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# VISUAL SYSTEM AND NEUROENDOCRINE HYPOTHALAMUS DEVELOPMENT: ROLES FOR THE GUIDANCE CUE NETRIN-1 AND ITS RECEPTOR DCC

by

**Michael S. Deiner**

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

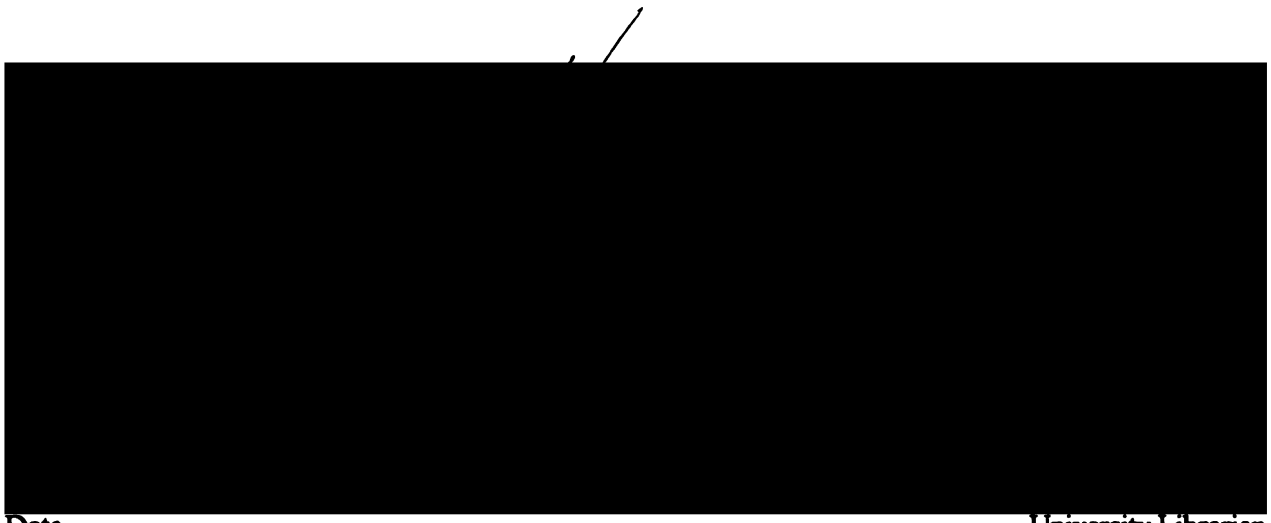
**Biomedical Science**

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA SAN FRANCISCO



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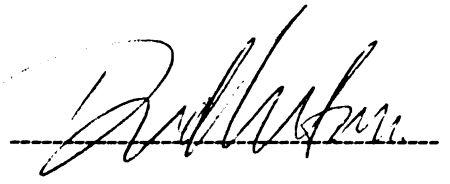
## ADVISOR'S STATEMENT

Regarding previous publications and material with multiple authors

Chapter 2 has been previously published (Deiner, M.S., T.E. Kennedy, A. Fazeli, T. Serafini, M. Tessier-Lavigne and D.W. Sretavan. 1997. Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. Neuron 19(3):575-589). Copyright is held by Cell Press, and it is reproduced with permission (see page v). This work was published in collaboration with Drs. Tim Kennedy, Tito Serafini, Amin Fazeli, Marc Tessier-Lavigne and David Sretavan. Michael Deiner was the primary investigator and author.

Chapter 3 has been submitted for publication (Deiner, M.S. and D.W. Sretavan. 1999. Altered midline axon pathways and ectopic neurons in the developing hypothalamus of netrin-1 and DCC deficient mice).



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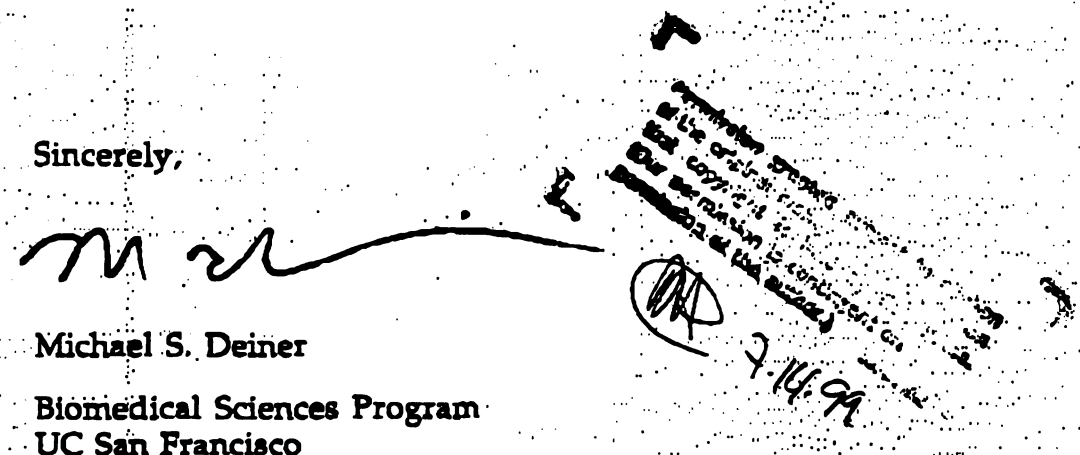

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**VISUAL SYSTEM AND NEUROENDOCRINE HYPOTHALAMUS DEVELOPMENT: ROLES FOR THE GUIDANCE CUE NETRIN-1 AND ITS RECEPTOR DCC**

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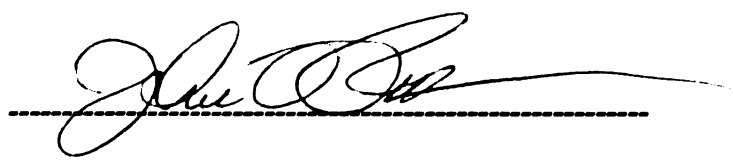

**John L. Rubenstein**
**Chair, Ph.D. Thesis Committee**

## ABSTRACT

During development, embryonic retinal ganglion cell (RGC) axons must extend toward and grow through the optic disc to exit the eye into the optic nerve. In the embryonic mouse eye, we found that immunoreactivity for the axon guidance molecule netrin-1 was specifically on neuroepithelial cells at the disk surrounding exiting RGC axons, and RGC axons express the netrin receptor, DCC (deleted in colorectal cancer). *In vitro*, anti-DCC antibodies reduced RGC neurite outgrowth responses to netrin-1. In netrin-1- and DCC-deficient embryos, RGC axon pathfinding to the disc was unaffected; however, axons failed to exit into the optic nerve, resulting in optic nerve hypoplasia. Thus, netrin-1 through DCC appears to guide RGC axons locally at the optic disc rather than at long range, apparently reflecting the localization of netrin-1 protein to the vicinity of netrin-1-producing cells at the optic disc.

Further along the visual pathway, RGC axons in netrin-1 or DCC deficient mice grow in unusually angular trajectories within the ventral hypothalamus, often forming the optic chiasm in an abnormally posterior position. In heterozygous *Sey*$^{neu}$ mice which also have small optic nerves, RGC axon trajectories appear normal indicating the altered RGC axon trajectories in netrin-1 and DCC mutants are not secondarily due to optic nerve hypoplasia. Intrinsic hypothalamic patterning is also affected in netrin-1 and DCC mutants including a severe reduction in posterior axon projections of gonadotropin-releasing hormone neurons. In addition to axon pathway defects in mutants, anti-diuretic hormone and oxytocin neurons are found ectopically in the ventromedial hypothalamus, apparently no longer confined to the supraoptic nucleus. In sum, netrin-1 and DCC, presumably through direct interactions, govern both axon pathway formation and neuronal position during hypothalamic development and loss of netrin-1 or DCC function affects both visual and neuroendocrine systems. Netrin protein localization also indicates that unlike in more caudal CNS, guidance about the hypothalamic ventral midline does not require midline expression of netrin.

Optic nerve hypoplasia and hypothalamic developmental abnormalities can now be added to corpus callosum defects in netrin-1 and DCC mutant mice. This trio of seemingly disparate phenotypes is reminiscent of the human congenital multisystem syndrome of septo-optic dysplasia.

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# Chapter 1

# Thesis Introduction

A functional nervous system is crucial for our survival, allowing us to sense and respond appropriately to stimuli in our environment. Given the large number of cues and responses (both external and internal) that must be processed on a daily basis, it is not too surprising that the mammalian nervous system is quite complex, consisting of large numbers of neurons precisely connected to their targets. Each neuron typically has multiple dendrites which receive signals from upstream cells or sensory organs and an axon which sends outgoing signals to downstream targets such as other neurons. A crucial requirement for proper nervous system function therefore is that each neuron is connected properly to receive signals and to send signals to correct target neurons. A key area of investigation in the field of developmental neurobiology is to explore the mechanisms which ensure a migrating axon growing out from the cell body chooses the correct path to allow it to eventually reach and form connections with its final target. In my thesis work, I have examined this issue in the developing visual and neuroendocrine systems.

Over a century ago, Santiago Ramon y Cajal proposed a number of different models of how cells and axons are guided along the route to their targets during development. Central to his ideas was that axons were extensions protruding away from a cell body and that growth cones at their growing end could interact with chemicals in the environment that either promoted growth in general (chemotrophism) or promoted growth directionally towards a concentrated target source (chemotropism), eventually bringing the axon to its final target (Ramon y Cajal, 1892). Cajal proposed this chemical guidance cue model as an addition to Wilhelm His' model that some axons may simply follow

the path of least resistance to reach their target (mechanical guidance) (reviewed in Hamburger, 1980).

Cajal's concept of an axon with a growth cone as an outgrowth from the cell body was subsequently proven true by the pioneering tissue culture work of Ross G. Harrison (Harrison, 1910) and (reviewed in Hamburger, 1980). Subsequently, the first identification of molecules which could affect axon outgrowth came from the tissue culture work of Rita Levi-Montalcini and colleagues in the 1950s which demonstrated that a specific protein called nerve growth factor (NGF) could promote axon outgrowth from sensory and sympathetic neurons (Levi-Montalcini et al., 1954). Later studies also demonstrated that NGF could promote directional growth (Menesini Chen et al., 1978). The importance of this work was that it showed protein molecules could act as both trophic and tropic substances for guidance of growing axons, as originally proposed by Cajal (Levi-Montalcini, 1979). Along with secreted diffusible proteins such as NGF, subsequent studies have also shown that substrate bound proteins such as laminin (Timpl et al., 1979) can also promote axon outgrowth (Baron-Van Evercooren et al., 1982) and, if presented non-uniformly, can even promote directional outgrowth along specific pathways (Hammarback et al., 1985). In the last few decades, a large number of growth promoting and attracting axon guidance cues have been found (reviewed in Tessier-Lavigne and Goodman, 1996). Furthermore, a number of guidance cues that can inhibit and even repel the growth of axons have also been found (for example see Walter et al., 1987; also reviewed in Tessier-Lavigne and Goodman, 1996).

Together with these and many other studies, a modern understanding of how guidance cues might work has evolved. One current view suggests that cues either allow or prohibit axon growth locally when presented as a cell surface-bound substrate or that cues either attract or repel growing axons over long distances when presented as a concentration gradient (see figure 1-1, figure modified from Tessier-Lavigne and Goodman, 1996). For any given cue, an interaction between the guidance cue ligand in the environment and sensory receptor proteins expressed by a migrating cell or at the growth cone is thought to activate intracellular signaling cascades that results in the appropriate changes in cell migration or growth cone behavior, causing guidance. Ultimately, a number of different positive and negative cues laid out in the appropriate pattern could guide a cell or axon along a specific pathway to the correct target.

Remarkably, through the discovery and study of axon guidance cues and receptors in multiple model systems, it is now clear that guidance cue and receptor homologues often have similar functions in the developing nervous systems of many different animals (reviewed in Tessier-Lavigne and Goodman, 1996). As an example, the guidance cue netrin and its receptors have been studied in nematodes (Ishii et al., 1992), in vertebrates (Serafini et al., 1994; Serafini et al., 1996; Strähle et al., 1997) and in the fruitfly (Harris et al., 1996; Mitchell et al., 1996) (reviewed in Guthrie, 1997). In vertebrates, netrins are secreted from cells and can interact with netrin receptors expressed on growing axons and on migrating cells to cause migration towards a concentrated long range netrin source (Kennedy et al., 1994; Keino et al., 1996; Serafini et al., 1996; Fazeli et al., 1997). Similar roles exist in nematodes and fruitflies. A structural depiction of netrin-1 and one of its receptors, DCC and an example of how they

are thought to function to guide one class of vertebrate spinal cord axons is shown in figure 1-2.

Although the mechanisms proposed to guide migrating cells and axons are commonly described in terms of simple definitive categories, the boundary between these categories is becoming increasingly blurred as studies begin to show a given cue may act in a different manner depending on the particular neuron and region of the nervous system. For example, in addition to acting as a long-range attractive cue for migrating cells or axons, netrin may also act as a short-range growth promoting cue (Wadsworth et al., 1996; Deiner et al., 1997) and can even repel, rather than attract, migration of certain axons and cells (Colamarino and Tessier-Lavigne, 1995; reviewed in Guthrie, 1997). Furthermore, for any specific type of migrating cell or axon, in general we are not yet able to define the complete set of cues needed for proper pathway formation. A major goal in the field of axon guidance today is to first elucidate all of the contributing cues in a specific migration pathway and then to learn the logic and rules of how individual axons integrate all of these cues and mechanisms to follow the correct pathway to targets during development.

In my thesis work I have focused on how a series of guidance cue mechanisms could act in concert in the early developing mouse visual system. Most vertebrates rely on their sense of vision to carry out tasks basic for day to day survival and in order for this system to function properly, axons of retinal ganglion cells must be connected appropriately to convey visual information from the eye to the brain. During development, one of the earliest pathfinding tasks that retinal ganglion cell axons must undergo occurs when the axons extend within the retinal eye cup from the cell body towards the exit point, called

the optic disc to then leave the eye to enter the optic nerve *en route* to the brain. This is a precise system in that axons are not sent outward towards the periphery or along indirect routes towards the disc, suggesting that axon guidance mechanisms must be playing a role (Silver and Sidman, 1980; Halfter et al., 1985).

Several studies have begun to dissect out some of the mechanisms and molecules involved in this process (depicted in fig. 1-3). Cues which have been proposed to direct axons towards the optic disc involve an inhibitory outer boundary of proteoglycans that directs initial axon elongation centrally (Brittis et al., 1992) as well interactions of cell adhesion molecules of the immunoglobulin superfamily (Brittis et al., 1995; Brittis and Silver, 1995). Once at the disc though, the cues and mechanisms involved in guiding axons out of the eye into the nerve are not known.

In chapter two of my thesis, I present several lines of evidence that suggest netrin-1 acting through DCC is involved in guidance of retinal ganglion cell axons at the disc. This includes *in situ* data showing suggestive patterns of netrin and DCC protein expression, *in vitro* results showing a retinal neurite outgrowth response to netrin and *in vivo* phenotypic analysis in mouse embryos lacking either netrin-1 or DCC, depicting axon guidance errors at the optic disc. In chapter three, I present similar lines of evidence suggesting netrin-1 and DCC are also involved further along the visual system in the developing optic chiasm of the ventral diencephalon region, also known as the hypothalamus. As part of chapter three, I also present another component of my thesis work demonstrating that netrin-1 and DCC are involved not only in visual system pathway formation in the hypothalamus but also in several aspects of hypothalamic neuroendocrine system development. Finally, in chapter four I

describe a human disorder called septo-optic dysplasia (Skarf and Hoyt, 1984) and depict how it consists of symptoms that are quite similar to the phenotypes I have observed in mice missing either netrin-1 or DCC (described in chapters two and three). I then describe the design and current progress of an ongoing collaboration asking if mutations in netrin-1 or DCC could therefore be involved in certain cases of human septo-optic dysplasia.

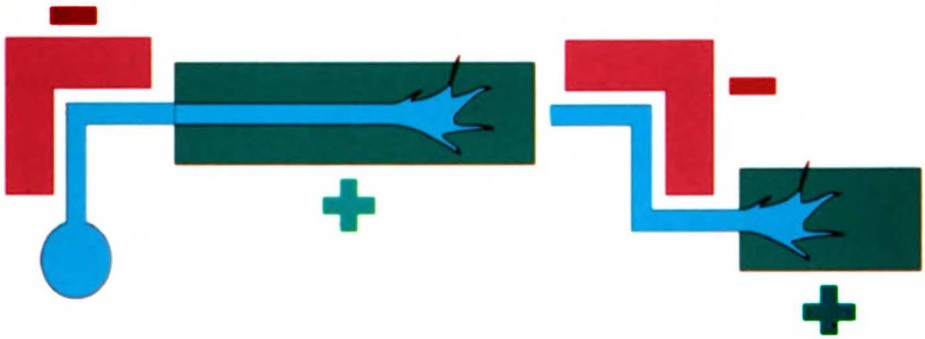
In summary, my thesis work has begun to help us better understand the many cues and mechanisms used for guiding RGC axons in the early developing visual system as well as for guiding neurons of the neuroendocrine hypothalamus. Netrin-1 and DCC, presumably through direct interactions, should now be considered as major players in these processes. Having stated this though, a second finding of my work is that not all RGC axons require netrin-1 and DCC interactions to exit the eye and not all neuroendocrine neurons require netrin-1 and DCC to develop proper connections. This suggests that other guidance cue molecules and receptors must also be involved. The goal of future studies will be to determine what these other cues and mechanisms are and how, along with netrin and DCC interactions, all of these cues might act in concert to achieve the ultimate goal of properly wired and functional visual and neuroendocrine systems.

FIGURE LEGENDS

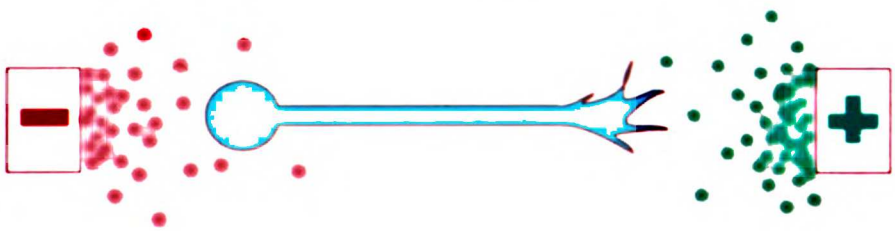
**Figure 1-1: Guidance Cue Mechanisms**

A growing neuron (light blue) extends an axon with a motile sensory apparatus at its tip (a growth cone). Receptors on the growth cone are thought to respond to cues in the environment that activate intracellular signal transduction pathways resulting in behavioral changes in the extension of a growth cone and thus guiding axon growth. Similar concepts apply to migrating cells. Guidance cues fall into several different categories: **(Top)** Inhibitory, short range cues (red) prevent growth into the region where they are expressed, while permissive short-range cues (green) allow for growth. **(Bottom)** Long range cues depicted as secreted from a source appear as a gradient. Interaction of the ligand with receptors in growth cones either causes increased growth into regions of higher ligand concentration (up a gradient towards the source) or increased growth into regions of lower ligand concentration (down a gradient away from the source). Proper migration of a single cell or axon probably requires multiple guidance mechanisms acting at different points along the route of migration. This figure is a modification of a figure from (Tessier-Lavigne and Goodman, 1996).

## SHORT RANGE (CELL SURFACE)



## LONG RANGE (DIFFUSIBLE)



(modified from Tessier-Lavigne and Goodman, 1996)

**Figure 1-1**

**Figure 1-2: Netrin-1 is a guidance cue and DCC is a netrin receptor**

Netrin-1 (red) is a secreted molecule with a globular, EGF repeat and basic c-terminal domains. The globular and EGF repeat domains, are homologous to domains 6 and 5 of the laminin beta 2 chain. The netrin receptor DCC (green) is a member of the immunoglobulin superfamily with 4 Ig, 6 FNIII repeat, a transmembrane and DCC cytoplasmic domains. The schematic of a cross section of the developing spinal cord summarizes the model based on both *in vitro* and *in vivo* evidence that expression of DCC on axons of dorsal commissural neurons causes them to send axons towards the ventral midline in response to a concentration gradient of netrin emanating away from the floorplate.

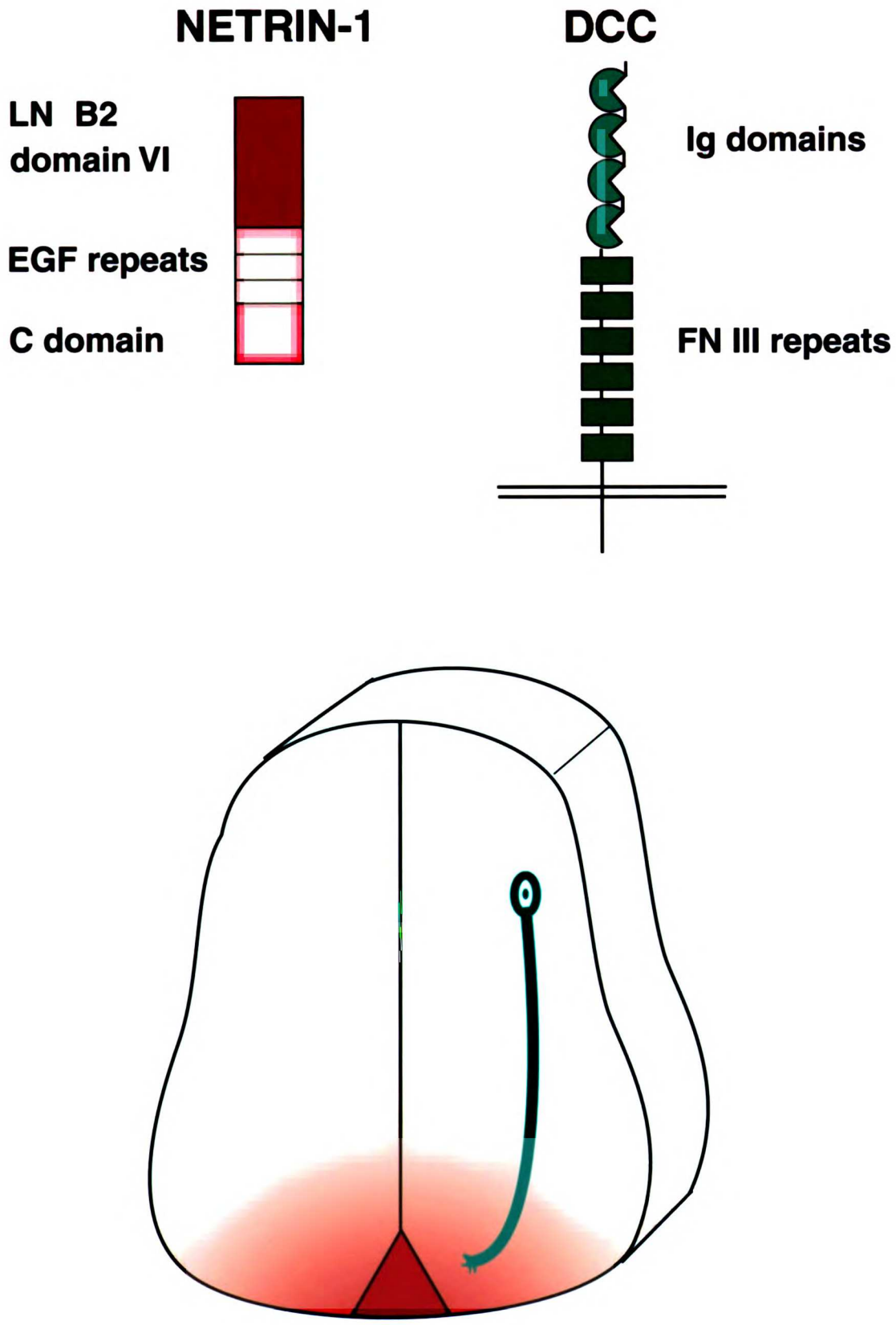

NETRIN-1
DCC
LN B2
domain VI
EGF repeats
C domain
Ig domains
FN III repeats

**Figure 1-2**

**figure 1-3: Eye development and proposed axon guidance mechanisms**

Developing retinal ganglion cell axons extend within the retinal eye cup from the cell body towards the exit point, called the optic disc (**OD**) to then leave the eye to enter the optic nerve *en route* to the brain. An inhibitory outer boundary of chondroitin sulfate proteoglycans (**CSPG**) has been proposed to direct initial axon elongation centrally (Brittis et al., 1992). Cell adhesion molecules (**CAM**) of the immunoglobulin superfamily including the retinal ganglion cell marker, L1 have been proposed to guide axons to the optic disc (Brittis et al., 1995; Brittis and Silver, 1995). Once at the disc though, the cues and mechanisms involved in guiding axons out of the eye into the nerve are not well known.

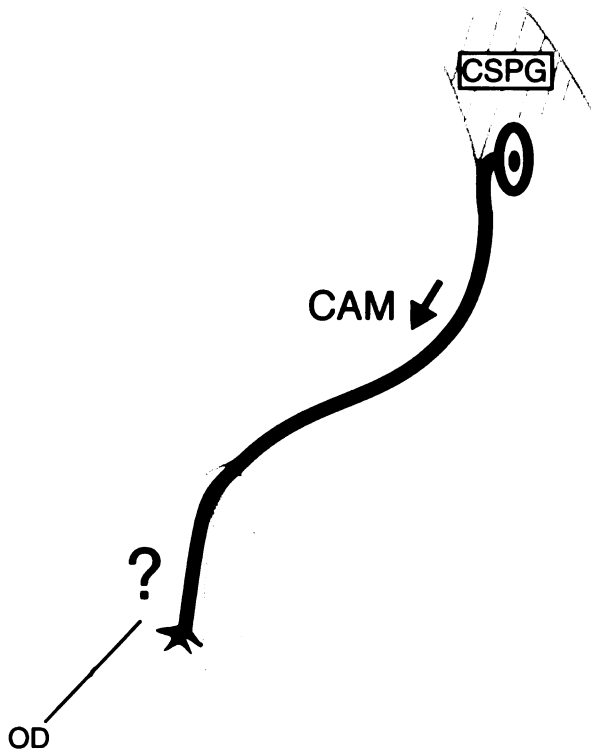



**Figure 1-3**

# Chapter 2

## Netrin-1 and DCC Mediate Axon Guidance Locally At The Optic Disc: Loss of Function Leads To Optic Nerve Hypoplasia.

SUMMARY

Embryonic retinal ganglion cell (RGC) axons must extend toward and grow through the optic disc to exit the eye into the optic nerve. In the embryonic mouse eye, we found that immunoreactivity for the axon guidance molecule netrin-1 was specifically on neuroepithelial cells at the disk surrounding exiting RGC axons, and RGC axons express the netrin receptor, DCC (deleted in colorectal cancer). *In vitro*, anti-DCC antibodies reduced RGC neurite outgrowth responses to netrin-1. In netrin-1- and DCC-deficient embryos, RGC axon pathfinding to the disc was unaffected; however, axons failed to exit into the optic nerve, resulting in optic nerve hypoplasia. Thus, netrin-1 through DCC appears to guide RGC axons locally at the optic disc rather than at long range, apparently reflecting the localization of netrin-1 protein to the vicinity of netrin-1-producing cells at the optic disc.

INTRODUCTION

During development, axon pathfinding mechanisms work to ensure that RGC axons grow along specific pathways to innervate appropriate visual processing centers in the CNS. One of the first pathfinding tasks required for the proper development of RGC axon projections occurs within the eye as RGC axons find their way from their site of origin to enter the optic nerve *en route* to the brain. The morphogenetic events involved in early eye formation that set the stage for axon exit from the retina have been well described. In vertebrates, the early optic cup forms as an extension of the CNS and remains attached to the

brain via the optic stalk (see Barishak, 1992; Kaufman, 1992 for review). Invagination of the ventral aspect of both the optic cup and optic stalk forms a continuous groove called the optic fissure. Subsequently, the two sides of the fissure fuse giving rise to an eye cup with the optic stalk extending from its back end (posterior pole). At this stage RGC axons begin to enter the optic stalk which is then referred to as the optic nerve. As more RGC axons exit, the axons and the surrounding local retinal cells at the retina/optic nerve junction together constitute the optic disc.

In the mouse, the process of pathfinding within the eye occurs from approximately E11 to E18 as RGC are born in a central to peripheral gradient (Drager, 1985). The earliest axons originate from RGCs in dorsal central retina near the optic fissure and exit the eye at E11.5. Axons of later generated RGCs in successively more peripheral retinal regions have to travel a greater distance to exit through the disc. Regardless of their site of origin, all RGC axons exhibit two highly stereotyped behaviors. First, newly formed RGC axons appear always to orient in the direction of and grow without deviation straight to the fissure or later towards the disc (Silver and Sidman, 1980; Halfter et al., 1985). Second, upon reaching the fissure or disc, all RGC axons successfully exit to enter the optic nerve (Goldberg and Coulombre, 1972; Halfter and Deiss, 1984). Both pathfinding features occur essentially without error during development.

Several mechanisms have been proposed to explain the directed RGC axon outgrowth to the disc. First, a chondroitin sulfate proteoglycan has been proposed to act as an inhibitory barrier to prevent initial axon growth in the wrong direction towards the periphery. In addition, straight axonal growth towards the disc appears to require interactions with other axons via

immunoglobulin superfamily (IgSF) members such as L1 and NCAM (Brittis et al., 1995; Brittis and Silver, 1995), although interactions with other cell-surface or extracellular matrix components may also be involved (Halfter and Deiss, 1984; Reichardt et al., 1992). In addition to substrate bound cues, a long range chemoattractive cue has been proposed to guide RGC axons towards the fissure or disc (Ramon y Cajal, 1972), although existence of such a cue has not been demonstrated (Halfter and Deiss, 1984; Halfter and Deiss, 1986; Brittis and Silver, 1995; Halfter, 1996).

The mechanisms that guide RGC axons into the nerve after they reach the fissure or disc are not well understood. Neuroepithelial cells at the fissure have processes which form intercellular spaces that are proposed to channel RGC axons into the nerve (Silver and Sidman, 1980; Krayanek and Goldberg, 1981). In SEM studies, RGC growth cones are seen in contact with the glial cell processes that enwrap RGC axon bundles in the optic nerve (Krayanek and Goldberg, 1981; Silver and Sapiro, 1981; Silver and Rutishauser, 1984). Although these observations suggest interactions involving RGC axons and glial cells at the fissure or disc are involved in guidance into the nerve, the molecules required for this to occur are currently not known. Of note, these optic fissure and optic stalk glial cells express the transcription factor Pax2 (Dressler et al., 1990; Nornes et al., 1990; Otteson et al., 1998). Mutations in the Pax2 gene affects eye structure, further underscoring the importance of these cells in developmental processes in the eye (Favor et al., 1996; Torres et al., 1996).

In this paper, we have investigated the role of the axon guidance molecule netrin-1 in RGC axon pathfinding within the retina and at the early disc. In the vertebrate nervous system, netrin-1's function as an axon guidance cue is best

understood in terms of development of commissural axon projections from dorsal spinal cord to the ventral midline floor plate. Netrin-1 protein is secreted by floor-plate cells and promotes commissural axon outgrowth from dorsal spinal cord explants in collagen gels as well as turning of commissural axons within explants towards an exogenous netrin source (Kennedy et al., 1994; Serafini et al., 1994). The commissural axon outgrowth response to netrin is partially blocked upon addition of a function blocking antibody against DCC, an IgSF member which appears to serve as a netrin receptor (Chan et al., 1996; Keino-Masu et al., 1996; Kolodziej et al., 1996) and is present on commissural axons (Keino-Masu et al., 1996). It is thought that a gradient of netrin protein with peak levels of expression at the floor plate acts as a long range diffusible cue to attract commissural axons via DCC towards the ventral midline. This model is supported by the observations that commissural axons in netrin-1 (Serafini et al., 1996) and DCC deficient mice (Fazeli et al., 1997) do not follow normal ventrally directed trajectories towards the midline floor plate.

Currently it is not known whether netrin proteins or DCC play a role in RGC axon guidance in the retina; although data have hinted that they could be involved as netrin-1 mRNA is expressed in the optic stalk (Kennedy et al., 1994; Serafini et al., 1996), DCC is expressed in the eye (Keino-Masu et al., 1996) and *in vitro*, netrin-1 promotes neurite outgrowth from retinal explants (Wang et al., 1996) and can rapidly orient the direction of *Xenopus* retinal ganglion cell growth cones toward a pipette ejecting netrin-1 (de la Torre et al., 1997). Here, we have analyzed netrin-1 and DCC involvement in embryonic mouse retinal axon outgrowth *in vitro*, investigated the *in vivo* localization of both proteins in the embryonic retina, and analyzed RGC axon guidance defects in both netrin-1

deficient embryos and in embryos lacking functional DCC. The results show that netrin-1 plays a critical role in visual system development by DCC-mediated guidance of embryonic RGC axons through the early optic disc into the optic nerve. Furthermore, our results suggest that in the mouse visual system, netrin-1 may guide axons through a relatively short range guidance mechanism.

## RESULTS

### Localization of Netrin Protein in the Embryonic Mouse Retina

Immunolabelling using a polyclonal netrin antibody revealed the presence of netrin protein in E11-E14 mouse retinas. At E11, netrin was found associated with cells lining the optic fissure (fig. 2-1b). (In this paper, we refer to these cells as glial cells, following the convention of most authors.) At E14, after the fissure has fused and a distinct optic disc is present, netrin protein was found associated with glial cells in the optic disc (fig. 2-1c). In sections of the retina cut in the plane of the optic nerve, these cells appeared as two triangular shaped groups on either side of the axons exiting through the disc. As a group, these netrin positive cells formed a cuff around the exiting RGC axons. Using an anti-Pax2 antibody, immunostaining showed that the netrin positive cuff of glial cells corresponded in location with Pax2-expressing cells at the disc (fig. 2-1f). Although netrin was clearly expressed at the developing optic fissure and disc, we did not detect an obvious gradient of netrin expression extending away from these regions within the eye. The spatial distribution of netrin protein in the eye was almost identical to the netrin-1 gene expression pattern as determined by anti-ß-galactosidase immunoreactivity (fig. 2-1e) in a line of mice in which the endogenous netrin-1

promoter drives expression of a netrin/ ß-galactosidase fusion protein (Skarnes et al., 1995; Serafini et al., 1996).

The netrin polyclonal antibody used in this study was raised against a peptide corresponding to sequence conserved in chick netrin-1, chick netrin-2 and mouse netrin-1 (Kennedy et al., in preparation). However, the netrin protein in the eye is most likely netrin-1, given the pattern of netrin-1 gene expression and the finding that netrin-2 mRNA does not appear to be expressed in the eye (Hao Wang and M.T.L., unpublished data). Therefore, we will refer to the netrin protein in the eye as netrin-1.

The netrin-1 positive glial cells that surround exiting RGC axons at the disc have processes which may serve as potential growth surfaces for retinal axons leaving the retina. This can best be seen in a cross section through the disc cut perpendicular to the axis of the optic nerve (see shaded box in E14 schematic in fig. 2-1a indicating plane of section). Sections cut in this plane (fig. 2-1g) show RGC axons are segregated into bundles by the glial cell processes which are arranged radially around the developing hyaloid artery at the center of the optic disc. Netrin-1 protein was associated with these glial cell processes (fig 2-1h).

Other than immunostaining at the optic fissure or the disc there was no detectable netrin-1 immunostaining in other regions within the retina or in the retinal pigment epithelium. However, netrin-1 was found associated with glial cells in the E11 optic stalk and the E14 optic nerve (fig 2-1b and data not shown). Developmentally, netrin-1 immunostaining at the optic disc and optic nerve was barely detectable above background by E16. Although not quantified, the levels of netrin-1 protein present at the optic fissure or disc appeared lower than at the

floor plate of the hindbrain when compared in the same horizontal tissue sections (compare fig. 2-1b and 2-1i).

**The Netrin Receptor DCC Is Present On Retinal Ganglion Cell Axons**

Using a monoclonal antibody directed against DCC, we observed immunostaining on the cell bodies of E14 RGCs as well as all along their axons within the optic fiber layer, through the disc and into the optic nerve (fig. 2-1j and data not shown). The pattern of DCC immunoreactivity was very similar to that of L1 (fig. 2-1l), another IgSF member which, in the retina, is known to be present only on RGCs and their axons during development (Bartsch et al., 1989). DCC was detectable on RGCs and their axons around the time of fissure closure at E12 (not shown), and was therefore present on some of the earliest RGC axons leaving the eye.

**Netrin-1 Promotes Retinal Neurite Outgrowth in a Dose Dependent Manner**

The immunohistochemical findings described above are consistent with the possibility that netrin-1 plays a role in intra-retinal RGC axon pathfinding. To investigate this possibility further, we first tested whether netrin-1 can affect outgrowth of neurites from retinal explants *in vitro* using collagen gel assays. Conditioned medium from a stably-transfected 293 cell line secreting netrin-1 (Shirasaki et al., 1996) or purified recombinant netrin-1 protein promoted increased retinal neurite outgrowth from E14 explants obtained from all quadrants of the retina, consistent with published results (Wang et al., 1996).

Neurites responding to netrin-1 were immunopositive for L1, a molecular marker for RGCs axons (fig 2-2a to 2-2c).

To determine the dose response relationship of RGCs to netrin-1, E14 retinal explants were cultured for 24 hours in collagen gels with different concentrations of purified recombinant netrin-1 (fig. 2-2d). Under control conditions without netrin-1, the average total neurite bundle length per explant was 472 ± 114 μm (n=44 explants). The addition of netrin-1 promoted neurite outgrowth in a dose dependent manner with a peak response at 100 ng/ml netrin-1. Compared to control, 100 ng/ml netrin-1 increased the average total neurite bundle length per explant to 1716±348 μm, (n=44, $p<0.001$). At higher netrin-1 concentrations (≥ 500 ng/ml), a reduction in the neurite outgrowth response was observed (for example, at 1000 ng/ml, average total neurite bundle length per explant was 907±228 μm, n=44). Both the presence of a peak response at 100 ng/ml and decreased netrin-1 effectiveness at higher concentrations are similar to what has been reported for commissural neurons (Serafini et al., 1994).

To test whether netrin-1 could promote neurite outgrowth during the earliest stages of RGC axon exit at the fissure, explants of different ages were grown in control or netrin-1 conditioned media for 3-5 days (see methods). The netrin-1 induced outgrowth response at E11.5-12.5 (10.8 fold above baseline) was quite similar to that at E14 (10.7 fold) (see Table 2-1). Taken together, the presence of netrin-1 at the fissure or disc at E11.5 to E14 and the fact that retinal explants from these ages had similar outgrowth responses to netrin-1 are consistent with a role for netrin-1 during the initial period of retinal axon growth at the fissure and early disc. We found, however, that E16 retinal explants also had a similar

response (10.5 fold) although the E16 disc region had barely detectable levels of netrin-1 protein.

**Embryonic RGC Axons Respond to Netrin-1 via DCC**

Based on the expression of DCC on RGC axons we tested whether the *in vitro* outgrowth response elicited by netrin-1 could be mediated through an interaction with axonal DCC (fig 2-2e to 2-2i). Using an anti-DCC antibody previously shown to reduce the outgrowth of rat commissural neurons in response to netrin-1, we found the mean retinal neurite outgrowth response to netrin-1 (1978±217 μm, n=90 explants) was significantly reduced in the presence of 5 μg/ml anti-DCC antibody (962±163 μm, n=90, $p<0.001$), but not in the presence of a control (anti-*trp*E) antibody (1596±177 μm, n=90, $p>0.5$). To rule out the possibility that the anti-DCC antibody had non-specific inhibitory effects on neurite outgrowth, explants were grown in collagen alone for up to 30 hours with and without antibody. No significant difference in the mean amount of retinal neurite outgrowth was apparent between collagen alone (454±272 μm, n=24) or in the presence of 5 μg/ml anti-DCC antibody (845±284 μm, n=24, $p>0.05$). Although the anti-DCC antibody significantly reduced retinal neurite outgrowth, it did not completely block netrin-1's effects. It is possible that higher antibody concentrations would fully prevent outgrowth or that this antibody might not completely block DCC function. Alternatively, mouse RGC axons might express additional netrin-1 receptors other than DCC.

**Netrin-1 Deficient Mice Have Optic Nerve Hypoplasia and Hypopigmented RPE Streaks**

To determine whether netrin-1 plays a role in RGC axon guidance in the retina *in vivo*, we examined the eye development and intra-retinal RGC axon pathfinding in netrin-1 deficient mouse embryos. While there was no difference in overall size and shape of the eyes between E15-16 wild-type (wt) and netrin-1 deficient (mutant) littermates, two striking abnormalities were observed. In wt eyes, the retinal pigment epithelium (RPE) surrounding the back of the eye appeared as a homogenous layer with speckled pigmentation (fig. 2-3a). In contrast, in mutant eyes, embedded within the RPE was a loose network of hypo-pigmented streaks (fig. 2-3b, arrowheads) which extended from the ciliary margin at the periphery to the optic disc at the posterior pole. Often, the dorsal region of the eye opposite the closed fissure had an absence or lower number of hypopigmented streaks compared to other regions (not shown).

The second abnormality observed was that the optic disc size was reduced in the eyes of netrin-1 mutant animals compared to littermates (compare fig. 2-3a to 2-3b, arrows), suggesting the presence of a smaller optic nerve. This was confirmed by anterogradely labeling axons in the optic nerve with DiI crystals placed in the optic disc (see fig 2-3c and 2-3d) or by histological examination of optic nerves in 1 μm thick plastic sections (see fig 2-3e to 2-3f). Both techniques showed a decrease in the number of RGC axons in the mutant optic nerve, which when occurring in the presence of a normal sized eye, is known as optic nerve hypoplasia (reviewed in Brodsky et al., 1996). A reduction of axons in the optic nerve was already seen at E12.5 (compare fig. 2-3g & 2-3h), a time when the

earliest generated RGC axons normally grow out of the eye. This finding indicated that the optic nerve hypoplasia in mutant mice was most likely due to primary failure of RGC axons to exit the retina, and not secondarily due to dying back of RGC axons which had initially entered the optic nerve.

**Normal Cellular Organization of the Retina, Optic Disc, and Optic Nerve in Netrin-1 Mutants**

Since optic nerve hypoplasia can in principle result from either RGC axon guidance defects or abnormal retinal/optic disc development, the degree of cellular differentiation within the mutant retina and disc was examined. In 1 μm thick retinal sections, the cytoarchitectural organization and location of the retinal neuroepithelial cell layer, the RGC cell layer and the optic fiber layer (see fig 2-4a and 2-4b), as well as the lens and blood vasculature (not shown) were indistinguishable between wt and netrin-1 mutant littermates. Quantitative analysis revealed no significant difference ($p>0.5$, student's unpaired t-test) in the mean retinal cell densities between E12.5 wt (22,653 ±2,704 cells/$mm^2$) and mutant littermate retinas (20,969 ± 2,041 cells/ $mm^2$). At E15.5, RGC densities were also not significantly different ($p>0.5$, t-test) between wt (13,915 ± 1,613 RGC/$mm^2$) and mutant littermates (13,204 ± 528 RGC/ $mm^2$), and no significant differences in the thickness of the RGC layer or the entire retina were found ($p>0.05$, t-test).

Histological analysis also revealed no evidence of abnormal optic disc development in netrin-1 mutant eyes which could have blocked axon exit and

resulted in optic nerve hypoplasia. The timing of optic fissure closure was not different between wt and mutant animals and, other than a smaller number of axons in the optic disc and optic nerve at E12.5 and E15.5, we found no noticeable differences in the cytoarchitectural appearance of wt and mutant fissures, discs, and optic nerves (see fig 2-3e-h ). Of note, the cuff of Pax2 positive cells normally seen surrounding the exiting RGC axons in the wt disc (fig 2-1f or 2-4c) was still present in the mutant disc (see fig 2-4d). However, in horizontal sections, the two triangular groups of Pax2 positive cells were more closely spaced, consistent with the smaller number of axons exiting through the disc. Overall, the findings of normal retinal and optic disc cellular differentiation and normal eye cup morphogenesis indicate that netrin-1 is not involved in cell patterning in the developing mouse eye.

**Local RGC Axon Guidance Defects At The Optic Disc**

The finding of normal cell density in the RGC layer and no other apparent structural defects suggested that the presence of fewer axons in the netrin-1 mutant optic nerve resulted from incorrect RGC axon guidance in the retina. We therefore examined intra-retinal RGC axon trajectories by placing small DiI crystal in the peripheral portions of fixed retinas of E15-16 wt and mutant littermates (see schematic in fig. 2-5a). Retinas were then flatmounted to visualize trajectories of the labeled axons. In all wt retinas, a cone of labeled axons extended within the optic fiber layer from the labeling site and headed directly toward the disc, where axons disappeared upon exiting through the disc into the optic nerve (fig. 2-5b, dashed circle). In mutant retinas (n=7), labeled RGC axons also extended from

the labeling site within the optic fiber layer directly to the vicinity of the disc (fig 2-5c), indicating that the normal mechanism of guidance to the region of the optic disc may not involve netrin-1. However, once at the disc (fig 2-5c, dashed circle), many labeled axons were splayed out and headed abnormally into other regions of the retina without exiting into the optic nerve. This local axon guidance defect at the disc was observed as early as E12.5 when disorganized bundles of axons seemingly protruded away from the disc into other retinal regions (compare fig.2-5d & 2-5e). These findings indicate that netrin-1 is not required for retinal axon guidance to the disc but is required locally at the disc for proper exit of RGC axons from the eye.

**Ectopic Axon Bundles and RPE Streaks in Netrin-1 Mutant Eyes**

Axon bundles that failed to exit at the optic disc appeared to grow ectopically into other regions in netrin-1 mutant eyes. In wt retinas, L1-immunopositive RGC axons were found in the optic fiber layer and at the disc where they exited the eye into the optic nerve (fig. 2-5f). In contrast in mutant retinas, ectopic RGC axon bundles were not restricted to the optic fiber layer and were observed penetrating through the thickness of the retina, sometimes into the subretinal space in between the retina and the RPE (fig. 2-5g and 2-5h). L1-labeled ectopic bundles (fig 2-5i, arrowheads) could be followed into the RPE (fig. 2-5i, arrow), pushing apart and inserted in between the pigmented cells. These ectopic axon bundles in the RPE were the likely source of the hypo-pigmented streaks present in the mutant embryonic eyes. DiI placed in mutant retinal wholemounts (as described above), sometimes retrogradely labeled ectopic

bundles which failed to exit at the disc and projected to the labeling site. These were seen in a focal plane below the optic fiber layer. The fact that in mutant retinas the DiI crystal labeled both axons that extended normally towards the disc as well as axons from other retinal regions that had grown past the disc to the labeling site may explain why axon bundles between the labeling site and the disc sometimes appeared less distinct in the mutant retina (compare fig. 2-5b and 2-5c).

**Adhesion Molecule Expression and *In vitro* RGC Responses are Unaffected In Netrin-1 Mutants**

Previous studies have implicated two IgSF molecules L1 (Brittis et al., 1995) and, to a lesser extent, NCAM (Thanos et al., 1984; Halfter and Deiss, 1986; Brittis et al., 1995), in intra-retinal axon guidance. Immunolabeling in netrin-1 mutant retinas showed that L1 was still present on RGC axons at levels comparable to wt retinas (compare fig 2-5g and 2-5f). Likewise, in mutant embryos, NCAM was expressed on RGC axons and other retinal cells in a pattern similar to wt embryos (compare fig 2-6b & 2-6a). Thus, RGC axon guidance defects noted in netrin-1 mutants was unlikely to result from secondary alterations in retinal expression of these cell adhesion molecules.

The presence of RGC axons growing in ectopic locations away from the optic fiber layer in addition to their failure to exit the eye at the disc raised the possibility that the ability of RGC axons to interact with guidance cues may have been generally affected in mutant embryos. This was not likely, given that guidance to the vicinity of the disc (fig 2-5c) appeared normal and expression of

L1 and NCAM were not affected (see above). To test whether retinal axons were compromised in a general way in pathfinding ability, we investigated whether RGCs from mutant retinas could still respond to netrin-1 cues. Immunostaining revealed no obvious difference in expression of DCC on RGC axons in controls (fig. 2-6c) compared to mutant littermate retinas (fig 2-6d). DCC was also present on the ectopic RGC axons which extended abnormally out of the disc into the mutant retina (6d, arrowheads). Thus, reduced netrin-1 expression did not lead to observable changes in expression of one of its known receptors on RGC axons. Mutant retinal neurites were also still capable of responding to netrin-1 in a collagen matrix *in vitro* (fig. 2-6e). Baseline neurite outgrowth in collagen alone did not differ statistically between retinal explants from wild-type, heterozygous, and mutant animals (not shown), and explants of all genotypes, responded to 100 ng/ml netrin-1 with similar 6-fold increases in amount of neurite outgrowth from baseline. These findings demonstrated that the response mechanisms to collagen and netrin-1 leading to neurite outgrowth were not affected in netrin-1 mutant retinas and, taken together with the ability of RGC axons to head straight for the optic disc in netrin-1 mutant retinas, indicated that there was no generalized defect in RGC axon interaction with guidance cues.

**Optic Disc Axon Guidance Defects and Optic Nerve Hypoplasia in Embryos Lacking Functional DCC**

Further support for a model of netrin-1 mediated RGC axon guidance locally at the optic disc was obtained from experimental observations of mouse embryos lacking DCC. As in the netrin-1 deficient embryos, all E15-16 DCC null

embryos examined (n=8) exhibited hypopigmented streaks in the RPE, small optic discs (figure 2-7b) and optic nerve hypoplasia (figure 2-7d). Furthermore, DiI labeling in the DCC null embryonic retinas (n=5) showed that RGC axons extended from the labeling site straight towards the optic disc but in the vicinity of the disc, axons failed to exit into the nerve and instead projected into other regions of the eye (figure 2-7f). As with eyes from wild-type animals, heterozygote embryos did not display any of these phenotypes (fig 2-7a,c,e). There was no obvious difference in the severity of the axon guidance defect at the disc in DCC null embryos compared to the netrin-1 mutant embryos. Thus, although DCC is expressed along RGC axons as they extend within the optic fiber layer towards the optic disc, DCC function is apparently not required within the retina for RGC axons to find their way to the disc but is necessary for axons to exit properly into the nerve. The fact that both netrin-1 and DCC deficient embryos exhibited an apparently identical defect suggests that netrin-1 mediated RGC axon guidance at the disc is largely governed by signaling involving axonal DCC.

## DISCUSSION

We have found that during embryonic retinal development, netrin-1 is localized to surfaces of the cells that form the optic fissure and early optic disc, raising the possibility that this guidance molecule is involved in RGC axon exit from the retina into the optic nerve. Three lines of evidence provided support for this hypothesis. First, RGC axons *in vivo* express DCC, a netrin-1 receptor (or receptor component), as they grow through the fissure or early disc region. Second, embryonic retinal neurites *in vitro* respond to netrin-1 in a dose dependent manner and this netrin response is reduced by blocking DCC function.

Third, analysis of intra-retinal axon trajectories in netrin-1 deficient embryos show that RGC axons still extend appropriately towards the disc but upon reaching its vicinity are unable to exit and instead grow ectopically within the eye. This inability of axons to exit the eye did not appear to result from altered retinal, disc or optic nerve development or from a general inability of RGC axons to respond to guidance cues. A similar axon guidance defect at the disc is also observed in embryos lacking functional DCC. Together, the data support a model in which RGC axon response to netrin-1 at the optic disc, mediated through DCC, is required for successful axon exit from the developing eye into the optic nerve. A summary of this model in the context of other proposed cues of intraretinal axon guidance is presented in figure 2-8.

**Guidance Roles of Netrin-1 in the Eye and in the Spinal Cord**

In the vertebrate eye, RGC axons all project towards the optic disc, where they change course to turn and project out of the eye along the axis of optic nerve towards the brain. This pathfinding behavior is somewhat analogous to that of commissural axons in the developing spinal cord which project to the ventral midline floor plate to turn and grow along the axis of the spinal cord towards the brain. The analogy is strengthened by the findings that in both systems netrin-1 is found along the axon path, that both RGC and commissural axons express DCC, and both show similar dose dependent neurite outgrowth responses to netrin-1. Netrin-1 can function *in vitro* as a long-range attractant to guide spinal cord commissural axons over a distance of 250 μm (Kennedy et al., 1994) and is required for guidance of commissural axons *in vivo* when these axons are within

150-250 μm of floor plate (Serafini et al., 1996). These observations initially raised the possibility that, as in the spinal cord, netrin-1 might function as a long range chemoattractant in the eye, drawing RGC axons towards the fissure or disc over several hundred micrometers.

However, two characteristics of netrin in the eye distinguished it from that in the ventral midline of the nervous system. First, the amount of netrin was much lower than in the floor plate of the hindbrain, when visualized by immunohistochemistry (Figure 2-1i). Secondly, we could not detect a gradient of netrin-1 protein extending away from the fissure or disc. Although our methods do not permit a determination of whether secreted netrin-1 is immediately bound on the netrin-1 producing cells, a comparison of the location of netrin-1 gene expression and anti-netrin immunoreactivity at the optic disc indicated that netrin-1 was found in the immediate vicinity of the cells producing this protein. We estimate that detectable netrin-1 immunoreactivity was found at most 2-3 cell diameters away from the producer cells. Since netrin-1 is a heparin-binding molecule that can associate with cell surfaces or the extracellular matrix (Serafini et al., 1994), one possible explanation for the lack of significant gradient of netrin-1 protein from the optic disc is that only a relatively low amount of netrin-1 may be secreted by optic disc cells and may be fully captured by netrin-1 binding sites in the local environment of the disc. Thus, the expression pattern of detectable netrin-1 protein appears more consistent with a role for netrin-1 in guiding RGC axons in the immediate vicinity of the optic disc. Our analysis of RGC axon trajectories in netrin-1-deficient and DCC-deficient animals is consistent with this expectation. Although we have not determined the precise site where RGC axons become misrouted, this misrouting clearly occurs much closer than 150 μm from

the disc, possibly even only when they encounter the netrin producing cells within the disc itself.

The fact that netrin-1 does not appear to act as a guidance cue operating over hundreds of micrometers towards the disc in the retina is in agreement with previous studies which have found no evidence for a long range chemoattractant emanating from the disc (Halfter and Deiss, 1984; Halfter and Deiss, 1986; Brittis and Silver, 1995; Halfter, 1996; and M.S.D. and D.W.S., unpublished). Previous studies have also noted changes in growth cone morphology and behavior as RGC axons enter the disc (Holt, 1989; Brittis et al., 1995), consistent with axon encounter of a new guidance molecule, possibly netrin-1, not present elsewhere in the retina. Netrin-1 is a candidate for this putative molecule, a possibility supported by the observation that netrin-1 increases the complexity and can cause a directional turning response of Xenopus retinal growth cones in cell culture (de la Torre et al., 1997). Thus, a comparison of the eye with spinal cord suggests that an individual guidance molecule, in this case netrin-1, may act either over a substantial portion of an axonal projection or instead only locally at a pathfinding decision point, depending on its distribution. How particular distributions of netrin are created and maintained in different areas remains to be determined.

**Netrin-1 Independent Guidance Out Of The Eye?**

We have observed that optic nerves are not completely missing in netrin-1 or DCC deficient embryos and a number of RGC axons do exit from the eye into the optic nerve (for example see fig. 2-3, fig. 2-7). In the netrin-1 deficient embryos, this could be explained by the fact that they still express a small amount

of netrin-1 (see Serafini et al., 1996), perhaps enough to guide some axons out of the eye. It is also possible that there may be separate populations of netrin-1 dependent and independent RGC axons, and netrin-1 independent axons continue to find their way out of the retina. If so, the netrin-1 independent population does not seem to correspond to RGCs born at a particular age, since in the netrin-1 mutant, early generated RGC axons are able to exit from the eye (fig. 2-3 ) and backfilling experiments show that later axons (E14) in the optic nerve originate from both central and peripheral retinal regions, indicating that axons from a range of ages were able to make their way out of the eye (M.S.D. and D.W.S., unpublished observations). Furthermore, the existence of a constant population of netrin-1 independent RGC axons should result in optic nerves of a fixed reduced size. This does not appear to be the case. In fact the degree of optic nerve hypoplasia in both the netrin-1 mutant and the DCC null embryos can vary from a mild reduction (figs. 2-3f and 2-5g) up to an almost completely missing nerve (figs. 2-6d). This variation can even be observed between the two optic nerves of a single embryo.

The differences in the number of RGC axons exiting the eye may be more easily explained by assuming that some degree of guidance information remains in the discs of mutant embryos. This guidance information might involve growth along intercellular channels, repulsion from peripheral retina or be supplied by the remaining small amount of netrin-1 (in netrin-1 deficient embryos), by a secondary netrin-1 receptor (in DCC null embryos), or by other neurite outgrowth promoting molecules at the disc such as R-cadherin (Redies and Takeichi, 1993). With severely reduced levels of netrin-1 or without functional DCC, these mechanisms presumably do not perfectly guide RGC axons through the disc,

resulting in some pathfinding errors. As later RGC axons fasciculate along earlier ones, followers of misrouted axons would also be misrouted. With this in mind, the degree of hypoplasia may simply depend on how early in development the first pathfinding mistakes were made by RGC axons. An eye in which mistakes occurred early would have more severe hypoplasia than one in which mistakes occurred late.

**Phenotypes From IgSF Perturbation, and Chx10, Pax2 Gene Disruptions**

Previous studies have implicated the IgSF molecule L1 in intra-retinal axon guidance. After blockade of L1 function using an L1 antibody applied to retinal eye cups maintained *in vitro*, RGC axons throughout the retina were noted to defasciculate and wander (Brittis et al., 1995), in contrast to the finding in netrin-1 mutant or DCC null mice where the splaying out of axons occurred only at the optic disc. The defasciculation after L1 blockade suggests that L1, unlike netrin-1 or DCC, is involved in proper fasciculation of axons as they grow towards the disc.

The role of a second IgSF molecule, NCAM, in intra-retinal pathfinding is less clear. Although application of anti-NCAM antibodies to developing chick eye cups *in vitro* have reportedly had minimal effects on retinal axon guidance (Halfter and Deiss, 1986; Brittis et al., 1995), a study using direct injections of an NCAM antiserum to the developing chick eye *in ovo* have reported abnormal axon trajectories (Thanos et al., 1984). It is worth noting that these abnormal axon trajectories were found at the disc and were remarkably similar to the splayed out retinal axon trajectories at the disc in netrin-1 mutant mice. It is not known

whether the NCAM antiserum used in that study would distinguish between NCAM and other IgSF members such as DCC. Nevertheless, NCAM expression appeared normal in netrin-1 mutant retinas, indicating that optic disc pathfinding errors in netrin-1 mutant mice did not result indirectly from altered NCAM function. Furthermore, NCAM antibody treatment leads to abnormal disc morphogenesis (Silver and Rutishauser, 1984) which was not observed in either netrin-1 mutant or DCC null embryos.

Mutations in two genes have recently been shown to result in abnormal optic nerve development. The homeobox gene Chx10 is the underlying gene defect in or-j mice (Liu et al., 1994; Burmeister et al., 1996) which have optic nerve aplasia. However, the lack of axons in the optic nerves of or-j mice is almost certainly due to retarded eye growth leading to microphthalmia and defects in early retinal cell differentiation (Robb et al., 1978; Silver and Robb, 1979). Although we have not investigated whether Chx10 is secondarily affected in netrin-1 mutant or DCC null mice, the fact that they both have normal size eyes and normal retinal differentiation makes this unlikely.

Although the antibodies we used did not permit co-localization of netrin-1 and Pax2 on individual cells, we found Pax2 and netrin-1 had coincident location on the same groups of cells at the E14 optic disc and that netrin-1 localization at the fissure and in the optic stalk is quite similar to the pattern of Pax2 expression (Torres et al., 1996; Otteson et al., 1998). This suggests a comparison between the phenotypes in netrin-1 and Pax2 mutant mice may be informative. Mice with a targeted disruption (Torres et al., 1996), chromosomal deletion (Keller et al., 1994; Otteson et al., 1998) or frameshift mutation (Favor et al., 1996) of the Pax2 gene, exhibit abnormal optic fissure closure which is thought to lead to reduced RGC

axon exit into the optic nerve and optic disc aplasia. In addition, other structural defects include abnormal retinal vasculature and abnormal RGC axon topography in the optic nerve. The fact that these structural defects including abnormal optic fissure closure were not seen in netrin-1 mutants along with the finding that Pax2 was still normally expressed in the cuff of cells at the optic disc in the netrin-1 mutant eyes, make it unlikely that the phenotypes seen in the netrin-1 mutant were due to effects on Pax2 expression.

**Optic Nerve Hypoplasia in Humans**

Optic nerve hypoplasia is thought to be the optic disc anomaly most often encountered by ophthalmologists (Brodsky et al., 1996) and is a significant cause of congenital blindness (Jan et al., 1977; Lambert et al., 1987). Generally, it is thought that optic nerve hypoplasia in patients with otherwise normally formed eyes results from either the failure of RGC differentiation during development resulting in fewer axons, or abnormal developmental events at visual targets in the CNS causing a secondary dying back of RGC axons which initially exited the optic disc into the optic nerve (reviewed in Brodsky et al., 1996). Our findings suggest that in mouse, a third cause of optic nerve hypoplasia is a developmental defect in RGC axon guidance out of the eye, reducing the number of axons in the nerve. It is not known whether similar axon guidance defects might also cause optic nerve hypoplasia in humans. Of note, a significant number of optic nerve hypoplasia cases in humans occur in the setting of septo-optic dysplasia (or De Morsier's syndrome), a clinical syndrome comprised of optic nerve hypoplasia and partial or total agenesis of the corpus callosum that can be associated with an

absent septum pellucidum and endocrine abnormalities (Skarf and Hoyt, 1984). The combination of optic nerve hypoplasia and absent corpus callosum is also found in both the netrin-1 deficient and the DCC null mice (Serafini et al., 1996; Fazeli et al., 1997; and this study).

**Conservation of Mechanisms In Eye Development**

Studies have shown that the expression and function of netrin-1 and DCC homologues in the formation of the ventral midline commissures appear to be conserved from *C. elegans* to vertebrates, including rodents (reviewed in Keynes and Cook, 1996). The results here indicate that in rodents, DCC mediated axonal guidance by netrin-1 has also been recruited as a mechanism contributing to the proper development of eyes, homologues of which do not exist in C. elegans. Although eyes in different taxa vary greatly in structure and embryological development (reviewed in Land and Fernald, 1992), it is thought that eye development in all these organisms is governed by a highly conserved regulatory gene pathway involving Pax-6. It remains to be seen whether axon guidance molecules such as netrin-1 and DCC have also been similarly conserved in their function and play a role in the formation of connections between photoreceptive organs and the brain across different taxa.

## METHODS

### Antibodies

The primary antibodies used in this study for immunolabeling were: 1) anti-L1 (rabbit polyclonal, diluted 1:650, gift of C. Lagenauer (Chung et al., 1991), 2) anti- Pax2 (rabbit polyclonal, diluted 1:300, gift of Greg Dressler (Puschel et al., 1992), 3) anti- NCAM (mouse monoclonal (IgM Mab) 5A5 ascites, diluted 1:75, Developmental Hybridoma Bank (Dodd et al., 1988), 4) anti-DCC ( mouse Mab #AF5 (IgG1), used at 1 µg/ml, Oncogene Research Products), 5) anti-ß-galactosidase (rabbit polyclonal #7-063100), used at 3µg/ml, 5 Prime --> 3 Prime, Inc., and 6) anti-netrin (rabbit polyclonal #11760, diluted 1:50). Netrin polyclonal antibody 11760 was raised using a peptide antigen corresponding to sequence conserved in chick netrin-1, chick netrin-2 and mouse netrin-1 (Kennedy et al., in preparation).

For *in vitro* experiments involving retinal explants, anti-DCC mouse Mab #AF5 (IgG1) was special-ordered without Na-azide from Oncogene Research Products and used at 5 µg/ml. The control Mab used was an anti-*trp*E mouse Mab (IgG1, Oncogene Research Products, cat # OB01) purified using the same methods as the anti-DCC Mab and used at 5 µg/ml. *trp*E is a bacterial protein that is not expressed in mammalian cells and the anti-*trp*E Mab does not bind to any mouse tissue.

**Immunohistochemistry**

E11-E16 embryos (plug day = E0) were harvested from anesthetized C57/bl6 timed pregnant mice (Sretavan et al., 1994). For immunostaining on 10 μm cryostat sections, the heads were fixed in 4% paraformaldehyde in 0.1M phosphate buffer pH 7.25 for 1 hr. at 4 °C, infiltrated overnight in 30% sucrose/PBS, embedded in OCT (Baxter), and stored at -80°C until use. For immunostaining on paraffin sections, heads were fixed using Carnoy's fixative (Bancroft and Stevens, 1982) and then embedded in EM-400 wax (Surgipath) until use.

Netrin immunostaining was carried out using 5-10 μm thick paraffin sections. Antigenicity was significantly enhanced by boiling the tissue for 10 min. in 0.01M Citrate buffer at pH=6.0 as described by Kennedy et al. (in preparation). After cooling to RT, sections were dipped once in 0.1% triton X-100/PBS, and then incubated for 1 hr. in blocking solution ( 5% Milk, 1% glycine, 3% Heat Inactivated Normal Donkey Serum (HINDS) and 0.01% Na-azide in 0.1M Tris buffered saline). Sections were then incubated with primary antibody for 1 hr, washed 5 times (20 min./wash), incubated with the secondary antibody for 3 hours and then washed 5 times (20 min./wash) before coverslipping. All incubations and washes were carried out at RT in the presence of blocking solution.

Immunolabeling of L1 and Pax2 were carried out on cryostat sections preblocked in HINDS or Heat Inactivated Normal Goat Serum (HINGS) for 20 min. Incubations with primary antibody were performed at RT for 1-2 hr followed by 5 washes in 0.1 M PBS (4 min./wash). Secondary antibody was

applied for 1-2 hr. at RT, the sections were then washed 5 times before coverslipping. All antibody incubations were carried out in the presence of 1% HINDS or 1% HINGS for blocking.

Identical methods were used to label DCC using the anti-DCC mouse Mab with the following modification. To decrease the amount of background staining associated with the use of an anti-mouse IgG secondary antibody in embryonic mouse retinal tissues, unconjugated goat Fab fragments against mouse IgG (H+L) were used to block endogenous mouse IgG in retina tissues prior to addition of primary antibody. The Fab fragments (Jackson Immunoresearch, # 115-007-003) were used at a concentration of 15 μg/ml. in 0.05% TritonX-100/PBS for 2 hours at RT. This additional blocking step binds endogenous IgG and decreases its recognition by the conjugated secondary anti-mouse IgG antibody.

For immunofluorescence, sections were labeled with the appropriate secondary antibodies conjugated to either FITC or Cy3 (Jackson Immunoresearch). For visualization with diaminobenzidine, antibody labeled sections were treated as previously described (Sretavan et al., 1994).

Neurites from retinal explants in collagen gels were labeled using an anti-L1 antibody using wholemount immunohistochemistry as previously described (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995) , except that washes were in 50 ml of PBS and all antibody incubations also contained 1% HINDS.

### *In vitro* Outgrowth Assays

Collagen gels were made using bovine collagen (Vitrogen-100, Celtrix Pharmaceuticals) diluted to 1 mg/ml in media and the pH adjusted to 7.25 using

sterile filtered NaOH (5M). All manipulations with collagen were carried out on ice (< 4 °C ) prior to addition of explant tissues. For experiments using purified recombinant netrin-1, collagen was made with fresh culture media consisting of DME-H21, 5% FCS , 2 mM glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. For dose-response experiments, purified recombinant netrin-1 was added to the 1 mg/ml collagen solution to obtain final netrin-1 concentrations of 50-1000 ng/ml. For DCC function blocking experiments, either anti-DCC or the control antibody, anti *trp* E, were added to the collagen solution to achieve a final concentration of 5 µg/ml. 200 µl of collagen solutions containing netrin-1 and/or antibodies were then aliquotted into each well of an eight-well chamberslide (Nunc) and maintained at 4 °C until the addition of explants.

Conditioned media from a stably-transfected netrin-1-secreting 293 cell line (Shirasaki et al., 1996) was used for testing the netrin-1 responsiveness of retinal explants of different ages. 7-15 ml of supernatant harvested from confluent 150 mm plates of either mock-transfected or netrin-1-secreting 293 cells was spun down at 100 x G to pellet large cell debris. To further enrich for netrin-1, small molecules (less than 50 kDa) were then removed by filtration through Centriprep-50 concentrators (Amicon) leaving 2-3 ml of retentate. Fresh culture media (1-2 ml) was then added to the retentate to obtain a final volume of 4 ml which was then used to dilute collagen to 1 mg/ml before adjusting pH. 300 µl aliquots of the collagen solutions were then added to each well of eight-well chamberslides. Since approximately 20 µg of netrin-1 is in the supernatant of confluent plates (C. Mirzayan personal communication), the final concentration of netrin-1 in these experiments was estimated to be 400-800 ng/ml.

For all *in vitro* experiments, retinal explants were obtained as described previously (Sretavan et al., 1994) with the additional step of removing approximately the central 1/5 of the retina using a glass micropipette to punch out any optic disc tissue that contained netrin-1. The number of explants obtained per retina ranged from 6-8 explants per E11.5 retina up to 15-20 explants per E16 retina. In a given experiment, the measured sizes of explants used in all conditions were not significantly different. Explants from all quadrants were pooled together and 4-5 retinal pieces were then added to the chamberslide wells containing collagen. Chamber slides were then placed at 37 °C for 30 min. until the collagen hardened at which time 50 µl of culture media was then added. Retinal explants were kept at 37 °C, in 5% $CO_2$ for 23-26 hours before fixation using 4% PFA, pH=7.25. In experiments using cell line conditioned media, 250 µl of media was added to the hardened collagen and explants grown for 3-5 days prior to fixation. Of note, the dose response, DCC blocking and conditioned media experiments were repeated three times on separate days and equal numbers of explants at all conditions were tested on each day.

The amount of neurite outgrowth per explant was quantified using either Nerolucida™ image tracing/data quantification software or by tracing explant neurites on a video monitor, converting the tracings to digital images, and quantifying the amount of outgrowth based on the number of black pixels using the public domain NIH Image program (http://rsb.info.nih.gov/nih-image/). The neurite lengths per explant were arrived at using pixel values obtained from tracings of known lengths. Of note, all neurite tracing was done without knowledge of the experimental conditions of explants. Given that under any

given experimental condition, the variation of neurite outgrowth from explants did not show a normal distribution, for all *in vitro* outgrowth experiments, the statistical significance of differences between any two conditions was analyzed using the Mann-Whitney U test, a nonparametric analogue to the *t* test for two independent samples.

**Netrin-1 and DCC Deficient Mice**

Production, breeding and genotyping of the netrin-1 deficient mouse was as described previously (Serafini et al., 1996). L1, Pax-2, NCAM, and ß-galactosidase immunohistochemistry and *in vitro* collagen outgrowth experiments/analysis using tissue from these litters was carried out identically as above with C57/bl6 embryonic tissue. Production, breeding and genotyping of the DCC deficient mouse was as described previously (Fazeli et al., 1997).

**Retinal DiI Labeling In Both Netrin-1 and DCC Deficient Embryos And Littermates**

Anterograde labeling of axons in the optic nerves with DiI (D-282 Molecular Probes), was carried out as described (Sretavan et al., 1994). For intraretinal labeling of RGC axon projections to the optic disc in E15-16 embryos (fixed in 4% PFA), a glass micro-pipette was used to insert a small DiI crystal near to the ciliary margin of the retina. An incubation period of 6-8 hr. at 37°C was sufficient to label axons all the way to the disc. To visualize the intraretinal trajectories of labeled axons, retinas were removed from embryos, opened up

after making 5-7 radial cuts and then flatmounted under a glass coverslip. DiI fluorescence was visualized using rhodamine fluorescence optics.

**Histological Analysis of Netrin-1 Mutant Embryos and Littermates**

Embryonic netrin-1 heads were harvested and fixed using 1/2-strength Karnovsky's fixative and then embedded in an epon/araldite resin (1:2) (for details see Bancroft and Stevens, 1982). 1μm thick serial sections were cut with glass knives using a Sorvall MT-2B ultramicrotome. Sections were then stained with 1% toluidine blue/sodium borate and coverslipped with DePeX (BDH). For quantitative analyses of cell layer thickness and cell densities in mutant and wt retinas of littermates, comparisons were made at corresponding retinal sites in wt and mutant eyes as defined by equal distances from the periphery and equal sizes of the vitreal spaces. All measurements of cell density or cell layer thickness were done before knowing the genotypes. At E12.5 when the RGC layer is still ill-defined, retinal cell density was calculated by counting the number of cell nuclei within a 140 μm x 140 μm box spanning the entire retinal thickness from the RPE to the optic fiber layer. The data represent the average counts obtained at 3 matched sites for each genotype. At E15.5, cell density in the morphologically distinguishable RGC layer was measured at 2 matched sites in each of two wt and two mutant retinal sections. Using these sections, the average thickness of the undifferentiated neuroepithelial and RGC cell layers were calculated from measurements made in corresponding sites.

ACKNOWLEDGEMENTS

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TABLES AND FIGURES

**Table 2-1: Developmental Age and Netrin-1-Induced Outgrowth**

| Age | - Net | + Net | p value |
|---|---|---|---|
| E11.5-12.5 | 109±68<br>n=26 | 1181±432,<br>n=24 | <0.05 |
| E14 | 1164±460,<br>n=21 | 12492±3261,<br>n=21 | <0.005 |
| E16 | 127±70,<br>n=24 | 1331±576,<br>n=24 | <0.05 |

The mean neurite bundle lengths per explant from eyes of three different ages grown in the presence of netrin-1 conditioned medium (+ Net) or parental cell line control conditioned medium ( -Net). Values given are in μm ± S.E.M., n=number of explants examined, p values were determined by the Mann-Whitney U test (see methods). Note that although the baseline outgrowth from E14 explants was higher than from the other two ages, netrin-1 still promoted a similar 10-fold increase in outgrowth. The amount of netrin-1 promoted neurite outgrowth observed in these experiments using conditioned media (3-5 days *in vitro*) was generally more than that seen in experiments using purified recombinant netrin-1 (1 day *in vitro*).

**Figure 2-1: Immunolocalization of Netrin-1, DCC and Pax2**

(a) Schematic diagram of sections through E11 and E14 retinas. RET, retina; OF, optic fissure; OS, optic stalk; OD , optic disc; ON, optic nerve. Panels b-f, i-l are high magnification views of the optic fissure or optic disc from sections cut within the plane of the page as depicted in the schematic. Panels g, h are high magnification views of the optic disc cut in sagittal section (cut through the axis of the optic nerve in the plane depicted by the shaded box in the schematic, perpendicular to the plane of the page). White dashes in b-f and j-l outline the parts of the retinas present in each panel.

(b) Horizontal section of an E11 retina showing netrin-1 (red) localized to cells along the optic fissure and the optic stalk. OF, optic fissure; OS, optic stalk.

(c) Section through an E14 retina showing that netrin-1 is present on two triangular shaped groups of cells flanking the RGC axons that exit through the disc. Blood cells (arrowhead) are non-specifically labeled and can be seen without primary antibody (see 1d).

(d) An adjacent E14 retinal section processed identically but without addition of primary antibody (secondary alone). Arrowhead points to non-specifically labeled blood cells.

(e) Horizontal section of an E14 optic disc showing ß-galactosidase immunoreactivity (red). In this embryo, ß-gal expression was under control of the

endogenous netrin-1 promoter (Skarnes et al., 1995; Serafini et al., 1996), thus cells that express the netrin-1 gene are ß-gal positive. Note that the netrin protein distribution in the eye (shown in 1c) was localized to and did not extend beyond the same triangular shaped cell groups at the disc in which the netrin-1 gene was expressed.

(f) Horizontal section through an E14 retina showing Pax2 immunoreactivity (red) on two triangular shaped groups of cells flanking the RGC axons exiting through the optic disc, in a pattern similar to netrin expression (compare to 1c, 1e).

(g) 1 µm-thick toluidine blue stained sagittal section through the disc. Glial cells (arrows) send processes (arrowheads) towards the centrally located hyaloid artery (A) segregating retinal axons into bundles (*).

(h) Sagittal section through the E14 disc showing netrin-1 immunoreactivity (red) in a pattern reminiscent of the glial cell processes in figure 2-1g. DAPI (dark blue) labeling shows retinal neuroepithelial and vascular cell nuclei.

(i) Horizontal section through the head of an E11 embryo showing the floor plate (FP) of the hindbrain. In the same section, the intensity and amount of netrin-1 protein labeling in the hindbrain floor plate was higher than in the eye (compare 1i to 1b, photos taken with same camera exposure time).

(j) E14 horizontal section showing DCC (red) immunoreactivity on RGC axons in the optic disc.

(k) An adjacent retinal section processed identically except without anti-DCC antibody.

(l) A second adjacent section shows L1 immunolabeling (green) on retinal axons at the optic disc.

Scale bars = 25 μm, except for g = 50 μm.

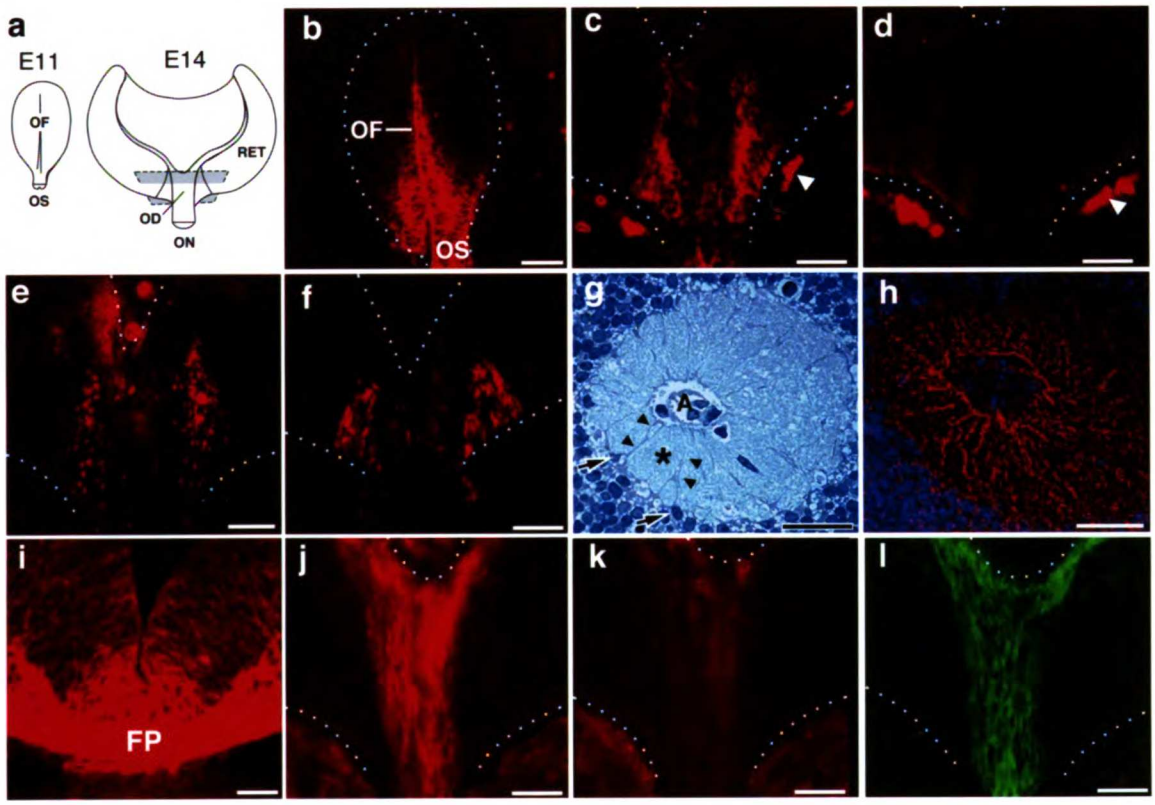


**Figure 2-1**

**Figure 2-2: Netrin-1-induced L1-Positive RGC Neurite Outgrowth is Dose Dependent and is Reduced by Blocking DCC Function**

(a) E14 Retinal explant grown in collagen alone and immunostained with an anti-L1 antibody.

(b) E14 Retinal explant grown in collagen and recombinant netrin-1, immunostained with an anti-L1 antibody. Netrin promotes the outgrowth of L1 positive neurites (most likely RGC axons).

(c) E14 Retinal explant grown in collagen and processed identically except without anti-L1 antibody. Arrows point to unlabeled neurites.

(d) Graph of E14 neurite outgrowth in response to recombinant netrin at different concentrations (see methods for details of data analysis). X axis is netrin-1 concentration (in log scale). Y axis is the average total neurite bundle length per explant. Retinal neurites responded to netrin-1 in a dose dependent manner with a peak response above baseline at 100 ng/ml netrin ($p<.001$). At higher netrin concentrations tested, a less potent neurite outgrowth response was observed. Fig 2-2a and b show explants with the mean amount of neurite outgrowth under conditions of collagen alone and with 100 ng/ml netrin added, respectively.

(e) The retinal neurite outgrowth response to 100 ng/ml netrin was significantly reduced in the presence of 5 μg/ml anti-DCC antibody ($p<0.001$), but not of a control (anti-trpE) antibody ($p>0.5$).

(f-i) Summary presentations of neurite outgrowth from each of the conditions shown in fig. 2-2e. Tracings of the neurites from individual explants (90 explants per condition) were stacked together: (f) collagen alone , (g) collagen with 100 ng/ml netrin, (h) collagen with 100 ng/ml netrin and 5 μg/ml anti-DCC antibody, (i) collagen with 100 ng/ml netrin and 5 μg/ml anti-trpE antibody.

Scale bars in 2a-c = 100 μm, scale bar for 2f-i = 200 μm.

Error bars = standard error of the mean.

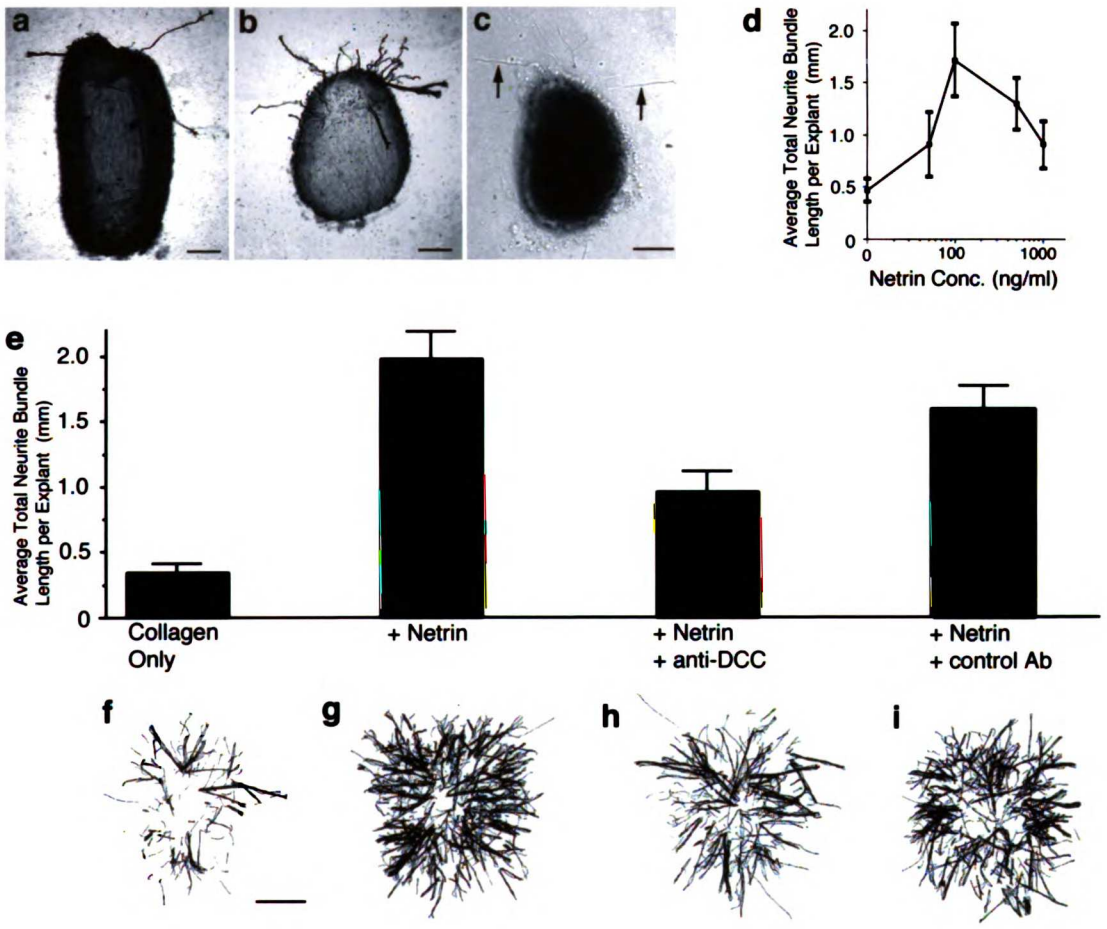
a
b
c
d
Average Total Neurite Bundle Length per Explant (mm)
2.0
1.5
1.0
0.5
0
0
100
1000
Netrin Conc. (ng/ml)
e
Average Total Neurite Bundle Length per Explant (mm)
2.0
1.5
1.0
0.5
0
Collagen Only
+ Netrin
+ Netrin + anti-DCC
+ Netrin + control Ab
f
g
h
i

**Figure 2-2**

**Figure 2-3: Netrin-1 Deficient Mice Have Optic Nerve Hypoplasia and Hypopigmented RPE Streaks**

(a) In E16 wt eyes, the retinal pigment epithelium (RPE) appeared as a homogenous layer with speckled pigmentation interrupted only at the posterior pole by the axons exiting the eye through the optic disc (arrow).

(b) In an E16 mutant littermate, a loose network of hypo-pigmented streaks (arrowheads) can be seen within the RPE. The size of the optic disc (arrow) was also reduced (compare fig. 2-3a to 2-3b).

(c) Anterograde DiI labeling of axons in the optic nerve of an E16 control embryo.

(d) DiI Labeling in a netrin-1 mutant littermate showed fewer axons in the optic nerve.

(e) 1 μm thick plastic horizontal sections of an E15.5 optic nerve from a control animal.

(f) A similar section from a netrin-1 mutant littermate showed a smaller optic nerve (optic nerve hypoplasia).

(g) A sagittal section through an E12.5 optic nerve in a control animal. At this age, the early generated RGC axons are found as bundles (for examples see asterisks) in the ventral 2/3 of the optic nerve.

(h) A similar section from a netrin-1 mutant littermate showed fewer axons in the optic nerve. Notice that, as in a normal nerve, the RGC axon bundles in the mutant optic nerve were ventrally located and separated into bundles by processes of glial cells (as in 3g).

Scalebars = 200 μm (a-d), 50 μm (e,f) and 20 μm (g,h).

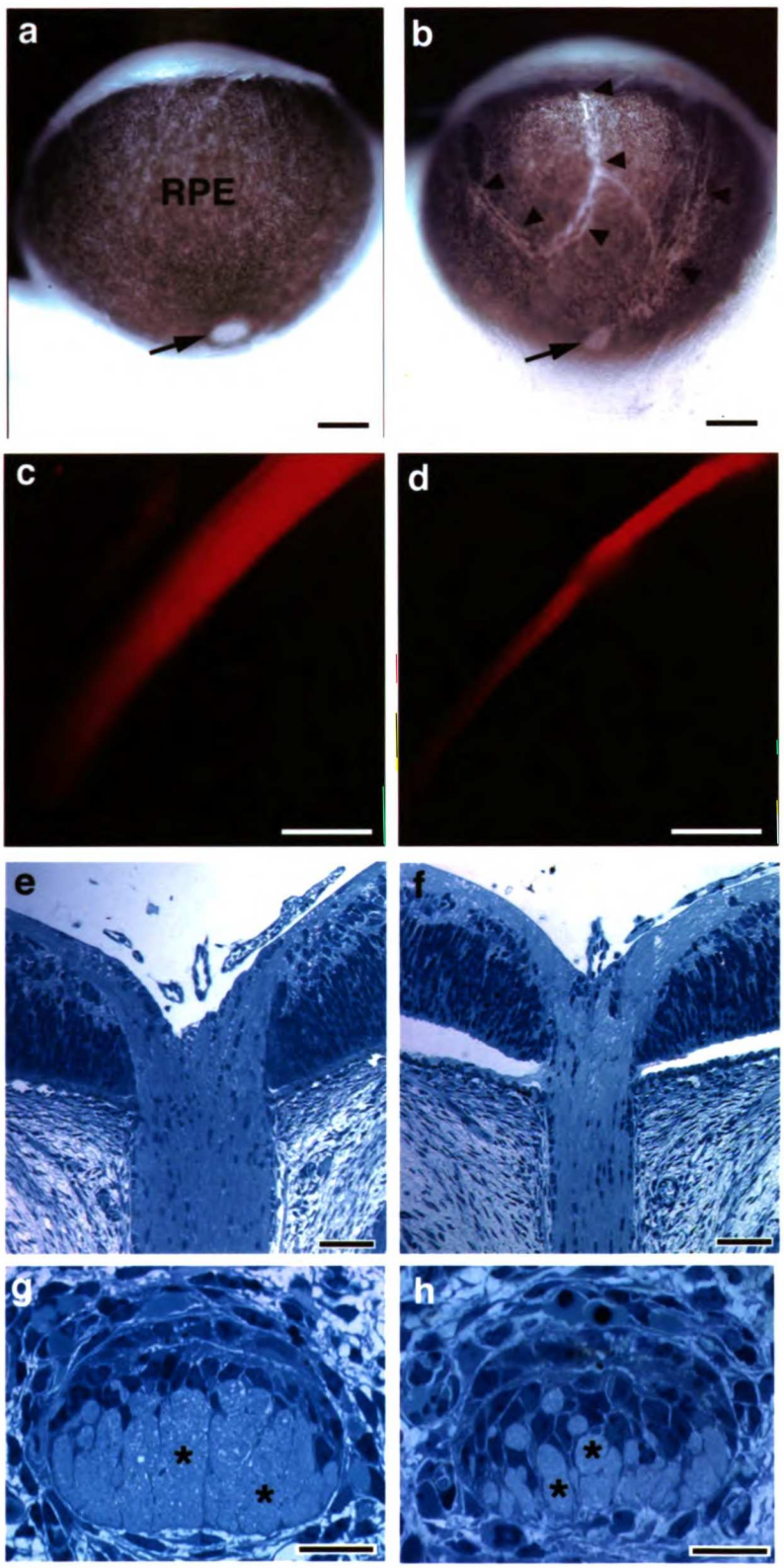


**Figure 2-3**

**Figure 2-4: Cellular Organization in the Retina and Optic Disc of Netrin-1 Deficient Embryos.**

(a): 1 μm thick retinal section from an E15.5 wild-type embryo. NE, neuroepithelial cell layer; RGC, retinal ganglion cell layer; F, optic fiber layer.

(b) A similar retinal section from a netrin-1 mutant littermate. The cytoarchitectural organization and location of the retinal layers were indistinguishable between wt and mutant. There were no significant differences in the thicknesses of cell layers or cell densities (see results).

(c) Horizontal retinal section through the optic disc region of an E15 control embryo showing Pax2 immunoreactivity in two triangle-shaped cell groups (arrowheads) flanking the RGC axons exiting at the optic disc.

(d) Horizontal retinal section through the optic disc region of an E15 netrin-1 mutant littermate showing the presence of triangular-shaped groups of Pax2 positive cells (arrowheads). The Pax2 positive groups of cells were more closely spaced, corresponding to the smaller number of axons exiting through the optic disc.

Scalebars=25 μm.

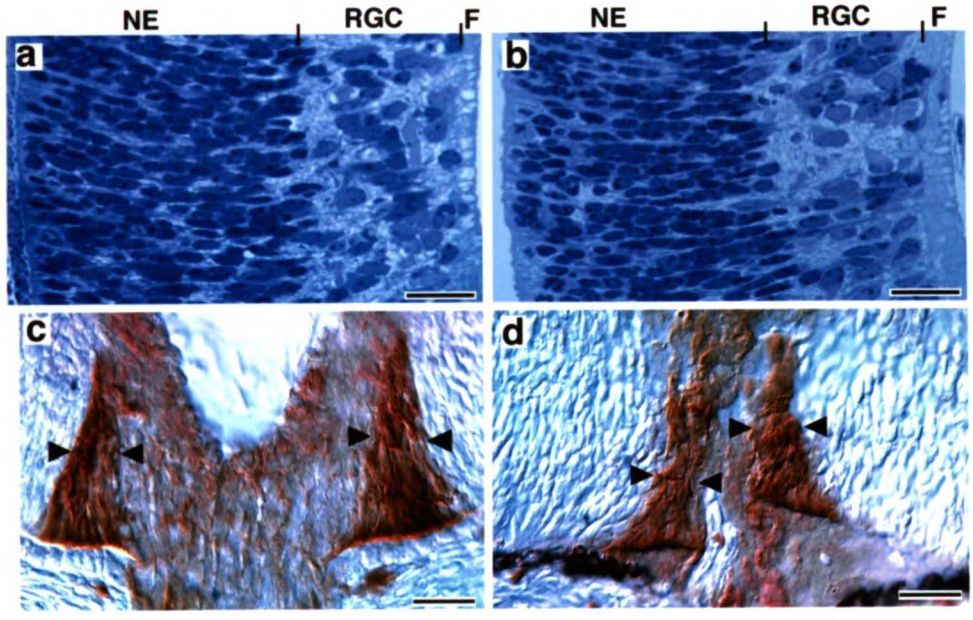


**Figure 2-4**

**Figure 2-5: Axon Guidance Defects at the Optic Disc and Ectopic Axon Bundles in Mutant Embryos.**

(a) Schematic diagram of a retinal flatmount showing the position of a small DiI crystal (asterisk) placed in peripheral retina and the resulting labeled axons projecting towards the optic disc.

(b) In an E15.5 wt retina, DiI labeled axons extended from the labeling site (asterisk) directly toward the optic disc (approximate location indicated by dashed circle), where they disappeared upon exiting through the disc into the optic nerve.

(c) In a netrin-1 mutant littermate retina, labeled RGC axons also extended from the labeling site (asterisk) in a straight fashion directly to the optic disc. However, once at the disc (approximate location indicated by dashed circle), many labeled axons splayed out without exiting into the optic nerve and headed abnormally into other retinal regions.

(d) 1 μm thick sagittal sections of the optic disc from an E12.5 control embryo. RGC axon bundles separated by glial cell processes were cut in cross section and had an orderly, regular appearance.

(e) A similar section of an E12.5 netrin-1 mutant littermate optic disc showed disorganized RGC axon bundles. Some projected away (arrowheads) from the optic disc into other regions of the retina.

(f) An E14 wt retina showing L1 immunopositive RGC axons (red) in the optic fiber layer and exiting at the optic disc.

(g) In an E14 netrin-1 mutant littermate, RGC axons exhibited a degree of L1 immunoreactivity comparable to control embryos. However, L1 positive RGC axon bundles were not restricted to the optic fiber layer and instead penetrated through the thickness of the retina. Notice the smaller number of RGC axons exiting into the optic nerve.

(h) A higher magnification view of the dotted boxed area on the left side in 5g. An ectopic bundle (arrowheads) penetrated all the way through the retina into the subretinal space (S).

(i) A double exposure high magnification view of the dotted boxed area on the right side in 5g. (retina-phase contrast; RGC axons-Rhodamine fluorescence). An ectopic L1 labeled axon bundle (arrowheads) extended from the subretinal space into the RPE (arrow), inserting in between pigmented RPE cells. Ectopic axon bundles in the RPE are likely to be the source of the hypo-pigmented streaks present in the mutant embryonic eyes (described in fig 2-3).

Scalebars=100 μm (b,c,f,g) and 25 μm (d,e,h,i).

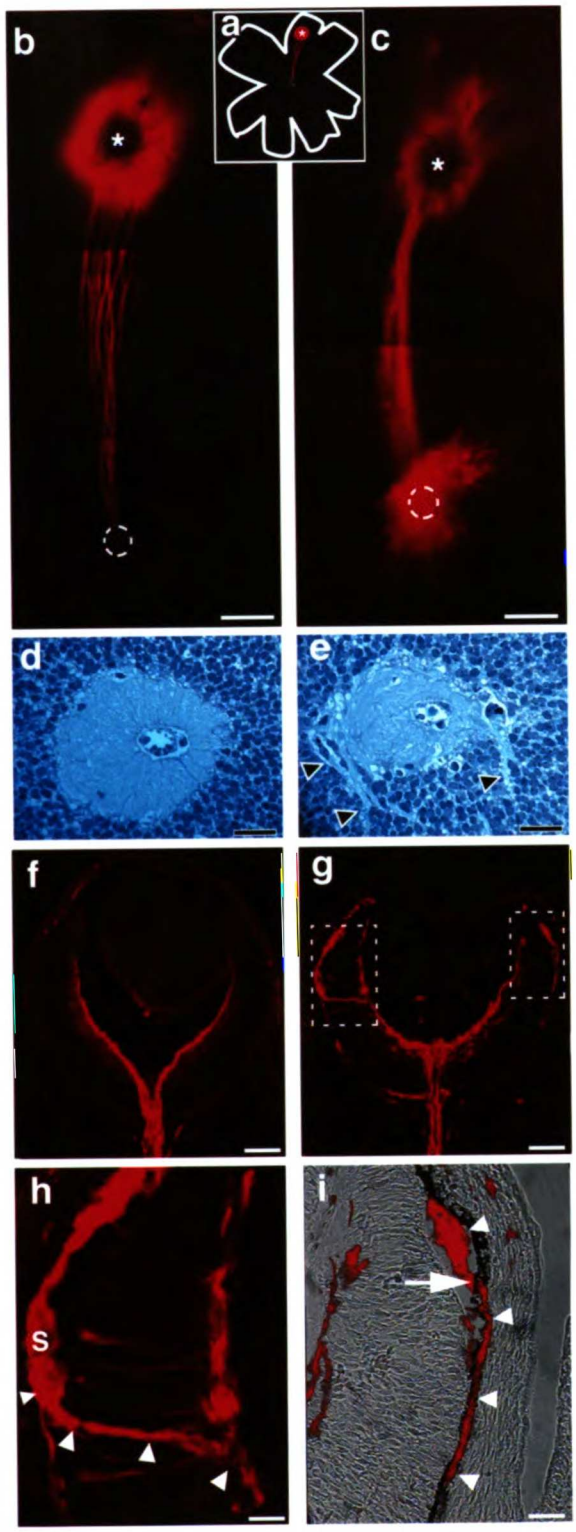


**Figure 2-5**

**Figure 2-6: The Netrin-1 Deficiency Does Not Alter Retinal DCC or NCAM Expression or Outgrowth Responses to Netrin-1.**

(a) A horizontal retinal section from an E14 control embryo showing NCAM expression (white) on both RGC axons and other retinal cells.

(b) A horizontal retinal section from an E14 netrin-1 mutant littermate showing comparable levels of NCAM expression on retinal cells and RGC axons, many of which failed to exit through the optic disc in this section.

(c) A horizontal retinal section from an E14 control embryo showing DCC expression on RGC axons. No other retinal cell types were labeled.

(d) A horizontal retinal section from an E14 netrin-1 mutant littermate showing comparable levels of DCC expression on RGC axons. Note DCC positive RGC axons which extended abnormally out of the optic disc into the retina (arrowheads).

(e) Comparison of netrin-promoted neurite outgrowth from E14 wild-type, heterozygous, and netrin-1 mutant retinal explants in collagen. NET, plus 100 ng/ml netrin; C, collagen alone. In collagen alone, control and mutant littermate explants had similar mean amounts of baseline neurite outgrowth per explant ($p>0.4$, Mann-Whitney, data not shown). Graph shows explants of all genotypes responded to 100 ng/ml netrin with an almost identical 6 fold increase in the amount of neurite outgrowth above their baseline.

Scalebars=25 μm (a-d).

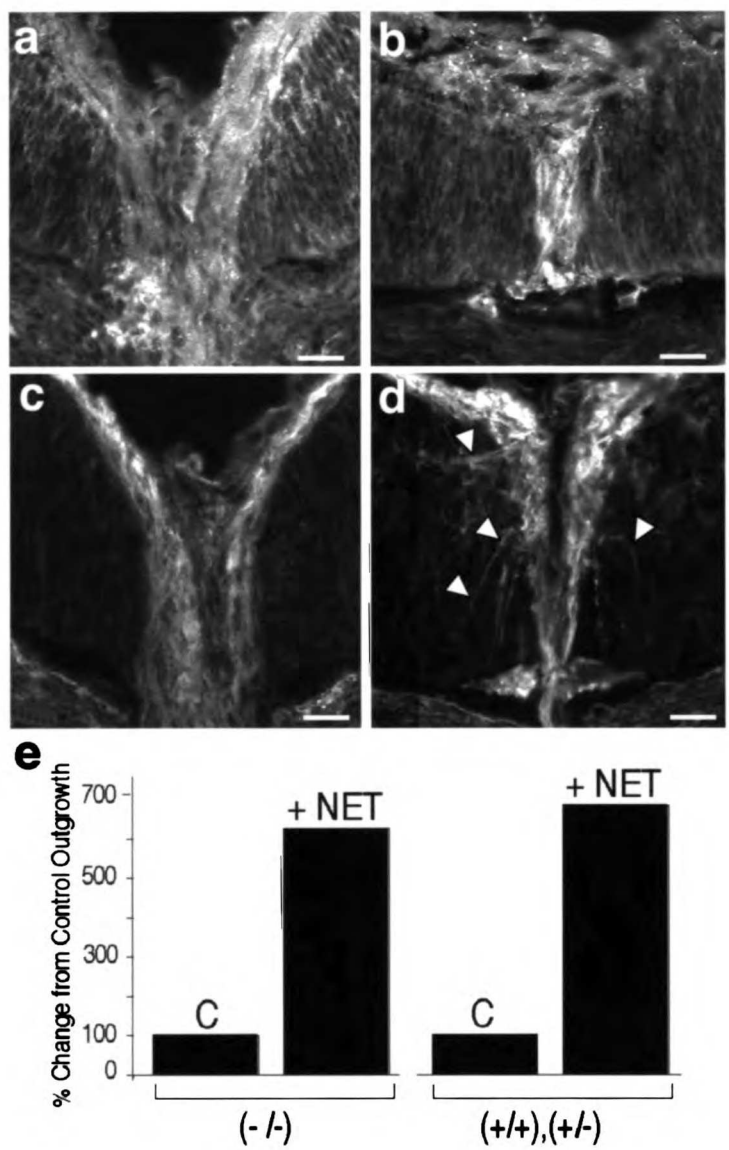
a
b
c
d
e
% Change from Control Outgrowth
700
500
300
100
0
+ NET
+ NET
C
C
(- /-)
(+/+),(+/-)

**Figure 2-6**

**Figure 2-7: Embryos Lacking Functional DCC Have Hypopigmented RPE Streaks, Optic Nerve Hypoplasia and Axon Guidance Defects at the Optic Disc**

(a) In an E15.5 heterozygote eye, the RPE appears normal, interrupted only where axons exit the eye through the optic disc (arrow) into the optic nerve.

(b) In an E15.5 DCC null littermate eye, hypo-pigmented streaks (arrowheads) within the RPE and a reduced optic disc size (compare fig. 2-7a to 2-7b, arrows) were seen.

(c) DiI labeled axons in the optic nerve of an E15.5 heterozygote embryo.

(d) DiI Labeling in a DCC null littermate showed fewer axons in the hypoplastic optic nerve.

(e) A flatmount of an E16 heterozygote retina, showing DiI labeled axons extended from the peripheral labeling site directly toward the optic disc (approximate location indicated by dashed circle), where they disappeared upon exiting through the disc into the optic nerve.

(f) In a DCC null littermate retina, labeled RGC axons also extended from the labeling site towards the optic disc. However, once at the disc (approximate location indicated by dashed circle), many labeled axons failed to exit into the nerve and splayed out past the disc into other retinal regions.
Scalebars=200 μm (a-b), 100 μm (c-f).

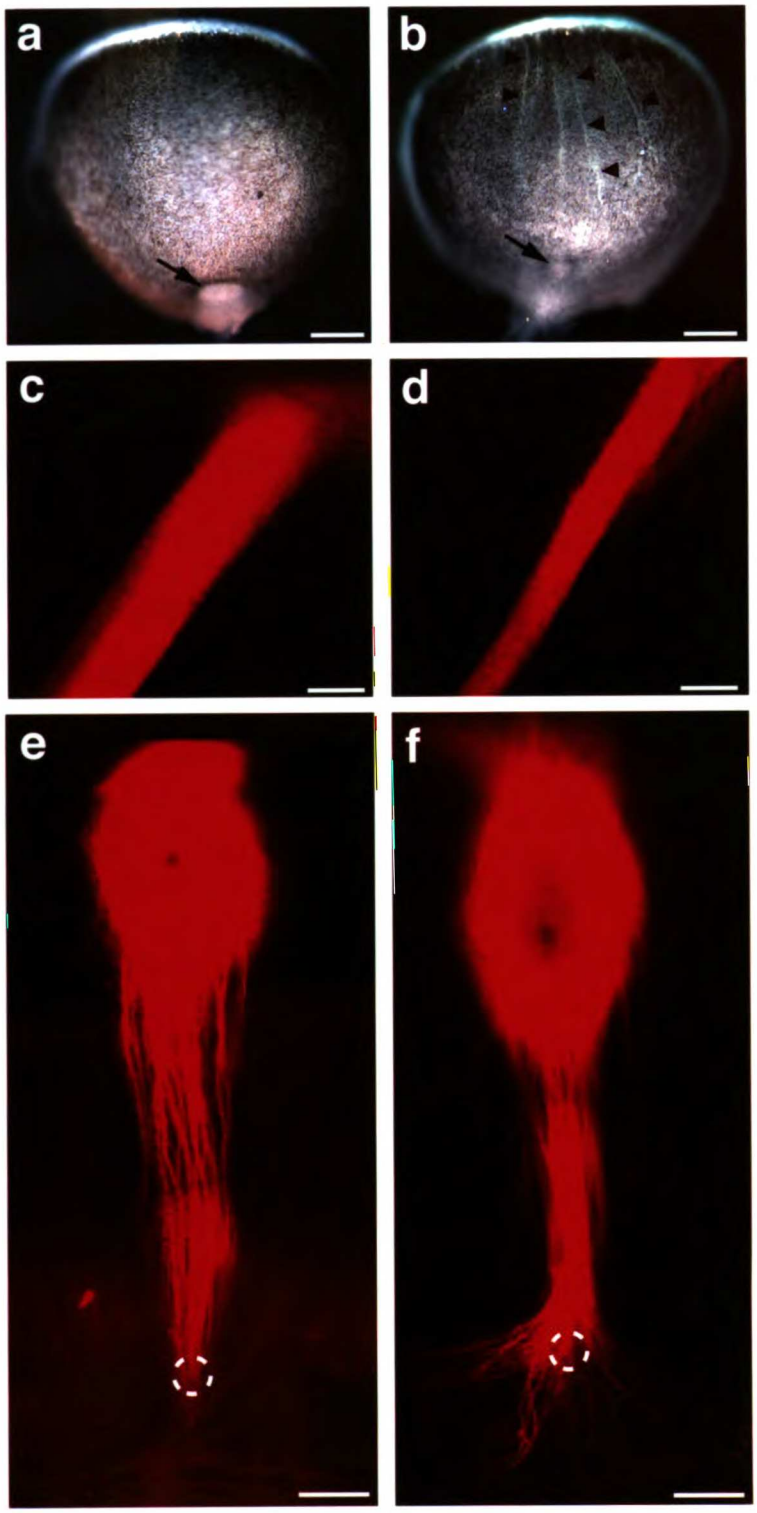


**Figure 2-7**

**Figure 2-8: An updated model of intraretinal mechanisms of axon guidance**

In summary, all of our evidence points to a model where netrin (red) acts at the optic disc as a short range cue for DCC (green) expressing retinal ganglion cell axons specifically to guide them at this exit point from the eye (dashed box), although the details of how a netrin/DCC interaction triggers the appropriate growth cone pathfinding behavior changes remains unknown. The cues previously shown to direct axons towards the optic disc involve an inhibitory outer boundary of chondroitin sulfate proteoglycans that directs initial axon elongation centrally as well interactions of cell adhesion molecules of the immunoglobulin superfamily such as L1. Our results are consistent with a model where cues other than netrin such as these act to guide axons from the cell bodies to the disc and at the optic disc, netrin acts as one of the last steps to guide axons out of the eye.

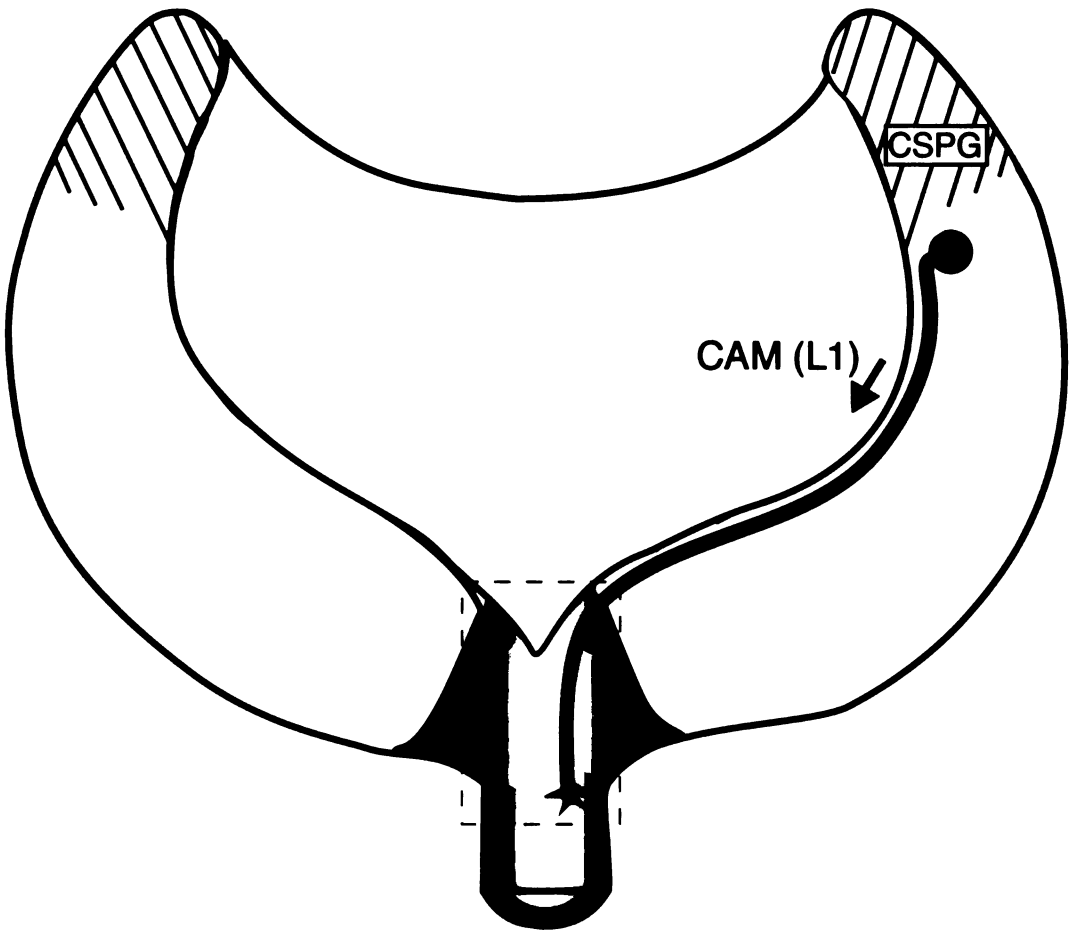


**Figure 2-8**

# Chapter 3

## Altered Midline Axon Pathways and Ectopic Neurons in the Developing Hypothalamus of Netrin-1 and DCC Deficient Mice

ABSTRACT

Proper optic nerve formation in mouse involves interactions between netrin-1 at the optic disc and the netrin-1 receptor DCC (deleted in colorectal cancer) expressed on retinal ganglion cell (RGC) axons. Deficiency in either protein causes RGC pathfinding defects at the disc leading to optic nerve hypoplasia (Deiner et al., 1997). Here we show that further along the visual pathway, RGC axons in netrin-1 or DCC deficient mice grow in unusually angular trajectories within the ventral hypothalamus, often forming the optic chiasm in an abnormally posterior position. In heterozygous *Sey*$^{neu}$ mice which also have a small optic nerve, RGC axon trajectories appear normal indicating the altered RGC axon trajectories in netrin-1 and DCC mutants are not secondarily due to optic nerve hypoplasia. Intrinsic hypothalamic patterning is also affected in netrin-1 and DCC mutants including a severe reduction in the posterior axon projections of gonadotropin-releasing hormone neurons. In addition to axon pathway defects in mutants, anti-diuretic hormone and oxytocin neurons are found ectopically in the ventromedial hypothalamus, apparently no longer confined to the supraoptic nucleus. In sum, netrin-1 and DCC, presumably through direct interactions, govern both axon pathway formation and neuronal position during hypothalamic development and loss of netrin-1 or DCC function affects both visual and neuroendocrine systems. Netrin protein localization also indicates that unlike in more caudal CNS, guidance about the hypothalamic ventral midline does not require midline expression of netrin.

## INTRODUCTION

Despite the importance of the hypothalamus in neuroendocrine and autonomic function, little is known of the developmental mechanisms that give rise to the complex patterns of neuronal connectivity in this anterior CNS region. Most neurons destined to become part of the neuroendocrine hypothalamus are born starting around E10.5 and subsequently migrate away from the ventricular zone to form hypothalamic neuronal groups (nuclei) distinguished by expression of specific neuropeptides or hormone releasing factors (Altman and Bayer, 1978a; Altman and Bayer, 1978b). Many of these neurons then send axons posteriorly to the median eminence (ME), infundibulum, or posterior pituitary to regulate hormonal release into the bloodstream (for review see Swanson and Sawchenko, 1983; Ganten et al., 1986; Swanson, 1986). As these events take place, RGC axons enter the hypothalamus from the optic nerves, cross the ventral midline to form a commissure called the optic chiasm (chiasm), and then continue onward to form the optic tracts. Given that development of hypothalamic neurons and RGC axon pathways are spatiotemporally intermixed, similar mechanisms and molecular cues may govern both visual and neuroendocrine development in this part of the CNS.

Caudal to the hypothalamus, cell differentiation and axon guidance about the ventral midline of the developing hindbrain and spinal cord appear to be organized by activities derived from midline structures such as the notochord and floorplate (reviewed in Dodd et al., 1998). An important CNS ventral midline guidance cue is Netrin-1, a diffusible protein secreted by ventral midline floorplate cells that can attract commissural axons (Kennedy et al., 1994; Serafini

et al., 1994; Shirasaki et al., 1996) and animals lacking netrin-1 or DCC, a netrin-1 receptor, are missing or have severely reduced ventral midline commissures (Serafini et al., 1996; Fazeli et al., 1997). Given that the notochord and floorplate are not clearly morphologically defined at the ventral diencephalon midline compared to in the hindbrain and spinal cord, it is not known whether axon guidance and cell patterning about the ventral hypothalamic midline involves cues such as netrin-1 or uses novel mechanisms.

Here we investigated whether netrin-1 or DCC are involved in mouse hypothalamic development. The results show that netrin-1 and DCC are required for development of RGC axon trajectories during chiasm formation and for development of GnRH axon pathways. Furthermore, in the absence of netrin-1 or DCC, ADH and oxytocin neurons of the supraoptic nucleus (SON) appear to migrate abnormally into the chiasm region. Thus, patterning of neuronal connectivity about the ventral hypothalamic midline is governed by netrin-1 and DCC. However, unlike in the spinal cord and hindbrain, netrin does not appear to be expressed at the ventral hypothalamic midline and therefore cannot be utilized by the nervous system as a midline cue at this location. Hypothalamic developmental abnormalities can now be added to corpus callosum defects (Serafini et al., 1996; Fazeli et al., 1997) and optic nerve hypoplasia (Deiner et al., 1997) in netrin-1 and DCC mutant mice. This trio of seemingly disparate phenotypes is reminiscent of the human congenital multisystem syndrome of septo-optic dysplasia (Skarf and Hoyt, 1984; Brodsky et al., 1996).

RESULTS

**Netrin-1 and DCC expression in the developing hypothalamus**

Immunostaining using a polyclonal pan-netrin antibody (from T. E. Kennedy, see Deiner et al., 1997), revealed netrin protein localization in discrete regions within the E12-16 embryonic mouse hypothalamus including in bilateral patches extending inward from the pial surface to deeper zones within the hypothalamus (fig. 3-1a, c, d) and in the ventricular zone (fig. 3-1 e). In contrast, DCC was much more widely expressed throughout the hypothalamus but was noticeably absent at a few locations including the ventricular zone (fig. 3-1g-i). Below, we more fully describe the patterns of netrin and DCC expression in relation to the development of RGC, gonadotropin-releasing hormone (GnRH), anti-diuretic hormone (ADH), and oxytocin neurons. This is followed by an analysis of how the development of these neurons and their axons are affected when netrin-1 or DCC function is disrupted.

**Relationship to RGC axons and chiasm neurons**

At E12.5, the first RGC axons have arrived at the ventral hypothalamus and have begun to form the chiasm. As the RGC axons enter the midline region, they encounter optic chiasm neurons which are found along the posterior boundary of the future chiasm and are organized into an inverted V-shaped array (reviewed in Mason and Sretavan, 1997). These embryonic optic chiasm neurons express the cell surface protein CD44 (Sretavan et al., 1994) and the SSEA-1

epitope (Marcus et al., 1995) and send processes dorsally into the tract of the post optic commissure (TPOC) (Easter et al., 1993; Sretavan et al., 1994). During development, RGC axons grow through the chiasm midline region along the anterior edge of this CD44/SSEA positive neuronal population (referred to in this paper as chiasm neurons) and RGC axons forming the optic tract grow immediately anterior to chiasm neuron axons suggesting chiasm neurons form a posterior border for the RGC axons (Sretavan et al., 1994; Marcus and Mason, 1995). Experimental evidence also shows that chiasm neurons are required for RGC axons to cross the chiasm midline (Sretavan et al., 1995).

Starting from E12 onwards, netrin was expressed bilaterally along the optic nerves (see fig. 3-2a) and just posterior to the attachment point of the optic nerves to the brain, lateral to the midline (see fig. 3-1c). The location of this lateral zone of netrin protein expression roughly correlated with the location of the chiasm neuron axons and the future position of the initial portions of the optic tracts. By E14-16, though similar in overall pattern, the levels of netrin expression were greatly reduced. During RGC axon ingrowth and chiasm formation, netrin was not detected at the ventral midline in the hypothalamus (fig. 3-1a-f). The early pattern of hypothalamic netrin protein expression reported here is similar to that of netrin mRNA (T. Serafini, personal communication) suggesting the antibody accurately represents netrin protein distribution.

RGC axons expressed DCC during their course within the optic nerve (fig. 3-2c), the chiasm, and the optic tract (fig. 3-2q, 3-2t). Chiasm neurons and their axons also expressed DCC (fig. 3-2f to 3-2k) and therefore like RGC axons may also be capable of responding to netrin. The expression data (summarized in fig. 3-2l) indicate that RGC axons do not grow towards the midline and form the

chiasm in response to netrin secreted from the hypothalamic midline. However, the bilateral patches of netrin expression in the optic nerve and the vicinity of the initial optic tract region suggest that netrin and DCC interactions could mediate some aspects of RGC or chiasm neuron axon pathway development in the ventral hypothalamus.

**GnRH neurons and axon pathways**

GnRH neurons originate in the olfactory placode, migrate along olfactory axons to the mediobasal olfactory bulb (Schwanzel-Fukuda and Pfaff, 1989; Wray et al., 1989; Livne et al., 1993), where they enter the brain and take up final residence in several hypothalamic regions including the medial septum, diagonal band and preoptic areas. From ages E13.75 through birth (Wu et al., 1997), GnRH neurons residing in these regions send axons posteriorly along both ventro- and dorsomedial routes (Hoffman and Gibbs, 1982) to terminate in the median eminence (ME) lateral contact zone and subsequently regulate gonadotropin release. Netrin was found at the ventral medial septal and presumptive diagonal band regions (see fig. 3-1a), where some migrating GnRH neurons take up final residence in the brain. In addition, bilateral patches of netrin protein were present in regions adjacent to the ventricles along the anterior-posterior length of the hypothalamus (see fig. 3-1e) as well as in the lateral posterior regions of the presumptive ME (see fig. 3-1f); a pattern reminiscent of the pathway taken by GnRH axons. Many GnRH neurons and their axons expressed DCC (fig. 3-2 m-o). The expression of DCC on GnRH neurons (see summary fig. 3-2p) together with the presence of netrin protein at the final destination of migrating GnRH neurons

and along GnRH axon pathways suggests that both GnRH neuron migration and/or axon guidance could depend on netrin and DCC interactions.

**Netrin, DCC, and supraoptic nucleus (SON) development**

ADH and oxytocin neurons destined to form the SON are thought to arise in the ventral diencephalon ventricular zone and migrate laterally to form a well-defined nucleus that is bounded along its ventromedial border by the optic tract (Karim and Sloper, 1980; Okamura et al., 1983). Axons of ADH and oxytocin neurons within the SON extend ventrocaudally to the midline region and then grow posteriorly through the ME internal zone into the posterior pituitary. There, ADH and oxytocin released into the circulation regulate blood pressure, osmolality and volume (ADH), as well as parturition and lactation (oxytocin). At E14, when the SON is just beginning to form, netrin was expressed within the SON (see fig. 3-2r), while DCC was expressed in the surrounding tissue (see fig. 3-2q). This pattern was found in subsequent stages of development after many ADH neurons had appeared in the SON (see fig. 3-2t-v). The absence of DCC expression in the SON was verified using both polyclonal and monoclonal anti-DCC antibodies. Thus developing neurons within the SON are notable for the absence of DCC even though netrin is apparently produced within the SON itself.

**Abnormal RGC axon trajectories in netrin-1 and DCC mutants**

We investigated and compared hypothalamic development between netrin-1 deficient and DCC deficient mice to identify developmental events

dependent on netrin-1 and DCC interactions. Based on the pattern of netrin-1 and DCC expression and given that RGC axons respond to netrin-1 *in vitro* (Wang et al., 1996; de la Torre et al., 1997; Deiner et al., 1997), we first examined chiasm formation. To do this, RGC axons were labeled with DiI and their trajectories in ventral hypothalamus were visualized in wholemount preparations. Images of wildtype and mutant RGC axon pathways were aligned for analysis using the anterior edge of the pituitary and the ventral midline as markers. In netrin-1 deficient and in DCC deficient mice, a smaller number of RGC axons appeared to reach the chiasm, consistent with the fact that fewer RGC axons exit the eye in these embryos (Deiner et al., 1997). Upon entering the hypothalamus, RGC axons grew along abnormally angular trajectories in all netrin-1 and DCC mutant embryos compared to wildtype littermates (see fig. 3-3a to 3-3c) and sometimes appeared to cross the midline at a more posterior position (seen in fig. 3-3a and 3-3b but not 3-3c).

To quantitatively analyze the angular RGC axon pathway defects, we measured two RGC axon turning angles in wildtype and mutant littermates (see fig. 3-3d). As this phenotype appeared quite similar in both the netrin-1 and DCC mutants (compare fig. 3-3a to 3-3b), we pooled the results from both sets of mutants. In E12 to E15 mutants, both turning angles were greater compared to wildtype littermates. For example at E14, compared to wildtype, the turning angles in mutants were on average 24 degrees greater at angle 1 (fig. 3-3e, black bars) and 12 degrees greater at angle 2 (fig. 3-3e, gray bars).

During normal development, early arriving RGC axons run in close apposition to chiasm neurons. As many more RGC axons enter the hypothalamus to form the chiasm, the main bundle of RGC axons is increasingly separated from

the chiasm neurons (Sretavan et al., 1994; Marcus and Mason, 1995), as if RGC axons are added anteriorly, but a number of RGC axons remain posteriorly (see fig. 3-3f, asterisk). In some netrin-1 and DCC mutant embryos the RGC axon bundle appeared to cross the midline at a posteriorly shifted position (fig. 3-3a and 3-3b). This position most likely falls within the region where the small number of posterior axons are normally found in the wildtype chiasm. This suggests that the apparent posterior position of the RGC axon bundle in mutants may have resulted from a reduced addition of axons anteriorly. Of note, RGC axons in netrin-1 or DCC deficient embryos entered the hypothalamus at approximately the correct position and despite the abnormally angular trajectories about the midline, RGC axons appeared to readjust and form the optic tract at a normal position in mutants (3a-c). This suggests that the RGC axon pathway developmental events requiring netrin-1 and DCC interactions are specifically localized to the region of the chiasm.

**Angular RGC axon trajectories are independent of a small optic nerve**

Given that a smaller number of RGC axons exit the eye in netrin-1 or DCC mutants, we examined whether abnormal RGC axon chiasm trajectories could simply be a consequence of optic nerve hypoplasia. To investigate, we studied chiasm development in *Sey*$^{neu}$ mice which have a mutated Pax6 gene (Hill et al., 1991), a paired box transcription factor expressed early in eye development (Walther and Gruss, 1991). *Sey*$^{neu}$/ + heterozygotes have a small eye which we reasoned should lead to reduced numbers of RGC axons in the optic nerve. This was confirmed in our analysis which showed that the diameter of the optic nerve

in heterozygotes was reduced by 20-30% compared to normal (see fig. 3-3g). In no case however, did we observe the abnormally angular RGC axon trajectories as measured by angles 1 and 2 (see fig. 3-3g and 3-3h). Therefore, a small optic nerve by itself does not lead to the abnormal RGC axon trajectories in the chiasm region. Instead, abnormal RGC axon trajectories in netrin-1 and DCC mutants are more likely due to the disruption of local hypothalamic developmental events dependent on netrin-1 and DCC function.

**Development of chiasm neurons**

One local neuronal population which can influence RGC axon trajectories is chiasm neurons. Given that chiasm neurons express DCC, and netrin was expressed in the region traversed by their axons, it was possible that defects in chiasm neuron development in DCC or netrin–1 mutants may have secondarily caused the abnormal RGC axon trajectories. Analysis of chiasm neuron development following CD44 immunostaining and DiI labeling in E12 littermates showed, however, that these neurons were arrayed normally in an inverted V-shape (compare fig. 3-4a to 3-4b) and sent axons dorsally in mutants, similar to the pattern seen in wildtype embryos (compare fig. 3-4c to 3-4d, DiI not shown; also see (Sretavan et al., 1994). The relationship of chiasm neurons to DiI-labeled RGC axons was analyzed at E14 in sagittal section. In mid-sagittal sections in both wildtype and mutant embryos, CD44 positive neurons at the midline appeared as curve-shaped cell groups (compare fig. 3-4e to 3-4f, arrows). Furthermore, the distance between these cell groups and the most posterior extent of the RGC axon bundle was roughly the same in wildtype (fig. 3-4g) and mutant

(fig. 3-4h) littermates. These results suggested that altered RGC axon trajectories in mutants were not secondarily due to grossly abnormal chiasm neuron development.

**Reduced GnRH axon projections**

At the ventral hypothalamus, in addition to angular RGC axon trajectories, the GnRH axon pathway was also severely affected in netrin-1 and DCC mutants. In wildtype animals, most anteriorly located GnRH neurons send axons posteriorly through the region of the organum vasculosum of the lamina terminalis (OVLT) just anterior to the chiasm and then along both ventral and dorsal routes adjacent to the third ventricle to reach the ME (Hoffman and Gibbs, 1982). In DCC mutants at P0, the location and numbers of GnRH axons in the OVLT appeared roughly similar to wildtype animals (compare fig. 3-5a to 3-5e), but the posterior axon projections to the ME were severely reduced (compare fig. 3-5c to 3-5g). However, the few axons that did reach the ME appeared to terminate in the appropriate position of the lateral ME contact zone. Similar findings were seen in netrin-1 mutants, although the number of GnRH axons in the OVLT appeared reduced in some netrin-1 mutants (OVLT, compare fig. 3-5b to 3-5f; ME, compare fig. 3-5d to 3-5h). Other than the GnRH axon pathway, axons of corticotropin-releasing hormone (CRH) neurons (compare fig. 3-5j to 3-5k) which also grow into the medial ME contact zone and ADH neurons (compare fig. 3-5l to 3-5m) which grow through the ME internal zone to the infundibulum, were not noticeably affected. This appropriate CRH and ADH axon growth suggests ME development was quite normal and rules out the possibility of a

general disruption of all axon growth through this region in mutants. A reduced GnRH projection to the ME was seen in netrin-1 and DCC mutants as early as E15 (not shown) when the first GnRH axons are thought to reach the ME. This indicates the reduced GnRH axon projection was not secondarily due to axons dying back, but more likely reflects an initial failure of the GnRH axons to reach their target.

Since RGC axons forming the chiasm are positioned close to the path of GnRH axons, altered RGC axon trajectories in the ventral hypothalamus may have lead to the reduced posterior GnRH axon projection. This possibility was examined in GAP-43 deficient mice in which RGC axon growth at the chiasm is disrupted to a greater extent than in netrin-1 or DCC deficient embryos (Strittmatter et al., 1995; Sretavan and Kruger, 1998) due to an initial failure of RGC axon progression from the chiasm region into the optic tracts (Kruger et al., 1998). In GAP-43 mutants, GnRH axon projections to the ME were not reduced (compare fig. 3-5n to 3-5o), suggesting that developmental events other than abnormal RGC axon growth at the chiasm were responsible for the reduced GnRH axon projections in netrin-1 and DCC mutants.

**Ectopic ADH and oxytocin neurons**

In addition to axon pathway defects, ectopic neurons were found in the ventral hypothalamus of both netrin-1 and DCC deficient mice. In wildtype animals at P0, ADH and oxytocin immunopositive neurons are found within the SON (arrows in fig. 3-6a, oxytocin not shown) which is bounded ventromedially by the optic tract. In both netrin-1 and DCC deficient animals, we observed trails

of ADH neurons just beneath the ventrolateral hypothalamus pial surface extending from the SON to the ventral midline (fig. 3-6b, arrowheads). These ectopic ADH neurons had leading and trailing processes (fig. 3-6c) and resembled migrating neurons (for example see Edmondson and Hatten, 1987). Some ectopic ADH neurons were found immediately anterior to the chiasm, appearing as bilateral clusters centered about the ventral midline (see fig. 3-6d,g). Three dimensional reconstructions in fig. 3-6e and 3-6f show the positions of all ADH positive neurons in a wildtype compared to a DCC mutant littermate. This pattern of ectopic ADH neurons was seen at E15, during the period of active neuron migration into the developing SON (Altman and Bayer, 1978a; Altman and Bayer, 1978b; Karim and Sloper, 1980; Okamura et al., 1983); raising the likelihood that ectopic neurons represent a failure of ADH neurons to properly migrate to or properly terminate migration within the SON. As with ADH neurons, ectopic oxytocin neurons in P0 mutant embryos were also present as clusters centered at the ventral midline anterior to the mutant chiasm (see fig. 3-i and j).

Other than the presence of ectopic ADH and oxytocin neurons, hypothalamic neuronal migration and the positions of hypothalamic nuclei did not appear to be generally disrupted in netrin-1 or DCC deficient embryos. For example, as shown above, CD44 neurons were found in their appropriate location (see fig. 3-4). Furthermore the paraventricular nucleus (PVN), which also normally contains ADH and oxytocin neurons (for review see Swanson and Sawchenko, 1983; Swanson, 1986), appeared normal in size and location in wildtype (fig. 3-6k,l) compared to mutants (fig. 3-6m,n) and trails of ectopic ADH or oxytocin neurons originating from the PVN were not observed. This suggests

that ADH and oxytocin neurons belonging to the PVN develop independently of netrin-1 and DCC interactions.

**The optic tract and ectopic ADH neurons**

The apposition of the SON and optic tract RGC axons (see fig. 3-2 q-v) suggests that RGC axons might normally serve as a barrier against abnormal neuron migration out of the SON. Fewer optic tract RGC axons in netrin-1 or DCC mutants (due to optic nerve hypoplasia) might then allow ectopic migration ventromedially out of this nucleus. To investigate, we examined the distribution of ADH neurons in E18 GAP-43 deficient embryos which have only very few RGC axons in the optic tracts (Sretavan and Kruger, 1998). In these mutant animals, ADH neurons were confined to the SON as in wildtype littermates, (compare fig. 3-6o to 6p), indicating that a reduced number of optic tract RGC axons is probably not the cause of ectopic ADH neurons in netrin-1 or DCC deficient embryos.

**Pituitary Development in Netrin-1 and DCC mutants**

Since normal pituitary development and function depends on proper innervation from specific hypothalamic neurons (for example see Schonemann et al., 1995; Michaud et al., 1998) and references therein), we examined pituitary cell differentiation and hormone production as a means of revealing additional possible defects in hypothalamic neurons, especially neurons for which there were no convenient markers. As newborn mutants usually died within 24 hours, we only examined pituitaries in animals up to age P0. In netrin-1 deficient and in

DCC deficient mutants, pituitary size and shape appeared grossly normal (compare figure 3-7a,c (wildtype) to 3-7b,d (mutant); also see fig. 3-4e vs. 3-4f). Innervation of the posterior pituitary by ADH immunopositive axons revealed no obvious abnormalities (compare fig. 3-5l to 3-5m). In pituitaries of wildtype compared to mutant littermates, the expression patterns of adrenocorticotropic hormone (ACTH) (fig. 3-7a,b) and growth hormone (GH) (fig. 3-7c,d) as well as luteinizing hormone and follicle stimulating hormone (not shown) appeared normal. In mutants at P0, circulating hormone levels of ACTH and GH were not reduced compared to wildtype littermates (see fig. 3-7e,f). This finding suggested that although ACTH levels are thought to be affected in part by ADH (Molitch, 1995), the presence of ectopic ADH neurons did not noticeably affect ACTH levels at P0. Furthermore, although we did not directly examine growth-hormone releasing hormone (GHRH) neuron development in mutant animals, the observation of normal GH expression in the anterior pituitary and normal serum GH levels suggested that GHRH neuron differentiation, innervation and regulation of GH levels was most likely not significantly affected in mutants (Godfrey et al., 1993; Lin et al., 1993; Li et al., 1996). In summary, although netrin-1 and DCC deficient animals exhibit specific defects in hypothalamic development, this was not associated with obvious pituitary or neuroendocrine abnormalities within the scope of our analysis at E12-P0.

## DISCUSSION

Results from this study demonstrate that specific aspects of mouse hypothalamus development require the guidance molecule netrin-1 and the netrin receptor DCC (see summary fig. 3-8). The similarities in phenotype in both netrin-1 and DCC mutants suggests this is through direct netrin-1 and DCC interactions. In the absence of netrin-1 and DCC, RGC axons are affected together with GnRH, ADH, and oxytocin neurons. While previous studies have identified important regulatory genes involved in hypothalamic cell fate specification (Schonemann et al., 1995; Kimura et al., 1996; Torres et al., 1996; Macdonald et al., 1997; Michaud et al., 1998; Dale et al., 1999), to our knowledge, this is the first report describing some of the guidance molecules involved in the patterning of axon pathways and the determination of neuronal positions in the developing mouse hypothalamus.

### Netrin-1 and DCC interactions and RGC growth in the developing hypothalamus

The mechanism by which the lack of netrin-1 and DCC interactions lead to the abnormally angular RGC axon trajectories remains unknown. *Sey*$^{neu}$/+ heterozygous mice which have a small optic nerve do not exhibit abnormal RGC axon trajectories within the hypothalamus indicating that optic nerve hypoplasia alone does not cause this phenotype. Previous studies show that chiasm neurons are involved in chiasm formation (reviewed in Mason and Sretavan, 1997) and in netrin-1 and DCC mutants, abnormal trajectories could result if the development

of these neurons was affected. However, this does not appear to be the case since chiasm neuron development and the relationship of RGC axons to these neurons appears unaffected in mutants. Although a simple model is that netrin-1 is required to guide DCC expressing RGC axons in the chiasm region, this guidance model cannot involve a normal midline focus of netrin expression since netrin was not observed at the hypothalamic midline during the period of RGC axon ingrowth and chiasm formation. Our findings support the notion that netrin-1 and DCC interactions govern axon guidance about the ventral midline in both anterior and posterior CNS commissures. However, while in the spinal cord netrin-1 is required for commissural axon guidance to the midline floorplate, in the hypothalamus, deficient netrin-1 function alters RGC axon trajectories but is not required for RGC axons to reach or cross the midline.

**Disrupted GnRH neuron axon projections**

The results indicate that GnRH neuron migration and/or axon pathfinding require netrin-1 and DCC interactions. In DCC mutants, GnRH innervation of the OVLT appeared relatively unaffected suggesting that a substantial number of GnRH neurons migrated appropriately into the hypothalamus and that GnRH axon pathfinding was not generally disrupted. Given that individual GnRH axons reportedly form *en passant* synapses in the OVLT and then project into the ME (Hoffman and Gibbs, 1982), the relative lack of GnRH axons posteriorly in the ME suggests a disruption of GnRH axon pathfinding after growth through the OVLT. If so, this implies GnRH axons require DCC function not for OVLT innervation but specifically for pathfinding in

more posterior parts of the hypothalamus. Recent cell culture studies suggest that a diffusible guidance cue may attract GnRH axons to the ME (Rogers et al., 1997). Whether this cue is netrin-1, signaling through DCC, remains to be tested. Compared to DCC mutants however, the GnRH innervation of the OVLT appeared reduced in netrin-1 mutants. This suggests that netrin-1, independent of interactions with DCC, may also be required either to ensure proper GnRH axon targeting of the OVLT or proper GnRH neuron migration into the hypothalamus.

Based on the reduced GnRH axon projection, one might predict that netrin-1 or DCC deficiency would disrupt the regulation of gonadotropin release, leading to hypogonadism. If true, this would indicate that altered GnRH neuron development can lead to hypogonadism both through the failure of proper GnRH neuron migration into the CNS as in Kallman's syndrome (Schwanzel-Fukuda et al., 1989), as well as through the disruption of GnRH axon pathway formation.

**Ectopic ADH and oxytocin neurons**

During normal development, ADH and oxytocin neurons are thought to originate in the region of the ventral diencephalic sulcus and then migrate laterally to the PVN and SON (Altman and Bayer, 1978a; Altman and Bayer, 1978b). In mutants, the SON was flanked ventromedially by a trail of ectopic ADH neurons, and both ADH and oxytocin neurons were abnormally located in the region of the chiasm. However, the PVN appeared unaffected with no apparent trails of ectopic neurons. Thus, ADH and oxytocin expressing neurons were not all affected equally in the netrin-1 and DCC deficient state.

One model of ADH and oxytocin neuron migration is that netrin-1 in the SON attracts migrating ADH and oxytocin neurons, and in mutants some neurons cannot therefore find their way to the SON and migrate to ectopic positions instead. However, based on the trail of ectopic neurons leading back to the SON, we believe it is more likely that netrin-1 and DCC interactions are required to confine ADH and oxytocin neurons to the SON and absence of these interactions in mutants leads to inappropriate migration of neurons out of the SON. A caveat to either model is that DCC was not expressed by neurons in the SON. It is possible that DCC was expressed by the SON neurons and was downregulated early on, or that an unconventional netrin-1 and DCC interaction occurs in which DCC in the surrounding tissue serves as a "barrier" guidance cue signaling via netrin-1 to prevent neuronal migration out of the SON. Although an unconventional cell non-autonomous role such as this for DCC and netrin-1 interactions has not been demonstrated, unconventional cell non-autonomous roles for DCC and netrin-1 have been demonstrated in other systems (Gong et al., 1999). An alternative that cannot be ruled out is that an unknown cell type normally prevents migration out of the SON and its development is somehow affected in netrin-1 and DCC deficient mutants.

**A shared feature of all developmental defects**

Although the three phenotypes identified involve neurons originating from separate regions of the CNS, they all exhibit developmental defects around the ventral hypothalamus/chiasm midline region. This includes the presence of ectopic ADH and oxytocin neuron clusters, angular RGC axon trajectories, and

severely reduced GnRH axon projections. The fact that all these developmental defects overlap anatomically at the ventral hypothalamic midline around the chiasm region allows for the possibility that one particular developmental defect in this region may have secondarily caused one or more of the other defects. For example, the presence of ectopic ADH neurons at the hypothalamic midline might alter RGC axon trajectories at the chiasm or the ability of GnRH neurons to send axons posteriorly through this region.

**Relationship to Septo-Optic Dysplasia (SOD)**

In the adult, acquired disorders affecting the hypothalamus often involve the visual system due to the location of the chiasm on the ventral hypothalamic surface (for review see Siatkowski and Glaser, 1995). This study shows that development of the visual and hypothalamic neuroendocrine systems are also linked via shared guidance molecules and suggests that mutations affecting guidance molecules could lead to congenital disorders affecting both vision and neuroendocrine function. The occurrence of hypothalamic abnormalities (shown in this paper), optic nerve hypoplasia (Deiner et al., 1997) and an absent corpus callosum (Serafini et al., 1996; Fazeli et al., 1997) in the netrin-1 or DCC deficient mice is phenotypically similar to the human congenital disorder of SOD. This raises the possibility that SOD may be linked to mutations in developmental genes such as netrin-1 or DCC. Although most cases of SOD have been described in the literature as largely sporadic in nature (Harris and Haas, 1972; Brodsky et al., 1996), a recent finding suggests that certain cases of SOD are caused by a mutation in the regulatory gene, Hesx1 (Dattani et al., 1998). In the mouse, Hesx1

is expressed in presumptive forebrain and Rathke's pouch (which forms the anterior pituitary) and mice missing Hesx1 have severely affected eye, pituitary, and prosencephalon development with accompanying abnormalities of the corpus callosum, septum pellucidum and anterior and hippocampal commissures. This phenotypic combination is remarkably similar to, but more severe than that observed in DCC or netrin-1 mutant mice. This suggests it may be worth examining if netrin-1 or DCC genes are regulated by Hesx1 and if they may be mutated in certain cases of SOD.

## METHODS

**Netrin-1, DCC, Pax6 and GAP-43 Deficient Mice:**

Production, breeding and genotyping of these mutant mouse strains was as previously described: netrin-1 deficient mice (Skarnes et al., 1995; Serafini et al., 1996); DCC deficient mice (Fazeli et al., 1997); GAP-43 deficient mice (Strittmatter et al., 1995; Kruger et al., 1998); Pax6 $Sey^{neu}/+$ mice (Hill et al., 1991). The processing of tissue from these litters for immunostaining and for DiI labeling of RGC axons was carried out identically as described below for C57/bl6 mice.

**Orientation Terminology:**

During mouse embryogenesis around E8, a 90° turn occurs along the dorsal - ventral axis to create the cephalic flexure such that rostral CNS anterior to the flexure comes to lie ventrally. Given that the present study describes results after the formation of the flexure and around birth, we have adopted the orientation terminology used conventionally in both the vision and the neuroendocrine literature. Anterior therefore refers to the direction towards the nose and dorsal is towards the top of the head.

**Antibodies:**

Anti-DCC (rabbit polyclonal, 1:3000, gift of Dr. E. Fearon, U. of Michigan Medical Center (Reale et al., 1996); anti-DCC (mouse Mab, 5 µg/ml, Pharmingen 15041A, San Diego, CA); anti-pan-netrin (rabbit polyclonal #11760, 1:50, raised using a peptide antigen corresponding to sequence conserved in chick netrin-1, chick netrin-2 and mouse netrin-1 (T. Kennedy and M. Tessier-Lavigne, in preparation; also see (Deiner et al., 1997); anti-CD44 (rat Mab KM201 (IgG), 1:3,

(Miyake et al., 1990); Anti-GnRH (rabbit polyclonal, 1:2500, Gift from W. Vale, Salk Institute, #PBL, L45); anti-CRH (rabbit polyclonal, 1:500, Gift from W. Vale, #PBL, rC70); anti-ADH (rabbit polyclonal, 1:250, Chemicon, #AB937, #AB1565, Temecula, CA); anti-ACTH (rabbit polyclonal, 1:1200, gift of National Hormone and Pituitary Program (NHPP), lot #AFP39013082); anti-GH (guinea pig polyclonal, 1:1200, gift of Dr. A.F. Parlow, Harbor-UCLA Medical Center, lot # AFP11121390); anti-FSH (rabbit polyclonal, 1:1000, gift of Dr. A.F. Parlow, lot # AFP131078); anti-LH (guinea pig polyclonal, 1:1000, gift of NHPP, lot # AFP22238790GPOLHBB).

**Immunohistochemistry:**

E12-18 embryos (plug day = E0) were harvested from anesthetized C57/bl6 timed pregnant mice (Sretavan et al., 1994). For immunostaining using 10-20 μm coronal cryostat sections, E12-18 heads were fixed in 4% paraformaldehyde in 0.1M phosphate buffer pH 7.25 (PFA) for 1 hr. at 4 °C, infiltrated overnight in 30% sucrose/PBS, embedded in OCT (Baxter, McGaw Park, IL), and stored at -80°C until use. P0 animals were first perfusion fixed with 4% PFA, immersion fixed in 4% PFA 1-2 days, and infiltrated 1-5 days in 30% sucrose/PBS before embedding in OCT. For immunostaining using paraffin sections, heads were fixed using Carnoy's fixative (Bancroft and Stevens, 1982) and then embedded in EM-400 wax (Surgipath, Richmond, IL) until use. For immunostaining using 100 μm thick vibratome sections, heads were fixed in 4% PFA, RGC axons were labeled with DiI as described below, and heads were then embedded in 3% agarose in PBS.

Immunolabeling of DCC, CD44, ADH, GnRH, CRH, GH, ACTH, FSH and LH was carried out on cryostat or paraffin sections preblocked in Heat Inactivated

Normal Goat or Donkey Serum (HINGS or HINDS respectively) for 20 min. Incubations with primary antibody were performed at room temperature (RT) for 1-2 hr. followed by 5 washes in 0.1 M PBS (4 min./wash). Secondary antibody was applied for 1-2 hr. at RT, the sections were then washed 5 times before coverslipping. All antibody incubations were carried out in the presence of 0.1% triton X-100 and either 1% HINDS or 1% HINGS for blocking. In some cases, a second 10% HINGS or HINDS 20 min. blocking step was carried out before addition of the secondary antibody. Netrin immunolabeling in 5-10 μm thick paraffin sections was carried out as described previously (Deiner et al., 1997). For visualization of primary antibodies using immunofluorescence, sections were labeled with the appropriate secondary antibodies conjugated to either Cy2 or Cy3 (Jackson Immunoresearch, West Grove, PA). For visualization using alkaline-phosphatase, sections were labeled with the appropriate AP-tagged secondary antibody and incubated with the color substrate, BM Purple (Boehringer, Indianapolis, IN). CD44 immunostaining in wholemounts was as described in (Sretavan et al., 1994) using the Vectostain-ABC kit (Vector Laboratories, Burlingame, CA). CD44 immunostaining using 100μm thick sagittal sections was also as described in (Sretavan et al., 1994) except that all antibody incubations, washes and reactions were carried out in 24 well plates.

**DiI Labeling and Angle Measurements of RGC Axons:**

Anterograde labeling of axons in the optic nerves with DiI (D-282 Molecular Probes, Eugene, OR), was carried out as described in (Sretavan et al., 1994). After dissecting overlying tissue to reveal labeled axons on the hypothalamic surface, preparations were aligned for imaging using the anterior

edge of the pituitary and the midline as landmarks. Labeled axons were imaged as described in the next section below. Using these images, the amount of axon turning at two sites (designated angles 1 and 2, depicted in fig. 3-3) was measured, in degrees, using Adobe Photoshop. Measurements were made without knowledge of genotype and similar values were obtained when an image was re-measured on separate days and by different observers. Angles 1 and 2 were measured in individual embryos from E12.5-15.5. Individual results from each animal were pooled into wildtype, heterozygous, and homozygous to obtain a mean turning angle expressed in degrees. The results from netrin-1 and DCC deficient animals were combined. At all ages, mean turning angle in mutants was always greater (sharper posterior turn in mutants for angle 1 and sharper anterior turn in mutants for angle 2) compared to mean turning angles measured in wildtype littermates.

**Digital Imaging:**

DiI and Cy3 fluorescence was visualized using rhodamine fluorescence optics, Cy2 using fluorescein optics. Images of immunolabeling in tissue sections and wholemounts were captured digitally using a Photometrics PXL2 cooled CCD camera or Optronics color CCD camera and processed for presentation using Adobe Photoshop and/or Illustrator. For all wildtype and mutant image pairs, images were collected using the same camera exposure times and processed simultaneously in Adobe Photoshop, allowing comparison between images. For 3-dimensional serial section reconstruction, ADH neurons in every 3rd 20 μm section were imaged digitally, and Adobe Illustrator was used to trace the position of labeled neurons. Serial sections were then assembled into one single

image file.

**GH and ACTH Assays:**

P0 pups were decapitated and 20-30 _l of blood collected in the presence of 1 _l of anti-coagulant. Older ages were not examined as mutants die soon after birth. Blood was immediately centrifugated for 5 minutes at 3000 x g, serum was collected, either diluted in water (ACTH assay) or undiluted (GH RIA) and snap frozen. ACTH levels were assayed at the UCSF Clinical Laboratories, Department of Laboratory Medicine using an antibody based chemiluminescence assay system, the ACTH 100T kit from Nichols Institute Diagnostics (cat. # 60-4175, San Juan Capistrano, CA). GH levels were measured by radioimmunoassay using I-125 mouse growth hormone, antiserum to rat growth hormone and a mouse growth hormone standard generously supplied by Dr. A. Parlow.

ACKNOWLEDGMENTS:

We thank Wylie Vale, A. Parlow, Lawrence Frohman, Eric Fearon, Tim Kennedy, Mark Fishman, Tito Serafini, Amin Fazeli and Marc Tessier-Lavigne for reagents and mice. Thanks to Selna Kaplan, Lita Ramos, Eric Birgbauer, Chris Severin, Judy Mak and Amanda Kahn for technical assistance and contributions. The ACTH, LH and FSH antibodies were a generous gift of the National Hormone and Pituitary Program, the National Institute of Diabetes and Digestive Kidney research, the National Institute of Child Health and Human Development, and the US Department of Agriculture. We also thank Michelle Bland, John Rubenstein, and Richard Weiner for their insightful comments. This research was supported by NIH EY10688 and TMMS grants EY 02162 & EY 07120

FIGURE LEGENDS

**Fig. 3-1: DCC and Netrin expression in the E12 mouse hypothalamus.** The schematic diagrams on the left column show the eyes, optic nerves and ventral hypothalamus. The red rectangle indicates the anterior-posterior hypothalamic level at which tissue sections were obtained for netrin (middle panel) and DCC (right panel) immunostaining. Tissue sections separated by 20-30 _m were used for netrin and DCC immunostaining at each level. In all panels, dorsal is at the top and the scale bar (shown in panel a) represents 200 _m. ( **a-f)** Netrin (arrowheads) was found in bilateral patches at different sites along the anterior-posterior length of the hypothalamus. (**a**) Netrin expression at the ventral medial septal and diagonal band region. The area of the dotted rectangle is shown at higher magnification in the inset at the upper right. Staining in blood cells and vessels is non-specific. **(b)** Note the absence of netrin at the ventral midline region. **(c,d)** Netrin is expressed at the ventral-lateral hypothalamic regions corresponding roughly to where axons of chiasm neurons (see fig. 3-2 ) grow dorsally to join the TPOC and where the initial portions of the optic tract will later develop. There is no apparent ventral midline netrin expression. **(e)** Netrin expression in the ventricular zone. The area of the dotted rectangle is shown at higher magnification in the inset at the upper right. Such bilateral patches of netrin were found in the ventricular zone region scattered throughout the anterior-posterior length of the hypothalamus. **(f)** Netrin expression in the presumptive lateral posterior ME region. **(g-l)** DCC expression at different levels along the anterior-posterior length of the hypothalamus. DCC was found in specific domains distributed throughout the hypothalamus but was absent from

the ventricular zones. Abbreviations: DB, diagonal band; MS, medial septum; POA, preoptic area; OC, future chiasm region; OT, future region of the optic tract; ME, median eminence.

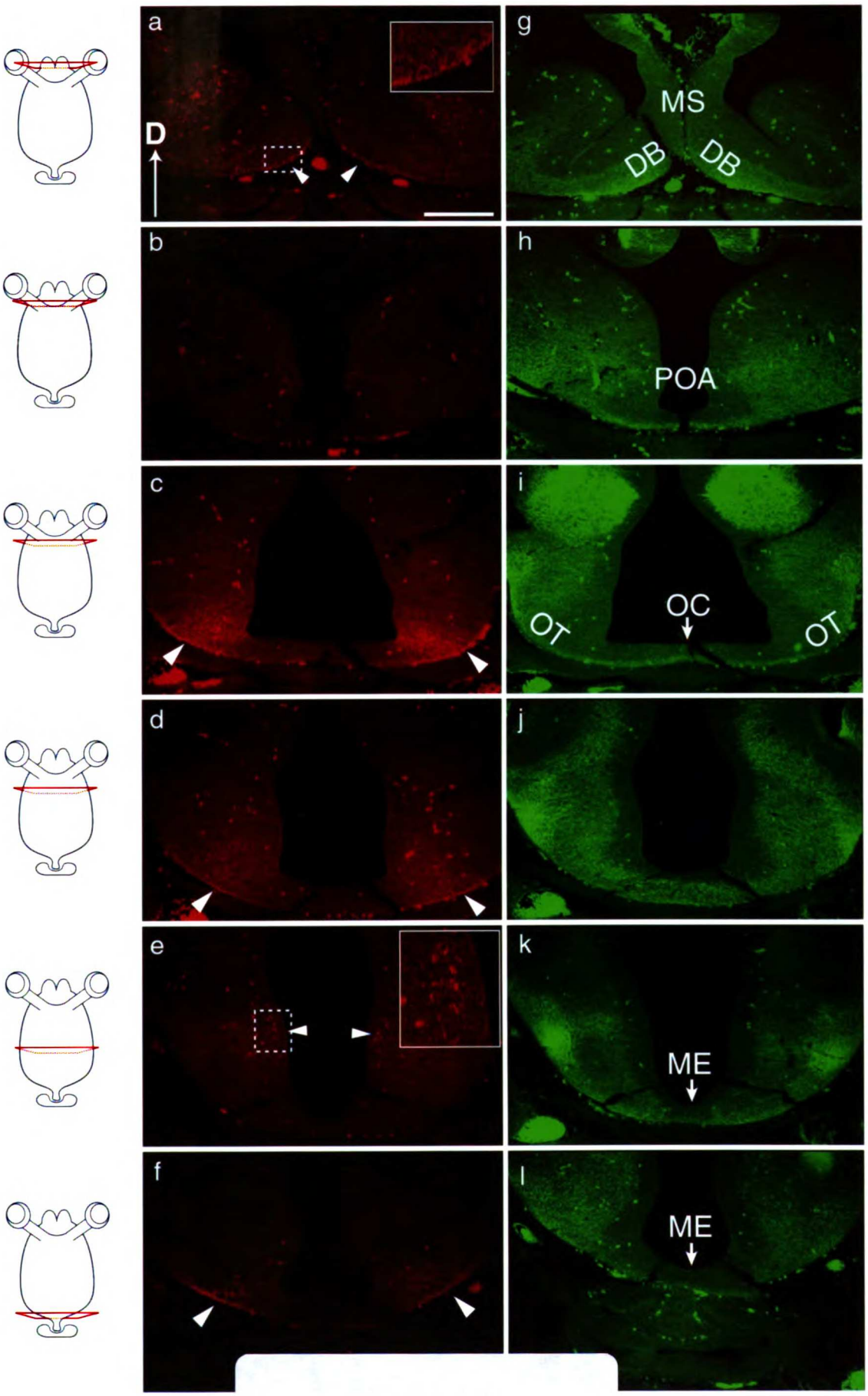


**Figure 3-1**

**Fig. 3-2: Relationship of Netrin and DCC expression to RGC axons, chiasm neurons, GnRH and ADH neurons.** **(a)** Netrin expression in the optic nerve at E14 (the retina is towards the top). **(b)** Immunostaining of an adjacent optic nerve section with anti-netrin antibody omitted. Note non-specific staining of blood cells. **(c)** DCC expression on RGC axons within the E14 optic nerve. **(d)** Immunostaining of an adjacent optic nerve section with anti-DCC antibody omitted. **(e)** E12.5 horizontal section dorsal to where the optic nerves attach to the hypothalamus shows netrin (dark blue) bilaterally in the approximate future region of the optic tract (see also fig. 3-1c and d). **(f)** E12.5 coronal section through the region of CD44 immunopositive chiasm neurons (red). **(g)** The same section as in panel f shows that DCC (green) is expressed by many neurons in this area. **(h)** Overlay of images in panels f and g showing colocalization of CD44 and DCC on neurons in this area. Most CD44 positive cells express DCC. **(i)** CD44 expression (red) on a dorsolaterally projecting axon of a chiasm neuron. **(j)** The same section as panel i showing expression of DCC (green) in the same region. **(k)** Overlay of images in panel i and j showing DCC expression on CD44 positive axons. **(l)** Summary diagram of netrin expression (red) with respect to the developing RGC axon and chiasm neuron pathway. DCC expression on RGC axons and chiasm neurons and axons is depicted in green. **(m)** E18 Sprague-dawley rat, coronal sections through the POA showing DCC immunopositive neurons and axons. The inset shows the approximate region shown in panels m-o. Similar results were seen in E16 mouse POA (not shown) **(n)** The same section as in panel m showing GnRH expression (red) in a neuron cell body (*) and axon (arrowheads). **(o)** Overlay showing cellular co-localization of DCC and GnRH. **(p)** Summary diagram showing netrin expression (red) in relationship to the

positions of GnRH neurons and to GnRH axon pathways. DCC expression on GnRH neurons and their axons is depicted as green. **(q)** E14 coronal section at the level of the SON showing DCC expression (green) in the tissue surrounding but not within the SON. The inset depicts the approximate region shown in higher magnification in panels q-s. **(r)** An adjacent section to panel q, showing netrin expression (red) within and just outside of the SON. **(s)** Superimposition of panels q and s. **(t)** E16 coronal section showing DCC expression in tissue surrounding the SON (arrow), including the region of the optic tract (asterisk). **(u)** Adjacent section showing ADH immunopositive neurons (red) within the SON. **(v)** Superimposition of panels t and u. Abbreviations: POA, preoptic area; SON, supraoptic nucleus. Scale bars: a-d, 25 μm; e, 200 μm; f-h, 10 μm; i-k, 1 μm; m-o, 10 μm; q-v, 100 μm.

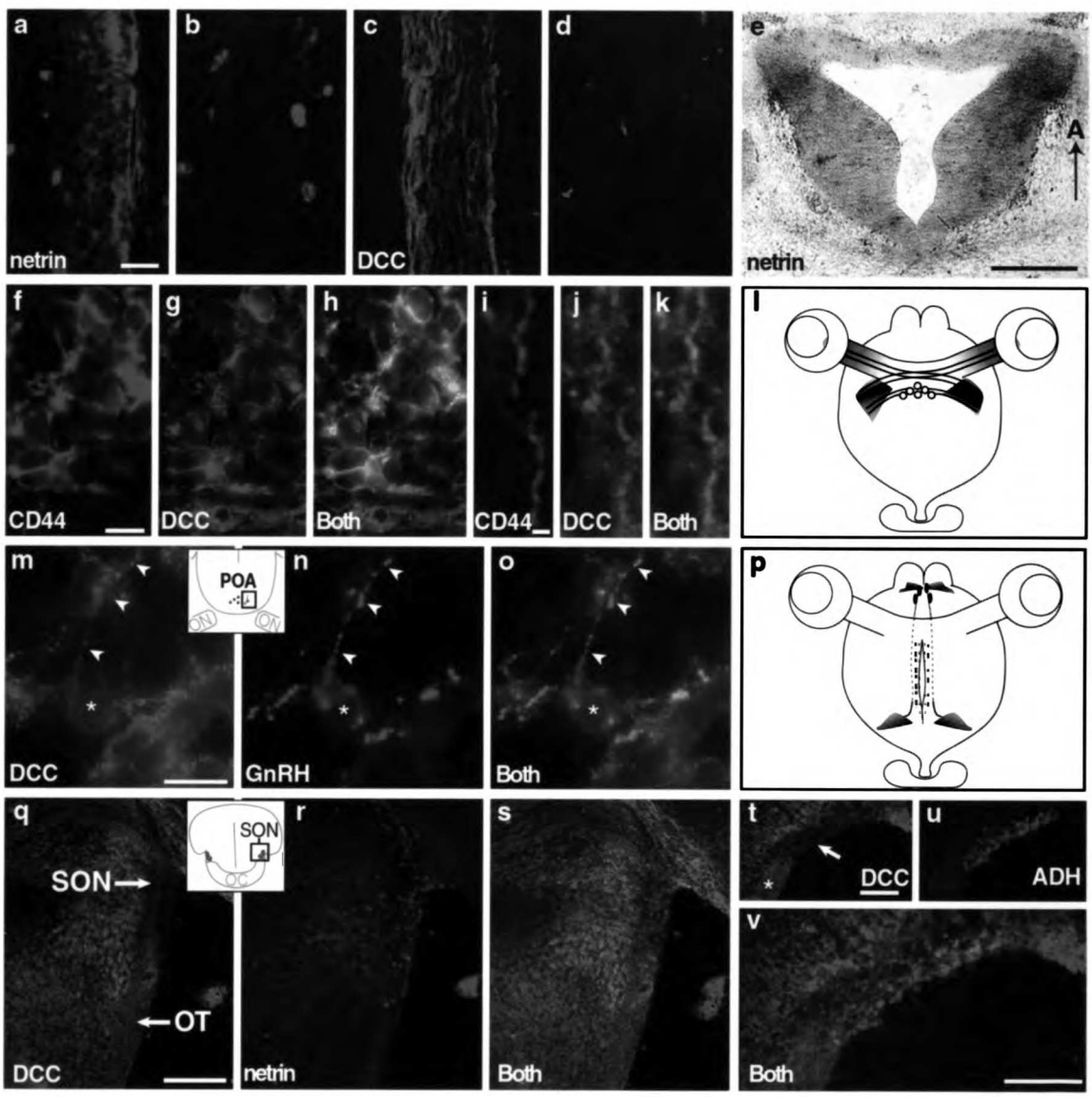
a
b
c
d
e
netrin
DCC
netrin
A
f
g
h
i
j
k
l
CD44
DCC
Both
CD44_
DCC
Both
m
n
o
p
POA
ON
ON
DCC
GnRH
Both
q
r
s
t
u
v
SON
SON
OT
DCC
netrin
Both
DCC
ADH
Both

**Figure 3-2**

**Fig. 3-3: Altered RGC axon trajectories at the ventral hypothalamus of DCC or Netrin-1 deficient mouse embryos.** **(a-c)** Each panel shows superimposed images of DiI labeled RGC axons at the ventral hypothalamic region in wildtype (red) and mutant (green) littermate embryos. The retina of origin is towards the upper left and the optic tract is towards the bottom right. (a, b E14; c, E14.5 embryos.) **(d)** Schematic diagram showing the location of the angles 1 & 2 measured in wildtype and mutant embryos. **(e)** Graph showing angles 1 & 2 in netrin-1 and DCC mutant embryos (data combined) compared to wildtype embryos throughout the period E12.5 to E15.5. The amount in degrees by which angles 1 & 2 in mutants exceed angles 1 & 2 in wildtype embryos is plotted on the y-axis. Black bars, angle 1; gray bars, angle 2. **(f)** In the chiasm of wildtype embryos, a subset of RGC axons (*, shown as a negative image to highlight axons) crosses the midline posterior to the main RGC axon bundle. The inset shows the outline of the main bundle in the chiasm (dark gray) and the red box indicates the approximate region from which the image in panel f was taken. In many cases of netrin-1 or DCC mutant embryos, RGC axons crossed the midline at a point that correlates with this posterior region. **(g)** DiI labeled RGC axons at the ventral hypothalamus of E14 wildtype littermates (red) and Pax6 *Sey*$^{neu}$/+ heterozygous embryos (green). Although optic nerve size in *Sey*$^{neu}$/+ heterozygous embryos is reduced, abnormally angular RGC axon trajectories were not observed. **(h)** Graph showing that angles 1 and 2 in *Sey*$^{neu}$/+ heterozygous animals were very similar to those present in wildtype animals. Scale bars: a-c, g, 250 μm; f, 100 μm.

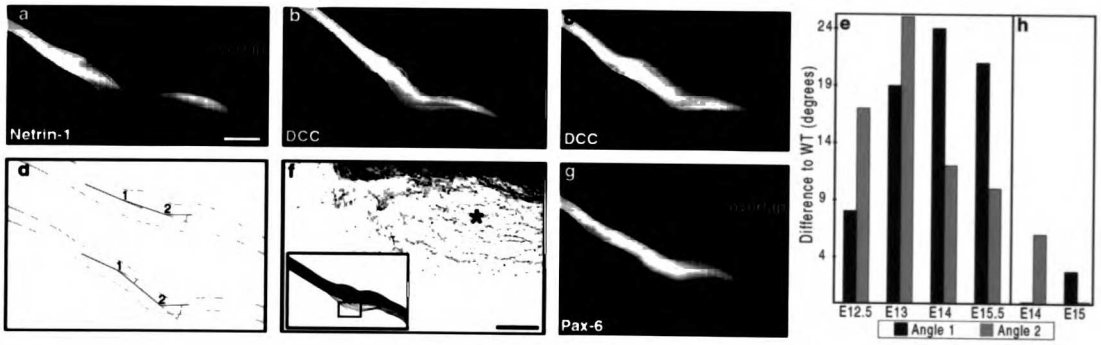
a
Netrin-1
b
DCC
c
DCC
d
1
2
1
2
f
*
g
Pax-6
e
h
24
19
14
9
4
Difference to WT (degrees)
E12.5
E13
E14
E15.5
E14
E15
Angle 1
Angle 2

**Figure 3-3**

**Fig. 3-4: Chiasm neuron development in DCC or Netrin-1 deficient mouse embryos. (a, b)** E12 hypothalamic wholemount preparations showing CD44 immunostaining of the inverted v-shaped array of chiasm neurons (arrowheads) in heterozygous (a) and netrin-1 mutant (b) littermates. **(c, d)** CD44 immunostaining of E12.5 coronal sections in wildtype (c) and netrin-1 mutant littermates (d) showing CD44 positive neuron cell bodies ventromedially (asterisks) and their axons (arrowheads) which project dorsolaterally. **(e, f)** DiI labeled RGC axons and CD44 labeled chiasm neurons in midsagittal sections of E14 wildtype (e) and DCC mutant littermates (f). In both, the RGC axons appear as red bundles while the labeled CD44 neurons appear as a light brown curve-shaped group (black arrows). In panels e and f, sections through the posterior (*) and anterior (**) pituitary are visible on the right side of the panels. Very dark black cells are blood cells. In wild-type animals, the distance between the posterior edge of the CD44 neurons and the anterior edge of the pituitary was somewhat variable. A similar degree of variability was seen in the mutants. **(g,h)** Tracings showing the relationship between the posterior portion of the RGC axon bundles and the CD44 neuron array in midline sagittal sections of several wildtype (g) and DCC deficient embryos (h). RGC axons in DCC mutants were found in approximately the same position and did not appear closer to CD44 neurons compared to wild-type. Abbreviations: A, anterior; D, dorsal; Scale bars: a-f, 200 μm.

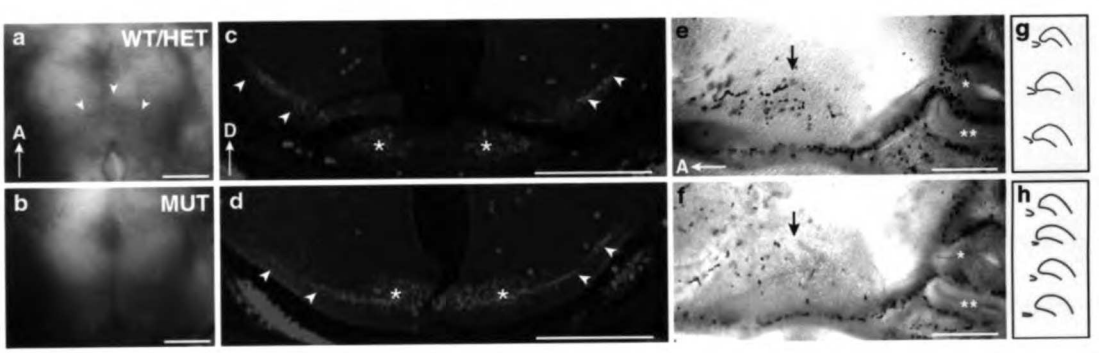


**Figure 3-4**

**Fig. 3-5: GnRH neurons axon pathways in wildtype and DCC or Netrin-1 deficient P0 littermates (coronal sections). (a-h)** Wildtype newborn animals (left 2 columns) and their mutant littermates (right 2 columns). **(a, b)** Examples of GnRH axons in the OVLT of wildtype littermates from a DCC (panel a) and Netrin-1 (panel b) litter. **(c, d)** Examples of GnRH axons in the ME regions of the same wildtype animals shown in panels a and b, respectively. **(e, f)** Examples of GnRH axons in the OVLT of DCC (e) and netrin-1 (f) deficient animals. The number of OVLT GnRH axons in DCC deficient animals appears quite similar to wildtype (compare to panels a and b). The number of OVLT GnRH axons in netrin-1 deficient animals appears reduced compared to wildtype (compare to panels a and b). **(g, h)** Examples of GnRH axon projections in the ME regions of DCC (g) and netrin-1 (h) deficient animals (same animals as shown in panels e and f, respectively). The GnRH axon projections are severely reduced compared to wildtype but terminate in the appropriate lateral ME contact zone regions (see c, d). **(j, k)** Unlike GnRH axons, axons of CRH positive neurons in wildtype (j) and mutant (k) appear to grow equally well into the medial ME contact zone (panel k is same animal as in panel g). **(l, m)** Axons of ADH positive neurons, which also grow through the ME internal zone to the infundibulum appeared similar in wildtype (l) compared to DCC mutant (m) infundibulum. **(n, o)** Compared to wildtype (n), GnRH positive axon projections were not reduced in an E18 GAP-43 deficient littermate (o). RGC axon growth in the chiasm is severely disrupted in GAP-43 deficient embryos. In all panels, dorsal is at the top. Abbreviations: OVLT, organum vasculosum of the lamina terminalis; ME, median eminence; Inf, infundibulum; G43, GAP-43. Scale bars: a,b,e,f are 50 μm; c,d,g,h are 200 μm; j-o, 200 μm.

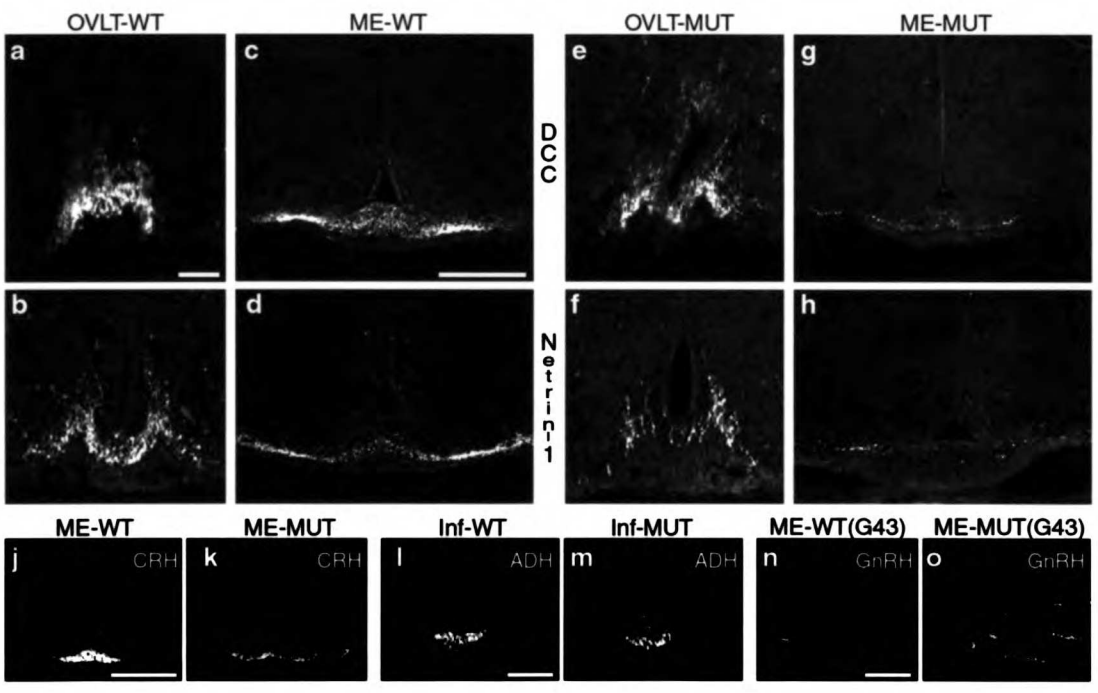
OVLT-WT
ME-WT
OVLT-MUT
ME-MUT
a
c
e
g
DCC
b
d
f
h
Netrin-1
ME-WT
ME-MUT
Inf-WT
Inf-MUT
ME-WT(G43)
ME-MUT(G43)
j CRH
k CRH
l ADH
m ADH
n GnRH
o GnRH

**Figure 3-5**

**Fig. 3-6: Ectopic ADH and oxytocin neurons in DCC or Netrin-1 deficient P0 littermates (coronal sections). (a)** In a typical P0 wildtype animal, ADH positive cells are confined laterally in the SON (arrows). **(b)** In a DCC mutant littermate, ADH positive neurons are found within the SON region (arrows) and ectopically in a trail (arrowheads) extending from the SON to the ventral midline. **(c)** Under higher magnification, ectopic ADH neurons (arrowheads) have leading and trailing processes reminiscent of migrating neurons. **(d)** Ectopic ADH neurons are found as bilateral cell clusters centered about the midline (arrow) in the region anterior to the chiasm. **(e, f)** The position of ADH positive neurons (red) in individual coronal sections from wildtype (e) and DCC mutant (f) littermates were traced and tracings from serial sections were stacked to form 3-D reconstructions. The 3-D reconstructions show the ventral surface of the brain as if the observer is viewing from the pituitary (posterior) towards the chiasm (anterior). The asterisk marks the location of ectopic neurons with migrating profiles (shown in panel c). The arrow points to the area containing ADH neuronal clusters at the midline (shown in panel d). **(g)** Ectopic ADH neuron clusters are also found at the midline (arrow) in the region anterior to the chiasm in netrin-1 mutants. **(h, i)** In contrast to a wildtype littermate (h), in the mutant (i) ectopic oxytocin neurons are found in the region anterior to the chiasm and form bilateral clusters at the midline (arrow). The arrowhead points to the optic nerve, just entering the brain. **(j)** Higher magnification view of ectopic oxytocin neuron clusters in the region anterior to the chiasm in a DCC mutant (arrow indicates midline). **(k, m)** The approximate number and distribution of ADH neurons in the PVN appeared similar in wildtype (k) and mutant (m) littermates. **(l, n)** The approximate number and distribution of oxytocin neurons in the PVN appeared

similar in wildtype (l) and mutant (n) littermates. **(o, p)** ADH positive neurons are normally restricted to the SON in E18 wildtype (o) or GAP-43 deficient (p) embryos even though RGC axon growth into the optic tracts in these mutant embryos is severely reduced. In all panels (except e, f) dorsal is at the top. Abbreviations: Ant., anterior; D, dorsal; Post., posterior; G43, GAP-43. Scale bars: a-b, 500 μm; c, 50 μm; d,g,j , 25 μm; h-i, 250 μm; k-n, 250 μm;. o-p, 250 μm.

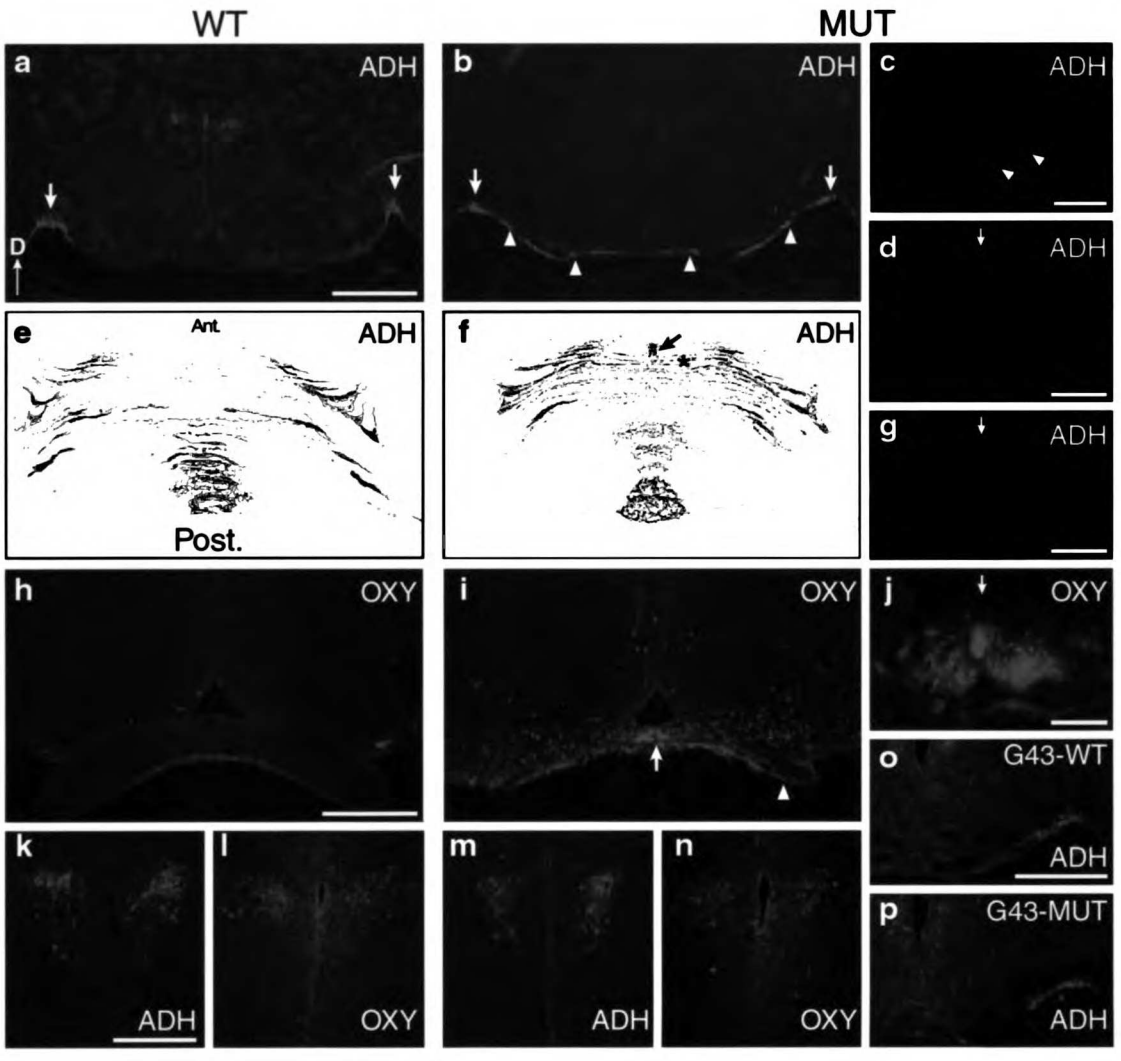
WT
MUT
a
ADH
D
b
ADH
c
ADH
d
ADH
e
Ant.
ADH
Post.
f
ADH
g
ADH
h
OXY
i
OXY
j
OXY
o
G43-WT
ADH
p
G43-MUT
ADH
k
ADH
l
OXY
m
ADH
n
OXY

**Figure 3-6**

**Fig. 3-7: Pituitary development in DCC or Netrin-1 deficient mice. (a-d)** Coronal sections of the pituitary gland at P0. The overall size and shape of the pituitary is not different between wildtype (left) and mutant animals (right). Mutants have normal organization of the posterior pituitary, intermediate lobe, pituitary cleft and anterior pituitary. Scale for a-d, 200 μm. **(a)** ACTH positive neurons in the intermediate lobe of a wildtype animal. **(b)** ACTH neurons are found in a similar pattern in a netrin-1 deficient littermate. **(c)** GH positive neurons are located in anterior pituitary in wildtype animals. **(d)** GH positive neurons are found in a similar pattern in a netrin-1 deficient littermate. **(e)** Plasma ACTH levels in P0 animals. Values (ng/L) were obtained for animals from three different DCC litters, and the values obtained for animals of each genotype were pooled. The numbers in parentheses indicate the number of animals of each genotype. Older ages were not examined as mutants die postnatally. Error bars indicate S.E.M. **(f)** Plasma GH levels in P0 DCC litters. Values (expressed as cpm) were obtained for animals from three different DCC litters, and the values for animals of each genotype were pooled. Abbreviations: PP, posterior pituitary; IL, intermediate lobe; PC, pituitary cleft; AP, anterior pituitary; ACTH, adrenocorticotropic hormone; GH, growth hormone.

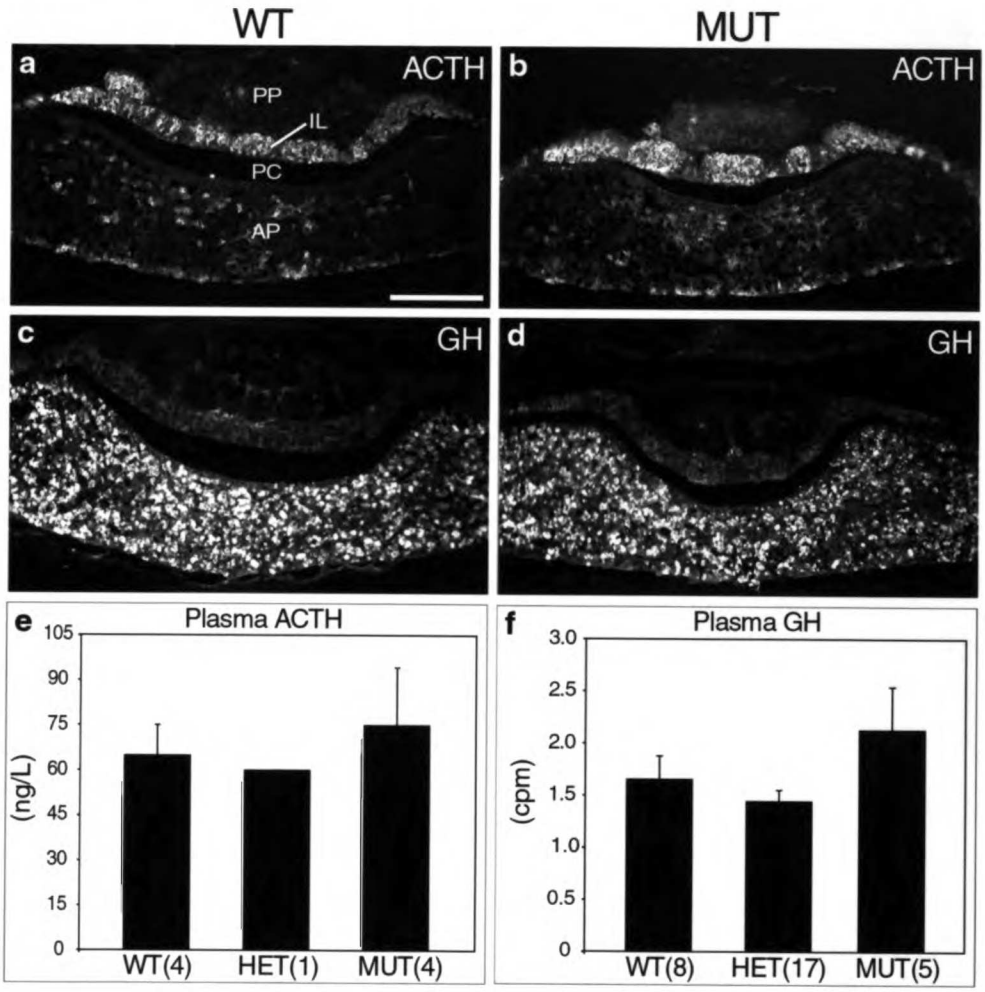
WT
MUT
a
ACTH
PP
IL
PC
AP
b
ACTH
c
GH
d
GH
e
Plasma ACTH
(ng/L)
105
90
75
60
45
30
15
0
WT(4)
HET(1)
MUT(4)
f
Plasma GH
(cpm)
3.0
2.5
2.0
1.5
1.0
0.5
0
WT(8)
HET(17)
MUT(5)

**Figure 3-7**

**Fig. 3-8 : Summary of defects in RGC axon trajectories and in development of GnRH, ADH, and oxytocin neurons in DCC or Netrin-1 deficient animals.**

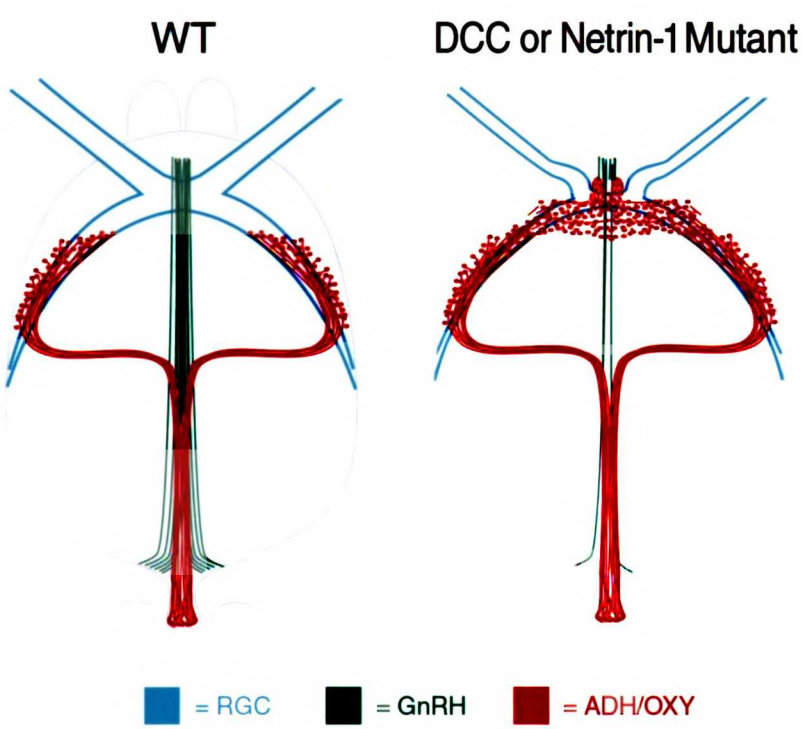


**Figure 3-8**

# Chapter 4

## Future Directions: Analysis of Gene Mutations in Patients with Septo-optic Dysplasia

ABSTRACT

In humans, optic nerve hypoplasia is characterized by a reduced number of retinal ganglion cell (RGC) axons in the nerve in the setting of otherwise normal eye development. Despite the fact that optic nerve hypoplasia often severely compromises vision and is thought to be the most commonly encountered optic disc anomaly, little is known about the genetic defects underlying this disorder (Brodsky et al., 1996). The optic nerve joins the brain in a region called the hypothalamus which, along with RGC axons, consists of the neuroendocrine system neurons and is crucial for regulating a large number of autonomic physiologic processes. Developmental disorders of the neuroendocrine hypothalamus exist in humans and the genetic defects underlying many of these disorders are not known. Ours and previous studies have found that mice missing netrin-1 or DCC genes have optic nerve hypoplasia, neuroendocrine system abnormalities and absent or missing commissures including the corpus callosum. This trio of seemingly disparate phenotypes is reminiscent of the human congenital multisystem syndrome of Septo-Optic Dysplasia (SOD) which consists of optic nerve hypoplasia associated with neuroendocrine and corpus callosum abnormalities (Skarf and Hoyt, 1984; Brodsky et al., 1996). A recent study reported that one sibling pair out of a larger group of SOD patients being tested had a mutation in the human homeobox gene Hesx1 (Dattani et al., 1998), suggesting mutations in other genes may also be linked to certain cases of SOD. In the study described here, we test the hypothesis that netrin-1 or DCC might play guidance roles in humans by assaying for potential mutations in netrin-1 or DCC genes in patients diagnosed with SOD.

## INTRODUCTION

In development, one of the earliest pathfinding tasks RGC axons must accomplish is to extend towards and then grow through the optic disc, exiting the eye into the optic nerve. In chapter 2 of my thesis I demonstrated that in embryonic mouse eye development, netrin-1 at the disc contributes to the critical task of guiding RGC axons out of the eye into the optic nerve, and RGC axons respond to netrin using the netrin receptor, DCC. In both netrin-1 and DCC deficient mouse embryos generated by targeted mutagenesis, RGC axon guidance up to the optic disc was unaffected. However, axons arriving at the disc failed to exit into the optic nerve, giving rise to optic nerve hypoplasia.

Human optic nerve hypoplasia is the most common optic disc anomoly encountered in ophthalmologic practice and is thought to be a significant cause of congenital blindness in children (Brodsky et al., 1996). Generally, it is thought that optic nerve hypoplasia in patients results from either the failure of RGC differentiation during development resulting in fewer axons, or abnormal developmental events at visual targets in the CNS causing a secondary dying back of RGC axons which initially exited the optic disc into the optic nerve (reviewed in Brodsky et al., 1996). Our findings suggest that in mouse, a third cause of optic nerve hypoplasia is a developmental defect in RGC axon guidance out of the eye, reducing the number of axons in the nerve. It is not known whether similar axon guidance defects might also cause optic nerve hypoplasia in humans. Of note, a significant number of optic nerve hypoplasia cases in humans occur in the setting of septo-optic dysplasia (SOD, or De Morsier's syndrome), a clinical syndrome comprised of optic nerve hypoplasia and partial

or total agenesis of the corpus callosum that can be associated with an absent septum pellucidum and neuroendocrine abnormalities (Hoyt et al., 1970; Skarf and Hoyt, 1984).

In chapter 3 of my thesis I demonstrated that netrin and DCC also play a role in governing normal mouse neuroendocrine hypothalamic development. The combination of neuroendocrine developmental abnormalities (chapter 3 above), optic nerve hypoplasia (Deiner et al., 1997) and chapter 2 above), and absent corpus callosum (Serafini et al., 1996; Fazeli et al., 1997) found in both the netrin-1 deficient and the DCC null mice is therefore remarkably similar to the symptoms found in SOD. This suggested the possibility that netrin-1 or DCC genes might be mutated in certain cases of SOD.

Although most cases of SOD have been described in the literature as largely sporadic in nature (Harris and Haas, 1972; Brodsky et al., 1996), a recent study showed that in one sibling pair, SOD was caused by a mutation in the regulatory gene, Hesx1 (Dattani et al., 1998). Findings from that study also suggested that SOD is heterogenous in origin and since mutations in Hesx1 were not found in the vast majority of SOD patients, mutations in other genes probably underlie different cases of SOD. Mice missing Hesx1 had a phenotypic combination that was remarkably similar to, but more severe than that observed in DCC or netrin-1 mutant mice. As a transcription factor located in regions of netrin and DCC expression, Hesx1 could potentially directly regulate netrin-1 or DCC expression such that altered Hesx1 function could therefore alter netrin-1 or DCC expression. Taken altogether, the netrin-1, DCC and Hesx1 studies suggested it was worth examining if human netrin-1 or DCC genes were mutated in certain cases of SOD.

To do so, I set up a collaborative study with clinicians in the pediatric ophthalmology and endocrinology departments at UCSF to test the hypothesis that alterations in the genes encoding netrin-1 or DCC might underlie SOD in humans. The study was also established to further investigate the frequency by which SOD results from Hesx1 gene mutations. A proposal for this study has been approved by the UCSF committee on human research (CHR approval #H80041428401).

## METHODS

Methods involve the collection of peripheral blood samples, isolation of genomic DNA, Southern DNA blots to detect gene deletions or re-arrangements, and Single Strand Conformational Polymorphism (SSCP) (Orita et al., 1989) or direct sequencing analysis to identify single base pair mutations.

**Patient recruitment and inclusion criteria:**

The patient population being screened presently consists of roughly 50 patients from the pediatric ophthalmology and endocrinology services at UCSF. The two target patient populations consist of patients with optic nerve hypoplasia alone and SOD patients diagnosed with optic nerve hypoplasia, neuroendocrine abnormalities and agenesis of the corpus callosum, a combination of symptoms which most closely resembles the known phenotypes in mice with targeted mutations in the netrin-1, DCC and Hesx1 genes.

**Genomic DNA isolation:**

A peripheral blood sample (2-3 ml) was obtained from patients at the blood draw service of the UCSF medical center. Genomic DNA was isolated by lysis of white blood cell nuclei followed by high salt precipitation using the Genomic DNA Wizard kit (Stratagene). Blood samples and DNA were stored at -20°C until use. Approximately 5 ug were used for each restriction digest and Southern blot analysis and 50-100 ng were used for each PCR reaction.

**cDNA probes and PCR primers:**

The cDNA probe for netrin-1 was obtained from Exelixis Inc. and made available to this project by a Materials Transfer Agreement with UCSF. The cDNA probe for DCC was obtained from Dr. Eric Fearon. Primers, (based on published primer sequences or designed based on sequences of netrin-1, DCC and Hesx1 genes) were ordered from Gibco/BRL and conditions were worked out to generate PCR products ranging from 200-350 bp, the ideal fragment sizes for fluorescence SSCP analysis (see below) and/or direct sequencing. For SSCP, fluorescently labeled primers were also purchased through Gibco/BRL.

**Screening for genetic defects:**

Three methods were used to look for mutations in the genes encoding netrin-1 DCC and Hesx1 in the study patient population: (1) To analyze for large gene deletions or rearrangements, Southern blots using a defined set of restriction enzymes and radiolabeled cDNA probes were used. To analyze for single base pair changes, (2) SSCP or (3) direct genomic sequencing was used. All methods

are considered standard techniques for the investigation of genetic mutations in known genes.

Direct sequencing of PCR products was carried out at the Beckman Vision Center core DNA sequencing facility using an ABI sequencing apparatus. SSCP analysis was conducted in two steps. The first involved PCR of 200-350 bp fragments using fluorescently labeled PCR primer pairs. The PCR products were then sent to the Genome Analysis Center at the Cancer Research Institute Mt. Zion Campus and subjected to SSCP analysis. SSCP at the Genome Analysis Center was carried out using an ABI sequencing apparatus which allows a significant reduction in gel running time as well as more sensitive and reproducible DNA running patterns through the use of fluorescently labeled probes.

## CURRENT RESULTS

A report of my current progress, analysis and findings for this project is given below and in table 4-1. So far, 46 patient DNA samples have been successfully obtained from patient blood samples. DCC genomic sequence has been examined for possible gross rearrangements or deletions using southern blot for 20 patients, and 26 of the 29 exons in DCC have been examined for possible small point mutations using SSCP for 25 patients. Netrin-1 genomic sequence has been examined for possible gross rearrangements or deletions using southern blot for 20 patients and 75% of exon 1 has been processed for examination of possible small point mutations using direct DNA sequencing for 34 patients. Hesx1 has been processed for examination of possible small point

mutations using direct sequencing for 1 of the 4 Hesx1 exons for 34 patients. Although no gross rearrangements or mutations have been found so far, for several patients we have found new polymorphic markers (silent mutations) of DCC. The lab plans to continue analysis of patient DNA for mutations and to generate working primers for PCR-sequencing on the two remaining DCC exons, one remaining netrin-1 exon and three remaining Hesx1 exons.

## FUTURE PLANS FOR STUDY AND POTENTIAL SIGNIFICANCE

This project came about as a result of findings from my thesis work and from previous publications. Although I have provided a first step in this project by setting up and gaining approval for the study, obtaining patient DNA, designing protocols for mutation analysis and beginning mutation analysis for some patients, the work will continue to be carried out by members of the lab as an ongoing project as more samples are obtained for analysis. In the future, continued sequence analysis of patient DNA samples, looking for possible netrin-1, DCC and Hesx1 mutations, will allow us a clearer understanding of whether mutations in these genes are or are not commonly linked to optic nerve and neuroendocrine disorders in humans, including SOD.

My thesis work has contributed to a better understanding of which cues are used for guiding RGC axons in the early developing visual system as well as for guiding neurons of the neuroendocrine hypothalamus. *In vivo* in mice generated by homologous recombination, complete removal of Netrin-1 or DCC leads to altered optic nerve and neuroendocrine formation due to guidance and neuron positioning errors, presumably through loss of direct netrin-1 and DCC

interactions. Although these findings suggest netrin-1 and DCC should now be considered significant players in neuronal development of both visual and neuroendocrine systems, studies in knockout mice do not contribute to an understanding of the specific domains of the netrin-1 or DCC proteins that are functionally important for axon guidance in these systems. Our hope is that, if detected in humans with SOD, small mutations in netrin-1 and DCC genes will help identify important functional regions on these molecule. This in turn will help to uncover the basic biology of how these guidance molecules and their receptors work at a molecular level.

My thesis work has also shown that netrin-1 and DCC, along with other previously reported cues, are still only part of the whole picture of how RGC axons are guided within the eye and brain and of how neuroendocrine neurons are guided within the hypothalamus during development. In other words, not all RGC axons require netrin-1 and DCC interactions to exit the eye and not all neuroendocrine neurons require netrin-1 and DCC to develop proper connections. Hopefully, future studies *in vitro* and in animal models will identify other cues and mechanisms which are involved and how all of these cues work together to achieve the ultimate goal of properly wired and functional visual and neuroendocrine systems. As more is known from basic science laboratory work about these other guidance cues, we may be able to screen for mutations in these genes as other potential causes of congenital optic nerve and neuroendocrine disorders in human SOD patients.

**Table 4-1: Methods and current results of analysis for gene mutations in 46 Patients with SOD**

| | | DCC | Netrin-1 | Hesx1 |
|---|---|---|---|---|
| Southern Blot | # tested | 20 patients | 20 patients | ND |
| | Results of Analysis | normal | normal | NA |
| SSCP | # tested | 26 of 29 exons, 25 patients | ND | ND |
| | Results of Analysis | 3 silent mutations | NA | NA |
| Sequencing | # sequenced | ND | 1 of 2 exons, 34 patients | 1 of 4 exons, 34 patients |
| | Results of Analysis | NA | NC | NC |

**ND** (not done yet ), **NA** (not applicable), **NC** (not completely analyzed yet)

# Chapter 5

# Thesis Overview and Concluding Remarks

## Overview

The results from my thesis work have expanded our understanding of the roles of an axon guidance molecule netrin-1 and of its receptor DCC for proper vertebrate nervous system development. Now, along with their known role in midline commissural axon guidance (Kennedy et al., 1994; Keino et al., 1996; Serafini et al., 1996; Fazeli et al., 1997), my studies have demonstrated that netrin-1 acting through DCC can provide guidance in non-commissural regions of the developing visual and neuroendocrine systems. Furthermore, the results also suggest that the mechanisms of how netrin-1 and DCC interact to provide guidance may vary between these different systems. In addition, my thesis findings help to provide a more complete picture of the cascade of guidance cues which directs RGC axon growth along the proper route from the eye to the brain to form this major sensory pathway, the visual system. This work also represents one of the first attempts to elucidate the role of any specific axon guidance cue for proper neuroendocrine hypothalamus development. Finally, results from this thesis demonstrate for the first time that optic nerve hypoplasia and hypothalamic neuroendocrine system disorders in mice can be caused by deficient axon guidance cue function during development; pointing to the possibility that axon guidance defects may also be responsible for similar birth defects in humans. In the remainder of this chapter, all of these key points are explored in more detail.

## DCC and netrin-1: multiple guidance roles and mechanisms

In the vertebrate nervous system, netrin-1's function as an axon guidance cue is probably best understood in terms of development of commissural axon

projections from dorsal spinal cord to the ventral midline floor plate. The current view, based on both *in vitro* and *in vivo* studies suggests that a gradient of netrin protein with peak levels of expression at the ventral midline floor plate acts as a *long range diffusible* cue to attract commissural axons via DCC towards the ventral midline (Kennedy et al., 1994; Keino et al., 1996; Serafini et al., 1996; Fazeli et al., 1997). In contrast, results from this thesis suggest that in the embryonic mouse eye, netrin-1 may guide axons through a relatively *short range* guidance mechanism since the limited expression of netrin-1 protein and the altered trajectories of RGC axons in netrin-1 or DCC-deficient animals are consistent with a role for netrin-1 in guiding RGC axons in the immediate vicinity of the optic disc. Similar phenotypes in both netrin-1 and DCC mutants suggests this short range guidance is via direct netrin-1 and DCC interactions.

In the hypothalamus, unlike in the spinal cord, netrin-1 is not expressed at the ventral midline and in the absence of netrin-1 or DCC, commissural (RGC) axons follow an abnormally angular route but are still able to grow towards and across the ventral midline. These results suggest the contribution of netrin-1 and DCC interactions to commissure formation differs in the optic chiasm vs. in the spinal cord. Furthermore, in the hypothalamus, netrin-1 and DCC interactions appear to be involved in formation of posteriorly directed ventral midline axon projections (GnRH neurons) and in lateral positioning of neuronal cell bodies (ADH and oxytocin neurons), roles not yet demonstrated elsewhere in the vertebrate brain or in the developing spinal cord for these guidance cues.

Overall, these results indicate that diffusible cues such as netrin-1 can act at both long and short range. Furthermore, they demonstrate that guidance towards or away from a ventral midline source is just one of a number of different locales where netrin-1 and DCC interactions direct axon growth and cell migration in the developing nervous system.

**A cascade of guidance cues in the visual system**

A number of studies have shown that individual axon guidance cue molecules can, in general, either be permissive or non-permissive substrates for axon growth or they can be attractive or repulsive to growing axons (reviewed in Tessier-Lavigne and Goodman, 1996). In most of these studies, the effect of a single guidance cue molecule on one particular type of neuron has been investigated. In the developing brain though, the situation is probably much more complex and a number of different cues are most likely involved, often simultaneously, in guiding a particular growing axon. A current issue in the field of axon guidance is therefore to understand how a number of different cues using different guidance mechanisms can function together to guide any specific growing axon all along it's pathway from the cell body to the target. A necessary step towards this goal is to first define the molecular road map of guidance cues in an experimentally accessible axon pathway. In this light, my results have contributed to a growing body of knowledge of how a number of different cues, now shown in this thesis to include netrin-1 and DCC, act in concert to guide RGC axons along the mouse visual system pathway from the eye to the brain.

My results also imply that there are still unknown cues that must also help guide RGC axons both within the eye as well as in the brain. Future studies may be able to further elucidate the nature of these cues by investigating components of the RGC axon pathway that appear independent of known cues. For example, some RGC axons are still able to exit the eye through the optic disc into the nerve in the absence of netrin-1 or DCC, suggesting some guidance information remains in the discs of netrin-1 or DCC mutants. Similarly, RGC axons still cross the ventral hypothalamus midline to enter the optic tracts in netrin-1 or DCC mutants suggesting other cues must guide RGC axons across the midline.

Future studies can also now begin to address how receptor ligand interactions of the many already-known cues, including netrin-1 interacting with DCC, lead to the appropriate intracellular signaling cascades to elicit the correct RGC axon growth cone behaviors for guidance. Furthermore, studies can now be geared towards understanding how the RGC axonal growth cone is able to properly integrate and interpret these many signaling cascades, sometimes simultaneously, to allow for correct guidance all along the route to final targets. Knowing more about these aspects of RGC axon guidance may also help in understanding how other critical CNS system axons are guided by potentially similar cascades of multiple cues during development and may ultimately be of therapeutic use in future approaches to repair these pathways if damaged.

**Axon guidance cues in hypothalamus development**

Previous to this thesis work, despite the critical importance of the hypothalamus in neuroendocrine and autonomic function, little was known of the developmental mechanisms that give rise to the complex patterns of neuronal connectivity in this anterior CNS region. Results from this thesis provide some initial insight into this issue and demonstrate that proper formation of GnRH neuron axonal projections and proper positioning of ADH and oxytocin neurons in the developing hypothalamus requires the guidance molecule netrin-1 and its receptor DCC. One area for future study is to now experimentally investigate the mechanisms of how netrin-1 and DCC interactions are required. As my results are just the first example of a role for guidance cues, another future goal is simply to investigate whether other guidance cues are also involved in guiding migrating axons and cells in the developing hypothalamus and to determine what they are.

**Relationship of guidance cues to disorders of optic nerve and hypothalamus**

In the adult human, acquired disorders affecting the hypothalamus often involve the visual system due to the location of the chiasm on the ventral hypothalamic surface (for review see Siatkowski and Glaser, 1995). This thesis shows that development of the visual and hypothalamic neuroendocrine systems in mouse are also linked via shared guidance molecules and suggests that mutations affecting guidance molecules could lead to congenital disorders affecting both vision and neuroendocrine function in humans. The occurrence of hypothalamic abnormalities, optic nerve hypoplasia and an absent corpus callosum (Serafini et al., 1996; Fazeli et al., 1997) in the netrin-1 or DCC deficient mice is phenotypically similar to the human congenital disorder of Septo-Optic Dysplasia (SOD) (Hoyt et al., 1970; Skarf and Hoyt, 1984). This raises the possibility that SOD may be linked to mutations in developmental genes such as netrin-1 or DCC. Our ongoing research looking for potential mutations in netrin-1 or DCC will reveal whether this possibility is a likely one or not. In the future, studies *in vitro* and *in vivo* may also identify other guidance cues and mechanisms which are required during development to achieve the ultimate goal of properly wired and functional visual and neuroendocrine systems. As more is known from basic science laboratory work about these other guidance cues, we may be able to screen for mutations in these genes along with netrin-1 and DCC as potential causes of congenital optic nerve and neuroendocrine disorders in human SOD patients.

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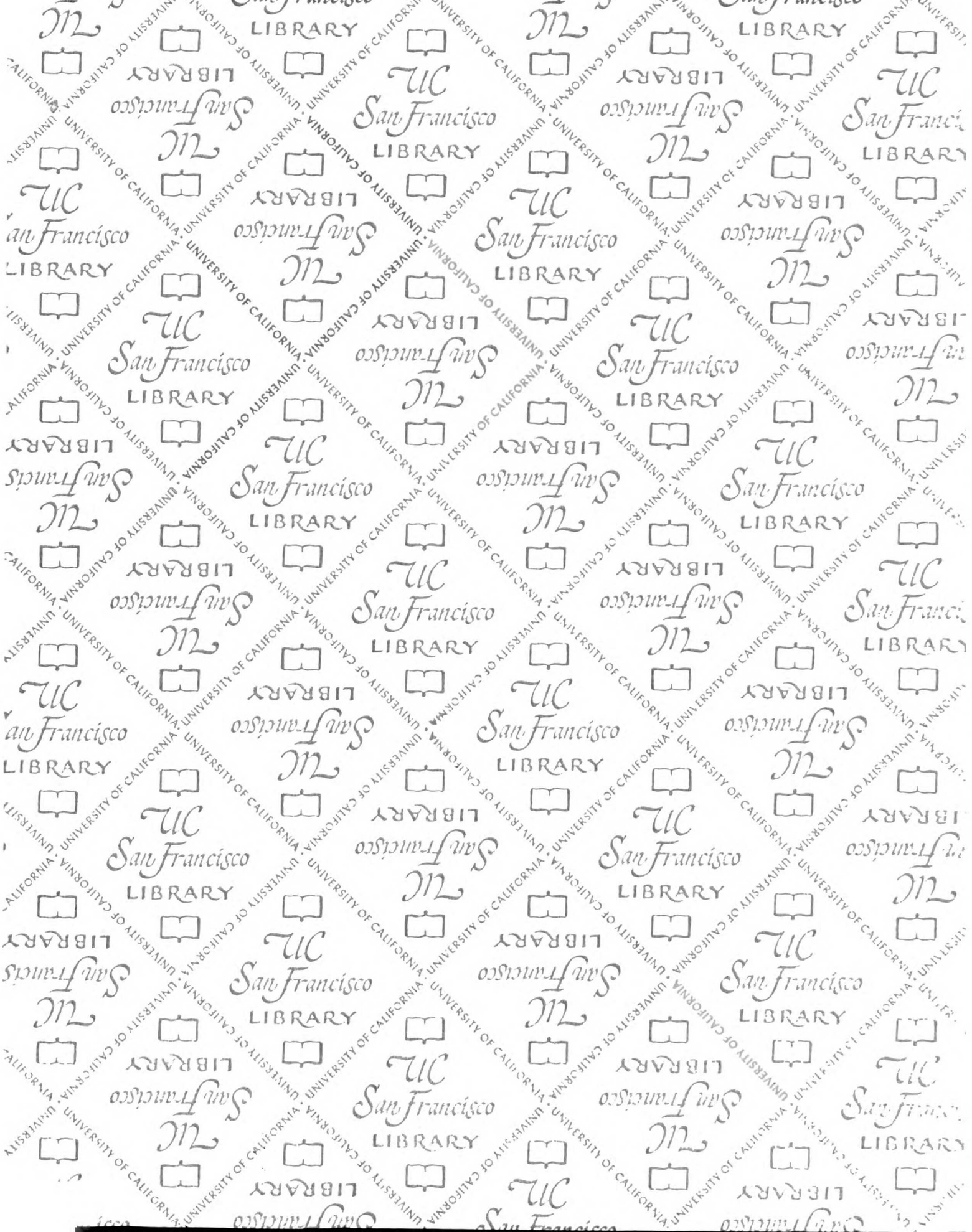
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