellent force on the extending growth cones. Short-range cues, in the form of non-diffusible molecules bound to cell surfaces or to the extracellular matrix (ECM), may direct axon growth by inhibiting extension in inappropriate directions (contact repulsion), or by providing a favorable substrate for growth (contact attraction). Such locally-acting cues are thought to be involved in processes such as selective fasciculation of extending axons to pre-existing axon tracts (reviewed in Tessier-Lavigne and Goodman, 1996). In contrast to these contact-mediated guidance cues, axon migration can also be guided by long-range gradients of diffusible molecules, a mechanism known as chemotropism. This mechanism implies that growth cones respond at a distance to these gradients by either turning toward the source of chemoattractant or, conversely, by growing away from a source of chemorepellent. In practice, experimental evidence indicates that extending axons *in vivo* are subjected to the combined effect of these four classes of guidance cues, and that the resultant pathway selection reflects a balanced response to all environmental factors.

One family of axon guidance molecules, the netrins, appears to function in more than one type of guidance mechanism. These laminin-related secreted proteins have been shown to mediate both the attractive guidance of axons to intermediate targets, as well as the inhibitory activity of repellent tissues in vertebrates, *Caenorhabditis elegans* and

Drosophila (Tessier-Lavigne and Goodman, 1996). In the vertebrate central nervous system (CNS), netrin-1 has been shown to mediate at least in part the attractive activity of the ventrally-located floor plate on the ventral migration of commissural axons (Tessier-Lavigne et al., 1988; Placzek et al., 1990a; Kennedy et al., 1994; Serafini et al., 1994; Shirasaki et al., 1995; Serafini et al., 1996; Shirasaki et al., 1996). Likewise, netrin homologues are known to direct the guidance of axons to the ventral midline in both *C. elegans* and *Drosophila* (Hedgecock et al., 1990; McIntire et al., 1992; Harris et al., 1996; Mitchell et al., 1996; Wadsworth et al., 1996). Experiments have also shown that the netrins can act as chemorepellents for axons that normally project away from the ventral midline in both *C. elegans* and vertebrates (Hedgecock et al., 1990; Colamarino and Tessier-Lavigne, 1995; Wadsworth et al., 1996; Varela-Echavarria et al., 1997). Moreover, netrins have also been implicated in the guidance of a number of axons in regions of the embryo apart from the ventral midline of the nervous system (Kennedy et al., 1994; Mitchell et al., 1996; Serafini et al., 1996; Wang et al., 1996; Lauderdale et al., 1997; Livesey and Hunt, 1997; Métin et al., 1997; Richards et al., 1997; Strähle et al., 1997), including the developing visual system (de la Torre et al., 1997; Deiner et al., 1997).

Recent studies have shown that the chemotropic ability of netrin-1 to guide axon growth at a distance may be due to a direct effect of netrin on the extending growth cone: *Xenopus* retinal and spinal growth cones turned rapidly, usually within minutes, toward the source of a gradient of soluble netrin-1 in a cell-free environment *in vitro* (de la Torre et al., 1997; Ming et al., 1997). These experiments argue that, at least in this *in vitro* setting, growth cones are capable of responding to a gradient of netrin-1 in solution, rather than absorbed to the substrate. Nevertheless, molecules such as nerve growth factor (NGF) and laminin are known to affect neurite outgrowth *in vitro* both as soluble and substrate-bound molecules (Gundersen and Barrett, 1979; Gundersen, 1985; Rivas et al., 1992). Therefore, it remains possible that *in vivo* netrin-1 acts as a substrate-bound guidance cue,

particularly given the non-specific association of netrins with cell membranes (Kennedy et al., 1994; Serafini et al., 1994; Mirzayan, 1997).

Although in both the developing spinal cord and the developing retina axons are attracted to a netrin-1-secreting intermediate target (the floor plate and the optic disc, respectively), there appear to be differences in mechanism of netrin-1 action in each case. In contrast with the spinal cord where there is good evidence for long-range gradients of netrin-1 protein (MacLennan et al., 1997, and T.E. Kennedy and M. Tessier-Lavigne, manuscript in preparation), immunological studies of netrin-1 protein distribution in the mouse retina argue for a short-range role of netrin-1 in the guidance of retinal ganglion cell (RGC) axons in the vicinity of the optic disc (Deiner et al., 1997). Furthermore, transplantation experiments in chick embryonic retinae have failed to demonstrate a long-range effect of the optic disc on RGC axon guidance (Halfter, 1996). These results, along with the observation that retinal axons migrate along, rather than toward, cells expressing netrin-1 in the optic nerve and in the optic tract (de la Torre et al., 1997; Deiner et al., 1997; Lauderdale et al., 1997; MacDonald et al., 1997; Strähle et al., 1997), raise the possibility that netrin-1 may guide RGC axons by a short-range, if not contact-mediated, mechanism in the developing visual system.

In order to address this question, we have once more focused our attention on the development of the optic projection in *Xenopus*. As mentioned above, netrin-1 is known to be expressed in the developing visual system in a number of vertebrate species (Kennedy et al., 1994; Serafini et al., 1996; de la Torre et al., 1997; Deiner et al., 1997; Lauderdale et al., 1997; MacDonald et al., 1997; Strähle et al., 1997), can promote the outgrowth and turning of retinal axons *in vitro* (Wang et al., 1996; de la Torre et al., 1997; Deiner et al., 1997), and has been implicated in the guidance of retinal ganglion cell axons out of the retina (Deiner et al., 1997). We show here that, as in other species (Lauderdale et al., 1997; Strähle et al., 1997), retinal ganglion cell axons grow in the optic tract along a population of *netrin-1*-expressing cells. To examine the role played by netrin-1 in the

establishment of the optic tract, we have taken advantage of an *in vivo* embryological preparation which allows us to expose growing RGC fibers in the optic tract to a variety of reagents (Chien et al., 1993). This exposed brain preparation has previously been used to investigate the role of a variety of factors, including growth factors and proteoglycans, as well as the cell biology involved in the guidance of RGC axons *in vivo* (Chien et al., 1993; McFarlane et al., 1995; Worley and Holt, 1996; Walz et al., 1997). We find that the addition of ectopic netrin-1, or of a truncated form of netrin-1 lacking the charged carboxy-terminal domain, disrupts the migration of retinal ganglion cell axons in the optic tract and leads to a failure of these fibers to properly recognize their targets in the optic tectum. Furthermore, we find that substrate-bound netrin-1 is as efficient at promoting retinal neurite outgrowth *in vitro* as soluble protein. Together, these findings raise the possibility that discrete tracts of substrate-bound netrin-1 protein in the ECM may provide an instructive cue for axon guidance, and suggest a possible role for netrin-1 in the guidance of RGC axons in the optic tract.

Results

Ectopic Netrin-1 or Netrin-1(VI-V) Disrupt RGC Axon Guidance in the Optic Tract

Recent experiments have implicated netrin-1 and its receptor Deleted in Colorectal Cancer (DCC) in the guidance of retinal ganglion cell (RGC) axons out of the developing retina (Wang et al., 1994; de la Torre et al., 1997; Deiner et al., 1997). In addition, *netrin-1* is also expressed in the optic tract of a variety of vertebrate embryos (Kennedy et al., 1994; Lauderdale et al., 1997; Strähle et al., 1997), including *Xenopus* (Figure 3-4H; de la Torre et al., 1997), raising the possibility that netrin-1 may also guide retinal axons from the optic chiasm to their targets in the optic tectum. In order to perturb potential guidance cues provided by the localization of endogenous netrin-1 protein, exogenous netrin was applied to the entire optic tract using an exposed brain preparation (Figure 4-1A; Chien et al., 1993). Two isoforms of netrin-1 protein were used for these experiments (Figure 4-1B): a full length form of the mature chick netrin-1 protein, referred to as netrin-1, and a truncated form of chick netrin-1 lacking the highly charged C-terminal domain, netrin-1(VI-V) (Mirzayan, 1997). Netrin-1(VI-V) has previously been shown to act as an antagonist of the spinal commissural axon outgrowth-promoting activity of netrin-1 *in vitro* (Mirzayan, 1997). Netrin-1 protein isoforms were applied to the exposed left side of the brain of stage 33/34 embryos at a time when the first RGC axons have entered the optic chiasm (Holt, 1984). Embryos were incubated under control or experimental conditions until reaching stage 40, at which stage most RGC axons have reached their targets in the midbrain (Holt, 1984). The optic projection was then visualized by filling the axons with horse radish peroxidase (HRP; see Experimental Procedures).

Antibody staining against the myc epitope tags reveals that, over the course of the experiment, netrin-1 and netrin-1(VI-V) bind to the basal surface of the exposed neuroepithelium (Figures 4-1C and 4-1D, respectively). Although the netrin-1 isoforms do

not penetrate the neuroepithelium to the same extent as other proteins (e.g. FGF-2; McFarlane et al., 1995), using high concentrations of the proteins (1-100 μg/ml), we were able to detect anti-myc staining up to the depth at which axons within the optic tract are extending (~10 μm; Silver and Rutishauser, 1984; Bovolenta and Mason, 1987; Easter and Taylor, 1989; Holt, 1989). Although these protein concentrations are approximately one thousand-fold higher than those used to stimulate outgrowth of commissural or retinal axons *in vitro* (Serafini et al., 1994; Shirasaki et al., 1995; Shirasaki et al., 1996; Wang et al., 1996; de la Torre et al., 1997; Deiner et al., 1997; Métin et al., 1997; Ming et al., 1997), it is possible that the actual concentration in the vicinity of the retinal growth cones is closer to the physiologically relevant range.

RGC axons normally follow a stereotypical path along the optic tract to reach their targets in the optic tectum (Figure 4-2A). These axons exit the optic chiasm in a tight bundle, then extend dorsally through the forebrain, make a 45° dorso-posterior turn in the mid-diencephalon toward the midbrain, and finally enter the tectum where they defasciculate and arborize before innervating their targets (Figure 4-2B). The optical projection of embryos incubated under control conditions was not different from that observed in unoperated embryos (data not shown). However, several types of pathfinding errors occur in embryos exposed to either netrin-1 or netrin-1(VI-V) (Figures 4-2C - F). These errors are summarized in Table 4-1 and described in further detail below.

Failure to Remain in the Optic Tract. The most common pathfinding error involves retinal fibers leaving the optic tract before reaching the tectum, and extending abnormally into the forebrain (Table 4-1). RGC fibers normally remain in a tight cohesive bundle as they extend through the optic tract (Figure 4-2A). However, in the presence of ectopic netrin-1 or netrin-1(VI-V) these axons tend to defasciculate and migrate into the anterior forebrain (Figures 4-2C - D and 4-3C - F). In some instances, these axons are seen leaving the optic tract in bundles, occasionally entering the anterior commissure (Figures 4-2E - F). In some individuals, these axons then cross over the dorsal midline to the

contralateral forebrain forming a commissure anterior to the pituitary (Figures 4-2E - G). However, once in the opposite forebrain, these axons tend to stall (Figure 4-2F). In addition, some retinal axons are observed to migrate deep into the diencephalon, away from their normal position at the pial surface of the brain (Silver and Rutishauser, 1984; Bovolenta and Mason, 1987; Easter and Taylor, 1989; Holt, 1989) (Figure 4-1E).

Failure to Terminate and Arborize in the Optic Tectum. Another type of mistargeting phenotype involves retinal axons that pathfind correctly to the region of the optic tectum, but fail to recognize their target. These fibers either continue to elongate beyond the tectum, extending posteriorly toward the hindbrain (Figures 4-3C and 4-3F) or turn sharply dorsally along the border of the tectum and migrate toward the dorsal midline (Figures 4-2C - D). In several embryos, these retinal axons migrate across the dorsal midline into the contralateral midbrain, forming a new commissure in the anterior midbrain, and go on to innervate the contralateral tectum (Figures 4-2E - G and 4-3G - H).

Formation of an Abnormal Ipsilateral Projection. Finally, in a significant number of embryos, retinal fibers are seen abnormally exiting from the optic chiasm into the ipsilateral optic tract and extending toward the ipsilateral optic tectum (Table 4-1; Figure 4-2H). Such a projection is very unusual since most frogs, including *Xenopus*, do not form an ipsilateral projection to the optic tectum until after metamorphosis. This mistargeting phenotype does not appear to be an HRP labeling artefact, such as the inadvertent labeling of retinal fibers left over from the removed contralateral eye, since it occurs very rarely in control embryos (Table 4-1).

Dose Dependence of Netrin-1- and Netrin-1(VI-V)-Induced Pathfinding Errors

Table 4-1 shows that the proportion of optic projections with targeting errors increases with the concentration of netrin-1 or netrin-1(VI-V) in the culture medium. However, it is important to note that we are unable to estimate the concentration of netrin protein within

the neuroepithelial tissue. In order to measure the degree to which these pathfinding mistakes were dependent on the concentration of exogenous protein, we quantified the extent of defasciculation in control and experimental optic projections. Two different methods were used to gauge defasciculation. First, the width of the optic tract was measured mid-way between the optic chiasm and the rhombic isthmus (Figure 4-4A; see Experimental Procedures). Normally, the optic projection remains compact throughout the forebrain, and then spreads and arborizes once it reaches the tectum. However, in both netrin-1- and netrin-1(VI-V)-treated brains, the mean mid-optic tract width of the optic projection is greater than in the control and increases with increasing concentration of exogenous protein (Figures 4-4B and 4-4C, respectively).

The second method we used to estimate the degree of defasciculation was based on measuring the surface area of the entire optic tract. Using camera lucida drawings, we measured the area of the optic projection, and normalized it to the area occupied by a region of the brain anterior to a line drawn between the rhombic isthmus and the cranial flexure in order to account for variability in brain size (Figure 4-5A; see Experimental Procedures). Using this method of quantitation, we found a clear effect of netrin-1(VI-V) on the defasciculation of retinal axons (Figure 4-5C). However, in contrast to the results with the measurements of the mid-optic tract width, we were unable to demonstrate an effect of netrin-1 above background (Figure 4-5B). This may reflect in part the fact that a smaller number of embryos was analyzed using the area ratio method.

Substrate-Bound Netrin-1 Stimulates *Xenopus* Retinal Neurite Outgrowth *In Vitro*

Soluble netrin-1 protein stimulates retinal neurite growth in a number of vertebrate species (Wang et al., 1996; de la Torre et al., 1997; Deiner et al., 1997), and can elicit a chemotactic response from *Xenopus* retinal and spinal neurites *in vitro* (de la Torre et al., 1997; Ming et al., 1997). However, the observation that retinal fibers in the optic tract

appear to migrate along, rather than toward, a population of netrin-1-expressing cells (Figure 4-1A; Kennedy et al., 1994; de la Torre et al., 1997; Deiner et al., 1997; Lauderdale et al., 1997; Livesey and Hunt, 1997; Strähle et al., 1997), as well as the fact that ectopic netrin-1 or netrin-1(VI-V) can disrupt retinal axon guidance in exposed *Xenopus* brain (this study), suggests that netrin-1 may also function as a substrate-bound cue for the guidance of retinal axons *in vivo*.

In order to begin addressing this possibility, we examined whether netrin-1 bound to the substrate could stimulate the outgrowth of neurites in dissociated *Xenopus* retinal cultures. These mixed cultures are expected to contain RGCs as well as other retinal cell types. As previously described, soluble netrin-1 (300 ng/ml) strongly stimulates retinal neurite growth on a substrate of laminin at sub-optimal concentrations (5 μg/ml; see Experimental Procedures), but does not enhance growth on poly-L-ornithine (Figures 3-5A and 4-6, and de la Torre et al., 1997). However, neurite outgrowth was strongly stimulated when neurons were grown on glass coverslips coated with a mixture of laminin and netrin-1 (0.5 μg/ml and 10 μg/ml, respectively; see Experimental Procedures), compared to a substrate of laminin alone (Figure 4-6). Supplementing the culture medium with additional netrin-1 (300 ng/ml) did not further stimulate neurite outgrowth on the laminin/netrin-1 substrate (Figure 4-6). Interestingly, substrate-bound netrin-1 alone does not strongly stimulate neurite outgrowth (Figure 4-6). This observation raises the possibility that extending growth cones may require an appropriate substrate to extend on (e.g. laminin) before responding to the outgrowth-promoting activity of netrin-1. Alternatively, these results may indicate a synergistic effect between the netrins and laminin.

These data suggest that netrin-1 may be able to stimulate neurite outgrowth when bound to the substrate. This hypothesis is supported by the observation that netrin proteins strongly associate with cell membranes (Kennedy et al., 1994; Serafini et al., 1994, and Figures 4-1C and 4-1D of this study), and has led to the proposal that the netrins may act

as substrate-bound guidance cues, rather than freely diffusible proteins to guide axons *in vivo* (discussed in Kennedy et al., 1994; Serafini et al., 1996; Tessier-Lavigne and Goodman, 1996; de la Torre et al., 1997; Deiner et al., 1997; MacLennan et al., 1997). It should be noted, however, that it is in principle possible that the substrate-bound netrin-1 may become soluble over the course of the experiment and act on the neurites as a diffusible factor. Alternatively, it is equally possible that the neurite outgrowth-promoting activity of soluble netrin-1 is the result of protein that becomes deposited on the growth substrate during the experiment. Although the coverslips used in these dissociated retinal cultures were both extensively washed and blocked to prevent non-specific binding (see Experimental Procedures), we cannot formally rule out either one of these possibilities at this time.

Discussion

There is considerable evidence that axons in the developing nervous system are guided to their intermediate and final targets through a variety of short- and long-range, attractive and repulsive mechanisms (reviewed in Tessier-Lavigne and Goodman, 1996). One family of axon guidance molecules, the netrins, were originally purified from embryonic chick brains based on their ability to stimulate the outgrowth of commissural axons *in vitro* (Serafini et al., 1994). In addition to this outgrowth-promoting activity, *in vitro* culture experiments have demonstrated that a point source of netrin-1, such as heterologous cells expressing recombinant protein, is also capable of reorienting the growth of commissural and cortical axons *in vitro* (Kennedy et al., 1994; Shirasaki et al., 1995; Shirasaki et al., 1996; Métin et al., 1997). Recent experiments have extended these findings by showing that netrin-1 acts directly on the extending growth cone to induce turning *in vitro*, and that this chemotropic activity is mediated at least in part by the netrin receptor DCC (de la Torre et al., 1997; Ming et al., 1997). Moreover, netrin-1 is also capable of repelling certain classes of axons at a distance (Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997). Thus, the netrins can act as bifunctional axon guidance cues. In this study, we present evidence that suggests the netrins may not only affect the paths of axons at a distance, but may also guide their migrations through short-range interactions and be involved in target recognition as previously shown in *Drosophila* (Harris et al., 1996; Mitchell et al., 1996).

The Role of Netrin-1 in the Developing Optic Tract

Retinal axons are guided along the pathway to their final targets by a range of axon guidance mechanisms. First, cell adhesion molecules (CAMs) such as N-Cadherin appear to play an important role in axon initiation and extension (Riehl et al., 1996). Local cues in the form of intercellular spaces (Silver and Sidman, 1980; Krayanek and Goldberg, 1981; Silver and Sapiro, 1981; Silver and Rutishauser, 1984), gradients of inhibitory

proteoglycans (Brittis and Silver, 1995) and other axons (Brittis et al., 1995; Brittis and Silver, 1995) direct RGC axons to the optic disc where netrin-1 has been implicated in the decision of these axons to leave the retina (Deiner et al., 1997). Studies in *Xenopus* have shown that within the optic tract, RGC fibers appear to be guided by stable, non-diffusible cues associated with the neuroepithelium (Harris, 1989). These may include factors associated with heparan sulfates, removal of which leads to defects in RGC axon guidance (Walz et al., 1997). Finally, several families of receptor protein tyrosine kinases (RPTKs) and their ligands, including members of the fibroblast growth factor (FGF) and Eph families, have been implicated in retinal axon target recognition and topographic mapping at the optic tectum (reviewed in Tessier-Lavigne and Goodman, 1996).

Our findings complement these previous studies, and suggest two potentially new roles for netrin-1 in the formation of the retino-tectal projection in *Xenopus*. As in other species, we find in *Xenopus* that RGC axons migrate over a band of *netrin-1*-expressing cells in the region of the optic tract. Exposure of this region to ectopic netrin protein leads to severe pathfinding errors by migrating RGC axons. Previous embryological studies where portions of the optic tract were rotated demonstrated the existence of local, non-diffusible cues that regulate the migration of retinal fibers (Harris, 1989). These findings were supported by the observation that in embryos where the tectum was removed early, retinal fiber migration was normal until reaching the location of the missing target, arguing against the existence of long-range target-derived chemoattractants (Reh et al., 1983). Taking these data into account, our results are consistent with netrin-1 playing a role in the local non-diffusible cues of the optic tract. The facts that netrins are known to associate strongly with cell surfaces, particularly with surface-associated proteoglycans (Serafini et al., 1994; Mirzayan, 1997), and that retinal axon pathfinding errors in the presence of exogenous netrin protein are similar to those observed in heparitinase-treated brains (Walz et al., 1997) further supports this hypothesis. However, no direct information is available on the localization of netrin-1 protein in the region of the optic tract. Furthermore, this

model in which netrin-1 provides a favorable corridor for retinal axons from the optic chiasm to the tectum fails to explain the apparent polarity of migration inherent to the optic tract observed in the embryological rotation experiments (Harris, 1989). An alternative explanation for the pathfinding errors observed in brains exposed to exogenous netrin protein is based on the previously reported ability of netrin-1 to induce branching and increase the extension rate in retinal neurites in vitro (Chapter Three; de la Torre et al., 1997). It is possible that the observed mistargeting is the result of an increse in colateral branch formation accompanied by increased growth rates. To address this issue, we would have to examine the behaviour of individual axons in the exposed brain preparations. Unfortunately, the method of axon labeling used in this study (anterograde transport of HRP) does not allow for this degree of resolution.

The Role of Netrin-1 in Target Recognition at the Optic Tectum

In addition to the potential role of netrin-1 in the optic tract, the observation that RGC axons exposed to exogenous netrin protein bypass or overshoot the tectum suggests that netrin-1 may also be involved in target recognition. Genetic studies in *Drosophila* have previously implicated netrins in the selection of discrete muscle targets by subsets of innervating motor neurons (Harris et al., 1996; Mitchell et al., 1996). The bypass phenotype of retinal axons exposed to ectopic netrin-1 described here is strikingly similar to that observed in embryos where the optic tract has been exposed to exogenous FGF-2 or where FGF signaling in RGC axons has been impaired by the expression of a dominant-negative FGF receptor (McFarlane et al., 1995; McFarlane et al., 1996). FGF-2 is highly expressed along the pathway of RGC axons from the chiasm to the optic tectum. However, the region of the optic tectum shows little or no expression of FGF-2. It has been suggested that the steep decreasing gradient of FGF-2 encountered by retinal axons invading the tectum may signal entry into the target region (McFarlane et al., 1995; McFarlane et al., 1996). Thus, in either the absence of FGF signaling or in the presence of

exogenous FGF-2, the normal decrease in FGF signaling at the tectum is lost and retinal axons bypass their target.

Likewise, netrin-1 appears to be expressed at lower levels in the tectum than in the optic tract (de la Torre et al., 1997; Lauderdale et al., 1997; MacDonald et al., 1997; Strähle et al., 1997). This sudden decrease in netrin signaling, perhaps in coordination with the decrease in FGF signaling or the presence of other signals (e.g. members of the Eph family; see Tessier-Lavigne, 1995), may trigger target recognition by the retinal fibers. Alternatively, one can propose a mechanism in which netrin-1 and FGF-2 simply stimulate axon growth in the optic tract; lower levels of netrin-1 protein in the tectum decrease axon extension rates and allow axons to detect target recognition signals. Application of exogenous netrin-1 or FGF-2 to the optic projection over-stimulates the extension mechanism, causing the axons to grow past their target. Both FGF-2 and netrin-1 have been shown to stimulate retinal neurite growth in vitro (McFarlane et al., 1995; de la Torre et al., 1997) One way to address this question would be to prevent netrin-1-mediated signaling in the retinal fibers: if retinal axons that still reach the tectum under those circumstances are incapable of recognizing the target, as is the case with FGF signaling(McFarlane et al., 1996), this would argue in favor of netrin-mediated target recognition.

There are several possibilities for blocking netrin-1 signaling in RGC axons. First, function-blocking antibodies against netrin-1 or a netrin receptor could be used in exposed brain preparations to prevent receptor-ligand binding. Such an approach is complicated by the fact that large amounts of antibodies are necessary to block protein function *in vivo* (Stoeckli and Landmesser, 1995, C.E. Holt, personal communication). An alternative would be to express dominant-negative netrin receptors in RGC axons to block signaling, as was done with the FGF receptor (McFarlane et al., 1996). However, netrin-1 signaling is required for RGC axons to exit the retina, and little is known of the signaling mechanisms of receptors such as DCC or UNC-5, making such an approach difficult at this

time. Another possibility would be to create a targeted disruption of the *netrin-1* locus in mice such that *netrin-1* expression in the developing optic tract is abolished. Finally, one could expose retinal axons in an exposed brain preparation to an antagonist of netrin-1 signaling. If netrin-1(VI-V) truly acts as an antagonist of netrin-1 signaling in retinal axons, the data presented here, which shows that both exogenous netrin-1 and netrin-1(VI-V) cause similar defects in retinal axon pathfinding, argue in favor of a model in which retinal fibers, normally primed to perceive changes in netrin-1 levels, recognize their entry into the tectum by the sudden decrease in netrin-1 concentration. However, the mechanism of action of netrin-1(VI-V) remains unclear, particularly in light of the fact that although this isoform has no outgrowth-promoting activity on commissural axons, it is capable of causing the turning of these axons *in vitro* (Mirzayan, 1997).

Although we have emphasized the similarity in the target recognition defect of retinal axons in brains exposed to FGF-2 or netrin, it is also important to point out that the pathfinding errors observed in netrin-exposed optic projections (e.g. abnormal anterior extensdion of axons into the telencephalon) are not seen in FGF-treated brains. Indeed, the only defects in retinal axon migrations in FGF-2-treated brains are observed at the optic tectum. It is interesting to note that these differences between the response to netrin and FGF-2 in the developing brain are also reflected in the activity of these molecules on retinal neurites in vitro. In particular, although both netrin-1 and FGF-2 can stimulate outgrowth of retina neurites in vitro, only netrin-1, and not FGF-2, can cause the rapid turning of retinal growth cones when presented in a defined gradient from a micropipette (V.H. Höpker, personal communication; de la Torre et al., 1997; Ming et al., 1997).

Short-Range, Contact-Mediated Guidance of RGC Axons by Netrin-1

It is well established that a gradient of soluble netrin-1 protein can act at a distance to direct the growth of axons *in vitro* (discussed in Tessier-Lavigne and Goodman, 1996; de la Torre et al., 1997). Nevertheless, the mechanism of netrin-1 action *in vivo* remains

unclear. Both netrin-1 and netrin-2 associate strongly with cell surfaces (Serafini et al., 1994; Mirzayan, 1997), suggesting that a substantial fraction of these molecules would not be freely diffusible *in vivo*. However, it is likely that substrate-bound gradients of netrin protein would be capable of orienting axonal growth (discussed in Kennedy et al., 1994; Serafini et al., 1996; Tessier-Lavigne and Goodman, 1996; de la Torre et al., 1997; Deiner et al., 1997; MacLennan et al., 1997). Indeed, proteins such as laminin and nerve growth factor are capable of affecting neurite outgrowth *in vitro* as both diffusible and substrate-bound molecules (Gundersen and Barrett, 1979; Gundersen, 1985; Rivas et al., 1992).

Our finding that RGC axons migrate along a band of netrin-1-expressing cells in the optic tract suggests a new mechanism of action for netrin-1. Instead of being attracted or repelled by a source of netrin-1, these axons appear to be migrating along a corridor of a permissive or favorable substrate at a uniform concentration. Disruption of the RGC fibers' ability to detect this presumptive netrin-1 corridor, for instance by adding exogenous netrin-1 protein outside the optic tract, causes them to migrate away from their pathway. Moreover, the finding that substrate-bound netrin-1 can stimulate retinal neurite outgrowth as effectively as soluble protein argues that RGC axons can respond to netrin-1 in a substrate-bound form *in vivo*. However, these observations raise several important questions that are not addressed in the current study. First, it remains to be demonstrated that the netrin-1 added as a soluble protein to the dissociated retinal cultures does not associate with the substrate to stimulate neurite outgrowth. Domain VI of netrin-1 shares 40% amino acid sequence identity with domain VI of the laminin γ chain, which can mediate the Ca^{2+}-dependent oligomerization of laminin trimers into large networks (Yurchenco and Cheng, 1993). Although BSA is used to prevent non-specific binding with the substrate (see Experimental Procedures), it would not prevent this type of interaction. For the same reasons, it would also be important to demonstrate that substrate-bound netrin-1 remains attached to the coverslips during the culture period. Second, previous reports have implicated DCC in the response of retinal growth cones to netrin-1 *in*

vitro and *in vivo* (de la Torre et al., 1997; Deiner et al., 1997). Does DCC also mediate the potential short-range interaction with netrin-1? Some evidence in rodents suggests that the outgrowth-promoting and the chemotropic activities of netrin-1 may be mediated by different signaling pathways (Mirzayan, 1997). For instance, function blocking antibodies to DCC eliminate the response of commissural axons to the outgrowth-promoting activity of netrin-1, but do not block the chemotropic response (Keino-Masu et al., 1996). This may be due to poor penetration of the spinal cord explants by the antibody, or they may reflect differences in the receptor mechanisms mediating the two responses (discussed in Keino-Masu et al., 1996; de la Torre et al., 1997; Mirzayan, 1997). Indeed, additional netrin receptors, such as the DCC-related Neogenin and a vertebrate homologue of the *C. elegans* UNC-5 protein, are expressed in the retina (Vielmetter et al., 1994; Leonardo et al., 1997). However, little is known about the cell types expressing these receptor proteins.

Despite these questions, the data presented here provide evidence for the potential existence of two new functions for netrins in vertebrates. Although we present these functions in the context of retinal axons guidance, the comparison of *netrin* gene expression patterns to axon tracts in the developing nervous system, as well as the analysis of axon guidance defects in netrin-1- and DCC-deficient mice suggests that they may bear relevance to axon guidance events elsewhere in the developing vertebrate nervous system (Kennedy et al., 1994; Keino-Masu et al., 1996; Serafini et al., 1996; de la Torre et al., 1997; Fazeli et al., 1997; Lauderdale et al., 1997; MacDonald et al., 1997). Additional studies into these mechanisms, both *in vivo* and *in vitro*, will be required to determine how general they are, and whether they can be applied to other candidate guidance molecules.

Experimental Procedures

Embryos

Xenopus laevis embryos were obtained by *in vitro* fertilization as previously described (Cornel and Holt, 1992). Embryos were raised in 10% Holtfreter's solution (Holtfreter, 1943) between 15°C and 20°C, and staged (Nieuwkoop and Faber, 1967).

Whole-Mount *In Situ* Hybridization

Whole-mount *in situ* hybridization was performed as described in Hemmati-Brivanlou et al. (1990), with the modifications described in (Harland, 1991) using BM purple (Boehringer Mannheim) as substrate. A digoxigenin-labeled antisense riboprobe was generated by *in vitro* transcription of an EcoRI-linearized *Xenopus netrin-1* cDNA in pBluescript (Stratagene) using T7 RNA polymerase (Harland, 1991). Embryos were photographed in PBS or after clearing in 2:1 benzyl benzoate:benzyl alcohol. Following whole mount RNA *in situ* hybridization, some embryos were sectioned in paraffin. 10 µm serial sections were collected on Superfrost+ slides (Fisher), dewaxed in xylenes and mounted using Permount (Sigma). In addition some embryos were sectioned using a Vibratome (Oxford) as previously described (Kennedy et al., 1994).

Exposed brain preparations

Exposed brain preparations were performed as previously described (Chien et al., 1993). Stage 33/34 embryos were anesthetized in Modified Barth's Saline (MBS: 88 mM NaCl, 1 mM KCl, 0.7 mM $CaCl_2$, 1 mM $MgSO_4$, 5 mM HEPES pH 7.8, 2.5 mM $NaHCO_3$) supplemented with 0.4 mg/ml tricaine (ethyl 3-aminobenzoate methanesulfonic acid) (Sigma) and immobilized right side down in Sylgard-coated dishes using minutien pins (Ø = 0.2 mm; Fine Science Tools). Fine minutien pins (Ø = 0.1 mm) were then used to remove the skin and eye from the left side of the head, exposing the entire anterior brain to

the posterior midbrain. All embryos were exposed before being randomly distributed between conditions and allowed to develop to stage 40. Control and experimental conditions consisted of MBS + 0.1% BSA, supplemented with the netrin protein for the experimental solution. Following culture, RGC axons from the contralateral (right) eye were filled with ~30% horse radish peroxidase (HRP, type VI; Sigma) in 1% lysolecithin (Sigma) as previously described (Cornel and Holt, 1992). After ~30 minutes, embryos were fixed for 30 minutes at room temperature in 0.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4). Following fixation, the brains and spinal cords were dissected out as one piece, reacted with diaminobenzidine (Sigma) and mounted following dehydration with Permount (Sigma).

Recombinant netrin protein isoforms

Two netrin isoforms were used in this study. Full length recombinant chick netrin-1 protein and a truncated form of netrin-1 lacking the carboxy terminal domain C (netrin-1(VI-V); see Ishii et al., 1992; Mirzayan, 1997). Both isoforms were purified as previously described (Mirzayan, 1997).

Immunocytochemistry

Xenopus embryos were fixed overnight at 4°C in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5, transferred to 30% sucrose, imbedded in OCT at -70°C, sectioned (10 μm), and stained as described (Cornel and Holt, 1992). Prior to incubation with primary antibody, slides were boiled in a microwave for 10 minutes in 0.1 M sodium citrate, pH 8.0 to reveal masked epitopes (T.E. Kennedy and M. Tessier-Lavigne, in preparation). Monoclonal antibody 9E10 (Evan et al., 1985) was used to detect myc-epitope tagged netrin isoforms.

Quantitation of Mistargeting Phenotypes in Exposed Brains

Measurement of the mid-optic tract width of the optic projection. Quantitation of the optic projection width was carried out as previously described (McFarlane et al., 1995). Briefly, camera lucida drawings of mounted brains were scanned and analyzed on a Power Macintosh computer using NIH Image (NIH, Bethesda). Previously described macros were used to normalize brain size to a reference line drawn between the optic chiasm and the rhombic isthmus (Chien et al., 1993). The width of the optic projection was then measured where it intersected a circle centered on the optic chiasm and passing through the middle of the reference line (see McFarlane et al., 1995).

Calculation of the ratio of the areas of the optic tract and the forebrain. Camera lucida drawing of mounted brains were scanned and analyzed using a Power Macintosh computer. NIH Image (NIH, Bethesda) was used to calculate the area of the optic projection. To normalize for variations in brain size, this area was then divided by the area of the brain anterior to a line drawn between two clearly visible landmarks: the rhombic isthmus and the cranial flexure.

Measurement of Neurite Extension

Neurite lengths were measured by capturing phase-contrast images from a microscope (Axiophot, Zeiss) onto a computer (Power Macintosh, Apple) with a video camera (CCD-IRIS/RGB, Sony), and tracing the neurites using NIH Image (NIH, Bethesda). Statistical analysis was performed using GBSTAT software package.

Acknowledgments

I would like to thank E. Cornel for invaluable technical assistance with the exposed brain preparation; A. Faynboym for providing purified netrin-1 and netrin-1(VI-V) protein; L.E. Connolly, K. Brose and C.M. Mirzayan for comments on this manuscript, and members of the Tessier-Lavigne, Holt and Harris laboratories for helpful discussions. This work was

Table 4-1: Axonal Pathfinding Defects in Netrin-Treated Exposed Brain Preparations.

Protein	(μg/ml)	*n*	Type of Axonal Guidance Defect (%)				
			Abnormal anterior projection	De-Fasciculated Optic Tract	Dorsally crossing fibers	Abnormal ipsilateral projection	Stalled Optic Projection
none	n/a	*41*	7%	15%	2%	2%	12%
[glycerol] [a]	0.4% [a]	*18*	39%	11%	0%	11%	6%
netrin-1	0.1	*10*	50%	30%	0%	0%	22%
	1	*18*	22%	22%	0%	33%	22%
	5	*6*	50%	67%	50%	0%	17%
	10	*36*	67%	56%	17%	28%	19%
	20	*14*	79%	36%	21%	29%	14%
	50	*12*	83%	83%	33%	42%	0%
netrin-1(VI-V)	10	*5*	100%	40%	0%	0%	0%
	20	*18*	56%	50%	17%	17%	0%
	50	*13*	100%	85%	62%	31%	0%
	100	*30*	83%	87%	57%	23%	10%

Table 4-1: Axonal pathfinding defects in netrin-treated exposed brain preparations.

[a] Recombinant netrin-1, but not netrin-1(VI-V), is stored in 50% glycerol (Mirzayan, 1997). To control for the effect of this glycerol on RGC axon pathfinding, glycerol was added to control medium at the same concentration as the highest netrin-1 condition (glycerol concentration estimated at 0.4%).

Figure 4-1: Diagram of the Exposed Brain Preparation and Localization of Ectopic Netrin-1 and Netrin-1(VI-V).

(A) Diagram of the exposed brain preparation (Chien et al., 1993). The entire left optic tract of stage 33/34 embryos was exposed be removing the eye, skin and mesenchyme from the left side of the head. Embryos were incubated in control or experimental culture medium until reaching stage 40, when most RGC axons have reached the optic tectum. The optic projection was then visualized by filling the RGC axons with horse radish peroxidase (HRP). Dashed line indicates plane of section in panels (C) and (D).

(B) Diagram of the structures of netrin-1 and netrin-1(VI-V). The netrins are composed of three domains, of which the amino-terminal two (domains VI and V) are homologous to laminin (Ishii et al., 1992). Domain V is composed of three individual epidermal growth factor (EGF) repeats. The carboxy-terminal domain (domain C) is predicted to have a high net positive charge at physiological pH. As indicated in the diagram, netrin-1(VI-V) is a truncated form of netrin-1 lacking domain C. Both isoforms have been tagged at the carboxy-terminus with a myc epitope to facilitate detection of the ectopic recombinant proteins.

(C and D) Transverse sections through the diencephalon of embryos exposed to (C) 10 μg/ml netrin-1 and (D) 100 μg/ml netrin-1(VI-V) for 18 hours, and stained with an anti-myc monoclonal antibody. In both cases intense staining is seen on the exposed side of the brain, particularly along the exposed basal lamina (arrowheads). For position of section see dashed line in (A).

(E) Transverse section through the midbrain of an embryo exposed to netrin-1 showing deeply migrating retinal axons (arrowheads). Retinal axons have been filled with HRP and are visualized using an anti-HRP antibody.

di, diencephalon; e, eye; tec, optic tectum.

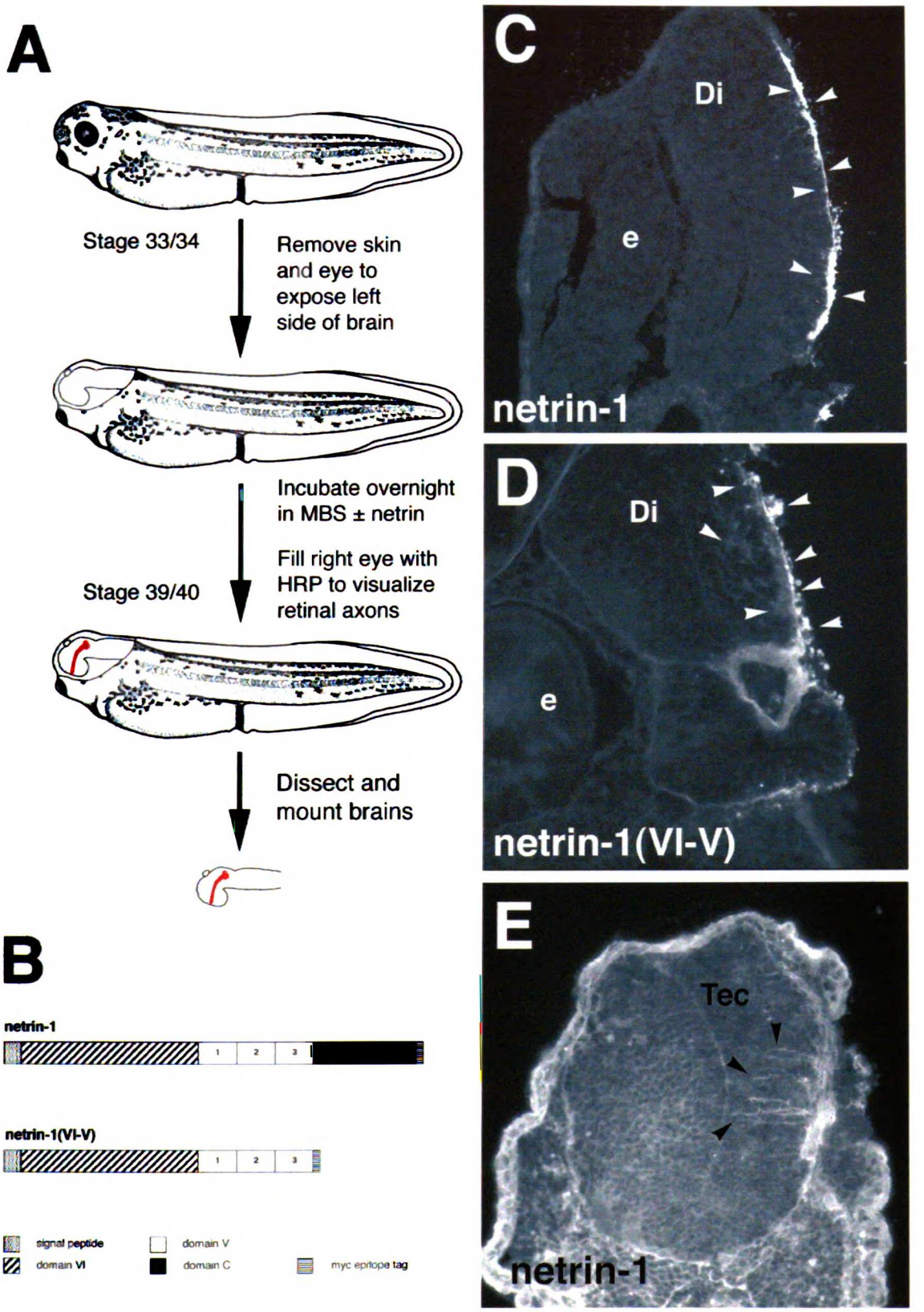

A
Stage 33/34
Remove skin and eye to expose left side of brain
Incubate overnight in MBS ± netrin
Fill right eye with HRP to visualize retinal axons
Stage 39/40
Dissect and mount brains
B
netrin-1
1
2
3
netrin-1(VI-V)
1
2
3
signal peptide
domain VI
domain V
domain C
myc epitope tag
C
Di
e
netrin-1
D
Di
e
netrin-1(VI-V)
E
Tec
netrin-1

Figure 4-2: Ectopic Netrin-1 and Netrin-1(VI-V) Disrupt RGC Axon Guidance *In Vivo*. Control retinal projections (A and B), and retinal projection in embryos exposed to netrin-1 (C, H) or netrin-1(VI-V) (D-G). All panels show a lateral view of the brain, except (G) which shows a dorsal view of a brain exposed to netrin-1(VI-V). Arrowheads in (A) indicate the borders of the optic tectum. Retinal axons in control embryos project to the tectum in a cohesive optic tract. In contrast, retinal axons in netrin-1- and netrin-1(VI-V)-treated embryos are often defasciculated (C-E) and grow abnormally into the anterior forebrain (arrowheads). In these embryos, retinal fibers are also seen projecting abnormally across the dorsal midline (E-G), either anterior (arrowheads) or posterior (arrows) to the pineal gland (see [A]). (E and F) Lateral views of the left (E) and right (F) sides of a brain exposed to netrin-1(VI-V). The asterixs (∗) show positions where retinal axons form two abnormal dorsal commissures that extend into the right (unexposed) side of the brain. (G) Dorsal view of another brain exposed to netrin-1(VI-V) showing retinal axons crossing the dorsal midline. The dotted line indicates the position of the dorsal midline. Retinal fibers are also observed failing to properly recognize and innervate the tectum (C-D; arrows).

hy, hypothalamus; oc, optic chiasm; ot, optic tract; pi, pineal; tel, telencephalon.

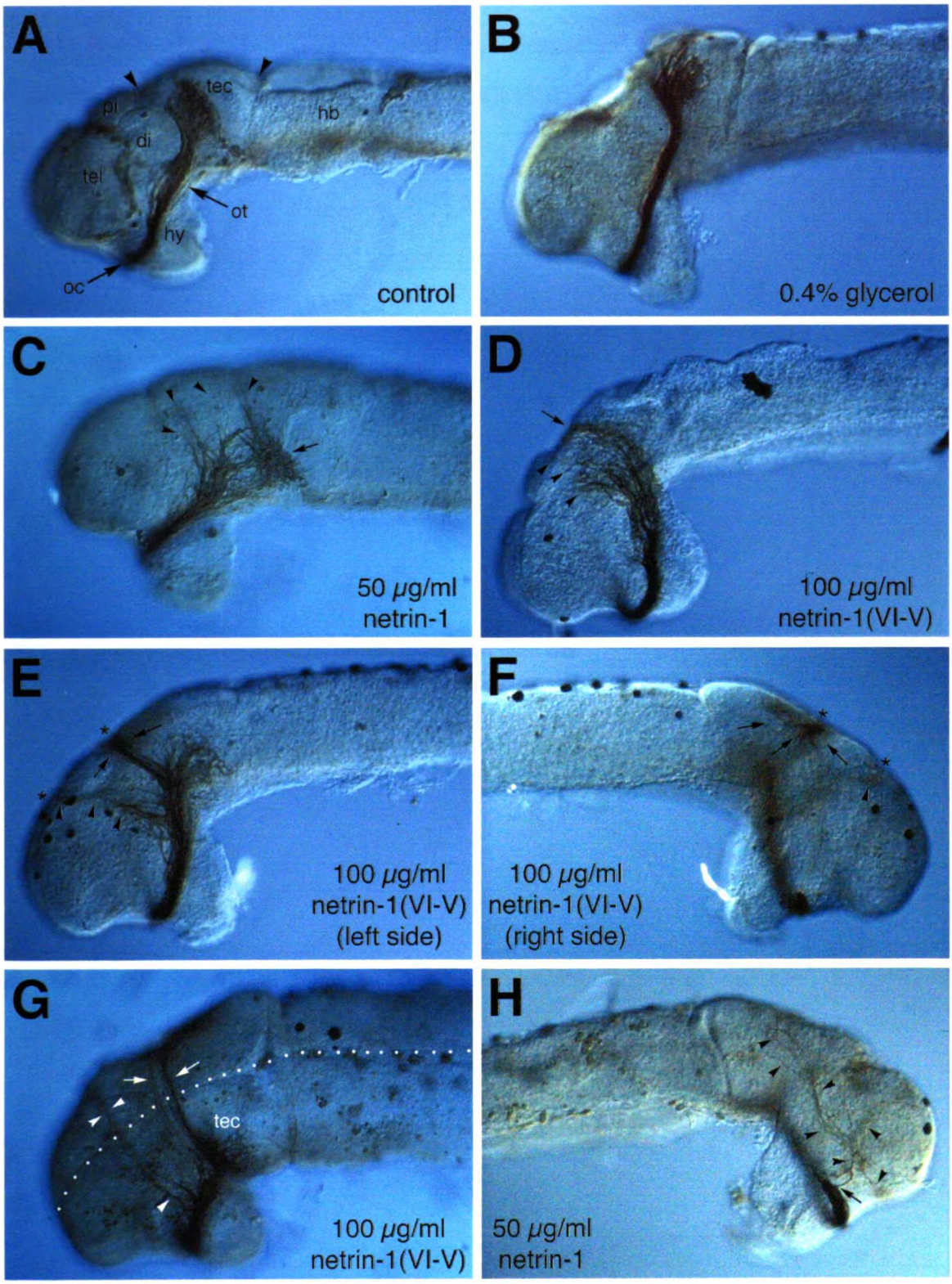

A
tec
pl
di
hb
tel
ot
hy
oc
control
B
0.4% glycerol
C
50 μg/ml
netrin-1
D
100 μg/ml
netrin-1(VI-V)
E
100 μg/ml
netrin-1(VI-V)
(left side)
F
100 μg/ml
netrin-1(VI-V)
(right side)
G
tec
100 μg/ml
netrin-1(VI-V)
H
50 μg/ml
netrin-1

Figure 4-3: Illustration of the pathfinding errors in netrin-1- and netrin-1(VI-V)-treated brains.

Camera lucida drawings of HRP-labeled optic projections in control brains (A-B) and brains exposed to netrin-1 (C, H) or netrin-1(VI-V) (D-G). Arrows indicate retinal axons bypassing the optic tectum

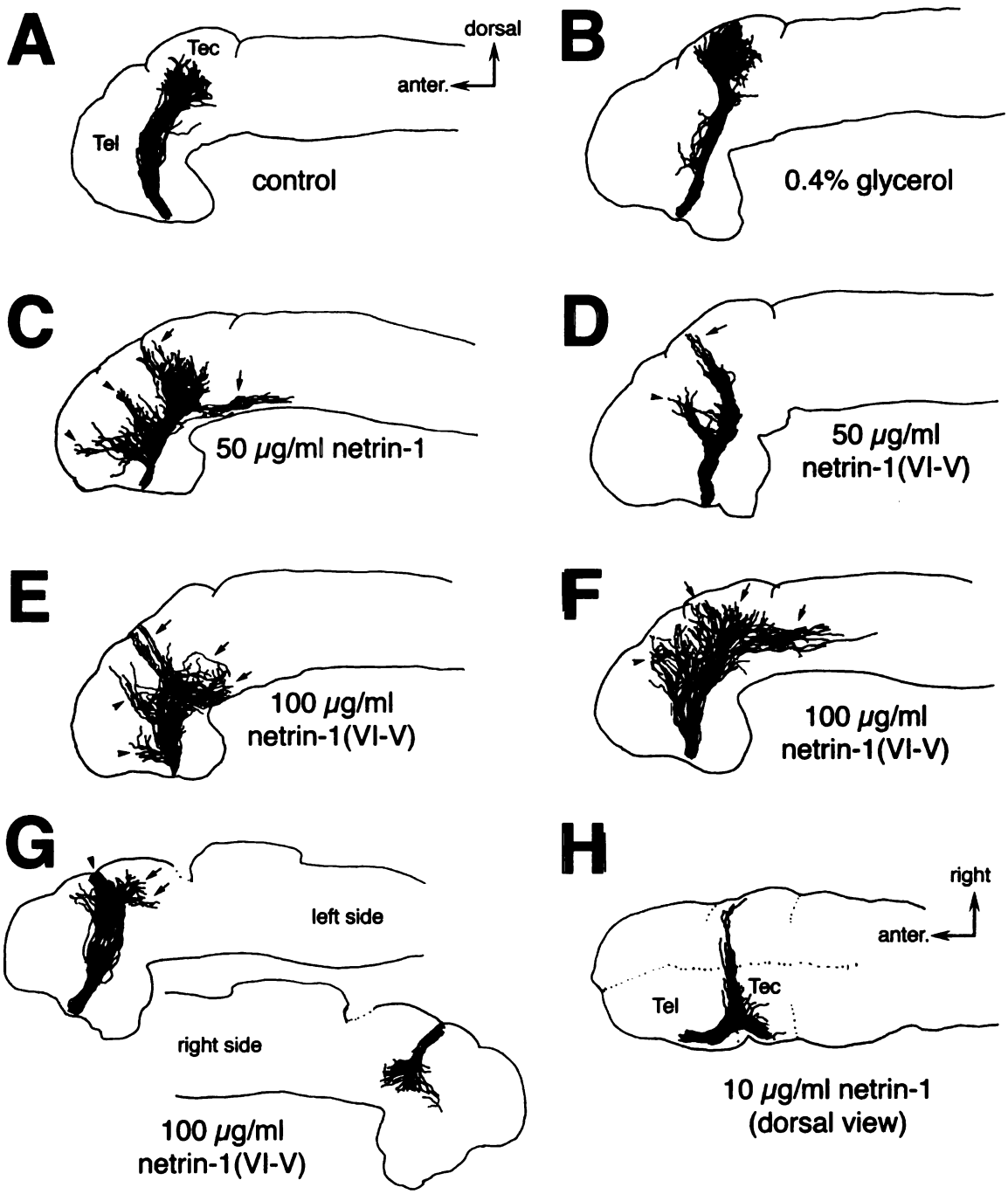

A
dorsal
Tec
anter.
Tel
control
B
0.4% glycerol
C
50 μg/ml netrin-1
D
50 μg/ml
netrin-1(VI-V)
E
100 μg/ml
netrin-1(VI-V)
F
100 μg/ml
netrin-1(VI-V)
G
left side
right side
100 μg/ml
netrin-1(VI-V)
H
right
anter.
Tec
Tel
10 μg/ml netrin-1
(dorsal view)

Figure 4-4: Quantitation of Mistargeting Phenotype by Mid-Optic Tract Width Measurements.

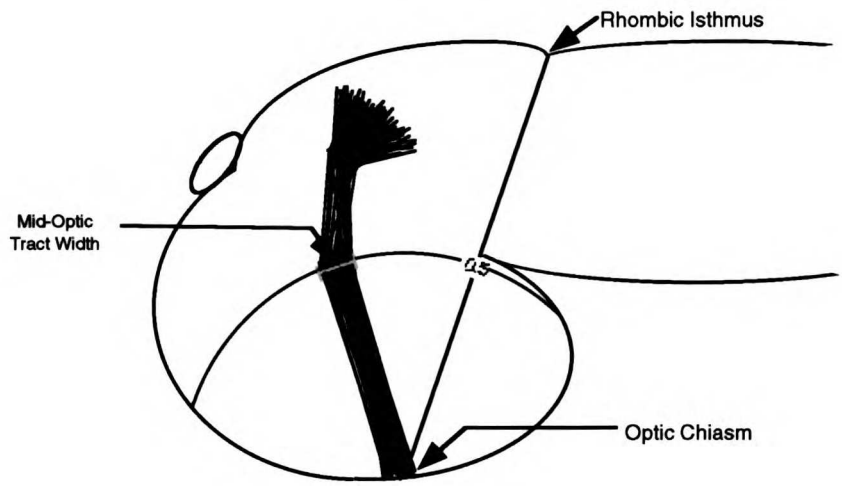
(A) Optic projection width was used to quantitate the defasciculation phenotype as described in the Experimental Procedures (see also McFarlane et al., 1995). The method of analysis is shown on a schematic (normal) brain. A standard reference line was drawn between the optic chiasm and the rhombic isthmus. A circle centered on the chiasm was drawn passing through the mid-way point of the reference line. The width of the optic tract was determined by measuring the arc string defined by the intersection of the optic projection and the circle (shown in yellow).

(B) Mean optic projection widths of netrin-1-treated brains compared to controls. Two control conditions are shown: "MBS alone" is standard saline culture medium (containing 0.1% BSA; see Experimental Procedures); "MBS+Glycerol" is standard culture medium supplemented with 0.4% glycerol to control for glycerol in netrin-1 storage solution (see Experimental Procedures).

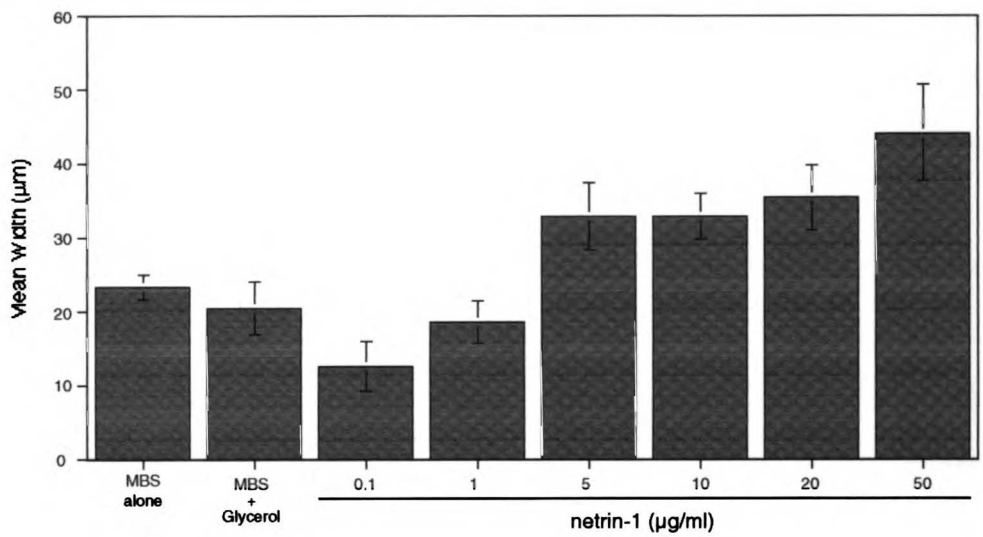
(C) Mean optic projection widths of netrin-1(VI-V)-treated brains compared to controls. Netrin-1(VI-V) is stored without glycerol, so the "MBS+Glycerol" control has been omitted.

The concentration of netrin-1 or netrin-1(VI-V) protein used in each experiment is indicated below each bar. Numbers in parenthesis indicate sample size (*n*) for each measurement. Error bars represent SEM.

A


Rhombic Isthmus
Mid-Optic
Tract Width
Optic Chiasm

B


Mean Width (µm)
0
10
20
30
40
50
60
MBS
alone
MBS
+
Glycerol
0.1
1
5
10
20
50
netrin-1 (µg/ml)

C

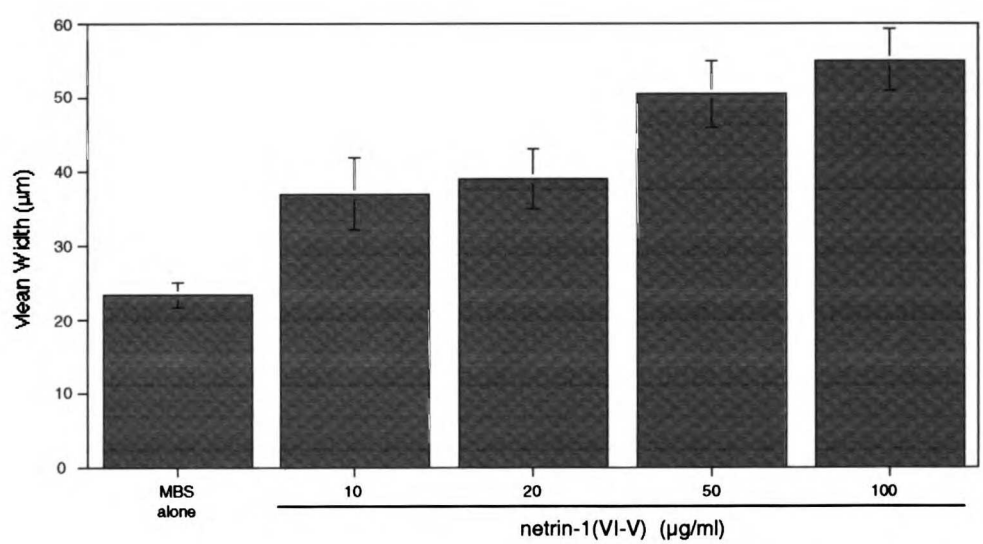

Mean Width (µm)
0
10
20
30
40
50
60
MBS
alone
10
20
50
100
netrin-1(VI-V) (µg/ml)

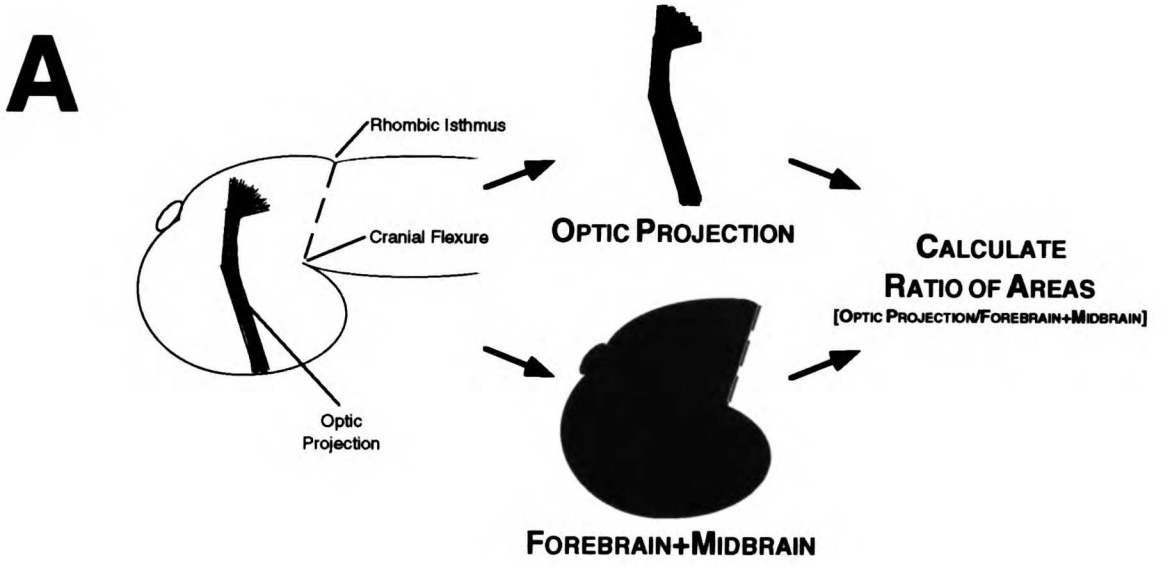
Figure 4-5: Optic Projection Area Ratio Measurements.

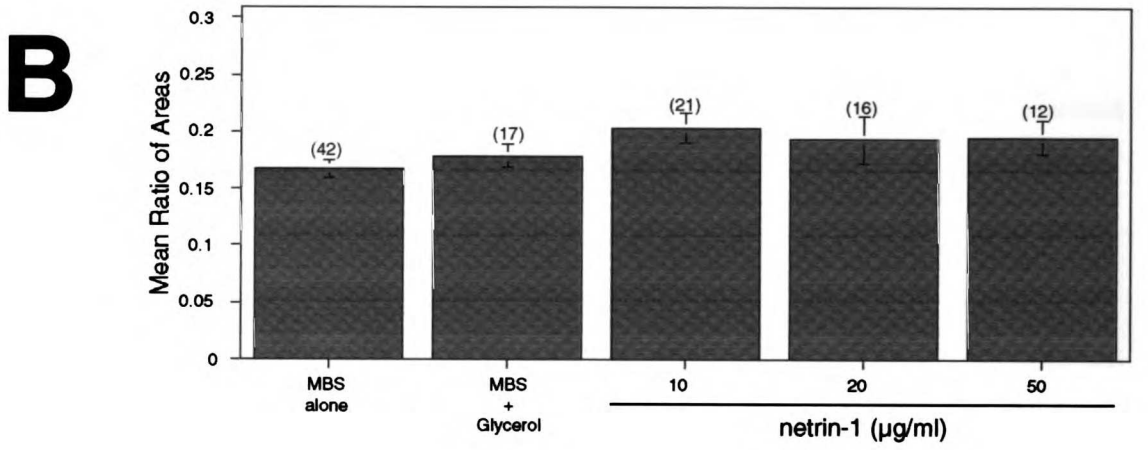
(A) To quantitate the lateral spread (defasciculation) of the optic projection in the exposed brain preparations, we compared the areas of the optic projection in control brains to those treated with netrin-1 and netrin-1(VI-V). In order to normalize for variations in brain size, the area of the optic projection was divided by the area of the region of the brain anterior to a line drawn between the rhombic isthmus and the cranial flexure. The mean value of this ratio was then presented for each experimental condition.

(B) Mean ratio of the areas of netrin-1-treated brains compared to controls. Two control conditions are shown: "MBS alone" is standard saline culture medium (containing 0.1% BSA; see Experimental Procedures); "MBS+Glycerol" is standard culture medium supplemented with 0.4% glycerol to control for glycerol in netrin-1 storage solution (see Experimental Procedures).

(C) Mean ratio of the areas of netrin-1(VI-V)-treated brains compared to controls. Netrin-1(VI-V) is stored without glycerol, so the "MBS+Glycerol" control has been omitted.

The concentration of netrin-1 or netrin-1(VI-V) protein used in each experiment is indicated below each bar. Numbers in parenthesis indicate sample size (*n*) for each measurement. Error bars represent SEM.


A
Rhombic Isthmus
Cranial Flexure
Optic
Projection
OPTIC PROJECTION
CALCULATE
RATIO OF AREAS
[OPTIC PROJECTION/FOREBRAIN+MIDBRAIN]
FOREBRAIN+MIDBRAIN


B
Mean Ratio of Areas
0.3
0.25
0.2
0.15
0.1
0.05
0
(42)
(17)
(21)
(16)
(12)
MBS
alone
MBS
+
Glycerol
10
20
50
netrin-1 (µg/ml)

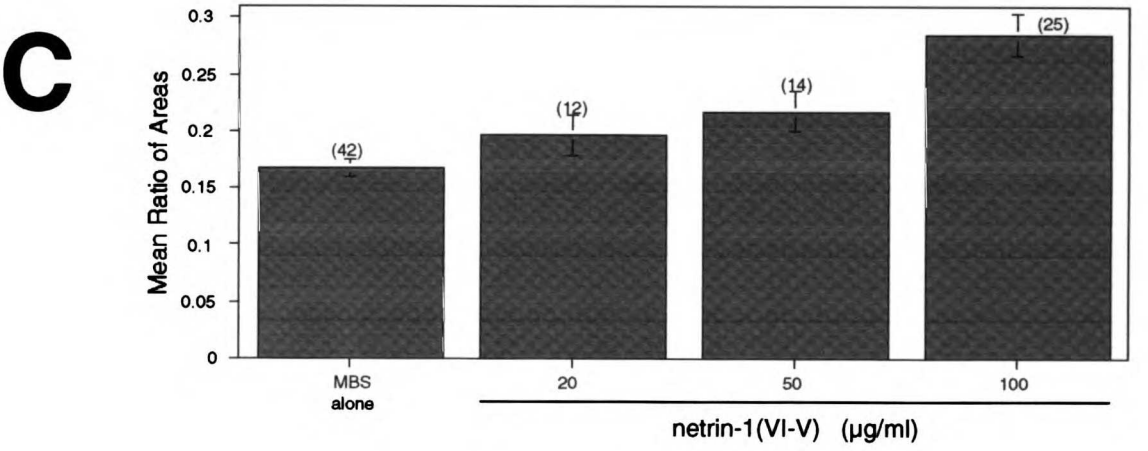
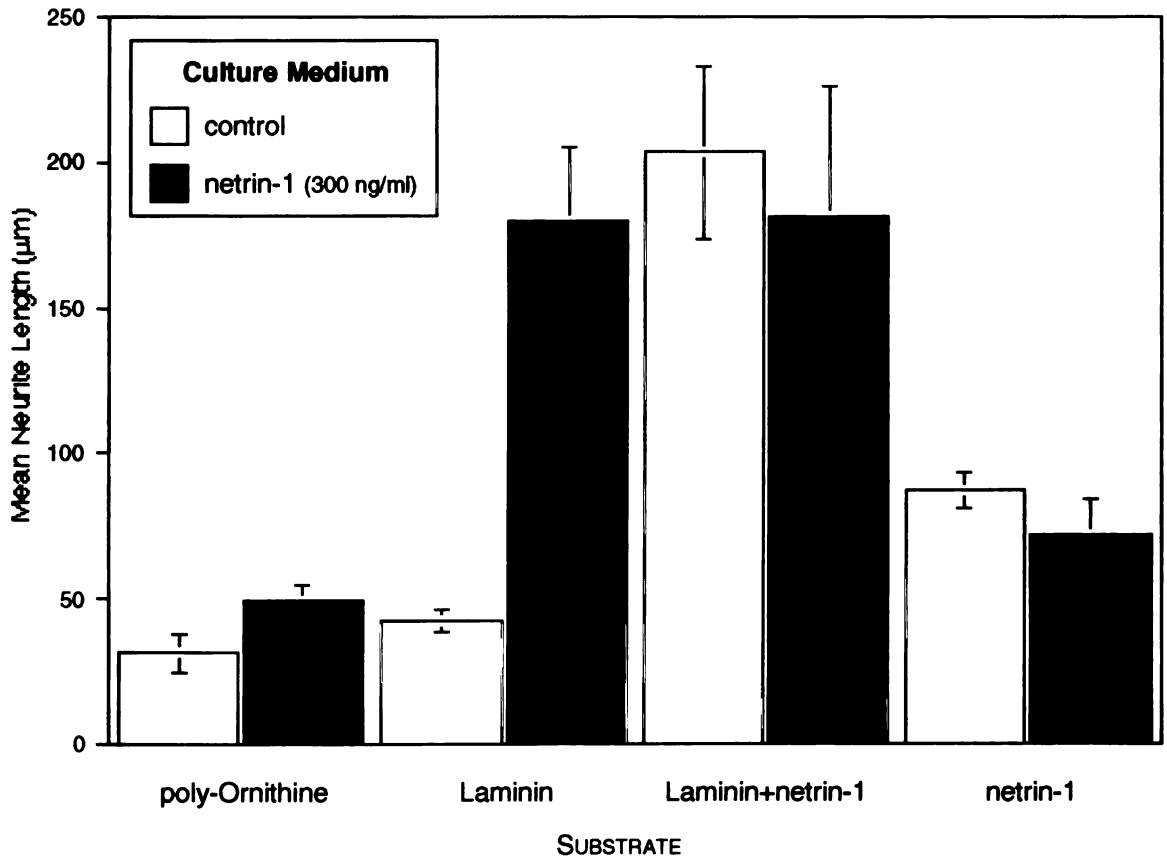

C
Mean Ratio of Areas
0.3
0.25
0.2
0.15
0.1
0.05
0
(42)
(12)
(14)
(25)
MBS
alone
20
50
100
netrin-1(VI-V) (µg/ml)

Figure 4-6: Substrate-Bound Netrin-1 Promotes Retinal Neurite Outgrowth *In Vitro*.

Dissociated retinal cells were grown on glass coverslips coated with various substrates, in the absence (white bars) or presence (gray bars) of soluble netrin-1 protein (300 ng/ml) in the culture medium. The length of the longest neurite of 30 randomly-selected neurons was measured and tabulated (see Experimental Procedures). On laminin alone (sub optimal concentration for growth; 0.5 μg/ml), soluble netrin-1 strongly stimulated neurite outgrowth. As previously reported, no significant stimulation of outgrowth by netrin-1 was observed on poly-L-ornithine alone (de la Torre et al., 1997). However, when the substrate contained laminin and netrin-1, neurite outgrowth was stimulated as strongly as on a laminin substrate in the presence of soluble netrin-1. No additional stimulation was observed by supplementing this substrate with soluble netrin-1. Finally, neurite outgrowth on netrin-1 alone was not significantly different than on poly-L-ornithine.

Neurite lengths are shown as the mean ± s.e.m. (in μm). Numbers in parenthesis represent the sample size (*n*) for each condition.


Culture Medium
control
netrin-1 (300 ng/ml)
Mean Neurite Length (µm)
250
200
150
100
50
0
poly-Ornithine
Laminin
Laminin+netrin-1
netrin-1
SUBSTRATE

Chapter Five

Summary and Conclusions

The growth of axons and the establishment of neuronal connections in the developing nervous system is governed by a multitude of short-range and long-range signals (Tessier-Lavigne and Goodman, 1996). Over the past two decades, we have witnessed impressive progress in the understanding of the molecular mechanisms of axon guidance. Not only have novel mechanisms been elucidated, but key molecular players have also been identified. One family of long-range guidance cues are the netrins, laminin-related molecules that act as diffusible chemoattractants and chemorepellents for a number of axonal migrations in vertebrates, *Caenorhabditis elegans* and *Drosophila* (Tessier-Lavigne and Goodman, 1996). In this thesis, I have presented evidence that implicates the vertebrate netrins, and particularly chick netrin-1, in guiding the circumferential migration of spinal commissural axons (Chapter Two; Kennedy et al., 1994). We have also found that netrin-1 can direct the growth of *Xenopus* retinal neurites *in vitro* by acting directly on the extending growth cone, and provide evidence that the netrin receptor Deleted in Colorectal cancer (DCC) mediates this chemotactic response (Chapter Three; de la Torre et al., 1997). Finally, additional studies in *Xenopus* suggest that, in addition to its ability to direct axonal migrations at a distance, netrin-1 may also act as a non-diffusible short-range guidance cue and target recognition signal for developing retinal axons (Chapter Four). These findings are striking because they suggest that the netrins, particularly netrin-1, are able to function in a number of different ways to guide developing axons to their intermediate and final targets.

The Netrins are Diffusible Chemotropic Molecules

Almost a century ago, Ramón y Cajal postulated that axons might be guided to their targets by diffusible chemotropic factors, and that, based upon the trajectories of spinal commissural axons *in vivo*, the floor plate might be a good candidate for a intermediate target producing such a signal (Ramón y Cajal, 1909). It was subsequently shown that the floor plate could indeed reorient the growth of commissural axons both *in vitro* and *in vivo*

(Tessier-Lavigne et al., 1988; Placzek et al., 1990a). Recently, two phylogenetically-conserved proteins purified from embryonic chick brain, netrin-1 and netrin-2, were shown to mimic two activities of the floor plate on commissural axons *in vitro*: the stimulation of outgrowth from spinal cord explants into a collagen matrix, and the reorientation of extending commissural axons within spinal cord explants (Kennedy et al., 1994; Serafini et al., 1994). Our finding that *netrin-1* is expressed by the floor plate during the period of commissural axon guidance (Chapter Two; Kennedy et al., 1994) argues that netrin-1 is responsible, at least in part, for the activities found in floor plate cells.

These observations raise several important questions. Netrin-1-induced axon turning has only been demonstrated *in vitro* within explants of neural tissue and over the course of 40 hours (Kennedy et al., 1994; Métin et al., 1997; Richards et al., 1997). Does netrin-1 act alone to direct the growth of developing axons, or are additional accessory factors required? A precedent for the requirement of such accessory factors is provided by the observation that netrin-1-induced outgrowth of commissural axons from young (E11), but not older (E13) rat spinal cord explants requires the function of Netrin Synergizing Activity (NSA) (Serafini et al., 1994). Moreover, floor plate cells from netrin-1-deficient mice can still reorient commissural axon growth in dorsal spinal cord explants (Serafini et al., 1996), implying the existence of additional floor plate-derived chemotropic factors. In order to address this question, we have taken advantage of an *in vitro* assay in which individual growth cones can be exposed to defined gradients of diffusible molecules created by pulsatile release of concentrated solutions from a micropipette (Lohof et al., 1992; Zheng et al., 1994). This experimental system has previously been used to examine the cellular and molecular response of growth cones to a variety of chemotropic molecules, including neurotransmitters and neurotrophins (Lohof et al., 1992; Zheng et al., 1994; Song et al., 1997). Using this assay, we were able to demonstrate that extending *Xenopus* retinal growth cones are capable of detecting and responding to a gradient of soluble netrin-1 by reorienting their growth toward the source of the gradient, in this case the micropipette

(Chapter Three; de la Torre et al., 1997). These experiments suggest that gradients of netrin-1 *in vivo* can provide the information necessary to guide the migrations of developing axons.

Receptor Mechanisms Involved in Detecting the Permissive and Instructive Activities of Netrin-1 on Developing Axons

In vitro studies using commissural, retinal and cortical axons have suggested that netrin-1 possesses two distinct activities that affect extending axons (Kennedy et al., 1994; Serafini et al., 1994; Shirasaki et al., 1995; Wang et al., 1996; de la Torre et al., 1997; Deiner et al., 1997; Métin et al., 1997; Richards et al., 1997). First, netrin-1 promotes the outgrowth of these axons either into a collagen matrix, or on two dimensional substrates. This "permissive" activity may allow axons to overcome inhibitory or unfavorable substrates, or may simply stimulate cellular axon extension mechanisms. However, this outgrowth activity does not appear to affect the directionality of axon growth. In contrast to this permissive activity, netrin-1 also possesses an "instructive" activity which is capable of determining the direction of growth. As summarized above, this instructive (chemotropic) activity appears to be the result of a direct action of netrin-1 on extending growth cones *in vitro* (Chapter Three; de la Torre et al., 1997). Interestingly, the chemotropic activity of netrin-1 can be either attractive or repulsive, depending on the responding axons (reviewed in Tessier-Lavigne and Goodman, 1996). However, it is not known whether the permissive activity of netrin-1 can also be inhibitory, for instance by inhibiting axon extension mechanisms in the growth cone.

Are these two activities important in the *in vivo* role of netrin-1? Analysis of the trajectories of commissural axons in netrin-1-deficient mice suggest that both the permissive and chemotropic activities of netrin-1 are important in guiding commissural axons to the floor plate (Serafini et al., 1996). In the absence of endogenous netrin-1, commissural axons appear unable to enter the ventral spinal cord, specifically the

developing motor column, and become severely disorganized (Serafini et al., 1996). This observation suggests netrin-1 might first function as a permissive cue, allowing commissural axons to extend into the ventral spinal cord. However, commissural axon trajectories in these mutant embryos also provide *in vivo* evidence for a chemotropic function of netrin-1. Despite the fact that most axons do not grow into the ventral spinal cord, a small number of axons are able to enter the motor column and grow ventrally. However, these axons do not grow in a directed fashion toward the floor plate, following instead the lateral edges of the spinal cord. The failure of these axons to turn toward the floor plate suggests that a chemotropic activity of netrin-1 is important in guiding these axons to the ventral midline *in vivo*.

Given the importance of these two activities of netrin-1 *in vivo*, we sought to determine whether they might be mediated by the same or different receptor mechanisms in the extending growth cone. Genetic analysis of axon guidance defects in *C. elegans* originally identified the first member of the netrin family, UNC-6, and two candidate receptors that could potentially mediate the UNC-6/netrin signal: UNC-5 and UNC-40 (Hedgecock et al., 1990). Recent studies have identified vertebrate and *Drosophila* homologues of UNC-40 and UNC-5 (reviewed in Tessier-Lavigne and Goodman, 1996). Of particular interest, the vertebrate UNC-40 homologue Deleted in Colorectal Cancer (DCC), expressed on commissural axons, has been shown to bind netrin-1 and is required for the outgrowth-promoting activity of netrin-1 on these axons (Keino-Masu et al., 1996). Similarly, DCC is also required for the netrin-1-induced outgrowth of retinal axons *in vitro* (Chapter Three; de la Torre et al., 1997; Deiner et al., 1997), consistent with recent evidence implicating netrin-1 and DCC in the guidance of retinal axons out of the developing retina (Deiner et al., 1997). Thus, DCC appears to be part of the receptor mechanism that mediates the permissive activity of netrin-1.

However, previous experimental evidence on the role of DCC in mediating the chemotropic role of netrin-1 was ambiguous. Monoclonal antibodies that block DCC

function did not abolish commissural axon turning within dorsal spinal cord explants, even at antibody concentrations that inhibited outgrowth-promoting activity of netrin-1 on these axons (Keino-Masu et al., 1996). One possible explanation for this negative result is the failure of the antibodies to penetrate into the explants and completely block DCC function, a possibility supported by the observation that these antibodies decreased the distance over which netrin-1 could induce the turning of commissural axons in dorsal spinal cord explants (Mirzayan, 1997). Using a simplified assay in which growth cones were exposed to defined gradients of purified netrin-1 in a cell-free environment, we were able to demonstrate that DCC function was required in *Xenopus* retinal growth cones to mediate the chemotactic response of these axons to gradients of netrin-1 (Chapter Three; de la Torre et al., 1997). Thus, at least in this *in vitro* setting, DCC is required for the response of retinal axons to both the outgrowth-promoting and chemotropic activities of netrin-1. Nevertheless, it remains to be demonstrated that commissural axons in DCC-deficient mice, which exhibit similar pathfinding defects as in netrin-1-deficient mice (Fazeli et al., 1997), are impaired in their ability to turn toward a point source of netrin-1 *in vitro*. Indeed, it remains possible that additional molecules, including perhaps some of the vertebrate UNC-5 homologues known to be expressed in the developing spinal cord and retina (Leonardo et al., 1997), may also be involved in the chemotropic response to the netrins.

Additional Mechanisms of Netrin-1 Action in Axon Guidance

The experiments described above demonstrated that the netrins, particularly netrin-1 in vertebrates, act as diffusible guidance cues to guide developing axons at a distance. Diffusible gradients of these bi-functional guidance molecules are able to attract and repell axons in vertebrates and *C. elegans* (reviewed in Tessier-Lavigne and Goodman, 1996). In the spinal cord, for instance, commissural axons are attracted to the ventral medline by gradients of netrin-1 produced by ventrally-located floor plate cells (Kennedy et al., 1994; Serafini et al., 1994; Serafini et al., 1996). Netrin-1 produced by floor plate cells also

appears to repel trochlear motor axons at a distance in the developing brain (Colamarino and Tessier-Lavigne, 1995). In addition, analysis of axon trajectories in mice deficient for netrin-1 or the netrin receptor DCC have implicated these molecules in a number of axon guidance events (Serafini et al., 1996; Fazeli et al., 1997), including the guidance of retinal ganglion cell (RGC) axons out of the retina (Deiner et al., 1997). In Chapter Four, I have presented experiments that suggest that netrin-1 may also play a role in the guidance of *Xenopus* RGC axons from the optic chiasm to the optic tectum. However, RGC axons in the optic tract migrate on, rather than toward or away from netrin-1 expressing cells (Chapters Three and Four; de la Torre et al., 1997; Lauderdale et al., 1997; Strähle et al., 1997). Our finding that substrate-bound netrin-1 is capable of stimulating *Xenopus* retinal neurite outgrowth to the same extent as soluble netrin-1 (Chapter Four) hints at a potentially new function for netrin-1 in axon guidance: short-range axon guidance mediated by the direct contact of the extending axon with a corridor of non-difusible netrin protein. Thus, there may be two different types of postive (attractive) netrin guidance cues for axons in the developing nervous system (Figure 5-1): (i) long-range, diffusible gradients that direct axon growth by chemotaxis (e.g. commissural axon guidance to the floor plate), and (ii) short-range, physical pathways marking attractive corridors to the target.

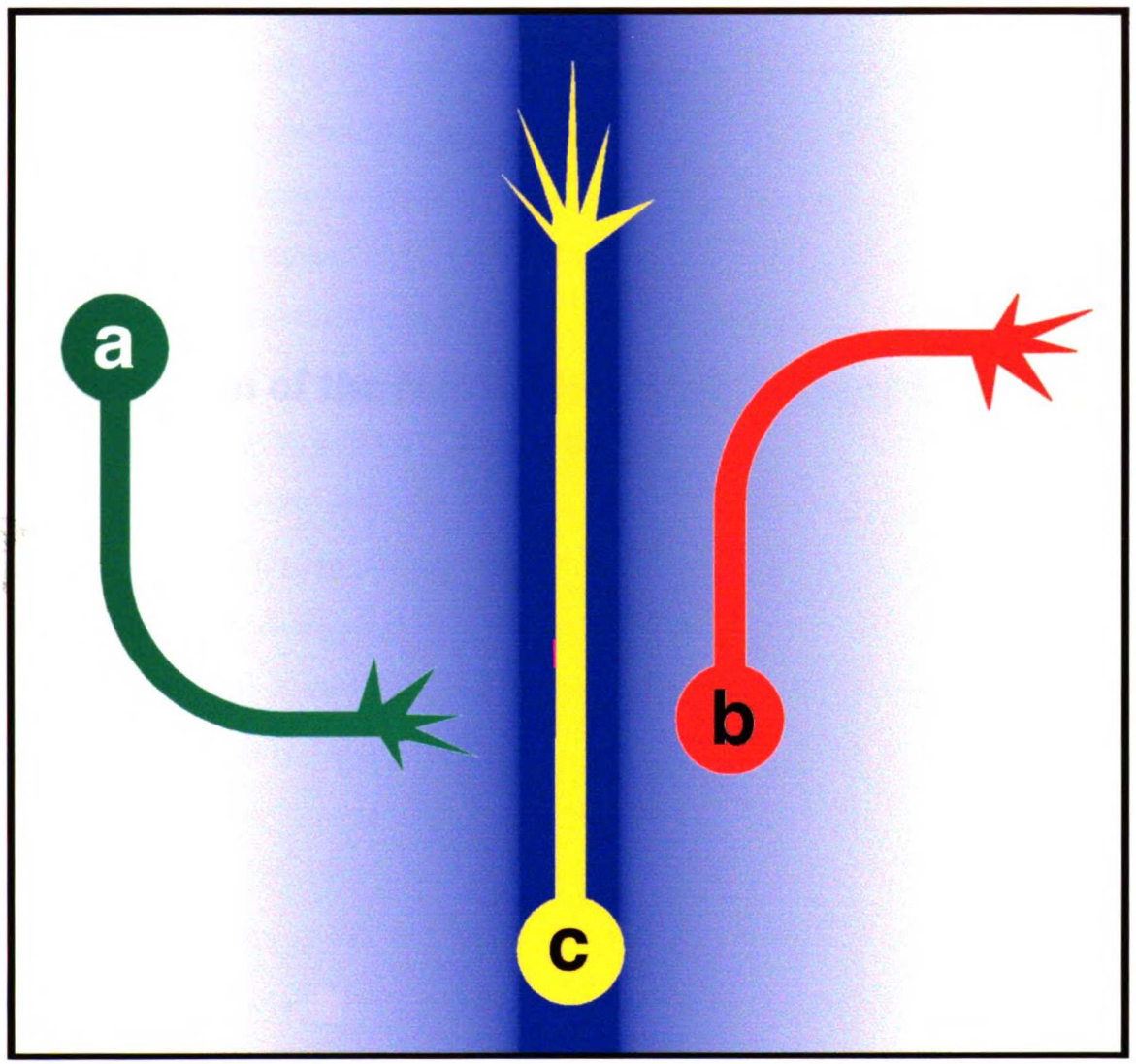
The experiments presented in Chapter Four have also indicated that netrin-1 may play an important role in RGC axons recognizing their targets in the optic tectum. This observation is supported by findings in *Drosophila* where perturbation of *netrin* gene function leads to defects in motor axon target recognition (Harris et al., 1996; Mitchell et al., 1996). As summarized above, netrin-1 may be functioning in the optic tract by establishing a corridor of attractive, substrate-bound netrin-1 on which RGC axons prefer to extend (see Chapter Four). However, upon reaching the end of the optic tract in the developing midbrain, retinal axons encounter the netrin-1-poor environment of the optic tectum. This sudden decrease in netrin signaling may directly indicate arrival in the target or may activate receptors for other target recognition signals (e.g. members of the Eph

family of signaling molecules; see Tessier-Lavigne, 1995 for review). Additional factors such as FGF-2 (McFarlane et al., 1995; McFarlane et al., 1996), which is also expressed at high levels in the optic tract but not in the tectum, have also been implicated in the recognition of the optic tectum by retinal axons. It is possible that the coordinated decrease in netrin and FGF signaling at the optic tectum triggers the expression of target recognition mechamisms; in the absence of a decrease of either signal, axons bypass the target.

The results presented in Chapter Four raise many interesting questions. For instance, can a patterned substrate of netrin-1 be used to guide axonal migrations? Previous studies have shown that molecules such as laminin and nerve growth factor (NGF) can promote the outgrowth and influence the direction of growth of axons *in vitro* both as soluble and substrate-bound factors (Gundersen and Barrett, 1979; Gundersen, 1985; Rivas et al., 1992). This issue is important given the known affinity of the netrins for cell surfaces (Serafini et al., 1994; Mirzayan, 1997). Another important question is whether DCC is also required for the migration of axons along such attractive corridors of netrin-1. Using the *in vitro* assays described in this thesis we are now in a position to begin answering these questions. In addition, these assays can be used to address more mechanistic questions. For instance, how does the extending growth cone "read" the gradient information? What molecules are involved in tying this perception of extracellular guidance information with the cytoskeletal processes of growth and turning? How do growth cones adjust their sensory machinery upon reaching an intermediate target? How do the netrins signal recognition of an axon's final target? Although recent studies have begun to address these issues (Ming et al., 1997; Song et al., 1997; Shirasaki et al., 1998), many questions still remain. It is striking that a single molecule such as netrin-1 may have so many functions in the guidance of axons. Perhaps, by having a number of possible responses of developing axons to a given molecule, the organism can limit the number of guidance cues necessary for establishing a simple network of axonal tracts. By studying the many responses of axons to a multi-functional guidance cue such as netrin-1, we may

Figure 5-1: Diagram of the potential responses of developing axons to netrin-1.

Netrin-1 can act as a multi-functional guidance cue for developing axons. Extensive *in vivo* and *in vitro* evidence suggests that the netrins can serve as long-range diffusible chemoattractants and chemorepellents (neurons ***a*** and ***b***, respectively) (reviewed in Tessier-Lavigne and Goodman, 1996). In addition, axons may migrate along netrin-expressing cells by contact-mediated attraction (neuron ***c***) (see Chapter Four). Netrin protein is indicated in blue.


a
b
c

Appendix

Expression of the Netrins in the Developing Chick Embryo

Summary

The netrins, a novel family of laminin-related axon guidance molecules, can mimic the outgrowth-promoting and chemotropic activity of the floor plate on spinal commissural axons *in vitro*. Analysis of the expression pattern of *netrin-1* and *netrin-2* transcripts in the developing chick embryo has shown that these molecules are expressed in a wide variety of cell types within, but also outside of the developing central nervous system, suggesting potential roles for these molecules in a number of axon guidance and cell migration events. As a supplement to the patterns presented in Chapters Two and Three, we describe here in further detail the expression of *netrin-1* and *netrin-2* in the developing chick embryo. Of particular interest is the expression of both *netrin-1* and *netrin-2* in the developing visual system of the chick embryo, in a pattern consistent with their playing a role in the guidance of retinal ganglion cell axons to their targets in the optic tectum. In addition, we find that netrins are expressed in a number of tissues, including developing muscle and the developing respiratory and gastro-intestinal tracts. These findings, supported by similar observations in other species, suggest a potential role for the netrins in a number of different axon guidance and cell migration events, including the formation of the retino-tectal projection.

Introduction

It has long been proposed that axons in the developing nervous system are guided to their synaptic targets through the action of long-range, diffusible chemotropic molecules (Ramón y Cajal, 1909). Embryological, genetic and culture experiments have now provided evidence for the operation of such guidance cues in numerous systems (reviewed by Tessier-Lavigne and Goodman, 1996). However, only recently have the molecular players involved in these long-range guidance events begun to be identified (Tessier-Lavigne and Goodman, 1996). One family of diffusible chemotropic factors, the netrins, have been implicated in directing the circumferential migration of commissural axons in the developing spinal cord (Kennedy et al., 1994; Serafini et al., 1994). These laminin-related proteins, originally identified from embryonic chick brain, were found to mimic the ability of the floor plate to promote the outgrowth and reorient the growth of spinal commissural axons *in vitro* (Tessier-Lavigne et al., 1988; Placzek et al., 1990a; Kennedy et al., 1994; Serafini et al., 1994). The expression of both *netrin* genes in the developing spinal cord, particularly the expression of *netrin-1* in the floor plate during the period of commissural axon growth (Kennedy et al., 1994), and the analysis of commissural axon pathways in mice lacking netrin-1 function (Serafini et al., 1996) argue that these proteins are responsible, at least in part, for the observed effects of the floor plate on the guidance of commissural axons.

Interestingly, experimental evidence also suggests that netrin homologues play a role in directing the growth of developing axons at a distance in *Caenorhabditis elegans* and *Drosophila*. In worms, the netrin homologue UNC-6 is expressed at the ventral midline and is responsible for guiding a number of circumferential axonal migrations(Hedgecock et al., 1990; Ishii et al., 1992; Wadsworth et al., 1996). Likewise, expression of Netrin-A and Netrin-B at the midline of the *Drosophila* central nervous system (CNS) is required for the proper migration of axons that normally project to the

midline (Harris et al., 1996; Mitchell et al., 1996). In addition to their role in axonal attraction, the netrins have also been implicated in mediating the repulsion of axons at a distance in *C. elegans* and vertebrates (Hedgecock et al., 1990; Ishii et al., 1992; Colamarino and Tessier-Lavigne, 1995; Wadsworth et al., 1996; Varela-Echavarria et al., 1997). Moreover, mutations in unc-6 have been shown to disrupt the migration not only of axons, but also of motile mesodermal precursor cells as well (Hedgecock et al., 1990; Ishii et al., 1992; Wadsworth et al., 1996). These findings raise the possibility that netrins may do more in vertebrate embryos than simply mediate the guidance of commissural axons to the floor plate.

In order to investigate other potential roles of netrin-1 and netrin-2, we have studied their expression in the developing chick embryo. These studies are meant to complement those previously described in Chapter Two in chick (Kennedy et al., 1994) and Chapter Three in *Xenopus* (de la Torre et al., 1997). Here we present data showing that both *netrin* genes are expressed in a wide variety of tissues and cell types, both within and outside the developing nervous system. In particular, it is interesting to note the expression of both netrins in the developing visual system. These observations, along with similar findings in other species (Kennedy et al., 1994; Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1996; Wadsworth et al., 1996; de la Torre et al., 1997; Deiner et al., 1997; Lauderdale et al., 1997; Livesey and Hunt, 1997; Strähle et al., 1997), suggest that netrins may direct a large number of axon guidance and cell migration events in the developing embryo.

Results

Netrin-1 and *Netrin-2* Are Expressed in the Developing Visual System

As early as stage 12 (Hamburger and Hamilton, 1951), *netrin-1*-expressing cells can be detected in the developing optic cup and stalk (Figures 6-1A and 6-1B). By stage 15, large amounts of *netrin-1* transcripts are seen in the ventral retina and in the region of the choroid fissure (Figure 6-1C - E). This region progressively narrows to from the optic disc, the exit point from the retina for retinal ganglion cell (RGC) axons. At this stage, *netrin-1* transcripts can also be detected in the optic stalk where RGC axons grow to form the optic nerve, and in the caudal diencephalon where these axons form the optic tract (Figure 6-1E). At later stages (embryonic day 10; E10), both *netrin-1* and *netrin-2* transcripts are detected in the ventricular region of the lateral ventricles of the midbrain (Figures 6-1F and 6-1G), a region of the brain corresponding to the optic tectum. Thus, it appears that the netrins, primarily *netrin-1*, are expressed along the retino-tectal pathway taken by RGC axons during their migration to their targets in the optic tectum.

Netrin Expression in Axial and Paraxial Mesoderm

From the early stages of gastrulation (stage 4), *netrin-1* is expressed in axial mesoderm (Figure 6-2A). These *netrin-1*-expressing cells appear to be part of the migratory head process mesoderm. By stage 7, *netrin-1* staining can be seen in the notochord underlying the developing neural tube from cranial levels to Hensen's node (Figures 6-2B and 6-2C). At this stage, *netrin-1* is also expressed in the paraxial mesoderm. In the pre-somitic mesoderm, *netrin-1* is expressed diffusely throughout the mesenchyme (Figure 6-2B and 6-2C). As the first few somites epithelialize, *netrin-1* expression remains strong throughout the somite (Figures 6-2B and 6-2D). However, by stage 11 expression of *netrin-1* transcripts become progressively localized to the dermamyotome and is lost in the anterior portions of the notochord (Figures 6-2B and 6-2E). *Netrin-2* transcripts are

also detected in the developing somite, particularly in the dermamyotome, and to a lesser degree in the sclerotome (Figure 6-2F). At late stages (stage 31) *netrin-1* expression is detected in migrating myoblasts at trunk levels (Figures 6-2G), and in condensing muscle tissue (data not shown).

Other Regions of *Netrin-1* and *Netrin-2* Expression

Northern analysis indicates that *netrin-2* is expressed in the developing respiratory and gastro-intestinal tracts (Kennedy et al., 1994). RNA *in situ* hybridization reveals that at stage 31, *netrin-2* expressed in a ring of indistinguishable mesenchymal cells surrounding the epithelium of the trachea, and in a group of mesenchymal cells running just ventral to the epithelium of the esophagus (Figure 6-2H).

Discussion

Previous studies have implicated the vertebrate netrins in the guidance of commissural axons to the floor plate, an intermediate target in the ventral neural tube (Kennedy et al., 1994; Serafini et al., 1994; Shirasaki et al., 1995; Serafini et al., 1996; Shirasaki et al., 1996). In addition, recent evidence has also implicated netrin-1 and the netrin receptor Deleted in Colorectal Cancer (DCC) in a number of axon and cell migrations in the developing and adult brain, including the migration of RGC axons out of the retina and to their targets in the midbrain (Serafini et al., 1996; de la Torre et al., 1997; Deiner et al., 1997; Fazeli et al., 1997; Livesey and Hunt, 1997, and Chapter Four). However, these studies have focused primarily on the function of the netrins and their receptors in the developing CNS. In this Appendix, we present expression data suggesting that the netrins may be involved in numerous processes outside the central nervous system. Since the involvement of netrin-1 in the formation of the retino-tectal projection has been discussed extensively in Chapters Three and Four, we will focus here on other regions of netrin expression and potential netrin involvement.

Netrin Expression in the Developing Somite and Muscles

Some evidence has been provided in vertebrates for the involvement of diffusible chemoattractants in the guidance of spinal motor neurons to their targets in the limb or axial musculature (for review, see Tosney, 1991; Eisen, 1994). For instance, hepatocyte growth factor/scatter factor (HGF/SF) has recently been shown to be a diffusible attractant for limb motor neurons (Ebens et al., 1996). Other studies in chick embryos have suggested that the dermomyotome, which does not express HGF/SF, may produce a chemoattractant for epaxial motor neurons (Tosney, 1987). Likewise, myoblasts have been shown to secrete an activity that promotes the outgrowth of *Xenopus* spinal neurites from neural tube explants *in vitro* (McCaig, 1986). Molecular candidates for these

activities have yet to be identified. Netrins are expressed in myotomal cells and muscle precursors in vertebrates as well as in *Drosophila* (this study; Kennedy et al., 1994; Harris et al., 1996; Mitchell et al., 1996; Serafini et al., 1996; Livesey and Hunt, 1997). Overexpression of Netrin-A and Netrin-B in *Drosophila* embryos disrupts the guidance of motor neurons to their target muscles (Harris et al., 1996; Mitchell et al., 1996). However, while the lack of Netrin-A and Netrin-B function leads to defects in motor neuron guidance in *Drosophila* (Harris et al., 1996; Mitchell et al., 1996), in contrast, no alteration of spinal motor neuron projections was observed in netrin-1-deficient mice (Serafini et al., 1996). It is possible that netrin-2 may guide these axons, and that due to overlapping expression and redundant function, no phenotype was observed in the single *netrin-1* mutant. Alternatively, netrin expression in developing myotome or muscles may serve to direct cell migrations to sites of muscle condensation or myotube formation.

Expression of *Netrin-2* in the Developing Gastro-Intestinal Tract

During the development of the gastro-intestinal system, neural crest cells from sacral and vagal levels migrate to and colonize the embryonic gut (reviewed in Gershon et al., 1993). Little is know about the mechanisms guiding these cells to the developing gastro-intestinal tract, or about the subsequent innervation of the enteric nervous system (ENS). In chick embryos, this process begins around stage 21 and continues through stage 33 (Newgreen et al., 1996). It is therefore striking to find that *netrin-2* expression coincides with the period of neural crest invasion. These finding suggest that a potential role for the *netrin-2* expression in the developing gut is to guide the migration of these neural crest cells to their targets. Similarly, netrin-1 is reported to be expressed in the developing chick heart (Kennedy et al., 1994), another organ colonized by the neural crest. This observation suggests that netrin-1 may also play a role in attracting neural crest cells, or afferent innervation from the vagal nerve, to the developing heart.

However, it is important to state that there is as of yet no experimental evidence for the existence of such neural crest chemoattractants in either the developing heart or gut.

Other Possible Roles for Netrins

Previous reports also indicate that the netrins are expressed in tissues such as the developing heart, spleen and gonads (Kennedy et al., 1994).. As described above, in tissues such as the developing heart, the netrins may play a role in guiding migratory neural crest cells or innervation of the peripheral nervous system. Similarly, organs such as the spleen or the developing gonads are the site of extensive cell migrations. For instance, it is possible that the netrins play a role in the migration of germ cell precursors to the developing gonads. Unfortunately, the role of the netrins in these systems cannot be easily addressed *in vivo* due to the death of netrin-1-deficient mice perinatally (Serafini et al., 1996). Nevertheless, these data strongly suggest that the netrins are involved in a number of developmental processes throughout the developing embryo.

Experimental Procedures

RNA *in situ* hybridization

White leghorn chicken embryos (Feather Hill Farms, Petaluma, Ca.) were incubated at 38°C. Embryos were dissected from surrounding membranes in ice-cold L-15 medium, fixed overnight in 4% paraformaldehyde (PFA) in DEPC-treated PBS at 4°C, and washed once in DEPC-treated PBS at 4°C for 1 h. *In situ* hybridization was performed as follows.

For whole-mount *in situ* hybridization, embryos before stage 15 were dehydrated through a PBS/methanol series, stored in methanol at -20°C, and processed as described by Coutinho et al. (1992) with the following modifications: (1) the proteinase K (PK) treatment was reduced to 15 min. at 37°C, and embryos were subsequently refixed 30 minutes in 4% PFA/0.2% glutaraldehyde in PBS; (2) the antibody was pre-adsorbed to acetone embryo powder (Wilkinson and Nieto, 1993) in 10% heat-inactivated goat serum, 0.1% Triton-X100 in PBS. Embryos from which the CNS was to be excised were refixed for only 10 min. in 4% PFA after PK treatment; at the end of the entire whole-mount procedure, the CNS was teased out using tungsten needles. Prior to mounting, embryos were passed through 30%, 50% and 80% glycerol. Embryos were sectioned at 20-30 μm on a vibratome, as previously described (Kennedy et al., 1994).

For *in situ* hybridization on sections, the brachial region of St. 15 and older embryos was fixed as described above, incubated overnight at 4°C in 30% sucrose in DEPC-treated PBS, transferred to OCT compound for 1-4 h at 4°C, and embedded in OCT at -70°C. 10μm cryostat sections were collected on Superfrost Plus slides (Fisher) and stored at -80°C. *In situ* hybridization using digoxygenin-labeled probes was performed as described (Schaeren-Wiemers and Gerfin-Moser, 1993). *In situ* hybridization using ^{33}P- or ^{35}S-labeled probes was performed as described (Frohman et al., 1990).

For both netrin-1 and netrin-2, several antisense riboprobes were prepared from the coding region and from the 3' untranslated region. For netrin-1, a probe corresponding to the first 900 bp of coding sequence was routinely used; similar results were obtained with a probe corresponding to domain C (455 bp). For netrin-2, a 2.1 kb probe comprising 1.1 kb of coding and 1.0 kb of 3' UTR sequences was routinely used; similar results were obtained with a 455 bp probe corresponding to 3' UTR sequences. Fragments were subcloned into pBluescript (Stratagene), or amplified by PCR and subcloned into pCR (Invitrogen); plasmids were linearized with appropriate enzymes and transcribed with T7 or SP6 RNA polymerase, as appropriate. Probes were used at a final concentration of 200 ng/ml. Prior to whole-mount *in situ* hybridization, large (>900 bp) probes were base-hydrolyzed to an average probe size of ~200 bp.

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Figure 6-1: Expression of Netrin-1 and Netrin-2 in the Developing Visual System.

Whole embryos (E), transverse sections of embryos (A-D) or coronal sections of E10 brains (F-G) were hybridized with antisense netrin-1 (A-F) or netrin-2 (G) RNA probes. Digoxygenin-labeled probes were used for panel E; in all other panels, ^{35}S-labeled probes were used. In all panels, dorsal is up. Anterior is left in E.

(A-B) Transverse section through the diencephalon and optic vesicle of a stage 12 embryo hybridized with a netrin-1 probe, viewed in phase contrast (A) and dark field illumination (B). Note staining in the optic cup and the optic stalk.

(C-D) Transverse section through the eye of a stage 15 embryo hybridized with a netrin-1 probe, viewed in phase contrast (C) and dark field (D). Netrin-1 is expressed in both the ventral neural retina (nr) and pigmented epithelium (rpe).

(E) Lateral view of the brain of a stage 15 embryo hybridized with a netrin-1 probe. Note expression in the ventral retina, adjacent to the choroid fissure and in the optic stalk

(E) Transverse vibratome section through the eye and diencephalon of a stage 15 embryo hybridized with a netrin-1 probe. Note staining in the choroid fissure, optic stalk and at the optic chiasm.

(F-G) Coronal sections through the midbrain of an E10 chick brain hybridized with netrin-1 (F) and netrin-2 (G) probes. Note *netrin-1* expression in the ventral and lateral ventricular zone of the lateral ventricles (F), compared to the staining throughout the ventricular zone of the lateral ventricles for *netrin-2* (G).

cf, choroid fissure; di, diencephalon; le, lens; mb, midbrain; os, optic stalk; ov, optic vesicle; tel, telencephalon.

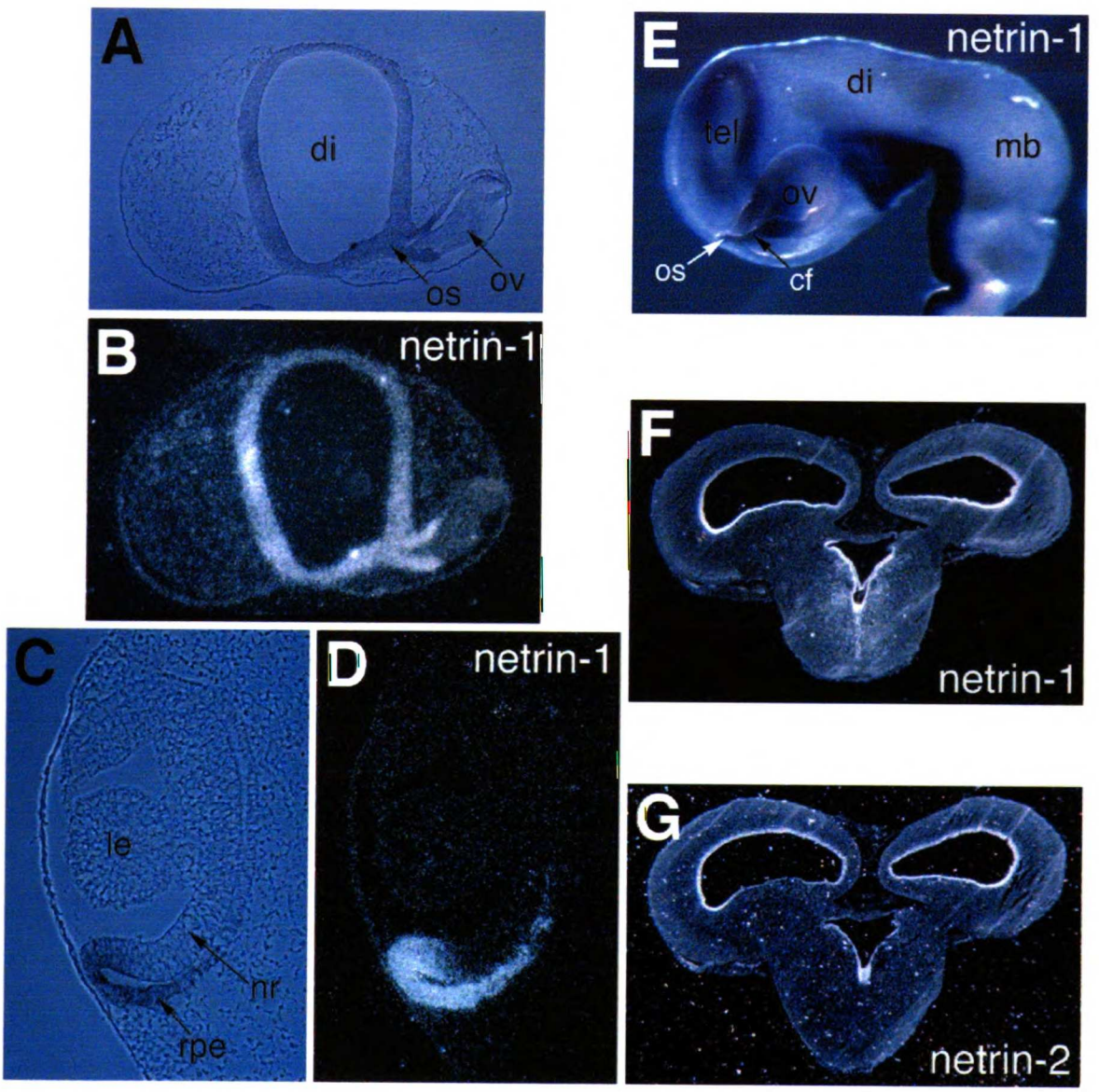
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Figure 6-2: Expression of Netrin-1 and Netrin-2 in Axial and Paraxial Mesoderm.

Whole embryos (A-E) or transverse sections of embryos (F-H) were hybridized with digoxygenin-labeled antisense netrin-1 (A-E, G) or netrin-2 (F, H) RNA probes. In panels A and B, anterior is up; in C-I dorsal is up.

(A) Dorsal view of a stage 4 embryo hybridized with a netrin-1 probe. Note netrin-1 expression in the head process mesoderm anterior to the primitive streak (arrowhead).

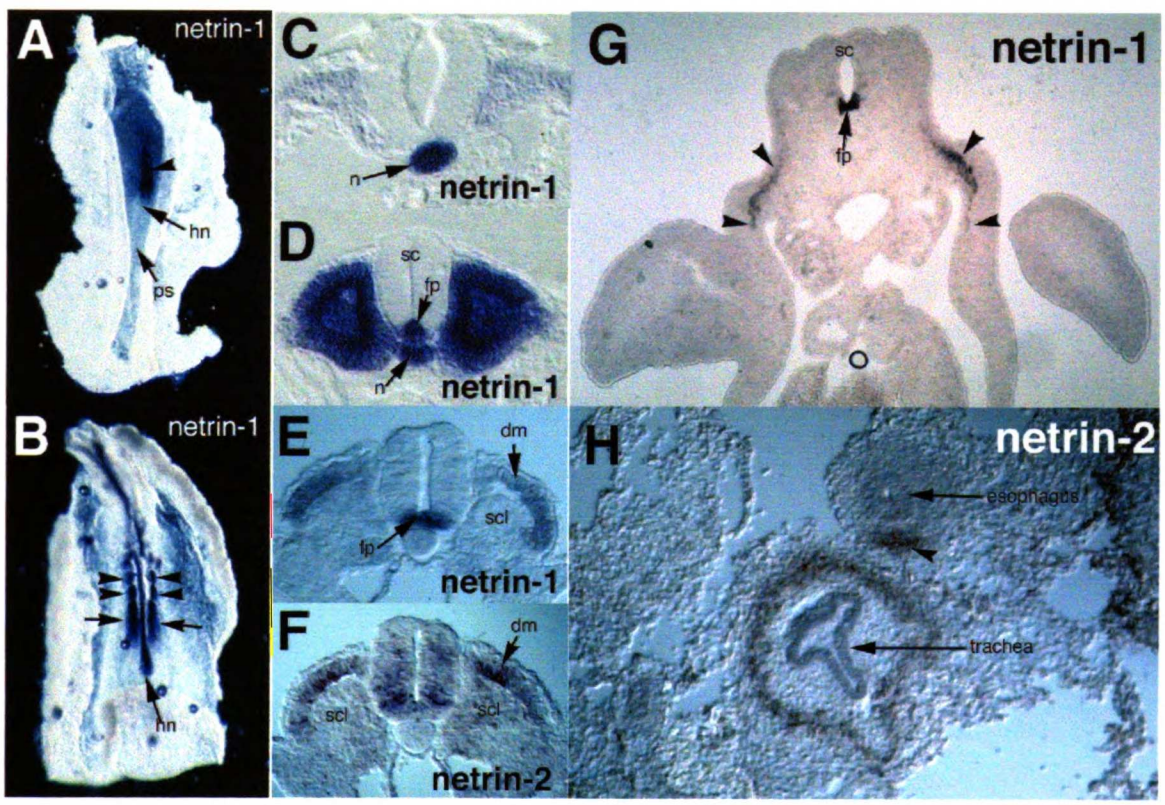
(B) Dorsal view of a stage 7 embryo hybridized with a netrin-1 probe. Note staining in axial and paraxial mesoderm (arrowheads).

(C-F) Transverse vibratome sections through stage 11 (C-D) and stage 15 (E-F) embryos hybridized with netrin-1 (C-E) or netrin-2 (F) probes.

(G) Transverse sections through the brachial region of a stage 31 embryo hybridized to a netrin-1 probe. Note netrin expression in the migrating myoblast cells.

(H) Transverse section through the brachial region of a stage 31 chick embryo showing *netrin-2* is expression in mesenchymal cells surrounding the trachea and just ventral to the esophagus.

dm, dermomyotome; fp, floor plate; hn, Hensen's node; n, notochord; ps, primitive streak; sc, spinal cord; scl, sclerotome.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esophagus
trachea

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Cuando yo muera quiero tus manos en mis ojos:
quiero la luz y el trigo de tus manos amadas
pasar una vez más sobre mí frescura:
sentir la suavidad que cambió mi destino.

Quiero que vivas mientras yo, dormido, te espero,
quiero que tus oídos sigan oyendo el viento,
que huelas el aroma del mar que amamos juntos
y que sigas pisando la arena que pisamos.

Quiero que lo que amo siga vivo
y a ti te amé y canté sobre todas las cosas,
por eso sigue tú florecienco, florida,

para que alcances todo lo que mi amor te ordena,
para que pasee mi sombra por tu pelo,
para que así conozcan la razón de mi canto.

Pablo Neruda, *Cien Sonetos de Amor.*