Netrin and netrin receptor function in glial motility and myelination

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ABSTRACT

Netrin-1 and its receptors play crucial roles during embryogenesis, guiding axon and neuronal cell migration. Here, roles of netrins and their receptors in glial function were investigated. In the embryonic spinal cord, netrin-1 expressed at the ventral midline orients axon extension. Spinal oligodendrocyte precursor (OP) cells are born close to, and migrate away from, the ventral midline. We find that OPs express DCC and UNC5 netrin receptors and, in an *in vitro* microchemotaxis assay, are repelled by a netrin-1 gradient. In the absence of netrin-1 or DCC function *in vivo*, fewer OPs migrate from the ventral to the dorsal embryonic spinal cord, consistent with netrin-1 acting as a repellent guidance cue for these cells.

In the adult CNS, oligodendrocytes continue to express DCC and UNC5 receptors, and upregulate netrin-1 expression. Our findings indicate that netrin-1 and its receptors are localized to paranodal axo-glial junctions, specialized cell-cell adhesions between non-compact myelin loops and axons. In myelinating cerebellar slice cultures derived from neonatal $DCC^{-/-}$ and $netrin\text{-}1^{-/-}$ mice, paranodes develop and mature normally but later become disorganized, resulting in loss of domain segregation at the nodal region. These data suggest that netrin-1 and DCC are essential for the maintenance of paranodal junctions, and may be indicative of a wider role in mediating cell-cell contacts in the adult.

Netrin-1, DCC, and UNC5 homologues have also been identified as putative tumor suppressors, and their expression is downregulated in many cancers, including glial tumors. In our studies, netrins were found to act as autocrine factors that restrain human glioblastoma cell migration, slowing cell movement and inhibiting the formation of focal contacts associated with lamellipodial protrusion and membrane extension. DCC and UNC5 homologues have previously been proposed to inhibit tumorigenesis by inducing cell death when unbound by netrin. However, we found no evidence of increased cell death in the absence of netrin function in oligodendrocyte precursors, oligodendrocytes or glioma cells. Instead, we find that netrins act as long-range guidance cues during glial precursor migration during development, while acting at short distances to stabilize cell-

cell and cell-matrix interactions of mature glia and glial tumor cells, maintaining tissue organization and preventing inappropriate cell motility.

RÉSUMÉ

Nétrine-1 et ses récepteurs jouent des rôles cruciaux durant le développement neuronal, guidant la migration des axones et des neurones. Cette thèse investigue les différents rôles des nétrines et de leurs récepteurs dans la fonction gliale. Dans le cordon médullaire embryonnaire, nétrine-1 exprimée au niveau de la linge médiane ventrale et oriente l'extension des axones. Des cellules spinales précurseurs d'oligodendrocytes (POs), naissent près de la lingne médiane ventrale et s'en eloignant durant le développement neuronal. Nos résultats démontrent que les POs expriment les récepteurs de nétrine DCC et UNC5 et que, suite à une analyse de migration *in vitro*, sont repoussés par un gradient de nétrine-1. *In vivo*, l'absence de fonction de nétrine-1 ou de DCC résulte en une diminution de la migration des POs des régions ventrales aux régions dorsales du cordon médullaire embryonnaire, confirmant ainsi que nétrine-1 agit en tant que signal répulsif pour ces cellules.

Dans le système nerveux central de l'adulte, les oligodendrocytes continuent à exprimer les récepteurs DCC et UNC5, et commencent à exprimer nétrine-1. Nos résultats indiquent que nétrine-1 et ses récepteurs sont localisés aux jonctions paranodales, qui sont des adhérences spécialisées entre les axones et les boucles de myéline non-compactes des oligodendrocytes. Dans les cultures cérébelleuses organotypiques myélinisées dérivées de souris néonatales $DCC^{-/-}$ ou nétrine-$1^{-/-}$, les jonctions paranodales se développent et maturent normalement. Cependant, elles se désorganisent rapidement, ayant pour résultat la perte de ségrégation fonctionnelle des domaines dans la région nodale. Ces données démontrent que nétrine-1 et DCC sont essentiels à l'entretien des jonctions paranodales, et suggèrent un rôle plus vaste dans l'entretien général des contacts intercellulaires chez l'adulte.

Nétrine-1, DCC et les homologues d'UNC5 ont également été identifiés en tant que suppresseurs putatifs de tumeurs, et leur expression est diminuée dans nombreux de cancers, y compris les tumeurs gliales. Nos études ont démontré que nétrine agit en tant que facteur autocrine qui ralentit la migration des cellules de glioblastome humaines, ralentissant leur mouvement et empêchant la formation des contacts focaux associés avec la projection de lamellipodes et l'extension des membranes. Il a été précédemment proposé que les homologues DCC et UNC5 empêchent la formation de tumeurs en

induisant la mort cellulaire lorsque ces récepteurs ne sont pas liés à nétrine. Cependant, nous n'avons trouvé aucune évidence d'augmentation de mort cellulaire en l'absence de nétrines fonctionnelles chez les POs, les oligodendrocytes ou les cellules de gliome. Nous avons plutôt constaté que, durant le développement, les nétrines agissent à la fois comme signaux de longue portée durant la migration des précurseurs de cellules gliales, et comme signaux de courte portée pour stabiliser les interactions cellule-cellule et cellule-matrice des cellules gliales matures et de cellules gliales cancéreuses, contribuant ainsi au maintien de l'organisation tissulaire et prévenant une motilité cellulaire inappropriée.

LIST OF FIGURES AND TABLES

LIST OF ABBREVIATIONS

ACh	acetylcholine
ADH	anti-diuretic hormone
APC	adenomatous polyposis coli
Apaf1	apoptosis protease releasing factor-1
BSA	bovine serum albumin
cAMP	cyclic adenosine monophosphate
cGMP	cyclic guanosine monophosphate
cdc42	cell division cycle 42
CGE	caudal ganglionic eminence
CGT	ceramide galactosyl transferase
CNP	2',3'-cyclic nucleotide 3'-phosphodiesterase
CNS	central nervous system
CST	ceramide sulfotransferase
Cx	connexin
CXCL	cys-X-cys chemokine ligand
CXCR	cys-X-cys chemokine receptor
DAPK	death-associated protein kinase
DB	DCC-binding
DD	death domain
DCC	deleted in colorectal cancer
DCC_{FB}	DCC function-blocking antibody
DIG	detergent-insoluble glycosphingolipid-enriched complex
DIP13α	DCC-interacting protein 13α
DISC	death-induced signaling complex
DIV	days *in vitro*
DMEM	Dulbecco's modified Eagle medium
DNA	deoxyribonucleic acid
DRG	dorsal root ganglion
E(#)	embryonic day #
ECD	extracellular domain
ECM	extracellular matrix
ena/VASP	enabled/vasodilator-stimulated phosphoprotein
ERK	extracellular-signal regulated kinase
FA	focal adhesion
FAK	focal adhesion kinase
FC	focal complex
FERM	4.1/ezrin/radixin/moesin
FGF	fibroblast growth factor
HRP	horseradish peroxidase
GABA	gamma-aminobutyric acid
GAP	GTPase activating protein
GalC	galactocerebroside
GnRH	gonadotrophin releasing hormone
GEF	GTPase effector protein

GFP	green fluorescent protein
GPI	glycosyl phosphatidyl inositol
GROα	growth related oncogene α
GRP	glial-restricted precursor
GTP	guanosine triphosphate
HA	hemagglutinin
HGF/SF	hepatocyte growth factor/scatter factor
ICD	intracellular domain
ID	interloop densities
Ig	immunoglobulin
IP_3	inositol triphosphate
kDa	kiloDaltons
LGE	lateral ganglionic eminence
LHRH	leuteinizing hormone releasing hormone
LOH	loss of heterozygosity
LPA	lysophosphatidic acid
MAG	myelin-associated glycoprotein
MAL	myelin and lymphocyte protein
MAPK	mitogen-activated protein kinase
MAX-1	required for motor neuron axon guidance-1
MBP	myelin basic protein
MGE	medial ganglionic eminence
MS	multiple sclerosis
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N-WASP	neuronal Wiskott-Aldrich syndrome protein
NCAM	neural cell adhesion molecule
Net_{FB}	netrin function-blocking antibody
nfc	neurofascin
NFH	neurofilament 200 kDa
NFM	neurofilament 145 kDa
NGF	nerve growth factor
NGL-1	netrin-G1 ligand-1
NOR	node of Ranvier
NrCAM	neural-glial-related cell adhesion molecule
OLDEM	oligodendrocyte medium
OP	oligodendrocyte precursor
OSP	oligodendrocyte specific protein
P(#)	postnatal day #
p75NTR	p75 neurotrophin receptor
PAGE	polyacrylamide gel electrophoresis
PAK	p21-activated kinase
PDGF	platelet-derived growth factor
PDZ	PSD-95/Dlg/ZO1
PBS	phosphate-buffered saline
PFA	paraformaldehyde
PI3K	phosphatidylinositol 3-kinase

PICK1	protein interacting with c-kinase 1
PIP_2	phosphatidylinositol biphosphate
PITPα	phosphatidylinositol transfer protein α
PKA	protein kinase A
PKCα	protein kinase Cα
PLCγ	phospholipase Cγ
PLP	proteolipid protein
PNS	peripheral nervous system
PS	periaxonal space
PSA-NCAM	polysialic acid-neural cell adhesion molecule
PSD-95	95 kDa post-synaptic density protein
rac1	ras-related C3 botulinum toxin substrate 1
RGM	repulsive guidance molecule
rhoA	ras homologue A
robo	roundabout
ROCK	rho kinase
RT-PCR	reverse transcriptase polymerase chain reaction
SACMN	spinal accessory motor neuron
Scrb	scribble
SD	standard deviation
SEM	standard error of the mean
sema	semaphorin
SH2	src homology 2
shh	sonic hedgehog
tag1	transient axonal glycoprotein
TB	transverse band
TGF	transforming growth factor
TNF	tumor necrosis factor
TNFR	tumor necrosis factor receptor
TUNEL	terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling
UNC	uncoordinated
VEGF	vascular endothelial growth factor
ZU5	ZO-1 and UNC5-like

CONTRIBUTION OF AUTHORS

CHAPTER 1

Sections of the literature review (including Figure 1.3) were adapted from:

Jarjour, A.A. and Kennedy T.E. (2004). Oligodendrocyte precursors on the move: mechanisms directing migration. *Neuroscientist* 10(2): 99-105.

I wrote the manuscript in collaboration with T.E. Kennedy.

Contribution to figures:
A.A. Jarjour: Figures 1.1, 1.2, 1.3, 1.4, 1.5
S.W. Moore: Figures 1.1, 1.2

CHAPTER 2

Jarjour, A.A., Manitt C., Moore, S.W., Thompson K.M., Yuh, S., Kennedy, T.E. Netrin-1 is a chemorepellent for oligodendrocyte precursor cells in the embryonic spinal cord. *J. Neurosci.* 23(9): 3735-3744. Article reproduced with permission.

The rationale for this project was developed by T.E. Kennedy and me. I performed every experiment reported in this paper. In situ hybridization probes were prepared by C. Manitt. Recombinant netrin-1 was prepared and purified by S.W. Moore. Experiments carried out by K.M. Thompson and S. Yuh contributed to the rationale of this study; however, specific experimental results were not included in the final manuscript. I wrote the manuscript in collaboration with T.E. Kennedy.

Contribution to figures:
A.A. Jarjour: Figures 2.1, 2.2, 2.3, 2.4, 2.5, 2.6, 2.7; Tables 2.1, 2.2, 2.3

CHAPTER 3

Jarjour, A.A., Rajasekharan, S., Baker, K.A., Mui, J., Vali, H., Kennedy, T.E. Organization of paranodal junctions in CNS myelin requires DCC and netrin-1. Manuscript in preparation.

The rationale for this project was developed by T.E. Kennedy and me. All experiments were performed by me except the dissection, sectioning, and immunohistochemistry steps in the analysis of netrin and receptor expression at the paranode in adult rat spinal cord, which was performed by K.A. Baker. Preparation of tissue for electron microscopic analysis was performed by J. Mui of the Faculty for Electron Microscopy Research in the department of Anatomy and Cell Biology, McGill University. H. Vali provided guidance concerning electron microscopic analysis. I wrote the manuscript in collaboration with T.E. Kennedy.

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CHAPTER 4

Jarjour, A.A., Durko M., Luk, T.L., Shekarabi, M., Kennedy, T.E. Inhibition of Glioblastoma Cell Motility by Netrin: A Novel Autocrine Mechanism Regulating Tumor Cell Migration. Manuscript submitted to the Molecular Biology of the Cell.

The rationale for this project was developed by M. Durko, T.E. Kennedy, and me. All experiments were performed by me alone, with the exception of the characterization of netrin and netrin receptor mRNA and protein expression, which were performed in collaboration with M. Durko. Experiments carried out by T.L. Luk and M. Shekarabi contributed to the rationale of this study; however, specific experimental results were not included in the final manuscript. I wrote the manuscript in collaboration with T.E. Kennedy.

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CHAPTER 1: LITERATURE REVIEW

During embryonic development, members of the netrin family of guidance proteins are expressed in restricted regions of the embryonic central nervous system (CNS). Netrins direct the migration of neuronal precursor cells and their axons, and are critically important for the complex task of correctly wiring the mammalian brain. The vast majority of the cells in the CNS, however, are glia. Dubbed "nervenkitt" (meaning "nerve glue") by Rudolf Virchow, who believed that they were the brain's connective tissue, glial cells were long considered to function as mere structural support (reviewed by Somjen, 1988). Glial cells have since, especially in recent years, been established as active regulators of CNS development and function (summarized by Gage and McAllister, 2005). Among their number are oligodendrocytes, the myelinating cells of the CNS, whose function increases the efficiency of communication between neurons. In the adult CNS, oligodendroglia express netrin-1, which is enriched in non-compacted myelin membranes specialized for axonal contact. Loss of netrin or netrin receptor expression is frequently observed in highly invasive glial tumors. Taken together, these observations suggest that netrin and its receptors are important for the function of CNS glia (reviewed by Baumann and Pham-Dinh, 2001; reviewed by Barallobre et al., 2005).

I. STRUCTURE AND FUNCTION OF NETRINS AND THEIR RECEPTORS

A great deal has been learned about the functions of netrins and other guidance cues over the past decade, but the idea that migrating cells and axonal growth cones are directed to their targets by guidance cues is far older. Just prior to the turn of the century, Santiago Ramón y Cajal announced his 'Neurotropic theory', proposing that target cells secrete substances that guide growth cones. Similarly, he envisioned, neuronal cells could be guided by mechanisms of 'positive chemotaxis' (attraction) or 'negative chemotaxis' (repulsion) (Ramón y Cajal, 1892). A specific example he offered of such guidance was the migration of commissural neuron axons in the embryonic spinal cord, which he suggested were directed towards their destinations in the ventral midline by a cue secreted by the floor plate (Ramón y Cajal, 1909).

While Ramón y Cajal eventually recognized that it would be difficult to provide data to support his ideas and reluctantly changed the course of his investigations, the

development of new experimental approaches over the next century eventually allowed others to provide empirical support for his theories. Lumsden and Davies (1983) used collagen gel co-cultures to demonstrate that trigeminal sensory axons could extend from a tissue explant towards explants of whisker epithelium, their final target *in vivo*. Shortly thereafter, work from the laboratory of Tom Jessell provided evidence for the ideas put forth by Ramón y Cajal years earlier, showing that floor plate explants could attract commissural axons from explants of dorsal spinal cord (Tessier-Lavigne et al., 1988; Placzek et al., 1990). Simultaneously, studies were being carried out by David Hall's laboratory on *C. elegans* mutants generated in an earlier mutagenic screen for 'uncoordinated' phenotypes. Hedgecock and colleagues (1990) identified a gene called *unc6*, encoding a secreted laminin-like protein, that was required for circumferential axon migration (Brenner, 1974; Hedgecock et al., 1990). Soon after, work by Marc Tessier-Lavigne's group identified the secreted factors that promoted commissural axon outgrowth as the vertebrate homologues of UNC6. These proteins were dubbed the 'netrins', from the Sanskrit *netr*, meaning 'one who guides' (Serafini et al., 1994; Kennedy et al., 1994).

A. Netrins

In the years since their discovery, several netrin family members have been identified in a multitude of species. Netrins typically contain approximately 600 amino acids, have molecular masses of ~75 kDa, and their secondary structure, which is highly conserved, consists an amino-terminal signal peptide and three domains (VI, V, and C). Domains VI and V, which comprise roughly three quarters of the netrin sequence, are homologous to laminins. Specifically, domain VI is homologous to the globular domain VI of laminin, while domain V, which can be divided into sub-domains V-1, V-2, and V-3, resembles the epidermal growth factor (EGF) repeats of the eponymous domain in laminin (Serafini et al., 1994). The C domain has no homology to laminin and is the region of netrin that is most variable between family members. It shares homology with tissue inhibitors of metalloproteinases, secreted frizzled-related proteins and complement proteins, though the functional significance of these relationships remains unclear. The C domain contains many basic amino acids and binds heparin with high affinity, raising the

possibility that this domain may mediate binding to negatively-charged factors present at the cell surface or in the extracellular matrix (ECM) (Banyai and Patthy, 1999; Kappler et al., 2000; reviewed by Manitt and Kennedy, 2002; reviewed by Barallobre et al., 2005).

The netrins can be divided into three categories: Netrins 1-3, netrin-4, and the netrin-G subfamily (netrin-G1 and G2, Fig 1.1A). Key differences exist between netrins 1-3 and other netrin family members. The VI and V domains of netrins 1-3 are homologous to those of the laminin γ chain, while those of netrin-4 (also called β-netrin) more closely resemble the corresponding domains of the laminin β chain (Fig 1.1B, Yurchenco and Wadsworth, 2004). The VI and V domains of netrin-G subfamily members share slightly greater similarity with the γ chain than the β chain, but have hallmarks of both. The most significant difference between netrin-Gs and secreted netrins is at the C-terminus, which in the netrin-G subfamily is rich in hydrophobic residues and is associated with the plasma membrane by a glycosyl phosphatidylinositol (GPI) linkage (Nakashiba et al., 2000).

i. Netrins 1, 2, and 3

Netrins 1, 2, and 3 comprise the vast majority of family members identified to date, and have been identified in an array of vertebrate and invertebrate species including *C. elegans* (Ishii et al., 1992), *C. intestinalis* (sea squirt, Hotta et al., 2000), the medicinal leech *H. medicinalis* (Gan et al., 1999), *Drosophila* (Harris et al., 1996; Mitchell et al., 1996), amphioxus (lancet, Shimeld, 2000), *P. Marinus* (sea lamprey, Shifman and Selzer, 2000), zebrafish (Strahle et al., 1997; Lauderdale et al., 1997), *Xenopus* (de la Torre et al., 1997), chicken (Kennedy et al., 1994), mouse (Serafini et al., 1996), rat (Manitt et al., 2001), and human (Van Raay et al., 1997; Meyerhardt et al., 1999). Netrin-1 and netrin-3 have been identified in mammals, while netrin-2 is found in chicken and zebrafish, but has no known mammalian orthologue (Serafini et al., 1994; Park et al., 2005). The distribution of netrin-1 in mammals appears to correspond with the combined distributions of netrin-1 and netrin-2 in chicken, suggesting that it may also assume the role of netrin-2 during mammalian development (Serafini et al., 1996).

In the developing CNS, *netrin-1* is expressed prominently at the ventral midline, and is also expressed in other regions such as the retina, striatum, substantia nigra, and cerebellum (Kennedy et al., 1994; Livesey and Hunt, 1997). Netrin-1 is also expressed in

many tissues outside the CNS during development including heart, tongue, lung, inner ear, intestine, mammary gland, pancreas (Kennedy et al., 1994; Livesey and Hunt, 1997; Salminen et al., 2000; Srinivasan et al., 2003; De Breuck et al., 2003; Jiang et al., 2003). Mice lacking functional netrin die hours after their births. In the absence of a functional *netrin-1* gene, the spinal ventral commissure fails to form, as do the more rostral corpus callosum and hippocampal commissure, establishing netrin-1 as a key regulator of commissural axon organization (Serafini et al., 1996). Netrin-1 is also required for normal axon migration of other regions of the CNS. In its absence, the axons of retinal ganglion cells cannot exit the optic nerve, resulting in hypoplasia (Deiner et al., 1997). Netrin-1 is also required for the proper development of entorhino-hippocampal, CA3-to-CA1, and reciprocal hippocampo-septal and septo-hippocampal connections in the developing hippocampus, and for the projection of gonadotropin-releasing hormone (GnRH) neuron axons (Deiner and Sretavan, 1999; Barallobre et al., 2000; Pascual et al., 2004). Further *in vitro* studies established that netrin-1 is a bifunctional cue, providing empirical support for what had been hinted at by the phenotype of *C. Elegans unc6* mutants, in which both dorsoventral and ventrodorsal axonal projections are aberrant (Hedgecock et al., 1990). Netrin attracts some axons, such as those of spinal commissural neurons, inferior olivary neurons and thalamo-cortical neurons (Kennedy et al., 1994; Metin et al., 1997; Causeret et al., 2002) and repels others, such as spinal accessory, trochlear and cranial motor axons (Colamarino and Tessier-Lavigne, 1995; Guthrie and Pini, 1995; Dillon et al., 2005).

As implied by the presence of cell migration defects affecting both neurons and non-neuronal cells in nematode *unc6* mutants (Hedgecock et al., 1990), netrin-1 also guides the migration of neuronal precursor cells in vertebrates. *In vitro*, netrin-1 can attract inferior olivary neurons and pontine neurons, and repel striatal neuron precursors, cerebellar granule cells, and spinal accessory motor neurons (Alcantara et al., 2000; Hamasaki et al., 2001; Causeret et al., 2002; Dillon et al., 2005). *In vivo*, netrin-1 is required for the attractant guidance of migrating leuteinizing hormone-releasing hormone (LHRH) neurons from the vomeronasal organ to the forebrain, and for the migration of antidiuretic hormone (ADH) and oxytocin neurons to the developing supraoptic nucleus and for the repellent guidance of spinal accessory motor neurons (Deiner and Sretavan, 1999; Schwarting et al., 2004; Dillon et al., 2005). Netrin-1 is also necessary for the

formation of pontine nuclei and the inferior olivary complex, suggesting that it may regulate their migration in vivo as well (Bloch-Gallego et al., 1999).

Considerably less is known about netrin-3 than netrin-1. In the developing CNS, *netrin-3* expression is restricted to spinal motor neurons and the thalamus, and its function in these structures is unknown. *In vitro* netrin-3 can attract spinal commissural axons, though with less efficiency compared to netrin-1, and repel trochlear motor axons, but little is known about the *in vivo* relevance of these findings (Wang et al., 1999). Netrin-3 is also expressed outside the CNS in the developing cranial and dorsal root ganglia, limb buds, bowel, pancreas, and muscle (Wang et al., 1999; Puschel, 1999; Jiang et al., 2003).

ii. Other netrins

Netrin-4 is abundant throughout the developing CNS, including the olfactory bulb, retina, and ventricular regions of the brain. In the spinal cord, *netrin-4* expression is initially restricted to the floor plate, like netrin-1, but is rapidly upregulated throughout the gray matter during embryogenesis. Postnatally, *netrin-4* expression is upregulated in the olfactory bulb, cerebellar granule neurons, hippocampal neurons, and cortical neurons. Outside the CNS, *netrin-4* expression is found in the developing dorsal root ganglia, pancreas, intestine, kidney, lung, and spleen, and in the adult intestine, spleen, kidney, ovary, heart, lung, spleen, and vascular basement membranes (Koch et al., 2000; Yin et al., 2000; Liu et al., 2004b; Zhang et al., 2004). Interestingly, Zhang and colleagues (2004) reported the expression of an alternatively spliced netrin-4 that lacks a signal peptide, suggesting that it is not secreted.

The GPI-linked netrin-G subfamily consists of two genes (netrin-G1 and netrin-G2) encoding nine alternatively spliced isoforms. Unlike the secreted netrins, netrin-Gs are specific to mammals, as no orthologues exist in the genomes of *C. elegans* or *Drosophila*. Netrin-Gs are expressed throughout the brain, but regions of netrin-G1 and netrin-G2 expression are generally non-overlapping. Little is known about their function, but netrin-G1 and netrin-G2 substrates can promote neurite outgrowth from dissociated cortical and thalamic neurons (Nakashiba et al., 2000; Nakashiba et al., 2002).

B. Netrin receptors

In their 1990 publication describing the *C. elegans unc6* mutant, Hedgecock and colleagues also described two mutants, *unc5* and *unc40*, with similar, but more restricted, circumferential migration phenotypes. While both dorsal and ventral migrations were disturbed in *unc6* mutants, only dorsal migrations (away from sites of unc6 expression) were disturbed in *unc5* mutants, while ventral migrations were primarily disturbed in *unc40* mutants. For this reason, it was hypothesized that *unc40* and *unc5* encoded guidance receptors that respond to unc6 (Hedgecock et al., 1990). Following the discovery of vertebrate netrins, vertebrate homologues of UNC40 and UNC5 were identified, and were found to act as netrin receptors (Fig. 1.1C). The canonical netrin receptors in mammals are UNC40 homologues Deleted in Colorectal Cancer (DCC) and neogenin, and the UNC5 homologues (UNC5A, B, C, D), though other proteins have also been proposed to act as netrin receptors, as described below (Keino-Masu et al., 1996; Leonardo et al., 1997). All of these receptors are bound by netrins 1 and 3. Netrin-1 and netrin-3 bind neogenin and UNC5 homologues with roughly the same affinity, but the DCC-binding affinity of netrin-1 is approximately four-fold greater than that of netrin-3 (Wang et al., 1999). Netrin-G1 and netrin-G2 do not bind DCC, neogenin, or UNC5 homologue receptors, but may instead themselves act as part of a receptor complex for netrin-G1 ligand (NGL-1) (Nakashiba et al., 2002; Lin et al., 2003). No binding of netrin-4 to canonical netrin receptors (or any other receptor) has been reported in the literature.

i. DCC family

DCC family receptors identified to date include DCC and neogenin in vertebrates, UNC40 in *C. elegans*, and frazzled in *Drosophila* (Chuong et al., 1994; Vielmetter et al., 1994; Cooper et al., 1995; Keino-Masu et al., 1996; Kolodziej et al., 1996; Chan et al., 1996; de la Torre et al., 1997). These are type-I transmembrane proteins characterized by the presence of four immunoglobulin (Ig) domains and six fibronectin type III repeats in their extracellular domains (ECDs), and by a unique C-terminus characterized by conserved regions P1, P2, and P3 (Kolodziej et al., 1996; Hong et al., 1999).

In the developing CNS, *dcc* is expressed is multiple regions throughout the brain, including the cortex, striatum, hippocampus, olfactory bulb, hypothalamus, and cerebellum, and in the dorsal spinal cord. Outside the CNS, it is also expressed in neurons of the developing peripheral and enteric nervous systems, and in the developing bladder

(Keino-Masu et al., 1996; Gad et al., 1997). The phenotypes observed in $DCC^{-/-}$ mice closely resemble those of mice lacking functional netrin-1. $DCC^{-/-}$ mice die shortly after their births. Reminiscent of the defects in ventrally-projecting circumferential axon migration observed in the C. elegans *unc40* mutant, spinal commissural axon extension was aberrant in $DCC^{-/-}$ animals. DCC mutants, like netrin-1 mutants, also lack the corpus callosum and hippocampal commissure, and display optic nerve hypoplasia and abnormal migration of GnRH neuron axons (Hedgecock et al., 1990; Deiner et al., 1997; Fazeli et al., 1997; Deiner and Sretavan, 1999). In mice lacking *dcc* expression, defects in LHRH, GnRH, oxytocin, pontine, and spinal accessory motor neuron migration are similar to those observed in netrin-$1^{-/-}$ animals, and pontine nuclei are again absent, providing evidence that DCC is also required for the guidance of cell migration by netrin-1 (Fazeli et al., 1997; Deiner and Sretavan, 1999; Schwarting et al., 2001; Schwarting et al., 2004; Dillon et al., 2005).

In vitro, inhibition of DCC function prevents netrin-induced commissural axon outgrowth and attractant turning of *Xenopus* spinal neuron axons to netrin-1 (Keino-Masu et al., 1996; Ming et al., 1997). Similarly, blocking DCC function inhibits attractant cell migration of striatal neurons (Hamasaki et al., 2001). Together, these data provide direct evidence that DCC is required for netrin-1 mediated attraction. The intracellular P3 domain is particularly important for DCC-mediated chemoattractive responses to netrin. P3 is required for DCC multimerization, and for DCC-mediated chemoattraction in *Xenopus* spinal neurons (Hong et al., 1999; Stein et al., 2001). The importance of the DCC P3 domain *in vivo* is reinforced by studies of the naturally-occurring DCC^{kanga} mutant (so named because of its hopping gait), that expresses a truncated mRNA lacking exon 29, that encodes the P3 domain. DCC^{kanga} mice live into adulthood, but display many of the same phenotypes seen in *dcc* deletion mutants: The corpus callosum, anterior commissure and pontine nuclei are missing, and midline crossing does not occur, suggesting that commissural neurons do not cross the midline (Finger et al., 2002).

DCC plays a key role in repellent guidance as well. Studies of *Drosophila* mutants suggest that DCC is required for long-range, but not short-range repellent axon guidance (Keleman and Dickson, 2001). Inhibition of DCC function in *Xenopus* spinal neurons also prevented the turning of axons away from a netrin-1 source. The P1 domain of the DCC

intracellular domain (ICD) was required for repulsion of axons by netrin-1 (Hong et al., 1999).

Considerably less is known about neogenin, the other DCC family member that has been identified in vertebrates. In the developing CNS, *neogenin* is expressed in cortex, striatum, hippocampus, olfactory bulb, thalamus, hypothalamus, midbrain, cerebellum, and retina. Neogenin expression is also observed in cranial and dorsal root ganglia of the PNS. Unlike DCC, extensive neogenin expression has been observed in other tissues. These include heart, bladder, gut, pancreal, and mammary epithelia, mesenchymal cells of the lung and kidney, and in differentiating skeletal muscle and cartilage (Gad et al., 1997; Srinivasan et al., 2003; Fitzgerald et al., 2006). No guidance function in response to netrin has yet been identified for neogenin *in vitro*, and no axon guidance phenotype was observed in mice lacking a functional *neogenin* gene (Leighton et al., 2001). However direct evidence has been provided indicating that neogenin does have a guidance function in response to another ligand, repulsive guidance molecule (RGM), a protein originally identified as an axon guidance molecule in the visual system. *In vitro*, RGM can repel retinal axons and collapse growth cones in a neogenin-dependent manner, though no evidence supporting a functional interaction *in vivo* has yet been reported (Rajagopalan et al., 2004; Matsunaga and Chedotal, 2004).

ii. UNC5 homologues

The four vertebrate homologues of *C. Elegans* UNC5 are type-I transmembrane proteins. Their ECDs are comprised of two Ig repeats and two thrombospondin type-I domains. Intracellularly, UNC5 receptors include a ZU5 domain, which is also found in tight junction protein zona occludens-1 and in ankyrins 1-3, a DCC-binding (DB) motif, and a death domain (DD) (Hofmann and Tschopp, 1995; Leonardo et al., 1997; Schultz et al., 1998; Hong et al., 1999; Engelkamp, 2002).

The expression of the four UNC5 homologues in the embryo varies considerably. Developmental *unc5a* expression is largely restricted to the CNS, and is found in spinal motor columns, hippocampus, entorhinal cortex, retina, and the cerebellar granule cell layer. *Unc5b* expression is found in the developing roof plate of the spinal cord, retina, and in the external germinal layer, molecular layer, and inner granule cell layer of the cerebellum, consistent with expression throughout granule cell differentiation and

migration. Outside the CNS, *unc5b* is expressed in developing mammary tissue, limb buds, and inner ear, but its expression is most prominent in vascular tissue (Leonardo et al., 1997; Engelkamp, 2002; Lu et al., 2004). In the developing CNS, expression of *unc5c*, originally identified as *rostral cerebellar malformation* (*rcm*), is observed in Purkinje and granule cell precursors of the developing cerebellum, olfactory bulb, hippocampus, pontine, olivary nuclei, and spinal roof plate and motor columns. *Unc5c* is also detected outside the CNS in migrating neural crest cells, and in a variety of developing tissues including limb buds, gut, kidney, lung, gonad, spleen, bladder, bone, and cartilage (Ackerman et al., 1997; Przyborski et al., 1998). Finally, *unc5d* is expressed in developing limb buds, inner ear, mammary tissue, but does not appear to be abundant in the CNS (Engelkamp, 2002).

Mice lacking *unc5b* expression in an inbred background die during embryogenesis when the neural tube fails to close, and die a few days later from heart failure when outbred. Extensive abnormalities are observed in the vasculature of these animals, including aberrant migration of vascular endothelial tip cells and excessive vessel branching (Lu et al., 2004). UNC5C mutant mice, unlike netrin-1, DCC, and UNC5B mutants survive until adulthood, though they are ataxic. As their original moniker implies, these animals show extensive cerebellar abnormalities including dramatically reduced cerebellar size and complexity, including ectopically positioned granule and Purkinje neurons (Ackerman et al., 1997). Analysis of UNC5C mutant chimeras suggests that aberrant granule cell localization is a direct consequence of the mutation, while the Purkinje neuron phenotype is a consequence of abnormal granule cell positioning and the resulting lack of cerebellar boundaries (Goldowitz et al., 2000). The phenotypes of UNC5A and UNC5D mutant mice have not yet been reported.

Early hints regarding the nature of the response to netrin mediated by the UNC5 family receptors were provided by the aforementioned *C. Elegans* experiments performed by Hedgecock and colleagues (1990). Their findings indicated that ventro-dorsal, but not dorso-ventral, axon guidance is affected in *unc5* mutants. Further nematode studies revealed that ectopic *unc5* expression in neurons that normally project ventrally or longitudinally resulted in their axons migrating dorsally in an UNC6-dependent manner (Hamelin et al., 1993). These observations, coupled with their demonstration that netrins

could also function as repellent cues for the axons of trochlear motor neurons in explants of rat hindbrain-midbrain junction, led Colamarino and Tessier-Lavigne (1995) to hypothesize that UNC5 receptors mediate repellent responses to netrin. This was demonstrated directly by experiments in which the attractant responses of migrating *Xenopus* spinal neuron axons were converted to repulsion following ectopic UNC5 expression (Hong et al., 1999).

Long-range repellent responses to netrin-1 require physical interaction between the ICDs of DCC (through its P1 domain) and UNC5 homologues (Hong et al., 1999). The identity of the cytoplasmic region of UNC5 receptors that is required for this interaction is disputed. Transfection experiments in *Xenopus* suggested that it was through the conserved DB domain, while later *C. elegans* data suggested that the interaction occurs in a region between the transmembrane domain and the ZU5 domain (Hong et al., 1999; Merz et al., 2001; Killeen et al., 2002). The requirement of DCC for repellent responses to netrin is not absolute, however. Several reports from *C. elegans* and *Drosophila* have provided evidence that UNC5 family receptors can respond to netrin-1 independently of UNC40/Frazzled, and evidence has been provided that the role of DCC family receptors in repellent guidance may be to increase the sensitivity or efficacy of UNC5 receptor-mediated responses to netrin. The resulting model is one in which short-range responses to netrin are mediated by UNC5 alone, while both UNC5 and DCC are required for long-range guidance (Colavita and Culotti, 1998; Hong et al., 1999; Merz et al., 2001; Keleman and Dickson, 2001; Huang et al., 2002). A recent report describing signaling events downstream of netrin receptors, described below, appears to support this idea (Li et al., 2006).

iii. Other netrin receptors

Other cell surface proteins have been suggested to act as netrin receptors. It has been proposed that netrin-1 binds and activates adenosine receptor A_{2B}, leading to cyclic AMP (cAMP) production, and that addition of an A_{2B} antagonist inhibits netrin-1-mediated commissural axon outgrowth from rat spinal cord explants (Corset et al., 2000). These findings were corroborated by Shewan and colleagues, showing that exposure to these same A_{2B} antagonists inhibits pathfinding of young *Xenopus* retinal axons (Shewan et al., 2002). However, significant doubts linger about the identity of A_{2B} as a netrin

receptor because of a third report, which called into question the data described in these studies. Many issues raised concern the secondary effects of the A_{2B} inhibitors used. These factors were shown to inhibit axon elongation and turning of *Xenopus* spinal neuron axons in response to three guidance factors other than netrin-1, suggesting that they act nonspecifically. When axon outgrowth in response to netrin-1 was investigated in rat spinal cord explants, use of one A_{2B} inhibitor at the (abnormally high) concentrations employed in the first study resulted in decreased axon outgrowth, but was accompanied by considerable necrotic cell death in the explants. The authors concluded that the reduced outgrowth observed was the result of a toxic effect of the inhibitor. Additionally, the authors failed to detect A_{2B} mRNA in rat spinal cord by either RT-PCR or *in situ* hybridization (the authors of the first study had demonstrated A_{2B} expression in explants by immunocytochemistry), suggesting that the detection of A_{2B} in these cells may have been artifactual (Stein et al., 2001). Thus, support for the role of A_{2B} as a netrin receptor is tenuous.

Evidence has also been provided that integrins also act as netrin receptors. Yebra and colleagues (2003) reported that $\alpha6\beta4$ and $\alpha3\beta1$ integrins mediated the adhesion and migration of pancreatic epithelial cells to substrates of netrin-1, and that netrin-1 physically interacts with integrin subunits. Little is known to date about the nature of netrin-integrin associations, whether or not netrins can bind other integrins, or about the possible involvement of canonical netrin receptors.

C. Signaling pathways activated by netrin

Central to intracellular events underlying guidance responses of migrating cells and axons is the organization of the actin cytoskeleton. The growth cone and the leading edge of migrating cells are characterized by lamellipodia and filopodia, complex and dynamic actin-based membrane protrusions (reviewed by Suter and Forscher, 2000). These structures are highly sensitive to their environment: Contact of even a single filopodium with a high-affinity substrate is sufficient to reorient an entire growth cone (O'Connor et al., 1990). In recent years, much has been learned about the intracellular mechanisms responsible for netrin-mediated guidance, pertaining particularly to remodeling of the actin cytoskeleton. Of particular importance to the regulation of the

actin cytoskeleton both in neuronal and non-neuronal cells is the Rho family of small GTPases. The three best-characterized family members are Ras-related C3 botulinum toxin substrate 1 (rac1), cell division cycle 42 (cdc42), and ras homologue A (rhoA). When in the active GTP-bound form, rac1, cdc42 and rhoA regulate the formation of lamellipodia, filopodia, and of stress fibers and adhesion foci, respectively (reviewed by Hall, 1998). It has been hypothesized that attractant cues primarily activate rac1 and cdc42, resulting in the formation of lamellipodial and filopodial protrusions and a simultaneous decrease in retrograde flow and actin depolymerization, resulting in the generation of actin filaments and axon extension (reviewed by Mueller, 1999; reviewed by Dickson, 2001). This has been observed, for instance, in the attractant response to netrin-1 (Shekarabi et al., 2005). Repellent guidance cues are believed to act primarily through the activation of rhoA, which inhibits growth and induces collapse, as has been reported for ephrin-A5 signaling (Wahl et al., 2000), or in response to stimulation with the bioactive lipid lysophosphatidic acid (LPA) (Kozma et al., 1997; Yuan et al., 2003; reviewed by Mueller, 1999).

i. Attractant guidance

A growing body of literature has implicated rho GTPases in signaling responses downstream of netrin-DCC binding. Ectopic DCC expression in NG108-15 or HEK293 cells in the presence of netrin-1, resulted in increased surface area and filopodial number. These increases could be blocked by inhibitors of rac1 and cdc42, respectively. Rac1 and cdc42 activity are also required for netrin-1-induced neurite outgrowth from N1E-115 cells. Exposure of DCC-expressing cells to netrin-1 resulted in an increase of GTP-bound cdc42 and rac1, demonstrating that netrin-1, via DCC, activates these rho family GTPases (Shekarabi and Kennedy, 2002; Li et al., 2002b). A similar mechanism was later demonstrated to act downstream of netrin and DCC in rat commissural neurons (Shekarabi et al., 2005). RhoA is also likely to be involved in chemoattraction to netrin, though its role is less well understood. Inhibition of either rhoA or its effector rho kinase (ROCK) results in increased netrin-1-induced, DCC-mediated neurite outgrowth from both N1E-115 cells and precerebellar neurons. Interestingly, rhoA activity was also required for nuclear translocation during the migration of precerebellar neurons, raising

the possibility that rhoA may play a particularly significant role in cell migration in response to netrin (Li et al., 2002b; Causeret et al., 2004).

In addition to demonstrating that DCC activates rho family GTPases in neurons, Shekarabi and colleagues (2005) also demonstrated that netrin-1 binding to DCC results in the recruitment of a protein complex to the DCC ICD (Shekarabi et al., 2005). This complex includes the adaptor protein nck1, which binds DCC constitutively via its first and third SH3 domains, and is required for netrin-1-induced, DCC-mediated, neurite outgrowth from N1E-115 cells (Li et al., 2002a). Netrin-1 binding results in the recruitment of neuronal Wiskott-Aldrich syndrome protein (N-WASP) to the DCC ICD complex and the recruitment and subsequent activation of rac1, cdc42, and p21-activated kinase (pak1), a downstream effector of these two GTPases. The recruitment of pak1 to the complex occurs through nck1, and this association is required for netrin-1-induced growth cone expansion (Shekarabi et al., 2005). N-WASP is also likely recruited to the complex via nck1, as these proteins have previously been reported to bind each other (Rohatgi et al., 2001). Cdc42 and rac1 are recruited to the complex by associating with both pak1 and N-WASP (Shekarabi et al., 2005). Focal adhesion kinase (FAK) and src family kinases are also possible participants in this complex. FAK has also previously been shown to associate with nck1 (Schlaepfer et al., 1997) and constitutively associates with the DCC ICD. Netrin-1 induces FAK phosphorylation and activation, leading to the activation of src family kinases including src and fyn (Li et al., 2004; Liu et al., 2004a). FAK and src family kinase activity have been shown to promote the phosphorylation of the DCC ICD in the presence of netrin-1, and are required for rac1 activation, neurite outgrowth from N1E-115 cells, and attractant turning of *Xenopus* retinal ganglion cell axons (Meriane et al., 2004).

One role of this complex may be to link netrin-DCC signaling to the actin cytoskeleton. Arp2/3, a protein complex that nucleates and crosslinks actin filaments, binds the C-terminus of N-WASP (Mullins, 2000). The N-terminus of N-WASP binds F-actin, suggesting that DCC, through its ICD-associated complex, functions as a transmembrane bridge linking extracellular netrin-1 to the cytoskeleton (Shekarabi et al., 2005). Proteins of the enabled/vasodilator-stimulated phosphoprotein (Ena/VASP) family are also likely involved in regulation of the F-actin cytoskeleton downstream of netrin

and DCC. Ena/VASP proteins prevent the capping of F-actin, allowing for further filament elongation and promoting filopodia formation (reviewed by Krause et al., 2003). *C. Elegans* Ena/VASP protein UNC34 is required for chemoattraction to UNC6, and Ena/VASP proteins are also required for netrin-induced filopodia formation in mouse hippocampal neurons (Gitai et al., 2003; Lebrand et al., 2004). Previous studies have suggested that WASP and Ena/VASP family members can associate either directly (Castellano et al., 2001), or indirectly through a scaffold protein (Salazar et al., 2003), raising the possibility that Ena/VASP proteins may also be associated with the DCC-ICD complex.

Other signaling pathways have also been implicated in chemoattractant responses to netrin. Activation of the mitogen-activated protein kinase (MAPK) pathway has been reported to be required for netrin-mediated neurite outgrowth and chemoattraction (Forcet et al., 2002). In cultures of spinal commissural neurons, MAPKs extracellular signal-regulated kinase-1 and -2 (ERK1/2) associated with DCC, and addition of netrin-1 resulted in DCC-dependent ERK activation. MAPK activation has implicated in netrin-induced local protein synthesis within growth cones, which has been hypothesized to mediate responses to netrin (Campbell and Holt, 2001; Campbell and Holt, 2003). It has been reported, however, that in lung endodermal cells, netrins can also suppress ERK activity, most likely through a receptor other than DCC (Liu et al., 2004b). Also required for turning for Xenopus spinal neurons to netrin-1 is the co-activation of signaling molecules phosphatidylinositol-3 kinase (PI3K) and phospholipase C-γ (PLCγ, Ming et al., 1999). Netrin-1 binding to DCC promotes the recruitment of phosphatidylinositol transfer protein-α (PITPα) to the DCC P3 domain, and increases its activity, increasing the synthesis of phosphoinositides such as phosphatidylinositol biphosphate (PIP_2). PLCγ, which is phosphorylated and activated in the presence of netrin-1, hydrolyzes PIP_2 to inositol triphosphate (IP_3) and diacylglycerol (DAG), and IP_3 promotes Ca^{2+} release from intracellular stores (Xie et al., 2005; Xie et al., 2006). Increases in intracellular Ca^{2+} are required for turning of Xenopus spinal neurons towards sources of netrin-1 (Hong et al., 2000).

ii. Repellent guidance

Considerably less is known about repellent guidance by netrin. In *Drosophila*, DCC function is required for long-range, but not short-range, repellent effects of netrin (Keleman and Dickson, 2001). In cultures of mouse dorsal spinal cord neurons, transfection with DCC greatly increased tyrosine phosphorylation of the UNC5 ICD by src and FAK, resulting in physical association between the UNC5 ICD and src. This interaction required the SH2 domain of src, and tyrosine residues within the ZU5 domain of the UNC5 ICD. Phosphorylation of UNC5 at tyrosine residues between the ZU5 and DB domains, one of the sites phosphorylated by src, was required for netrin-1-induced shrinkage of COS cells, suggesting that phosphorylation of the UNC5 ICD is required for the receptor to induce cytoskeletal collapse (Li et al., 2006). Src signaling is also required for UNC5-mediated repellent axon and gonadal cell migration in *C. Elegans* (Lee et al., 2005b). Together, these findings suggest that while UNC5 homologues can mediate repellent responses to netrin in the absence of DCC function, the presence of DCC enhances the repellent effect by promoting UNC5 ICD phosphorylation by src and/or FAK. It is conceivable that such a mechanism could increase the sensitivity of migrating growth cones or cells expressing DCC and UNC5 to netrin relative to growth cones or cells expressing UNC5 alone, allowing those expressing both to be repelled by netrin gradients from greater distances away from the netrin source. However, src and FAK signaling are also required downstream of DCC in the context of attraction, suggesting that additional signals are also necessary to specify repulsion (Li et al., 2004).

In both C. elegans and mammalian cells, tyrosine phosphorylation within the ZU5 domain is also required for the association of the UNC5 homologue ICD with tyrosine phosphatase SHP2, which, like src, binds via a SH2 domain (Tong et al., 2001). The exact function of SHP2 in UNC5-mediated signaling is unknown, but it has been shown to regulate rhoA activity and regulate the organization of focal adhesions and cell migration (Yu et al., 1998; Schoenwaelder et al., 2000).

iii. Modulation of netrin signaling

Various molecules have been demonstrated to influence the cellular response to netrin. The best characterized of these is cyclic AMP (cAMP). Ming and colleagues (1997) reported that addition of a cyclic AMP analog enhanced attractive turning of *Xenopus* spinal neurons towards low concentrations of netrin-1. Increased intracellular

cAMP and resultant PKA activation were later demonstrated to result in increased abundance of cell surface DCC, promoting attractant responses to netrin-1 (Bouchard et al., 2004). In addition, PKA phosphorylates ena/VASP proteins, providing another possible mechanism by which cAMP agonists may promote attraction to netrin. Ming and colleagues (1997) also found that PKA inhibition resulted in the conversion of attractant turning responses to netrin-1 to repellent ones. Substrates of laminin-1 were found to decrease intracellular cAMP concentration, converting the response of *Xenopus* retinal neurons from attraction to repulsion (Hopker et al., 1999). This finding suggests that cAMP levels and the directional response to netrin-1 *in vivo* may vary depending on the particular ECM molecules encountered by a cell.

Findings in *Xenopus* spinal neurons suggest cAMP does not act alone, and that cyclic GMP (cGMP) also modulates the response to netrin-1. Nishiyama and colleagues (2003) provided evidence that the major determinant of the direction of growth cone turning towards or away from netrin is the intracellular ratio of cAMP to cGMP, which modulates the activity of L-type Ca^{2+} channels. Specifically, higher cAMP:cGMP ratios results in high channel activity, increased Ca^{2+} influx, and turning towards a source of netrin-1, likely mediated by calcium-calmodulin-dependent protein kinase II. Lower ratios resulted in low channel activity, decreased Ca^{2+} influx, and turning away from a netrin-1 source, likely mediated by calcineurin phosphatase and phosphatase-1 (Nishiyama et al., 2003; Wen et al., 2004).

Low intracellular cAMP does not always result in a repellent response to netrin, however. A recent report by Moore and Kennedy (2006) suggests that the influence of cAMP on the response of a given cell to netrin depends on the compliment of netrin receptors it expresses. In spinal commissural neurons expressing DCC but not UNC5 homologues, PKA inhibition decreased the sensitivity of chemoattractive turning to netrin-1, but did not result in repulsion. Because the *Xenopus* spinal neurons assayed in the study by Ming and colleagues (1997) were almost certainly a heterogeneous population whose expression of netrin receptors almost certainly varies, it is difficult to re-interpret their findings in this light. Retinal neurons in many species are, however, known to express both DCC and UNC5 receptors, though UNC5 expression in *Xenopus* has not yet been demonstrated (Deiner et al., 1997; de la Torre et al., 1997; Petrausch et

al., 2000; Ellezam et al., 2001; Shewan et al., 2002). Low PKA may therefore result in a switch from an attractive to a repellent response to netrin-1 only when UNC5 family members are present (Moore and Kennedy, 2006).

The responsiveness of cells expressing UNC5 receptors to netrin-1 can also be altered. Regulation of receptor localization to the cell surface regulates the repellent response to netrin as well. Protein interacting with C-kinase 1 (PICK1) binds the C-termini of UNC5A at the plasma membrane and protein kinase Cα (PKCα) into this complex, resulting in receptor internalization from the plasma membrane. Consequently, UNC5A-mediated growth cone collapse is inhibited, and chemorepellent turning is converted to chemoattraction (Williams et al., 2003b; Bartoe et al., 2006). UNC5B has been reported to bind and segregate inhibitory G protein $G_{i\alpha 2}$, resulting in the liberation of adenyl cyclase and an increase in intracellular cAMP, an interaction that is strengthened in the presence of netrin-1 (Komatsuzaki et al., 2002). These interactions may be components of inhibitory pathways that regulate the repellent response to netrin. Altering responsiveness to netrin may be particularly important when migrating axons or cells reach intermediate choice points where their trajectories must be reoriented.

A putative positive regulator of UNC5 function is the cytoplasmic adaptor protein MAX1. Mutations of *unc6* and *unc5* were found to enhance the *max1* mutant motor axon migration phenotype in *C. Elegans*, and mutations of the Band 4.1/ezrin/radixin/moesin (FERM) domain of MAX1 abolished this effect. However, while *C. Elegans* UNC5 and MAX1 proteins co-localize in neuronal processes *in vivo* and human UNC5B and MAX1 co-localize in cultured COS cells, no physical interaction could be demonstrated between them, suggesting that MAX1 modulates UNC5 signaling indirectly. Interestingly, mutations of *unc40* do not enhance the max1 phenotype, suggesting that MAX1 may be specifically involved in DCC-independent repellent actions of UNC5 (Huang et al., 2002).

D. Netrins regulate tissue morphogenesis during development

In recent years, netrins have been found to play key roles in adhesion and morphogenesis of multiple non-CNS tissues. In the developing mammary gland, netrin-1 is expressed by prelumenal epithelial cells, and its receptor neogenin by cap cells of

terminal end buds. In the absence of either netrin-1 or neogenin, the organization of the cell layers in terminal end buds is disrupted, suggesting that netrin-neogenin interactions were required for adhesive interactions between these cells. This is likely a direct effect on cell-cell adhesion: Addition of exogenous netrin-1 protein to cultured neogenin-transfected COS cells, or to neogenin-expressing L1 mouse fibroblasts, resulted in neogenin-dependent cell aggregation (Srinivasan et al., 2003).

In the developing lung epithelium, *netrin-1* and *netrin-4*, and netrin receptors *dcc* and *unc5b*, are expressed in proximal lung epithelia but, unlike in the mammary gland, not in the terminal regions of the buds. Functional experiments employing explant cultures of endodermal epithelium provide evidence that netrin-1 and netrin-4 prevent bud formation, and that these effects are DCC-independent. Based on these observations, the authors hypothesized that netrin protein is deposited in basement membranes, where it facilitates the outgrowth of buds towards mesodermal sources of FGF10, and prevents ectopic budding (Liu et al., 2004b). Similarly, netrin-1 is expressed by pancreatic epithelial progenitor cells, and is localized to basement membranes. Pancreatic progenitors were shown to adhere to a substrate of netrin-1, and migrate towards increasing netrin-1 concentrations. These cells do not express DCC or neogenin, and these responses depend instead on the α6β4 and α3β1 integrins. Though the effect on morphogenesis was not investigated in this tissue, the distribution of netrin protein and its adhesive effects on pancreatic progenitor adhesion hint at a possible role in the pancreatic morphogenesis (Yebra et al., 2003). Though the roles of netrin in lung and pancreatic development remain to be demonstrated *in vivo*, these findings provide evidence that netrins mediate short-range interactions that are distinct from their better understood roles in long-range guidance.

E. Netrin-1 and its receptors are tumor suppressors

Due in large part to the neonatal lethality of the netrin-1 and DCC loss-of-function phenotypes and embryonic lethality of mice lacking UNC5B function, the vast majority of research concerning netrin and netrin receptor function has centered on the function of these proteins during embryonic development. Netrins and their receptors continue to be expressed at high levels in the adult both inside and outside the CNS, and lost or reduced

expression of DCC, UNC5 homologues, and netrin-1 have all been associated with tumorigenesis in many tissues.

i. DCC

Years prior to its identification as a netrin receptor, the *dcc* gene was first described as a candidate tumor suppressor gene present on chromosome 18q21, a region affected by allelic loss in the majority of colorectal cancers (Vogelstein et al., 1988; Fearon et al., 1990; Hedrick et al., 1994). For years thereafter, DCC's candidacy as a tumor suppressor was often called into question. Reasons for this included the presence of other candidate tumor suppressor genes, notably members of the SMAD family (Thiagalingam et al., 1996; Takaku et al., 1998); and a paucity of identified point mutations in *dcc* coding sequences (Miyake et al., 1994). Furthermore, no apparent predisposition towards tumorigenesis was observed in adult $DCC^{+/-}$ mice, and no increase in the incidence of tumor formation was detected in DCC knockout mice. Conclusions drawn from the latter analysis, however, were complicated by the finding that mice lacking DCC die shortly after birth and tumors may not have had time to develop (Fazeli et al., 1997).

Since then, however, substantial evidence supporting a role for DCC in suppressing tumor dissemination has accumulated. *Dcc* expression is detected in neurons and glia in the adult CNS, but is also found in many non-neural tissues (Hedrick et al., 1994; Reale et al., 1994). *Dcc* expression is reduced or absent in many types of cancers that occur both inside and outside the CNS. These include colorectal (Kikuchi-Yanoshita et al., 1992; Saito et al., 1999; Meyerhardt et al., 1997), esophageal (Miyake et al., 1994), pancreatic (Simon et al., 1994; Hilgers et al., 2000), cervical (Enomoto et al., 1995; Kersemaekers et al., 1999), ovarian (Saegusa et al., 2000), testicular (Murty et al., 1994; Strohmeyer et al., 1997), gastric, mammary, prostate (Reale et al., 1994; Meyerhardt et al., 1997), neuroblastoma (Reale et al., 1996; Reyes-Mugica et al., 1997; Reyes-Mugica et al., 1998; Ekstrand et al., 1995), and glioma (Scheck and Coons, 1993; Reyes-Mugica et al., 1997; Nakatani et al., 1998; Hara et al., 2001).

In many cases, reduced or lost DCC expression was reported to be infrequent in early stage tumors, but correlated with aggressive invasion and metastasis. In colorectal cancer, DCC expression is high in normal mucosa, adenoma, and intramucosal carcinoma (stage 0), is downregulated in almost all invasive cancers (stages 1-4), and is absent in almost 100% of liver metastases. Loss of DCC expression is also a predictor of poor patient prognosis for patients with stage II or III colorectal cancer (Kikuchi-Yanoshita et al., 1992; Jen et al., 1994; Shibata et al., 1996; Saito et al., 1999; Aschele et al., 2004). A similar correlation of loss or reduced DCC expression with patient prognosis was observed for tumors arising from CNS glia. While glioma cells almost never metastasize outside the CNS, loss of DCC expression correlated strongly with the development of glioblastoma multiforme, the most highly invasive form of glioma, with tumor recurrence, and with poor patient prognosis (Reyes-Mugica et al., 1997; Nakatani et al., 1998). Functional studies have also provided further evidence that DCC is a tumor suppressor. Ectopic expression of *dcc* in tumor cells reduced tumorigenicity (Klingelhutz et al., 1995; Velcich et al., 1999; Kato et al., 2000), and expression of DCC antisense RNA in multiple types of tumor cells resulted in a faster growth rate, anchorage independence, and tumorigenicity when these cells were transplanted into nude mice (Narayanan et al., 1992). Together, these data suggest that DCC loss of heterozygosity (LOH) contributes to tumor progression, and that its identity as an important predictor of patient prognosis makes it an important potential therapeutic target.

ii. UNC5 homologues

Evidence exists that the other major family of netrin receptors, the UNC5 homologues, are also tumor suppressors. UNC5 homologues, particularly UNC5B, are expressed at high levels in both neurons and glia in the adult CNS and in other tissues including thyroid, kidney, ovary, uterus, stomach, colon, lung, spleen, bladder, and breast (Ackerman et al., 1997; Ellezam et al., 2001; Manitt et al., 2004; Thiebault et al., 2003). Substantially decreased *unc5 homologue* gene expression was observed in ovarian, colon, rectal, lung, and kidney tumors; and to a lesser extent in breast, uterine, stomach, and thyroid tumors. Analysis for individual *unc5 homologue* expression revealed that decreased expression and allelic loss were more frequent for *unc5a* and *unc5c* than

unc5b; and multiple *unc5c* mutations were detected in colorectal tumors (Thiebault et al., 2003). Epigenetic mechanisms also likely modulate UNC5 homologue expression. Tanikawa and colleagues have provided evidence that *unc5b* is a direct transcriptional target of p53, suggesting that the loss of p53 activity seen in many cancers may be responsible for decreased expression of UNC5B protein (Tanikawa et al., 2003).

iii. Netrin-1

In addition to its roles in development, netrin-1 is widely expressed by neurons and glia in the CNS of adult mammals (Manitt et al., 2001; Ellezam et al., 2001) and in other tissues such as heart, small intestine, colon, liver, prostate, and pancreas (Meyerhardt et al., 1999; De Breuck et al., 2003; Latil et al., 2003; Mazelin et al., 2004). Identification of its receptors as tumor suppressors in numerous tissues raised the possibility that netrin-1 could play a similar role. Consistent with this theory, human netrin-1 expression was substantially reduced in prostate tumors (Latil et al., 2003). Furthermore, absent or reduced expression of *netrin-1* and the presence of missense mutations in the human *netrin-1* gene have been reported in brain tumors, including neuroblastoma and glioblastoma (Meyerhardt et al., 1999).

Taken together, the observations that netrin-1, DCC, and unc5 homologues are expressed in many adult tissues, and that their expression is absent or decreased in multiple tumor types, suggest that netrin and its receptors play significant roles as tumor suppressors both in the CNS and in other tissues.

F. Netrin-1 and its receptors as mediators of cell death

For nearly a decade, very little was known about the mechanism underlying DCC's anti-tumorigenic properties. Based on the homology between the extracellular domain of DCC and those of homophilic adhesion molecules such as neural cell adhesion molecule (NCAM), it was proposed that DCC may promote cell-cell adhesion, likely through a heterophilic interaction with an unknown binding partner (Chuong et al., 1994). Others provided evidence that DCC regulated proliferation and differentiation, though some studies showed DCC expression associated with proliferating cells (Chuong et al., 1994), and others with differentiated cells (Lawlor and Narayanan, 1992; Hedrick et al.,

1994; reviewed by Cho and Fearon, 1995). No proposed anti-tumorigenic mechanism for DCC has been widely accepted until recently. Many reports, primarily from the groups of Patrick Mehlen and Dale Bredesen, have provided evidence that netrin-1 and its receptors act as tumor suppressors by regulating cell death. These findings, they suggest, imply that loss of netrin-1, DCC, or UNC5 homologue expression in tumor cells renders them resistant to apoptosis.

i. DCC as a 'dependence receptor'

In 1998, it was proposed by Mehlen and colleagues that, in addition to its roles in guidance, DCC functions as a 'dependence receptor', inducing apoptosis in the absence of its ligand netrin-1, while inhibiting apoptosis when bound by its ligand. When full-length DCC was ectopically expressed in HEK293T cells increased cell death occurred, which could be blocked by co-expression or addition of netrin-1 conditioned medium. DCC-induced cell death was blocked by caspase inhibitors, suggesting that death was apoptotic. Cleavage at aspartic acid residue 1290 (D1290) of the DCC intracellular domain (ICD) was also required for killing in 293T cells and may be mediated, in part, by caspases (Mehlen et al., 1998).

Two major signaling pathways have been reported to underlie apoptotic killing. They are known as the extrinsic pathway (also known as the death receptor pathway) and the intrinsic pathway (also called the mitochondrial pathway). Activation of the extrinsic pathway occurs when apoptotic stimuli outside the cell, such as death receptor ligands, bind their receptors on the cell surface, while the intrinsic pathway is typically engaged in response to signals from inside the cell, such as DNA damage, growth factor deprivation, or oxidative stress (reviewed by Jin and El Deiry, 2005). Common to both pathways are the involvement of caspases, a family of cysteine proteases that act as the 'executioners' of the apoptotic process by cleaving specific target proteins, allowing for its orderly destruction of the cell (reviewed by Goodsell, 2000).

Apoptotic killing through the extrinsic pathway involves the formation of a death-inducing signaling complex (DISC) following the binding of death-inducing ligands such as nerve growth factor (NGF) and tumor necrosis factor (TNF) superfamily members. The DISC includes caspase-8, an 'initiator' caspase, whose activation is followed by the activation of caspase-3, caspase-6, and caspase-7, the 'effector' caspases, which carry out

the cell's death program (reviewed byAshkenazi and Dixit, 1998). The intrinsic apoptotic pathway is activated when signals from within the cell result in the release of cytochrome c from the mitochondria. Cytochrome c associates with apoptosis protease releasing factor-1 (Apaf1) and the uncleaved initiator caspase, procaspase-9, to form another complex, the 'apoptosome'. This association results in the cleavage and activation of caspase-9 and subsequent activation of caspase-3 (Shi, 2002; Jin and El Deiry, 2005). Caspase-3 is critical for the terminal events of apoptosis downstream of both the intrinsic and extrinsic pathways (Lakhani et al., 2006).

Further overexpression studies by Forcet and colleagues (2001) revealed that caspase-9 and procaspase-3 both associate with the DCC ICD. The interaction with caspase-9 is located upstream of the cleavage site and is required for DCC-induced cell death. Interaction between caspase-9 and the DCC ICD is reduced in the presence of netrin-1, resulting in decreased apoptosis. Procaspase-3 associates with the C-terminal portion that is released following proteolysis, and its interaction with the DCC ICD is dependent on either the presence of netrin-1 or the mutation of D1290, a residue required for cleavage. This means, paradoxically, that procaspase-3 can only bind DCC under conditions where DCC can not induce cell death. This led the authors to speculate that in the presence of netrin, a pool of caspase-3 is unavailable for apoptotic signaling, and that caspase-3 sequestration may contribute to the antiapoptotic activity downstream of netrin-bound DCC.

This mechanism is distinct from the intrinsic pathway because neither cytochrome c release nor Apaf-1 is required for DCC-induced killing, and from the extrinsic pathway, because caspase-8 is not required. These findings led Forcet and colleagues to propose that DCC-induced killing in the absence of netrin-1 occurs by a novel apoptotic signaling pathway. Mehlen and Furne (2005) have proposed the following 'speculative model': In the absence of netrin-1, caspase-9 associates with the DCC ICD and promotes the activation of caspase-3, resulting in caspase-mediated cell death. They further suggest, based on the report of Forcet and colleagues, that the interaction between caspase-9 and DCC is indirect. The authors speculate that the adaptor protein involved may be DCC-interacting protein 13α (DIP13α), which interacts with the DCC ICD upstream of the

cleavage site and is required for DCC-induced killing (Liu et al., 2002). An interaction between DIP13α and caspases has not yet been reported.

ii. Mammalian UNC5 homologues as 'dependence receptors'

While the DCC ICD contains no motifs that hint at its death-promoting function, the intracellular domains of UNC5 netrin receptors contain a death domain, a conserved sequence found in death receptors including Fas and the tumor necrosis factor receptor (TNFR) (Hofmann and Tschopp, 1995; reviewed by Ashkenazi and Dixit, 1998). Similar to their findings with DCC, Mehlen and colleagues reported that transient transfection of 293T cells with full-length *unc5a*, *unc5b*, or *unc5c* resulted in significantly increased cell death, which was blocked by the presence of netrin-1 protein. The investigators concluded that death occurred by apoptosis because caspase activation was increased following transfection with UNC5B, and cell death was inhibited in the presence of a caspase inhibitor. As was observed with DCC, the UNC5B ICD is cleaved following transfection, resulting in the shedding of a C-terminal fragment containing the death domain. Unlike DCC, UNC5 homologues were cleaved at DXXD motifs; classical sites of caspase proteolysis (Thornberry et al., 1997). UNC5A, UNC5B, and UNC5C are all caspase substrates *in vitro*, and UNC5B cleavage in transfected cells did not occur when caspase activity was inhibited. Inhibition of UNC5B cleavage by mutation of the cleavage site resulted in a partial, but not complete, reduction in UNC5B-induced cell death (Llambi et al., 2001); suggesting that UNC5 ICD cleavage facilitates, but is not required for, UNC5-induced killing. Interestingly, the pro-apoptotic role of UNC5s had previously been hinted at by Hong and colleagues (1999), who used UNC5 constructs lacking the death domain for their studies of UNC5 function in the guidance of commissural axons, because transfection with sequences encoding the full-length protein resulted in the death of many of the transfected neurons.

Despite the common presence of a death domain in the ICDs of UNC5 family members, and the observation that all three can be cleaved by caspases, other reports suggest that apoptotic signaling downstream of UNC5 homologues varies. Llambi and colleagues (2005) have shown that UNC5B, but not UNC5A or UNC5C, interacts with death-associated protein kinase (DAPK) through its death domain, and that disruption of the UNC5B-DAPK interaction results in decreased apoptosis. DAPK, like the UNC5

homologues, has also been proposed to act as a tumor suppressor; promoting apoptosis through its serine-threonine kinase activity (Inbal et al., 1997; Cohen et al., 1999). Consistent with their potential involvement in a common tumor suppressive mechanism are the observations that DAPK requires p53 function for cell death induction (Raveh et al., 2001), and that UNC5B is transcriptionally regulated by p53 (Tanikawa et al., 2003). Association with UNC5B in the absence of netrin-1 decreases the inhibitory autophosphorylation of DAPK, while its catalytic activity is decreased in the presence of netrin-1. The mechanism by which UNC5B decreases DAPK phosphorylation is, however, unknown.

Another report, from the laboratory of Lindsay Hinck, suggests that the differences between the roles of UNC5 homologues in apoptosis may be even greater. Williams and colleagues (2003) provide evidence that UNC5A is a far more potent promoter of apoptosis than UNC5B or UNC5C. The investigators demonstrated that apoptosis following UNC5A overexpression is dependent on the interaction of its intracellular ZU5 domain with NRAGE. NRAGE has previously been demonstrated to be involved in apoptotic signaling by associating with the neurotrophin receptor p75NTR (Salehi et al., 2000). The interaction between UNC5A and NRAGE is specific, as NRAGE only weakly associates with UNC5B, and does not bind UNC5C at all. Like p75NTR (Coulson et al., 2000), the UNC5A ICD contains a juxtamembrane PEST sequence, a stretch of amino acids rich in proline, glutamic acid, serine, and threonine, that is required for UNC5A-NRAGE binding and UNC5A-induced apoptosis. The ICDs of UNC5B and UNC5C do not contain a PEST sequence, which may explain their relative inability to bind NRAGE or induce apoptosis. Williams and colleagues also reported that, contrary to what was reported previously by Llambi and colleagues (2001), deletion of the death domain had no effect on either UNC5A-mediated killing, or on the NRAGE-UNC5A interaction. This is not surprising because, while death domains were so named because of their presence in the ICDs of various death receptors, various lines of evidence suggest that their presence does not necessarily imply a role in apoptotic signaling (reviewed by Feinstein et al., 1995).

iii. Netrin-1, its receptors, and trophism in vivo

As multiple lines of *in vitro* evidence suggest that netrin receptors induce apoptosis in the absence of ligand, it would be expected that cell survival, and not just guidance, is aberrant in mice lacking netrin-1 or its receptors. In the embryonic neural tube, where the roles of netrin-1 in guidance of axon cell migration have been extensively characterized, many cell populations have been shown to express DCC and UNC5 netrin receptors (Keino-Masu et al., 1996; Leonardo et al., 1997; Jarjour et al., 2003; Tsai et al., 2003; Dillon et al., 2005). In netrin$^{-/-}$ mice, DCC and/or UNC5-expressing pontine (Yee et al., 1999) and inferior olivary neurons (Bloch-Gallego et al., 1999) show both guidance defects and decreased cell numbers relative to their wild-type littermates, though no increase in cell death was reported. Llambi and colleagues (2001) subsequently provided evidence for increased TUNEL labeling (which detects DNA fragmentation in apoptotic cells), in the hindbrains of netrin-1$^{-/-}$ mice. The authors suggested that the increase in TUNEL labeling was predominant in cells expressing DCC or UNC5B, consistent with the hypothesized death-inducing roles of these receptors. Mazelin and colleagues (2004) extend these findings to the colon where, they hypothesize, netrin-1 expressed at the base of intestinal crypts promotes the survival of early, proliferating cells but not differentiated, non-proliferating cells that have migrated towards the lumen, where less netrin is present. The investigators demonstrate that, in mice engineered to overexpress netrin-1 throughout the intestine, fewer apoptotic crypt cells are observed. Overexpression of netrin-1 was also associated with the formation of spontaneous pre-cancerous intestinal epithelial growths (adenomas) in some mice. When netrin-1 was overexpressed in an adenomatous polyposis coli (APC) mutant background, which predisposes the animals to develop adenomas, more severe adenomas were observed. The netrin-overexpressing *apc*$^{-/-}$ animals also occasionally developed severe invasive carcinomas that did not occur in *apc*$^{-/-}$ mutants that expressed netrin-1 normally. The investigators concluded that these phenotypes were the result of disregulated cell survival due to the excess netrin-1 present (Mazelin et al., 2004). Taken together, these data suggest regulating cell death signaling may underlie the tumor suppressive roles of netrin-1 and its DCC and UNC5 receptors *in vivo*.

II. OLIGODENDROGLIAL DEVELOPMENT AND CNS MYELINATION

The initial characterization of oligodendrocytes in the early 1920s was the subject of much debate, and marks one of the few instances were the ideas of Ramón y Cajal proved incorrect. While Ramón y Cajal believed that glial cells (astroglia) and their processes could insulate nerve cells, he thought that myelin was a 'more perfect' insulation than could be provided by glia. He described the adendritic and apolar-appearing cells in the CNS white matter as the "third element", and speculated that they may be responsible for central myelin (Ramón y Cajal, 1913). His student Pio del Río-Hortega later determined that this "third element" consisted of two distinct process-bearing cell types that had previously been inadequately stained. They were identified as microglia and oligodendrocytes, which were at first called 'intrafasicular glia' (del Río-Hortega, 1922). Work by Wilder Penfield confirmed the identity of oligodendrocytes, and further suggested that they were the myelin forming cells of the CNS (Penfield, 1924). Ramón y Cajal eventually ceded that microglia were a separate cell type, but not oligodendroglia (Ramón y Cajal, 1920). Such was the magnitude of this disagreement between one-time student and teacher that it is said to have led to a break in their relationship (Somjen, 1988).

The emergence of myelin, a highly-specialized multilamellar membrane formed by oligodendrocytes in the CNS and Schwann cells in the PNS, was a seminal event in the evolution of the vertebrate nervous system. In the CNS, the myelin sheath elaborated by an oligodendrocyte functions as an electrical insulator that promotes the rapid propagation of axon potentials without requiring large-diameter axons, meaning that many rapidly-conducting axons could be compacted into a limited space (reviewed by Baumann and Pham-Dinh, 2001).

Although myelinating oligodendrocytes are present throughout the adult CNS, oligodendrocyte precursor cells (OPs) arise only in specific regions of the developing CNS, ventricular or subventricular domains next to regions of Sonic hedgehog expression (Orentas et al., 1999; Davies and Miller, 2001; Nery et al., 2001). From these sites close to the ventral midline of the developing CNS, they migrate significant distances to reach their axonal targets and populate the nascent white matter. These cells then proliferate, differentiate, establish contact with, and wrap target axons (Fig. 1.3), forming one of the most extensive intercellular specializations in the CNS.

A. Origins of OP cells

The origin of the oligodendrocyte lineage has been extensively studied, and has historically been the source of vigorous debate within the field (Richardson et al., 2000; Spassky et al., 2000; Miller, 2002; Gabay et al., 2003; Noble et al., 2004; Richardson et al., 2006). Recently, many advances have been made in understanding the lineage relationships between neurons, OPs, and astrocytes (Rowitch, 2004; Richardson et al., 2006). The current model is based on evidence that the generation of neuronal precursor cells and OPs is closely linked, and that bipotential precursor cells give rise to both in the presence of the morphogen sonic hedgehog (shh). Expression of olig2, a transcription factor upregulated by sonic hedgehog, defines a group of precursor cells in the ventral pMN domain of the embryonic spinal cord that gives rise to both motor neurons and oligodendrocytes (Zhou et al., 2001). Olig2 expression is required for the development of all pMN progeny (Lu et al., 2002; Takebayashi et al., 2002). Not all oligodendrocytes in the spinal cord arise from the pMN domain, however. In mice lacking the expression of transcription factors nkx6.1 and nkx6.2, which regulate the expression of olig2, the pMN domain does not give rise to ventral OPs and motor neurons. Some cells expressing olig2 and OP marker PDGF-alpha receptor (PDGFαR) do, however, still appear in the absence of nkx6.1 and nkx6.2. These OPs, which appear later than the pMN derived cells, expressed dbx1 and pax7, markers of dorsal domains of the neural tube normally associated with the generation of interneurons and astrocytes. It was concluded that a second 'wave' of OPs originates dorsally (Cai et al., 2005; Vallstedt et al., 2005). However, in wild-type animals, dorsally derived OPs, which are derived from radial glia, contribute a relatively low percentage of total spinal cord oligodendrocytes. Estimates of the exact percentage vary, ranging from under 5% to as much as 15% (Fogarty et al., 2005; Richardson et al., 2006).

Recent fate-mapping studies suggest that OPs also originate in multiple 'waves' from multiple regions in anterior regions of the CNS. The first PDGFαR-positive OPs originate in the neuroepithelium of the medial ganglionic eminence (MGE) and disperse into all regions of the embryonic forebrain. A second ventrally-originating wave of OPs then arrives from the lateral and caudal ganglionic eminences (LGE-CGE). Late during

embryogenesis, a third population of OPs generated within the cortex itself populates the forebrain. These dorsally-derived OPs appear to replace the MGE-derived cells, as the earliest-arriving cells are eliminated by adulthood (Kessaris et al., 2006; reviewed by Richardson et al., 2006).

While the studies described above firmly established that OPs originate both dorsally and ventrally in the brain and spinal cord, the existence of multiple oligodendrocyte lineages had been proposed years earlier. In 1995, Zalc, Thomas, and colleagues proposed that the expression of DM-20, an alternatively spliced form of proteolipid protein (PLP), defines an early-occurring OP population (Timsit et al., 1995). The early PLP/DM-20-expressing cells do not express PDGFαR, suggesting that they do not require PDGF-AA for their proliferation and survival, unlike the vast majority of oligodendrocytes. The PDGF-independence of these cells was demonstrated explicitly using dissociated CNS cultures prepared from transgenic embryos in which the PLP promoter drives expression of an antibiotic resistance gene and β-galactosidase. When cells expressing the transgene were selected for and allowed to differentiate in vitro, the vast majority became oligodendroglia, even when PDGFαR function was inhibited (Spassky et al., 1998; Spassky et al., 2001; reviewed by Le Bras et al., 2005).

However, the idea that PLP/DM-20 expression defines a distinct oligodendroglial lineage remains controversial. While this cell population possesses the capacity to differentiate into oligodendroglia, it is worth noting that the *in vitro* differentiation experiments were carried out under culture conditions favourable to oligodendrocyte differentiation (Bottenstein and Sato, 1979; Eccleston and Silberberg, 1984). It has been suggested that the early-appearing population of PLP/DM-20-positive may consist of multipotent progenitors (Yu et al., 1994; Richardson et al., 2000), raising the possibility that the PLP/DM-20-positive population may give rise to cell types other than oligodendrocytes *in vivo*.

An alternative model of oligodendrocyte specification proposes that OPs arise from glial-restricted precursors (GRPs). GRPs differ from OPs in their responses to trophic factors, mitogens, differentiation factors, and their capacity to give rise to type-1 astrocytes (Rao et al., 1998; Gregori et al., 2002). When they were first described, the principal difference between GRPs and OPs was that GRPs could be isolated from both

the dorsal and ventral embryonic spinal cord, while OPs were believed to arise specifically from the ventral spinal cord *in vivo* (Gregori et al., 2002; Warf et al., 1991; Pringle and Richardson, 1993). A later investigation revealed that dissociated cultures prepared from immature CNS can, regardless of their origin, can be 'dorsalized' or 'ventralized' depending on culture conditions. For this reason, it was suggested that GRPs are generated *in vitro* by deregulating normal dorsoventral patterning, and may not exist, much less contribute to OP development, *in vivo* (Gabay et al., 2003). With the recent reports that some OPs do, in fact, have a dorsal origin, it is apparent that yet more investigation is necessary to resolve questions about the lineage relationships between oligodendroglia and other cells of the CNS.

B. Trajectories of OP migration

The early OP populations that populate the developing cortex originate in the medial ganglionic eminence (MGE) and, to a lesser extent, the lateral ganglionic eminence (LGE) and anterior entopeduncular area. OPs first populate more ventral regions of the telencephalon, and then migrate dorsally into the developing cortex (Spassky et al., 1998; Sussel et al., 1999; He et al., 2001; Olivier et al., 2001). OPs originating in the subventricular zone of the ventral diencephalon populate the developing thalamus and hypothalamus (Pringle and Richardson, 1993). In the metencephalon, OPs first appear in a small focus in the ventricular zone adjacent to the floor plate and subsequently migrate dorsally and caudally to populate the pons and cerebellum (Ono et al., 1997a). The cues that direct OP migration in these regions of the developing CNS are not well understood. Furthermore, because the final population of cortical oligodendrocytes are derived from a later-arriving precursor population originating within the cortex itself (reviewed by Richardson et al., 2006), the relative importance of the cues controlling earlier waves of precursor migration is unclear.

OP migration has been extensively studied in both the developing optic nerve and spinal cord. OPs that populate the optic nerve originate at the ventral midline of the third ventricle just dorsal to the optic chiasm. They then migrate from the chiasm into the nerve, populating it along its entire length (Ono et al., 1997b; Small et al., 1987). The optic nerve head appears to prevent migrating OPs from invading the retina (Ffrench-

Constant et al., 1988; Morcos and Chan-Ling, 2000). In the embryonic spinal cord, OPs originate in the ventral ventricular zone, at two foci located on either side of the midline, slightly dorsal to the floor plate (Pringle and Richardson, 1993; Yu et al., 1994). Although OPs migrate from the ventral midline throughout the spinal cord (Ono et al., 1995; Calver et al., 1998), the precise paths taken are not well characterized. Radial glia may contribute to directing OPs laterally (Diers-Fenger et al., 2001). Glial precursor cells have been observed to migrate along white matter tracts in slice culture preparations (Kakita and Goldman, 1999), but OPs do not migrate exclusively along pre-existing axon tracts in either the optic nerve or spinal cord (Ueda et al., 1999; Miller et al., 1997; Calver et al., 1998). This observation led to the suggestion that secreted long-range chemotropic cues might play a key role (Miller et al., 1997).

C. Cues that Regulate OP Motility

Multiple factors that influence OP motility have been identified using *in vitro* assays. These include components of the extracellular matrix and secreted factors. It has been useful to catalogue proteins that direct axon extension into four classes: substrate-bound attractants and repellents, which act as short-range guidance cues, and secreted attractants and repellents, which act as long-range cues (Tessier-Lavigne and Goodman, 1996). Substrate-bound short-range cues remain associated with the surface of the cells that secreted them or the neighbouring extracellular matrix (ECM), acting in the immediate vicinity of their source. Long-range cues, in contrast, may function many cell diameters away from the cells that produce them. The same terminology can be applied to cues that guide OP migration (Jarjour and Kennedy, 2004; De Castro and Bribian, 2005). Factors that have been reported to direct OP migration are summarized in Figure 1.4.

i. Substrate-bound cues

It has been proposed that substrate-bound cues influence the migration of OPs *in vivo* by regulating their adhesive interactions with axonal tracts, astrocytic surfaces, and the ECM (reviewed by De Castro and Bribian, 2005). Most such factors have been identified on the basis of their influencing the migration of OPs in culture. Using the agarose drop assay, Milner and colleagues (1996) found that substrates of laminin, fibronectin, vitronectin, or 'astro-glial matrix' (ECM secreted by astrocytes *in vitro*)

promote OP migration, while a collagen substrate inhibits migration. In transfilter migration assays, membranes coated with fibronectin or laminin-2 (merosin) promoted OP migration, while a coating of tenascin-C inhibited migration (Frost et al., 1996; Kiernan et al., 1996). In contrast, no stimulation of OP migration was found when OPs were confronted with gradients of soluble fibronectin or laminin-1 in transfilter assays (Armstrong et al., 1990). These findings provide evidence that substrate bound fibronectin or laminin-1 promotes OP migration, but suggest that gradients of these cues do not guide OPs. It remains to be determined if substrate bound gradients of these cues can direct OP migration.

$\alpha_V\beta1$ integrins are expressed by migratory OPs and bind fibronectin and vitronectin, both of which are expressed in developing white matter tracts during development (Sheppard et al., 1991; Pons and Marti, 2000). OP migration on astro-glial matrix was blocked by peptides inhibiting α_V or $\beta1$ integrin function (Milner et al., 1996). Unlike fibronectin and vitronectin, laminin-1 is restricted to basement membranes and capillary walls in both the developing and mature CNS (Hunter et al., 1992), suggesting a limited influence on OP migration *in vivo*.

Recent *in vitro* studies have hinted at roles in OP migration for another well-established family of guidance molecules, the ephrins. The ephrin ligands and their Eph receptors are a large group of receptor tyrosine kinases involved in neural development (reviewed by Wilkinson, 2001). Ephrins and their ligands signal bidirectionally. "Forward" signaling through Eph receptors and "reverse" signaling through transmembrane ephrin ligands regulate motility in many contexts including axon guidance, cell migration and angiogenesis (reviewed by Davy and Soriano, 2005). Eph receptors are extensively involved in the development of the visual system, as they are expressed by retinal ganglion cells and regulate retinocollicular mapping (reviewed by McLaughlin et al., 2003). Prestoz and colleagues (2004) have reported that OPs migrating into the optic nerve express ephrins B2 and B3, and selectively associate with EphB-coated substrates *in vitro*. They have proposed that interactions between ephrins and Eph receptors present on retinal ganglion cell axons regulate the distribution of OPs in the optic nerve . Conversely, a review by Cohen (2005) references unpublished observations suggesting that OPs express EphB receptors, and their migration is restrained by EphrinB

ligands *in vitro*. Considerable further investigation remains to be carried out before the roles of ephrins and Eph receptors in the regulation of OP migration are understood.

Little is known about the roles that these substrate-bound cues play in the intact CNS. A limited role in oligodendroglial development *in vivo* has been established for tenascin-C. Mutant mice lacking tenascin-C exhibit increased rates of OP migration in the developing optic nerve and reduced rates of OP proliferation. No phenotype was detected later in development, suggesting that there is compensation for these defects. Tenascin-C is also expressed at the optic nerve head and may contribute to preventing OP migration into the retina, but OPs are not detected in the retina in tenascin-C null mice, suggesting that other cues may share this function (Garcion et al., 2001).

ii. Secreted Cues

In addition to cues associated with cell surfaces or ECM, many secreted factors can guide the migration of OPs. These include many with known roles in the guidance of migrating axons and cells, including many growth factors. PDGF-A and basic fibroblast growth factor (bFGF or FGF-2) promote OP migration in both agarose drop and transfilter migration assays (Milner et al., 1996; Armstrong et al., 1990; Simpson and Armstrong, 1999). Evidence obtained using both assays suggests that while PDGF-A is a more potent attractant than FGF-2, the effects of PDGF-A and FGF-2 are not additive. This suggests that both factors may stimulate migration through similar signal transduction mechanisms (Simpson and Armstrong, 1999). Published reports are not unanimous on this point, however. Osterhout and colleagues (1997) reported that introduction of a dominant-negative FGF receptor resulted in the inability of OPs to migrate *in vivo*, even though PDGF signaling remained intact . Because FGF-2 has been reported to inhibit oligodendroglial differentiation (McKinnon et al., 1990), the inability to migrate may be secondary to accelerated differentiation and consequent decreased motility in these cells. Further differences between migration in response to the two growth factors are reported by Zhang and colleagues (2004), who observed that OP attraction to PDGF, but not FGF, requires that they express polysialic acid-neural cell adhesion molecule (PSA-NCAM).

During OP migration, PDGF-A is expressed by astrocytes in the optic nerve (Mudhar et al., 1993; Fruttiger et al., 2000) and by neurons in the developing brain and

spinal cord (Yeh et al., 1991; Hutchins and Jefferson, 1992). Reduced numbers of OPs and oligodendrocytes are found in PDGF-A knockout mice (Fruttiger et al., 1999), but because PDGF regulates OP proliferation in addition to guidance (Noble et al., 1988), it is difficult to differentiate between its trophic and tropic effects. FGF-2 is unlikely to play a major role in OP migration *in vivo*, as migration is largely complete prior to maximal FGF-2 expression (Murtie et al., 2005).

Other growth factors have also been reported to influence OP migration *in vitro*. OPs express met, the receptor for hepatocyte growth factor/scatter factor (HGF/SF) (Kilpatrick et al., 2000). HGF/SF promotes OP migration in the transfilter migration assay (Yan and Rivkees, 2002). Furthermore, application of HGF/SF induces OP process extension, and increased actin and β-tubulin expression (Yan and Rivkees, 2002). Vascular endothelial growth factor (VEGF) has been reported to attract migrating undetermined neural progenitors, including cells that may later differentiate into oligodendroglia, in an FGF-dependent manner (Zhang et al., 2003). However, roles for HGF/SF and VEGF in oligodendroglial development *in vivo* have not yet been established.

Another family of proteins originally described as axon guidance cues that have been implicated in guiding OP migration are the semaphorins, a large family of secreted, transmembrane, GPI-linked proteins defined by an amino-terminal 'sema' domain. The extensively characterized secreted class 3 semaphorins act primarily as secreted repellent cues but also function as attractants for some axons (reviewed by Raper, 2000). Like netrin-1, semaphorin-3A (sema3A) is expressed at the optic chiasm (Sugimoto et al., 2001) and in the ventral spinal cord (Messersmith et al., 1995) during OP migration. Class 3 semaphorins act through a receptor complex consisting of neuropilins (Raper, 2000) and plexins (Tamagnone et al., 1999). While expression of plexin family members by OPs has not been reported, cultured OPs express neuropilin family members, as do OPs migrating in the developing optic nerve, but expression is reduced as they differentiate (Sugimoto et al., 2001; Spassky et al., 2002; Cohen et al., 2003). Of interest, OPs isolated from post-natal rat brain express multiple class 3 semaphorins, membrane spanning class 4, 5, and 6 semaphorins, and a membrane tethered GPI-linked class 7 semaphorin (Cohen et al., 2003). Sema5A is expressed only by oligodendrocyte lineage

cells, and inhibits the outgrowth of retinal ganglion cell axons *in vitro*, suggesting that sema5A may be a glial cue that inhibits regeneration of CNS axons following injury (Goldberg et al., 2004). Very little is known about the functions of other semaphorins expressed by OPs.

Three reports have investigated the role of class 3 semaphorins in OP migration, but the results of these studies vary. Evidence has been presented that Sema3A repels OPs migrating from explants of embryonic mouse optic nerve (Spassky et al., 2002) or has no effect on OPs migrating from explants of neonatal rat optic nerve (Sugimoto et al., 2001). Consistent with a repellent action, OPs derived from neonatal rat brain avoid substrate stripes of Sema3A, 3B, 3C, and 3F (Cohen et al., 2003). Spassky et al. (2002) also provide evidence that Sema3F attracts OPs migrating from optic nerve explants. Differences in age, species, and experimental protocol may account for the heterogeneity of these observations. The contribution of semaphorins to OP guidance *in vivo* is currently not clear.

iii. Chemokines

While the factors described above guide migrating OPs, Tsai and colleagues (2002) have provided evidence that expression of the chemokine CXCL1 by astrocytes in the developing white matter instructs migrating OPs to stop. Addition of exogenous CXCL1 inhibited OP migration within explants of embryonic spinal cord and blocked OP migration to PDGF-A in a transfilter migration assay. CXCL1 signals via the chemokine receptor CXCR2, which is expressed by OPs both *in vitro* and *in vivo*. In mice lacking CXCR2, oligodendrocytes are abnormally concentrated at the outermost edges of the cord, consistent with the hypothesis that white matter derived CXCL1 acts via CXCR2 to halt OP migration (Tsai et al., 2002).

Another chemokine, CXCL12, appears to play a radically different role. CXCL12 exerts an attractant response on OPs in the microchemotaxis assay. This response is mediated by a second chemokine receptor, CXCR4, which is expressed at a high level by motile precursors but whose expression level decreases as cells differentiate into oligodendrocytes. CXCL12 is expressed by vascular endothelia and the pia mater in the developing spinal cord, and mice lacking CXCR4 expression show decreased

ventrodorsal OP migration, suggesting that CXCL12 promotes OP dispersal *in vivo* as well as *in vitro* (Dziembowska et al., 2005).

D. Oligodendroglial proliferation and differentiation

While proper migration of OPs is necessary for CNS myelination, the vast majority of the total OPs in the developing CNS are produced by the proliferation of precursors arriving in the developing white matter. A number of mechanisms exist that regulate proliferation and cell survival, restricting both OP and oligodendrocyte number, ultimately matching the number of oligodendrocytes produced with the number of axons to be myelinated. Many of these same factors controlling proliferation, and others, also regulate the differentiation of oligodendroglia from early progenitors into more mature progenitors, then into immature, non-myelinating oligodendrocytes, and finally into mature, myelinating oligodendrocytes (reviewed by Miller, 2002).

i. Regulation of OP number

Crucial to generation of the proper number of oligodendroglia are growth factors. Principal among them are platelet-derived growth factor-A (PDGF-A) and fibroblast growth factor-2 (FGF-2), which are often used in tandem to promote OP proliferation *in vitro* (McKinnon et al., 1990; Bogler et al., 1990). Their contributions to OP development *in vivo* are distinct, however.

PDGF-A is critical to early oligodendroglial development, and is expressed throughout the developing CNS (Yeh et al., 1991). The earliest OPs are small, bipolar cells recognized by the monoclonal antibody A2B5 that express high levels of PDGFαR, making them highly proliferative in the presence of PDGF-A (Raff et al., 1984; Noble et al., 1988; Pringle et al., 1992). In PDGF-$A^{-/-}$ mice, very few OPs and, consequently, oligodendrocytes developed throughout the CNS (Calver et al., 1998; Fruttiger et al., 1999). Conversely, when PDGF-A was overexpressed in developing spinal cord or optic nerve, an increased number of OPs were produced. The number of proliferating cells increased proportionally with mitogen supply both *in vitro* and *in vivo*, suggesting that the supply of PDGF-A limits OP number (Fruttiger et al., 2000; van Heyningen et al., 2001). PDGF-A is thus a powerful mitogen during early oligodendroglial development.

Growth factors do not act alone to regulate OP proliferation, as other factors have been shown to modulate PDGF signaling. In addition to arresting OP migration, CXCL1 (also called GROα) acts synergistically with PDGF-A to promote OP proliferation *in vitro* (Robinson et al., 1998). Increased OP number in the *jimpy* mutant spinal cord, where CXCL1 expression is upregulated, and decreased numbers of oligodendrocytes in the $CXCR2^{-/-}$ spinal cord suggest that CXCL1 plays a similar role *in vivo* (Wu et al., 2000; Tsai et al., 2002). Thus, the role of CXCL1 in oligodendrocyte development may be to act as a developmental switch, prompting OPs to respond to PDGF as a proliferative, and not a migratory, cue once they have reached their destinations in the presumptive white matter. The transition from migration to proliferation as OPs mature is accompanied by changes in integrin expression. Downregulation of β1 expression, accompanied by a concomitant increase in β3 expression, correlates with reduced motility and increased proliferation in cells cultured on integrin ligand substrates (Milner et al., 1996; Blaschuk et al., 2000; Garcion et al., 2001).

Integrins are key modulators of growth factor-induced OP proliferation. Such modulation is necessary for the response to PDGF-A *in vivo*, because the physiological concentration of PDGF in the embryonic CNS (<1 ng/ml, van Heyningen et al., 2001) is insufficient to induce OP proliferation *in vitro*. Culturing OPs on substrates of ECM ligands that bind $\alpha_v\beta3$ integrin sufficiently increased the sensitivity of these cells to low PDGF-A concentrations to allow proliferation, suggesting that the appropriate ECM environment is required for PDGF-induced OP proliferation *in vivo* (Baron et al., 2002). Accordingly, mice deficient in tenascin-C, an $\alpha_v\beta3$ ligand, show reduced OP proliferation *in vivo* (Garcion et al., 2001). Neurotrophin-3 and insulin-like growth factor also act as mitogens for OPs, and further potentiate PDGF-induced proliferation *in vitro* (Barres et al., 1994; Jiang et al., 2001), though their roles *in vivo* are less clear.

OP number is also regulated negatively, independently of any soluble factor, by feedback inhibition that inhibits precursor proliferation at high cell densities (Zhang and Miller, 1996). Increased OP density *in vitro* resulted in increased expression of cell cycle inhibitor $p27^{kip1}$, decreased cyclin A expression, and changes in Rb phosphorylation, all of which are reversed when cells are re-cultured at lower densities (Nakatsuji and Miller,

2001). Density-dependent feedback inhibition in OPs proliferating *in vivo* has not yet been confirmed, however.

ii. Oligodendrocyte differentiation

During maturation from the early to late OP stage, A2B5+ cells become multipolar and begin to express antigens recognized by the O4 monoclonal antibody while losing proliferative responses to PDGF. Cells then differentiate into immature oligodendrocytes, downregulating A2B5 antigen and upregulating galactocerebroside (Hart et al., 1989; Bansal and Pfeiffer, 1992; Fok-Seang and Miller, 1994). FGF-2 can promote the proliferation of both OPs and immature oligodendrocytes in culture (Gard and Pfeiffer, 1993; Fok-Seang and Miller, 1994), but also regulates oligodendrocyte differentiation. Exposure to FGF-2 inhibits differentiation of cultured OPs, maintaining the cells as immature precursors, and withdrawal of PDGF-A and FGF-2 from OP cultures induces rapid cell cycle exit and differentiation (McKinnon et al., 1990; Raff et al., 1990). *In vivo*, FGF-2 is expressed throughout the developing CNS (Ernfors et al., 1990). While no change in OP proliferation was observed FGF-2 null mice, fewer oligodendrocytes were produced due to premature differentiation, consistent with observations *in vitro* (Murtie et al., 2005).

The response to FGF-2 is mediated by different FGF receptors (FGFRs) throughout development. FGFR1 is expressed at all stages of oligodendrocyte development, and like PDGFαR, its expression is upregulated by FGF-2 (McKinnon et al., 1990; Bansal et al., 1996). FGFR2 expression is low in OPs and increases following differentiation into oligodendroglia. FGFR3 expression peaks in mature OPs immediately prior to differentiation, leading to the suggestion that this receptor mediates the differentiation-inhibiting role of FGF-2 (Bansal et al., 1996). However, Oh and colleagues (2003) reported delayed oligodendroglial differentiation and myelination in the absence of FGFR3 expression but no change in OP number, OP proliferation, or oligodendrocyte survival; leading them to conclude that FGFR3 promotes oligodendroglial differentiation. This conclusion is inconsistent with the observed phenotype in FGF-$2^{-/-}$ mice (Murtie et al., 2005). Murtie and colleagues also reported that the timing of FGF-2 expression was inconsistent with the defects in oligodendroglial development in FGFR3 knockout mice. Together with the observation that the affinity of

this receptor for FGF-2 is much lower than for FGF-1 (Ornitz and Leder, 1992), these findings suggest that FGFR3 does not mediate the response of OPs to FGF-2 *in vivo*. Recently, other FGF family members have been demonstrated to have varying effects on oligodendroglial proliferation and maturation *in vitro* (Fortin et al., 2005), though their roles *in vivo* are not yet understood.

Other factors also regulate oligodendroglial maturation. Transforming growth factor β (TGF-β) and its receptors are expressed by cultured OPs, and inhibit proliferation while promoting differentiation *in vitro* (McKinnon et al., 1993). As with migration and proliferation, α_V integrin is involved in this step of development, this time complexed with the β5 subunit. Following a final burst of proliferation, $\alpha_V\beta3$ integrin expression is downregulated and $\alpha_V\beta5$ expression is upregulated just prior to final differentiation. Blocking $\alpha_V\beta5$ function inhibits differentiation *in vitro* (Milner and Ffrench-Constant, 1994; Blaschuk et al., 2000). Another integrin, $\alpha_V\beta8$, is expressed throughout oligodendroglial development. Its expression is high in OPs, decreases during periods of proliferation (coincident with times of peak $\alpha_V\beta3$ expression) and increases again in differentiating and differentiated oligodendrocytes. Unlike the other integrins expressed by oligodendroglia, the expression of the β8 subunit is downregulated in the presence of neurons. Its function is unclear (Milner et al., 1997).

iii. Control of oligodendrocyte number and final differentiation

Studies of the influence of growth factors on oligodendroglial development revealed a disconnect between the number of OPs and the final number of oligodendrocytes generated. During the normal development of the oligodendrocyte lineage as many as 50% of all oligodendrocytes *in vivo* are eliminated by apoptosis, suggesting that a surfeit of OPs is initially produced. The cells usually die soon after differentiation, prior to upregulating the expression of myelin genes such as myelin basic protein (MBP) and proteolipid protein (PLP, Barres et al., 1992). When excess OPs and immature oligodendrocytes are produced in mice engineered to overexpress PDGF-A, increased proliferation is compensated for by increased elimination of immature oligodendrocytes by programmed cell death. The result is a final number of mature, myelin-forming cells not significantly different from that observed in wild-type animals. This implies that separate mechanisms regulate OP and oligodendrocyte number, and that

signals controlling the survival of oligodendrocytes override those controlling proliferation (Calver et al., 1998).

The survival of recently-differentiated oligodendrocytes is restricted by competition for available axonally-derived survival factors. Some of these trophic factors are secreted, such as PDGF-A and neuregulin (also known as glial growth factor), and others, such as laminin-α2, are associated with the axon surface (Barres et al., 1992; Barres and Raff, 1999). Neuregulin promotes survival of mature OPs and oligodendrocytes, but not early OPs, *in vitro*. In spinal cord explants derived from neuregulin$^{-/-}$ mice, oligodendrocytes fail to develop, a phenotype that could be rescued by addition of exogenous neuregulin (Vartanian et al., 1999). Neutralization of endogenous neuregulin function in the optic nerve resulted in increased oligodendroglial death, while normally-occurring death was decreased when additional neuregulin was provided (Fernandez et al., 2000). Curiously, while neuregulin promoted the survival of oligodendrocytes *in vitro*, it also increased proliferation and inhibited differentiation of OPs (Canoll et al., 1996). Similarly, as described above, PDGF-A is also a mitogen and migratory cue for immature OPs. This raises the question: What is responsible for the switch between the disparate roles played by growth factors during early and late oligodendroglial development?

The answer again lies with integrin function; specifically that of α6β1 integrin. Activation of α6β1 switches the signaling response of oligodendroglia to PDGF-A from proliferation to survival while simultaneously increasing the sensitivity of the cells to PDGF. Similarly, though likely by a different intracellular mechanism, pro-proliferation, anti-differentiation signaling induced by neuregulin is inhibited in favor of a survival-promoting response, and final differentiation into mature, MBP-expressing oligodendrocytes (Frost et al., 1999; Colognato et al., 2002; reviewed by Baron et al., 2005). Accordingly, inhibition of α6β1 integrin function *in vitro* greatly decreases oligodendrocyte survival while blocking the activity of α_V integrins, which are involved in migration and proliferation, does not (Frost et al., 1999). Similarly, in CNS tissue derived from mice lacking α6 integrin, fewer oligodendrocytes are generated; the result of a substantial increase in cell death (Colognato et al., 2002).

The importance of α6β1 integrin function to the later stages of oligodendrocyte development implicates its ligands, members of the laminin family that contain the α2 chain such as laminin-2, laminin-4, and laminin-13, in the regulation of oligodendrocyte development. Previous findings had indirectly suggested that substrate-associated factors such as laminins play a role in oligodendrocyte survival. It was observed to be greatly enhanced by oligo-axonal contact *in vitro*, as this effect is seen when the cells are co-cultured with purified neurons, but not in the presence of neuron-conditioned medium (Barres et al., 1993). This trophic effect can be mimicked by substrates of α6β1 ligands, but not α_V integrin ligands (Frost et al., 1999). *In vivo*, Laminin-α2 is expressed in myelinating axon tracts in the CNS (Colognato et al., 2002) and, consistent with this, the CNS of laminin-α2$^{-/-}$ mice contains fewer mature oligodendrocytes relative to wild-type littermates, and is hypomyelinated (Chun et al., 2003). Some evidence also exists that laminin-α2 and α6β1 integrin promote the maturation of immature oligodendrocytes into mature, myelinating oligodendroglia, and the formation of myelin membrane sheets (Colognato et al., 2002). It is difficult, however, to differentiate between effects on survival, differentiation, and myelin formation *in vivo*.

Interestingly, as with netrin receptors, Src family kinases (in this case lyn and fyn) are key mediators of signaling downstream of integrins in oligodendroglia. Lyn associates with $\alpha_V\beta3$ integrin and is required for enhancement of PDGF-induced proliferation. Upon ligand binding to α6β1 integrin *in vitro*, lyn is released from its association with the integrin complex and inactivated while fyn associated with α6β1 integrin is activated. Fyn, and not lyn, was shown to be required for multiple α6β1 integrin-mediated responses including the switch from proliferative to trophic signaling downstream of neuregulin, increased oligodendrocyte survival, late differentiation to MBP+ stages, and the extension of myelin-like membranes (Colognato et al., 2004). This interaction likely occurs in vivo as well, as the hypomyelination phenotype in the CNS of fyn$^{-/-}$ mice closely resembles that observed in the absence of laminin-α2 (Umemori et al., 1994; Sperber et al., 2001; Chun et al., 2003).

Finally, it must be noted that the relative importance of these proteins to myelination is not identical in all regions of the CNS. The hypomyelination phenotype observed in mice lacking laminin-α2 or fyn is restricted to the forebrain and optic nerve,

while myelination in the caudal spinal cord is normal; correlating with regions of high and low laminin-α2 expression, respectively (Umemori et al., 1994; Sperber et al., 2001; Colognato et al., 2002). It is possible that different ECM molecules may influence survival and late differentiation in rostral and caudal regions of the CNS. Alternatively, these differences may also arise from variances in gene expression among oligodendroglial lineages.

E. Regulation of axo-glial contact at the CNS paranode

Following initial contact between the oligodendrocyte process and the target axon, the process extends, spiraling around the axonal surface to form a multilamellar myelin sheath encircling one axonal segment. During myelination, overlying layers of oligodendrocyte membrane become compacted, extruding cytoplasm towards the edges of each myelinated segment. Certain proteins become enriched within these cytoplasm-rich regions of non-compacted myelin membranes, termed the paranodal loops. Many of these proteins are involved in intercellular adhesion, resulting in the close association between the paranodal myelin membrane and the axonal membrane (also called the axolemma). In mature myelin, the paranodal loops are arranged in a roughly regularly spaced and symmetric pattern; with paranodal loops from the edges of adjacent myelin segments surrounding a short, unmyelinated portion of axon, called the node of Ranvier (NOR). This results in the division of each nodal region into three distinct domains: The node itself, the paranode, and the juxtaparanode (Peles and Salzer, 2000; Salzer, 2003). Domain organization in the CNS and PNS is nearly identical (reviewed by Poliak and Peles, 2003), but for technical reasons, much of the investigation in this field has been carried out in peripheral myelin. For this reason, many of the studies referred to below were carried out in PNS. Instances where known differences exist between CNS and PNS will be noted specifically.

i. The node of Ranvier

Conduction along myelinated axons is saltatory, jumping from one NOR to the next (reviewed by Baumann and Pham-Dinh, 2001). Critical to the establishment of saltatory conduction are the presence of high concentrations of voltage gated Na^+ channels at the initial axonal segment (the unmyelinated portion of the axon between the neuronal cell

body and the first myelinated segment), and the NORs (reviewed by Peles and Salzer, 2000). Na^+ channels clustered at the NOR are part of multiprotein complexes present that also contain neural-glial-related cell adhesion molecule (NrCAM), and the 186 kDa isoform of neurofascin (nfc186), and Na^+ channels physically associate with the latter (McEwen and Isom, 2004). While nfc186 is required for assembly of the nodal complex (Sherman et al., 2005), NrCAM is not (Lustig et al., 2001; Custer et al., 2003). Also present in these complexes are ankyrinG and βIV-spectrin, two proteins believed to link these complexes to the cytoskeleton (Kordeli et al., 1990; Davis et al., 1996; Tait et al., 2000; Jenkins and Bennett, 2002; Komada and Soriano, 2002).

While the assembly of nodal complexes has not been extensively investigated in CNS, it is better understood in PNS. In PNS, where Schwann cell-axon contact is required to induce the formation of nodal complexes (Peles and Salzer, 2000), gliomedin present on Schwann cell microvilli is a ligand for axonal NrCAM and nfc186, and these proteins accumulate before Na^+ channels (Custer et al., 2003). Association of gliomedin with NrCAM and nfc186 is necessary and sufficient to induce Na^+ channel clustering in cultured DRG axons *in vitro* (Eshed et al., 2005). For this reason, it is hypothesized that sites of gliomedin binding to NrCAM and nfc186 'nucleates' the formation of Na^+ channel-containing nodal protein complexes. However, while the composition of CNS nodal complexes is similar to those in PNS, their formation in the CNS appears to be dependent on a factor or factors secreted by oligodendrocytes, but not oligo-axonal contact or myelination (Kaplan et al., 1997; Kaplan et al., 2001), and the specific identity of the required inducer or inducers remains unknown.

ii. The paranode

Flanking the NOR are paranodal domains, where non-compacted loops of paranodal myelin contact the axolemma. The tight association between axonal and glial membranes at the paranodal junction is believed to act as a barrier between the nodal domain and the juxtaparanodal domain, isolating electrical activity at the NOR from the internode and preventing the lateral diffusion of axonal proteins among domains (Pedraza et al., 2001). Ultrastructurally, the points of contact between oligodendrocyte and axolemmal membranes are characterized by the presence of electron-dense ridges called transverse bands (TBs) which resemble structures seen in septate junctions of *Drosophila*

epithelial and glial cells, which maintain cell-cell contacts required for the integrity of the blood-nerve barrier (Tao-Cheng and Rosenbluth, 1983; Baumgartner et al., 1996). The axonal protein caspr (also called caspr1, paranodin, or ncp1) is concentrated at the paranode during myelination (Einheber et al., 1997; Menegoz et al., 1997). Caspr forms a complex in *cis* with the GPI-linked protein contactin (Rios et al., 2000), and this association is required for the localization of caspr to the axonal membrane (Faivre-Sarrailh et al., 2000). The recruitment of the caspr-contactin complex to the paranodal domain is dependent on the presence of the 155 kDa isoform of neurofascin (nfc155), which is localized to the myelinating processes of oligodendrocytes and Schwann cells. Nfc155 is believed to form a complex with caspr and contactin in *trans*, and all three are essential for the formation of normal paranodal axo-glial junctions in both the CNS and PNS (Tait et al., 2000; Bhat et al., 2001; Boyle et al., 2001; Charles et al., 2002; Sherman et al., 2005). Cytoskeletal protein 4.1B, which is also enriched at paranodes, binds a juxtamembrane FERM domain in the caspr ICD and is believed to be another member of this complex. Its function downstream of caspr is poorly understood, but it has been hypothesized to link the paranodal complex to the cytoskeleton (Ohara et al., 2000; Gollan et al., 2002).

In the absence of caspr or contactin the paranodal complex does not form, and the paranodal junction is disordered (though more so in the CNS than PNS). Ultrastructurally, CNS paranodal loops are disorganized and no longer closely apposed to the axolemma, and TBs are no longer visible. Disruption of paranodal junctions does not prevent formation of the nodal complex, as nodal Na^+ channel clustering still occurs in their absence, though not as tightly. However, juxtaparanodal proteins such as K^+ channels (discussed further below) become abnormally localized to the paranode, and are no longer segregated from nodal proteins. Loss of separation between the node and regions of the axon covered by compact myelin results in severely decreased conduction velocities (Bhat et al., 2001; Boyle et al., 2001). In the neurofascin knockout, expression of both nfc186 and nfc155 is lost, resulting in the loss of both paranodal junctions and nodal complexes. Restoration of nfc155 extracellular domain expression to the knockout animals rescued the recruitment of the caspr-contactin complex to the paranode, but did

not rescue the clustering of nodal Na^+ channels (Sherman et al., 2005), providing further evidence that formation of the paranodal and nodal complexes is largely independent.

Other proteins, including ceramide galactosyl transferase (CGT) and ceramide sulfotransferase (CST), enzymes required for the synthesis of myelin lipids galactocerebroside and sulfatide respectively; myelin and lymphocyte protein (MAL), and 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNP) are required for the maintenance, but not the initial formation, of paranodal junctions (Dupree et al., 1998; Dupree et al., 1999; Ishibashi et al., 2002; Rasband et al., 2005; Schaeren-Wiemers et al., 2004). All four proteins have been reported to be localized to, or have an involvement in the organization of, protein-lipid microdomains (also called lipid rafts) in myelinating glia. Galactocerebroside, sulfatide, and CNP are major components of lipid rafts in oligodendroglia (Kramer et al., 1997; Taylor et al., 2002). MAL is a proteolipid that associates with lipid raft-forming glycosphingolipids (Frank et al., 1998) and, based on its established role in apical protein targeting in epithelial cells (Cheong et al., 1999), has been hypothesized to play a role in sorting and trafficking of lipid rafts and raft-associated proteins in oligodendrocytes (Schaeren-Wiemers et al., 1995).

The importance of lipid raft organization for maintenance of the paranodal region is likely due in part to the importance of raft domains for nfc155 function. Nfc155 protein present at the paranode is partitioned into detergent-insoluble glycosphingolipid-enriched complexes (DIGs), suggesting that it is localized to lipid rafts (Schafer et al., 2004), likely because it is palmitylated (Ren and Bennett, 1998). In $CGT^{-/-}$ or $MAL^{-/-}$ mice, nfc155 is no longer sorted into lipid rafts, raising the possibility that the failure of paranodal maintenance in these animals stems from the mislocalization of nfc155 (Menon et al., 2003; Schafer et al., 2004; Schaeren-Wiemers et al., 2004). In wild-type animals, nfc155 only becomes raft-associated in the adult, after the paranodal region has developed. This may explain why these mutations affect maintenance of the junctions, and not their formation (Schaeren-Wiemers et al., 2004).

iii. The juxtaparanode

As its name suggests, the juxtaparanodal domain is located under the compact myelin immediately adjacent to the innermost paranodal loop. Like the node and paranode, it too is defined by the presence of an axolemmal protein complex. Central to

this complex are delayed rectifier K^+ channels, which are clustered at the juxtaparanode. These channels have been proposed to provide a means for K^+ ions to flow from the axoplasm to the periaxonal space, preventing hyperexcitation and backfiring following nodal Na^+ influx, and maintaining internodal resting potential (Wang et al., 1993; Chiu et al., 1999; Vabnick et al., 1999). Also present in the complex are the adhesion molecule transient axonal glycoprotein (tag1), and caspr2 (Traka et al., 2002; Poliak et al., 1999). Caspr2 is structurally similar to caspr, except for the presence of a cytoplasmic PDZ-binding domain, through which it indirectly associates with K^+ channels, likely through PDZ domain-containing adaptor proteins (Poliak et al., 1999). The complex is also associated with overlying oligodendroglial membrane by homophilic tag1 binding (Traka et al., 2003). Both caspr2 and tag1, which directly interact, are required for K^+ channel accumulation at the juxtaparanode. Surprisingly, however, nerve conduction velocities were not affected in caspr2$^{-/-}$ mice, suggesting that clustering of axonal K^+ channels at the juxtaparanode is not a requirement for normal saltatory conduction (Traka et al., 2003; Poliak et al., 2003).

Like the caspr-containing paranodal complex, the juxtaparanodal complex is likely tethered to the cytoskeleton via protein 4.1B (Denisenko-Nehrbass et al., 2003). However, the association of this juxtaparanodal complex with the axonal cytoskeleton may not be as rigid as that of the nodal complex, as caspr2, K^+ channels, and protein 4.1B become aberrantly located along the axolemma when paranodal junctions are disrupted (Poliak et al., 2001; Bhat et al., 2001). Also present at the juxtaparanode, positioned in the adaxonal myelin membrane over the juxtaparanodal K^+ channel-containing complex, is gap junction protein connexin 29 (Cx29). Cx29 has been hypothesized to form membrane channels that allow for the removal of K^+ ions from the periaxonal space into the overlying glial cell (Altevogt et al., 2002). Other connexins, Cx32 and Cx47, allow excess K^+ ions to flow across adjacent paranodal loops and away from the paranodal region (Kamasawa et al., 2005).

Figure 1.1. Structure of netrins and their receptors.

A) Conserved structures of netrins. All netrins contain conserved VI and V domains. The carboxy terminal C domain found secreted netrins (netrin-1, 2, 3, and 4) contains several positively charged, basic residues. The carboxy terminus of netrin-G subfamily members is rich in hydrophobic amino acids and is tethered to the plasma membrane through a GPI linkage. B) The VI and V domains of netrins 1, 2, and 3 are homologous to the VI and V domains of the laminin γ chain, while the VI and V domains of netrin-4 more closely resemble the eponymous domains in the laminin β chain. C) DCC and UNC5 family members are the canonical netrin receptors. In mammals, the DCC family includes DCC and neogenin, and the UNC5 family includes UNC5A, B, C, and D. A) was adapted from a figure by T.E. Kennedy. B) and C) were adapted from a figure by S.W. Moore.

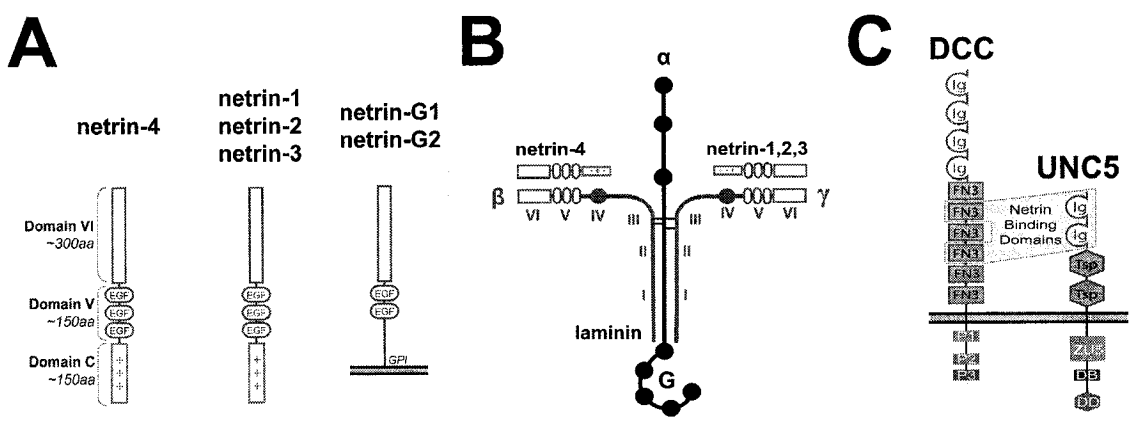

A
netrin-4
netrin-1
netrin-2
netrin-3
netrin-G1
netrin-G2
Domain VI
~300aa
Domain V
~150aa
Domain C
~150aa
EGF
GPI
B
α
netrin-4
netrin-1,2,3
β
γ
VI V IV III
III IV V VI
II
I
laminin
G
C
DCC
UNC5
Ig
FN3
Netrin
Binding
Domains
TSP
DB
DD

Figure 1.2. Attractant and repellent signaling responses to netrin.

A) Summary of the attractant signaling response to netrin. Netrin-DCC binding has been proposed to result in receptor multimerization, localization to lipid rafts, and recruitment of a bevy of signaling molecules including the tyrosine kinases FAK and fyn, resulting in tyrosine phosphorylation of the DCC ICD. Also recruited are many proteins involved in remodeling the actin cytoskeleton, including rho GTPases cdc42 and rac1, which are activated in response to netrin. B) Summary of repellent signaling responses to netrin. Short-range repellent responses to netrin do not require DCC (left). Netrin binding to UNC5 results in tyrosine phosphorylation, mediated at least in part by src, and the recruitment of tyrosine phosphatase SHP2. DCC-independent repulsion may also indirectly involve max1. Long-range repulsion requires the association of UNC5 receptors with DCC upon netrin binding, and potentiates UNC5 tyrosine phosphorylation and the recruitment of FAK and src family kinases relative to the DCC-UNC5 complex (right). Adapted from a figure by S.W. Moore.

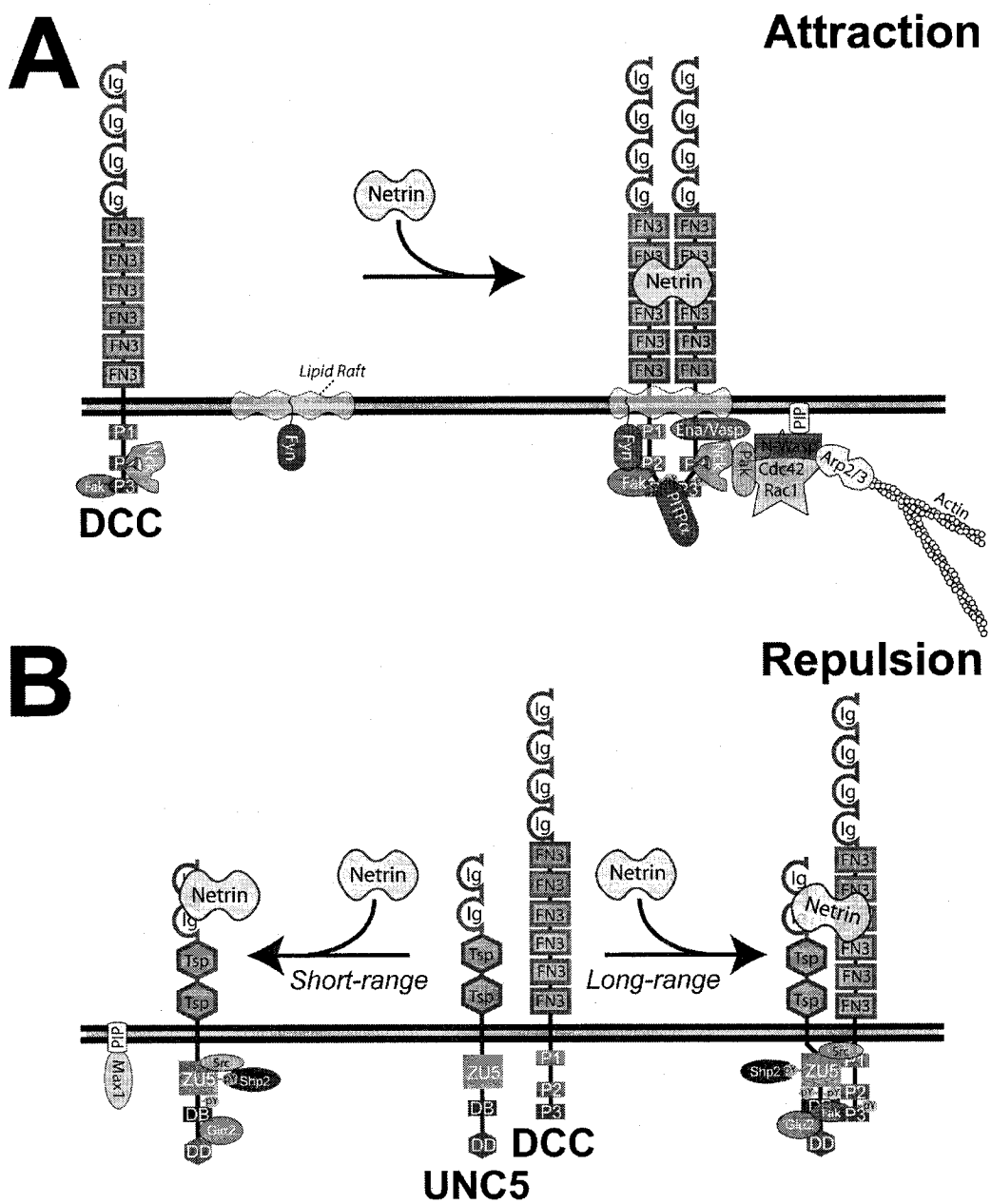

A
Attraction
Netrin
Lipid Raft
DCC
Fyn
Ena/Vasp
PIP
N-WASP
Cdc42
Rac1
Arp2/3
Actin
B
Repulsion
Short-range
Long-range
Netrin
Tsp
FN3
Ig
PIP
Max1
Src
ZU5
Shp2
DD
DCC
UNC5

Figure 1.3. Oligodendrocyte maturation in the developing spinal cord.

In the developing spinal cord (and in other regions of the CNS), oligodendrocyte precursor cells are born at restricted foci, and then disperse throughout the developing tissue. The cells then proliferate, differentiate into oligodendrocytes, and establish contact with target axons. Because more oligodendrocytes are generated than are needed for myelination, extraneous oligodendrocytes are eliminated by programmed cell death.

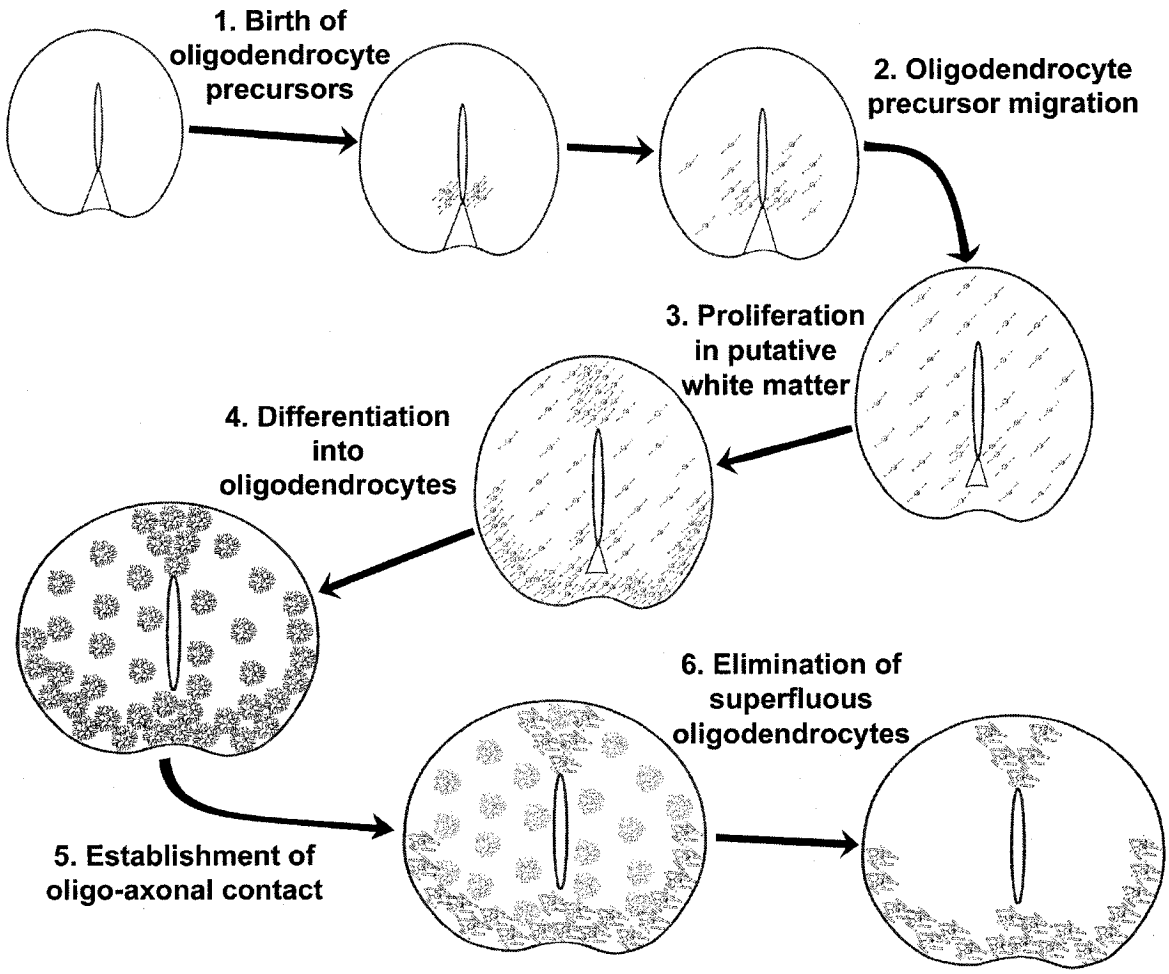
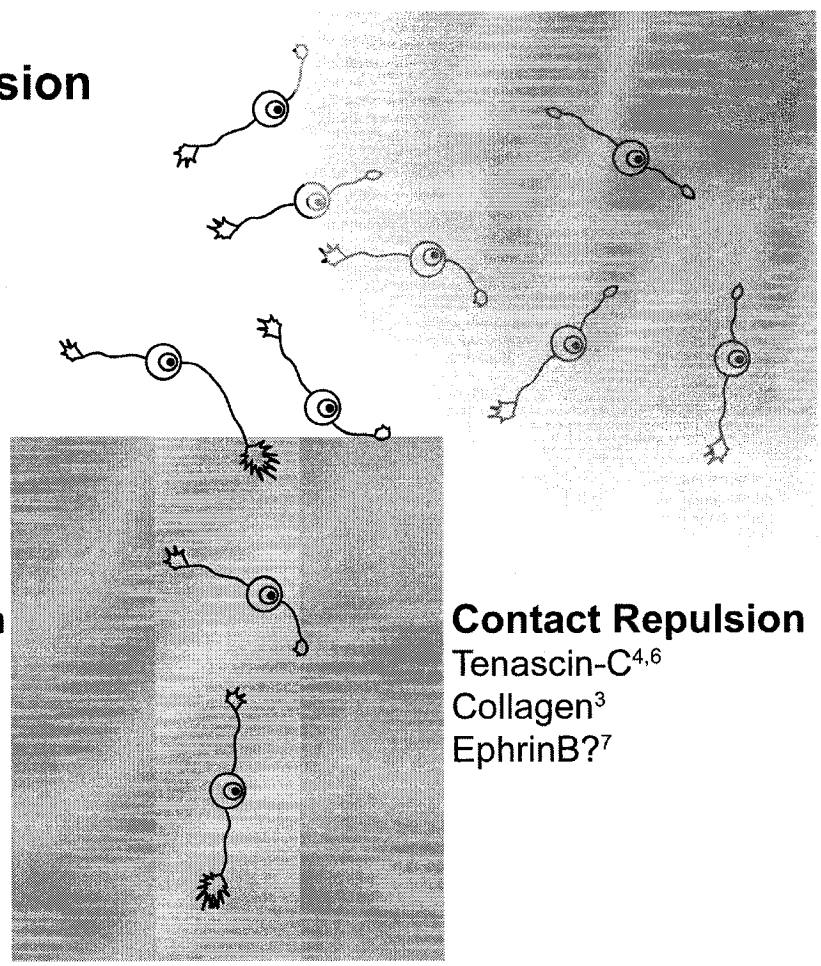

1. Birth of oligodendrocyte precursors
2. Oligodendrocyte precursor migration
3. Proliferation in putative white matter
4. Differentiation into oligodendrocytes
5. Establishment of oligo-axonal contact
6. Elimination of superfluous oligodendrocytes

Figure 1.4. Guidance cues for migratory oligodendrocyte precursor cells.

Guidance cues implicated in OP migration include long-range attractant and repellent cues, and short-range (contact-mediated) attractants and repellents. Attractant cues encompass a range of factors including chemotropic attractants and migration-promoting permissive cues, whereas repellent cues consist of a range of factors including chemotropic repellents and nonpermissive cues that inhibit migration. EphrinB and EphB are qualified with a '?' because of conflicting reports regarding their roles in regulating OP migration. References (denoted in superscript) correspond to: 1) Spassky et al. (2002), 2) Cohen et al. (2003), 3) Milner et al. (1996), 4) Frost et al. (1996), 5) Prestoz et al. (2004), 6) Kiernan et al. (1996), 7) Cohen (2005), 8) Armstrong et al. (1990), 9) Simpson and Armstrong (1999), 10) Yan and Rivkees (2002), 11) Dziembowska et al. (2005).

Chemorepulsion

Semaphorin 3A[1,2]
Semaphorin 3B[2]
Semaphorin 3C[2]
Semaphorin 3F[2]

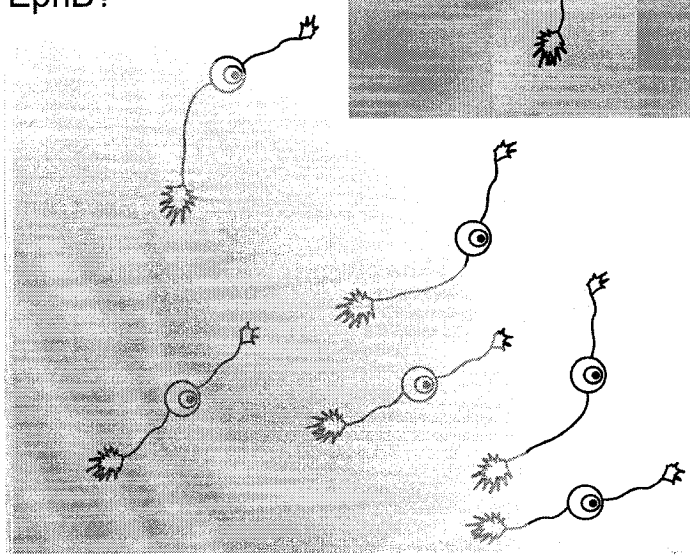


Contact Attraction

Laminin-1[3]
Laminin-2[4]
Fibronectin[3,4]
Vitronectin[3]
EphB?[5]

Contact Repulsion

Tenascin-C[4,6]
Collagen[3]
EphrinB?[7]

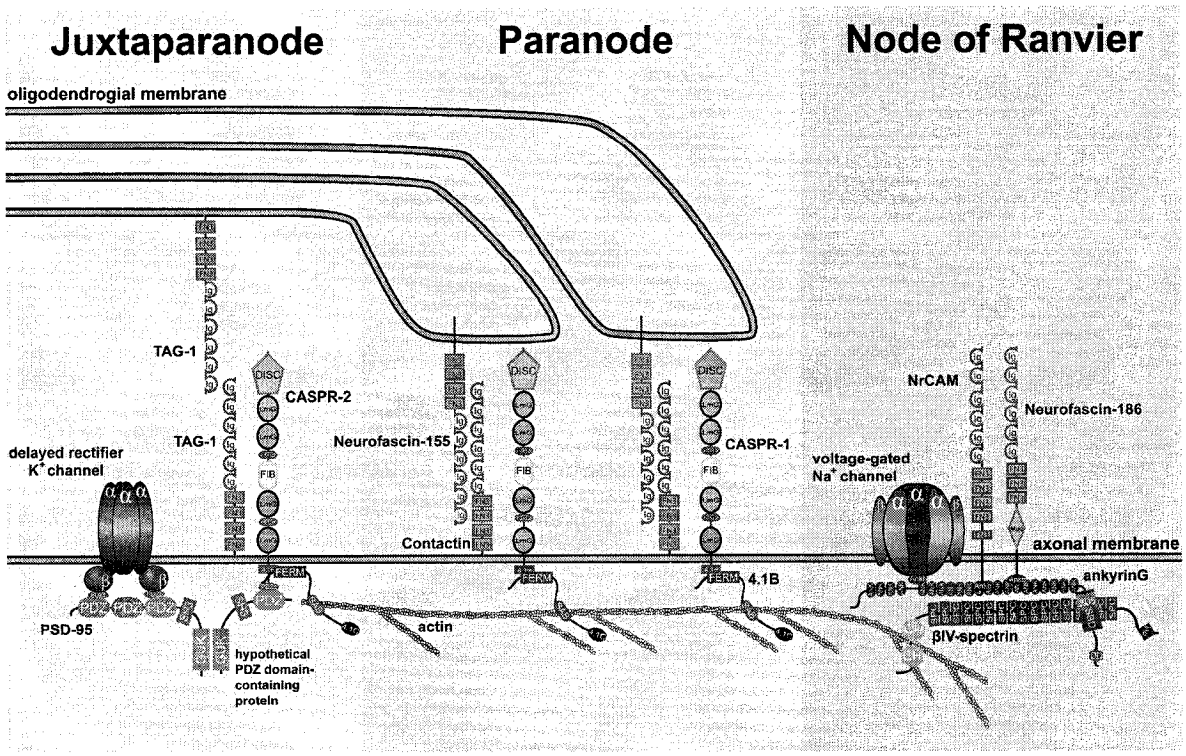


Chemoattraction

PDGF-A[3,8,9]
FGF-2[3,8]
HGF/SF[10]
Semaphorin 3F[1]
CXCL12[11]

Figure 1.5. Molecular composition of the CNS node of Ranvier.

The nodal, paranodal, and juxtaparanodal domains are characterized by distinct sets of molecules. At the node of Ranvier (right) Na^+ channels are complexed with NrCAM and neurofascin-186. This nodal complex is anchored to the actin cytoskeleton through ankyrinG and βIV-spectrin. At the paranodal domain (middle), oligodendroglial protein neurofascin-155 associates with axonal proteins contactin and CASPR-1. 4.1B associates with the CASPR-1 ICD, and links the paranodal complex to the cytoskeleton. At the juxtaparanodal domain (left), delayed rectifier K^+ channels are complexed with axonal CASPR-2 and TAG-1, which homphilically binds TAG-1 present in the overlying oligodendroglial membrane. The interaction between CASPR-2 and K^+ channels is believed to be mediated by PSD-95 and another unidentified PDZ domain-containing protein. Domain abbreviations: ANK, ankyrin; Ankb, ankyrin-binding; CH, calponin-homology; DD, death domain; DISC, discoidin-like domain; EGF, epidermal growth factor; FERM, 4.1/ezrin/radixin/moesin; FIB, fibrinogen-like; FN3, fibronectin-III-like; 4.1C, 4.1 cytoplasmic terminal; 4.1m, 4.1-binding motif; GUK, guanylate kinase homology; Ig, Immunoglobulin; LmG, laminin G; Muc, mucin-like; PDZ, PSD-95/Dlg/ZO-1; PDZb, PDZ-binding; PH, pleckstrin homology; SAB, spectrin/actin-binding; SBD, spectrin-binding; SH3, src homology 3; SPC, spectrin; ZU5, ZO-1 and UNC5-like.


Juxtaparanode
Paranode
Node of Ranvier
oligodendrogial membrane
TAG-1
CASPR-2
TAG-1
delayed rectifier
K+ channel
Neurofascin-155
Contactin
CASPR-1
4.1B
NrCAM
Neurofascin-186
voltage-gated
Na+ channel
axonal membrane
ankyrinG
βIV-spectrin
PSD-95
hypothetical
PDZ domain-
containing
protein
actin
DISC
FIB
FERM

RESEARCH RATIONALE AND OBJECTIVES

Like maturing neurons, developing glial cells are born, migrate, proliferate, differentiate, extend processes, and establish and maintain contacts with neighboring cells. When I began my doctoral studies, it had been established that netrins act over long distances to guide the migration of neuronal cells and extending axons by either attracting or repelling them. It had also been reported that netrins act as short-range cues in some instances, directing retinal ganglion cell axons to exit the retina in mammals, and regulating neuromuscular synapse formation by *Drosophila* motor axons (reviewed by Kennedy, 2000). However, knowledge of the roles played by netrins and their receptors in glia, which account for the majority of the cells in the CNS, was limited.

Work in our laboratory had revealed that in the adult spinal cord, both oligodendrocytes and neurons express *netrin-1* (Manitt et al., 2001). This finding, and the knowledge that close lineage relationships exist between certain types of neurons and oligodendroglia (Richardson et al., 2000), prompted us to investigate the roles played by netrin-1 during oligodendrocyte development, both during the early long-distance migrations of OP cells (Chapter 2), and at small distances in the organization of axo-glial contacts (Chapter 3).

Finally, it had previously been reported that *netrin-1, dcc, and unc5 homologue* expression is lost or reduced in tumor cells, including glially-derived tumors, and that loss of DCC function correlates with increased metastasis and invasion (Reyes-Mugica et al., 1997; Meyerhardt et al., 1999; Saito et al., 1999; Thiebault et al., 2003). These observations led us to investigate whether netrin-1 and DCC regulate the migration of glioma cells (Chapter 4).

The specific aims of the research described in this thesis were:

1) To investigate if cells that are the precursors of CNS glia are, like neuronal cells and axons, guided in their migration by netrin-1. In particular, we wished to determine if the netrin-1 gradient that guides dorso-ventral commissural axon migration in the ventral spinal cord also influences the ventro-dorsal migration of OP cells (Chapter 2). Our findings indicate that OP cells express DCC and UNC5 homologue netrin receptors, and

that netrin-1 is required for OP migration in the embryonic spinal cord. Based on data obtained using an *in vitro* microchemotaxis assay, we found that netrin-1 guides OP migration by a chemorepellent mechanism, likely by inducing cytoskeletal collapse.

2) To identify the role of netrin-1 and its receptor DCC in oligodendroglia, particularly in non-compact myelin membranes (Chapter 3). These studies provide evidence that netrin and its receptors are present at CNS paranodal axo-glial junctions. Using cerebellar organotypic cultures prepared from netrin-1 and DCC mutant neonates, we show that paranodal junctions develop and mature in the absence of these proteins, but these junctions later become disorganized, suggesting that netrin-1 and DCC are required for their maintenance.

3) To investigate the role of netrins in glial tumor cell migration (Chapter 4). We obtained data suggesting the existence of two distinct influences of netrins on glioma cells: Netrin-1 and netrin-3 act as autocrine or paracrine inhibitors of cell motility, through a mechanism not requiring DCC. Netrin-1 also acts through DCC to inhibit tumor cell migration in the presence of laminin. In addition, our data suggests that netrin does not influence apoptosis, consistent with the regulation of motility and not survival underlying the tumor suppressive actions of netrins and their receptors.

PREFACE TO CHAPTER 2

During embryonic spinal cord development, netrin-1 expressed at the floor plate acts as a bi-functional guidance due, directing axon migration both dorsally and ventrally depending on the compliment of receptors expressed (reviewed by Kennedy, 2000). During this period of netrin-1 expression, cells that are precursors of oligodendrocytes are generated at the ventral midline, just dorsal to the site of netrin-1 expression. These cells then proceed to migrate away from their sites of birth and populate the developing spinal cord (Warf et al., 1991; Ono et al., 1995), and it was hypothesized that a chemorepellent cue may be responsible (Miller et al., 1997). Considering that spinal oligodendrocytes and motor neurons are believed to be derived from a common lineage (reviewed by Richardson et al., 2000), and that the axons of trochelar motor neurons were known to be repelled by netrin-1 (Colamarino and Tessier-Lavigne, 1995), it was reasonable to hypothesize that netrin-1 at the ventral midline may also direct the migration of OP cells.

The aims of this study were to:

1) Determine if OP cells express netrin receptors

2) Investigate the possibility that netrin-1 can influence the migration of OP cells and, if such a role were found to exist, to gain some insight into the underlying mechanism of guidance

3) Examine the distribution of OP cells in the embryonic spinal cords of mice lacking netrin-1 or DCC expression to determine if netrin-1 influences these cells *in vivo*

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CHAPTER 2

Journal of Neuroscience
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Netrin-1 is a chemorepellent for oligodendrocyte precursor cells in the embryonic spinal cord

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Abbreviated title: Netrin-1 repels oligodendrocyte precursors.

Key words: oligodendrocyte precursor cell, cell migration, netrin-1, DCC, unc5h1, embryonic spinal cord, multiple sclerosis, apoptosis, dependence receptor.

Glossary of abbreviated terms: DCC, Deleted in Colorectal Cancer; DCC_{FB}, DCC function blocking antibody; UNC5H, Unc-5 homolog; PDGF, Platelet-derived growth factor; MTT, (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; OP, oligodendrocyte precursor

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I. ABSTRACT

Netrin-1, secreted by floor plate cells, orients axon extension in relation to the ventral midline of the embryonic spinal cord. Oligodendrocyte precursor (OP) cells are born close to the ventral midline and migrate away from the floor plate. Here we show that OP cells, identified by expression of the PDGFα receptor, express the netrin receptors *dcc* and *unc5h1,* but do not express netrin-1. Using a microchemotaxis assay, we demonstrate that migrating OPs are repelled by a gradient of netrin-1 *in vitro*. Furthermore, application of netrin-1 to OPs *in vitro* triggers retraction of OP processes. In the absence of netrin-1 or DCC function *in vivo*, fewer OP cells migrate from the ventral to the dorsal embryonic spinal cord, consistent with netirn-1 acting as a repellent. In addition to their role regulating cell movement, DCC and UNC-5 homologues have been suggested to function as pro-apoptotic dependence receptors, triggering cell death in the absence of netrin-1. In contrast, we report no evidence of increased OP cell death *in vivo* or *in vitro* in the absence of either netrin-1 or DCC. These findings indicate that netrin-1 is a repellent cue for migrating OPs in the embryonic spinal cord.

II. INTRODUCTION

During the development of the central nervous system (CNS), many neural cell types migrate great distances to reach their final destinations. While neuronal migration has been studied extensively, the cues that direct the migration of oligodendrocyte precursors (OP) are not well understood. Several factors that influence OP motility *in vitro* have been identified. Basic fibroblast growth factor (bFGF or FGF-2) and the platelet-derived growth factor A chain are soluble chemoattractants for OPs (Armstrong et al., 1990; Milner et al., 1996; Simpson and Armstrong, 1999), and reduced numbers of OPs(Armstrong et al., 1990; Simpson and Armstrong, 1999) and oligodendrocytes have been found in *PDGF-A* knockout mice (Fruttiger et al., 1999). However, because PDGF-A is also a mitogen for OPs (Noble et al., 1988; Calver et al., 1998),(Calver et al., 1998) this phenotype may be due to deficient OP migration, proliferation, or both. Substrates of the extracellular matrix (ECM) proteins laminin, fibronectin, or vitronectin promote OP migration (Milner et al., 1996) while tenascin C and collagen are non-permissive substrates for migrating OPs (Kiernan et al., 1996; Milner et al., 1996; Milner et al., 1996). The rate of OP migration increases in tenascin-C null mice, but tenascin C does not appear to direct OP cell migration (Garcion et al., 2001). Sugimoto et al. (2001) have provided evidence that both Sema3A and netrin-1 are chemorepellents for OP cells migrating from explants of newborn rat optic nerve . In contrast, using a similar *in vitro* assay, Spassky et al. (2002) conclude that netrin-1 attracts OP cells migrating from explants of embryonic optic nerve . Spassky et al. (2002) also provide evidence for Sema3F attracting migrating OPs, and, in agreement with Sugimoto et al. (2002), that Sema3A is a chemorepellent for these cells. While these experiments address OP migration *in vitro*, if these cues direct OP migration *in vivo* is not clear.

In the embryonic spinal cord, OPs originate in the ventral ventricular zone, at two foci located on either side of the midline, slightly dorsal to the floor plate (Pringle and Richardson, 1993; Yu et al., 1994; Ono et al., 1995; Orentas and Miller, 1996; Yu et al., 1994; Ono et al., 1995; Orentas and Miller, 1996). Beginning at ~E12.5 in the mouse, OPs disperse throughout the developing spinal cord, migrating away from the ventral midline (Calver et al., 1998). This trajectory suggests that cues produced by floor plate cells may repel migrating OP cells.

We postulated that netrin-1 might function as a repellent for OP cells in the embryonic spinal cord. Netrin-1 is produced at the ventral midline of the embryonic neural tube where it repels some types of migrating axons and attracts others (Kennedy et al., 1994; Colamarino and Tessier-Lavigne, 1995; Varela-Echavarria et al., 1997; Kennedy et al., 1994; Varela-Echavarria et al., 1997). Netrin-1 also directs the circumferential migration of neuronal precursor cells (Varela-Echavarria et al., 1997; Przyborski et al., 1998; Alcantara et al., 2000; Hamasaki et al., 2001; Przyborski et al., 1998; Hamasaki et al., 2001; Alcantara et al., 2000). Here we show that netrin-1 is expressed by floor plate cells as OP cells migrate away from the ventral midline of the developing spinal cord. A netrin receptor complex composed of DCC and an UNC-5 homolog mediates the repellent response to netrin-1 (Hong et al., 1999). We report that both *dcc* and *unc5h1* are expressed by migrating OP cells in the embryonic spinal cord. To test the hypothesis that netrin-1 might influence OP motility, we used an *in vitro* microchemotaxis assay and found that a gradient of netrin-1 repels the migration of cultured OP cells. Application of netrin-1 to OP cells *in vitro* caused the retraction of OP processes, consistent with a repellent function. Furthermore, we report that the distribution of OP cells is disrupted in the spinal cords of mouse embryos lacking DCC or netrin-1. Importantly, the change in cell distribution occurs without a change in cell number, indicating that the absence of netrin-1 or DCC does not affect cell survival. These findings indicate that netrin-1 functions as a repellent guidance cue for OP cell migration in the embryonic spinal cord.

III. MATERIALS AND METHODS

Animals and Oligodendrocyte Precursor Cell Culture

Sprague Dawley rat pups and pregnant Balb/c mice were obtained from Charles River Canada (QC). Mice heterozygous for netrin-1 or DCC function were obtained from Marc Tessier-Lavigne (Stanford) and Robert Weinberg (Harvard), respectively. All procedures with animals were performed in accordance with the "Canadian Council on Animal Care" guidelines for the use of animals in research. Oligodendrocyte precursor cells were obtained from mixed glial cultures derived from the cerebral cortices of P0 rat pups as described (Armstrong, 1998).

Antibodies, Immunocytochemistry, and Immunohistochemical Quantification

The following antibodies were used: monoclonal anti-NG2 (Chemicon, CA), anti-DCC intracellular domain (anti-$DCC_{IN,}$ G97-449), anti-DCC function blocking (DCC_{FB}, AF5, Calbiochem, CA); polyclonal anti-PDGFαR (C-20, Santa Cruz Biotech, CA), and anti-netrin PN2 (Manitt et al., 2001). A2B5 hybridoma was provided by V.W. Yong (U. Calgary).

For live labeling with A2B5, cells were incubated for 30 min at 4°C with hybridoma supernatant. Unbound antibody was washed away with DMEM. Cells were then washed with PBS, fixed with 4% paraformaldehyde (PFA), permeabilized with PBS containing 0.25% Triton X-100, and blocked with 3% heat-inactivated horse serum (HS)/1% bovine serum albumin/0.1% Triton X-100. Cells were then incubated with anti-DCC or anti-netrin PN2 diluted in blocking solution. NG2 labeling was similarly carried out on fixed cells without the live labeling step. Primary antibodies were detected with secondary antibodies coupled to Cy3 or Alexa 488 (Molecular Probes). Nuclei were stained with Hoechst dye.

For immunohistochemical analyses, netrin-1 or DCC heterozygous mice were crossed and E15 embryos obtained (plug date designated E1). Embryos were frozen in 2-methyl butane (Fisher) chilled in liquid nitrogen, then mounted individually in optimal cutting temperature compound (O.C.T. Tissue Tek, Sakura Finetek, CA), 6 μm cryostat

sections of the spinal brachial enlargement cut, mounted onto slides (Superfrost Plus, Fisher) and fixed by immersion in 4% PFA, 15% picric acid (pH 8.5) in PBS (45 min, rt). The sections were rinsed in PBS, permeabilized with 0.5% Triton X-100 in PBS, and rinsed in PBS. To enhance antigenicity, sections were immersed in boiling PBS in a microwave oven for 11 min., cooled, and blocked (5% HINHS, 1% BSA in PBS; 1 hr, rt). Sections were then incubated with anti-PDGFαR (1:1000) in blocking solution (overnight, 4°C) and visualized using Alexa 488-conjugated secondary antibody. Sections were stained with Hoechst dye to visualize nuclei. Fluorescence was visualized using a Carl Zeiss Axiovert 100 microscope, a Magnafire CCD camera (Optronics, CA), and Northern Eclipse Software (Empix Imaging, ON). To analyze the distribution of OPs, each spinal cord section was photographed in four quadrants using a 20 X objective. The four images were assembled into a single image of a complete spinal cord using Photoshop (Adobe, CA). The length of the dorsal-ventral axis was measured along the midline from the top of the roof plate to the base of the ventral commissure, and each cord divided into thirds. A cell was counted only if anti-PDGFαR staining encircled a blue Hoechst-stained nucleus. Due to this criterion, fewer cells were counted in this analysis per section of cord relative to previous studies using PDGFαR mRNA *in situ* hybridization (Calver et al., 1998). Three sections were counted per embryo. Non-adjacent sections were counted to avoid counting the same cell more than once.

Transfilter microchemotaxis assay

OP-enriched cell suspensions were plated at a density of 1.25 x 10^5 cells/ml on poly-D-lysine-coated polycarbonate transwell culture inserts (6.5 mm diameter with 8 μm pore size, Corning). 100 μl of cell suspension was used per filter, and the filters placed in the wells of a 24-well tissue culture plate over 600 μl of medium. OLDEM was the base medium used for all assay conditions (DMEM, 5 μg/ml insulin, 100 μg/ml transferrin, 30 nM sodium selenite, 30 nM triiodothyronine, 6.3 ng/ml progesterone, 16 μg/ml putrescine, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM glutamax). Cells were allowed to migrate for 16 hrs at 37°C, cells on the upper side of the filter were then scraped off, and the cells attached to the lower side of the filter fixed with 4% paraformaldehyde/0.1% glutaraldehyde (30 min, rt). Filters were then rinsed with PBS,

and cell nuclei stained with Hoechst dye. Cell nuclei were imaged using epiflorescence. For each transwell assay, a single image of the filter was captured using a 4 X objective and nuclei counted using Northern Eclipse software. Where pooled results are presented, the value 'percent migration vs control' (Fig. 2.3C) for a given trial represents the number of cells migrated in that condition expressed as a percentage of the mean number of cells migrating in control conditions.

Analysis of OP morphology

Dissociated OP cells were plated in OLDEM at a density of 2.5 x 10^4 cells/well in an 8-well plastic chamber slide pre-coated with poly-D-lysine. Cells were maintained in culture overnight, and the medium was then replaced with either fresh OLDEM (as a control) or OLDEM plus the factors being tested. Following incubations of 30 minutes, 2 hours, or 16 hours, the cells were fixed and labeled with rhodamine-conjugated phalloidin, anti-PDGFαR (visualized using Alexa 488-conjugated secondary antibody), and Hoechst as described above. The surface area, length of longest process, and number of major processes of PDGFαR-positive OPs were measured using rhodamine-phalloidin staining and Northern Eclipse software. Surface area corresponds to the two-dimensional surface area of the entire OP cell, including the cell body and all processes. The length of the longest process is the measurement from the base of the longest process to its tip. A major process refers to a terminal process whose length exceeded the maximum diameter of the cell body.

MTT assay

Dissociated OP cells were plated in OLDEM at a density of 1 x 10^4 cells/well in a 96-well tissue culture plate pre-coated with poly-D-lysine. Cells were grown in culture for 16 hrs, followed by one additional hr in the presence of 0.5 mg/ml MTT (Sigma). The medium was then aspirated, and the cells dissolved in isopropanol (100 µl/well). Absorbance of the converted dye (ΔO.D.) was measured at 570 nm with background subtraction at 630 nm (Denizot and Lang, 1986).

Statistical analyses

All comparisons were carried out by ANOVA with Fisher's Least-Significant-Difference post-hoc test, and were performed using SYSTAT software (SPSS, Il).

In situ hybridization

Sense and antisense cRNA probe pairs corresponding to *netrin-1* (Manitt et al., 2001), *dcc* (Keino-Masu et al., 1996), *unc5h1* and *unc5h2* (Leonardo et al., 1997), and *unc5h3* (Ackerman et al., 1997) were used. Cryostat sections of E15 mouse spinal cord were cut and fixed, synthesis of digoxigenin (DIG) labeled probes, and hybridization, were carried out as described (Manitt et al., 2001). For *in situ* hybridization analysis of expression *in vitro*, OP cells were cultured using Lab-Tek chamber slides (Fisher) and fixed with 4% PFA. Hybridization was carried out overnight at 57°C (*netrin-1*), 57°C (*dcc*), 57°C (*unc5h1*), 60°C (*unc5h2*), and 57°C (*unc5h3*), followed by a stringent wash in 2X SSC for 1 hr at 65°C. Slides were blocked (0.5 % blocking powder in 1 X PBS; NEN, MA) for 30 min rt. Hybridization was detected using a peroxidase-coupled antibody against DIG (Roche, QC). Incubation with anti-PDGFαR was carried out during this step. *In situ* signals were amplified using the TSA-Indirect (ISH) Tyramide Signal Amplification kit (NEN, MA), and visualized using Alexa 594-conjugated streptavidin. Alexa 488-conjugated anti-rabbit IgG secondary antibody was used to visualize PDGFαR immunostaining.

IV. RESULTS

Netrin-1 is expressed at the ventral midline of the developing spinal cord during oligodendrocyte precursor migration.

In the embryonic spinal cord, OP cells are born in the ventricular zone just dorsal to the floor plate. These cells then migrate away to populate all regions of the cord (Pringle and Richardson, 1993; Yu et al., 1994; Ono et al., 1995; Orentas and Miller, 1996; Diers-Fenger et al., 2001; Yu et al., 1994; Ono et al., 1995; Orentas and Miller, 1996; Diers-Fenger et al., 2001). OP cells first appear in the embryonic mouse spinal cord at ~E12.5 and are distributed throughout the mouse brachial spinal cord by E15 (Pringle and Richardson, 1993; Calver et al., 1998; Calver et al., 1998). *Netrin-1* is known to be expressed in the floor plate and ventral neuroepithelium of the E11.5 spinal cord (Serafini et al., 1996), but expression had not been examined later in development during OP cell migration. Using *in situ* hybridization analysis we show that at E15, floor plate cells continue to express *netrin-1* as OPs migrate through the neuroepithelium (Fig. 2.1A). Double labeling with an antibody against the PDGFαR, a marker specific for OP cells in the embryonic spinal cord, indicated that OPs do not express detectable levels of *netrin-1* at E15 (Fig. 2.1A, B). Thus, expression of *netrin-1* is temporally and spatially consistent with it having a role as an OP repellent.

Oligodendrocyte precursor cells express the netrin receptors *dcc* and *unc5h1*, but not *unc5h2* or *unc5h3* in the E15 mouse spinal cord.

DCC and UNC-5 homolog family members mediate the chemorepellent response of migrating neurons and axons to netrin-1 (Hedgecock et al., 1990; Hamelin et al., 1993; Keleman and Dickson, 2001; Hong et al., 1999; Przyborski et al., 1998; Goldowitz et al., 2000; Hamasaki et al., 2001; Hamelin et al., 1993; Keleman and Dickson, 2001; Hong et al., 1999; Przyborski et al., 1998; Goldowitz et al., 2000; Hamasaki et al., 2001). By labeling OP cells with anti-PDGFαR in combination with *in situ* hybridization analysis we investigated the expression of *dcc*, *unc5h1*, *unc5h2*, and *unc5h3* by OPs in E15 spinal cord. *Dcc* and *unc5h1* expression was detected in most, if not all, PDGFαR-positive OPs (Fig. 2.1C-F), supporting the hypothesis that these cells could respond to netrin-1.

Unc5h2 and *unc5h3* expression was not detected in oligodendrocyte precursors at E15 (not shown).

Netrin-1 repels migrating oligodendrocyte precursors *in vitro*.

To directly test the hypothesis that netrin-1 repels OP migration, cultures enriched for OP cells were prepared from the cerebral cortices of newborn rat brain as described (Armstrong, 1998). OP cells were identified using the A2B5 monoclonal antibody (Raff et al., 1983), polyclonal antibodies against NG2 (Stallcup and Beasley, 1987), or polyclonal antibodies against PDGFαR, all markers of OPs but not mature oligodendrocytes. Double labeling with these markers and either antibodies against netrin or DCC, or *in situ* hybridization for unc-5 homolog expression was carried out 24 hrs after plating OP cells. These findings indicated that under these conditions *in vitro,* all OP cells express DCC (Fig. 2.2A, B) and *unc5h1* (Fig. 2.2C, D) but not netrin-1 (Fig. 2.2E). These results are consistent with our findings in the E15 spinal cord (Fig. 2.1).

We then determined if netrin-1 influences OP migration using a transfilter microchemotaxis assay (described in Falk et al., 1980), an established method of analyzing OP cell motility (Armstrong et al., 1990; Simpson and Armstrong, 1999; Frost et al., 2000; Simpson and Armstrong, 1999; Frost et al., 2000). Cells were plated onto the top surface of a polycarbonate filter containing pores 8 μm in diameter that was suspended in the well of a tissue culture plate (Fig. 2.3A). Putative tropic factors can be tested by adding them to the medium beneath the filter, to the cell suspension prior to plating on top of the filter, or to both the top and bottom compartments. Cells migrating into a pore from the top of the filter can be challenged with an increasing gradient of the putative cue (cue on bottom), a decreasing gradient of the cue (cue on top), or an equal concentration of the cue on both sides (cue on top and bottom). Cells initially adhere to the upper surface of the filter. During migration, either spontaneously or in response to an added factor, some cells enter a pore and move to the lower side of the filter. At the end of the assay, which lasts 16 hrs, cells that remain on the upper side of the filter are scraped off and the cells that have migrated to the lower side of the filter are fixed in place, stained, and counted. An attractant in the bottom chamber enhances migration from the top to the bottom of the filter. Conversely, a repellent cue in the lower chamber will

reduce migration from the top to the bottom. A cue that has a kinetic effect will similarly influence migration irrespective of being placed in the bottom, top, or both chambers.

Figure 2.3B presents data from a single representative trial using the microchemotaxis assay. In the absence of any added cue, 187 ± 16 (mean ± SEM per 4x field) OP cells spontaneously migrated to the bottom of the filter. PDGF-AA (20 ng/mL), a known OP cell chemoattractant, increased migration (372 ± 25 cells) when added to the bottom compartment. In contrast, when OPs were challenged with 100 ng/ml netrin-1 in the bottom compartment, migration decreased significantly (100 ± 7 cells). Addition of 100 ng/ml netrin-1 to the top compartment caused an increase in the number of cells migrating to the lower side of the filter (223 ± 21 cells; Fig. 2.3B). When the results from multiple experimental trials were pooled (Fig. 2.3C), the increase in the number of cells migrating away from netrin-1 in the top compartment was found to be significant. Interestingly, when OPs were exposed to netrin-1 (100 ng/ml) added to both the top and bottom compartments (Fig. 2.3B) migration was reduced (95 ± 8 cells) to a level not significantly different from that obtained with netrin-1 in the lower chamber alone. Immunostaining the cells plated on the top of the filter, or the cells that migrated to the lower side of the filter, demonstrated in both cases that ~90% of the cells present were A2B5 or PDGFαR positive (not shown).

DCC contributes to mediating both attractant and repellent responses of neuronal growth cones to netrin-1 (Hong et al., 1999). To determine if DCC is required for the repellent response of OP cells to netrin-1, OP cells were challenged with netrin-1 in the lower chamber in the presence of a DCC function blocking antibody added to the top and bottom chambers (2.7 μg/ml, DCC_{FB}). Addition of DCC_{FB} blocked the response to netrin-1, producing migration not significantly different from control (Fig. 2.3C).

We carried out a dose response analysis of the repellent action of netrin-1 using the transfilter migration assay. Cells were challenged with increasing concentrations of netrin-1, from 3 ng/ml to 200 ng/ml, added to the bottom chamber. Increasing concentrations of netrin-1 resulted in fewer cells migrating across the filter (Fig. 2.3C). These values were best fit using a sigmoidal curve and the EC_{50} for the repellent response of OP cells to netrin-1 determined to be ~20 ng/ml (Fig. 2.3D).

Netrin-1 has been proposed to have trophic effects (Mehlen et al., 1998; Forcet et al., 2001; Llambi et al., 2001; Forcet et al., 2001; Llambi et al., 2001) in addition to its well-documented function as a tropic guidance cue. This raised the possibility that netrin-1 might influence transfilter migration through an effect on cell survival, and not cell motility. We directly tested if the presence or absence of netrin-1 influences the survival or proliferation of OP cells *in vitro*. We determined if netrin-1 influences OP survival or proliferation over a period of 16 hrs in culture, the duration of the migration assay, using MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), as previously described (Denizot and Lang, 1986; Richter-Landsberg and Vollgraf, 1998; Pang et al., 2000; Richter-Landsberg and Vollgraf, 1998; Pang et al., 2000). Quantification of the amount of metabolized MTT indicated that there was no difference between cells cultured without netrin-1 and cells cultured with 38 ng/ml, 100 ng/ml, 200 ng/ml netrin-1, or 100 ng/ml netrin-1 and 2.7 μg/ml DCC_{FB} (Fig. 2.3E). These observations indicated that the presence or absence of netrin-1 does not affect the survival of these cells. In contrast, increased MTT conversion was detected in the presence of 20 ng/ml PDGF-AA (Fig. 2.3E). However, the ~30% increase in dye production is not sufficient to account for the ~75% increase in the number of cells detected on the underside of the filter at the end of the migration assay, consistent with PDGF being both a mitogen and a chemoattractant for OPs, as previously described (Noble et al., 1988; Armstrong et al., 1990; Calver et al., 1998; Milner et al., 1996; Simpson and Armstrong, 1999; Calver et al., 1998; Milner et al., 1996; Armstrong et al., 1990; Simpson and Armstrong, 1999).

Netrin-1 induces retraction of oligodendrocyte precursor cell processes.

Partial collapse of the cytoskeleton has been proposed to contribute to the turning response made by axonal growth cones to repellent guidance cues (Luo et al., 1993). To test the hypothesis that the repellent action of netrin-1 might trigger cytoskeletal collapse and process retraction, OPs were incubated *in vitro* with 100 ng/ml netrin-1 for 30 minutes, 2 hours, and 16 hours. OPs were then labeled with an antibody against PDGFαR and for filamentous action (F-actin) using rhodamine-coupled phalloidin. Addition of netrin-1 caused a rapid and persistent decrease in OP surface area, process length, and process number at all time points. Addition of 2.7 μg/ml DCC_{FB} blocked the effect of

netrin-1 at all time points examined. Application of DCC_{FB} alone had no effect (Fig. 2.4, Table 2.1).

Aberrant distribution of oligodendrocyte precursors in mice lacking netrin-1 or DCC.

To determine if netrin-1 contributes to directing OP migration *in vivo*, we examined the distribution of OP cells in E15 mouse embryos lacking functional netrin-1 or DCC. Although the optic nerve has been widely used as a model system to study oligodendrocyte development, in netrin-1 or DCC knockout mice the axons of retinal ganglion cell neurons do not enter the optic nerve, producing optic nerve hypoplasia (Deiner et al., 1997). We therefore focused our analysis of the distribution of OP cells *in vivo* on the E15 spinal cord. Heterozygous netrin-1 or DCC mice were crossed, producing litters containing wild type, heterozygote, and homozygous loss of function embryos. Quantitative comparisons were performed within litters to maintain a precise age-match between embryos.

Because development of the oligodendroglial lineage progresses along a rostral-caudal gradient in the spinal cord (Foran and Peterson, 1992; Hajihosseini et al., 1996; Hajihosseini et al., 1996), tissue sections were collected exclusively from the brachial enlargement. OPs were detected with anti-PDGFαR. For quantification, the image of each cross section of the spinal cord was divided into dorsal, medial, and ventral thirds along the dorsal-ventral axis (Fig. 2.5A). A cell was counted only if anti-PDGFαR staining encircled a blue Hoechst-stained nucleus (Fig. 2.5B). This analysis indicated that in embryos homozygous for loss of netrin-1 or DCC function, significantly fewer OP cells were present in the dorsal third of the spinal cord. A corresponding increase was found in the number of cells present in the ventral third of the embryonic spinal cord in the absence of netrin-1 or DCC function (Figs. 2.6, 2.7, Table 2.2). Importantly, the total OP cell number in sections of homozygous netrin-1 or DCC loss of function embryos was not significantly different from their heterozygote or wild-type littermates (Fig. 2.7B, D). To age match the embryos as precisely as possible, the data presented in Fig. 2.7 and Table 2.2 is restricted to embryos derived from a single litter. The same total number of PDGFαR positive cells was not always found in E15 spinal cords when compared

between litters, likely reflecting the lack of a precise age match. However, analysis of multiple litters always revealed the phenotype reported in Fig.2.7 and Table 2.2. These results indicate that the absence of netrin-1 or DCC function produces a dramatic change in the distribution of these cells, consistent with DCC being required to mediate a repellent response of OP cells to netrin-1.

V. DISCUSSION

Netrins are a family of secreted proteins that function as tropic guidance cues directing cell and axon migration. We have recently reported that netrin-1 is expressed by mature myelinating oligodendroglia in the adult spinal cord (Manitt et al., 2001). This prompted us to investigate the possibility that netrin-1 might contribute to oligodendrocyte development. Here, we show that migrating OP cells in the embryonic spinal cord express *dcc* and *unc5h1*. Furthermore, we report that in the absence of netrin-1 or DCC function, fewer OP cells are found in the dorsal embryonic spinal cord, with a corresponding increase in the ventral spinal cord. The total number of OP cells present in a section of spinal cord remains the same, supporting the conclusion that this phenotype is the result of disrupted OP migration, and not due to altered proliferation or cell death. The repellent response to netrin often involves both DCC and an UNC-5 homolog family member (Colavita and Culotti, 1998; Hong et al., 1999; Keleman and Dickson, 2001; Hong et al., 1999; Keleman and Dickson, 2001). Our analysis of the effect of loss of DCC function, either due to gene knockout *in vivo* or using a function blocking antibody *in vitro*, indicates that OPs require DCC to be repelled by netrin-1.

In contrast, Spassky *et al.* (2002) have recently reported that an aggregate of cells expressing netrin-1 exerts a modest chemoattractive effect on OP cells migrating from explants of embryonic rat optic nerve *in vitro*. Several possibilities may account for the discrepancy between these results and our conclusion that netrin-1 functions as a repellent for OPs. Multiple lineages of OP cells have been described (Spassky et al., 1998; Fu et al., 2002; Spassky et al., 1998) and it may be the case that OP cells migrating from explants of optic nerve are different from OP cells in the embryonic spinal cord. Secondly, cues presented with netrin-1 can influence the response to netrin-1 (Hopker et al., 1999). Such cues present in the optic nerve explant, or secreted by the netrin-1 producing cells, may switch the response of the OP cells to netrin-1 from repulsion to attraction. Importantly, the results of the transfilter migration assays reported here minimize the contributions of additional environmental factors by challenging OP cells with purified netrin-1 protein. In agreement with our findings, Sugimoto et al. (2001) have provided evidence that netrin-1 is a repellent cue for glial precursor cells, including OPs, migrating out of explants of newborn rat optic nerve. Spassky et al. (2002) suggest

that the age of the explanted optic nerve may account for the discrepancy between these results, but this remains to be resolved. No direct evidence had been provided that netrin-1 influences OP cell migration *in vivo*. Here, the results of our *in vitro* analysis are consistent our findings *in vivo*, indicating that netrin-1 is a repellent for migrating OP cells in the embryonic spinal cord.

Relatively little is known about the migratory paths taken by OPs as they disperse throughout the developing spinal cord. While radial glia may contribute to directing OPs laterally (Diers-Fenger et al., 2001), the ventro-dorsal migration of OP cells is poorly understood. It was suggested that OPs might migrate dorsally along commissural axons (Miller, 1996); however, the absence of migrating streams of OPs along these axons argues against this (Miller et al., 1997; Calver et al., 1998; Calver et al., 1998). Alternatively, it has been proposed that OPs might be directed by cues that either attract them dorsally or repel them from the ventral embryonic spinal cord (Miller et al., 1997). Our finding that netrin-1 repels OP migration *in vitro* supports the conclusion that a repellent action of netrin-1 directs migrating OP cells into the dorsal embryonic spinal cord.

Loss of DCC or netrin-1 function does not induce OP cell death.

Both DCC and UNC-5 homologues have been suggested to function as pro-apoptotic dependence receptors, causing cell death in the absence of netrin-1 (Mehlen et al., 1998; Forcet et al., 2001; Llambi et al., 2001; Forcet et al., 2001; Llambi et al., 2001). Convincing evidence; however, has not been provided that cells expressing physiologically relevant levels of netrin receptors die, either *in vitro* or *in vivo*, as a result of the absence of netrin. We show that OP cells express *dcc* and *unc5h1*, but not *netrin-1*. The absence of netrin-1 or DCC caused a defect in migration, but no effect on cell survival was observed *in vivo* or *in vitro*, indicating that neither DCC nor UNC5H1 functions as a pro-apoptotic dependence receptor in these cells.

Tropism, Repulsion, and Collapse.

Using the transfilter microchemotaxis assay, we found that netrin-1 placed in the bottom chamber reduced the number of OPs migrating to the lower side of the filter,

suggesting that netrin-1 is a repellent for OP cells. Consistent with this, netrin-1 in the upper chamber increased the number of OP cells migrating through the filter, indicating that these cells preferentially migrate down a gradient of netrin-1. Interestingly, an equal concentration of netrin-1 in the top and bottom compartments reduced migration to a level similar to that produced by netrin-1 on the bottom alone. If netrin-1 produced a purely tropic effect on OP migration, it might be expected that surrounding the cells with a uniform concentration of netrin-1 would have no effect on motility. The observation that a uniform concentration of netrin-1 causes the cells to become less motile indicates that netrin also exerts a kinetic effect on OP motility. However, when netrin-1 is placed only in the upper compartment and a high concentration of netrin-1 surrounds the cells, the cells do not freeze, but given the opportunity to escape from netrin-1, they migrate to the lower side of the filter. These results show that netrin-1 inhibits OP motility, but also suggest that if an OP cell finds itself in the midst of a gradient of netrin-1, its movement will be asymmetrically inhibited, and the cell will move in the direction of less inhibition.

These findings suggest that asymmetric limited collapse of the OP cytoskeleton and withdrawal of OP cell processes may underlie the repellent response of OP cells to a gradient of netrin-1. Partial collapse has been proposed as a mechanism underlying turns made by axonal growth cones in response to repellent guidance cues. For example, semaphorins were first identified in vertebrates on the basis of their ability to cause growth cone collapse (Luo et al., 1993). Although a repellent cue can cause the complete collapse of a neuronal growth cone (Luo et al., 1993), encountering a local source of the same repellent, such as a microscopic bead coated with the cue, may only induce partial collapse (Fan and Raper, 1995). In this case, the edge of the growth cone contacting the bead may withdraw, but the growth cone will continue to extend away from the cue (Luo and Raper, 1994). Our demonstration that netrin-1 induces a rapid and persistent retraction of OP cell processes is consistent with the hypothesis that a gradient of netrin-1 may direct OP cell migration by triggering asymmetric collapse the OP cytoskeleton.

In the chemotaxis assay, although migration toward netrin-1 is significantly reduced when compared with control, migration was not completely blocked. Our findings are consistent with a model in which netrin-1 reduces, but does not completely suppress spontaneous oligodendrocyte motility. If a cell is to move from a high

concentration toward a lower concentration of a repellent cue, the collapsing action of the cue cannot be so potent that it inhibits motility entirely, otherwise the cell will never escape the high concentration of the cue. This interpretation is in agreement with the observations that netrin-1 induces partial, but not complete, withdrawal of OP cell processes, that netrin-1 does not completely block OP migration in the transfilter assay, and that a uniform concentration of netrin-1 presented on the top and bottom of the chemotaxis chamber inhibits migration to an extent similar to presentation of netrin-1 in the bottom chamber alone.

Oligodendrocytes, but not oligodendrocyte precursors, express netrin-1 *in vivo*.

Here we show that *netrin-1* is not expressed by OP cells. We have recently reported that *netrin-1* is expressed by myelinating oligodendrocytes in the adult mammalian spinal cord (Manitt et al., 2001). While OPs are capable of migrating great distances and remyelinating axons when transplanted into either demyelinated lesions or mutant animals lacking normal myelination (Gumpel et al., 1989; Groves et al., 1993; Warrington et al., 1993; Groves et al., 1993; Warrington et al., 1993), OPs transplanted into appropriately myelinated regions migrate very little (O'Leary and Blakemore, 1997). Furthermore, in a study using co-culture of oligodendroglia and the CG4 OP-like cell line (Louis et al., 1992), the extending processes of CG4 cells collapsed when they contacted oligodendrocyte processes (Jefferson et al., 1997). These findings suggest that netrin-1 produced by mature oligodendroglia *in vivo* may inhibit the migration of OPs into regions where sufficient numbers of oligodendrocytes are present and locally contribute to appropriately spacing them along the axon.

Myelination is essential for proper CNS function. In demyelinating diseases, such as multiple sclerosis (MS), even focal myelin loss can result in impairment (Orentas and Miller, 1998). The evidence presented here indicates that netrin-1 is an essential cue that directs migrating OP cells during neural development. Further understanding the fundamental mechanisms that direct the development and maturation of oligodendrocytes will provide insight into developing strategies that aim to promote remyelination in the context of demyelinating diseases.

Figure 2.1. Oligodendrocyte precursors express *dcc* and *unc5h1*, but not *netrin-1*, *in vivo*.

Double-label *in situ* hybridization-immunohistochemical analyses of coronal sections of E15 mouse spinal cord. Cell nuclei are stained blue with Hoechst dye. (A and B) *In situ* hybridization identifies *netrin-1* expressing floor plate cells (red). Panel B illustrates that the PDGFαR immuno-positive OPs in panel A (green) do not express *netrin-1*. Panels C and D show ventral spinal cord white matter and motoneurons at the edge of the gray matter. Panel C shows that PDGFαR immuno-positive OPs (green) express *unc5h1* (red, *in situ* hybridization). Panel E presents a four-fold magnification of one cell from panel C, illustrating double-labeling. Panel D shows that PDGFαR immuno-positive OPs (green) express *dcc* (red, *in situ* hybridization). Panel F depicts a four-fold magnification of one cell from panel D, illustrating double-labeling. No *in situ* hybridization signal was detected in PDGFαR positive OPs (green) using either the *unc5h1* or the *dcc* control sense hybridization probes (not shown). Arrowheads in panels C and D indicate double-labeled cells. The large PDGFαR negative *dcc* and *unc5h1* positive cells in the gray matter are motoneurons. Objective magnification: (A and B) 20X, (C-F) 40X. Scale bars: (A and B) 40 μm, (C and D) 20 μm, (E and F) 5 μm.

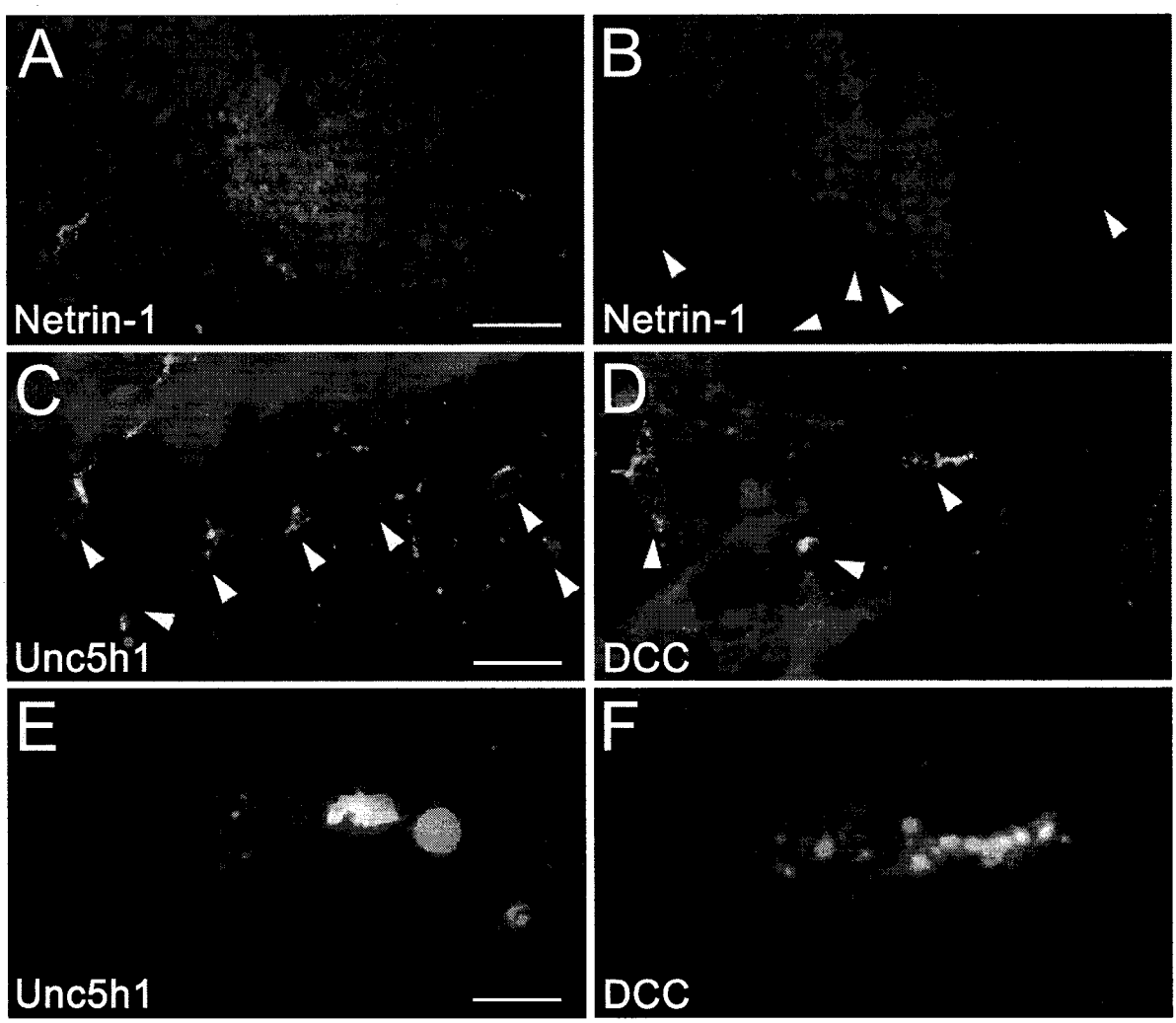
A
Netrin-1
B
Netrin-1
C
Unc5h1
D
DCC
E
Unc5h1
F
DCC

Figure 2.2. Oligodendrocyte precursors express *dcc* and *unc5h1*, but not *netrin-1*, *in vitro*.

(A) OPs co-immunolabel with antibodies against the OP marker NG2 (green) and the netrin receptor DCC (red). Panel B illustrates the same cell as in (A), showing only the DCC immunostaining (red) and Hoechst (blue). Panel C shows cells double-labeled with anti-PDGFαR (green) and with unc5h1 anti-sense RNA probes (red). Panel D illustrates the same cell as in (C), showing only the unc5h1 anti-sense signal. The corresponding unc5h1 sense RNA probe produced no signal (not shown). Panel E illustrates that OPs, immunolabeled with the A2B5 monoclonal antibody (red) in a mixed glial culture are not labeled by an antibody against netrin-1 (green). Objective magnification: (A, B, C and D) 100X, (E) 40X. Scale bar corresponds to 10 μm in all panels.

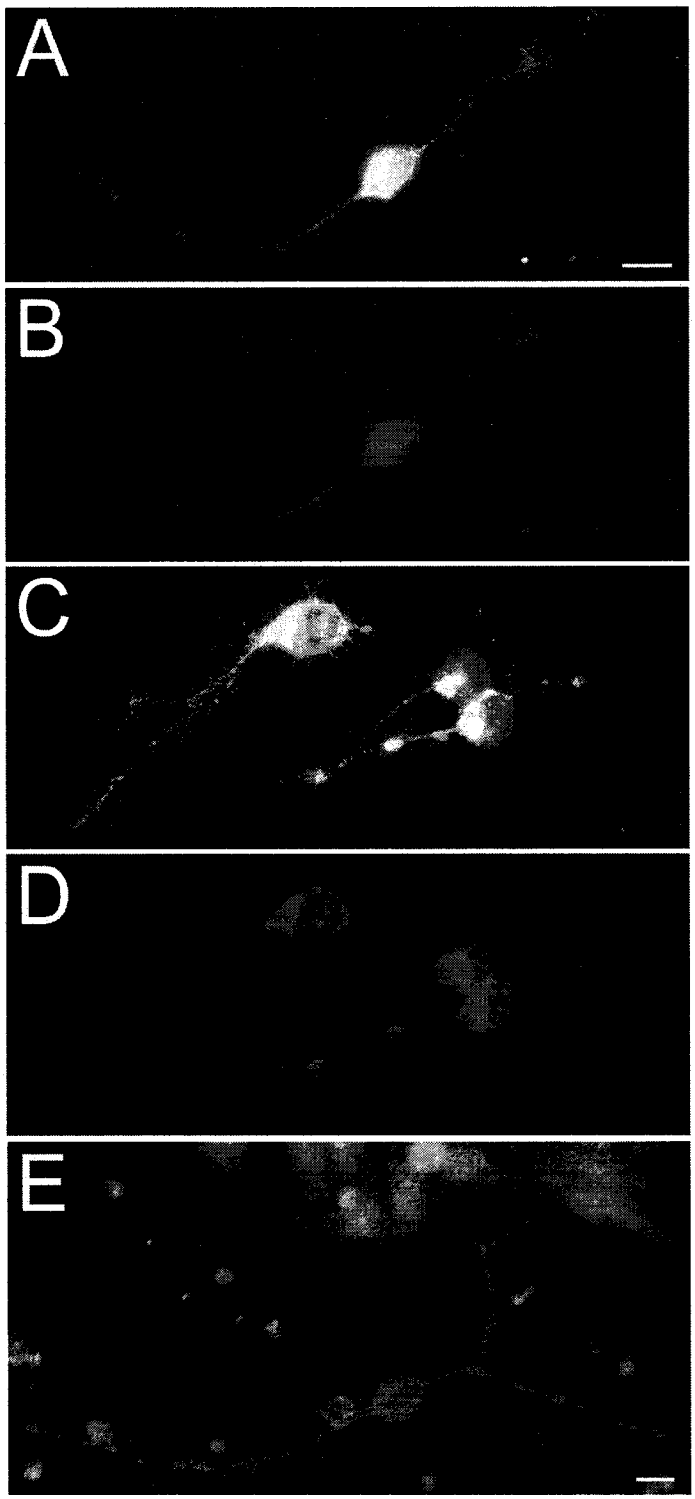
A
B
C
D
E

Figure 2.3. Netrin-1 is a chemorepellent for oligodendrocyte precursor cells *in vitro*. (A) Illustration of the transfilter microchemotaxis assay. (B-D) Netrin-1 repels OP cells. Panel B shows the data from one representative trial of this experiment. 100 ng/ml netrin-1 in the bottom compartment (NB100) significantly reduced cell migration compared to control. Increased migration to the bottom chamber was seen when 100 ng/ml netrin-1 was added to the top compartment (NT100, B and C). The OP chemoattractant PDGF-AA (20 ng/ml, bottom) was used as a positive control (PB20). For each condition shown in B, n=4 wells per condition. Panel C presents results pooled from multiple experimental trials and expressed as percent of control. Increasing concentrations of netrin-1 (3-200 ng/ml, NB3 to NB 200) produce a graded increase in the repellent action of netrin-1. Addition of the DCC function blocking antibody (2.7 μg/ml) to the top and bottom chambers blocked the repellent action of netrin-1 in the bottom chamber (DCC_{FB} NB100). In the presence of DCC_{FB} without added netrin-1 the same number of cells migrated as did in control. In panel C, n=22 for control, n=8 for NB3, NB10, NB25, NB50, NB200, DCC_{FB}, DCC_{FB} NB100, and NT100, n=16 for NB100, and n=21 for PB20. (D) Analysis of the results presented in (C) indicated that ~20 ng/ml netrin-1 produced a half-maximal repellent effect of netrin-1. (E) The MTT assay indicated that application of netrin-1 at 0, 38, 100, or 200 ng/ml for 16 hrs does not affect OP survival (n=6). Addition of DCC_{FB} with 100 ng/ml netrin-1 also had no effect. PDGF-AA, an established mitogen for OP cells, resulted in an increased number of OPs after 16 hrs. Values shown are the mean ± SEM. *: $p < 0.05$; **: $p < 0.005$.

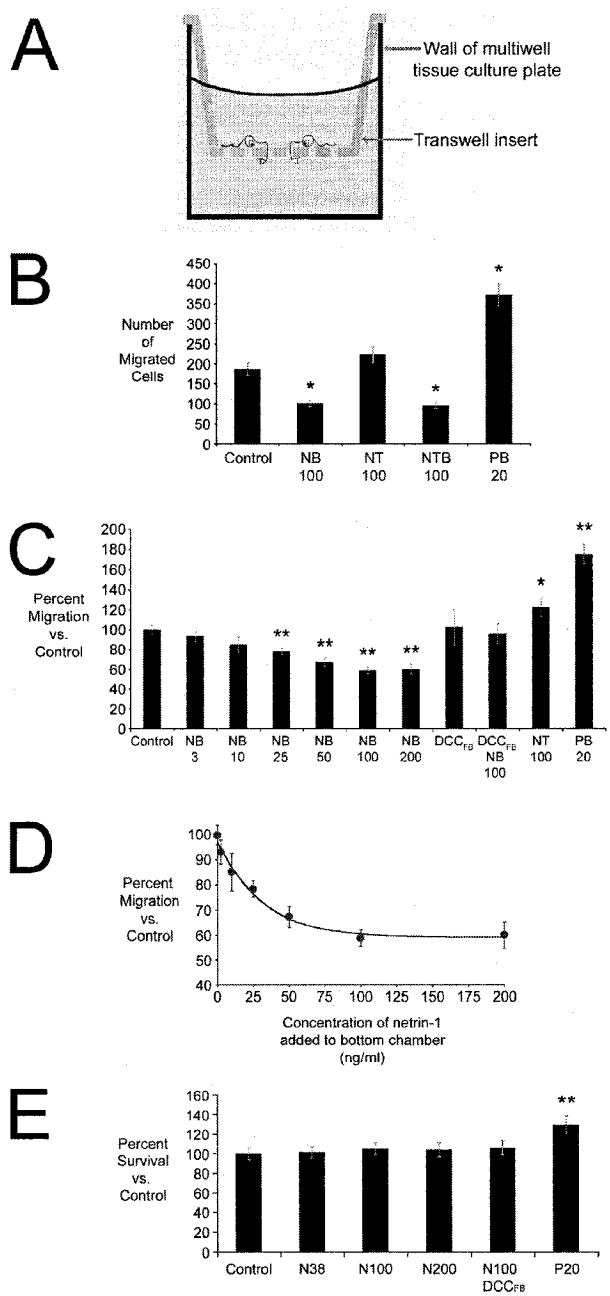
A
Wall of multiwell tissue culture plate
Transwell insert
B
Number of Migrated Cells
450
400
350
300
250
200
150
100
50
0
Control
NB 100
NT 100
NTB 100
PB 20
C
Percent Migration vs. Control
200
180
160
140
120
100
80
60
40
20
0
Control
NB 3
NB 10
NB 25
NB 50
NB 100
NB 200
DCC$_{FB}$
DCC$_{FB}$ NB 100
NT 100
PB 20
D
Percent Migration vs. Control
100
90
80
70
60
50
40
0 25 50 75 100 125 150 175 200
Concentration of netrin-1 added to bottom chamber (ng/ml)
E
Percent Survival vs. Control
160
140
120
100
80
60
40
20
0
Control
N38
N100
N200
N100 DCC$_{FB}$
P20

Figure 2.4. Retraction of OP processes induced by netrin-1.

OP cultures were exposed to netrin-1, DCC_{FB}, or both netrin-1 and DCC_{FB}, for 30 minutes, 2 hours, or 16 hours; fixed, and then stained for PDGFαR immunoreactivity and F-actin using rhodamine-coupled phalloidin. Phalloidin staining is shown in A, B, and C. Cell surface area, length of longest process, and number of major processes were then quantified. (A, B, C) Images of a control cell (A), and cells exposed to netrin-1 (B), or netrin-1 and DCC_{FB} (C) for 30 minutes. The cells illustrated in panels A, B, and C, have morphologies corresponding to the mean values shown in D, E, and F at the 30 min time point. Exposure of OP cells to netrin-1 for 30 minutes, 2 hours, or 16 hours results in decreased mean cellular surface area (D), length of the longest process (E), and number of major processes (F). Results obtained following incubation with DCC_{FB} or co-incubation of OPs with netrin-1 and DCC_{FB} did not differ significantly from control. Values shown are the mean ± SEM. *: $p < 0.05$; **: $p < 0.005$. Objective magnification is 40X. Scale bar corresponds to 10 μm.

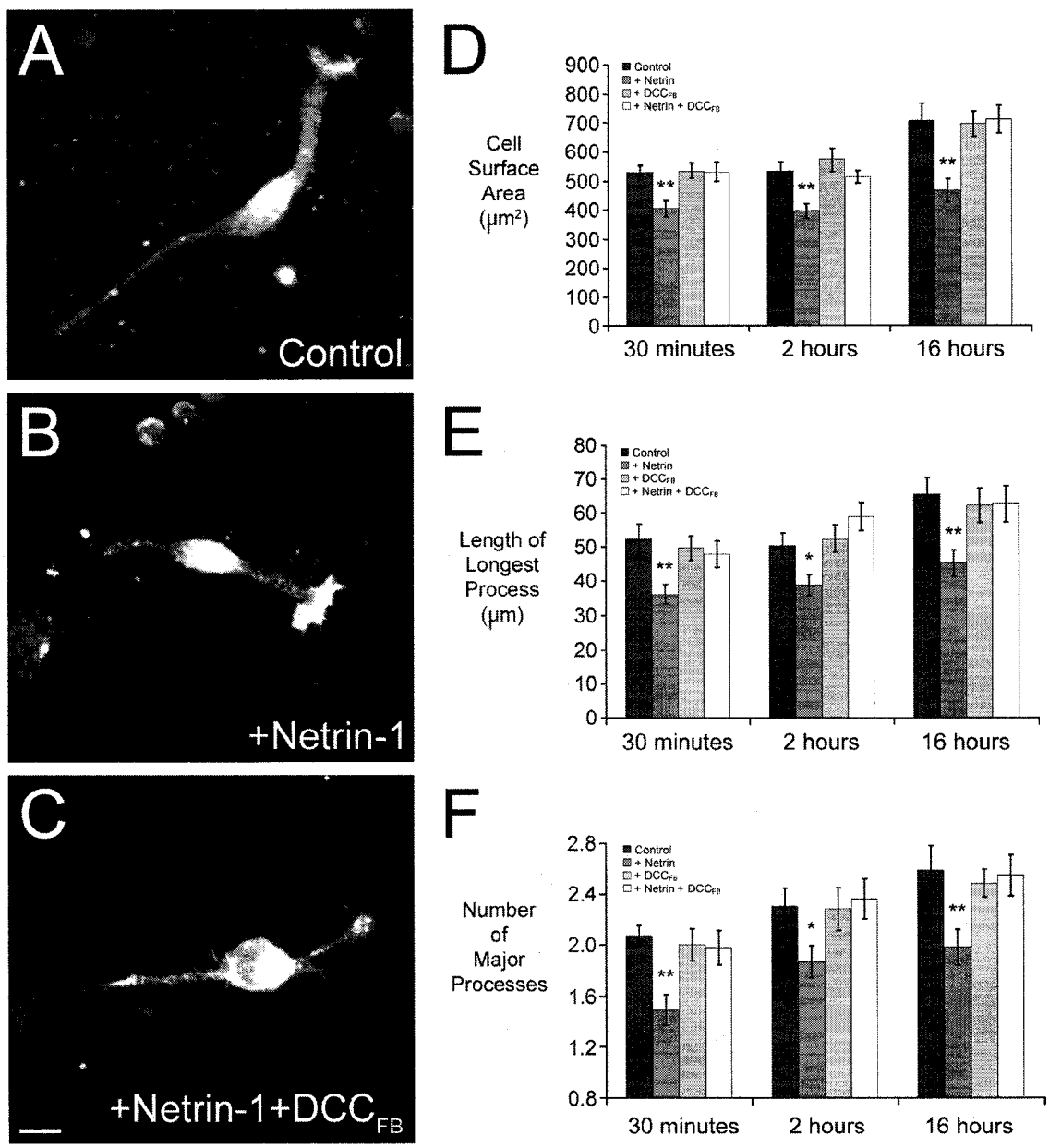
A
Control
B
+Netrin-1
C
+Netrin-1+DCC$_{FB}$
D
Cell Surface Area (μm^2)
Control
+ Netrin
+ DCC$_{FB}$
+ Netrin + DCC$_{FB}$
900
800
700
600
500
400
300
200
100
0
**
**
**
30 minutes
2 hours
16 hours
E
Length of Longest Process (μm)
80
70
60
50
40
30
20
10
0
**
*
**
30 minutes
2 hours
16 hours
F
Number of Major Processes
2.8
2.4
2.0
1.6
1.2
0.8
**
*
**
30 minutes
2 hours
16 hours

Figure 2.5. Analysis of OP cell distribution in E15 spinal cord.

(A) Coronal section of the brachial enlargement of an E15 spinal cord immunolabeled with anti-PDGFαR (yellow), and nuclei stained blue with Hoechst dye. For each section, four images were collected and tiled into a single image. The dorso-ventral height of the cord was measured at the midline, the cord divided into dorsal, medial, and ventral thirds, and the number of PDGFαR-positive OPs in each third counted. Only cells with Hoechst-positive nuclei encircled by PDGFαR immunoreactivity (B, arrowhead) were counted. Immunoreactivity not meeting this criterion (B, arrow) was excluded.

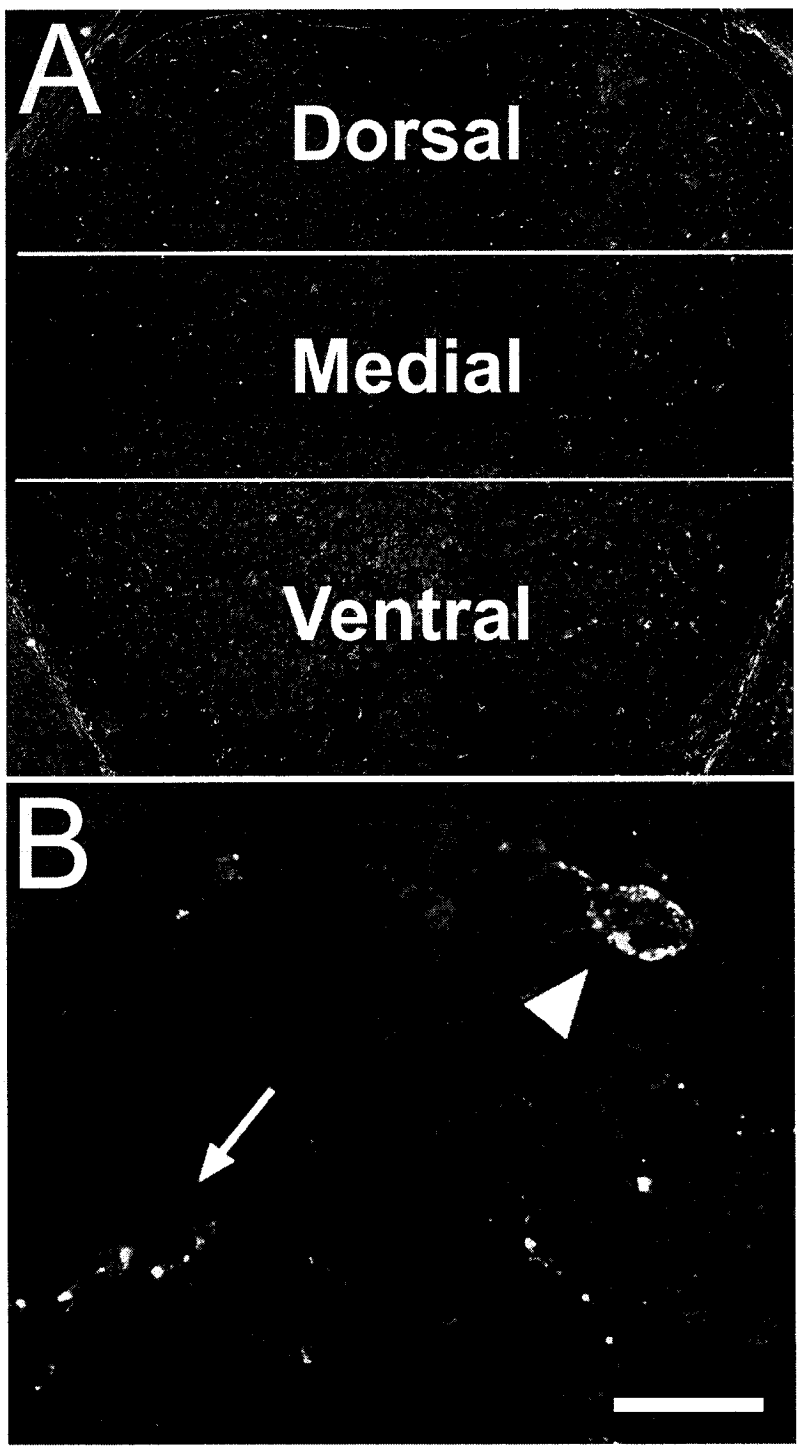
A
Dorsal
Medial
Ventral
B

Figure 2.6. Distribution of OP cells in E15 spinal cord sections.

The absence of netrin-1 or DCC function produces an aberrant distribution of OP cells in E15 spinal cord. Fewer PDGFαR-positive OP cells (arrowheads) were detected in the dorsal spinal cords of netrin-1 (B) or DCC (F) knockout (-/-) embryos compared to wild-type (+/+) littermates (A, E). Conversely, more OP cells were detected in the ventral spinal cords of netrin-1 (D) or DCC (H) knockout (-/-) embryos compared to their wild-type (+/+) littermates (C, G). 20 X objective, scale bar is 100 μm.

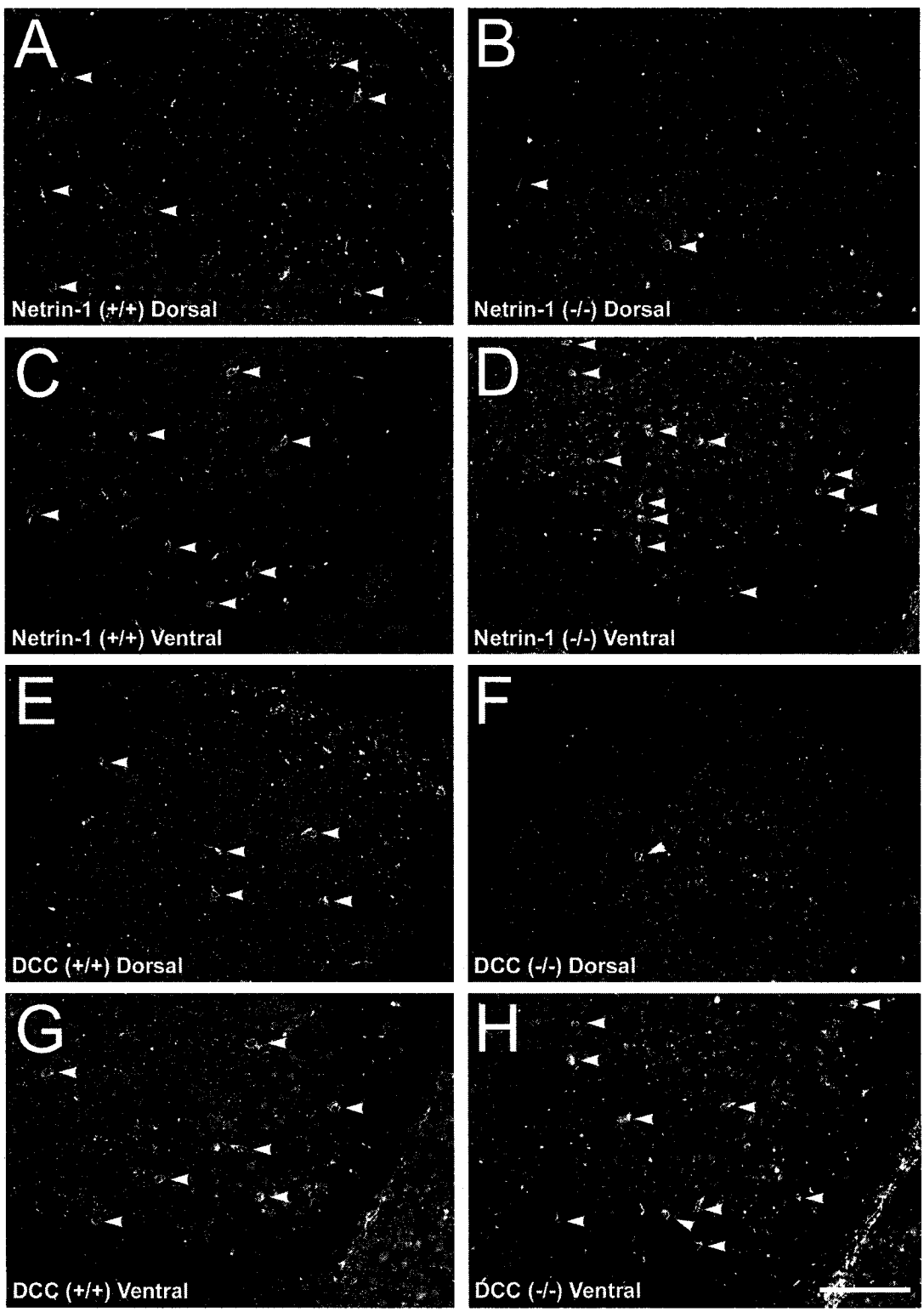
A
Netrin-1 (+/+) Dorsal
B
Netrin-1 (-/-) Dorsal
C
Netrin-1 (+/+) Ventral
D
Netrin-1 (-/-) Ventral
E
DCC (+/+) Dorsal
F
DCC (-/-) Dorsal
G
DCC (+/+) Ventral
H
DCC (-/-) Ventral

Figure 2.7. OP cell number is reduced in the dorsal spinal cord and increased in the ventral spinal cord of E15 mice lacking netrin-1 or DCC function.

(A, C) Quantification of the number of OP cells in the dorsal, medial, and ventral thirds of the spinal cords of netrin-1 (A) and DCC (C) wild-type, heterozygote, and homozygous loss of function embryos. As shown in Fig. 2.5, fewer OPs were detected in the dorsal spinal cords of the homozygous loss of function embryos, while a greater number of OPs were detected in the ventral spinal cords of these animals. (B, D) Total number of OPs counted in the spinal cords of netrin-1 (B) and DCC (D) wild-type, heterozygote, and mutant spinal cord sections. Values shown are the mean ± SD. *: $p < 0.05$; **: $p < 0.005$.

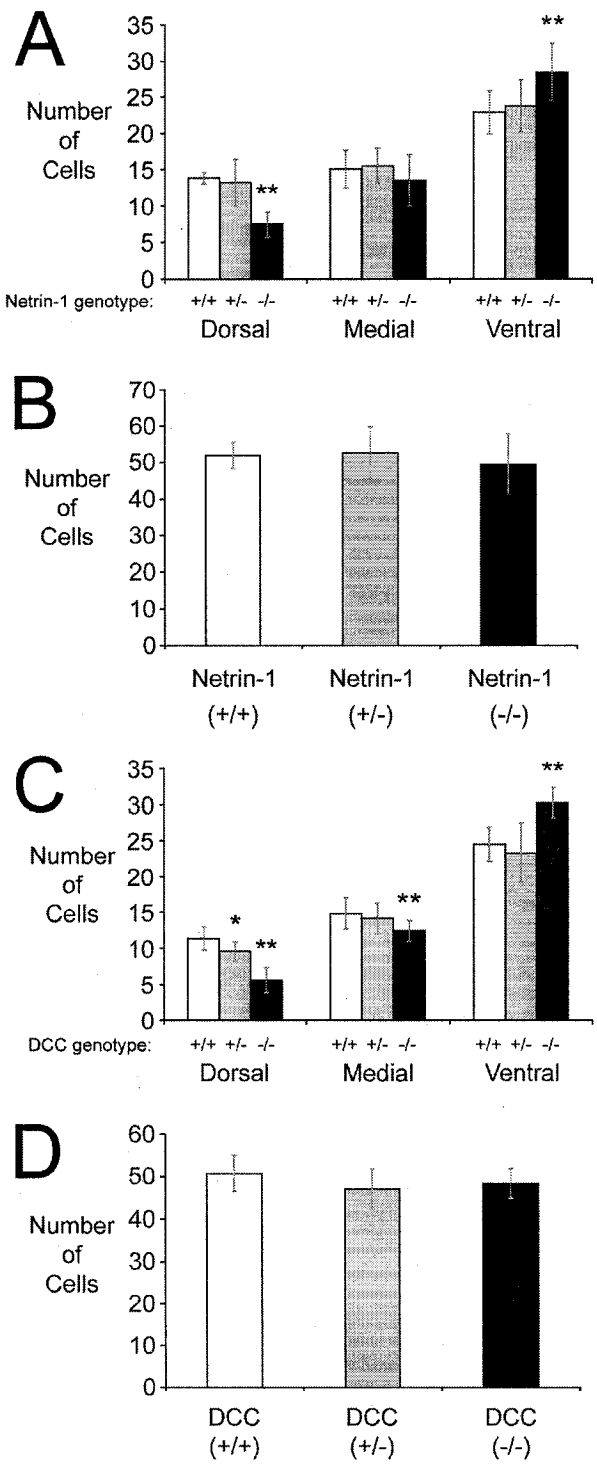
A
Number of Cells
35
30
25
20
15
10
5
0
**
**
Netrin-1 genotype:
+/+ +/- -/-
+/+ +/- -/-
+/+ +/- -/-
Dorsal
Medial
Ventral
B
Number of Cells
70
60
50
40
30
20
10
0
Netrin-1 (+/+)
Netrin-1 (+/-)
Netrin-1 (-/-)
C
Number of Cells
35
30
25
20
15
10
5
0
*
**
**
**
DCC genotype:
+/+ +/- -/-
+/+ +/- -/-
+/+ +/- -/-
Dorsal
Medial
Ventral
D
Number of Cells
60
50
40
30
20
10
0
DCC (+/+)
DCC (+/-)
DCC (-/-)

Table 2.1. Retraction of OP processes by netrin-1.

Surface Area (μm^2)

	30 minutes	2 hours	16 hours
Control	530.3±24.9	533.6±30.8	706.1±60.9
Netrin-1	406.2±27.7	396.9±25.3	468.1±38.4
DCC_{FB}	536.2±26.0	573.1±39.1	696.6±44.1
Net+DCC_{FB}	531.6±33.0	515.3±22.1	712.3±46.8

Length of Longest Process (μm)

	30 minutes	2 hours	16 hours
Control	52.4±4.2	50.3±3.8	65.4±4.9
Netrin-1	36.1±2.8	38.7±3.1	45.2±3.9
DCC_{FB}	49.7±3.6	52.3±4.0	62.1±5.1
Net+DCC_{FB}	47.9±4.0	58.7±3.9	62.5±5.4

Number of Major Processes

	30 minutes	2 hours	16 hours
Control	2.07±0.08	2.30±0.14	2.59±0.19
Netrin-1	1.49±0.12	1.87±0.12	1.98±0.14
DCC_{FB}	2.00±0.13	2.28±0.17	2.49±0.11
Net+DCC_{FB}	1.98±0.13	2.36±0.16	2.55±0.16

n

	30 minutes	2 hours	16 hours
Control	54	46	34
Netrin-1	57	53	56
DCC_{FB}	54	39	35
Net+DCC_{FB}	50	33	42

Netrin-1 induces a rapid and persistent retraction of OP processes, an effect that is DCC-dependant. OP surface area, process length, and process number (mean ± S.E.M.) were measured as described in Materials and Methods. n is the number of cells counted for a given condition and time point.

Table 2.2. Distribution of PDGFαR-positive OP cells in wild-type, netrin-1, or DCC-deficient E15 mouse spinal cord.

	Mean # of OP / region of s.c				
Genotype	**Dorsal**	**Medial**	**Ventral**	**Total**	**n**
Netrin-1$^{+/+}$	13.9±0.8	15.1±2.6	22.9±3.0	51.9±3.7	9
Netrin-1$^{+/-}$	13.2±3.2	15.6±2.4	23.8±3.6	52.5±7.2	18
Netrin-1$^{-/-}$	7.4±1.7	13.6±3.5	28.4±4.0	49.4±8.3	9
DCC$^{+/+}$	11.3±1.6	14.8±2.2	24.5±2.4	50.7±4.3	12
DCC$^{+/-}$	9.5±1.4	14.1±2.2	23.3±4.1	47.0±4.8	15
DCC$^{-/-}$	5.6±1.7	12.4±1.4	30.3±2.1	48.3±3.6	12

Spinal cord sections from E15 mouse embryos were collected and processed as described in Materials and Methods. Images of spinal cord sections were divided into dorsal, medial and ventral thirds, and the number of OPs in each third counted. An OP was only counted if PDGFαR immunostaining surrounded a Hoechst-stained nucleus. OP number is mean ± SD. n is tissue sections analyzed per condition.

PREFACE TO CHAPTER 3

The study described in Chapter 2 described the role of netrin-1 in the guidance of *dcc* and *unc5 homologue*-expressing OP cells. Previous work from our laboratory has demonstrated that following differentiation, oligodendrocytes continue to express *dcc* and *unc5 homologue* receptors (Manitt et al., 2004) and upregulate netrin-1 expression, which is enriched in non-compact myelin membranes (Manitt et al., 2001). Non-compact myelin membranes are predominantly found at sites of axo-glial contact such as the periaxonal space and paranodal region. In addition, netrin and DCC family netrin receptors have previously been reported to mediate cell-cell interactions (Chuong et al., 1994; Winberg et al., 1998; Srinivasan et al., 2003), leading to the hypothesis that netrin and DCC may be involved in regulating axo-glial interaction (Kennedy, 2000).

The aims of this study were to:

1) Determine if netrin-1 and its receptors are expressed at CNS paranodes

2) Investigate whether CNS paranodes are disrupted in the absence of netrin-1 or DCC and, if so, identify the consequences on domain segregation at the nodal region

CHAPTER 3

Editor in chief

Organization of Paranodal Junctions in CNS Myelin Requires DCC and Netrin-1

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Running Title: Netrin and DCC regulate CNS paranodal myelin

Key words: Myelin, axo-glial junction, paranode, septate-like junctions, Caspr, Neurofascin

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I. ABSTRACT

Netrin-1 and its receptors play crucial roles in axon guidance in the developing central nervous system, but are also widely expressed by multiple neuronal populations and oligodendrocytes in the adult CNS. Here, we describe a novel role for netrin in regulating the organization of adhesive complexes between oligodendrocyte processes and axons at the paranodal axo-glial junction. In myelinating cerebellar slice cultures derived from neonatal $DCC^{-/-}$ and netrin-$1^{-/-}$ mice, paranodes initially develop and mature normally but later become disorganized; with paranodal loops becoming detached from the axonal surface and from each other and transverse bands becoming less visible. Consequently, potassium channels become abnormally localized to the paranodal region in mutant slices, and the caspr immunoreactive domain expands longitudinally along the axon. These data suggest that netrin-1 and DCC are essential for the maintenance of paranodal axo-glial interactions, and may be indicative of a wider role in mediating cell-cell contacts in the adult.

II. INTRODUCTION

The netrin family of proteins are guidance cues for migrating axonal growth cones and cells during neural development. They are bifunctional; attracting some axons and cells and repelling others (reviewed by Barallobre et al., 2005). Oligodendrocyte precursors in the developing spinal cord express netrin receptors *dcc*, *unc5a*, and *unc5b*, and respond to netrin-1 expressed at the floor plate which guides the cells away from their ventral sites of origin (Jarjour et al., 2003; Tsai et al., 2003). In the adult, spinal oligodendrocytes themselves express netrin-1 and multiple netrin receptors (Manitt et al., 2001; Manitt et al., 2004), but the function for netrin-1 in the mature CNS has not been identified. Subcellular fractionation of adult rat spinal cord white matter indicated that netrin-1 is enriched in fractions containing non-compact myelin membranes (Manitt et al., 2001). Non-compact myelin fractions are typically characterized by the presence of proteins present at points of axo-glial contact, including the highly-specialized paranodal junction (Menon et al., 2003).

The functional division of myelinated axons into distinct domains is crucial for the establishment of saltatory conduction. Concentrated at the nodes of Ranvier are high densities of voltage-gated sodium channels, which depolarize the axonal membrane and generate the action potential (Waxman and Ritchie, 1993). The paranodal axo-glial junctions, where each layer of the myelin sheath terminates in a cytoplasm-filled membrane loop that tightly abuts the axon, flank the node. The paranode separates the node from the juxtaparanodal domain, the outermost region of the internode (reviewed by Poliak and Peles, 2003). Concentrated at the juxtaparanode are *Shaker*-type voltage-gated potassium channels (Wang et al., 1993). The paranode has been proposed to act as a barrier that separates sodium channels and juxtaparanodal potassium channels, as potassium channels are mislocalized to the paranodal region in mutant mice where the close association between the paranodal loops and the axon are disrupted, (Boyle et al., 2001; Bhat et al., 2001; Ishibashi et al., 2002; Dupree et al., 1999).

Here, we describe a novel role for netrin-1 and its receptor DCC in mediating axo-glial contact. We obtained evidence that DCC and UNC5 homologue netrin receptors and netrin- are enriched at oligodendroglial paranodes, and that in the absence of netrin-1 or DCC function, CNS paranodes become disrupted. Surprisingly, paranodes develop - and

mature - normally, but this organization is subsequently lost. To our knowledge, this is the first demonstration of this phenotype. Our findings implicate netrin-1 and DCC in the organization of the CNS paranodal axo-glial junction.

III. MATERIALS AND METHODS

Animals

Adult male Sprague-Dawley rats, 180-200g, and newborn CD1 mouse pups were obtained from Charles River Canada (QC). Mice heterozygous for netrin-1 or DCC function were obtained from Marc Tessier-Lavigne (Stanford) and Robert Weinberg (Harvard), respectively. All procedures with animals were performed in accordance with the "Canadian Council on Animal Care" guidelines for the use of animals in research.

Antibodies

The following primary antibodies were used in this study: Guinea pig polyclonal anti-Caspr, rabbit polyclonal anti-Caspr (a kind gift of Dr. David Colman, McGill University, Svenningsen et al., 2003), mouse monoclonal anti-DCC intracellular domain (DCC_{IN}, BD Biosciences Pharmingen, San Jose, CA), rabbit polyclonal anti-Kv1.2 (Alomone Labs, Jerusalem, Israel), mouse monoclonal anti-myelin basic protein (MBP; Chemicon International, Temecula, CA), rabbit polyclonal anti-MBP (Chemicon), rabbit polyclonal anti-netrin PN2 (Manitt et al., 2001), rabbit polyclonal anti-neurofascin NFC2 (a kind gift of Dr. Peter Brophy, University of Edinburgh, recognizes both 155 and 186 isoforms of neurofascin, Tait et al., 2000), mouse monoclonal anti-neurofilament 145 kDa (NFM; Chemicon), chicken polyclonal anti-NFM (EnCor Biotechnology Inc., Alachua, FL), chicken polyclonal anti-neurofilament 200 kDa (NFH; EnCor), mouse monoclonal anti-sodium channel (pan) (Na^+ch, Sigma), and rabbit polyclonal anti-UNC5 homologue (pan) (Tong et al., 2001; Manitt et al., 2004, a kind gift of Dr. Tony Pawson, Mount Sinai Hospital). Secondary antibodies used were Alexa 488-conjugated donkey anti-mouse and goat anti-rabbit, Alexa 546-conjugated goat anti-mouse and goat anti-rabbit, Alexa 633-conjugated goat anti-chicken and goat anti-mouse, and Alexa 647-conjugated donkey anti-rabbit from Molecular Probes (Eugene, OR), and rhodamine red x-conjugated donkey anti-guinea pig (Jackson ImmunoResearch, West Grove, PA).

Immunocytochemistry and confocal analysis of adult rat spinal cord

Adult rats were anesthetized with sodium pentobarbital (Somnotol; 65 mg/kg, i.p.; MTC Pharmaceuticals) and perfused transcardially with PBS, followed by 4%

paraformaldehyde in PBS at pH 7.0. Spinal cords were then equilibrated in 30% sucrose in PBS for 48 hours at 4°C, embedded in optimal cutting temperature compound (Sakata Finetek, Torrence, CA) and 18 μm longitudinal and coronal cryosections cut. Sections were rinsed in TBS and incubated in blocking solution (5% bovine serum albumin (Sigma), 0.3% Triton X-100 in TBS) for 1 hour at 4°C. Sections were then incubated in primary antibody diluted in blocking solution overnight at 4°C followed by secondary antibody diluted in 3% BSA in TBS for 70 minutes. Slices were then rinsed in ddH_2O, mounted on slides, and coverslipped using Gel Mount (Biomeda, Foster City, CA) for analysis by confocal microscopy. Images were captured using a Zeiss LSM 510 confocal microscope. In all cases, single optical slices were collected.

Cerebellar slice cultures

Cerebellar slice culture preparation was based on previously-published methods (Notterpek et al., 1993; Dusart et al., 1997). Briefly, following decapitation, brains were dissected out into ice-cold Hank's Balanced Salt Solution (HBSS) and 250 μm sagittal slices of cerebellum and attached brainstem were cut using a McIlwain tissue chopper. The tissue slices were placed on Millipore Millicell-CM organotypic culture inserts (Fisher) in medium containing 50% MEM with Earle's salts, 25% Earle's Balanced Salt Solution, 25% heat-inactivated horse serum (HIHS), 6.5 mg/ml glucose, and glutamax-II supplement with penicillin-streptomycin and amphotericin B (all purchased from Invitrogen, except glucose, which was purchased from Sigma). The membranes were transferred into fresh medium every two days.

Immunocytochemistry and confocal analysis of cerebellar slice cultures

Slices processed for immunolabeling were fixed while attached to membranes with 4% paraformaldehyde in phosphate-buffered saline (PBS) for 1 hour, rinsed in PBS for 10 minutes, and blocked with 3% HIHS, 2% bovine serum albumin (BSA), 0.25% Triton X-100 in PBS for 2 hours. Slices were then incubated in primary antibody 36-48 hours, washed once for 10 minutes and then thrice for 1 hour, and then incubated in secondary antibody overnight. Slices were then washed once for 10 minutes, twice for 1 hour, and once overnight. All antibody dilutions and washes were done in blocking

solution. Slices were then rinsed in ddH_2O, mounted on slides, and coverslipped using Gel Mount (Biomeda, Foster City, CA) for analysis by confocal microscopy.

Images were captured using a Zeiss LSM 510 confocal microscope. In all cases, single optical slices were collected. For analysis of nodal length, slices were labeled with mouse anti-MBP and rabbit anti-NFM antibodies. The plane chosen for imaging of each node was that where MBP immunoreactivity flanking the node was closest. For analysis of sodium and potassium channel distribution, slices were labeled with mouse anti-Na^+ch and rabbit anti-Kv1.2 antibodies. The plane chosen for imaging was that where nodal Na^+ch and juxtaparanodal Kv1.2 immunoreactivity was closest. The distance between Na^+ch and Kv1.2 channel expression, and the width of the Na^+ch channel immunoreactivity were analyzed. For analysis of neurofascin localization, the distance between the outermost edges of the region of neurofascin immunoreactivity was measured. The plane chosen for imaging was that in which the node of Ranvier most evenly bisects the region of neurofascin immunolabeling. For analyses of Caspr immunoreactivity, the width of each caspr expression domain and the distance between caspr domains across each node were measured. For analyses of neurofascin and Caspr localization, slices were also labeled with chicken anti-NFH and mouse anti-MBP antibodies. Distances were measured using LSM 510 Image Browser software.

Electron Microscopy

Slices and attached membrane were cut out from surrounding membrane and fixed overnight with 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer, osmicated with potassium ferrocyanide-reduced 1% osmium tetroxide solution for one hour, and then dehydrated with successive 10 minute rinses in 30, 50, 70, 80, 95, and 100% ethanol (three times). Tissue was then infiltrated with 1:1, 1:2, and 1:3 ethanol to epon blends, and then in pure epon, for one hour each, and then embedded in epon, tissue side down, in a plastic beam capsule. 70-100 nm sections were then cut onto 200 mesh copper grids and stained with 4% uranyl acetate for 5 minutes, followed by Reynolds's lead citrate for 3 minutes.

Images were observed using a transmission electron microscope at 80 kV using a JEM-2000FX (JEOL, Tokyo, Japan; used for all analyses except for 25 DIV netrin

cultures) or at 120 kV using a Tecnai 12 (FEI, Hillsboro, OR; 25 DIV netrin cultures) Gatan Bioscan CCD camera. For each condition, at least two slices from each of two animals were analyzed. For analyses of compact myelin, the width of the periaxonal space at each axon was measured, and the periodicity of compact myelin was calculated. For analyses of paranodal myelin; due to technical limitations encountered during the embedding process, only heminodes were analyzed. Each heminode was examined for the presence of four 'faults'. A heminode was credited with a fault if the majority of paranodal loops present lacked transverse bands (TBs); if neighboring paranodal glial membranes lacked electron density between apposed glial loops ('interloop densities') and these membranes were separated by two or more membrane widths; if at least one paranodal loop had detached from the axonal surface; or if at least one paranodal loop faced away from the axonal surface (see Fig. 3.7A).

IV. RESULTS

Localization of netrin-1 and netrin receptors in adult CNS white matter

Netrin-1, DCC and UNC5 homologues are all expressed by myelinating oligodendrocytes in the mature CNS (Manitt et al., 2001; Manitt et al., 2004). Furthermore, subcellular fractionation of adult CNS white matter indicates that netrin protein is associated with noncompact myelin, which includes periaxonal and paranodal membranes (Manitt et al., 2001). To further characterize the distribution of netrin and its receptors in CNS white matter, immunohistochemical analysis for netrin, DCC, and UNC5 protein was performed in sections of adult rat spinal cord. The PN2 antibody used to detect netrin protein recognizes netrin-1 and netrin-3, though the immunoreactivity observed was assumed to correspond to netrin-1 because detectable levels of *netrin-3* were not observed in the adult rat spinal cord (Manitt et al., 2001). Consistent with our fractionation analysis, netrin-1 and UNC5 immunoreactivity were found to co-localize with caspr at the paranode in longitudinal sections (Fig. 3.1A-C and M-O). DCC immunoreactivity was also observed in close proximity to the paranode (Fig. 3.1G-I). In axonal cross-sections, netrin-1 and UNC5 immunoreactivity is closely associated with, and partially overlaps, that for caspr (Fig. 3.1D-F, P-R), suggesting that netrin-1 and UNC5 protein are localized to regions where oligodendroglial paranodal loops closely appose the axonal surface. While DCC immunoreactivity was observed to closely surround the axon at the paranode, it did not co-localize with caspr (Fig. 3.1J-L), suggesting that its localization may be restricted to regions of the glial membrane that do not contact the axon. Based on these observations, we hypothesized that netrin-1 and DCC may play a role in regulating the organization of the paranodal axo-glial junction in the CNS.

Establishment of long-term myelinating cerebellar organotypic cultures

To determine the roles of netrin-1 and DCC in the organization the axo-glial junction, we sought to examine CNS white matter in the absence of *netrin-1* or *dcc* expression. However, mice lacking functional netrin-1 or DCC protein die within hours of birth (Fazeli et al., 1997; Serafini et al., 1996), before the majority of CNS myelin formation in rodents. To circumvent this early lethality, we established organotypic

cerebellar slice cultures derived from either *netrin-1* or *dcc* newborn knockout mice and their wild-type littermates.

In the developing rat cerebellum, the MBP-positive oligodendroglia are first observed at postnatal day 2 (P2). They increase in abundance throughout the cerebellar white matter by P7. Rare myelinated axon segments are first observed at P7, and increase in abundance by P12, with extensive myelination seen by P20 (Reynolds and Wilkin, 1988). A similar developmental time course is observed during cerebellar oligodendroglial development in mouse (Foran and Peterson, 1992). Oligodendroglial development in our cultures was similar to developmental time course observed *in vivo.* MBP-positive oligodendrocytes were visible at 3 days in vitro (DIV), and increased in number and process complexity between 3 and 7 DIV. Widespread MBP-positive myelin profiles were visible by 13 DIV, and myelination was extensive by 30 DIV (Fig. 3.2A-D). Using this method, we have maintained healthy myelinating cultures as long as 70 DIV. Myelin ultrastructure appeared normal, as clear major dense lines, interperiod lines and periaxonal spaces were evident in electron micrographs of myelin cross-sections, with the number of myelin wraps routinely exceeding 10 (Fig. 3.4).

Netrin-1 is essential for the migration of granule cell precursors during development, mediated at least in part by its receptor UNC5C. It has previously been reported that Purkinje cells in the postnatal rat cerebellum express *netrin-1*, *dcc*, and *unc5c* (Bloch-Gallego et al., 1999; Wehrle et al., 2005). In the adult rat spinal cord, oligodendrocytes express *netrin-1*, *dcc*, and multiple *unc5 homologues*, but principally *unc5b* (Manitt et al., 2004). In our cultures, netrin, DCC, and UNC5 homologue immunoreactivity was closely associated with both Purkinje neurons (Fig 3.2E-H) and oligodendrocytes (Fig 3.2H-J), suggesting that the expression of these proteins may be similar to what is observed *in vivo*. At the paranodal axo-glial junction, netrin and DCC immunoreactivity co-localized with that for caspr, as was observed *in vivo* (Fig. 3.3A-L). UNC5 immunoreactivity overlapped with that for caspr as well, but was also present along the axon outside of the paranodal region (Fig. 3.3M-R).

Compact myelin is largely normal in mature DCC and netrin-1 mutant slice cultures

To investigate whether netrin-1 and DCC are required for the proper organization of CNS myelin, cerebellar slice cultures were prepared from newborn netrin-1 and DCC

mutant animals and their wild-type littermates and maintained *in vitro* for 49 or 67 days, respectively. Extensive MBP-positive myelin profiles were visible in both netrin-1 and DCC cultures. In electron micrographs of cross sections of $DCC^{-/-}$ and netrin-$1^{-/-}$ slices (Fig. 3.4B,F), compact myelin appeared to be grossly normal, as was the case for slices obtained from wild-type animals (Fig. 3.4A, E). The width of the periaxonal space (PS; Fig. 3.4D, H) was unaffected in both $DCC^{-/-}$ and netrin-$1^{-/-}$ slices. Myelin periodicity was unaffected in netrin-1 mutant slices (Fig. 3.4G), though a slight increase was observed in $DCC^{-/-}$ slices (Fig. 3.4C).

Mature DCC and netrin mutant slice cultures exhibit abnormal paranodal myelin

The localization of netrin, DCC, and UNC5 proteins to paranodal myelin (Manitt et al., 2001, Fig. 3.1, 3.3), and previous reports describing a role for netrin in mediating short-range cell-cell and cell-matrix interactions (Winberg et al., 1998; Srinivasan et al., 2003; Liu et al., 2004b; Shekarabi et al., 2005) suggested that netrin-1 may mediate paranodal axo-oligodendroglial interactions.

The paranodal axo-glial junction is characterized ultrastructurally by terminal expansions of myelinating glia which form sequences of cytoplasm-containing loops of myelin membrane that tightly associate with each other and the axonal surface. In electron micrographs of myelinated axons, points of contact between paranodal myelin loops and the axolemma are characterized by TBs, and paranodal loops appear closely apposed to each other (reviewed by Pedraza et al., 2001). In electron micrographs of transverse-sectioned wild-type cerebellar slice cultures, TBs (Fig. 3.5C, 3.6C; black arrowheads) and interloop densities (IDs; Fig. 3.5C; white arrowhead) are readily observed at the vast majority of paranodes. In DCC and netrin-1 mutant slices, however, paranodes were frequently disordered (Figs. 3.5, 3.6). Specifically, TBs were often absent (and, when present, were frequently diffuse) and glial membranes not contacting the axon (Fig. 3.5D, 3.6D; black arrows) or neighboring glial loops (Fig. 3.5D; white arrow) were frequently observed. Everted glial loops, oriented away from the axon, were also common (Fig. 3.5E, 3.6E; black arrows). Occasionally, paranode-like structures were localized to internodal regions (Fig. 3.5F, 3.6F).

To quantify the differences observed between wild-type and DCC or netrin-1-deficient paranodes (at 67 and 49 DIV, respectively), heminodes were scored for the presence of each of four 'faults' (Fig. 3.7A): The absence of TBs (1), absence of IDs and abnormal separation between glial loops (2), detachment of paranodal loops from the axolemma (3), and the presence of everted loops (4). In both DCC (Fig. 3.7B) and netrin-1 (Fig. 3.7C) mutant slices, all four faults were far more frequent than in cultures obtained from wild-type littermates. On average, DCC mutant heminodes scored 1.78 faults (compared to 0.19 for wild-type heminodes; Fig. 3.7D). Netrin-1 mutant heminodes were more severely disordered, scoring a mean of 2.44 faults (compared to 0.27 for wild-type heminodes; Fig. 3.7E). When heminodes were binned according number of faults, over half of netrin mutant heminodes were classified as 'severely abnormal' (having 3-4 faults; Fig. 3.7G), compared to just under 30% of DCC mutant heminodes (Fig. 3.7F). The greater severity of the netrin-1 loss-of-function phenotype suggests that receptors other than DCC, perhaps the UNC5 homologues, are also required to mediate responses to netrin-1 at paranodal axo-glial junctions.

Paranode and juxtaparanodal domains are abnormal in netrin-1 and dcc mutant cerebellar slice cultures

In myelinated axons, the paranodal axo-glial apparatus has been described to act as a 'barrier' (reviewed by Pedraza et al., 2001), preventing the diffusion of potassium channels from the juxtaparanode into the paranode (reviewed by Rasband, 2004). In caspr$^{-/-}$ (Bhat et al., 2001), contactin$^{-/-}$ (Boyle et al., 2001), and CGT$^{-/-}$ (Dupree et al., 1998; Dupree et al., 1999) mice, all of which show ultrastructural disorganization at the paranodal axo-glial junction and lack TBs (Tao-Cheng and Rosenbluth, 1983), potassium channels are mislocalized into the paranodal region, consistent with a model in which the mature paranodal junction acts as a 'barrier',

To investigate whether the ultrastructural abnormalities observed in the netrin-1 or DCC-deficient slices result in a failure of the paranode to properly segregate nodal sodium and juxtaparanodal potassium channels, we immunolabeled our cultures with pan-Na^+ channel (Na^+ch) antibody and antibodies against potassium channel subunit Kv1.2 (Fig. 3.8B,D,I,K; red and green respectively) and measured the distance between the Na^+ch and Kv1.2-immunoreactive domains, and the width of the Na^+ch-immunoreactive

band. In netrin-$1^{+/+}$ and $DCC^{+/+}$ cultures, a clear gap was visible between nodal Na^+ch and juxtaparanodal Kv1.2 immunoreactivity (Fig. 3.8B,I). In netrin-$1^{-/-}$ and $DCC^{-/-}$ cultures, however, Kv1.2 immunoreactivity was often inappropriately localized to the paranode (Fig. 3.8D,K; arrowheads), and the mean distance between Na^+ch and Kv1.2 immunoreactivity was reduced in netrin-$1^{-/-}$ and $DCC^{-/-}$ relative to wild-type cultures (Fig. 3.8F,M). In addition, the Na^+ch-immunoreactive domain was widened in netrin-$1^{-/-}$ nodes (Fig. 3.8D; arrow) relative to netrin-$1^{+/+}$ nodes (Fig. 3.8G), but not in $DCC^{-/-}$ nodes (Fig. 3.8K,N). Taken together, these data suggest that the paranodal axo-glial junction fails to act as a barrier at netrin-$1^{-/-}$ and $DCC^{-/-}$ nodes of Ranvier, resulting in the inappropriate localization of ion channels. Furthermore, as suggested by the increased severity of the netrin-$1^{-/-}$ phenotype ultrastructurally, the failure of the paranodal barrier appears to be more severe in the absence of netrin-1, as the localization of Na^+ch protein is also affected.

Increased length of the node of Ranvier has previously been reported in $CGT^{-/-}$ and $MAG^{-/-}CGT^{-/-}$ mice (Dupree et al., 1998; Marcus et al., 2002). In $DCC^{-/-}$ cultures, nodal length was increased relative to that of $DCC^{+/+}$ nodes (Fig. 3.8H,J,L). Interestingly, this was not observed in netrin-$1^{-/-}$ cultures (Fig. 3.8A,C,E). This observation, combined with the increased periodicity of compact myelin in DCC-/- cultures (Fig. 3.4) implies, surprisingly, that part of the role DCC plays in myelin is netrin-1-independent.

Expression and localization of caspr and neurofascin in $DCC^{-/-}$ and netrin-$1^{-/-}$ nodes of Ranvier

Using antibodies recognizing either neurofascin (both 155 and 186 kDa isoforms; Fig. 3.9, 3.10A-D) or caspr (Fig. 3.9, 3.10E-H), we investigated whether the loss of netrin-1 or DCC affects the expression or localization of these proteins (Fig. 3.9, 3.10). Neurofascin localization does not appear to be altered in DCC (Fig. 3.9A-D) or netrin-1 slices (Fig. 3.10A-D), and the width of the neurofascin-immunoreactive domain is not changed in the $DCC^{-/-}$ (Fig. 3.9I) or netrin-$1^{-/-}$ (Fig. 3.10I) nodes of Ranvier. The possibility that the distribution of the 155 and 186kDa neurofascin isoforms are differentially regulated in the absence of netrin-1 or DCC cannot, however, be excluded. Caspr also continues to be expressed, but the caspr-immunoreactive domain flanking

DCC$^{-/-}$ and netrin-1$^{-/-}$ nodes of Ranvier is widened relative to cultures derived from their wild-type littermates (Fig. 3.9J, 3.10J) and the distance separating the caspr domains at the node is decreased (Fig. 3.9K, 3.10K).

One possible explanation for the widening of the caspr-immunoreactive domains in the absence of netrin-1 and DCC could be that the paranodal domain itself becomes elongated in the absence of netrin-1 or DCC. It has previously been proposed that nfc155 present on the glial membrane directs the formation of the paranodal axo-glial junction and associates, likely indirectly, in *trans* with an axonal complex containing caspr and contactin (Gollan et al., 2003). Nfc155 is required for the recruitment of the caspr-contactin complex to the paranode (Sherman et al., 2005). This is, however, inconsistent with our observations that the neurofascin-immunoreactive domain is not similarly widened, and that the regions of Kv1.2 and Na^+ch immunoreactivity are expanded. An alternative explanation is that, in the absence of netrin-1 or DCC, nfc155 is localized to paranodal loops normally, but the disengagement of the oligodendroglial membrane from the axonal surface disrupts the interactions between nfc155 and the caspr-contactin complex, freeing a proportion of these molecules to diffuse along the axon, away from the paranode.

Myelin formation and maturation is normal in DCC$^{-/-}$ and netrin-1$^{-/-}$ slice cultures

The observation of abnormal paranodal myelin at 67 DIV in DCC$^{-/-}$ cultures and 49 DIV in netrin-1$^{-/-}$ cultures, time points substantially later than the formation of superficially normal myelin in our culture system (Fig. 3.1), raises the question of whether the observed phenotype is the result of abnormal myelin formation or maintenance. To address this question, we carried out the same experiments using cerebellar slice cultures derived from newborn netrin-1$^{-/-}$ and DCC$^{-/-}$ mice and their wild-type littermates, and maintained *in vitro* for 25 days. At 25 DIV, paranodal axo-glial junctions in wild-type cultures had matured sufficiently such that TBs were present where each loop of glial membrane contacts the axon (Fig. 3.11A,F). In stark contrast to our observations at later time points, netrin-1$^{-/-}$ and DCC$^{-/-}$ paranodes were indistinguishable from netrin$^{+/+}$ and DCC$^{+/+}$ paranodes (Fig. 3.11B,G). When the presence of the 'faults' defined in Fig. 3.7A were quantified, no significant increase was seen in any in the

absence of either netrin-1 or DCC (Fig. 3.11C,H), and the mean number of faults per heminode did not significantly differ between netrin-$1^{-/-}$ or $DCC^{-/-}$ and wild-type cultures (Fig. 3.11D,I). When the heminodes were binned as described previously, the vast majority of both wild-type and mutant heminodes were classified as normal (no faults; Fig. 3.11E,J).

The lack of a visible phenotype in electron micrographs of 25 DIV netrin-$1^{-/-}$ or $DCC^{-/-}$ paranodes implies that the domain organization of the paranode may also be normal in the cultures at this age. As predicted, sodium and potassium channels are similarly segregated at netrin-1 (Fig. 3.12B,D) and DCC (Fig. 3.12I,K) wild-type and mutant nodes of Ranvier. The distance separating Na^+ch and Kv1.2-immunoreactive domains was unaltered in netrin-$1^{-/-}$ or $DCC^{-/-}$ slices (Fig. 3.12F,M), and no increase in the width of the Na^+ch-immunoreactive band was observed (Fig. 3.12G,N). Nodal length was also unchanged in the absence of netrin-1 or DCC at 25 DIV (Fig. 3.12A,C,E,H,J,L). As with the older cultures, neurofascin was present in both 25 DIV wild-type and mutant cultures, and its distribution was unaltered in the absence of netrin-1 or DCC (Fig. 3.13, 3.14A-D,I). The distribution of caspr at netrin-$1^{-/-}$ or $DCC^{-/-}$ paranodes at 25 DIV, however, was indistinguishable from that observed in wild-type cultures (Fig. 3.13, 3.14E-H,J,K), consistent with our observations that the paranodal region develops normally in the absence of netrin-1 or DCC. Taken together, these data suggest that the defects observed at the paranodal axo-glial junction in older netrin-$1^{-/-}$ and $DCC^{-/-}$ cultures is a consequence not of aberrant myelin formation, but of maintenance.

V. DISCUSSION

Netrins have been most extensively described as long-range tropic guidance cues directing cell and axon migration during embryonic development. In the adult CNS, expression of netrin-1 and its receptors is widespread in both neuronal and glial cells including oligodendrocytes (Manitt et al., 2001; Ellezam et al., 2001; Manitt et al., 2004). Fractionation of adult CNS white matter indicated that netrin protein is enriched in non-compact myelin membranes implicated in axo-glial contact (Manitt et al., 2001). Here, we show that netrin-1 and DCC and UNC5 homologue netrin receptors are enriched in the paranodal region both in the adult CNS *in vivo* and in organotypic cerebellar cultures. While paranodal myelin developed and matured normally in 25 day-old cultures derived from netrin-$1^{-/-}$ or $DCC^{-/-}$ neonates, the paranodal loops were disordered and domain organization disrupted in 49 to 67 day-old netrin or DCC mutant cultures. We concluded that netrin and DCC are required for the maintenance, but not the development of CNS paranodal axo-glial junctions.

Netrin-1 and DCC are required for the maintenance of CNS paranodes

We observed that in CNS-derived tissue, both myelination and formation of the paranodal axo-glial junction occurred normally in the absence of netrin-1 or DCC. It has been reported that DCC and UNC5 family members, both expressed by oligodendrocytes, are "dependence receptors"; inducing cell death in the absence of netrin-1 (Chen et al., 1999; Llambi et al., 2001; reviewed by Mehlen and Mazelin, 2003). In this light, the observation that myelination is grossly normal, especially in netrin-$1^{-/-}$ cultures is perhaps surprising, as it implies that massive oligodendrocyte death does not occur in the absence of netrin. We and others have previously reported that no increase in oligodendrocyte precursor cell death is observed in the netrin-$1^{-/-}$ spinal cord during development (Jarjour et al., 2003; Tsai et al., 2006), suggesting that DCC and UNC5 homologues may not induce cell death in the absence of netrin-1 in oligodendrocyte lineage cells.

The observation that paranodal loops form and mature normally but later become disordered in the absence of netrin-1 and DCC, resulting in a breakdown of domain segregation in the nodal region, is consistent with a role for these molecules in maintenance of the axo-glial junction. Reports documenting CNS paranodal phenotypes in $CGT^{-/-}$ (Dupree et al., 1998; Dupree et al., 1999; Marcus et al., 2002; Rasband et al.,

2003), $CST^{-/-}$ (Ishibashi et al., 2002), $CGT^{-/-}$:$MAG^{-/-}$ (Marcus et al., 2002), $MAL^{-/-}$ (Schaeren-Wiemers et al., 2004), and $CNP^{-/-}$ (Rasband et al., 2005) mice have also described these gene products as being either directly or indirectly required for the maintenance of the paranodal region. In all cases, the paranodal region begins to develop normally and then becomes progressively disordered, but the age of onset and severity of disorganization of the paranodal region varies considerably among them.

Significant differences exist, however, between previously reported maintenance phenotypes and those described here. Netrin-$1^{-/-}$ and $DCC^{-/-}$ paranodes form and mature normally; as caspr and neurofascin cluster and ordered TBs appear in a manner indistinguishable from what is observed in cultures derived from wild-type cerebella. It is only after the paranodes have matured that the axo-glial junction destabilizes. In all previous reports of paranodal maintenance phenotypes, TBs either do not form at all or develop abnormally. A second key distinction that can be made between the netrin-$1^{-/-}$ and $DCC^{-/-}$ phenotype and any other previously reported to our knowledge, is that neurofascin localization appears to be unaffected at longer time points, despite the ultrastructural defects and loss of domain segregation observed. The increase in the width of caspr immunoreactive domains is in all likelihood secondary to the detachment of a subset of paranodal loops from the membrane, where the resulting disengagement of the caspr-contactin complex from its glial binding partner results in lateral diffusion along the membrane. This interpretation carries with it the implication that the nfc155-caspr-contactin complex may be necessary, but not sufficient, for the maintenance of paranodal organization. While the netrin-$1^{-/-}$ or $DCC^{-/-}$ phenotypes observed at 49 or 67 DIV respectively are minor compared to those observed in the $caspr^{-/-}$, $contactin^{-/-}$, $neurofascin^{-/-}$, $CGT^{-/-}$, or $CST^{-/-}$ CNS, it is possible that the netrin-1 or DCC mutant phenotypes continue to increase in severity with age. While it is difficult, for technical reasons, to assess paranodal organization at later time points in organotypic cultures, studies using lineage-restricted netrin-1 or DCC knockout animals would address this possibility.

Another question that remains to be addressed is whether netrin and receptors play a similar role in PNS paranodal junctions. Netrin and DCC are both expressed by Schwann cells, consistent with this possibility (Madison et al., 2000; Moon et al., 2005).

However, paranodal loss-of-function phenotypes are typically less severe in the PNS, due perhaps to the added presence of the basal lamina (reviewed by Poliak and Peles, 2003).

Interdependent and independent roles of netrin and DCC

While the phenotypes observed in netrin-$1^{-/-}$ and $DCC^{-/-}$ cerebellar slice cultures exhibit extensive similarity, they are not identical. A minor increase in the periodicity of compact myelin and in nodal length are observed in $DCC^{-/-}$, but not netrin-$1^{-/-}$, slices. The mechanism by which this occurs is unclear, though netrin-1-independent actions of DCC have previously been reported (Gitai et al., 2003). The ultrastructural abnormalities observed in the netrin-1 mutant paranodes are more severe than those observed in the DCC mutants, as evidenced by the larger mean number of faults per paranode (compare Fig. 3.7D and E) and the greater number of paranodes scored to be 'severely abnormal' (compare Fig. 3.7F and G). In addition, while potassium channel localization is aberrant in both $DCC^{-/-}$ and netrin-$1^{-/-}$ paranodes, the sodium channel domain is also widened in the absence of netrin-1 (compare Fig. 3.8D,G to K,N). A spreading of the sodium channel domain has previously been reported at $caspr^{-/-}$ (Bhat et al., 2001) and $CGT^{-/-}$ mutant CNS paranodes but not, for example, in the MAL mutant (Schaeren-Wiemers et al., 2004). The increased severity of the netrin-$1^{-/-}$ phenotype suggests that the activity of netrin-1 at the paranode is mediated in part by a DCC-independent mechanism. UNC5 homologue protein is localized to the paranodal region both *in vivo* and in our culture system, making it a probable candidate to mediate the activity of netrin-1 at the paranode (Figs. 3.1, 3.2).

UNC5 receptors mediate repellent responses to netrin, acting either with DCC or independently of it (Hong et al., 1999; Killeen et al., 2002). Analyses in *Drosophila* suggest that DCC is required for long-range, but not short-range, repellent actions of netrin (Keleman and Dickson, 2001). While relatively little is known about UNC5 signaling, it has been proposed that DCC greatly facilitates, but is not required for, the phosphorylation of UNC5 by Src family kinases (Li et al., 2006), suggesting that the role of DCC in long-range repellent guidance may be to increase the sensitivity of UNC5-expressing cells to netrin. However, this study and others provide evidence that DCC is also involved in mediating some short-range actions of netrin (Deiner et al., 1997; Shekarabi et al., 2005).

Netrin, motility, and myelination

How, then, do netrin and its receptors contribute to the maintenance of paranodal axo-glial junctions? Most netrin-1 protein in the CNS is not freely soluble but membrane-associated (Manitt et al., 2001). Recently, a role for netrin and its receptors in mediating adhesive cell-cell interactions in the developing embryo has emerged. In the developing mammary gland netrin-1, acting through DCC homologue neogenin, mediates an adhesive interaction between cell layers (Srinivasan et al., 2003). Netrin-1 and netrin-4 have been reported to prevent inappropriate budding of the developing lung endoderm; a role mediated by UNC5B (Liu et al., 2004b). Adhesive roles for netrin-1 have also been reported in developing pancreas (Yebra et al., 2003), and in vascular smooth muscle cells (Park et al., 2004). Netrin-1 has also been demonstrated to act through DCC to mediate cell-substrate adhesion (Shekarabi et al., 2005). Netrins also mediate short-range interactions in the nervous system; acting as short-range targeting cues for motor axons at the drosophila nerve-muscle synapse (Winberg et al., 1998) and short-range guidance cues for retinal ganglion cell axons at the mammalian optic disc (Deiner et al., 1997).

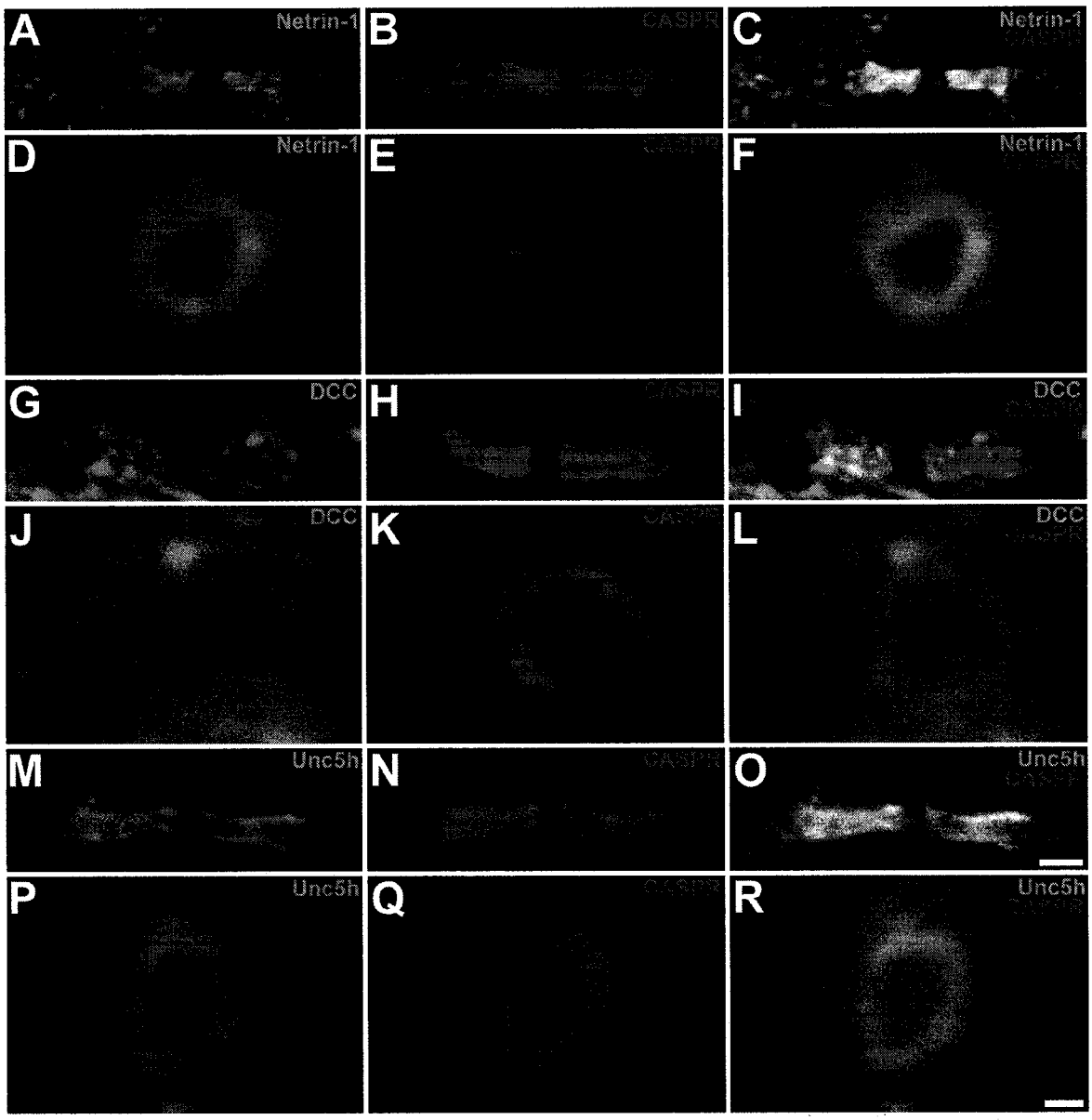
Studies of axon guidance in cell lines may provide insight into the mechanism by which netrin mediates paranodal axo-glial interaction. DCC expressed in both the embryonic CNS and in cell lines is localized to lipid rafts, and intact lipid rafts are required for netrin-1-mediated commissural axon outgrowth from spinal cord explants, and for repellent guidance of xenopus spinal neurons. (Guirland et al., 2004; Herincs et al., 2005). Src and fyn kinases have been implicated in netrin-induced signal transduction downstream of DCC (Li et al., 2004; Meriane et al., 2004), while src has also been reported to regulate the phosphorylation of UNC5B (Li et al., 2006). Together, these observations raise the possibility that DCC, like nfc155, may be concentrated in oligodendroglial raft domains at the paranode. Fyn is localized to lipid raft domains in bovine myelin and mouse oligodendrocytes (DeBruin et al., 2005) and has been co-purified from CNS in low-density fractions with caspr and nfc155 (Menon et al., 2003). If DCC is also localized to DIGs in paranodal myelin, it would be reasonable to hypothesize that fyn may act downstream of DCC. Interestingly, UNC5 homologues contain a ZU5 domain. While the function of this domain is currently unknown, that similar domains have been identified in tight junction proteins of the zona occludens family and actin-binding protein ankyrin-1 provide a possible, if tenuous, link between UNC5 homologues

and a role in mediating adhesion and cytoskeletal association (identified using InterPro software, Apweiler et al., 2001).

In summary, we propose that netrin-1 associated with oligodendroglial membranes regulates axo-glial interaction through DCC and another receptor, most likely one or more UNC5 homologues (Fig. 3.15). Immunoelectron microscopy will be necessary to confirm differential receptor localization and determine where netrin-1 accumulates ultrastructurally. Based on the apparent difference between DCC and UNC5 localization, it would be tempting to suggest that DCC may predominantly mediate interactions between glial loops and speculate that UNC5 homologues mainly mediate axo-glial interactions. However, the phenotypes observed in DCC and netrin-1 mutant slice cultures do not entirely support such a discrete division of function, as, for instance, abnormal separation between glial loops was more common in netrin-$1^{-/-}$ paranodes than in $DCC^{-/-}$ paranodes (Fig. 3.7). While this may indicate that other netrin receptors also regulate interactions between adjacent glial membranes, it may also be a simple consequence of the more severe phenotype observed in the absence of netrin-1. In addition to netrin-1 and DCC oligodendrocyte lineage-specific knockout experiments mentioned above, UNC5 homologue loss-of-function studies will also be required to understand the contribution that these receptors make to paranodal organization.

Figure 3.1. Expression of netrin, DCC and UNC5 protein at the CNS paranodal axo-glial junction

Triple-label immunohistochemical analysis of longitudinal (A-C, G-I, M-O) and coronal (D-F, J-L, P-R) sections of adult rat spinal cord. Caspr immunoreactivity was visualized using Alexa 546-conjugated secondary antibodies (red) and NFM immunoreactivity visualized using Alexa 633-conjugated secondary antibodies (pseudocoloured blue). Netrin-1, DCC, and UNC5 homologue (unc5h) proteins were visualized using Alexa 488-conjugated secondary antibodies (green). Netrin-1 (A-F) and unc5h (M-R) protein is localized to the paranodal axo-glial junction, where it is closely associated with, and partially overlaps, that for caspr. DCC protein (G-L) appears to surround the axon at the paranode, but does not co-localize with caspr to the same extent as netrin-1 and UNC5 homologues. A-C, G-I, M-O: 100x objective, digital zoom 4, scale bar = 2 μm. D-F, J-L, P-R: 100x objective, digital zoom 10, scale bar = 500 nm.


A
Netrin-1
B
CASPR
C
Netrin-1
D
Netrin-1
E
F
Netrin-1
G
DCC
H
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DCC
J
DCC
K
L
DCC
M
Unc5h
N
O
Unc5h
P
Unc5h
Q
R
Unc5h

Figure 3.2. Characterization of cerebellar organotypic cultures

Oligodendrocyte maturation in slices of newborn mouse cerebellum follows a similar time course to that observed *in vivo*. After 3 DIV, oligodendrocytes, immunolabeled with an antibody against MBP and visualized using Alexa 488-conjugated secondary antibodies (green) can be readily detected (A). They increase in number and complexity between 3 and 7 days in vitro (B). By 14 DIV, multiple myelinated axons are visible (C), and by 30 DIV, extensive myelination is observed throughout the white matter (D). E-G: Association of netrin and netrin receptor protein with Purkinje cells. Netrin (E), and unc5h (G) protein was closely associated with Purkinje neuron cell bodies. DCC (F) was associated with both the cell bodies and nuclei of Purkinje neurons. H-J: Association of netrin and netrin receptor protein with oligodendrocytes. Netrin (H) and Unc5h (J) immunoreactivity was associated with oligodendrocyte cell bodies, and DCC immunoreactivity was associated with both cell bodies and nuclei (I). A-D: 20x objective, scale bar = 100 μm. E-J: 100x objective, digital zoom 2, scale bar = 5 μm.

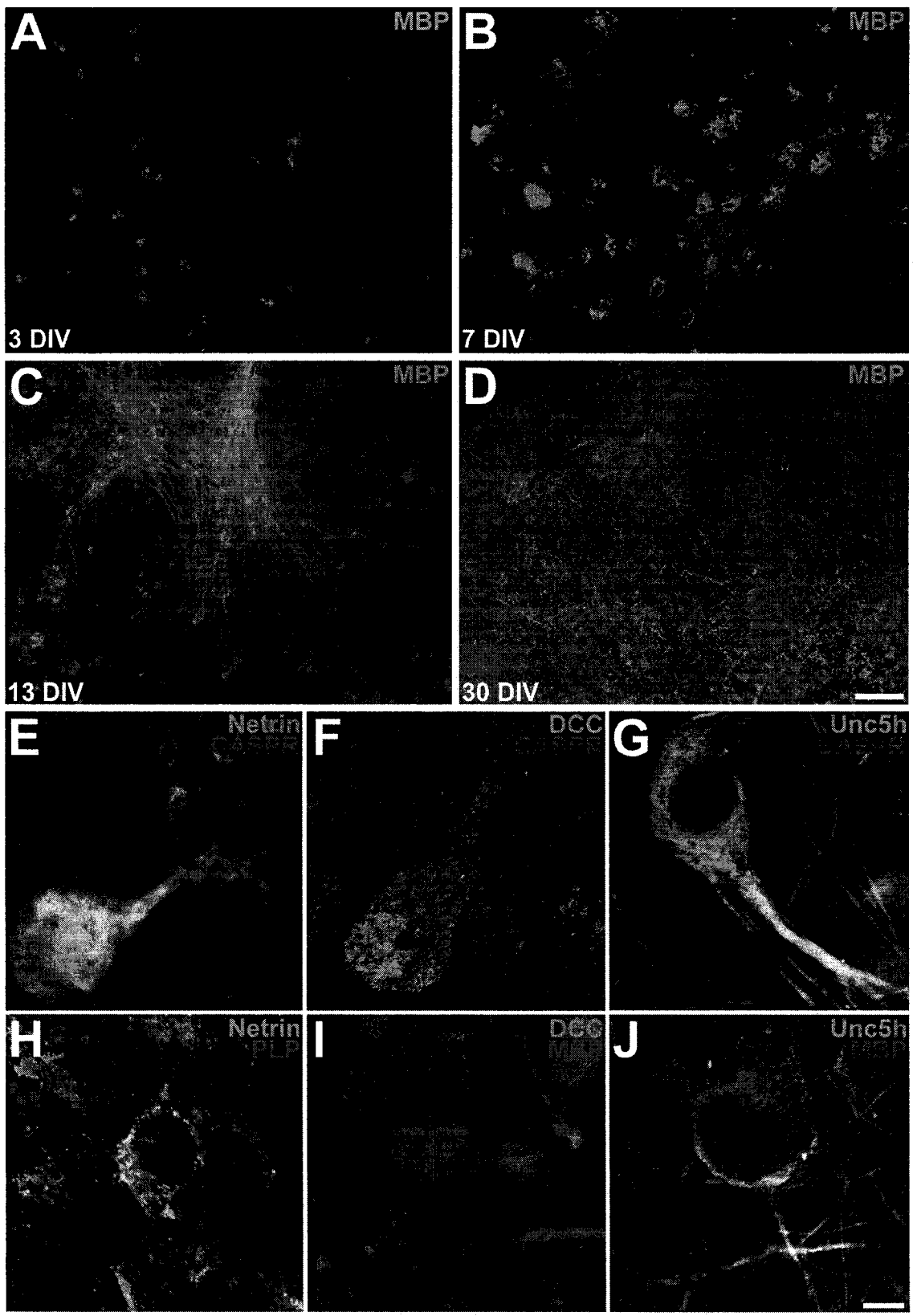
A
MBP
3 DIV
B
MBP
7 DIV
C
MBP
13 DIV
D
MBP
30 DIV
E
Netrin
F
DCC
G
Unc5h
H
Netrin
I
DCC
J
Unc5h

Figure 3.3. Distribution of netrin, DCC and UNC5 homologues in mature myelinated organotypic slice cultures

Triple-label immunohistochemical analysis of longitudinal (A-C, G-I, M-O) and cross-sectional (D-F, J-L, P-R) images of the paranode in mature cerebellar slice cultures. Caspr immunoreactivity was visualized using Alexa 546-conjugated secondary antibodies (red). PLP (C) and MBP (I, O) immunoreactivity was visualized using Alexa 633-conjugated secondary antibodies (pseudocoloured blue). Netrin-1, DCC, and UNC5 homologue (Unc5h) proteins were visualized using Alexa 488-conjugated secondary antibodies (green). Netrin-1 protein (A-F) is localized to the paranodal axo-glial junction, where it is closely associated with, and partially overlaps, that for caspr. UNC5 homologue immunoreactivity (M-R) also overlaps with that for caspr, but is also observed along the axon. DCC protein (G-L) appears to surround the axon at the paranode, but does not co-localize with caspr to the same extent as netrin-1 and UNC5 homologues. A-C, G-I, M-O: 100x objective, digital zoom 4, scale bar = 2 μm. D-F, J-L, P-R: 100x objective, digital zoom 10, scale bar = 500 nm.

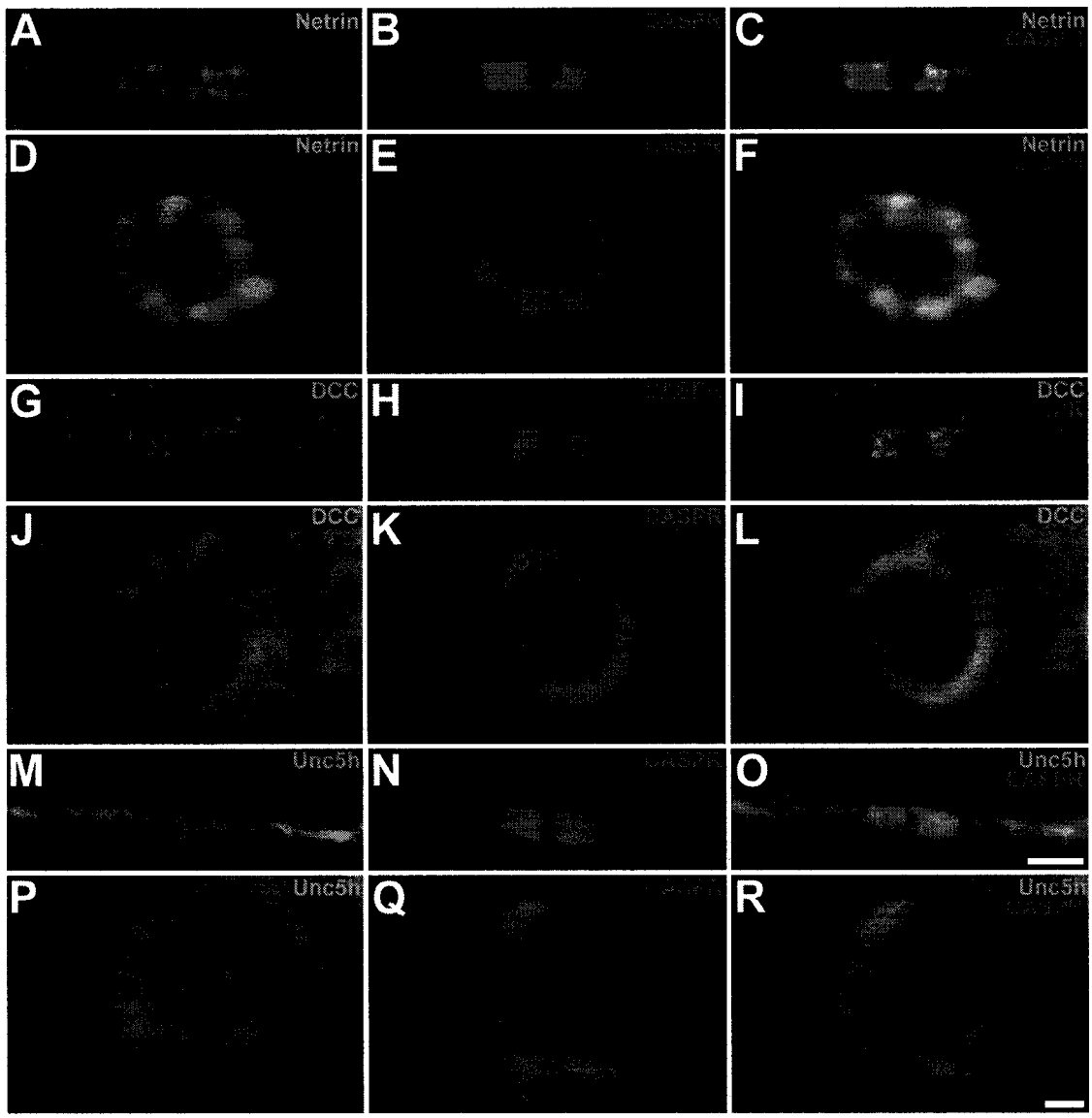
A
Netrin
B
C
Netrin
D
Netrin
E
F
Netrin
G
DCC
H
I
DCC
J
DCC
K
L
DCC
M
Unc5h
N
O
Unc5h
P
Unc5h
Q
R
Unc5h

Figure 3.4. Minor increases in periodicity are observed in DCC$^{-/-}$ compact myelin

Spacing between layers of the myelin sheath was analyzed by transmission electron microscopy in cross-sections cut from cerebellar slice cultures derived from DCC-or netrin-1-deficient animals (B, F respectively) and their wild-type littermates (A, E respectively). The width of the periaxonal space was unaffected in the mutant slices (D). While the periodicity of netrin-1$^{+/+}$ and netrin-1$^{-/-}$ myelin wraps was not significantly different, the absence of DCC expression resulted in a small but significant increase in the spacing between layers of compact myelin. Electron micrographs were imaged at 410000x. Ax: axon. PS: periaxonal space. Scale bar corresponds to 50 nm. *: $p < 0.05$.

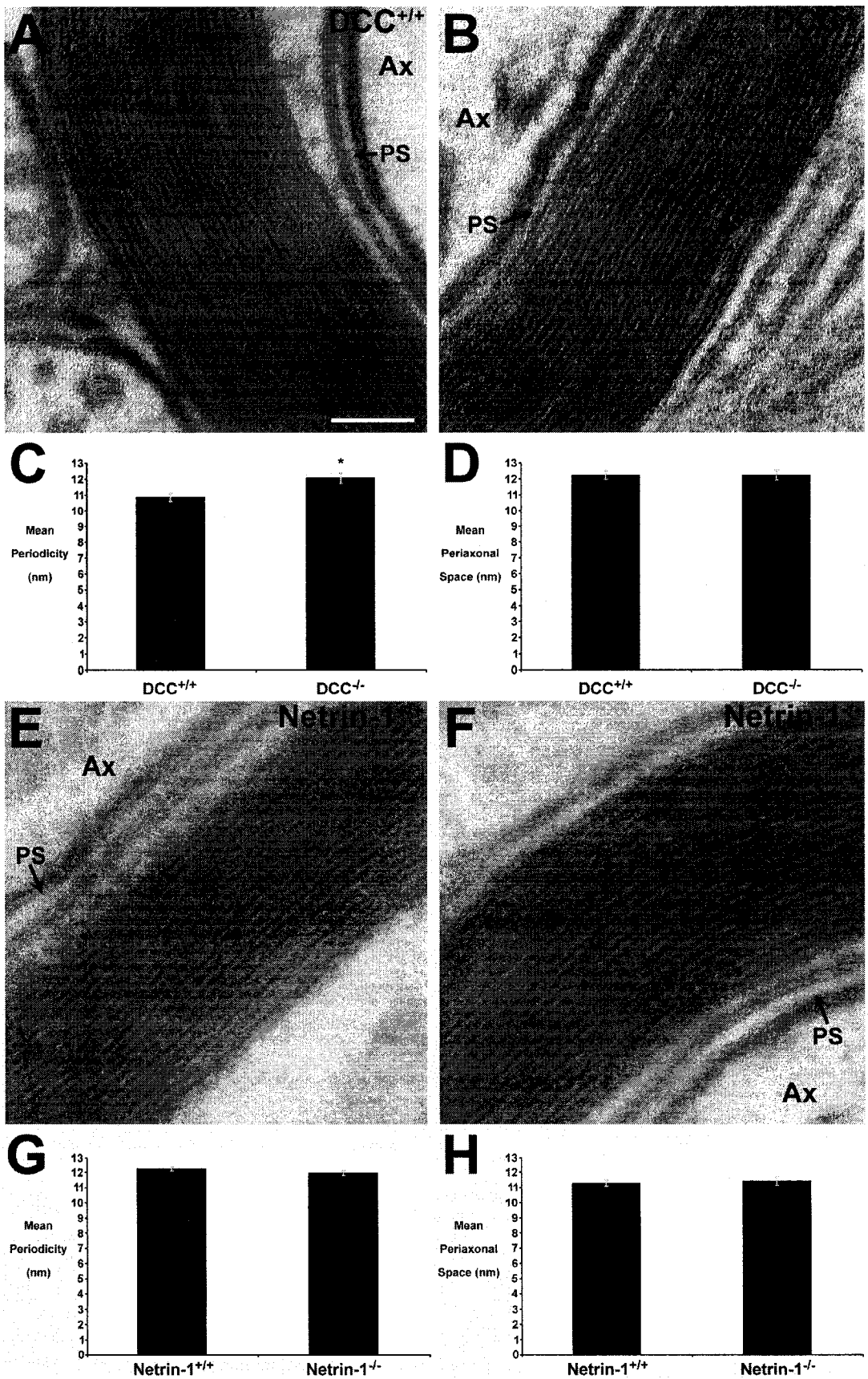
A
DCC+/+
Ax
PS
B
Ax
PS
C
Mean Periodicity (nm)
*
DCC+/+
DCC-/-
D
Mean Periaxonal Space (nm)
DCC+/+
DCC-/-
E
Netrin-1
Ax
PS
F
Netrin-1
PS
Ax
G
Mean Periodicity (nm)
Netrin-1+/+
Netrin-1-/-
H
Mean Periaxonal Space (nm)
Netrin-1+/+
Netrin-1-/-

Figure 3.5. Abnormal paranodal myelin in DCC knockout organotypic slice cultures
The organization of both paranodal and internodal myelin was examined using transmission electron microscopy in organotypic slices derived from newborn wild-type (A,C) or DCC knockout (B,D,E,F) mouse cerebellum maintained in culture for 67 days. In slices collected from wild-type animals, paranodal myelin was well organized and electron-dense transverse bands between the axonal and oligodendrocyte membranes (C, black arrowhead) and interloop densities between paranodal loops (C, white arrowhead) were present. In contrast, in slices lacking DCC, both transverse bands and interloop densities were frequently absent and paranodal loops were often disordered. This included separation from the axolemma (D, black arrows) and from each other (D, white arrows). Paranodal loops oriented away from the axon were also frequently observed in the knockout (E, arrow). Regions of myelin decompaction resembling paranodal loops were present in internodal myelin (F). A, B: 25000x, scale bar corresponds to 500 nm. C-F: 68000x, scale bar corresponds to 200 nm.

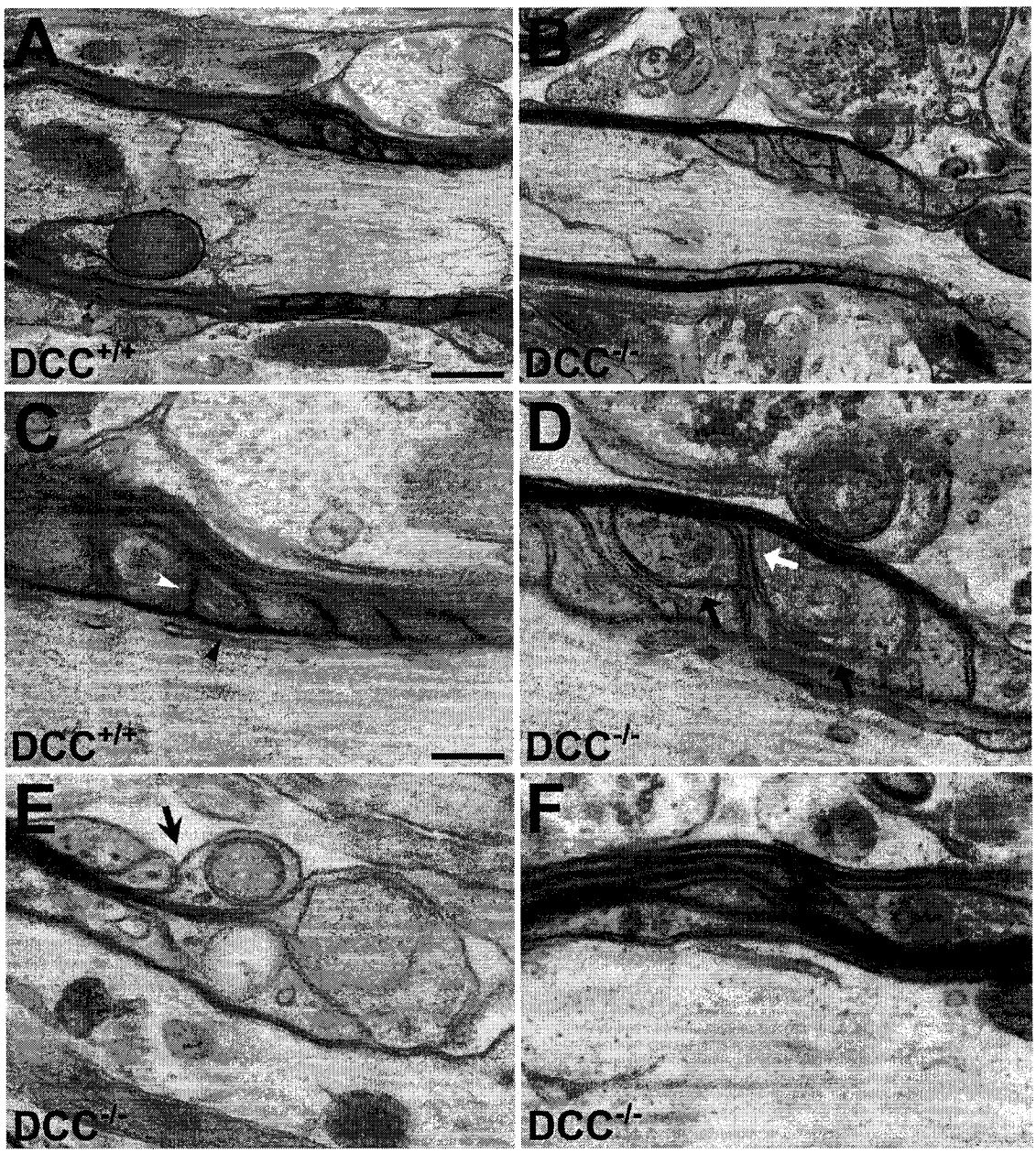
A
DCC+/+
B
DCC-/-
C
DCC+/+
D
DCC-/-
E
DCC-/-
F
DCC-/-

Figure 3.6. Abnormal paranodal myelin in netrin-1 knockout organotypic slice cultures

The organization of both paranodal and internodal myelin was studied by transmission electron microscopy in organotypic slices derived from newborn wild-type (A, C) or netrin-1-deficient (B,D,E,F) mouse cerebellum maintained in culture for 49 days. In slices collected from wild-type animals, paranodal myelin was well organized. Electron-dense transverse bands were observed linking the axonal and oligodendrocyte membranes (C, black arrowheads) and paranodal loops were closely apposed to each other. In slices collected from netrin-1 mutant mice, paranodal loops were often greatly disordered, separated from the axolemma, and lacking transverse bands and interloop densities (D, black arrows). As in the DCC mutant, paranodal loops inappropriately oriented away from the axon were observed (E, arrows). Regions of decompaction, resembling paranodal loops, oriented both towards and away from the axon were also observed in internodal myelin (F). A, B: 25000x, scale bars correspond to 500 nm. C-F: 68000x, scale bar corresponds to 200 nm.

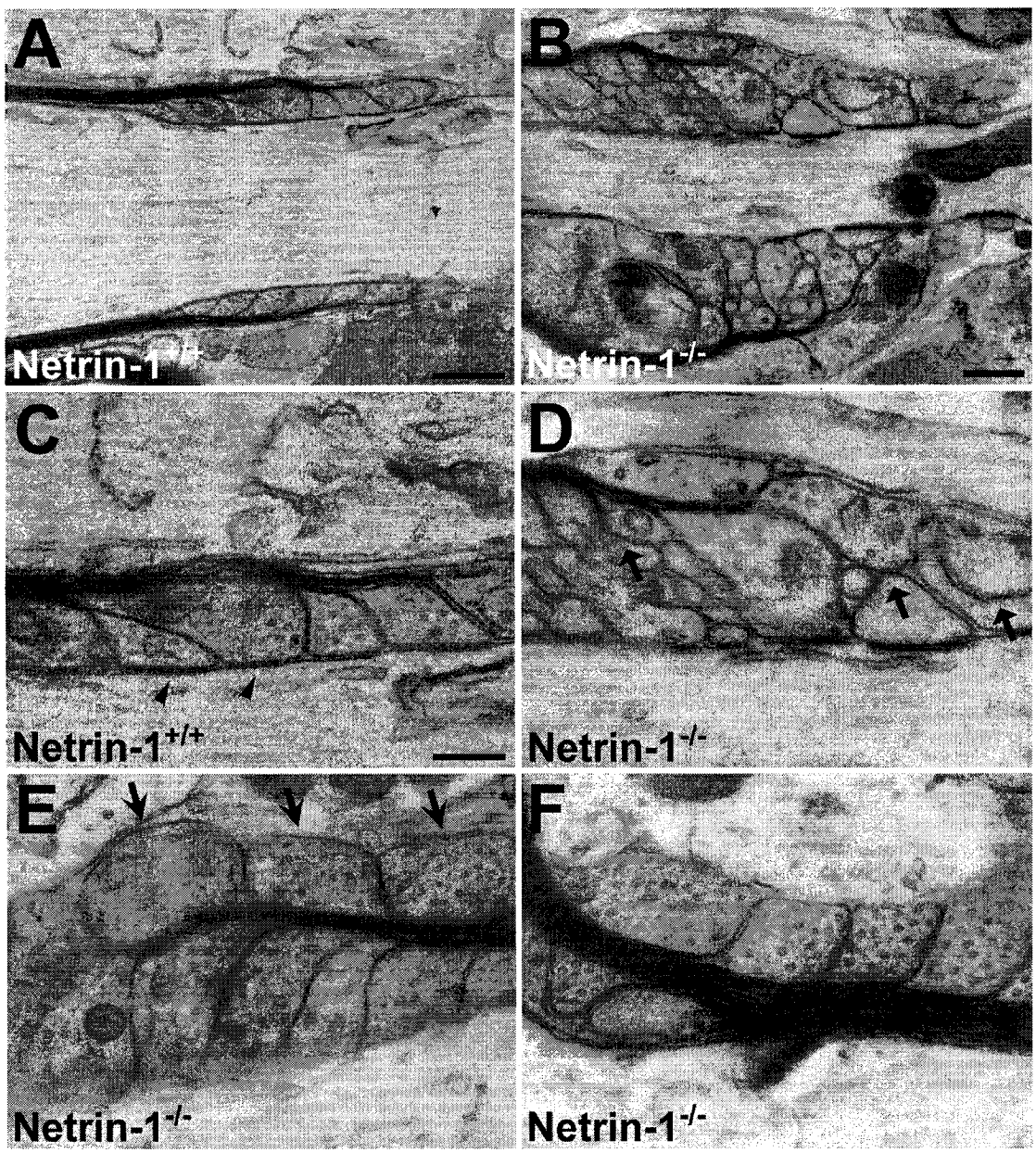
A
Netrin-1 +/+
B
Netrin-1 -/-
C
Netrin-1 +/+
D
Netrin-1 -/-
E
Netrin-1 -/-
F
Netrin-1 -/-

Figure 3.7. Quantification of paranodal defects in the absence of netrin-1 or DCC

The integrity of paranodal myelin was analyzed in electron micrographs of transverse sections of cerebellar slice cultures derived from DCC- (B,D,F) or Netrin-1-knockout (C,E,G) mice maintained in culture for 67 or 49 days, respectively. (A) Myelin heminodes were examined for four defects: Lack of transverse bands (1), lack of interloop densities (2), separation of paranodal myelin loops from the axonal surface (3), and inverted paranodal loops (4). Each observation was counted as one 'fault'. (B,C) In both netrin and DCC mutants, the incidence of each of the four defects was increased, as was the mean number of faults per heminode (D,E). DCC (F) and Netrin-1 (G) wild-type and mutant heminodes were classified as normal (0 faults), mildly abnormal (1 fault), moderately abnormal (2 faults), and severely abnormal (3-4 faults). Notably, while 97 % of $DCC^{+/+}$ heminodes and 97 % of $Netrin\text{-}1^{+/+}$ heminodes examined were classified as normal or mildly abnormal, not a single wild type heminode was severely abnormal. (one value of n corresponds to one heminode). OL: oligodendroglial membrane. AX: axolemma. TB: transverse bands. ID: Interloop densities. **: $p < 0.005$.

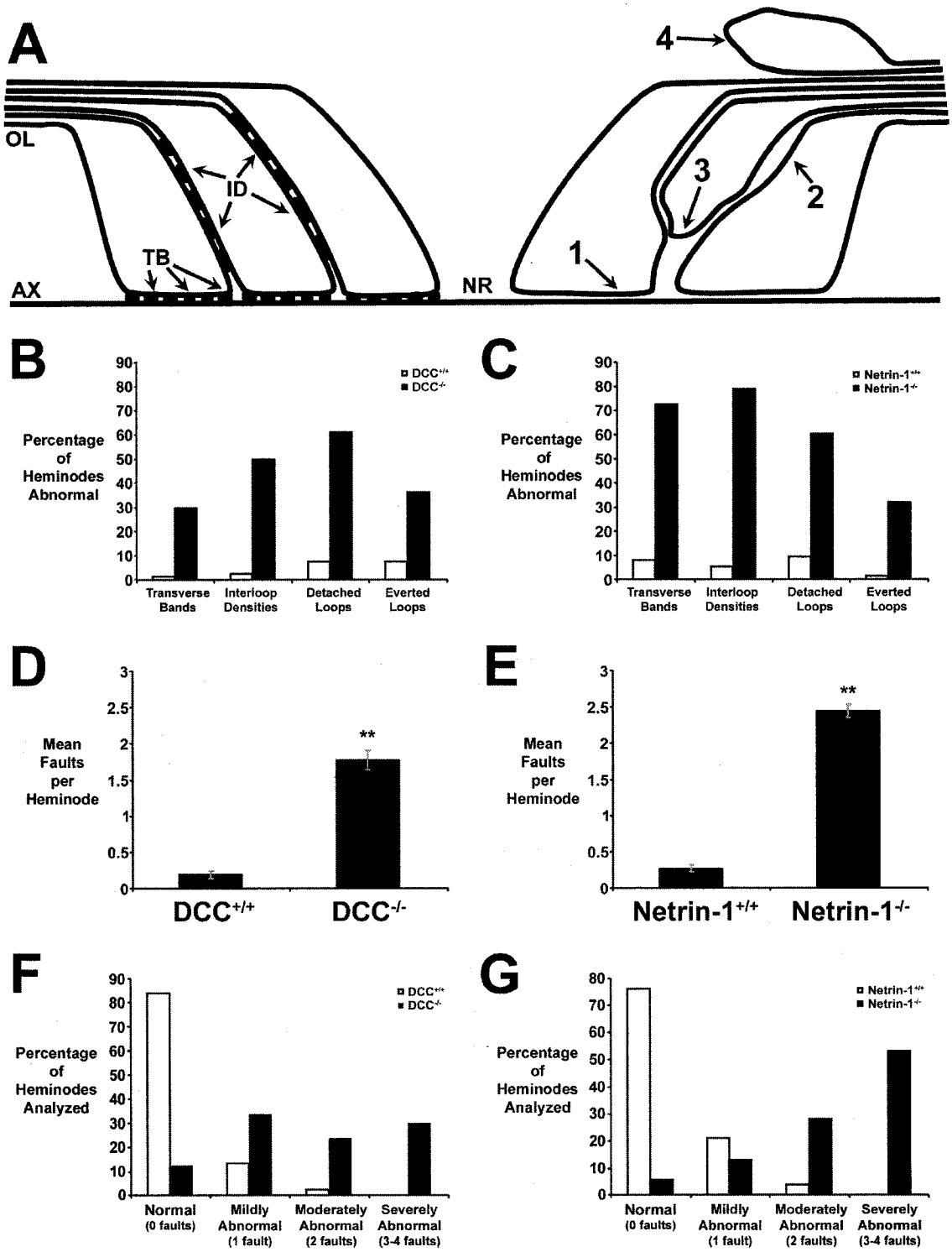
A
OL
AX
TB
ID
NR
1
2
3
4
B
Percentage of Heminodes Abnormal
DCC+/+
DCC-/-
Transverse Bands
Interloop Densities
Detached Loops
Everted Loops
C
Percentage of Heminodes Abnormal
Netrin-1+/+
Netrin-1-/-
Transverse Bands
Interloop Densities
Detached Loops
Everted Loops
D
Mean Faults per Heminode
**
DCC+/+
DCC-/-
E
Mean Faults per Heminode
**
Netrin-1+/+
Netrin-1-/-
F
Percentage of Heminodes Analyzed
DCC+/+
DCC-/-
Normal (0 faults)
Mildly Abnormal (1 fault)
Moderately Abnormal (2 faults)
Severely Abnormal (3-4 faults)
G
Percentage of Heminodes Analyzed
Netrin-1+/+
Netrin-1-/-
Normal (0 faults)
Mildly Abnormal (1 fault)
Moderately Abnormal (2 faults)
Severely Abnormal (3-4 faults)

Figure 3.8. Disruption of the domain organization of the node of Ranvier in 60 DIV netrin-1 and DCC knockout slice cultures

60 DIV cerebellar slice cultures double-labeled with antibodies against MBP and NFM (A,C,H,J), or Na+ch and Kv1.2 (B,D,I,K). MBP and Kv1.2 protein were visualized using Alexa 488-conjugated secondary antibodies (green). NFM and Na^+Ch were visualized using Alexa 546-conjugated secondary antibodies (red). The mean distance between regions of MBP immunolabeling across the node was unchanged in the absence of netrin-1 expression (A,C,E). Nodal length was increased in $DCC^{-/-}$ slices relative to $DCC^{+/+}$ slices (H, J, L). In cultures lacking netrin-1 (B,D,F) or DCC (I,K,M), we detect a reduced distance between Na^+ channels localized within the node of Ranvier and K^+ channels normally localized to the juxtaparanodal region. This decrease was due largely to the apparent 'leaking' of K^+ channels into the paranode, and occasionally the node itself (D,K; arrowheads). In netrin-$1^{-/-}$ (D,G), but not $DCC^{-/-}$ (K,N) slices, the width of the Na^+ch-positive domain was increased relative to control (arrow). 100x objective. A,C,H,J: digital zoom 5. B,D,I,K: digital zoom 4. Scale bars correspond to 2 μm. *: $p < 0.05$. **: $p < 0.005$.

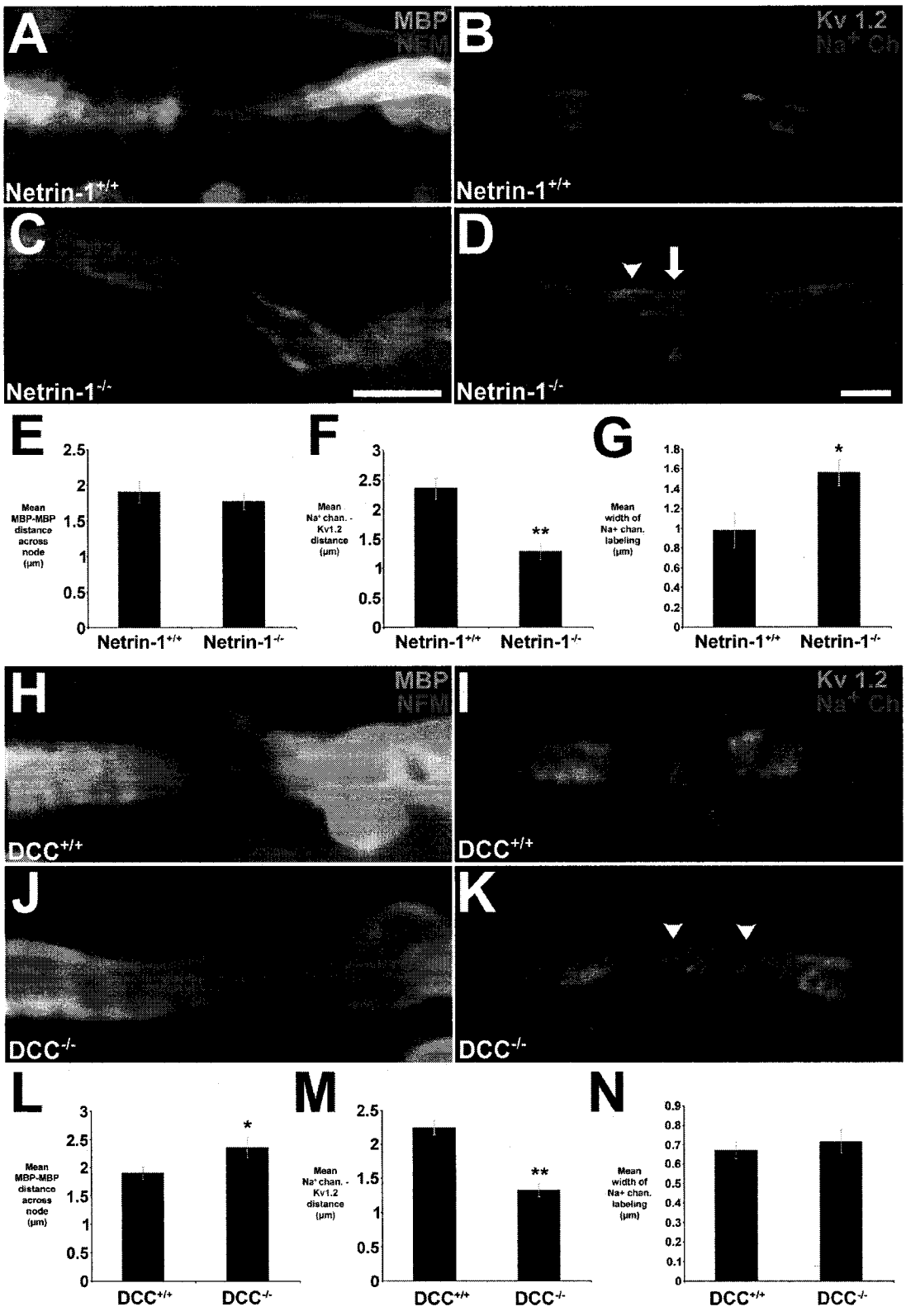
A
MBP
NFM
Netrin-1+/+
B
Kv 1.2
Na+ Ch
Netrin-1+/+
C
Netrin-1-/-
D
Netrin-1-/-
E
Mean MBP-MBP distance across node (µm)
2.5
2
1.5
1
0.5
0
Netrin-1+/+
Netrin-1-/-
F
Mean Na+ chan. - Kv1.2 distance (µm)
3
2.5
2
1.5
1
0.5
0
**
Netrin-1+/+
Netrin-1-/-
G
Mean width of Na+ chan. labeling (µm)
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Netrin-1+/+
Netrin-1-/-
H
MBP
NFM
DCC+/+
I
Kv 1.2
Na+ Ch
DCC+/+
J
DCC-/-
K
DCC-/-
L
Mean MBP-MBP distance across node (µm)
3
2.5
2
1.5
1
0.5
0
*
DCC+/+
DCC-/-
M
Mean Na+ chan. - Kv1.2 distance (µm)
2.5
2
1.5
1
0.5
0
**
DCC+/+
DCC-/-
N
Mean width of Na+ chan. labeling (µm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DCC+/+
DCC-/-

Figure 3.9. Caspr, but not neurofascin, distribution is altered in $DCC^{-/-}$ cultures at 60 DIV.

60 DIV cerebellar slice cultures were triple-labeled with antibodies against NFH, MBP, and either nfc (A-D) or caspr (E-H). MBP was visualized using Alexa 546-conjugated secondary antibodies (pseudocoloured green), NFH was visualized using Alexa 633-conjugated secondary antibodies (pseudocoloured blue), and nfc or caspr were visualized using Alexa 488-conjugated secondary antibodies (pseudocoloured red). The width of nfc-immunoreactive bands were unchanged between $DCC^{-/-}$ and $DCC^{+/+}$ nodal regions (I). The caspr immunoreactive domains were widened at $DCC^{-/-}$ paranodes relative to wild-type slices (J), and the distance between caspr-immunoreactive bands is decreased (K), suggesting that that caspr domain has expanded both in the directions of the internode and the node. 100x objective, digital zoom 4. Scale bar corresponds to 2 μm. *: $p < 0.05$.

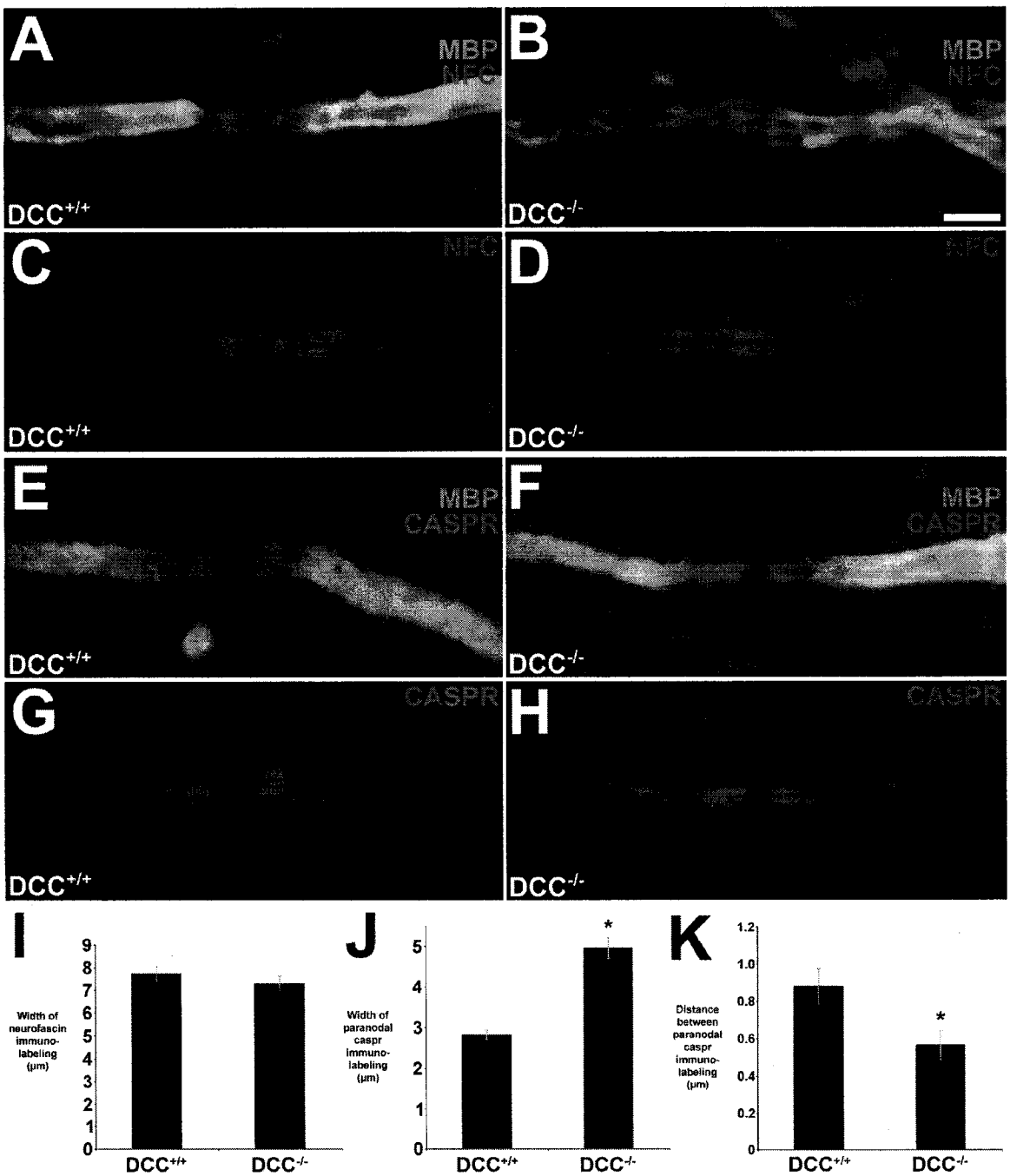
A
MBP
NFC
DCC+/+
B
MBP
NFC
DCC-/-
C
NFC
DCC+/+
D
NFC
DCC-/-
E
MBP
CASPR
DCC+/+
F
MBP
CASPR
DCC-/-
G
CASPR
DCC+/+
H
CASPR
DCC-/-
I
Width of neurofascin immuno-labeling (µm)
9
8
7
6
5
4
3
2
1
0
DCC+/+
DCC-/-
J
Width of paranodal caspr immuno-labeling (µm)
5
4
3
2
1
0
*
DCC+/+
DCC-/-
K
Distance between paranodal caspr immuno-labeling (µm)
1.2
1.0
0.8
0.6
0.4
0.2
0
*
DCC+/+
DCC-/-

Figure 3.10. Caspr, but not neurofascin, distribution is altered in netrin-1$^{-/-}$ cultures at 60 DIV.

60 DIV cerebellar slice cultures were triple-labeled with antibodies against NFH, MBP, and either nfc (A-D) or caspr (E-H). MBP was visualized using Alexa 546-conjugated secondary antibodies (pseudocoloured green), NFH was visualized using Alexa 633-conjugated secondary antibodies (pseudocoloured blue), and nfc or caspr were visualized using Alexa 488-conjugated secondary antibodies (pseudocoloured red). The width of nfc-immunoreactive bands were unchanged between netrin-1$^{-/-}$ and netrin-1$^{+/+}$ nodal regions (I). The caspr immunoreactive domains were widened at netrin-1$^{-/-}$ paranodes relative to wild-type slices (J), and the distance between caspr-immunoreactive bands is decreased (K), suggesting that that caspr domain has expanded both in the directions of the internode and the node. 100x objective, digital zoom 4. Scale bar corresponds to 2 μm. *: $p < 0.05$. *: $p < 0.005$.

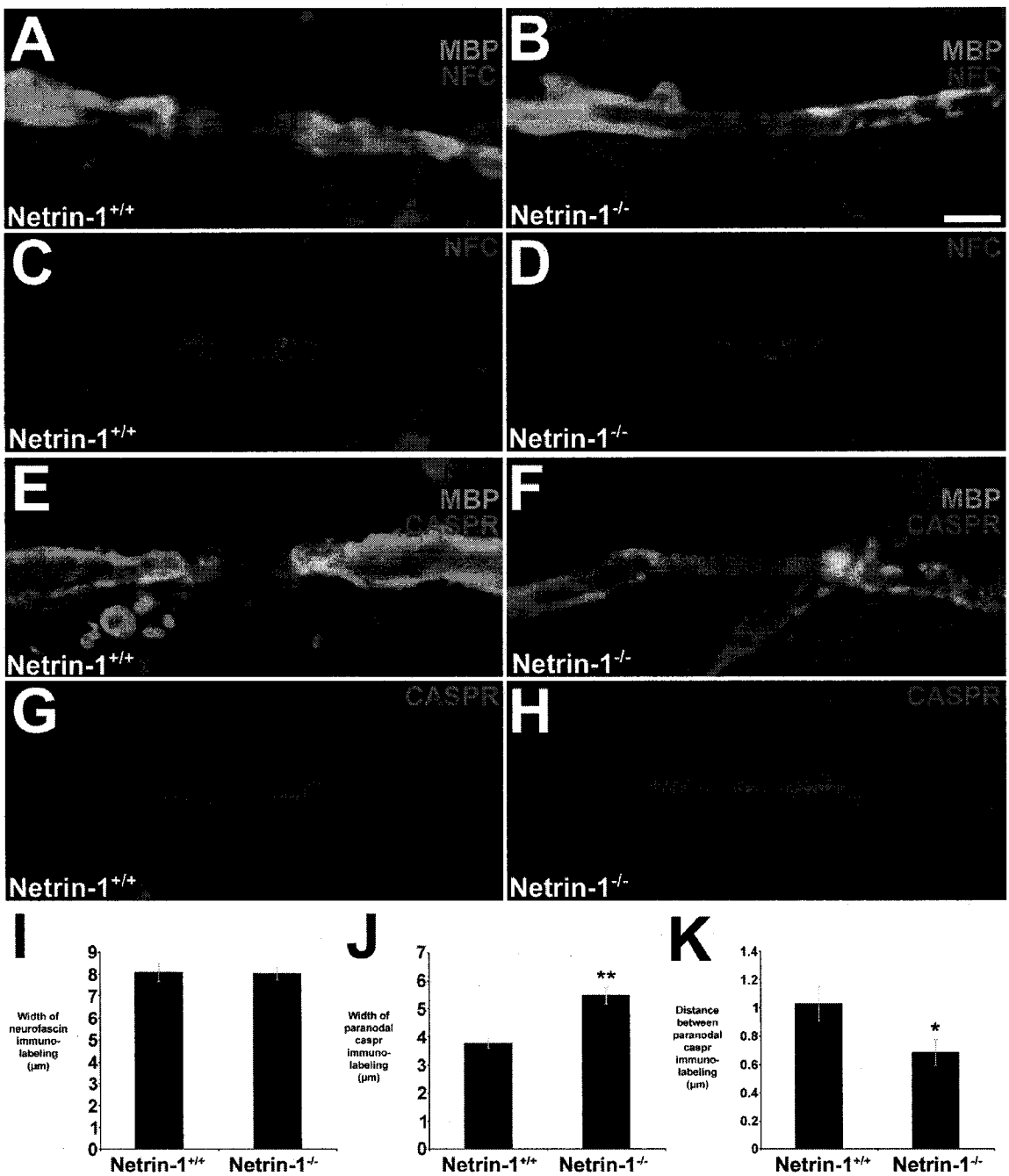
A
MBP
NFC
Netrin-1+/+
B
MBP
NFC
Netrin-1-/-
C
NFC
Netrin-1+/+
D
NFC
Netrin-1-/-
E
MBP
CASPR
Netrin-1+/+
F
MBP
CASPR
Netrin-1-/-
G
CASPR
Netrin-1+/+
H
CASPR
Netrin-1-/-
I
Width of neurofascin immuno-labeling (µm)
0
1
2
3
4
5
6
7
8
9
Netrin-1+/+
Netrin-1-/-
J
Width of paranodal caspr immuno-labeling (µm)
0
1
2
3
4
5
6
7
**
Netrin-1+/+
Netrin-1-/-
K
Distance between paranodal caspr immuno-labeling (µm)
0
0.2
0.4
0.6
0.8
1
1.2
1.4
*
Netrin-1+/+
Netrin-1-/-

Figure 3.11. Paranodal myelin is ultrastructurally normal at 25 DIV

The organization of paranodal myelin was studied by transmission electron microscopy in organotypic slices derived from newborn netrin-$1^{-/-}$ or $DCC^{-/-}$ animals and their wild-type littermates, and maintained in culture for 25 days (A,B; F,G respectively). Unlike what was observed in older slices, paranodal myelin was well-organized in both wild-type and mutant cultures. In almost every heminode studied at this age, TBs were observed linking the axonal and oligodendrocyte membranes and paranodal loops were closely apposed to each other. Detached and everted loops were very rarely observed at 25 DIV (C-E, H-J). 25000x, scale bars correspond to 500 nm.

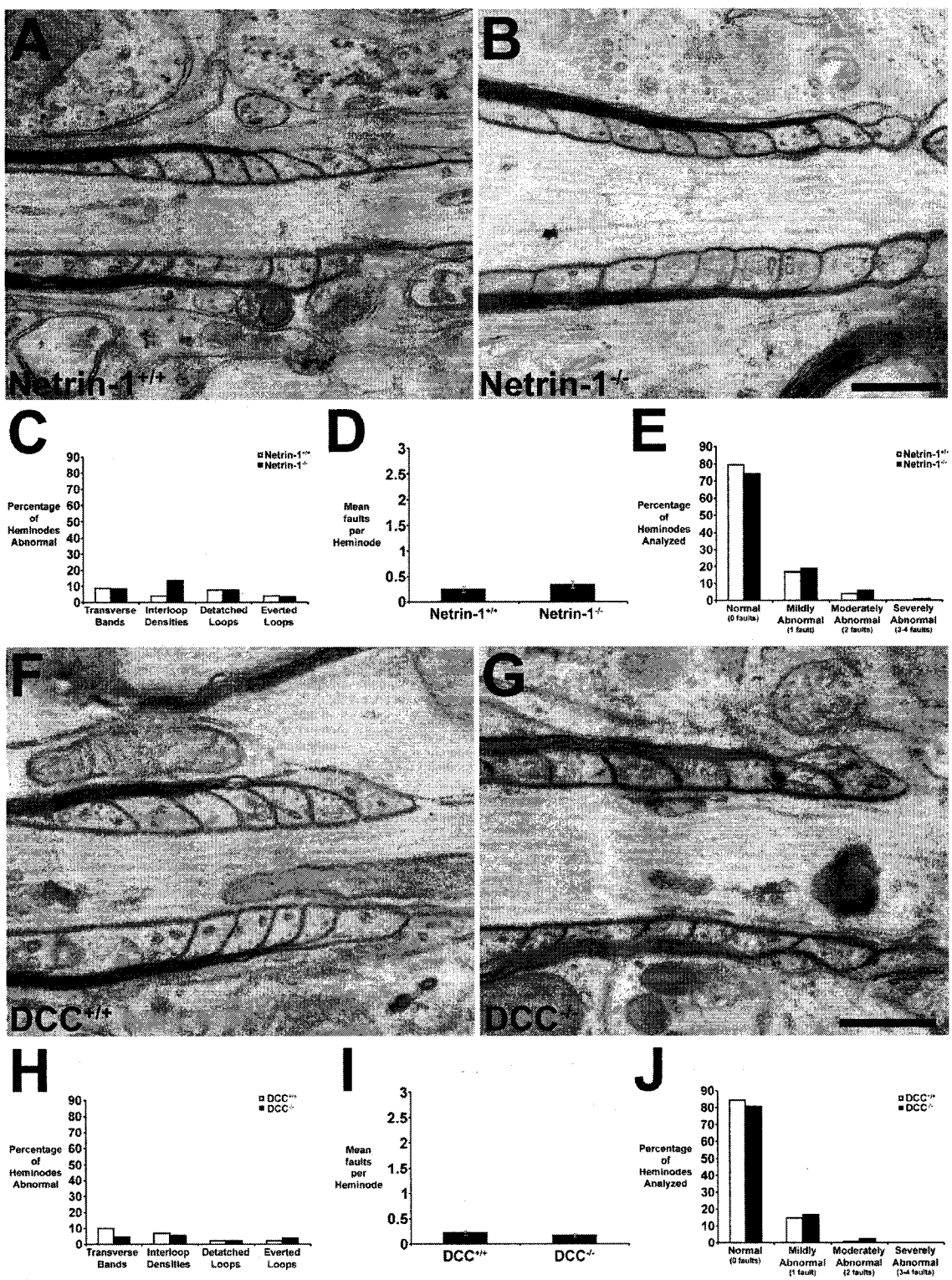
A
Netrin-1+/+
B
Netrin-1-/-
C
Netrin-1+/+
Netrin-1-/-
Percentage of Heminodes Abnormal
Transverse Bands
Interloop Densities
Detatched Loops
Everted Loops
D
Mean faults per Heminode
Netrin-1+/+
Netrin-1-/-
E
Netrin-1+/+
Netrin-1-/-
Percentage of Heminodes Analyzed
Normal (0 faults)
Mildly Abnormal (1 fault)
Moderately Abnormal (2 faults)
Severely Abnormal (3-4 faults)
F
DCC+/+
G
DCC-/-
H
DCC+/+
DCC-/-
Percentage of Heminodes Abnormal
Transverse Bands
Interloop Densities
Detatched Loops
Everted Loops
I
Mean faults per Heminode
DCC+/+
DCC-/-
J
DCC+/+
DCC-/-
Percentage of Heminodes Analyzed
Normal (0 faults)
Mildly Abnormal (1 fault)
Moderately Abnormal (2 faults)
Severely Abnormal (3-4 faults)

Figure 3.12. Preservation of nodal domain organization in 25 DIV netrin-1 and DCC knockout slice cultures

25 DIV cerebellar slice cultures double-labeled with antibodies against MBP and NFM (A,C,H,J), or Na+ch and Kv1.2 (B,D,I,K). MBP and Kv1.2 protein were visualized using Alexa 488-conjugated secondary antibodies (green). NFM and $Na^{+}Ch$ were visualized using Alexa 546-conjugated secondary antibodies (red). The mean distance between regions of MBP immunolabeling across the node was unchanged in the absence of netrin-1 (A,C,E) or DCC (H,J,L) expression at 25 DIV, unlike what was observed in more mature cultures. No change in distance between $Na^{+}Ch$ and Kv1.2 immunoreactivity was observed in either netrin-$1^{-/-}$ (B,D,F,G) or $DCC^{-/-}$ (I,K,M,N) cultures. 100x objective. A,C,H,J: digital zoom 5. B,D,I,K: digital zoom 4. Scale bars correspond to 2 μm.

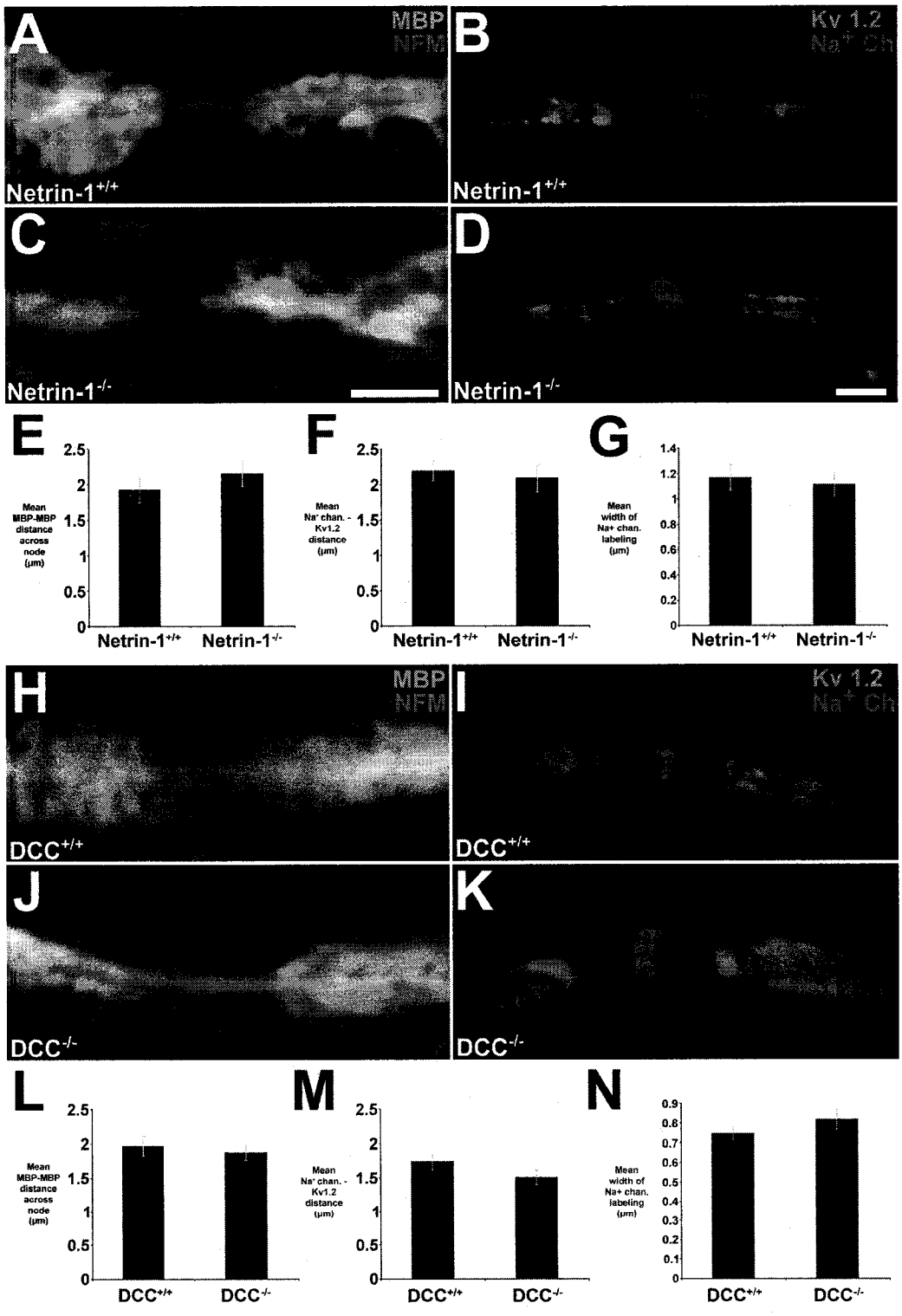
A
MBP
NFM
Netrin-1+/+
B
Kv 1.2
Na+ Ch
Netrin-1+/+
C
Netrin-1-/-
D
Netrin-1-/-
E
Mean MBP-MBP distance across node (µm)
2.5
2
1.5
1
0.5
0
Netrin-1+/+
Netrin-1-/-
F
Mean Na+ chan. - Kv1.2 distance (µm)
2.5
2
1.5
1
0.5
0
Netrin-1+/+
Netrin-1-/-
G
Mean width of Na+ chan. labeling (µm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Netrin-1+/+
Netrin-1-/-
H
MBP
NFM
DCC+/+
I
Kv 1.2
Na+ Ch
DCC+/+
J
DCC-/-
K
DCC-/-
L
Mean MBP-MBP distance across node (µm)
2.5
2
1.5
1
0.5
0
DCC+/+
DCC-/-
M
Mean Na+ chan. - Kv1.2 distance (µm)
2.5
2
1.5
1
0.5
0
DCC+/+
DCC-/-
N
Mean width of Na+ chan. labeling (µm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
DCC+/+
DCC-/-

Figure 3.13. Neither caspr nor neurofascin distribution is altered in $DCC^{-/-}$ cultures at 25 DIV.

25 DIV cerebellar slice cultures were triple-labeled with antibodies against NFH, MBP, and either nfc (A-D) or caspr (E-H). MBP was visualized using Alexa 546-conjugated secondary antibodies (pseudocoloured green), NFH was visualized using Alexa 633-conjugated secondary antibodies (pseudocoloured blue), and nfc or caspr were visualized using Alexa 488-conjugated secondary antibodies (pseudocoloured red). The width of nfc-immunoreactive bands were unchanged between $DCC^{-/-}$ and $DCC^{+/+}$ nodal regions (I). Caspr-immunoreactive domains were also unchanged at 25 DIV $DCC^{-/-}$ paranodes (J,K), unlike what was observed in older cultures. 100x objective, digital zoom 4. Scale bar corresponds to 2 μm. *: $p < 0.05$.

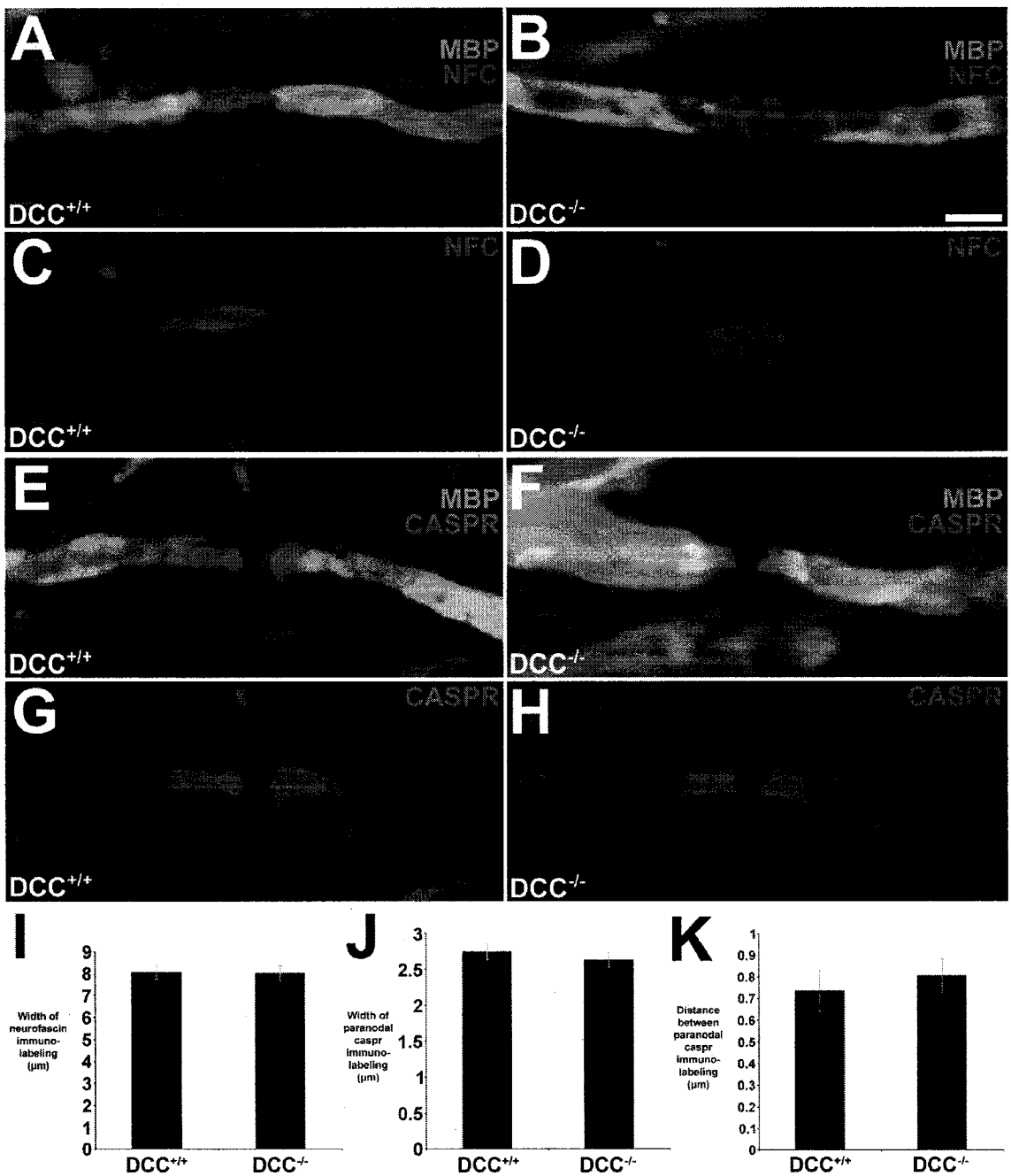
A
MBP
NFC
DCC+/+
B
MBP
NFC
DCC-/-
C
NFC
DCC+/+
D
NFC
DCC-/-
E
MBP
CASPR
DCC+/+
F
MBP
CASPR
DCC-/-
G
CASPR
DCC+/+
H
CASPR
DCC-/-
I
Width of neurofascin immuno-labeling (µm)
0
1
2
3
4
5
6
7
8
9
DCC+/+
DCC-/-
J
Width of paranodal caspr immuno-labeling (µm)
0
0.5
1
1.5
2
2.5
3
DCC+/+
DCC-/-
K
Distance between paranodal caspr immuno-labeling (µm)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
DCC+/+
DCC-/-

Figure 3.14. Neither caspr nor neurofascin distribution is altered in netrin-1$^{-/-}$ cultures at 25 DIV.

25 DIV cerebellar slice cultures were triple-labeled with antibodies against NFH, MBP, and either nfc (A-D) or caspr (E-H). MBP was visualized using Alexa 546-conjugated secondary antibodies (pseudocoloured green), NFH was visualized using Alexa 633-conjugated secondary antibodies (pseudocoloured blue), and nfc or caspr were visualized using Alexa 488-conjugated secondary antibodies (pseudocoloured red). The width of nfc-immunoreactive bands were unchanged between netrin-1$^{-/-}$ and netrin-1$^{+/+}$ nodal regions (I). Caspr-immunoreactive domains were also unchanged at 25 DIV netrin-1$^{-/-}$ paranodes (J,K), unlike what was observed in older cultures. 100x objective, digital zoom 4. Scale bar corresponds to 2 μm. *: $p < 0.05$.

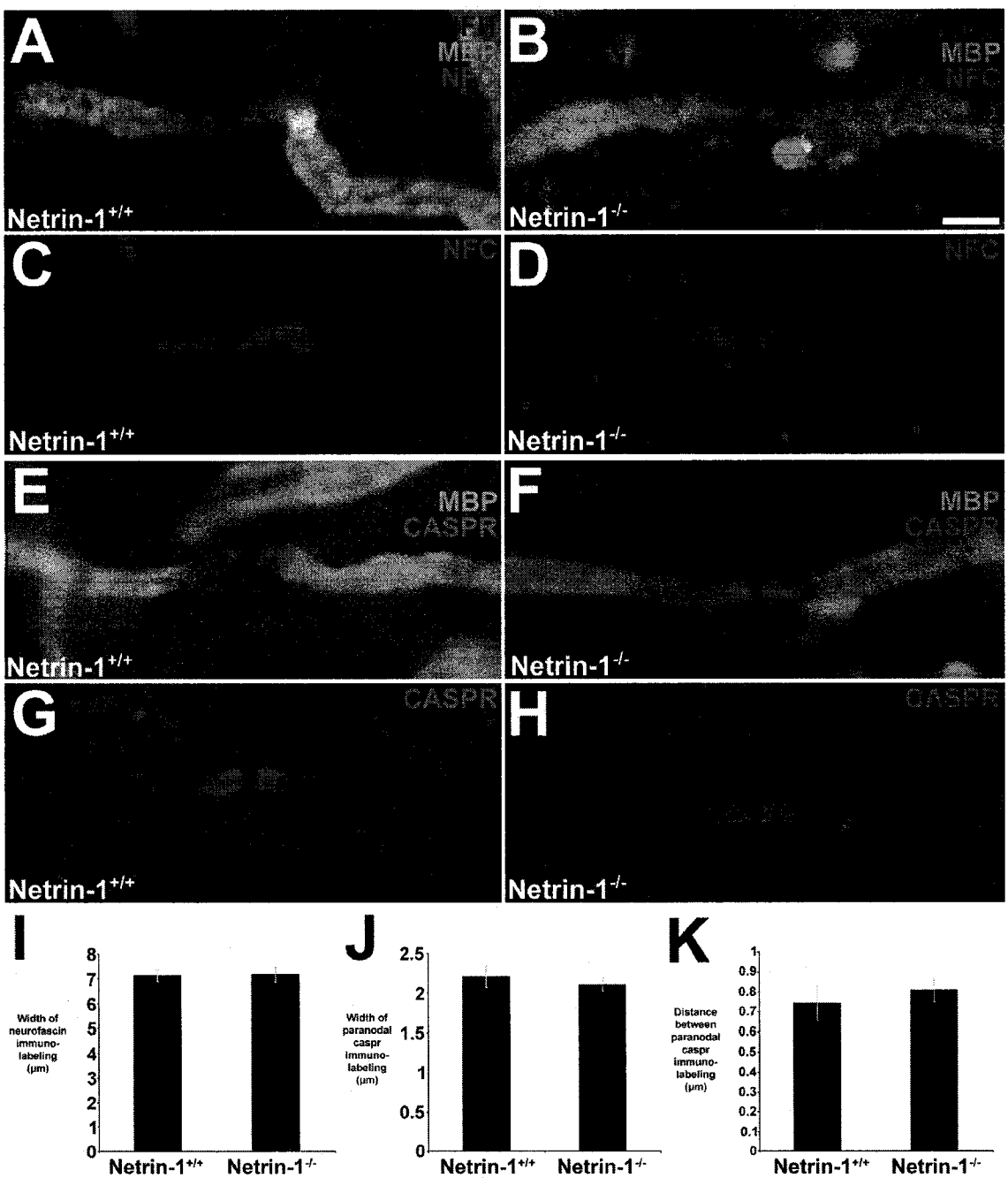
A
MBP
NFC
Netrin-1+/+
B
MBP
NFC
Netrin-1-/-
C
NFC
Netrin-1+/+
D
NFC
Netrin-1-/-
E
MBP
CASPR
Netrin-1+/+
F
MBP
CASPR
Netrin-1-/-
G
CASPR
Netrin-1+/+
H
CASPR
Netrin-1-/-
I
Width of neurofascin immuno-labeling (µm)
0
1
2
3
4
5
6
7
8
Netrin-1+/+
Netrin-1-/-
J
Width of paranodal caspr immuno-labeling (µm)
0
0.5
1
1.5
2
2.5
Netrin-1+/+
Netrin-1-/-
K
Distance between paranodal caspr immuno-labeling (µm)
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
0.8
0.9
1
Netrin-1+/+
Netrin-1-/-

Figure 3.15. Model of netrin-1 function at the paranodal axo-glial junction

Our data suggests a model in which netrin-1 localized to the paranodal axo-glial junction is membrane associated, and plays a role in maintaining the adhesion of paranodal loops to both the axonal surface and each other. These actions are mediated through DCC and other receptors, most likely members of the UNC5 family.

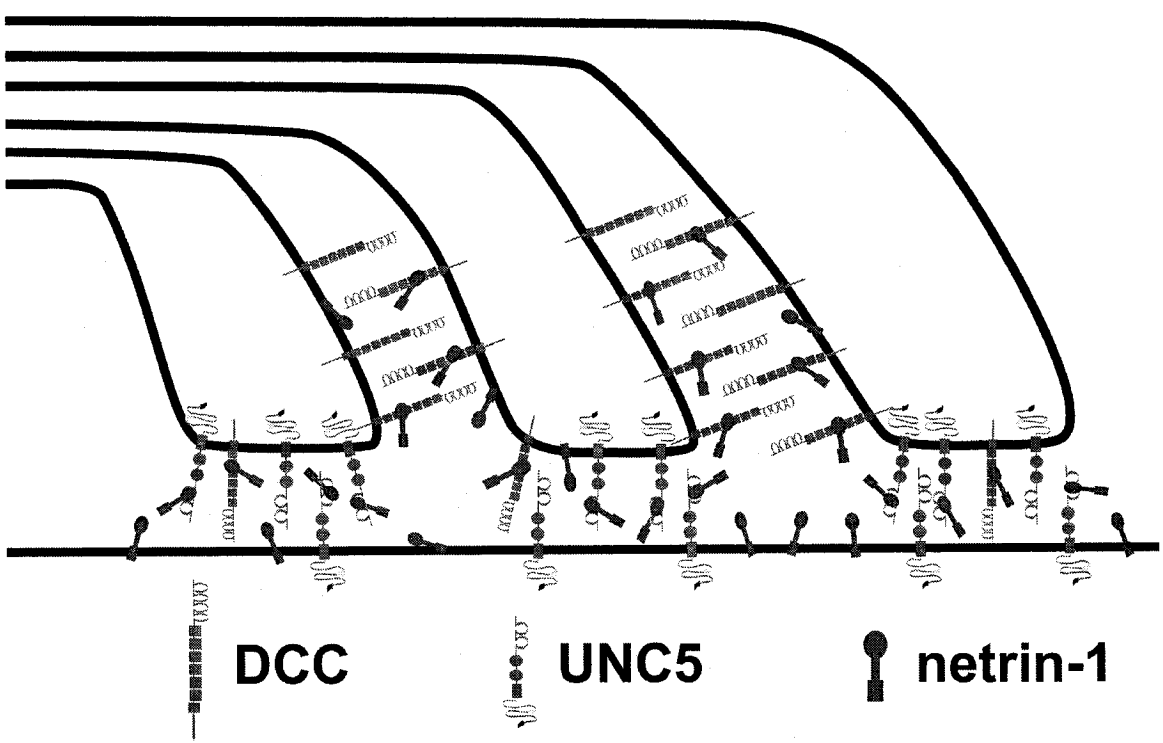
DCC
UNC5
netrin-1

Table 3.1. Organization of compact myelin in DCC and netrin-1 mutant cerebellar slice cultures

	Periaxonal space (μm)	Periodicity (wraps/μm)	n
$DCC^{+/+}$ 67 DIV	12.24±0.26	10.83±0.25	27
$DCC^{-/-}$ 67 DIV	12.22±0.21	12.04±0.35	32
Netrin-$1^{+/+}$ 49 DIV	11.29±0.21	12.24±0.13	58
Netrin-$1^{-/-}$ 49 DIV	11.43±0.29	11.96±0.15	42

As described in figure 3.4, the width of the periaxonal space is unaffected in both netrin-1 and DCC mutant slice cultures. Myelin periodicity is unchanged in netrin-1 mutant cultures, but a slight increase in periodicity is observed in DCC mutant cultures.

Table 3.2. Ultrastructural paranodal abnormalities in DCC$^{-/-}$ and netrin-1$^{-/-}$ cerebellar slice cultures

	% TB abnormal	% ID abnormal	% detached loops	% everted loops	mean faults/ heminode	n
DCC+/+ 25 DIV	10.0	6.9	2.3	2.3	0.22±0.04	130
DCC-/- 25 DIV	4.7	5.4	2.3	3.9	0.39±0.03	129
Netrin-1+/+ 25 DIV	8.8	3.9	7.8	3.9	0.24±0.05	102
Netrin-1-/- 25 DIV	8.5	13.7	7.7	3.4	0.33±0.07	117
DCC+/+ 67 DIV	1.2	2.5	7.5	7.5	0.18±0.05	81
DCC-/- 67 DIV	30.0	50.0	61.3	36.3	1.78±0.13	80
Netrin-1+/+ 49 DIV	8.0	5.3	9.4	1.4	0.27±0.05	113
Netrin-1-/- 49 DIV	72.7	79.1	60.4	31.7	2.43±0.09	139

	% Normal	% Mildly abnormal	% Moderately abnormal	% Severely abnormal
DCC+/+ 25 DIV	80.8	16.9	2.3	0.0
DCC-/- 25 DIV	84.5	14.7	0.8	0.0
Netrin-1+/+ 25 DIV	79.4	16.7	3.9	0.0
Netrin-1-/- 25 DIV	74.3	18.8	6.0	0.9
DCC+/+ 67 DIV	84.0	13.6	2.5	0.0
DCC-/- 67 DIV	12.5	33.8	23.8	30.0
Netrin-1+/+ 49 DIV	76.1	21.2	2.7	0.0
Netrin-1-/- 49 DIV	5.8	12.9	28.1	53.2

As described in figures 3.5, 3.6, 3.7, and 3.11, paranodal myelin in netrin-1 and DCC mutant cerebellar slice cultures is normal at 25 DIV, but becomes disorganized at later time points. n represents one heminode.

Table 3.3. Disrupted domain organization in older netrin-1$^{-/-}$ and DCC$^{-/-}$ cerebellar slice cultures

	NOR length (μm)	n	Na^+ch-Kv1.2 dist (μm)	n	Na^+ch domain width (μm)	n
DCC+/+ 25 DIV	1.96±0.14	39	1.74±0.12	86	0.75±0.03	43
DCC-/- 25 DIV	1.88±0.11	32	1.50±0.11	88	0.82±0.05	42
Netrin-1+/+ 25 DIV	1.93±0.18	35	2.19±0.14	70	1.17±0.10	35
Netrin-1-/- 25 DIV	2.15±0.18	23	2.08±0.18	66	1.11±0.09	29
DCC+/+ 60 DIV	1.90±0.11	30	2.24±0.10	56	0.67±0.23	28
DCC-/- 60 DIV	2.36±0.18	32	1.33±0.10	96	0.72±0.06	29
Netrin-1+/+ 60 DIV	1.90±0.15	26	2.34±0.18	56	0.98±0.09	28
Netrin-1-/- 60 DIV	1.77±0.11	34	1.28±0.13	58	1.56±0.22	29

	nfc label width (μm)	n	caspr label width (μm)	n	caspr-caspr dist (μm)	n
DCC+/+ 25 DIV	7.24±0.30	31	2.74±0.11	64	0.74±0.04	32
DCC-/- 25 DIV	6.95±0.35	30	2.63±0.10	68	0.81±0.08	34
Netrin-1+/+ 25 DIV	7.11±0.27	28	2.21±0.13	60	0.74±0.09	30
Netrin-1-/- 25 DIV	7.17±0.26	29	2.11±0.08	84	0.80±0.06	42
DCC+/+ 60 DIV	7.75±0.30	28	2.84±0.14	50	0.89±0.09	25
DCC-/- 60 DIV	7.34±0.33	28	4.99±0.28	54	0.57±0.08	27
Netrin-1+/+ 60 DIV	8.07±0.39	31	3.76±0.16	60	1.03±0.12	30
Netrin-1-/- 60 DIV	8.02±0.29	39	5.47±0.29	84	0.68±0.09	42

As described in figures 3.8-10 and 3.12-14, the domain organization of paranodal myelin is disrupted in 60 DIV, but not 25 DIV, cerebellar slice cultures. For quantification of NOR length, Na^+ch domain width, nfc label width and caspr-caspr distance, n represents measurements from one nodal region. For quantification of Na+ch-Kv1.2 distance and caspr label width, n represents each of two measurements obtained per nodal region.

PREFACE TO CHAPTER 4

Chapters 2 and 3 described the influence of netrin-1 on the function of glial precursor and mature glial cells, respectively. Gliomas, tumors arising from glial cells, have been hypothesized to be derived from either immature precursor cells, or as the result of de-differentiation of mature CNS glia (Dai et al., 2001; Hemmati et al., 2003). Netrin and netrin receptor expression is lost or reduced in multiple tumor types, including gliomas, leading to the suggestion that these proteins are tumor suppressors. In particular, loss of *dcc* expression correlates with the development of highly invasive glioblastoma (Reyes-Mugica et al., 1997; Meyerhardt et al., 1999; Thiebault et al., 2003). In recent years, discussion of the roles played by netrins and their receptors in tumorigenesis has centered on their proposed functions as regulators of apoptosis (Arakawa, 2004; Mehlen and Furne, 2005). As the results of our previous studies suggested that netrin-1 does not influence the survival of glia, but can influence their motility, we hypothesized that netrins and their receptors may regulate the migration of glial tumor cells.

The goals of this study were to:

1) Investigate whether netrins influence the migration of glioma cells and, if so, by what mechanism

2) Determine if netrin and their receptors regulate apoptosis in glioma cells, as predicted by the dependence receptor hypothesis

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CHAPTER 4

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Inhibition of Glioblastoma Cell Motility by Netrin:

A Novel Autocrine Mechanism Regulating Tumor Cell Migration

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I. ABSTRACT

Deregulation of mechanisms that control cell motility plays a key role in tumor progression by promoting tumor cell dissemination. Netrins and their receptors, Deleted in Colorectal Cancer (DCC) and UNC5 homologues, regulate cell and axon migration, and are implicated in tissue morphogenesis and tumorigenesis. Here we describe a novel function for netrin as an autocrine factor that restrains human glioblastoma cell migration. We provide evidence that netrin promotes the maturation of focal complexes, structures associated with cell movement, into focal adhesions. Consistent with this, netrin, DCC, and UNC5 homologues were associated with focal adhesions, but not focal complexes, in glioblastoma cells. Netrin receptors have been proposed to inhibit tumorigenesis by acting as dependence receptors, activating apoptosis in the absence of netrin; however, disrupting either netrin or DCC function did not increase glioblastoma cell death. These findings reveal a novel role for netrins as autocrine inhibitors of cell motility and identify them as potential therapeutic targets to regulate tumor cell migration.

II. INTRODUCTION

Cell migration is essential for normal embryonic development, wound healing, and immunity but can be devastating in tumor invasion and metastasis. Tumor progression is a multi-step process (Compagni and Christofori, 2000) and tumor cells acquire the ability to migrate far from their place of origin, in some cases metastasizing and invading surrounding tissue.

Netrins are secreted, laminin-related proteins that direct cell and axon migration during neural development. They are bifunctional, acting as either chemoattractants or chemorepellents for different cell types (reviewed by Manitt and Kennedy, 2002). Netrin-1 and netrin receptors, the UNC5 homologues and DCC, are expressed in many adult tissues (Fearon et al., 1990; Meyerhardt et al., 1997; Meyerhardt et al., 1999; Manitt et al., 2001; Thiebault et al., 2003; Manitt et al., 2004), but their function in the adult remains unknown. Netrin-1 is widely expressed by neurons and glia in the adult CNS (Manitt et al., 2001), and reduced expression has been documented in brain tumors, including glioblastoma (Meyerhardt et al., 1999). A role for netrins regulating tumor cell migration has not been established.

DCC is required for chemoattractant responses to netrin-1 (reviewed by Manitt and Kennedy, 2002). While substantial evidence points to an anti-oncogenic role for DCC, how the disruption of netrin signaling contributes to the malignant process is poorly understood. In colorectal cancer, allelic deletion involving chromosome 18q21 occurs in >70% of tumors (Vogelstein et al., 1988) and the *dcc* gene was first identified as a putative tumor suppressor from this chromosomal deletion (Fearon et al., 1990). Although no increased incidence of tumor formation was detected in DCC knockout mice (Fazeli et al., 1997), conclusions drawn from this study were complicated by the possibility that tumors may not have had time to develop due to the early post-natal lethality of DCC knockouts. *Dcc* expression is reduced in many cancers, including most high-grade gliomas (Ekstrand et al., 1995; Reyes-Mugica et al., 1997) and loss of DCC correlates with the development of highly invasive glioblastoma multiforme (Reyes-Mugica et al., 1997). Furthermore, ectopic expression of *dcc* in tumor cells reduced tumorigenicity (Klingelhutz et al., 1995), and expression of DCC antisense RNA in tumor cells resulted

in a faster growth rate, anchorage independence, and tumorigenicity when cells were transplanted into nude mice (Narayanan et al., 1992).

Chemorepulsion to netrin requires expression of an UNC5 homologue, and in some cases is also dependent on co-expression of DCC (reviewed by Manitt and Kennedy, 2002). Four UNC5 homologue family members, UNC5A-D, are expressed in mammals, UNC5A-D. Reduced expression of UNC5A, B and C has been detected in various cancers, suggesting that they may also function as tumor suppressors (Thiebault et al., 2003). DCC and UNC5 homologues have been proposed to suppress tumorigenesis through a dependence mechanism that triggers apoptosis in the absence of netrin (Arakawa, 2004); however, this dependence mechanism is not activated in several DCC and UNC5 homologue-expressing cell types, including glial precursor cells (Jarjour et al., 2003; Tsai et al., 2006).

Here we describe a novel role for netrin as an autocrine factor that restrains cell migration. U87, U343, and U373 human glioblastoma cell lines all express netrin receptors and either netrin-1 or netrin-3. In the absence of an applied gradient of netrin-1, disrupting autocrine netrin function increased the rate of cell migration. DCC was required for cells to respond to a gradient of netrin-1 as a chemoattractant; however in the absence of a gradient, DCC expression also slowed the rate of spontaneous cell movement. Netrin-1, netrin-3, DCC, and UNC5 homologues were detected in focal adhesions (FA), but not focal complexes (FC). Disrupting endogenous netrin promoted the formation of FCs and increased the rate of cell migration. These findings identify a function for autocrine netrin that regulates cell motility and suggest that netrin signaling might be manipulated to inhibit tumor cell migration and dispersion.

III. MATERIALS AND METHODS

Cells and cell culture

Human glioblastoma cell lines, U87, U343, U373 (ATCC, Rockville, MD) and astrocytes isolated from newborn mouse brain were grown as monolayer cultures in DMEM (Invitrogen, Burlington, ON), 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), glutamax-1 (Invitrogen) and penicillin/streptomycin.

Antibodies, Conditioned media, cell lysates, western blotting, and PCR

Antibodies against the following were used: cleaved caspase-3 (Asp175, mouse, Cell Signaling Technology, Beverly, MA); DCC (DCC_{IN}, mouse, G97-449; BD Biosciences Pharmingen, San Jose, CA; DCC_{GT}, goat, A-20; Santa Cruz Biotechnology, Santa Cruz, CA; function-blocking, DCC_{FB}, mouse, AF5; Calbiochem, La Jolla, CA); netrin-1 and 3 (PN2, rabbit, Manitt et al., 2001; netrin function-blocking ($Netrin_{FB}$, PN3, rabbit, Manitt et al., 2001; neogenin (rabbit, Santa Cruz Biotechnology); paxillin (mouse, BD Biosciences Pharmingen); pan-unc5h (rabbit, Tong et al, 2001, provided by Dr. Tony Pawson, Mount Sinai Hospital, Toronto, ON; preimmune rabbit IgGs (RbIgG; Invitrogen); and zyxin (rabbit, Abcam, Cambridge, MA).

Cultures were grown to 80% confluence and conditioned media collected following 48 hrs in serum-free DMEM. For lysates, cells were grown to 80% confluence, rinsed with PBS and lysed in 1 ml of hot sample buffer (60 mM Tris/HCl, pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT). For western blot analysis of cleaved caspase-3, cells were cultured at a density of 120,000 cells/well in a 12-well tissue culture plate. Nitrocellulose immunoblots were probed with DCC_{IN} (0.5 μg/ml), PN2 anti-netrin (4 μg/ml), anti-cleaved caspase-3 (1:1000), or anti-neogenin (0.4 μg/ml). After washing, membranes were incubated with HRP-coupled secondary antibodies and immunoreactivity visualized using chemiluminescence (NEN, MA).

PCR was carried out using standard methods. Primer sequences will be provided on request.

Transfilter chemotaxis assay

Cells were plated at a density of $4x10^5$ cells/ml on polycarbonate transwell culture inserts (6.5 mm diameter with 8 μm pore size, Corning). 100 μl of cell suspension was used per filter, and the filters placed in the wells of a 24-well plate over 600 μl of medium. DMEM with 0.2% BSA, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamax was the base medium used for all assay conditions. Following migration, cells on the upper side of the filter were scraped off, and the cells attached to the lower side of the filter fixed with 4% paraformaldehyde (PFA)/0.1% glutaraldehyde (30 min, 4°C). Filters were rinsed with PBS, cell nuclei stained with Hoechst dye. Four transwells were used per condition. Four images of each filter were captured using a 10 X objective and nuclei counted using Northern Eclipse software (Empix Imaging, TO). Where pooled results are presented, the value 'percent migration vs control' for a given trial represents the number of cells migrated in that condition expressed as a percentage of the mean number of cells migrating in control conditions. Recombinant netrin-1 protein was purified as described (Shekarabi et al., 2005) and used at a concentration of 100 ng/ml. Laminin-1 was used at 10 μg/ml (BD Biosciences, Bedford, MA). Net_{FB} and rabbit preimmune IgG (used as a control) were added at a concentration of 25 μg/ml. DCC_{FB} was added at a concentration of 10 μg/ml.

Plasmids and transfection

U343 and U373 cells were transfected using lipofectamine (Invitrogen) with expression constructs encoding either green fluorescent protein (GFP) alone or DCC tagged at its C-terminus with GFP (Shekarabi and Kennedy, 2002). Seventy-two hrs after transfection, the medium was changed to selection medium containing Geneticin (Invitrogen).

Confocal image analysis

10^4 cells were plated per well in chamber slides (Fisher) coated with 20 μg/ml poly-D-lysine (Sigma) at 4°C overnight, washed with Hanks buffered salt solution (Invitrogen) and allowed to dry. Cells were fixed in 4% PFA, 4% sucrose in PBS, and permeabilized with 0.25% Triton X-100 in PBS. Blocking was performed in 3% heat-inactivated normal goat serum, 2% BSA, and 0.125% Triton X-100 in PBS. Cells were

then incubated with anti-paxillin and anti-zyxin (Fig. 4.5), anti-paxillin and one of anti-netrin PN2, anti-UNC5, or anti-DCC_{GT}, or anti-zyxin and anti-DCC_{GT} (Fig. 4.6) diluted in blocking solution. Primary antibodies were detected with secondary antibodies coupled to Alexa 546 or Alexa 488 (Molecular Probes).

For imaging adhesive complexes, single confocal optical slices through the base of lamellipodia were collected. Identical settings were used for each condition examined for a given cell line. The outermost region of individual lamellipodial protrusions (excluding regions of paxillin or zyxin immunoreactivity contiguous with adhesive structures in the cell body) was outlined using Image J software (Rasband, 2006). Mask images were then generated representing either the regions staining with both paxillin and zyxin (using an 'AND' function) or representing the difference between the paxillin and zyxin images (the zyxin signal subtracted from the paxillin image). Images were adjusted to eliminate signal below a minimum value that was held constant across all images for each cell line. Signal in the 'AND' image corresponds to the area of each lamellipodium occupied by mature FAs that contain both paxillin and zyxin. To quantify FCs, the subtracted image representing paxillin staining without zyxin was filtered to exclude structures smaller than 3 pixels or larger than 40 pixels. The number of individual adhesions was then counted and the density of adhesions within each lamellipodium calculated. Netrin-1 was used at 100 ng/ml, Net_{FB} and rabbit preimmune IgG (as a control) at 25 μg/ml, and DCC_{FB} at 10 μg/ml.

Analysis of cell number and apoptosis

To investigate changes in cell survival or proliferation, cells were plated at a density of 30,000 cells per well in 8-well chamber slides (Fisher), allowed to settle for 2 hrs, treated as described for 16 hrs, fixed and stained with Alexa-488 phalloidin and Hoechst, and the number of live cells per 20 X field counted. To measure levels of apoptosis in these cultures, 120,000 cells were cultured in each well of a 12-well tissue culture plate, allowed to settle for 2 hrs, and treated as described for either 16 or 48 hrs. In all cases, the base medium used was DMEM with 2% FBS, penicillin/streptomycin, and glutamax-1.

IV. RESULTS

Glioblastoma cells express netrin and netrin receptors

To determine if netrins regulate glioblastoma cell migration, we first characterized netrin and netrin receptor expression in human astrocytoma cell lines U87, U343, and U373, and in cultures of astrocytes isolated from newborn rat cortex (Fig. 4.1A). Western blot analysis using an antibody that binds netrin-1 and netrin-3 (Manitt et al., 2001) detected a ~75 kDa band corresponding to full length netrin in conditioned medium collected from all cells tested. The DCC_{IN} antibody detected a ~185 kDa band, corresponding to DCC in astrocyte and U87 cell lysates. In contrast, DCC was not detected in lysates of U343 or U373 cells. The DCC homologue neogenin was expressed by astrocytes and all glioblastoma cell lysates.

RT-PCR (Fig. 4.1B) revealed *dcc* expression by U87 cells but not U343 or U373 cells, and *neogenin* and *unc5 homologue* expression by all three cell types. U87 cells express only *unc5b*, U343 cells express *unc5a, b*, and *c*, and U373 cells express *unc5a, b, c*, and *d*. *Netrin-1* expression was detected in U343 and U373 cells, and *netrin-3* expression in U87 cells. Netrin-1 and netrin-3 both bind DCC and UNC5 homologues and similarly evoke chemoattractant or chemorepellent responses (Wang et al., 1999).

As a result of finding that these cells express both netrins and netrin receptors, we then sought to determine if netrin might exert an autocrine influence on cell migration. We first assessed the relative motility of the three cell lines using a transfilter chemotaxis assay as described (Jarjour et al., 2003). Briefly, cells were cultured on the upper surface of a porous membrane (Fig. 4.1G) and allowed to migrate across. Following migration, cells remaining on the upper surface of the membrane were scraped off, and the cells that migrated to the underside fixed, stained, and counted. While this assay is most commonly employed to assess the migration of cells in response to a putative attractant or repellent cue, here we used it in the absence of added factors to compare the relative rates of spontaneous migration of the three glioblastoma lines. U343 and U373 cells, lacking DCC, migrated significantly faster than DCC expressing U87 cells (Fig. 4.1C). Notably, the U343 cells, which were derived from a grade IV glioblastoma multiforme (Nister et

al., 1987), migrated significantly faster than either the U87 or U373 cells, both of which were derived from less aggressive grade III astrocytomas (Ponten and Macintyre, 1968).

Autocrine netrin-1 inhibits U87 cell motility

U87 cells, which express DCC, migrate substantially more slowly than either U343 or U373 cells, which do not express DCC. We hypothesized that netrin and DCC expressed by U87 cells might exert a kinetic influence on the rate of cell movement, independent of netrin's influence on directional migration. We therefore tested the effect of blocking DCC and netrin function on the spontaneous rate of U87 cell migration. Addition of netrin function-blocking antibody (Net_{FB}) to both the top and bottom compartments, thereby disrupting autocrine netrin function, resulted in a striking greater than 25 fold increase in spontaneous migration across the filter relative to the number of cells migrating in either medium alone (Control), or in the presence of a control IgG (Fig. 4.1D). In contrast, the rate of spontaneous migration was not affected by addition of DCC function-blocking antibody (DCC_{FB}).

Autocrine netrin-1 inhibits migration of U373, but not U343, glioblastoma cells

Netrin's capacity to inhibit U87 cell motility in a DCC-independent manner led us to determine if a similar mechanism was active in U343 or U373 cells, which do not express DCC. The addition of netrin function-blocking antibody to both the top and bottom compartments of the transfilter assay significantly increased the rate of U373 migration (Fig. 4.1F), indicating that endogenous netrin-1 inhibits the rate of U373 migration.

Unlike U87 and U373 cells, blocking netrin function did not alter the rate of U343 cell migration (Fig. 4.1E). Grade IV glioblastoma multiforme-derived U343 cells were the most rapidly migrating of the three cell types examined (Fig. 4.1C) and while they express neogenin and UNC5 homologue netrin receptors, the absence of an increase in the rate of migration may be the result of more severe disruption of the mechanisms that regulate the motility of these cells.

Netrin-1 is a chemotropic attractant for U87 glioblastoma cells

Transfilter migration assays were then used to determine if DCC-expressing U87 cells respond to a gradient of netrin-1 as a chemoattractant. Addition of 100 ng/ml netrin-1 to the bottom compartment (NB) produced a significant increase in the number of U87 cells that migrated across the membrane relative to control (medium alone: Fig. 4.2A, 16 hr assay; Fig. 4.2B, 48 hr assay). In contrast, when netrin-1 was added to both the top and bottom compartments (NTB), migration was not significantly different from control. This indicates that U87 cells respond to a gradient of netrin-1 as a chemotropic attractant. When challenged with a gradient of netrin-1, with DCC_{FB} antibody in the top and bottom wells (NB DCC_{FB}), U87 cells migrated not significantly different from control, indicating that the tropic response of U87 cells to netrin-1 requires DCC. Neither U343 nor U373 cells, which do not express DCC, altered their migration in response to an exogenous gradient of netrin-1 (Fig. 4.2C), despite expressing neogenin and UNC5 homologue netrin receptors. These findings suggest that although these receptors may be sufficient to mediate autocrine inhibition of migration (Fig. 4.1F), they are insufficient for these cells to generate a chemotropic response to a gradient of netrin-1 (Fig. 4.2C).

Chemoattractant response of DCC-expressing U343 and U373 cells to a gradient of netrin-1

To further investigate the contribution of DCC to the regulation of cell motility, we reintroduced the *dcc* gene back into U343 and U373 cells by transfection with a cDNA encoding a DCC-GFP chimera (pDCC-GFP, described by Shekarabi and Kennedy, 2002). Expression of DCC by U343 and U373 cells was confirmed by western blot (Fig. 4.1A). Unlike the parental U343 and U373 cells lines, DCC-GFP expressing U343D and U373D cells migrated in response to an ascending gradient of netrin-1 (Fig. 4.2D, E), indicating that DCC transgene expression in these cells was sufficient to generate a chemotropic response to netrin-1. Like DCC-expressing U87 cells, the gain of function migration towards netrin-1 exhibited characteristics of true chemotropic attraction, as the cells only responded to a gradient. Uniform presentation of exogenous netrin-1 produced migration that was not significantly different from control. The DCC_{FB} antibody blocked the chemoattractant response of U343D and U373D cells, indicating that DCC is required for chemoattraction to netrin-1.

Consistent with the slow migration of DCC expressing U87 cells, the number of DCC-transfected U343 and U373 cells that migrated under control conditions was substantially reduced relative to that of the parental cells (Fig. 4.2D, E). These findings suggest that DCC expression decreases the motility of these cells; however, again consistent with the U87 cells (Fig. 4.1D), application of the DCC function-blocking antibody (DCC_{FB}) did not increase the rate of migration. In contrast, DCC_{FB} completely blocked the chemoattractant migratory response of the U87 cells, and the DCC-transfected U343 and U373 cells to a gradient of netrin-1. These findings are consistent with DCC expression engaging a mechanism that slows non-directional cell migration, but that is insensitive to DCC_{FB}.

Chemoattraction to netrin-1 is converted to repulsion by laminin-1

Laminin-1 converts the response of *Xenopus* retinal ganglion cell growth cones to netrin-1 from attraction to repulsion (Hopker et al., 1999). We therefore investigated the possibility that laminin-1 might influence the migratory response of U87 cells to a gradient of netrin-1 (Fig. 4.3). When U87 cells were challenged with an ascending gradient of laminin-1 (LB), the number of cells that migrated to the underside of the membrane increased. In a uniform concentration of laminin (LTB), U87 migration was not significantly different from control, indicating that a gradient of laminin-1, like netrin-1, is a chemoattractant for these cells. Interestingly, the combination of an ascending gradient of netrin-1 and a uniform concentration of laminin-1 (LTBNB) dramatically reduced the number of U87 cells that migrated to the underside of the membrane, suggesting that laminin-1 converted netrin-1 from an attractant to a repellent. Consistent with this, confronting cells with a descending netrin-1 gradient in the presence of a uniform concentration of laminin-1 (LTBNT), resulted in an increase in migration relative to controls. When the cells were simultaneously exposed to uniform concentrations of netrin-1 and laminin-1, (LTBNTB), fewer cells migrated to the underside of the membrane, indicating that the combined action of netrin-1 and laminin-1 exert a non-directional effect that inhibits U87 cell motility. These results are consistent with laminin-1 switching netrin-1 from an attractant to a repellent for U87 cells, as previously described for the axons of *Xenopus* retinal ganglion cells (Hopker et al., 1999). Addition

of DCC_{FB} antibody in the presence of a uniform concentration of laminin-1 and either an increasing gradient (LTBNB DCC_{FB}) or uniform concentration (LTBNTB DCC_{FB}) of netrin-1, produced migration that was not significantly different from control, indicating that the laminin-induced repellent response to netrin-1 requires DCC.

Netrin-1 and DCC do not affect U87, U343, and U373 cell survival or proliferation

DCC and UNC5 homologues have been proposed to function as dependence receptors, activating apoptosis in the absence of netrin-1 (Mehlen and Mazelin, 2003). This raises the possibility that the effects described above may be due to an influence on cell survival and not motility. Thus, we examined the consequences of manipulating netrin function on the survival of U87, U343 and U373 cells. No significant change in cell number (Fig. 4.4A), or activation of caspase-3, a sensitive indicator of apoptosis (Fig. 4.4B), was detected following 16 hrs treatment with exogenous netrin-1, laminin-1, or both; nor following disruption of netrin or DCC function using blocking antibodies. Further testing, by blocking netrin and DCC function for 48 hrs, again detected no increase in caspase-3 activation (Fig. 4.4C). In contrast, staurosporine, applied as a positive control, activated caspase-3 and caused extensive cell death (Fig. 4.4B, C). These findings are consistent with previous analyses of glial precursor cells, indicating that netrin-1 and DCC do not regulate oligodendrocyte precursor survival either *in vitro* or *in vivo* (Jarjour et al., 2003; Tsai et al., 2006), and they support the conclusion that the results found using the transfilter assays reflect changes in cell migration and not effects on cell survival or proliferation.

Endogenous netrin promotes the maturation of focal complexes into focal adhesions

Cell migration requires the formation of transient adhesive contacts with the extracellular matrix (ECM). Initial contacts occur at the leading edge of lamellipodia where integrins bind ECM ligands and recruit proteins such as vinculin and paxillin to form FC immature adhesive contacts (reviewed by Wozniak et al., 2004). The transition from FC to FA is marked by consolidation of the adhesive contact, an increase in size, and the recruitment of additional proteins including tensin and zyxin (Zaidel-Bar et al., 2003).

The effect of disrupting netrin function on adhesive complex formation in glioblastoma cells was investigated by examining the distribution of paxillin, which is present in both FAs and FCs, and zyxin, which is present in FAs but not FCs. The influence of netrin on FC formation was quantified by subtracting the distribution of zyxin immunoreactivity (Fig. 4.5C, H, M, R, W) from paxillin immunoreactivity (Fig. 4.5B, G, L, Q ,V) to create images representing regions of paxillin, but not zyxin localization (Fig. 4.5D, I, N, S, X). Using the 'paxillin minus zyxin' images, the density of FCs present in each lamellipodium was calculated. Exposure of U87 cells to a control preimmune antibody (RbIgG), DCC_{FB}, or netrin-1, resulted in no change relative to control. In contrast, application of $Netrin_{FB}$ resulted in increased FC density (Fig. 4.5Z). A similar increase was observed when netrin function was inhibited in U373 cells, but not U343 cells, in which FC density was high in all conditions examined (Fig. 4.5AA and AB, data not shown).

To determine if inhibiting endogenous netrin function influences FA density, images depicting regions of paxillin and zyxin colocalization were generated (Fig. 4.5E. J, O, T, Y). From the 'paxillin and zyxin' images, the density of FAs in each lamellipod was calculated. In U87 (Fig. 4.5AC) and U373 (Fig. 4.5AE) cells, addition of netrin function-blocking antibody resulted in decreased FA density. In all other conditions analyzed for U87 and U373 cells and in all conditions analyzed for U343 cells, no change in FA density was observed.

Notably, the increase in FC density and corresponding decrease in FAs correlates precisely with the changes in motility evoked by disrupting netrin function and measured using the microchemotaxis assay (Fig. 4.1). These data are consistent with a mechanism in which netrin promotes the maturation of FCs into FAs, and that these adhesive structures act to restrain cell movement.

Netrin and netrin receptors are localized to focal adhesions, but not focal complexes

We then investigated the possibility that netrin and netrin receptors might be localized to FCs or FAs and thereby directly influence their maturation. U87, U343, and U373 cells were labeled with the following antibodies: mouse monoclonal anti-paxillin and one of goat polyclonal anti-DCC, or rabbit polyclonal antibodies against netrin or

unc5 homologues. U87 cells were also labeled with goat polyclonal anti-DCC and rabbit polyclonal anti-zyxin (Fig. 4.6). In U87 cells netrin (Fig. 4.6A-C), DCC (Fig. 4.6M-O) and UNC5 homologue (Fig. 4.6G-I) immunoreactivity colocalized with large paxillin-positive foci characteristic of FAs (white arrowhead), but not smaller paxillin-positive structures characteristic of FCs (black arrowhead). In U343 and U373 cells, that lack DCC expression, netrin (Fig. 4.6D-F, P-R) and UNC5 homologue (Fig. 4.6J-L, V-X) immunoreactivity was similarly localized to FAs but not FCs. Consistent with localization to FAs, DCC and zyxin immunoreactivity colocalized in U87 cells (Fig. 4.6S-U). Colocalization with markers of FAs is consistent with netrins and netrin receptors regulating cell-substrate adhesion and motility.

V. DISCUSSION

Here we addressed the hypothesis that netrin may influence tumorigenesis by regulating glioblastoma cell migration. DCC and UNC5 homologues have been proposed to exert an anti-oncogenic effect by acting as pro-apoptotic dependence receptors, triggering cell death in the absence of ligand (reviewed by Mehlen and Fearon, 2004). Our current findings, and previous studies of glial precursor cells, found no evidence of a pro-apoptotic dependence function for netrin or its receptors either *in vivo* or *in vitro*. In contrast, these earlier studies indicated that netrin-1 regulates glial precursor cell migration during embryogenesis (Jarjour et al., 2003; Tsai et al., 2003; Tsai et al., 2006).

We provide evidence for a novel role for netrin as an autocrine inhibitor of tumor cell motility. Our findings indicate that DCC is required for glioblastoma cells to migrate directionally in response to a gradient of netrin-1. Ectopic DCC expression conferred on U343 and U373 cells the capacity to migrate in response to a netrin-1 gradient. DCC expression also slowed their rate of spontaneous migration, consistent with DCC restraining cell movement. The glioblastoma cell lines tested express either *netrin-1* (U343, U373) or *netrin-3* (U87). In the absence of exogenous netrin-1, disrupting endogenous netrin-1 or netrin-3 function dramatically increased the rate of cell movement. This was the case for U87 cells that express DCC and U373 cells that do not, indicating that in addition to DCC slowing glioblastoma cell migration, netrins influence glioblastoma cell motility through a DCC-independent mechanism. Consistent with increasing the rate of cell motility, disrupting endogenous netrin function increased the number of lammelipodial FCs, immature adhesive contacts that are associated with cell movement. Netrin, DCC, and UNC5 homologue immunoreactivity was co-localized with FA but not FC markers, suggesting that netrin may act at the FA itself to promote the maturation of FCs to FAs.

Netrin, focal adhesions, and cell motility

Netrin-1 signaling through DCC directs the organization of F-actin (Shekarabi and Kennedy, 2002), regulating the activation of Rho GTPases, PAK1, MAPK, FAK, and src family kinases (Shekarabi and Kennedy, 2002; Shekarabi et al., 2005; Forcet et al., 2002; Li et al., 2004; Ren et al., 2004; Liu et al., 2004a; Meriane et al., 2004). FAK and Src are

also activated downstream of UNC5 homologues in response to netrin (Tong et al., 2001; Li et al., 2006). Oligodendrocyte precursor cells, which express DCC and UNC5A, but not netrin, respond to a gradient of netrin-1 as a chemorepellent (Jarjour et al., 2003; Tsai et al., 2003). This is compromised by DCC function-blocking antibodies and in DCC knockout mice, consistent with DCC contributing to OP repulsion. Consistent with our finding that autocrine netrin slows glioblastoma cell migration, uniform application of netrin-1 to OPs inhibited cell migration and caused process retraction, a non-directional kinetic effect on motility (Jarjour et al., 2003). Although the precise relationship between netrin-mediated adhesion and netrin's role as a guidance cue directing motility remain to be determined, signaling mechanisms involved in netrin-mediated chemotropism are likely to play a role regulating netrin's influence on FA formation.

FAs are sites of interaction for many proteins (Wozniak et al., 2004). We provide evidence that netrin and netrin receptors are localized to FAs and may restrain cell movement by promoting their maturation. Numerous proteins present in FAs have been implicated in signaling downstream of netrin: FAK, Src, the Ena/VASP proteins (Lebrand et al., 2004), Rho-family GTPases Rac and Cdc42 (Shekarabi and Kennedy, 2002) and the GEF Trio (Forsthoefel et al., 2005). FAK is activated by autophosphorylation that creates a binding site for Src-family kinases. Association with FAK initiates a FAK-Src signaling complex. Extensive tyrosine phosphorylation is a key signaling event observed in focal adhesions, as it is thought to create 'docking' sites for recruitment of SH2 domain-containing proteins required for further signaling events (reviewed by Mitra et al., 2005). Recently, it has been reported that FAK and Src regulate the phosphorylation of UNC5B on multiple tyrosine residues upon netrin binding, and that following these phosphorylation events, Src associates directly with UNC5B via its SH2 domain. Interestingly, this is facilitated by, but does not absolutely require, DCC function (Li et al., 2006). Notably, FAK is required for the maturation of adhesive complexes (Sieg et al., 1999), and, together with Src, is essential for the normal turnover of FAs (reviewed by Mitra et al., 2005).

An emerging role for netrin in adhesion and tissue morphogenesis

The majority of netrin-1 protein is not freely diffusible in the CNS, but is bound to cell surfaces and extracellular matrix (Manitt et al., 2001; Manitt and Kennedy, 2002; Kennedy, 2000). We have shown that DCC binding immobilized netrin-1 mediates cell-substrate adhesion, consistent with a role for netrin mediating cell-cell and cell-matrix interactions (Shekarabi et al., 2005). Recent findings have identified key roles for netrins and netrin receptors during tissue morphogenesis (reviewed by Hinck, 2004), including development of the mammary epithelium (Srinivasan et al., 2003), pancreas (Yebra et al., 2003), lung (Liu et al., 2004b), and during angiogenesis (Lu et al., 2004; Park et al., 2004). Furthermore, overexpression of netrin-1 by cells in the intestinal epithelium of mice led to the formation of focal hyperplasias and adenomas (Mazelin et al., 2004). In this case, although these authors concluded that the phenotype induced is due to netrin-1 reducing cell death, they did not directly address the possibility that disruption of appropriate cell-cell interactions as a result of netrin-1 overexpression could be the primary cause underlying the disorganization of normal epithelial structure.

The models illustrated in figure 4.7 suggest that loss of netrin function may lead to disruption of appropriate cell-cell and cell-matrix interactions. We have provided evidence that in the presence of laminin-1, netrin-1 becomes a repellent for U87 cell migration, and that this requires DCC (Fig. 4.3). Importantly, the combined action of netrin-1 and laminin-1 may influence glioblastoma cell migration *in vivo*. Laminin-1 is restricted to basement membranes and capillary walls in developing and mature CNS (Yip and Yip, 1992; Gordon-Weeks et al., 1992; Hunter et al., 1992). Although deregulated cell migration makes an important contribution to the dissemination of tumor cells within the brain, metastasis of brain tumor cells outside the CNS is rare. Glioma cells are attracted to endothelial capillaries *in vitro* (von Bulow et al., 2001) and glioblastoma cells migrate in close association with capillary walls as they disseminate within the brain (Guillamo et al., 2001). Laminin-1 may facilitate this as it promotes glioma cell migration (Tysnes et al., 1997; Knott et al., 1998). Based on our evidence that laminin-1 biases cells to respond to netrin-1 as a repellent (Fig. 4.3, Hopker et al., 1999), the basal lamina may inhibit the migration of glioma cells expressing netrin-1 and DCC. However, in the absence of netrin function, our findings predict deregulation of this inhibition of migration, leading to laminin-1 in the basal lamina of blood vessels

becoming a permissive substrate that promotes tumor cell migration and dissemination to other brain regions (Fig. 4.7A). Correlated loss of DCC expression with tumor progression suggests that netrin and DCC play an important role in tissue maintenance in adulthood. We propose that appropriate cellular organization may be stabilized by autocrine and paracrine actions of netrin (Fig. 4.7B). Furthermore, loss of effective netrin signaling may disinhibit a mechanism that normally restrains cell migration and in the absence of netrin-mediated inhibition, local cues such as laminin, become potent promoters of migration.

Numerous cell types expressing netrin and netrin receptors in vivo have been described, however an autocrine function for netrin has not previously been reported. We provide evidence that autocrine expression of netrin restrains glioblastoma cell migration. These findings identify a novel netrin function that may contribute to the formation and maintenance of tissue organization, and identify netrin as a potential therapeutic target to inhibit tumor cell migration and dispersion.

Figure 4.1. Glioblastoma cell lines express netrins and their receptors: Endogenous netrin inhibits U87 and U373 cell migration. (A) Western blot analysis of cell lysates or conditioned media from astrocytes (Ast), U343, U373, and U87 cells. Molecular mass markers (kDa) are indicated to the left of each blot. Full-length netrin protein (~75 kDa) was detected in medium conditioned by each glioma cell line or by astrocytes (top panel). A band corresponding to full-length DCC protein (~185 kDa) was detected in whole cell lysates of astrocytes (Ast) and U87 cells, but not U343 and U373 cells. This blot was overexposed to reveal DCC in astrocytes and its absence in U343 and U373 cells. U343 and U373 cells transfected with pDCC-GFP express DCC-GFP chimeric protein, which migrates at a slightly higher molecular weight than endogenous DCC (middle panel). A ~190 kDa band, the molecular weight of full-length neogenin, was detected in lysates of all three cell lines (bottom panel, 30 μg protein/lane). (B) RT-PCR analysis of U87, U343 and U373 cell total RNA. (C) Transfilter microchemotaxis assays of U87, U343, and U373 motility. (D) U87 cell migration increased when 25 μg/ml netrin function-blocking antibody (Net_{FB}) was added to the top and bottom compartments, relative to medium alone (Control), or control antibody (Control IgG). 10 μg/ml DCC_{FB} did not increase migration. (F) Netrin function-blocking antibody (Net_{FB}) significantly increased U373 cell migration, but had no effect on U343 cell migration (E). (G) Schematic diagram of microchemotaxis assay. Number of cells migrated is per 10X objective field. Duration of microchemotaxis assays was 16 hrs. * $p < 0.05$ vs. control.

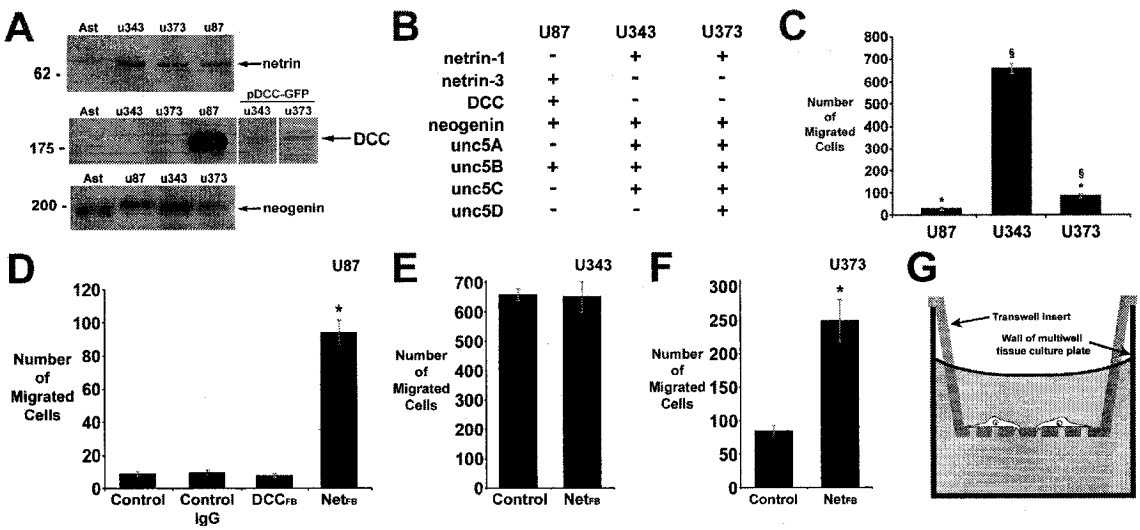

A
Ast u343 u373 u87
62 -
netrin
pDCC-GFP
Ast u343 u373 u87 u343 u373
175 -
DCC
Ast u87 u343 u373
200 -
neogenin
B
U87 U343 U373
netrin-1 - + +
netrin-3 + - -
DCC + - -
neogenin + + +
unc5A - + +
unc5B + + +
unc5C - + +
unc5D - - +
C
Number of Migrated Cells
U87 U343 U373
D
U87
Number of Migrated Cells
Control
Control IgG
DCCFB
NetFB
E
U343
Number of Migrated Cells
Control
NetFB
F
U373
Number of Migrated Cells
Control
NetFB
G
Transwell Insert
Wall of multiwell tissue culture plate

Figure 4.2. Netrin-1 is a chemoattractant for DCC-expressing glioblastoma cells. (A,B) Addition of 100 ng/ml netrin-1 to the bottom compartment (NB) of the transfilter microchemotaxis assay significantly increased U87 cell migration compared to control (medium alone). NB: netrin-1 bottom. NTB: netrin-1 top and bottom. NB DCC_{FB}: netrin-1 bottom, DCC function-blocking antibody. Similar results were obtained in assays lasting (A) 16 hours and (B) 48 hours. (C) A gradient of netrin-1 had no effect on the migration of U343 or U373 cells. (D) U343 cells transfected with a DCC expression construct (U343D control), reduced their rate of migration relative to the parental line (U343P). Increased migration of DCC-transfected U343 cells was evoked by a netrin-1 gradient (U343D NB), but not uniform netrin-1 (NTB). DCC_{FB} blocked this response (U343D DCC_{FB}). (F) Transfection of U373 cells with DCC produced responses similar to U343 cells, which mimic those seen in DCC expressing U87 cells. Number of cells migrated is per 10X objective field. 16 hr assays in all panels except (B). * $p < 0.05$ vs. control (A,B), U343P (D) or U373P (E). § $p < 0.05$ vs. U343D Control (E) or U373D Control (F).

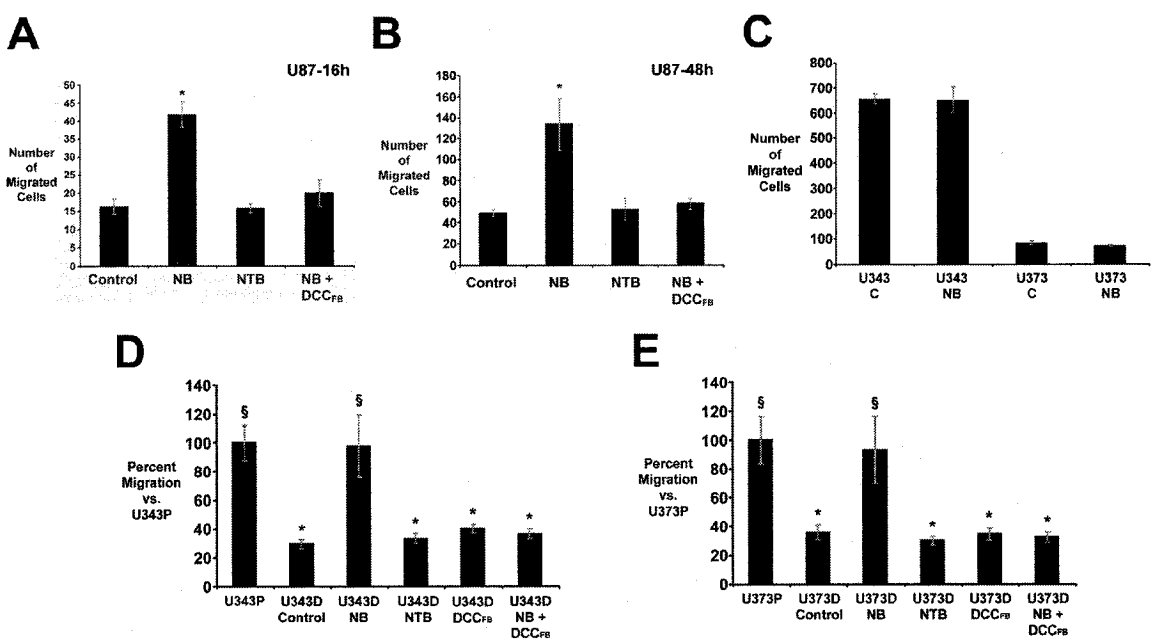

A
U87-16h
Number of Migrated Cells
Control
NB
NTB
NB + DCCFB
B
U87-48h
Number of Migrated Cells
Control
NB
NTB
NB + DCCFB
C
Number of Migrated Cells
U343 C
U343 NB
U373 C
U373 NB
D
Percent Migration vs. U343P
U343P
U343D Control
U343D NB
U343D NTB
U343D DCCFB
U343D NB + DCCFB
E
Percent Migration vs. U373P
U373P
U373D Control
U373D NB
U373D NTB
U373D DCCFB
U373D NB + DCCFB

Figure 4.3. U87 attraction to netrin is converted to repulsion by laminin-1

(A) U87 migration in the microchemotaxis assay challenged with an ascending gradient of laminin-1 (LB) increased relative to control (C). A uniform distribution of laminin-1 (LTB) does not increase U87 migration. An ascending gradient of netrin-1 and uniform laminin-1 (LTBNB), or uniform distributions of both netrin-1 and laminin-1 (LTBNTB), results in reduced U87 migration. Challenging cells with a descending gradient of netrin-1 with a uniform distribution of laminin-1 (LTBNT), evoked increased migration relative to control. Addition of DCC_{FB} to both the top and bottom compartments in the presence of a uniform distribution of laminin-1 and an ascending gradient of netrin-1 (LTBNB DCC_{FB}) or of uniform distributions of both netrin-1 and laminin-1 (LTBNTB DCC_{FB}) blocked the decrease in migration observed. (B) Schematic depicting migratory responses of U87 cells in (A). Migration assayed after 48 hrs. * $p < 0.05$ vs. control.

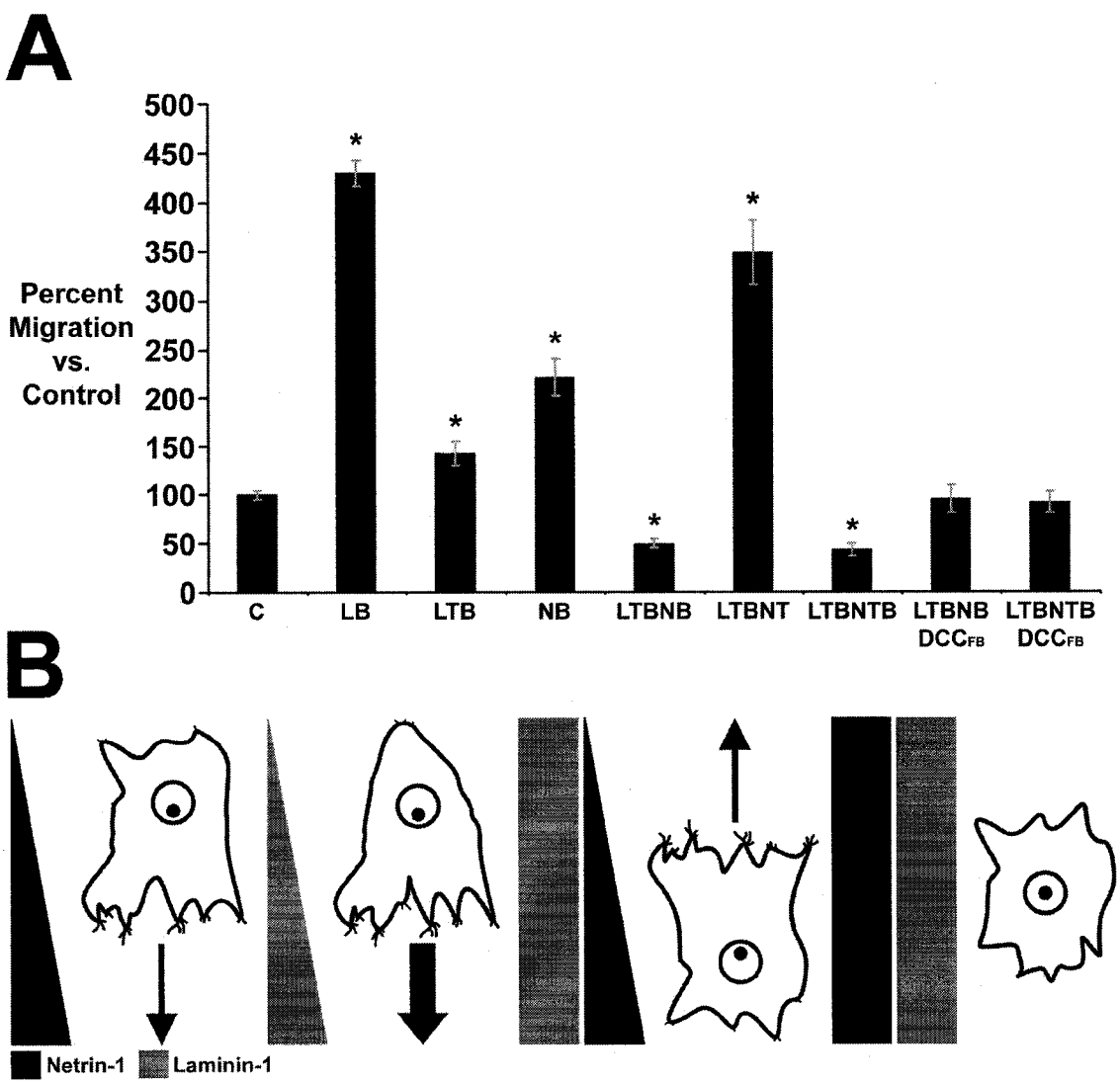

A
Percent Migration vs. Control
500
450
400
350
300
250
200
150
100
50
0
C
LB
LTB
NB
LTBNB
LTBNT
LTBNTB
LTBNB DCCFB
LTBNTB DCCFB
B
Netrin-1
Laminin-1

Figure 4.4. Neither netrin-1 nor laminin-1 influence the survival or proliferation of U87, U343, or U373 cells

(A) Cell viability was assessed by labeling F-actin with Alexa 488-conjugated phalloidin, nuclei with Hoechst, and counting. Addition of netrin-1, laminin-1, or both did not affect U87 cell viability. Neither 25 μg/ml Net_{FB} nor 10 μg/ml DCC_{FB} affected cell number. The number of U343 or U373 cells did not change following addition of 100 ng/ml netrin-1 or 25 μg/ml Net_{FB} (16 hr assay). (B) To further assess apoptotic cell death under the same conditions analyzed in panel A, cell lysates were analyzed by immunoblot for the active (cleaved) form of caspase-3. In all three cell lines, a 17 kDa caspase-3 band (black arrowhead) was only observed in lysates exposed to staurosporine, a potent inducer of apoptosis. The white arrowhead indicates a nonspecific 15 kDa immunoreactive band. (C) To determine if netrin acts as a 'dependence receptor' ligand, cells were treated with antibodies blocking either DCC or netrin function for 48 hours. As in panel B, only staurosporine treatment promoted cell death. Ponceau S staining demonstrates equal loading. Ctrl C control; Lam L laminin-1; Net N netrin-1; Net_{fb} N_{fb} netrin function-blocking antibody; DCC_{fb} D_{fb} DCC function-blocking antibody; LN laminin-1 and netrin-1; ND_{fb} Netrin-1 and DCC_{fb}; LND_{fb} Laminin-1 netrin-1 and DCC_{fb}; R pre-immune rabbit IgG; St staurosporine.

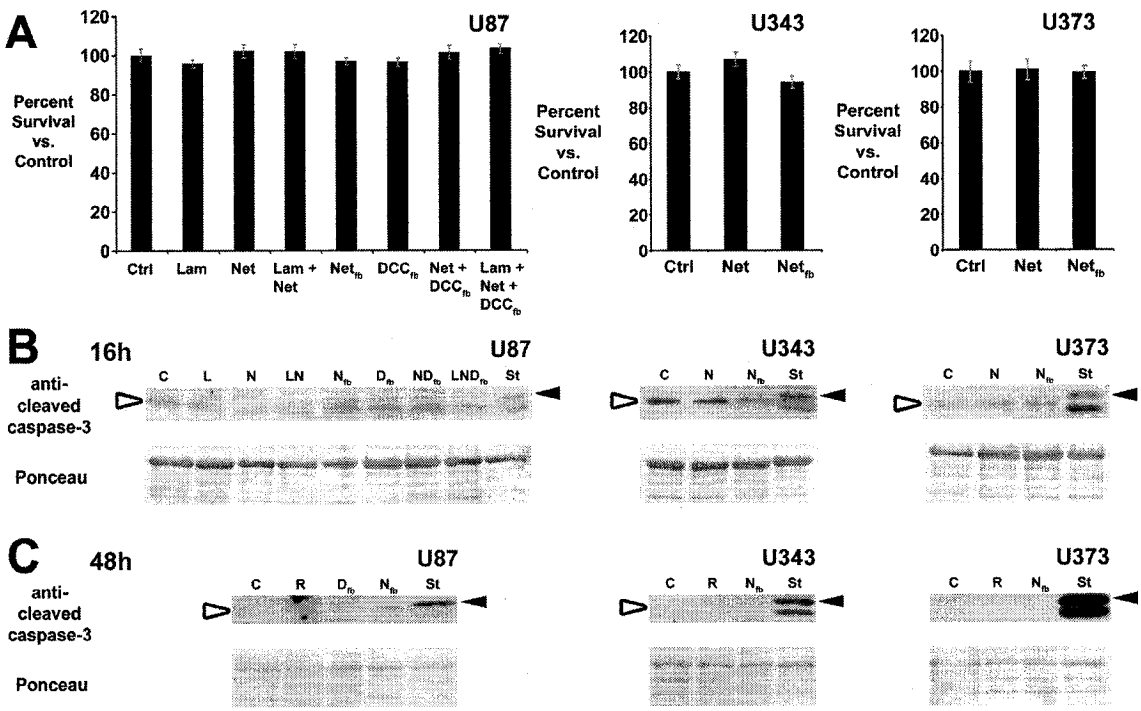

A
U87
U343
U373
Percent Survival vs. Control
120
100
80
60
40
20
0
Ctrl
Lam
Net
Lam + Net
Net$_{fb}$
DCC$_{fb}$
Net + DCC$_{fb}$
Lam + Net + DCC$_{fb}$
B
16h
anti-cleaved caspase-3
Ponceau
C
L
N
LN
N$_{fb}$
D$_{fb}$
ND$_{fb}$
LND$_{fb}$
St
C
N
N$_{fb}$
St
C
N
N$_{fb}$
St
C
48h
R
D$_{fb}$
N$_{fb}$
St
C
R
N$_{fb}$
St
C
R
N$_{fb}$
St

Figure 4.5. Disrupting netrin function increases the number of FCs and reduces the number of FAs in lamellipodial protrusions of U87 and U373, but not U343, cells. (A, F, K, P, U) U87 cells were labeled with antibodies against paxillin (green) and zyxin (red). FCs present in lamellipodia of U87 cells were identified and quantified by subtracting zyxin immunoreactivity (C, H, M, R, W) from paxillin immunoreactivity (B, G, L, Q, V), revealing localization of paxillin without zyxin (D. I, N, S, X). (Z) Density of paxillin+/zyxin- foci. FAs in U87 cell lamellipodia were identified and quantified by generating images of paxillin and zyxin co-localization (E, J, O, T, Y) and determining the density of paxillin+/zyxin+ foci (AC). 25 μg/ml control rabbit IgG (Rb IgG; K-O), 100 ng/ml netrin-1 (P-T) or 10 μg/ml DCC_{FB} (U-Y) resulted in no change in FC or FA density relative to control medium (A-E). 25 μg/ml Net_{FB} (F-J) significantly increased the density of FCs (Z) and decreased FA density (AC). FCs and FAs of U373 cells were similarly affected (AB, AE). FC of FA density was not altered by control antibody, netrin-1, or Net_{FB} in U343 cells (AA and AD). 100x objective, scale bar = 2 μm. * $p < 0.05$ vs. control.

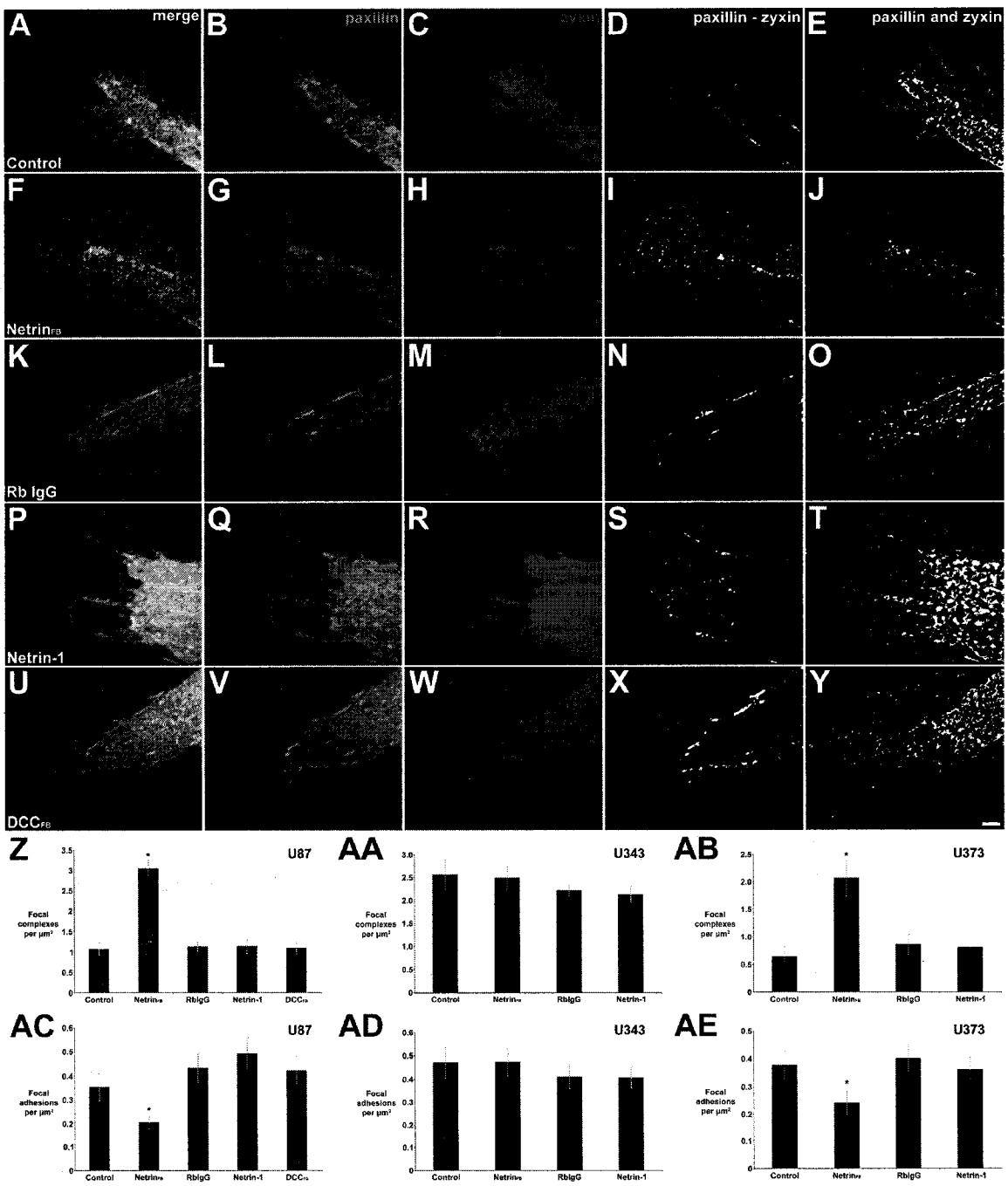

A
merge
B
paxillin
C
zyxin
D
paxillin - zyxin
E
paxillin and zyxin
Control
F
G
H
I
J
Netrin-1FB
K
L
M
N
O
Rb IgG
P
Q
R
S
T
Netrin-1
U
V
W
X
Y
DCCFB
Z
U87
Focal complexes per µm²
Control
Netrin-1FB
RbIgG
Netrin-1
DCCFB
AA
U343
AB
U373
AC
U87
Focal adhesions per µm²
AD
U343
AE
U373

Figure 4.6. Netrin and netrin receptors are localized to FAs but not FCs.

Labeling of U87, U343, and U373 cells with antibodies against paxillin (green) and netrin, DCC, and unc5 homologues (red; all panels except S-U) or zyxin (green) and DCC (red; S-U), and lamellipodia imaged. In U87 cells, small, paxillin-positive FCs localized at the lamellipodial edge were not netrin-positive (black arrowhead). Netrin immunoreactivity co-localizes with larger paxillin-positive structures located away from the lamellipodial edge (white arrowhead), consistent with FAs (A-C). UNC5 homologue (G-I) and DCC (M-O) immunoreactivity were similarly localized to FAs in U87 cells. DCC immunoreactivity also co-localized with zyxin-positive FAs (S-U). Similarly, in U343 and U373 cells, netrin (D-F, P-R) and UNC5 homologue (J-L, V-X) co-localize with FAs, but not FCs. 100x objective, scale bar = 2 μm.

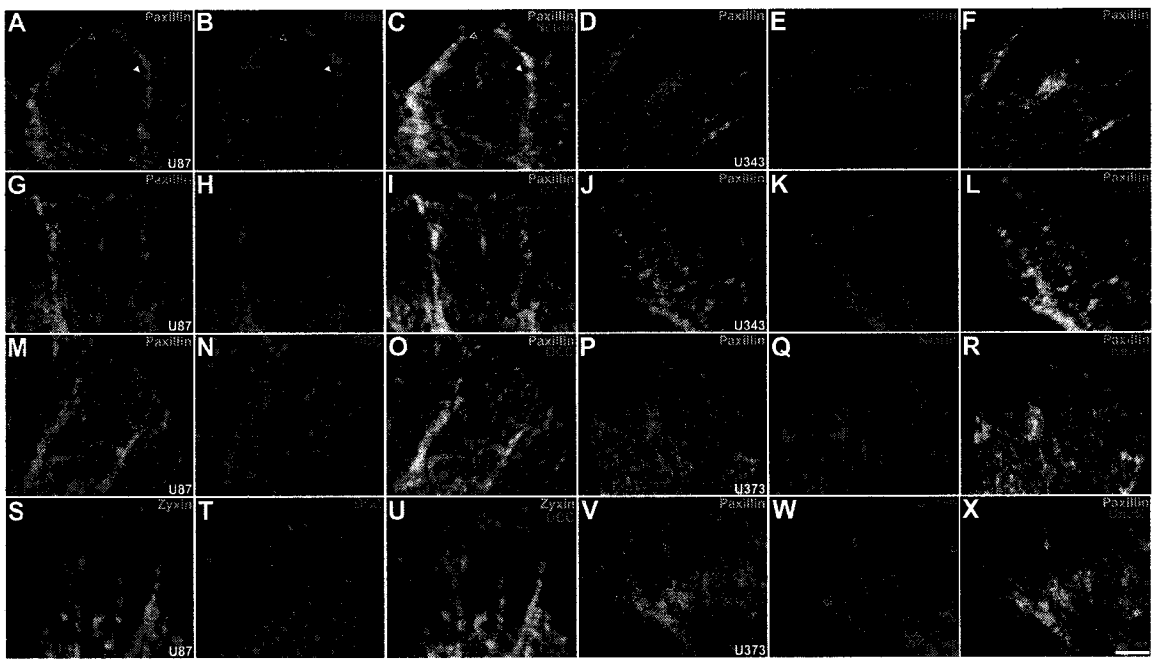
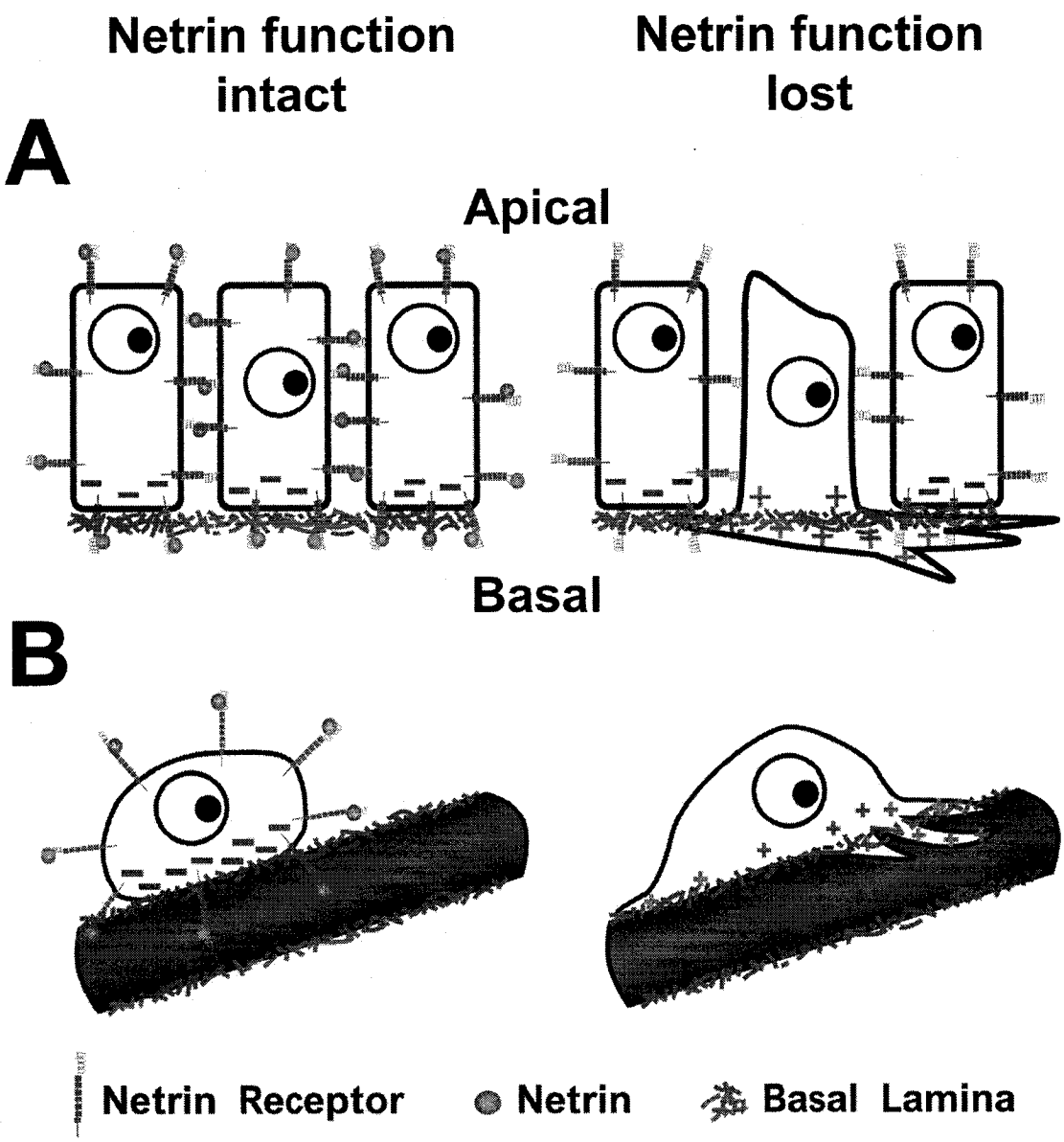

A
Paxillin
U87
B
C
Paxillin
D
Paxillin
U343
E
F
Paxillin
G
Paxillin
U87
H
I
Paxillin
J
Paxillin
U343
K
L
Paxillin
M
Paxillin
U87
N
O
Paxillin
P
Paxillin
U373
Q
R
S
Zyxin
U87
T
U
Zyxin
V
Paxillin
U373
W
X
Paxillin

Figure 4.7. Model of the tumor-suppressive activity of netrin

Model of tumor suppression by netrin *in vivo*. Upon encountering a laminin-1-rich basal lamina surrounding a blood vessel, glioblastoma cells expressing both netrin-1 and netrin receptors (A, left) are inhibited from migrating along the vessel by the combination of netrin-1 and laminin-1. Disruption of netrin signaling allows the cell to respond to laminin-1 as a permissive substrate, and migrate along the surface of blood vessels leading to tumor dissemination in the CNS. (B) We hypothesize that netrin may restrain cell motility and thereby contribute to maintaining the integrity of epithelial or endothelial cell layers in non-neural tissues.


Netrin function intact
Netrin function lost
A
Apical
Basal
B
Netrin Receptor
Netrin
Basal Lamina

CHAPTER 5: GENERAL DISCUSSION

Study of netrin function in the CNS has focused mainly on its role in axon guidance during embryonic development. The objectives of my research were to investigate the role of netrins and their receptors in glial cells. My findings demonstrate that OPs express both DCC and UNC5 homologue netrin receptors, and demonstrate that netrin and DCC are required for the guidance of these cells during their migration from their sites of origin in the ventral spinal cord to the developing spinal white matter (Chapter 2). Using a cerebellar slice culture system, I then investigated the roles of netrin and DCC in myelinating oligodendrocytes. I showed that netrins and their receptors are present at paranodal axo-glial junctions, and that in the absence of netrin-1 or DCC, myelination and the formation of paranodal axo-glial junctions occurs normally, but the junctions destabilize with time (Chapter 3). Finally, I investigated the role of netrin in glial tumor cells, and found that netrins inhibit glioma cell migration through an autocrine mechanism by promoting the maturation of focal adhesions, but do not affect apoptosis, as had previously been reported (Chapter 4). Together, my findings provide evidence for the involvement of netrins in glial function during development, adulthood, and in disease.

I. THE ROLE OF NETRIN-1 IN OP MIGRATION

In Chapter 2, we proposed that netrin-1 is a repellent cue for migratory OPs in the developing spinal cord, and that netrin repels OPs by a mechanism involving cytoskeletal collapse (Jarjour et al., 2003). Multiple investigations by other researchers describing the role of netrin-1 in the guidance of migrating OPs have also been published. While our conclusions and those reached by others are largely similar, discrepancies do exist between the findings.

A. Guidance of OPs by netrin-1: Attractant, repellent, or both?

In the embryonic mouse spinal cord, netrin-1 is produced by floor plate cells immediately ventral to the two foci where OPs originate, and is essential for the normal dispersal of spinal OPs (Jarjour et al., 2003; Tsai et al., 2003; Tsai et al., 2006). A similar repellent role for netrin-1 has been proposed for OPs that populate the optic nerve. These

cells are born at a focal point adjacent to the third ventricle, and migrate via the optic chiasm into the nerve, and in many species including mouse and rat, stop when they reach the retina (Ono et al., 1997b; Small et al., 1987; Gao et al., 2006). Netrin-1 is expressed by cells in the ventricular zone of the third ventricle, at the lateral and anterior edges of the optic chiasm, along the temporal edge of the optic nerve itself, and at the boundary of the retina and optic nerve (Deiner et al., 1997; Sugimoto et al., 2001; Spassky et al., 2002). OPs in both the embryonic spinal cord and optic nerve express both DCC and UNC5 homologue netrin receptors (Jarjour et al., 2003; Tsai et al., 2003; Spassky et al., 2002), which together mediate long-range repellent migratory responses to netrin in many cell types and organisms (Hong et al., 1999; Merz and Culotti, 2000; Keleman and Dickson, 2001), further suggesting that these cell populations are repelled by netrin.

Functional studies carried out both *in vitro* and *in vivo* support this proposed repellent role for netrin-1 as well. A source of netrin-1 repels OPs migrating from explants of either neonatal rat optic nerve or embryonic chick spinal cord (Tsai et al., 2003; Sugimoto et al., 2001) and a gradient of netrin-1 repels OPs in the transfilter migration assay (Tsai et al., 2003; Jarjour et al., 2003). Antibodies that block DCC function abolish the response to netrin-1 in both of these assays (Jarjour et al., 2003; Tsai et al., 2003). Furthermore, OP migration from the ventral to dorsal spinal cord is impaired in mice lacking either netrin-1 or DCC, (Jarjour et al., 2003; Tsai et al., 2006), consistent with netrin-1 being an essential repellent cue for migrating OPs *in vivo.* Thus far, it has not been possible to investigate the role of netrin-1 in regulating OP migration in the optic nerve *in vivo* because both netrin-1 and DCC knockout mice die at birth and the analysis is compromised by optic nerve hypoplasia in these animals (Deiner et al., 1997). However, the observed patterns of netrin-1 and netrin receptor expression and the data from functional studies carried out *in vitro* seem consistent with a scenario in which netrin-1 repels OPs away from the optic chiasm and into the optic nerve, and inhibits their inappropriate migration into the retina. Taken together, these data support a repellent role for netrin-1 in the guidance of OPs in the CNS.

Data regarding this conclusion, however, are not unanimous. Spassky and colleagues (2002) provide evidence that OPs migrating from explants of embryonic mouse optic nerve are attracted by netrin-1 *in vitro*. Their results also appear to suggest

that OPs entering the optic nerve initially express DCC only, allowing them to be attracted into the nerve, but later upregulate UNC5A expression, preventing the cells from entering the retina. One possible reason for the apparent discrepancy between these observations and those of Sugimoto and colleagues (2001) may be that Spassky and colleagues utilized optic nerve explants from transgenic mice in which OPs were identified by expression of proteolipid protein (*plp*). As was described in more detail in Chapter 1, it has been suggested that these cells represent either a separate oligodendrocyte lineage from PDGFαR-positive precursors (Le Bras et al., 2005), or an earlier stage of a common lineage (Richardson et al., 2000). Lineage differences are, however, unlikely to account for these differing migratory responses, because *plp*-positive precursors in the spinal cord are also repelled by netrin (Tsai et al., 2006), though it is possible that spinal and optic nerve *plp*-positive precursors respond differently to netrin.

A more probable explanation is that the discrepancies between the two studies may be the differences in the age of the explanted tissue or the experimental protocols used, as the experiments done by Sugimoto and colleagues (2001) were performed using older tissue; where it could be argued that UNC5A expression has already commenced, resulting in the observed repellent response to netrin. The proposed action of netrin as an attractant cue of optic nerve OPs remains problematic, however, as it is difficult to reconcile with the observation that optic nerve-bound OPs initially migrate away from the third ventricle, an abundant source of netrin-1 expression. While it is not impossible that these cells switch their response to netrin twice, from repulsion to attraction and back to repulsion, further investigation will be necessary for these observations to be reconciled.

B. What mechanism underlies repellent guidance of OP migration by netrin-1?

The leading process extended by an OP is similar in a number of respects to an axonal growth cone (Schmidt et al., 1997). Like axons, F-actin is concentrated at the flared leading edge during migration, and microtubules project from the cell body along the process (Simpson and Armstrong, 1999). Asymmetric collapse has been proposed as a mechanism underlying turns made by axonal growth cones in response to repellent guidance cues. Although a repellent cue can cause the complete collapse of a neuronal growth cone, a local source of the same repellent, such as a microscopic bead coated with

the cue, may only induce partial collapse (Fan and Raper, 1995). In this case, the edge of the growth cone contacting the bead may withdraw, but the growth cone will continue to extend away from the cue (Luo and Raper, 1994). In Chapter 2, I showed that addition of netrin-1 to OPs *in vitro* triggered a rapid and persistent decrease in cell surface area, process length, and process number (Jarjour et al., 2003). This is consistent with the idea that limited collapse of the OP cytoskeleton and withdrawal of OP processes may underlie the repellent response of OP cells in the presence of a netrin-1 gradient, and the decreased motility of OPs in the presence of a uniform concentration of netrin-1 (Jarjour et al., 2003: Chapter 2).

A recent report by Tsai and colleagues (2006) has extended our findings, but suggests a somewhat different mechanism of OPs migration in response to netrin-1. The investigators also observed disrupted OP dispersal in the spinal cords of *netrin-1*$^{-/-}$ mice, but did not observe decreased motility of purified rat OPs in the presence of a uniform concentration of netrin-1 *in vitro*. The exact reasons for the discrepancy between our findings and those of Tsai and colleagues (2006) remain to be determined. A strong possibility is that technical differences between the microchemotaxis assay, where cells are plated on poly-D-lysine-coated polycarbonate membranes and the time-lapse experiments performed by Tsai and colleagues, where cells were plated on glass surfaces, are responsible. It is also possible that the 'collapsed' appearance of the cells in the presence of netrin-1 that we have observed is not typical of netrin-mediated guidance *in vivo*, as Tsai and colleagues (2006) also observed that the absence of netrin-1 expression in murine spinal cord explant cultures resulted in altered cell polarity, but not shape. These experiments did, however, follow the movement of *plp*-expressing OPs, whose responses to netrin-1 may differ from cells of other OP populations.

Studies of other cues directing OP migration show similarly varied effects on the cytoskeleton. Time-lapse studies revealed that OP processes meandering towards stripes of Sema3B and 3C were repelled by partially collapsing the process and branching away from the stripes (Cohen et al., 2003). This is consistent with the hypothesis that a gradient of a chemorepellent factor may direct OP cell migration by triggering limited collapse of the cytoskeleton. Conversely, CXCL1 was not found to alter the morphology of migrating OPs (Tsai et al., 2002), suggesting that CXCL1 halts migration by a mechanism different

from that of the repellent cues. Further *in vitro* analysis suggested that CXCL1 increases the adhesiveness of OPs to the substrate, which may contribute to stopping their migration (Tsai et al., 2002).

C. Does netrin-1 influence remyelination?

Substantial evidence indicates that endogenous OPs have the capacity to proliferate, migrate, and myelinate axons in the adult human CNS (reviewed by Levine et al., 2001). It is not clear why remyelination is inhibited in demyelinating diseases such as multiple sclerosis. Remyelination does occur in early active MS lesions; however, the cellular pathology of a chronic MS lesion includes the loss of oligodendrocytes and myelin, absence of remyelination, and the loss of axons (Prineas et al., 2001). OPs are limited in number and do not appear to migrate a great distance into many demyelinated inactive lesions (Lucchinetti et al., 1999; Chang et al., 2002), though substantial evidence also suggests that in many cases it is an inability to differentiate, and not migrate, that causes remyelination to fail (Wolswijk, 1998; Kotter et al., 2006; reviewed by Franklin, 2002). Netrin-1 protein is abundant in normal adult CNS white matter (Manitt et al., 2001), suggesting that it may be present in myelin debris following demyelination. As oligodendrocyte membranes have been shown to inhibit oligodendrocyte precursor migration (Jefferson et al., 1997), the presence of netrin-1 in myelin debris following demyelination may contribute to the inability of precursor cells to repopulate demyelinated lesions. For this reason, neutralizing the action of netrin and other inhibitors of OP migration may be an important part of future approaches that aim to promote remyelination.

II. NETRIN AND THE ART OF MYELIN MAINTENANCE

Following migration from their sites of origin to developing white matter that is guided, at least in part, by netrin-1 (Jarjour et al., 2003: Chapter 2), DCC and UNC5 homologue-expressing OPs differentiate into oligodendrocytes, establish contact with axons in putative white matter, and myelinate them. During this time, oligodendrocytes themselves begin to express netrin-1. In CNS myelin, netrin is enriched in non-compacted myelin membranes, and netrin and its receptors are enriched at sites of axo-glial contact

at the paranodal junction (Manitt et al., 2001; Manitt et al., 2004). While netrin-1 and DCC are not required for formation and maturation of these structures, TBs disappear and paranodal loops later become disordered in their absence, suggesting that netrin-1 and DCC are required for the maintenance of CNS paranodal junctions (Chapter 3).

A. CNS paranodal maintenance phenotypes

Reports documenting CNS paranodal phenotypes in $CGT^{-/-}$ (Dupree et al., 1998; Dupree et al., 1999; Rasband et al., 2003), $CST^{-/-}$ (Ishibashi et al., 2002), $CGT^{-/-}$:$MAG^{-/-}$ (Marcus et al., 2002), $MAL^{-/-}$ (Schaeren-Wiemers et al., 2004), and $CNP^{-/-}$ (Rasband et al., 2005) mice have described these gene products as being required for the maintenance of paranodal junctions. In all cases, as with the netrin-$1^{-/-}$ and $DCC^{-/-}$ phenotypes, the paranodal loops begin to develop normally and then become progressively disordered. However, the age of onset and severity of disorganization of the paranodal region varies significantly between them.

In mice lacking the expression of CGT, which cannot synthesize sulfatide or galactocerebroside, the vast majority of still-immature paranodes in the spinal cord appear ultrastructurally normal at P15. However, by P23 and later, paranodes become progressively disordered, TBs do not appear, and nfc155, caspr and contactin no longer accumulate at paranodes. Most likely as a consequence of disrupted axo-glial adhesion, K^+ channels become aberrantly localized to the paranode (Dupree et al., 1998; Dupree et al., 1999; Marcus et al., 2002). $CGT^{-/-}$:$MAG^{-/-}$ mice show a more severe phenotype that manifests itself more rapidly than in the CGT single mutants (Marcus et al., 2002), while domain structure of the nodal region is similarly, but less severely, affected in $CST^{-/-}$ mice (Ishibashi et al., 2002). In CGT mutant animals, failure of paranodal junction maintenance likely occurs because lipid rafts cannot form normally because they lack major constituent lipids. The result is that nfc155, and likely other raft-associated oligodendroglial proteins normally present at the paranode, are improperly localized, resulting in the severe paranodal phenotype observed in animals lacking these lipids (Menon et al., 2003; Schafer et al., 2004).

Less severe maintenance phenotypes are observed in MAL and CNP knockout mice. Unlike what was observed in the $CGT^{-/-}$ CNS, nfc155 and caspr clustered at the

paranode and TBs formed in younger MAL mutants, though TBs were diffuse and disorganized and the number of caspr and nfc155-immunoreactive clusters decreased with age. K^+ channel protein also diffused laterally with time, but only in the direction of the internode, suggesting that the 'barrier' function of the paranodes that prevents lateral diffusion of juxtaparanodal complexes remained intact. The appearance of these phenotypes coincided with the expression of MAL, appearing by P16 in cerebellum and by P23 in corpus callosum (Schaeren-Wiemers et al., 2004). A possible explanation for the lesser severity of the $MAL^{-/-}$ phenotype relative to that seen in the $CGT^{-/-}$ CNS is that despite the absence of MAL, the relative abundance of GalC, sulfatide, and other myelin lipids is unchanged, suggesting that raft domains may still form to some extent. Though proteins that MAL would normally play a role in sorting to these domains would be less abundant, the localization of proteins that MAL does not sort would be unaffected. In addition, the absence of MAL resulted in only a 30% decrease in nfc155 protein in paranodal myelin, suggesting that some nfc155 function was retained.

Finally, in $CNP^{-/-}$ mice, the distribution of caspr is normal at 1 month of age, but the caspr-immunopositive domain extends into internodal regions in older animals by 3-4 months of age (Rasband et al., 2005), while normal TBs and only very minor disorganization of paranodes were observed in electron micrographs of $CNP^{-/-}$ CNS paranodes (Lappe-Siefke et al., 2003). The function of CNP at the paranode is unknown, though the maintenance phenotype observed has been hypothesized to be the result of disrupted protein turnover caused by aberrant organization of the microtubule cytoskeleton in $CNP^{-/-}$ oligodendrocytes (Lee et al., 2005a).

The maintenance phenotype that we observed in netrin-$1^{-/-}$ and $DCC^{-/-}$ paranodes differs from those described above in that the junctions destabilize only after normal paranodes with TBs have formed. Unlike what is observed in the absence of GalC and sulfatide (Marcus et al., 2002), neurofascin localization appears unaffected at longer time points, and the changes in caspr localization are subtle. In spite of this, K^+ channels are aberrantly localized to the paranode (though not to the same degree as in the $CGT^{-/-}$ mutant) and, in the netrin mutants, the Na^+ channel domain is widened. That the 'barrier' function of the paranodal junction can be compromised despite the relatively normal distribution of nfc155 and caspr suggests that either nfc155 and caspr-containing

paranodal complexes are insufficient for maintenance of paranodal junctions after a certain stage of maturation in the absence of netrin-1 or DCC, or that netrin-1 and DCC are somehow required for the function of these complexes. However, because very little is known about signaling downstream of nfc155, it is difficult to speculate about potential signaling interactions that may occur between it and netrin receptors. It is possible that both receptors are in close proximity to each other in paranodal membranes, because DCC is also localized to lipid raft domains (Guirland et al., 2004; Herincs et al., 2005). Like nfc155, DCC is palmitoylated in its transmembrane domain, a modification required for its partitioning into rafts (Herincs et al., 2005). Localization of DCC to lipid rafts appears to be ligand-independent. Disruption of raft domains disrupts both netrin-1-mediated commissural axon outgrowth and repellent turning of *Xenopus* spinal neurons (Guirland et al., 2004; Herincs et al., 2005). While it still unknown if netrin function generally requires the presence of intact lipid rafts, these observations raise the possibility that DCC, like nfc155, is mislocalized in $CGT^{-/-}$ mutants. If this were so, it would imply that the functions of netrin-1 and DCC, like those of nfc155 and its axonal binding partners, are compromised in the absence of CGT function.

In addition to the consequences of netrin-1 or DCC loss of function on axo-glial adhesion, we also observe a loss of adhesion between paranodal loops. While many proteins present in adhesive junctions (tight junctions and autotypic adherens junctions) between paranodal membranes in the PNS have been identified, these junctions remain poorly characterized in the CNS. OSP/claudin-11-deficient mice lack tight junctions between oligodendroglial membranes in compact myelin, but the paranodal regions appear ultrastructurally normal (Gow et al., 1999). E-cadherin function is required for autotypic adherens junction formation in PNS, but it is not expressed by oligodendrocytes (Fannon et al., 1995; Menichella et al., 2001; Poliak and Peles, 2003). Based on our observations, netrin-1 and DCC may play a role in regulating the interactions between CNS paranodal loops, though disrupted interactions between loops could also be a secondary consequence of disrupted axo-glial interactions.

B. Do netrin-1 and DCC regulate axo-glial adhesion?

The widespread expression of netrin-1 and its receptors by neurons and glia observed throughout the adult CNS, coupled with the discovery that the vast majority of netrin in the CNS is membrane-associated, led to the hypothesis that netrin-1 may mediate short-range adhesive interactions between neurons, and between neurons and oligodendrocytes (Kennedy, 2000; Manitt et al., 2001; Manitt et al., 2004). The observed netrin-1 or DCC loss-of-function phenotypes at the CNS paranodal junction described in Chapter 3 are consistent with such a role. Netrin-1 and DCC-family receptors have previously been demonstrated to mediate cell-cell adhesion both *in vitro* and *in vivo* in other tissues: Transfection of chicken fibroblasts with a construct expressing full-length DCC promoted cell-cell adhesion, while adhesions between endogenous DCC-expressing dissociated chicken skin epithelial cells or mesenchymal cells in skin explants were disrupted by exposure to antibodies raised against the DCC ECD (Chuong et al., 1994). These adhesive interactions were reported to be heterophilic, associating with an unknown 'receptor', as netrin-1 had not yet been identified as a ligand for DCC.

In vivo, *Drosophila netrin-b* expressed in muscle promotes the formation of neuromuscular synapses, independent of a long-range guidance function (Winberg et al., 1998). The involvement of netrin in synapse formation is particularly interesting, because of the many parallels that have been drawn between synapses and paranodal junctions (reviewed by Poliak and Peles, 2003). In mammals, netrin-1 and the DCC family member neogenin are required to stabilize adhesion between the prelumenal and cap cell layers during the development of the terminal end bud in the mammary gland (Srinivasan et al., 2003). The role of these proteins in cell-cell adhesion was verified *in vitro*: Addition of exogenous netrin-1 protein to cultured neogenin-transfected COS cells, or L1 mouse fibroblast cells, which endogenously express neogenin, also resulted in neogenin-dependent cell aggregation (Srinivasan et al., 2003). Interestingly, these effects of DCC family members and netrin-1 on cell-cell adhesion are likely evolutionarily conserved, as the original characterization of the *C. elegans unc6* and *unc40* mutants by Hedgecock and colleagues (1990) included a description of defects in uterine cell adhesion .

The mechanism by which netrin-1 and DCC may mediate adhesion at the paranode is unclear. Potentially relevant possibilities were raised by Slorach and Werb (2003) in relation to netrin-1/neogenin-mediated adhesion in the mammary gland. The

first is that netrin-DCC binding results in the direct recruitment of an adhesive complex. Alternatively, downstream signaling resulting from netrin-DCC interaction may regulate the expression of required adhesion molecules. A third possibility, inspired by a report from Hiramoto and colleagues (2000), is that DCC may 'present' netrin for binding by receptors on the axonal surface or on neighboring paranodal loops, as has been described to occur during netrin-mediated nerve fasciculation in *Drosophila* (Hiramoto et al., 2000). In all cases, the source of both proteins is likely oligodendroglial, based on the appearance of DCC immunoreactivity both *in vivo* and in our cultures (Figures 3.1, 3.3), and on the observation that netrin is localized to dendrites but not axons of differentiated neurons in culture (Tritsch and Kennedy, unpublished observations). Further investigation will be necessary to determine exactly how netrin-1 and DCC act to maintain the paranodal junction.

C. DCC-independent roles of netrin-1 at the paranode

The increased disorganization of netrin-$1^{-/-}$ paranodal junctions relative to $DCC^{-/-}$ paranodes suggests the involvement of another netrin receptor. UNC5 homologues, particularly UNC5B, are likely candidates because of their high level of expression in oligodendrocytes and localization to the paranodal junction (Figure 3.1, Manitt et al., 2004). In addition to their expression in oligodendrocytes, we detect UNC5 homologue protein on the axons of Purkinje cells in our cerebellar cultures (Figure 3.3), suggesting that netrin may influence axons in our cultures as well. This provides another possible explanation for the more severe phenotype observed in the absence of netrin-1 than in the absence of DCC, which was not observed to be present in axons in our cultures. This also raises the possibility that the role of netrin in paranodal maintenance may vary depending on the type of axon being myelinated.

III. NETRIN: SURVIVAL LIGAND OR MERE CONTEXT-DEPENDENCE?

The role of netrin-1 as a 'dependence receptor' ligand; rescuing DCC and UNC5 homologue-expressing cells from a certain death in its absence, has become accepted as a mechanism of action by netrin-1 and its receptors during development and later in life. (Mehlen and Bredesen, 2000; Bredesen et al., 2004; Arakawa, 2004; Porter and

Dhakshinamoorthy, 2004). It is thus surprising that the data described in Chapter 2 and Chapter 4, and to a lesser extent Chapter 3, appear to be wholly at odds with a trophic function of netrin.

A. Do endogenously-expressed DCC and UNC5 receptors induce apoptosis?

While evidence provided by multiple groups has established that ectopic expression of DCC and UNC5 homologue proteins can induce apoptosis, substantial differences exist between the published reports. While reports by Mehlen and colleagues have repeatedly shown that the presence of netrin-1 can rescue cells from death following ectopic DCC expression (Mehlen et al., 1998; Forcet et al., 2001), investigations by other groups suggest the opposite, that cells die following transfection regardless of whether netrin-1 is present or not (Chen et al., 1999; Shekarabi and Kennedy, 2002).

Accounts of UNC5-induced cell death show similar variation. While reports from Mehlen's laboratory claim that UNC5A, UNC5B and UNC5C can all induce apoptosis in 293T cells with similar efficacy, and that killing requires the death domain (Llambi et al., 2001; Llambi et al., 2005), experiments performed in COS7 cells from the laboratory of Lindsey Hinck suggests that UNC5A is a far stronger promoter of apoptosis than UNC5B or UNC5C, which promote cell death only weakly. Furthermore, Hinck's study suggests that the interaction between NRAGE and a PEST sequence present only in UNC5A is required for apoptosis, and that the death domain is not (Williams et al., 2003a). Perhaps even more confusingly, 293T cells endogenously express *unc5b* and weakly express *netrin-1* (Vereker and Barker, unpublished observations). This observation calls into question the conclusions reached by Llambi and colleagues (2001), who verified the expression of transfected UNC5 protein by immunoblot analysis for the attached hemagglutinin (HA) tag, but did not report what netrins or receptors were endogenously expressed by this cell line. Assuming that equal concentrations of DNA and the same promoter are used for each transfection, the presence of endogenous UNC5B in addition to transfected protein would seem to suggest that UNC5B may in fact possess weaker death-promoting ability than UNC5A or UNC5C. More importantly, the presence of endogenous netrin-1 was insufficient to prevent cell death following UNC5 transfection, in contrast with claims by the authors that netrin-1 is a survival-promoting 'dependence

receptor' ligand.

The discrepancies observed between results obtained by different groups may be due to variances between cell lines, expression constructs, the quantity of DNA used for transfection (which is too often not reported), transfection protocols, or reagents used by different groups. This variance makes it difficult to draw conclusions about how netrin receptors induce cell death, and what role, if any, netrin plays in this process.

The hypothesis that netrin receptors kill in the absence of ligand also implies that no cell that expresses endogenous netrin receptors but not netrin can survive *in vitro* in the absence of exogenous netrin, yet such cells do exist. Cultured rat OPs express DCC, UNC5A, and UNC5B, but not netrin-1 (Tsai et al., 2003; Jarjour et al., 2003: Chapter 2), yet survive in culture. In addition, no increase in viability is observed when these cells are cultured in the presence of purified netrin-1 for three days (Tsai et al., 2006). Liu and colleagues (2002) comment that IMR32 neuroblastoma cells also express high endogenous levels of DCC, but do not die by apoptosis (they imply, but do not state explicitly, that these cells do not express netrin). The authors suggest that killing may not occur because DCC does not interact with DIP13α in these cells, raising the possibility that unbound netrin receptors may only induce apoptosis in cell types containing the necessary apoptotic machinery. This suggests that OP cells may survive in the absence of netrin because they lack the downstream effectors responsible for netrin receptor-mediated killing in other cell types. Inconsistent with this, however, are data obtained using human glioma lines U87 and U373, and the endometrial cancer cell line HHUA. Forced expression of UNC5B induces cell death in U87 and U373 cells (Tanikawa et al., 2003), but when endogenous netrin function is blocked in these cells, the result was increased motility, not death (Chapter 4). While it has also been repeatedly suggested that the tumor suppressive actions of DCC are likely due to its ability to induce apoptosis (Mehlen and Fearon, 2004; Bredesen et al., 2004; Mehlen and Furne, 2005), this conclusion, too, may be questionable. In HHUA cells, which do not express netrin or DCC, forced DCC expression at high levels induces apoptosis while lower level DCC expression does not, yet lower level expression still suppressed tumorigenicity when the cells were transplanted into nude mice (Kato et al., 2000). This suggests that despite the capacity to do so, netrin receptors did not induce apoptosis in the absence of functional

ligand when expressed at lower, more physiologically relevant levels. Taken together, these observations are consistent with netrin receptors inducing cell death only when expressed beyond physiological levels, and call into question the importance of apoptotic regulation in tumor suppressive actions of netrin receptors.

B. Do DCC and UNC5 homologues regulate apoptosis *in vivo*?

Perhaps the most important question regarding the candidacy of DCC and UNC5 homologues as 'dependence receptors' is whether their hypothesized apoptosis-inducing roles are substantiated *in vivo.* If netrin-1 acts as a dependence receptor ligand *in vivo*, it would be expected that massive cell death would be observed in DCC and UNC5 homologue-expressing cells in its absence. While the migration of many axons and cells is aberrant in netrin-$1^{-/-}$ animals, mutant embryos appear grossly normal, suggesting that widespread apoptosis does not occur (Serafini et al., 1996). Decreased numbers of inferior olivary (Bloch-Gallego et al., 1999) and pontine (Yee et al., 1999) neurons in the netrin-$1^{-/-}$ hindbrain have been repeatedly cited as providing support for a 'dependence' mechanism (Bredesen et al., 2004; Mehlen and Thibert, 2004; Mehlen and Furne, 2005). In a recent review article, Bloch-Gallego and colleagues (2005) allude to unpublished data showing that inferior olivary neuron survival is unaffected in DCC mutant animals; consistent with DCC-induced cell death in the absence of ligand. The report by Yee and colleagues (1999), however, appears to have been misinterpreted, as the authors in fact suggest that the reduction in neuron number is a secondary consequence of aberrant migration, because $DCC^{-/-}$ mutant animals show a similar phenotype (Yee et al., 1999). Llambi and colleagues (2001) claim that TUNEL labeling, indicative of apoptosis, is increased in DCC and UNC5B-expressing cells in the hindbrains of netrin-$1^{-/-}$ animals. While a substantial increase in TUNEL labeling intensity is apparent throughout the mutant hindbrain, this includes many regions where neither DCC nor UNC5B is detected. This suggests that the apparent increase in apoptotic labeling may be artifactual. The inability of others to replicate the increased TUNEL labeling reported in netrin-$1^{-/-}$ mice is consistent with this (Lindsay Hinck, personal communication).

Other netrin-1 loss-of-function studies appear to provide evidence that netrin and its receptors regulate motility but do not directly regulate cell survival *in vivo*. We

reported in Chapter 2 that in the netrin-1$^{-/-}$ spinal cord, the distribution of OP cells was aberrant, but the total number of OPs was not significantly different from that seen in netrin-1$^{+/+}$ animals (Jarjour et al., 2003). However, Tsai and colleagues (2006) report that by birth the number of oligodendrocyte lineage cells in the spinal cord of netrin-1$^{-/-}$ mice is substantially reduced compared to wild-type littermates. Significantly, no increase in active caspase-3 or TUNEL-positive cells was observed in the knockout animals, suggesting that the failure of OP migration results in decreased proliferation, not increased apoptosis. The authors further suggest that perturbed axon extension observed in multiple inhibitory interneuron populations in the absence of netrin-1 (Serafini et al., 1996; Saueressig et al., 1999) may have a secondary effect on oligodendrocyte development, which has been shown to be regulated by direct synaptic signaling from hippocampal GABAergic interneurons (Lin and Bergles, 2004). Together, these data suggest that netrin-1 regulates the dispersal of OPs away from their sites of origin in the ventral spinal cord, but it is not necessary for their survival. Similarly, investigation of the role played by netrin-1 in angiogenesis reached a similar conclusion: In zebrafish embryos where *netrin-1a* expression is reduced by injection with antisense morpholino oligonucleotides, vascular morphogenesis occurred abnormally, but no change in cell number was observed (Lu et al., 2004).

Netrin receptor loss-of-function experiments provide additional evidence against a 'dependence' mechanism. While it would be expected that loss of a pro-apoptotic dependence receptor would result in increased cell survival, many cell populations show unchanged or even decreased numbers in the absence of netrin receptors. No increase in OP survival is observed in the spinal cord of DCC mutant mice (Jarjour et al., 2003: Chapter 2). Similarly, in UNC5B$^{-/-}$ mouse embryos and zebrafish embryos where *unc5b* expression was reduced as described above, vascular morphogenesis was aberrant, but no increases in cell survival were observed (Lu et al., 2004). In the developing DCC$^{-/-}$ mouse, the submucosal and pancreatic ganglia are entirely missing from the bowel and pancreas, respectively (Jiang et al., 2003). Additionally, decreased numbers of dopaminergic neurons are observed in the ventral tegmental area and substantia nigra in the brain (T.E. Kennedy, personal communication). In all cases, these observations appear

to weigh against the hypothesized roles of DCC and UNC5 homologues as dependence receptors.

Finally, while such a role might not be entirely surprising for DCC, which mediates chemoattractant responses towards netrin sources, the proposal that UNC5 homologues act as pro-apoptotic dependence receptors generates an immediate paradox. The dependence receptor model suggests that if a cell becomes lost and wanders away from a netrin-secreting target, it will be eliminated by an apoptotic mechanism induced by the absence of ligand (Llambi et al., 2001). However, if an UNC5 homologue appropriately directs a cell away from a source of netrin, the model also suggests that the cell will then be killed due to the absence of ligand. It seems unlikely that an UNC5 homologue would simultaneously function as a repellent guidance receptor and as a pro-apoptotic dependence receptor, mediating a response that leads cells to their death.

Based on these observations, it can be concluded that at the very least the prevalence of DCC and UNC5 death signaling in the absence of ligand has been overstated, and that at most, it operates in some cellular contexts and not others. Additionally, the possibility exists that evidence supporting the roles of DCC and UNC5 homologues as 'dependence receptors' may have limited physiological relevance, and that netrins and their receptors may play but a minor, indirect role in regulating cell survival *in vivo*. This does not, however, preclude the possibility that signal transduction mechanisms regulating apoptosis following netrin receptor overexpression *in vitro* may be involved in signaling downstream of netrin receptors expressed at physiological levels. One such example is provided by Campbell and Holt (2003), who report that caspase-3 activity is required for netrin-1-induced turning of Xenopus spinal neurons independent of cell-death induction. Further investigation, with a particular emphasis on the use of *in vivo* approaches, will be necessary to address these questions.

IV. NETRIN AND TUMOR SUPPRESSION: AN ALTERNATIVE HYPOTHESIS

The data presented in Chapter 4 provides evidence that netrin restrains glioma cell motility through an autocrine mechanism, suggesting that netrin and its receptors may act as tumor suppressors by preventing aberrant migration rather than by regulating apoptosis. These findings also address one of the logical inconsistencies of the

dependence receptor hypothesis: If netrin receptors are tumor suppressors, killing cells that have ventured into environments lacking netrin, inappropriate netrin expression would then be predicted to aberrantly promote survival. In this scenario, genes encoding netrins would be proto-oncogenes, as any mutation resulting in increased netrin expression would be expected to promote tumorigenesis. No such increase of netrin-1 expression in tumor cells has ever been demonstrated. The *netrin-1* gene, like that of its receptors, is inactivated in gliomas and other cancers, consistent with a role as a tumor suppressor (Meyerhardt et al., 1999; Latil et al., 2003). This implies that lost or reduced netrin or netrin receptor function in tumor cells most likely has similar, not opposing, consequences. This is consistent with what would be predicted by our findings, because loss of netrin, DCC, or UNC5 function would be expected to increase motility, though the receptors do not play identical roles.

The findings described in Chapter 4 suggest that netrins slow glioma cell migration, acting in an autocrine manner to influence cell-matrix adhesion. The increased number of focal complexes and corresponding decrease in focal adhesions when netrin function is inhibited implicates netrin in the maturation of adhesive complexes. Netrin, but not DCC, function-blocking antibodies increased cell motility and prevented focal complex maturation in U87 cells, and similar observations were made in U373 cells, which do not express DCC. Thus, it is probable that receptors other than DCC mediate this response to netrin.

Due to their well-characterized roles as netrin receptors and their presence in focal adhesions in the cell lines examined in this study, UNC5 homologues are likely candidates to mediate this response to netrin. However, the involvement of other receptors must be considered as well. $\alpha 6\beta 4$ and $\alpha 3\beta 1$ integrins, which have been reported to mediate adhesion to netrin-1 substrates in pancreatic epithelial cells *in vitro*, are expressed by U87 and U373 cells, respectively (Yebra et al., 2003; Kim et al., 2003; Yamamoto et al., 1997). Integrins are central components of adhesive complexes, and integrin clustering in response to ligand binding is required for the maturation of focal complexes into focal adhesions (reviewed by Wozniak et al., 2004), making them obvious candidates for such a role. The possibility that neogenin may also be involved regulating cell-matrix adhesion and inhibiting glioma cell migration also cannot be excluded, as it is

expressed by all three cell lines and plays a role in netrin-based cell-cell adhesion (Srinivasan et al., 2003). Further investigation will be necessary to identify the receptor or receptors involved.

What, then, has been learned about DCC function as a tumor suppressor from these studies? Perhaps the most important observation is that, as in *Xenopus* retinal ganglion cell neurons (Hopker et al., 1999), the presence of laminin-1 modulates the migratory response to netrin-1, resulting in DCC-dependent repulsion or inhibition of cell migration (Fig. 4.3). Glioma cells migrate as single cells along vascular basement membranes (Nagano et al., 1993; reviewed by Giese and Westphal, 1996), one of the few regions where laminin-1 is present in the adult CNS (Gladson, 1999). While the combined presence of laminin-1 and netrin-1, which is also present on the surface of blood vessels in the adult (Ly et al., 2005) would be inhibitory for the migration of DCC-expressing glioma cells, deletion of the region of chromosome 18q that includes the DCC gene would disinhibit this mechanism, rendering the vascular surface a favorable substrate for migration (Fig. 4.7A). This mechanism may also apply to other cancers. In the adult colon, netrin-1 protein distribution is restricted to the basal face of the cell (Mazelin et al., 2004). The combined presence of netrin-1 and laminin-1 present in the colonic basal lamina (Lohi et al., 1996) may then restrict the motility of DCC-expressing cells in the colon by a similar mechanism (Fig. 4.7B). This is consistent with and may explain the observations that lost or reduced DCC expression correlates with progression to more invasive phenotypes both in gliomas and in colon cancer (Reyes-Mugica et al., 1997; Aschele et al., 2004).

Finally, the possibility that DCC is involved in autocrine inhibition of glioma cell motility cannot be excluded. DCC is present in focal adhesions (Fig. 4.6) and the AF5 DCC function-blocking antibody used does not block either netrin-DCC binding or DCC-mediated adhesion of commissural neurons to a netrin-1 substrate (Keino-Masu et al., 1996; Shekarabi et al., 2005), suggesting that it may inhibit some functions of netrin but not others.

V. INTRACELLULAR SIGNALING RESPONSES TO NETRIN IN GLIA

While intracellular signaling downstream of netrin receptors has become better understood in recent years, the vast majority of what is known has been learned from studies of axon guidance. These signaling molecules are also likely to also function downstream of netrin in the contexts of OP migration, axo-glial adhesion, and glioma cell migration. In this section, I will use knowledge of netrin function in axon guidance to speculate about the signal transduction underlying the various roles that netrin plays in glial cells.

A. Repellent OP migration

In the embryonic spinal cord, netrin-1 secreted by the floor plate acts as a long-range cue, attracting commissural axons ventrally while repelling spinal accessory motor neuron (SACMN) axons and OP cells dorsally (Serafini et al., 1996; Jarjour et al., 2003: Chapter 2; Tsai et al., 2003; Dillon et al., 2005). Genetic evidence from *C. elegans* and *Drosophila*, as well as experiments studying turning responses of *Xenopus* spinal neurons, identified UNC5 as being required for netrin-mediated repulsion, and have implicated both DCC/UNC40/Frazzled and UNC5 in long-range repellent axon guidance (Colavita and Culotti, 1998; Hong et al., 1999; Keleman and Dickson, 2001). SACMNs and OPs express DCC and UNC5 netrin receptors, and both require DCC, as well as netrin-1, for their long-range guidance (Serafini et al., 1996; Jarjour et al., 2003: Chapter 2; Tsai et al., 2003; Dillon et al., 2005).

The data described in Chapter 2 suggests that netrin-1 requires DCC to repel OPs. Both the netrin-induced repellent response and decrease in cell surface area, process length, and process number were dependent on DCC function. DCC function has been hypothesized to be required for long-range repulsion in response to netrin gradients (Keleman and Dickson, 2001). DCC facilitates tyrosine phosphorylation of the UNC5 ICD by Src and FAK and its physical association with Src (Li et al., 2006), and studies in *C. elegans* have directly implicated Src in UNC5-mediated UNC6/netrin-directed repellent migration (Lee et al., 2005b). Src and FAK are also required for signaling downstream of DCC in the context of attractant guidance (Li et al., 2004), suggesting that additional, as-yet unidentified signals are also necessary to specify repulsion.

Due to their ubiquitous involvement in remodeling of the cytoskeleton during cell migration (reviewed by Raftopoulou and Hall, 2004), the likely downstream effectors of netrin-induced cytoskeletal collapse in OPs are the rho family GTPases. It has been hypothesized that attractive guidance cues activate rac1 and cdc42 while inhibiting rhoA, and that repellent cues do the opposite (Dickson, 2001). Consistent with this, rac1 and cdc42 are activated downstream of DCC and their activity is necessary for DCC-induced neurite outgrowth in a neuroblastoma cell line (Shekarabi and Kennedy, 2002). Inhibition of rhoA function potentiates the ability of DCC to induce neurite outgrowth (Li et al., 2002b), consistent with the possibility that DCC-mediated attraction to netrin involves rhoA inactivation.

The roles of rho GTPases in netrin-mediated repellent cell migration have not been established. Evidence obtained from studies of attractant guidance of precerebellar neurons (PCNs) by netrin-1 further suggest that while the roles of rho GTPases in cell migration and axon guidance may be similar, they are not identical. Rac1 and cdc42 activity were required for neurite outgrowth but not PCN migration, while inhibition of rhoA or ROCK promoted axon outgrowth, but inhibited PCN migration due to impaired nucleokinesis (Causeret et al., 2004). These findings suggest that process extension and cell body translocation are independently regulated in response to netrin-1, and that the activation and inhibition of the particular rho GTPases required for each may vary.

During the migration of bipolar OPs, the movements of the leading process, the cell body, and the trailing process must be coordinated. While the mechanisms responsible for netrin-mediated repellent guidance of cell migration likely differ from those controlling attractant responses, nucleokinesis must still occur, implying the possible involvement of rhoA. RhoA may also regulate cytoskeletal rearrangements within OP processes during repellent OP guidance. In *in vitro* experiments employing Xenopus spinal neurons, gradients of LPA, applied at concentrations insufficient to induce complete growth cone collapse, induced partial collapse, resulting in repellent turning away from the LPA source (Yuan et al., 2003). If rhoA is activated downstream of UNC5 and DCC in OPs, exposure to a netrin gradient would result in asymmetric rhoA activation across the cell. Additionally, it has been hypothesized that the distribution of rhoA activation is inverse to that of cdc42 and rac1 during cell migration (reviewed

byRaftopoulou and Hall, 2004). If this were also so, the result of exposure to a netrin-1 gradient would be that rho activity would be highest, and rac1 and cdc42 activity lowest, in regions of the cell exposed to a higher netrin-1 concentration, resulting in increased cytoskeletal collapse. Simultaneously, less rhoA activity and greater cdc42 and rac1 activity would result in decreased collapse in regions of the cell surrounded by a lower netrin-1 concentration. The result would be polarization of the cell and net displacement away from the netrin-1 source.

However, data obtained about the migration in response to class 3 semaphorins, which also repel OP migration through a mechanism involving cytoskeletal collapse (Sugimoto et al., 2001; Cohen et al., 2003) argue against such a neat division of rho GTPase function. Sema3 family members require rac1 to induce collapse in neuronal growth cones. Cdc42 and rhoA have also been implicated in sema3 signaling, but the reports describing their roles conflict (Jin and Strittmatter, 1997; Kuhn et al., 1999). Further investigation of the roles of rho GTPases in repellent guidance of cell migration by netrin will be necessary to understand how each contributes.

While most spinal OPs migrate dorsally from their sites of origin in the ventral spinal cord, a minority migrate ventrally into regions containing high concentrations of netrin-1, suggesting that OPs are not always repelled by netrin. By impinging on these pathways, factors present at the ventral midline may alter or silence the response of OPs to ventrally-enriched repellent guidance cues. Such a modulatory factor enriched at the ventral midline might cause OPs to become unresponsive or even attracted to otherwise inhibitory cues.

Various lines of evidence have demonstrated that cells can switch their responses to netrin. Findings in *Xenopus* spinal neurons suggest that the major determinant of the direction of growth cone turning towards or away from netrin is the intracellular ratio of cyclic AMP (cAMP) to cyclic GMP (cGMP) activity, where higher ratios result in turning towards a source of netrin-1, while lower ratios result in turning away from a netrin-1 source (Nishiyama et al., 2003). Increases in intracellular cAMP activate PKA, and result in increases in the abundance of cell surface DCC, promoting attractant responses to netrin-1 (Bouchard et al., 2004). Similarly, PKC and PICK1 increase the concentration of UNC5A at the cell surface (Williams et al., 2003b). The net response of cells expressing

both DCC and UNC5 receptors has been hypothesized to depend on a balance between DCC and UNC5 signaling, suggesting that any factor that either increases cAMP levels, or decreases PKC activity may play such a role. Another possibility is that a signal present in ventralmost white matter silences signaling responses to netrin. Candidates for such a role are the slit proteins, which bind robo family receptors, and are expressed at the ventral midline in the brain and spinal cord (Long et al., 2004). Binding of slits to roundabout (robo) receptors have been shown to result in the association of the C-termini of robo and DCC, resulting in the silencing of attractant responses to netrin (Stein and Tessier-Lavigne, 2001). It is not yet known, however, if repellent responses to netrin are silenced by slits, if oligodendroglial cells express robo proteins, or even if, in general, the mechanisms known to modulate the response of neuronal cells to netrin operate in oligodendroglia.

Another possible explanation for this apparent ability of certain OPs to migrate against a gradient of an ostensibly repellent cue is the heterogeneity that exists among OPs in the developing spinal cord, as sub-populations of OPs may respond differently to the guidance cues in their extracellular environment. This is unlikely, however, to account for the presence of OPs in the ventral-most regions of the cord, as PDGFαR-positive OPs are clearly visible in the ventral commissure at E15 (Fig. 2.1, 2.6), prior to the appearance of the vast majority of the second, dorsally-originating OP population. Additionally, most dorsally-originating OPs migrate into lateral, and not ventral regions of the cord (Cai et al., 2005; Vallstedt et al., 2005).

B. Paranodal adhesion

Current knowledge of intracellular signaling molecules acting downstream of cell surface proteins responsible for paranodal axo-glial adhesion, particularly within myelinating glia, is extremely limited. One of the few such proteins identified is the src family tyrosine kinase fyn, which co-fractionates with nfc155 from CNS tissue, suggesting that it is also present at the paranode. Fyn is localized to lipid raft domains in oligodendroglia, and like nfc155, its distribution is aberrant in $CGT^{-/-}$ mutants (Kramer et al., 1999; Menon et al., 2003; Colognato et al., 2004). Recently, it has been demonstrated that in commissural neurons, src family kinases phosphorylate the DCC ICD following

netrin-1 stimulation. Specifically, phosphorylation of Y1418 of the DCC ICD requires fyn, and is necessary for netrin-induced Rac1 activation and neurite outgrowth, and attractant turning of *Xenopus* retinal ganglion cell axons (Meriane et al., 2004). In addition, evidence from our own laboratory suggests that fyn and DCC associate in cultured oligodendrocytes (Rajasekharan and Kennedy, unpublished observations). Fyn also regulates the activation of rho GTPases downstream of integrins in oligodendrocytes and regulates oligodendrocyte process extension (Wolf et al., 2001; Liang et al., 2004). While the relation between oligodendrocyte process extension and paranodal adhesion is unclear, the rho family GTPases, particularly rac1 and cdc42, have been shown to promote cell-cell adhesion in response to cadherin binding (reviewed by Jaffer and Chernoff, 2004). While any suggestion that rac1 and cdc42 play such a role in netrin/DCC-mediated adhesion remains highly speculative, it is not unreasonable to suggest that a similar mechanism may underlie netrin and DCC function at the paranode.

Another possible role of rac1 and cdc42 at the paranode may be to regulate protein trafficking. In cultured neurons, cdc42 and its binding partner N-WASP are required for reorganization of the cortical actin network during exocytosis (Gasman et al., 2004), while rac1 has been reported to regulate exocytic membrane fusion (Humeau et al., 2002). Trafficking of myelin components is important for the maintenance of paranodal junctions, as indicated by the phenotype observed in animals lacking MAL (Schaeren-Wiemers et al., 2004). Impeded protein transport as a result of disruptions to the microtubule network may also underlie the paranodal phenotype observed in $CNP^{-/-}$ mice (Rasband et al., 2005; Lee et al., 2005a). Rac1 and cdc42 activity downstream of netrin-1 and DCC function may be required for the proper delivery of proteins and lipids necessary to maintain axo-glial adhesion.

Further insight into possible signaling interactions at the paranode can be gained by studying septate junctions in *Drosophila*. Septate junctions are points of cell-cell contact between epithelial cells, between axons and glia, and between glia which are believed to be evolutionarily related to paranodal junctions because of the many similarities that exist between them (reviewed by Bhat, 2003; Banerjee et al., 2006). Like paranodal junctions, they are characterized ultrastructurally by electron-dense structures similar to TB, and by the presence of *Drosophila* homologues of paranodal proteins caspr

(called neurexin-IV), contactin, and neurofascin (called neuroglian). All are localized to septate junctions and are required for their organization (Baumgartner et al., 1996; Faivre-Sarrailh et al., 2004; Genova and Fehon, 2003).

One of the few proteins required for *Drosophila* septate junction organization whose mammalian homologue has not yet been associated with a function at the paranode is scribble. Scribble is a large plasma membrane-associated protein involved in the regulation of cell polarity in *Drosophila* epithelial cells. It is localized to basolateral membranes and is required for the formation of septate junctions (Bilder and Perrimon, 2000; Bilder et al., 2003). In *circletail* mice, which lack functional mammalian scribble homologue scrb1, polarity defects are observed in planar cells of the cochlea, suggesting that scribble proteins may also regulate cell polarity in mammals (Murdoch et al., 2003; Montcouquiol et al., 2003). Scrb1 has been reported to form a complex with the rac1 and cdc42 GEF βPix and its binding partner GIT1. Association with scrb1 is required for membrane localization of βPix and promotes its association with Rho GTPases (Audebert et al., 2004).

The possible involvement of scribble proteins, and by extension βPix, in paranodal organization is of particular interest because recent findings in our laboratory suggest that βPix physically associates with DCC, and can increase its localization to lipid raft domains (Rodrigues and Kennedy, unpublished observations). DCC function can be influenced by mobilization of the receptor to the cell surface from an intracellular vesicular pool, which enhances DCC-mediated axon outgrowth to netrin-1 (Bouchard et al., 2004). The scrb1-βPix-GIT1 complex has been shown to regulate trafficking of the thyrotropin receptor in neurons by inhibiting receptor endocytosis and promoting its recycling upon ligand binding, resulting in increased presence of the receptor at the plasma membrane (Lahuna et al., 2005). It is not unreasonable to speculate that if scrb1 functionally interacts with DCC, the complex may similarly regulate DCC externalization. The potential influence of the scrb1-βPix-GIT1 complex on DCC function at the paranode may then involve both regulating the presentation of DCC at the plasma membrane, and promoting cdc42 and/or rac1 activation in response to netrin-DCC binding.

The significance of the possible involvement of scrb1 in DCC signaling would likely extend beyond cell-cell adhesion at the paranode. *Scrb1* has been identified as a putative tumor suppressor. As has been reported for *dcc* (Enomoto et al., 1995; Kersemaekers et al., 1999; Saito et al., 1999), *scrb1* expression is down-regulated in cancers of both the colon and cervix. Loss of *scrb1* expression also correlates with tumor progression, as its expression is most commonly lost in regions where epithelial cells lack polarity and tissue architecture is disorganized (Nakagawa et al., 2004; Gardiol et al., 2006). While it must be emphasized that any support for this connection between DCC and scrb1 function is entirely circumstantial at present, the implications of such an interaction would be significant. If scrb1 were shown to be involved in signaling events downstream of netrin and DCC, it could provide insight into the mechanisms of DCC-mediated cell-cell adhesion and tumor suppression, which may be one and the same.

Far more difficult to predict is the possible role played by UNC5 homologues at the paranode. As opposed to DCC family members, no evidence for an adhesive function of UNC5 homologues has been reported, though the ICD of UNC5 homologues contains a conserved ZU5 domain also present in the tight junction protein zona occludens-1 (ZO-1) (Itoh et al., 1997) and ankyrins 1-3. While very little is known about the function of the ZU5 domain in UNC5 homologues, it has been shown to function as a spectrin-binding domain in ankyrins. While one (unspecified) human UNC5 homologue tested did not bind β2-spectrin (Mohler et al., 2004), this observation hints at a possible role for the ZU5 domain in linking the UNC5 homologue ICD with the cytoskeleton. More precise localization of UNC5 protein at the paranode and UNC5 homologue loss-of-function studies are necessary to firmly establish a role for these proteins at the paranode.

C. Inhibition of glioma cell migration by netrin

Inhibition of netrin function in glioma cells increased their rate of migration, increasing the number of focal complexes and decreasing focal adhesions (Chapter 4), suggesting that autocrine or paracrine netrin stimulation promote the maturation of focal adhesions. This function of netrin does not require DCC, as application of a DCC function-blocking antibody had no effect on U87 cell migration, and was also observed to occur in U373 cells, which do not express DCC.

As described above, the rho GTPases are candidate downstream effectors of changes in cell-matrix adhesion regulated by netrin signaling. Data describing the function of rhoA in cell-matrix adhesions suggests parallel function with netrin in this context. Injection of Swiss 3T3 fibroblasts with constitutively active RhoA promoted the maturation of focal complexes into focal adhesions. Conversely, inhibition of the rhoA effector rho kinase (ROCK) results in decreased focal adhesion formation and a corresponding increase in focal complex number, reminiscent of what was observed following netrin inhibition and establishing a role for rhoA in the maturation of adhesive complexes (Rottner et al., 1999).

The observed presence of UNC5 homologue receptors in focal adhesions in both U87 and U373 cells (Fig. 4.6) raises the possibility that they may mediate this response to netrin. In this case, a possible signaling intermediary would be src, which can activate rhoA (Berdeaux et al., 2004). If such a mechanism is operant, blocking netrin function would decrease src activity downstream of UNC5 homologues, decreasing rhoA activity and the maturation of focal complexes into focal adhesions. However, activation of rhoA in response to src transfection was fairly weak, and other studies suggest that src acts within focal adhesions to phosphorylate and activate p190rhoGAP, which inactivates rhoA (Brouns et al., 2001; Volberg et al., 2001). If rhoA is activated downstream of UNC5 homologues, other signaling proteins would most likely be involved.

Another mechanism by which netrin may regulate cell-matrix adhesion is by directly binding integrins, as the α6β4 and α3β1 integrins have been proposed to act as netrin receptors in pancreatic progenitor cells (Yebra et al., 2003). The particular integrin recruited to focal complexes is dictated by the substrate encountered by the cell (Fath et al., 1989), suggesting that a cell encountering netrin could recruit α6β4 or α3β1. While short-term engagement of integrins by a substrate-associated ligand results in a transient decrease in rhoA activity, prolonged binding results in rhoA activation, resulting in the maturation of adhesive complexes (Ren et al., 1999). This has been directly demonstrated to occur downstream of α6β4 integrin (O'Connor et al., 2000), though β1 integrins appear to inactivate rhoA (Miao et al., 2002), suggesting that if netrin regulates cell-matrix adhesion through integrins, integrin-mediated effects may vary depending on cell type.

The role played by DCC in these cells remains unclear. DCC transfection decreased the motility of U343 and U373 cells (Fig. 4.1), yet the DCC function-blocking antibody AF5 did not increase U87 cell motility or influence adhesive complexes (Fig. 4.3, 4.5). This lack of an effect of the AF5 antibody does not entirely exclude DCC from having a possible role in autocrine/paracrine inhibition of motility by netrin, because it does not block the physical association of netrin and DCC, or DCC-mediated commissural neuron adhesion to a netrin substrate (Keino-Masu et al., 1996; Shekarabi et al., 2005). Use of other strategies to inhibit DCC function will be necessary to address this question.

A second possibility is that DCC expression influences cell-matrix adhesion indirectly by regulating gene expression. DCC can be proteolysed by a γ-secretase-like cleavage in N2a neuroblastoma cells, releasing a C-terminal fragment that could activate transcription of a reporter gene, suggesting that it may translocate to the nucleus (Taniguchi et al., 2003). While the physiological relevance of these observations is debatable, ectopic expression of DCC protein lacking the cytoplasmic domain in another neuroblastoma cell line, SJNB-8, resulted in decreased N-cadherin and α- and β-catenin mRNA and protein, resulting in diminished cell adhesion (Reyes-Mugica et al., 2001). If this indeed occurs physiologically, regulation of gene expression would provide another possible mechanism of tumor suppression by DCC.

CONCLUSIONS AND FUTURE DIRECTIONS

The data that I have described here represent the initial characterizations of three novel roles for netrins and their receptors in glial cells. A broad conclusion that can be drawn from my findings is that the role of netrin in the CNS changes dramatically with age. Netrin-1 is expressed at restricted regions of the embryonic CNS, where it guides motile axons and neuronal and glial cells. Conversely, in the adult CNS, netrin-1 is widely expressed in neurons and glia, promotes cell-cell and cell-substrate adhesion, and inhibits inappropriate motility.

Further investigation will be necessary to better understand these phenomena. While the UNC5 homologues' involvement in OP migration and in paranodal function has been inferred, it remains to be demonstrated directly. The function of UNC5 proteins will be more easily investigated as better reagents become available. The receptors that mediate autocrine inhibition of glioma cell migration by netrin – likely UNC5 homologues or integrins - also remain to be identified. Cultured OP cells are a potentially useful tool for studying signal transduction events activated by netrin in the context of repulsive migration, which are currently poorly understood. Similarly, the U87 and U373 glioma cell lines, which respond differently to netrins, can be used to investigate signaling events downstream of chemoattractive cell migration to netrin, and in netrin regulation of adhesive complexes. As netrins and their receptors have been implicated in many other types of tumors, examining the influence of netrin on the motility and adhesive properties of cells derived from colon or mammary tumors, for instance, would be informative. The generation of inducible *netrin-1*, *dcc*, or *unc5 homologue* mutant mice would be particularly useful for studying netrin and netrin receptor function in cancer, as it would circumvent the issue of neonatal lethality that has prevented the *in vivo* study of netrin function in the adult to date.

Such mouse strains would also be powerful tools for understanding netrin function at the paranode *in vivo*. If the observed phenotypes are similar to what was observed in cerebellar slice cultures, these strains could potentially be used to determine what comprises TBs. Since TBs are initially formed and then disappear in netrin and DCC mutants, a possible approach would be to purify axo-glial junctions from knockout mice

at stages of maturation before and after the appearance of junctional maintenance phenotypes, and then perform a differential proteomic analysis of the junctional components present.

A deeper understanding of how netrin function evolves with age and the underlying signaling events involved may be important in multiple disease-related contexts. In some cases it may be desirable to promote cell motility, designing strategies to increase OP migration in order to remyelinate demyelinated axons in multiple sclerosis or following CNS injury. In others, the objective would instead be to inhibit motility, preventing the devastating spread of glioblastoma cells throughout the brain, or to promote cell-cell adhesion, stabilizing repaired myelin sheaths. Further investigations stemming from these findings should result in a better understanding of both netrin and glial function.

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