A ROLE FOR UNC5 HOMOLOGUES IN AXON GUIDANCE AND BRANCHING IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM

Mohammed A. Ashour

Department of Neurology and Neurosurgery

Montreal Neurological Institute & Hospital

McGill University, Montreal, Quebec

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ABSTRACT

The proper wiring of the central nervous system is a crucial step for normal biological development. Netrin-1 belongs to a family of bifunctional guidance molecules that play a role in navigating growing axons either towards (chemoattraction) or away (chemorepulsion) from specific targets. There are four UNC5 homologues in mammals, which are related to the product of the *Unc*oordinated-5 gene, originally identified in the nematode worm *C. elegans*. These homologues bind netrin-1 and are thought to mediate chemorepulsion by forming a heterodimer with another netrin-1 receptor, DCC. It is not clear, however, whether mammalian UNC5s repel axons in an exclusively DCC-dependent fashion, or if chemorepulsion is possible in the absence of DCC. Here, we provide evidence that cortical neurons obtained from DCC knockout mice are capable of turning away from a gradient of netrin-1, indicating that DCC is not required to mediate chemorepulsion in response to a gradient of netrin-1. Furthermore, we explore a role for UNC5A in vivo vis-à-vis examining mossy fiber sprouting in the hippocampus, and provide preliminary data suggesting that the loss of UNC5A function in knockout mice does not result in enhanced mossy fiber sprouting in mice up to at least four months of age. Taken together, these novel findings contribute to our growing understanding of the function of the UNC5 protein family, and the role it plays in the development and maintenance of the mammalian central nervous system.

RESUMÉ

Le câblage approprié du système nerveux central est une étape cruciale pour le développement biologique normal. Comme membre d'une famille des molécules qui donne les directives à les axones qui dirigez leur environment, netrin-1 joue un rôle bifonctionnelle, alors qui il peut causer les axones croissant d'aller vers (chemoattraction) ou de s'éloigner (chemorepulsion) des cibles spécifiques. Un des récepteurs de netrin-1, Uncoordinated-5 (UNC-5), à l'origine identifié dans le nématode *C. elegans*, peut négocier le chemorepulsion en formant un heterodimer avec un autre récepteur de netrin-1, DCC. Bien qu'un rôle semblable ait été décrit pour les homologues mammifères d'UNC5 (UNC5A-D), il n'est pas clair s'ils repoussent des axones d'une mode DCC-dépendante, ou si le chemorepulsion est possible en l'absence du DCC. Ici, nous fournissons l'évidence qui les neurones corticaux obtenus des souris qui manque le gène DCC sont capables de s'éloigner d'un gradient de netrin-1, indiquant que le récepteur DCC n'est pas nécessaire pour le chemorepulsion négocier par les UNC5s en réponse à un gradient de netrin-1. En plus, par investiguer le rôle du UNC5A dans la régulation de croissance des fibres moussue du hippocampe, nous explorons un rôle *in vivo* pour le récepteur UNC5A. Nous fournissons des données préliminaires proposant que jusqu'à quatre mois d'âge, la perte de fonction d'UNC5A dans les souris ne résulte pas dedans plus de croissance dans les fibres examinés. Pris ensemble, ces résultats originaux contribuent à notre connaissance de la fonction de la famille de la protéine UNC5, et au rôle qu'elle joue dans le développement et l'entretien du système nerveux central mammifère.

ABBREVIATIONS

ADP:	Adenosine Diphosphate
ATP:	Adenosine Triphosphate
ANOVA:	Analysis of Variance
cAMP:	cyclic Adenosine Monophosphate
CFP:	Cyan Fluorescent Protein
CNS:	Central Nervous System
DAP:	Death Associated Protein
DB:	DCC-Binding Domain
DCC:	Deleted in Colorectal Cancer
DD:	Death Domain
DIV:	Day in Vitro
DSCAM:	Down Syndrome Cell Adhesion Molecule
ECM:	Extracellular Matrix
EGF:	Epidermal Growth Factor
FAK:	Focal Adhesion Kinase
FNIII:	Fibronectin type III repeat
GFP:	Green Fluorescent Protein
GPI:	Glycosylphosphatidylinositol
GTPase:	Guanine Triphosphate Protease
Ig:	Immunoglobulin
MTA:	Mean Turning Angle
PI3K:	Phosphoinositide-3-kinase
PIDD:	p53 Protein with Death Domain
PKC:	Protein Kinase C
PP2A:	Protein Phosphatase 2A
PNS:	Peripheral Nervous System
RGC:	Retinal Ganglion Cell
RGM:	Repulsive Guidance Molecule
Src:	Sarcoma
SEM:	Standard Error of the Mean
TSP:	Thrombospondin
UNC5:	Uncoordinated 5
UPA:	UNC5-PIDD-Ankyrin Domain
ZU5:	Zona Occludens-UNC5 Domain

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CHAPTER 1:

Literature Review

The development of the nervous system is marked by both progressive and regressive events (Low and Cheng, 2006). Progressive events, which usually take place relatively early in development, include neuronal proliferation, neurite outgrowth, and synapse formation. Collectively, these set up a broad pattern of neural connectivity. Subsequently, however, regressive events take place. These include: cell death, axon pruning, and synapse elimination. Cell death leads to the removal of the entire neuron and ultimately results in the loss of all neurites associated with the terminated neuron. In contrast, axon pruning enables the removal of misguided or profuse axon branches in the absence of cell death. These regressive events are vital for the refinement of the premature pattern into to a more elaborate, precise and mature circuitry (Vanderhaeghen and Cheng, 2010). Ultimately, it is the balance between progressive and regressive events that helps optimize the neural circuitry of the brain.

Growing axons in the developing nervous system depend on secreted guidance molecules in order to form precise synaptic connections with their appropriate targets (Kennedy, 2000). Axon guidance requires that receptor proteins found on the surface of the navigating growth cone (the dynamic, actin-rich structure extending from the axon that seeks out its synaptic target) interact with secreted or cell surface-bound ligands encountered *en route* to, or in the target field of those axons. Binding of a ligand to a receptor initiates intracellular signaling events that coordinate a series of complex morphological changes in the growth

cone. These changes cumulatively result in the growth cone either moving away from (chemorepulsion) or towards (chemoattraction) the guidance cue.

The secreted cues involved in promoting chemoattractive or chemorepulsive responses that take part in directing axonal growth can be broadly divided into two categories: short-range and long-range (Tessier-Lavigne and Goodman, 1996). A guidance cue is said to act at 'short-range' when it operates in the immediate vicinity of its cellular source; that is, either when it is attached to the surface of the secreting cell or very close to it. By contrast, 'long-range' cues function at a distance, typically stipulated as several cell diameters away from the secreting cell (Rajasekharan and Kennedy, 2009; Shen and Cowan, 2010). Although multiple families of axon guidance cues have been identified, five of these are particularly well-defined (Reviewed in: Shen and Cowan, 2010). These include the laminins, slits, ephrins, semaphorins and netrins. Broadly, activation of signaling pathways through these guidance cues typically leads to at least one of four outcomes: growth cone repulsion, collapse, attraction or extension, resulting from the regulation of the rate of axon extension through signaling events that act locally to modulate cytoskeletal dynamics in the growth cone (Bashaw and Klein, 2010)). Often, the phenomena of growth cone attraction and axon extension are observed together. Likewise, it is not uncommon for growth cone repulsion to accompany growth cone collapse. However, it is important to note that these pairs of events do not necessarily operate through the same signaling mechanisms (Rajasekharan and Kennedy, 2009).

I. AXON GUIDANCE CUES

LAMININS

Laminins are large, secreted trimeric proteins made up of an α-chain, a β-chain, and a γ-chain which are found in five, three, and three genetic variants, respectively (Nguyen and Senior, 2006). Fifteen different laminin isoforms exist, each assembled from various combinations of these 5α, 3β and 3γ chains and at least twelve different heterodimers have been documented in vivo (Aumailley et al., 2005). Laminins comprise a major component of the basal lamina (a thin fibrous sheet underlying the epithelium in the extracellular matrix (ECM) (Colognato and Yurchenco, 2000) and interact with multiple proteins, most notably the integrins – a large family of receptors in the ECM.

Laminins facilitate axonal extension in many different types of neurons from both the central and peripheral nervous systems (CNS and PNS, respectively). For example, depletion of laminin from periphel nerve myelin preparations has been shown to substantially reduce its axon growth capacity, suggesting that laminin is a key component of the peripheral nerve basal lamina, and that it is responsible for promoting regeneration (David et al., 1995). In addition, laminins influence neural crest cell migration, Schwann cell migration, axon extension, and nerve-muscle synapse formation (Colognato and Yurchenco, 2000).

SLITS

Over twenty years ago, a group of mutations were identified in *Drosophila melanogsater* to play a role in trafficking axons once they crossed to the contralateral side of the CNS during development (Anderson and Nusslein-Volhard, 1984). *Slit*, one of these mutations, was found to be an essential midline repellant that inhibits ispilaterally projecting neurons from approaching the midline (Kidd et al., 1999). It also prevents contralaterally projecting neurons from re-crossing it (Rajagopalan et al., 2000; Simpson et al., 2000).

Slit belongs to a family of related genes which encode a large secreted protein composed of a laminin G-domain, leucine rich repeats (LRR), and epidermal growth factor repeats (EGF) (Rothberg et al., 1990). The receptor for Slit, Robo, was identified from a mutation in the *Drosophila* gene, *Roundabout,* which generated a phenotype whereby axons that would otherwise project ipsilaterally and then contralaterally would instead cross and recross the midline indefinitely (Seeger et al., 1993).

To date, three Robo and Slit homologs have been identified in mammals: Robo1, Robo2 and Rig-1; and Slit1-3, respectively (Taguchi et al., 1996; Holmes et al., 1998; Itoh et al., 1998). In addition to regulating axon branching, Slits serve as guidance cues for axons in the olfactory bulb, retina, and the dentate gyrus of the hippocampus (Li et al., 1999; Erskine et al., 2000; Long et al., 2004).

EPHRINS

Eph receptors comprise the largest family of receptor tyrosine kinases in the mammalian genome (Himanen and Nikolov, 2003a). Ephrins are either transmembrane or GPI-linked proteins that bind EphB (EphB1-B6) and EphA (EphA1-A10) receptors, respectively (Himanen and Nikolov, 2003b; Aasheim et al., 2005). Expression of EphB receptors by retinal ganglion cells (RGCs) and ephrinBs in the tectum directs the formation of lateral-to-medial projections into the tectum (McLaughlin et al., 2003b). Analogously, graded expression of EphAs by the retinal ganglion cells and ephrinAs in the tectum results in the topographic projection of RGC axons along the tectal anterior/posterior axis (McLaughlin et al., 2003a). Despite being studied exstensively as chemorepellent axon guidance cues, ephrins are involved in contact-mediated repulsion and attraction, and have been noted to also influence synaptic plasticity, adhesive interactions between cells, vascular development, and cell migration (Holmberg and Frisen, 2002; Knoll and Drescher, 2002).

SEMAPHORINS

The semaphorin family is divided into eight subclasses, number from s1-7 and a viral class, 'V'. Semaphorins are short-range inhibitory signals that signal through multimeric receptor complexes (including plexins) and typically act as guidance cues for axonal growth cones (Kolodkin et al., 1997; de Wit and Verhaagen, 2003). All semaphorins are either secreted, transmembrane, or GPI linked proteins that share an extracellular "sema" domain comprised of 500

amino acids. Intracellular signaling is conducted by semaphorins when they form a complex with a neuropilin family member and a ligand-binding component (Tamagnone and Comoglio, 2000). Although they are most extensively studied for their role as repellents that affect fasciculation, branching, and axon steering (Kolodkin and Ginty, 1997), they have been shown to promote the outgrowth of grasshopper axons *in vivo*, and can be bifunctional, acting as either chemorepellents or chemoattractants (Wong et al., 1997; Song et al., 1998).

NETRINS

Of the well characterized and richly diverse aforementioned cues, the netrin family constitutes a particularly intriguing group of guidance molecules as they are able to function at both short and long range (Kennedy, 2000). Netrins belong to a family of secreted proteins that direct cell migration and axon extension during development (Kennedy et al., 1994; Serafini et al., 1994). These proteins were first purified from embryonic chick brain using commissural axon outgrowth from explants of embryonic rat dorsal spinal cord as an *in vitro* functional assay. The purified proteins were named netrin-1 and netrin-2 (Serafini et al., 1994), and were found to be homologous to a gene identified in *C. elegans* that is required for circumferential axon guidance, *unc-6*. In turn, this gene encodes a protein that shares sequence homology with laminins (Brenner, 1974; Hedgecock et al., 1990; Ishii et al., 1992).

Three secreted netrins (netrin-1, -3, and -4) have been identified in mammals, and all share the same basic structure. They are composed of approximately 600 amino acids and have a molecular mass of approximately 65-70 kilodaltons (kDa) (Matus et al., 2006). Netrin genes encode proteins that are made of three domains (VI, V, and C) and an amino terminal signal peptide characteristic of secreted proteins. Domains V and VI of netrins are homologous to domains V and VI found at the amino terminal ends of laminins (Kennedy, 2000). Two more netrins, (netrins G1 and G2) have been identified in mammals. Unlike netrins 1-4, netrin Gs are tethered to the plasma membrane and are not secreted (Rajasekharan and Kennedy, 2009). Netrin-1 is currently the most widely studied netrin in the mammalian CNS (Rajasekharan and Kennedy, 2009), and it is the focus of the project presented here.

II. THE ROLE OF NETRINS DURING DEVELOPMENT

In the embryonic nervous system, netrin-1 has been characterized as a primarily long-range, where a gradient of netrin-1 emanates from the floor plate of the neural tube and attracts extending commissural axons towards the ventral midline (Rajasekharan and Kennedy, 2009). In contrast, to the long-range function observed in the developing nervous system, in the adult mammalian CNS, netrin-1 is expressed by oligodendrocytes, which serve to myelinate the axons of the CNS (Manitt et al., 2001; Jarjour et al., 2008). Netrin-1 Embryonic spinal commissural axons in mice deficient in netrin-1 fail to extend appropriately in the embryonic spinal cord (Skarnes et al., 1995; Serafini et al.,

1996). In addition, the corpus callosum and the anterior and hippocampal commissures fail to form, indicating that netrin-1 is required for the normal development of multiple major axon tracts in the mammalian CNS.

UNC-6 and Netrins A/B are essential for directing cell and axon migration with respect to the ventral midline of the developing nervous system of *C. elegans* and *D. melanogaster,* respectively (Hedgecock et al., 1990; Ishii et al., 1992; Hamelin et al., 1993). This is analogous to mouse, where netrin-1 expressed by the floor plate plays a critical role in directing axon extension relative to the ventral midline of the embryonic spinal cord (Serafini et al., 1996). In addition, it has been found that several axon tracts in mouse require netrin-1 to cross from one side of the CNS to the other. This was evidenced by an observation that deficiency of netrin-1 disrupts the formation of major axon projections to the midline of the brain, as well as the hippocampal commissure and the corpus callosum (Serafini et al., 1996). It has also been shown that netrin-1 expression is required for the axons of the retinal ganglion cells to exit the retina and enter the optic nerve (Deiner et al., 1997), as well as for the guidance of dopaminergic axons within the ventral midbrain, the hippocampus and the thalamocortical projection (Braisted et al., 2000; Lin et al., 2005; Xu et al., 2010).

By comparison to netrin-1, which has also been implicated in tissue morphogenesis and cell-cell adhesion (Srinivasan et al., 2003; Jarjour et al., 2008), the functions of the other netrin family members are relatively poorly understood in the vertebrate nervous system. Roles for netrins-2 and -3 have

been identified in axon guidance; netrin-Gs have been implicated in synaptogenesis, and netrin-4 in various forms of tissue morphogenesis (Barallobre et al., 2005). It should be noted that the other netrin family members have well-described functions in angiogenesis, lung morphogenesis, vasculogensis, salivary gland formation, mammary gland branching, amongst others (Kim et al., 2006; Lejmi et al., 2008; Hoang et al., 2009; Nacht et al., 2009).

III. NETRIN RECEPTORS

DCC & NEOGENIN

Netrin-1 is a versatile, bifunctional ligand known to attract or repel different classes of neurons, and it is thought that this dichotomy of function is dependent on the type of receptor it binds (Colamarino and Tessier-Lavigne, 1995; Mitchell et al., 1996). The first netrin receptors to be identified were the Deleted in Colorectal Cancer (DCC) subfamily of proteins. These included Frazzled in *Drosophila;* and Neogenin and DCC in mammals (Keino-Masu et al., 1996). The role of *netrin-1* and *dcc* is so critical that loss of function mice not only show axon guidance defects in many tracts, but also exhibit complete loss of the corpus collosum and hippocampal comissure (Barallobre et al., 2005). For example, mutation of the *netrin-1* gene in mice results in severe defects in the projection of spinal cord commissural axons whereby these axons fail to reach the floor plate (Barallobre et al., 2005). Anatomically, DCC and Neogenin are both single-pass transmembrane proteins containing four immunoglobulin domains and six

fibronectin type III (FNIII) repeats in their extracellular domains, and three highly conserved intracellular domains named P1, P2, and P3 (Figure 1.0) (Keino-Masu et al., 1996; Hong et al., 1999). Of note, the fourth and fifth FNIII repeats in DCC are required to bind netrin-1 (See Figure 1.1, Model A)(Kruger et al., 2004), while the three intracellular domains are thought to have critical roles vis-à-vis the recruitment of molecules involved in signal transduction, the exact mechanism by which they regulate this cytoplasmic organization remains elusive (Li et al., 2004b). Knocking out *DCC* in mice resulted in a very similar phenotype to that generated in *netrin-1* deficient mice, supporting the conclusion that DCC is a receptor for netrin-1 (Serafini et al., 1996; Fazeli et al., 1997).

Like DCC, neogenin is capable of transducing signals elicited by netrin-1 (Gad et al., 2000; Srinivasan et al., 2003). These interactions have been implicated in tissue morphogenesis, myoblast differentiation, angiogenesis and, most recently, axon guidance (Monnier et al., 2002; Fitzgerald et al., 2007; Wilson and Key, 2007; Lejmi et al., 2008). Although Neogenin interacts with netrins 1-3, only netrins-1 and -2 have been shown to elicit functional activity through Neogenin (Kang et al., 2004). Neogenin is also a receptor for repulsive guidance molecule (RGM), a glycosylphosphatidylinositol (GPI)-linked glycoprotein with no apparent homology to other known axon guidance molecules (Monnier et al., 2002; Wilson and Key, 2007).

DSCAM

Recently, a type-1 transmembrane receptor, Down's syndrome cell adhesion molecule (DSCAM), has been shown to function as a receptor for netrin-1 that participates in netrin-dependent axon guidance during development (Yamakawa et al., 1998(Ly et al., 2008; Liu et al., 2009)). DSCAM (Figure 1) contains ten immunoglobulin (Ig) domains and six FNIII repeats in its extracellular domain (Yamakawa et al., 1998). Of these, Ig domains seven to nine have been shown to be both necessary and sufficient to directly bind netrin-1 (Ly et al., 2008).

Although the exact signaling mechanisms activated by netrin-1 through DSCAM are unknown, other studies have shown that human DSCAM directly binds to Pak1 and stimulates Pak1 phosphorylation and activity (Li and Guan, 2004). Interestingly, DCC recruits Pak1, amongst other molecules, into an intracellular signaling complex that promotes growth cone expansion, upon binding to netrin-1 (Shekarabi et al., 2005). Since DSCAM has been shown to collaborate with DCC in mediating turning responses to netrin-1, it would be interesting to speculate that Pak1 may play a role in the signaling events permitting this response (Ly et al., 2008).

UNC5 & Homologues

UNC-5 was originally identified in the nematode worm *C. elegans* as part of an effort to determine the molecules and receptors that guide cell movements

during neural development *in vivo* (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992). The first UNC5-like protein to be detected in mammals was encoded by the rostral cerebellar malfunction (rcm) gene, which was identified as a result of a spontaneous genomic mutation that results in uncoordinated behavior in mice (Ackerman et al., 1997). Two more vertebrate homologues were discovered at the same time – UNC5h1 and UNC5h2, based on their homology to *C. elegans* UNC-5 - and as such, Rcm was later renamed UNC5h3 (Leonardo et al., 1997). The fourth vertebrate UNC5 homolog, UNC5H4, was independently characterized five years later (Engelkamp, 2002). The mammalian UNC5 proteins - UNC5H1, 2, 3, and 4 – are interchangeably referred to as UNC5A, B, C, and D, respectively, with the latter notation dominating the current literature (Arakawa, 2004).

Since the discovery of the first three vertebrate UNC5s, UNC5 orthologs have been identified in a host of invertebrates, including *D. melanogaster* (Kim et al., 1999), , and the yellow fever mosquito, *A. aegypti* (Nene et al., 2007); and vertebrates, including: *X. laevis* (Anderson and Holt, 2002), zebrafish (Kaur et al., 2007), and the sea lamprey, *P. marinus* (Shifman and Selzer, 2000). It is now clear that UNC5s comprise a family of conserved UNC-6/netrin receptors that mediate a repulsive response to netrins (Hong et al., 1999). All UNC5s contain four conserved domains: thrombospondin (TSP) type 1 domain, ZU5 domain, Immunglobulin (Ig), and death domain (DD) (Wang et al., 2009a). Of all mammalian UNC5 homologues, UNC5D is by far the least understood, perhaps

due to its relatively different sequence homology to the other three mammalian paralogs. One speculation to address this peculiar finding is that the Deleted in Colorectal Cancer (DCC)-binding (DB) domain, a short intracellular segment required for interaction of UNC5 with DCC, was most divergent in UNC5D (Hong et al., 1999; Engelkamp, 2002).

All UNC5s are single pass type 1 transmembrane receptors (Llambi et al., 2005). Each member of the UNC5 family contains, from its amino to its carboxy terminal end; two Ig domains (Ig1 and Ig2, both of which are required to bind netrin) (Hong et al., 1999) two TSP type 1 domains (Hong et al., 1999), a single pass transmembrane domain, a ZU5 domain (named for its homology with a portion of Zona-Occludens-1 found in tight junctions (Leonardo et al., 1997)), a DB domain (also referred to as a UPA domain since it is conserved in UNC5s, PIDD and Ankyrins (Wang et al., 2009a) and a well defined DD (Hofmann and Tschopp, 1995; Ackerman et al., 1997; Leonardo et al., 1997). Intriguingly, the organizational pattern of the UNC5 cytoplasmic domains, which consist of the ZU5, UPA/DB and death domains, is also found in ankyrins as well as PIDD, both of which belong to families of scaffolding proteins. Ankyrins are involved in the assembly of specialized membrane structures that can include ion channels, cytoskeletal specializations, and cell adhesion molecules (Bennett and Healy, 2008). PIDD serve as molecular switches in modulating programmed cell death (Lin et al., 2000; Park et al., 2007; Cuenin et al., 2008).

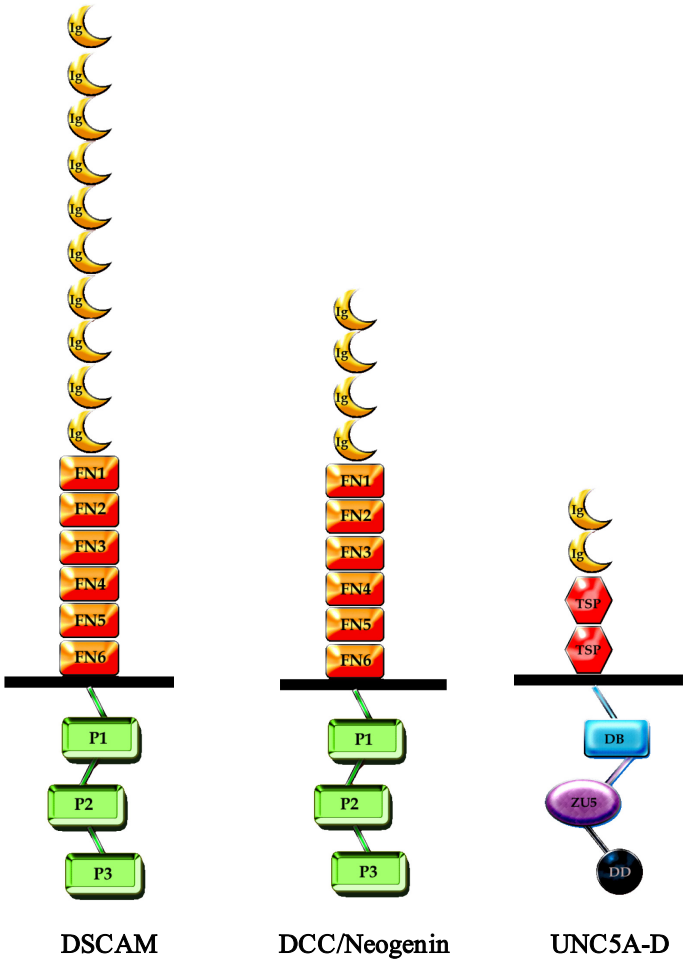

Figure 1.0: Illustrations of putative netrin-1 receptors, Down Syndrome's Cell Adhesion Molecule (DSCAM), Deleted in Colorectal Carcinoma (DCC), Neogenin, and the four mammalian homologues of Uncoordinated-5 (UNC5)

The crystal structure of the cytoplasmic portion of UNC5B was recently revealed, illustrating that the three distinctly folded cytoplamic domains form a structural supramodule, with the ZU5 domain binding both the DB/UPA and DD separately, forming an L-shape in which the DB/UPA and DD do not interact

with each other (Wang et al., 2009a). Peptide sequence alignment analysis revealed high conservation of the ZU5-DB/UPA-DD domain organization amongst different members of the UNC5 family as well as UNC5s from different species (Wang et al., 2009a). Furthermore, it was determined that the supramodule forms a closed confirmation which is likely homologous to ankyrins and PIDD, and it is believed that release of this closed confirmation leads to the activation of UNC5B, promoting apoptosis (Wang et al., 2009a).

The localization and function of UNC5 has been particularly well studied in the invertebrate nematode body wall (Hedgecock et al., 1990; Leung-Hagesteijn et al., 1992). For example, studies in which UNC5 was ectopically expressed in touch receptor neurons (which normally extend their pioneer axons longitudinally or ventrally on the epidermis in the presence of unc-6/netrin), showed pioneer axons and mesodermal cells migrating in a dorsal trajectory instead (Hamelin et al., 1993). Further studies demonstrated the importance for precise spatial and temporal expression of UNC5 in distal tip cells during ventral-to-dorsal migrations, in order for proper migration patterns to be induced (Hong et al., 1999). In *Drosophila*, UNC5 is normally expressed in the CNS segmental nerve motor axons (Keleman and Dickson, 2001) as well as by cardiovascular cells (Albrecht et al., 2011). However, *Drosophila* embryos with a homozygous mutation for UNC5 display severe defects in motor axon guidance, as well as a failure to properly form a heart lumen (Labrador et al., 2005; Albrecht et al., 2011).

UNC5s are expressed in vertebrates in different regions of the central nervous system and by different cell types throughout the entire body. In *Xenopus*, for example, UNC5 expression has been well characterized in the developing visual system (Anderson and Holt, 2002). In mammals, expression of UNC5 homologues has been described in a number of different tissues (see Table 1). Functionally, both invertebrate UNC5 and vertebrate UNC5s have been shown to interact with different chemotropic guidance cues in order to mediate mechanisms involved in cell proliferation (Lee et al., 2007); intracellular signaling (Tong et al., 2001; Kruger et al., 2004; Picard et al., 2009); axonal regeneration, outgrowth and guidance (Colavita and Culotti, 1998; Hong et al., 1999; Keleman and Dickson, 2001; Finger et al., 2002; Labrador et al., 2005; Bartoe et al., 2006; Manitt et al., 2006; Bouvree et al., 2008; Picard et al., 2009; Albrecht et al., 2010); cell migration (Hamelin et al., 1993; Ackerman et al., 1997; Su et al., 2000; Itoh et al., 2005; Dillon et al., 2007); tumor suppression (Bernet et al., 2007); and programmed cell death (Llambi et al., 2001; Thiebault et al., 2003; Williams et al., 2006; Tang et al., 2008; Wang et al., 2008; Mille et al., 2009).

UNC5 has been shown to bind netrin-1 directly, where both Ig domains of UNC5 are required to bind netrin (See Figure 1.1, model B) (Kruger et al., 2004).Netrin-1 stimulation of UNC5 is thought to activate RhoA and, to a much lesser extent Rac1 and Cdc42, eliciting intracellular signaling cascades (Picard et al., 2009).

Table 1.0: Expression patterns of mammalian UNC5 homologues.

RECEPTOR	SITE OF EXPRESSION (in mice)
UNC5A/H1	Forebrain, midbrain (Williams et al., 2003), hindbrain (Barrett and Guthrie, 2001), lungs, spinal cord, whisker follicles (Engelkamp, 2002)
UNC5B/H2	Forebrain, midbrain, hindbrain (Kaur et al., 2007), Lungs (Dalvin et al., 2003), eyes (Ellezam et al., 2001), heart, limb buds, ears, vascular system (Engelkamp, 2002), kidney (Wang et al., 2009b), liver (Zhang et al., 2009), placenta (Dakouane-Giudicelli et al., 2010)
UNC5C/H3	Cerebellar cortex (Ackerman et al., 1997), limb buds (Engelkamp, 2002), spinal cord (Finger et al., 2002), colon (Grady, 2007), and hindbrain (esp. cerebellum) (Przyborski et al., 1998).
UNC5D/H4	Cortex (Zhong et al., 2004), limb bud, mammary gland (Engelkamp, 2002)

IV. UNC5 FUNCTIONS

UNC5 PROMOTES CHEMOREPULSION

The simplest model representing the bifunctionality of netrin suggests that DCC mediates attraction, whereas UNC5s mediate repulsion. However, evidence suggests reality is more complex than this simple model. For example, in both *C. elegans* and vertebrates, many neurons express both *unc-5* family genes as well as *Dcc* family genes (Chan et al., 1996). In addition, it has also been demonstrated that expression of either mammalian UNC5A or *C. elegans* UNC5 in *Xenopus* spinal cord neurons is sufficient for their axons to be repelled by netrin-1 (Hong et al., 1999).

Not only does DCC mediate attractive signaling via netrin-induced homodimerization of DCC (Huber et al., 2003), but it has also been shown to participate in repulsive axon guidance by heterodimerizing with UNC5 (Hedgecock et al., 1990; Stein et al., 2001). Interestingly, stimulation of PKCα in mammalian hippocampal axons leads to the internalization of UNC5A, which prevents UNC5A-mediated growth cone collapse and converts netrin-1 stimulated chemorepulsion to attraction (Bartoe et al., 2006). Finally, there is some evidence to suggest that UNC5 may be capable of mediating repulsion without DCC, suggesting two potential models for netrin-1 induced chemorepulsion, though it remains unclear which model more accurately represents conditions in the mammalian nervous system (Figure 1.1) (Keleman and Dickson, 2001).

Furthermore, netrins can induce the cytoplasmic domains of DCC and UNC5 to associate, forming a receptor complex capable of switching attraction to repulsion (Hong et al., 1999). Both *in vitro* and *in vivo* studies demonstrated that the specificity of the repulsive responses to netrins is generated by the UNC5 cytoplasmic domain (Hong et al., 1999). This was conclusively proven when a truncated UNC5 molecule lacking an extracellular domain was shown to be sufficient to promote a repulsive response (Hong et al., 1999). More dramatically a chimeric UNC5-DCC molecule was developed, in which the extracellular portion of DCC was fused with the intracellular domain of UNC5, and the hybrid molecule still elicited similar levels of repulsion to a source of netrin-1 as did the wildtype UNC5 (Hong et al., 1999).

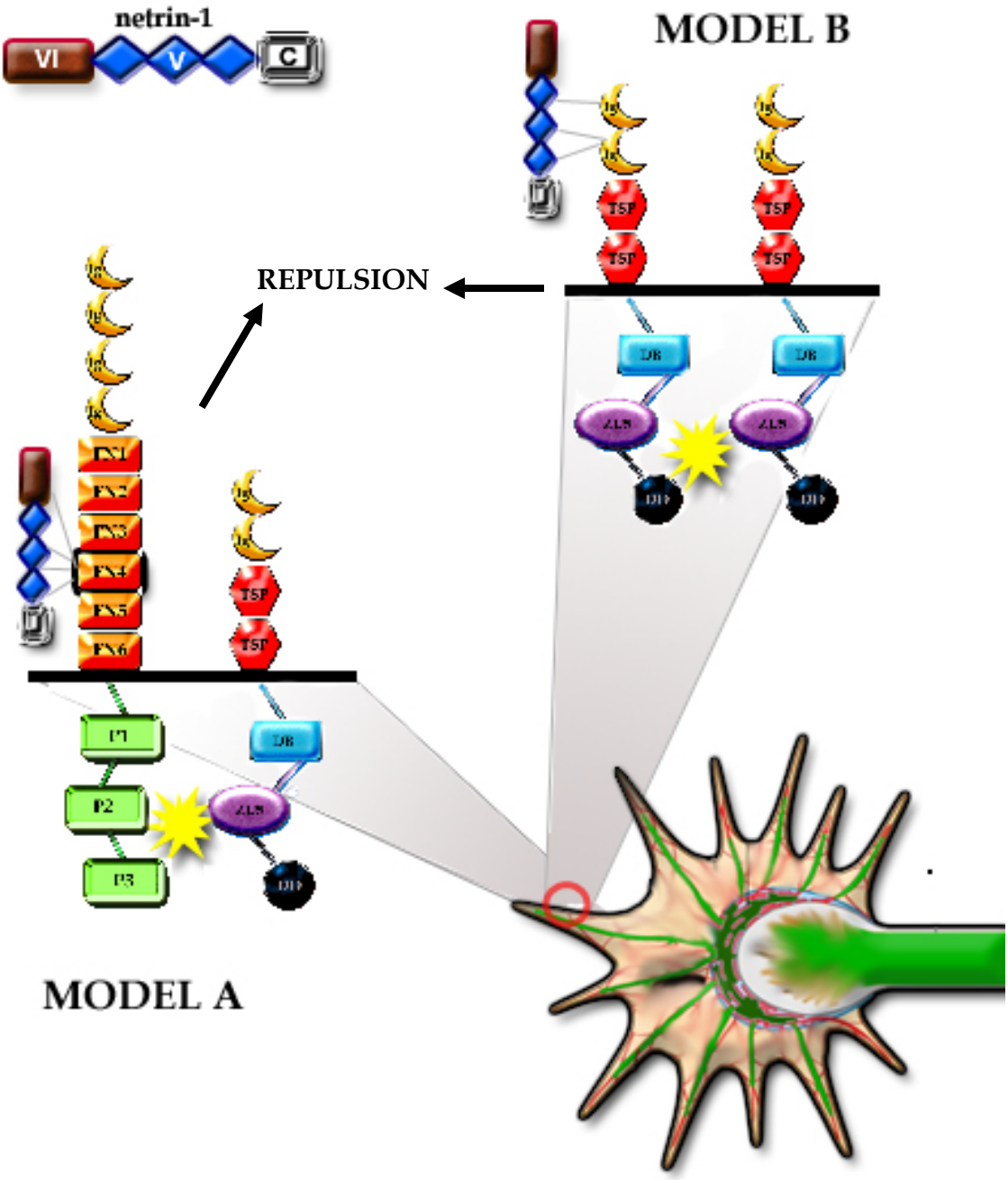

Figure 1.1: Two competing models representing netrin-1 induced chemorepulsion in the mammalian nervous system. *Model A:* DCC and UNC5 form a heterodimeric receptor that binds netrin-1 and transduces signaling cascades that ultimately lead to repulsion. Netrin-1 binds via its VI and V domains to either DCC's FN4 and FN5 repeats, or via its V and C domains to both of the Ig domains of UNC5.

Model B: UNC5 mediates chemorepulsion independent of DCC. In this case, it is not clear whether UNC5 molecules homodimerize with each other, or whether a single UNC5 receptor is sufficient to promote repulsive turning.

In order to propagate an attractive response, DCC forms both a physical and functional complex with focal adhesion kinase (FAK) and Src family members (Li et al., 2004b; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004). Activation of FAK and Src kinases by netrin stimulation induces phosphorylation of tyrosine residue Y1420 in DCC (Li et al., 2006). Mutation of the tyrosine phosphorylation site in DCC reduces the ability of DCC to mediate the netrin-stimulated attractive effects on spinal neurons in vitro (Li et al., 2006). It has also been established that tyrosine phosphorylation and tyrosine kinases play an important role in DCC mediated netrin signaling (Li et al., 2004b; Liu et al., 2004; Meriane et al., 2004; Ren et al., 2004).

UNC5-MEDIATED APOPTOSIS

Receptors that share the ability to induce cell death when they are expressed in a context in which their trophic ligands are unavailable are called dependence receptors (Mehlen and Bredesen, 2004). In addition to their role as chemorepellent netrin-1 receptors, UNC5A-C are thought to be dependence receptors with the capacity to induce apoptosis in the absence of netrin (Llambi et al., 2001). Although the mechanism underlying this function remains unclear, studies using UNC5B suggest that the intracellular domain of UNC5 interacts

with the serine/threonine kinase death-associated protein (DAP) kinase, and that in the absence of netrin-1, UNC5 dephosphorylates DAP kinase in a process mediated by protein phosphatase 2A (PP2A), which serves to trigger apoptotic events (Llambi et al., 2005; Guenebeaud et al., 2010). When netrin-1 is present, however, UNC5B interacts with the brain-specific GTPase PIKE-L, which triggers the activation of downstream kinase signaling events that ultimately inhibit UNC5B's pro-apoptotic activity (Tang et al., 2008).

Other studies have suggested that netrin-1 induces the multimerization of DCC and UNC5B, and that this multimerization is ultimately responsible for inhibiting the apoptotic activity of UNC5 (Mille et al., 2009). Finally, a pro-apoptotic role for UNC5 is supported by deletion studies, whereby loss of UNC5A in vivo is shown to decrease apoptosis and increase the number of neurons in the spinal cord, although this study did not find the UNC5A induced apoptosis was dependent on the absence of netrin-1, as predicted by the dependent receptor hypothesis (Williams et al., 2006). It is also thought that UNC5s act as tumor suppressors by inducing apoptosis of tumor cells in order to limit tumor development, which would otherwise proliferate in environments that lack netrin-1 (Grady, 2007). Additionally, UNC5 genes have been shown to be down-regulated in many cancers, particularly in colorectal cancer (Thiebault et al., 2003). For example, inactivation of UNC5C in mice has been associated with intestinal tumor progression (Bernet et al., 2007).

CHAPTER 2:

A Novel DCC-Independent Mechanism for Chemorepulsion in Mammalian Cortical Neurons

INTRODUCTION

Although mammalian UNC5 homologues are thought to multimerize with DCC in order to transduce a repellent response to netrin-1, an UNC5-sufficient model has been suggested in which the UNC5 receptor promotes short-range chemorepulsion independent of DCC (Keleman and Dickson, 2001; Merz et al., 2001; Picard et al., 2009; Wang et al., 2009a). It is interesting to note that this model, which extensively permeates the existing literature, is almost exclusively based on findings from two reports (Keleman and Dickson, 2001; Merz et al., 2001). The extent to which these findings have been generalized is quite striking, given that the actual conclusions reached by both groups were remarkably humble in scope and scarcely flexible to inter-species extrapolation. To illustrate this point, it is worth briefly examining both publications.

The first experiment, conducted in *Caenorhabditis elegans*, suggests that UNC-5 can partially transduce the signal by UNC-6, an ortholog of netrin, independently of UNC-40/DCC (Merz et al., 2001). This finding served to strengthen a previous report, which showed relatively weak defects in ventral-to-dorsal migrations caused by the *unc-40* mutations, suggesting that UNC-5 did not absolutely require UNC-40 to signal repulsion (Hedgecock et al., 1990). Although the authors state that, "in ectopic expression assays *in vitro* or *in vivo,* however, UNC-5 does absolutely require UNC-40/DCC for growth cone repulsion," they immediately follow this with an important disclaimer: "As we

have shown, however, this is not the case in cells that normally express UNC-5 and that are repelled by UNC-6 (Merz et al., 2001)". In other words, this report shows that, UNC-5 is capable of *transducing* the repulsive signal independently of UNC-40, but with some important limitations. First, it cannot be inferred on the basis of this data that UNC-5 functions in this manner when it binds netrin-1. Second, and more importantly, this UNC-5 function was only observed in an artificial context and does not occur in cells that express endogenous levels of UNC-5 (Merz et al., 2001).

The second experiment, performed using *Drosophila melanogaster,* demonstrated that short-range repulsion of commissural axons induced by Netrin and mediated by UNC5 did not require the DCC family member, Frazzled (Keleman and Dickson, 2001). Netrins in the *Drosophila* CNS are expressed at the midline and thought to act through Frazzled in order to guide commissural axons toward and across the midline (Harris et al., 1996; Kolodziej et al., 1996; Mitchell et al., 1996). Using a *UAS-Unc5* transgene and an *elav-GAL4* driver, Keleman and Dickson generated embryos that overexpressed UNC5 in all postmitotic neurons. This overexpression of UNC5 prevented commissural axons from crossing the midline, which was believed to be driven by Netrin and orchestrated through UNC5's interaction with Frazzled. To explore whether Netrin and Frazzled were in fact needed for this repulsive response, Keleman and Dickson generated embryos carrying both transgenes in the background of either '*Netrin deficiency*'

or '*frazzled null*' allelic combinations. As they expected, midline repulsion by Unc5 required Netrin. However, they were surprised to observe growth cone repulsion occuring independently of *frazzled.* This was taken as evidence that Frazzled, or DCC, is not required for short-range repulsion (Keleman and Dickson, 2001).

Although this experiment conveyed an unprecedented result, it did not prove that UNC5 acts alone to mediate chemorepulsion; it simply eliminated Frazzled as a potential co-receptor. In other words, it is plausible that UNC5 interacts with a different co-receptor in order to mediate the chemorepulsive response – a possibility the authors do not rule out (Keleman and Dickson, 2001). One possible candidate for this role as a co-receptor in mammals is neogenin, given its resemblance to DCC and its demonstrated role in chemorepulsion via the interaction with its ligand, repulsive guidance molecule A (RGMa) (Hata et al., 2009). Indeed, UNC5B has recently been shown to interact with neogenin via the intracellular molecule LARG, forming a co-receptor complex for RGMa (Hata et al., 2009)). However, since *Drosophila* do not express an orthologue of neogenin, it is possible that the result observed by Keleman and Dickson involves either UNC5 alone, or another co-receptor that is yet to be identified.

Thus, the primary aim of this project is to investigate whether UNC5 is capable of mediating a repulsive response to netrin-1 in the absence of DCC using mammalian cortical neurons.

To determine whether the conclusions reached on the basis of studies in *Drosophila* (Keleman and Dickson, 2001) as well as in *C. elegans* (Merz et al., 2001) apply to mammals, we set out to study the turning response of mammalian neurons using a chemotaxis assay that can produce a stable gradient of netrin-1. Thus, a reliable turning assay which produces a concentration gradient that reaches a linear steady state suitable for studying long-term chemotaxis had to be established in the Kennedy lab (Zicha et al., 1997).

Rationale for Using the Dunn Chemotaxis Chamber

In the past, chemotaxis has often been studied using a Boyden chamber or transwell assay (Jungi, 1975; Rhodes, 1982; Babich and Sinensky, 2001). However, both of these methods are based on scoring cells that have migrated into or across a filter membrane toward a source of putative chemotrophic factor (Chen, 2005), which is problematic as a measure of axon guidance. A second drawback to using these assays is that the local concentration gradients produced by the chemotrophic factor in and around the pores of the filter membrane are variable and difficult to quantify (Venge, 1979; Babich and Sinensky, 2001). A third limitation of these assays is that the motility of the cells cannot be observed directly and migration is thus only deduced from the final distribution of the cell population (Thomsen and Jensen, 1991; Connolly and Maxwell, 2002; Chen, 2005). To overcome these limitations, the Dunn chemotaxis chamber was developed. This chamber allows for the migratory behavior of cells to be directly

viewed, and also produces a highly stable, uniform gradient of known direction and magnitude (Dunn and Zicha, 1993; Zicha et al., 1997).

The Dunn chemotaxis chamber is a modification of the Zigmond chamber (Zigmond and Hirsch, 1973), which was designed to provide direct observation of slow-moving cells exposed to a concentration gradient that remains stable over long periods of time (Zicha et al., 1991; Dunn and Zicha, 1993). Since its development, the Dunn chamber has been used in the successful characterization of the migratory responses of a wide variety of cell types to various guidance cues, including: fibroblasts to platelet-derived growth factor (Zicha et al., 1991); human macrophages to colony-stimulating factor-1 (Jones et al., 2002); neutrophils to interleukin-8 (Zicha et al., 1998; Orr et al., 2003), microglia to adenosine triphosphate (ATP)/adenosine diphosphate (ADP)(Honda et al., 2001), and *Dictyostelium* to PI3K (Takeda et al., 2007), The Dunn chamber has also been applied to assays of neurite outgrowth, initially implicating retinoic acid as a chemotactic molecule in neural development (Maden et al., 1998).

The Dunn Chamber (Hawksley DCC100) is a glass slide that consists of two concentric rings separated by an annular bridge approximately 1 mm wide (See Figure 2.0) (Webb et al., 1996; Maden et al., 1998). In practice, the cover slip containing the growing cells is inverted and sealed completely on the top of the slide, covering the media-containing rings. The bridge is arranged such that there

is a 20 μm gap between it and the cover slip. The cells that lie directly above the bridge are viewed during the assay.

One of the wells (usually the inner well) is typically filled with the same medium in which the cover slips were bathed (the conditioned media), while the outer well is filled with the conditioned media plus the concentrated guidance cue to be used (in this case, netrin-1). The concentric layout results in the inner well (flanked by both types of media) maintaining a constant volume, and as long as there are no air bubbles in either well, the fluid's incompressibility prevents any media flow between the wells which could otherwise distort the diffusion gradient (Zicha et al., 1997).

Theoretically, a peptide of molecular weight between 35,000 daltons and 75,000 daltons (netrin has a molecular weight of 75 kiloDaltons) will form a linear gradient within 10 minutes and decay with a half life of 10 hours (Zicha et al., 1991). The specific concentration of the guidance cue to be used varies with cell-type, but typically will range between 150 μg/mL and 500 μg/mL (Keren et al., 2009). In the initial experiments carried out here, we first established a suitable concentration of netrin-1 for each cell type, and then carried out live imaging experiments.

MATERIALS & METHODS

Primary Cortical Neuron Cell Culture

All animal work was performed in accordance to the Canadian Council on Animal Care Guidelines. Wild type pregnant mice were obtained from Charles River (St. Constant, Canada), while DCC knockout embryos and wild type littermates were obtained from crosses conducted at the Animal Care Facility at the MNI. Special coated #3D glass coverslips designed for the Dunn chamber (Erie Scientific, Portsmouth, NH) were washed with ethanol and stabilized before being inserted into 6-well plates and coated with 5 mg/mL of

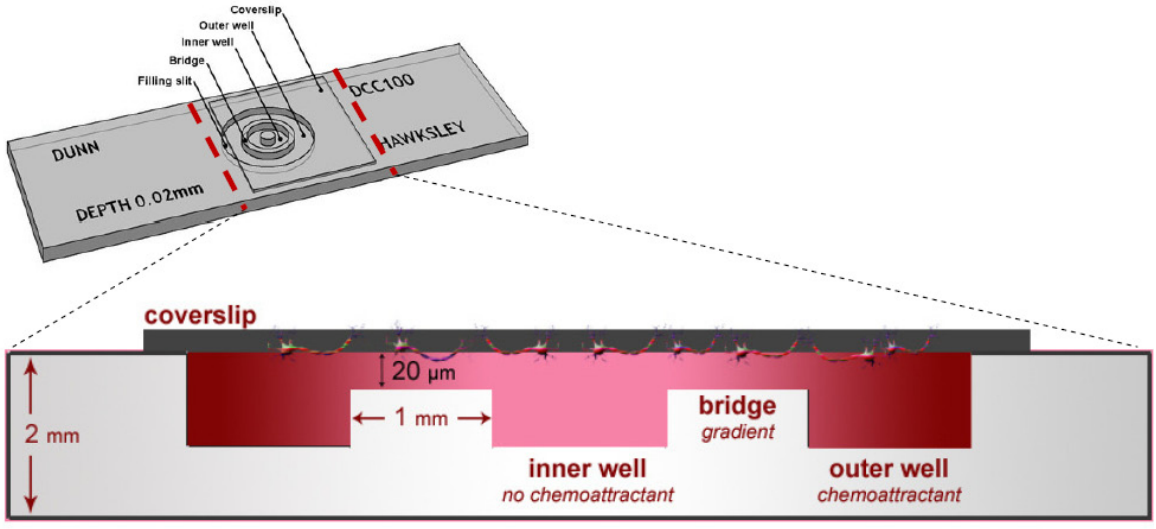

Figure 2.0: Schematic representation of the Dunn Chamber. Coverslip with cells of interest is placed on top of two concentric wells containing regular medium and conditioned medium including recombinant netrin-1. The concentric wells are separated by a bridge where live imaging takes place. *Adapted with modification from Dunn et al, 2011.*

poly-D-lysine (Sigma, St. Louis, MO) dissolved in calcium and magnesium chloride-rich Hank's Balanced Salt Solution (HBSS) for 2-3 hours. Cortices were dissected and immediately washed once in cold S-minimum essential medium (SMEM) free of L-glutamine and rich in Earle's salts, comprised of 10mM HEPES. The cortical neurons were trypsinized with SMEM solution composed of 50 µL of 4% DNase and 200 µL of 0.25% trypsin for 25 minutes at 37°C. Cells were then diluted in a solution containing neurobasal and fetal bovine serum (FBS) and counted before they were plated and cultured in a media consisting of Neurobasal supplemented with 2%B27, N-2 (a serum-free supplement), and penicillin/stremptomycin.

Neocortical neurons from E15-16 DCC knockout mice were prepared using the same protocol with some slight modifications. Each embryonic cortex was dissected, trypsinized, and triturated separately, and tail clips obtained from each embryo for genotyping. Only cells isolated from DCC knockout embryos were used for live imaging. See Figure 2.1 for an example of the genotyping.

Dunn Chamber Assembly

Primary cortical neurons were grown on PDL-coated 18 mm square #3D coverslips (Erie Scientific, Portsmouth, NH) for Dunn chamber axon guidance assays, at medium density (such that individual neurons could be isolated and imaged separately (approximately 150,000 cells/well in a 6-well plate.

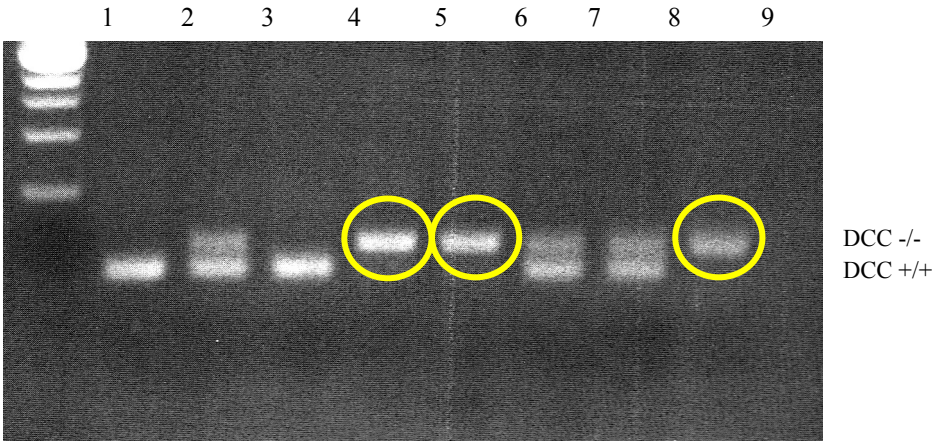

Figure 2.1: Genotyping results obtained from eight embryos derived from a cross between two adult mice heterozygous for DCC. Lane 1 is a 1kB ladder. Lanes 2 and 4 are wild-type DCC embryos; 3, 7 and 8 are heterozygotes; and 5, 6 and 9 are DCC knockout embryos, which were utilized in the Dunn chamber chemotaxis assay.

The Dunn chamber was assembled according to the protocol by Yam et al. (2009), which is modified from Zicha et al. (1997) and Wells and Ridley (2005). Specifically, the chamber was pre-washed once with Neurobasal alone and then twice with conditioned media. After the two-step washing procedure, conditioned media was added to fill the inner and outer concentric wells. A coverslip containing cortical neurons was then inverted onto the Dunn chamber and sealed on three sides using hot paraffin:beeswax:Vaseline, leaving one side with a narrow slit open at the edge to drain and refill the outer well. Excess media was removed by blotting and using a gel-loading tip, the conditioned

media from the outer well was removed through the filling slit, and the chemoattractant (netrin-1diluted in conditioned media to achieve the desired concentration) was added to the outer well. The filling slit was then sealed using the same wax mixture. In order to avoid changes in pH of the media, the Dunn chambers were assembled rapidly (<10 minutes).

Once the chamber was properly assembled, with no air bubbles present, imaging commenced within 5 min. Time-lapse phase contrast images were acquired from 5-10 different positions along the annular bridge of the chamber using a 40X fluotar LD objective every 30 seconds, for a minimum of 2 h. These time-intervals depend on cell type, and were determined empirically. In a chemotaxis assay examining the attraction of embryonic chick neural tube cells to retinoic acid, for example, recordings were taken at 2-minute intervals for a period of 72 hours (Webb et al., 1996).

Dunn Chamber Gradient Quantification

Tetramethylrhodamine-40 kDa dextran, a fluorescent compound that can be used to create concentration gradients of diffusible molecules for imaging, was kindly provided by Dr. Edward Ruthazer (purchased from Molecular Probes Invitrogen)). For netrin-1 gradient quantification experiments, tetramethylrhodamine-40 kDa dextran was placed in the outer well of the Dunn chamber and images of the gradient across the bridge were acquired with a 10X fluotar objective. Although 40 kDa dextran is not equivalent in size to netrin-1,

this approach is not unprecedented (for example, see (Yam et al., 2009)). Nevertheless, a similar experiment using a genetically-encoded fluorescent netrin-1 would be ideal.

Imaging was performed at multiple positions on a heated stage. However, since the gradient of netrin increases closer to the outside well and gradually decreases towards the inner well, it was important to consistently image axons that were in the same horizontal position along the annular ridge in order to ensure that the axons being imaged are exposed to approximately the same concentration of guidance cue at the beginning of the experiment.

Microscopy and Image Acquisition

Live cell imaging was performed at a stable temperature of 37 °C on a Zeiss Axiovert 200M microscope (Zeiss, Toronto, Ontario) equipped with an automated MS-2000 XYZ stage, a heated chamber and hydrating fluid (ASI, Eugene, OR). All images were captured via an Orca ER CCD camera (Hamamatsu) using Northern Eclipse software (Empix Imaging, Toronto, Ontario). All time-lapse phase contrast and/or epifluorescent images were acquired using a 40X objective. For every image captured, axons were temporarily exposed to light at 20% intensity for 450 milliseconds before XYZ stamping. A brief 15 second time delay took place between shots, where the light was off.

Quantification and Data Analysis

To quantify the movement towards or away from the source of netrin-1, a vector was drawn from the initial to final position on neurons that were selected for imaging. In turn, these cortical neurons were selected on the basis of several stringent criteria adopted from Yam et al. (2009). For example, axons that experienced no net growth, or turned back and folded on themselves, or made contact with debris/another cell body, or fasciculated with other axons, or retracted more than 10 μm from their initial position, were not included in the quantification. Thus, only single, non-fasciculated axons that experienced net growth over the duration of the imaging period were analyzed. For each axon quantified, the distal 10 μm of the axon at the first time point was tracked and defined as the initial position of the axon.

Next, the base of the growth cone was tracked for each time point to determine the trajectory, and all axon positions and trajectories were rotated and translated such that the initial axon segment started at Cartesian coordinate (0,0). Furthermore, since the absolute direction of the gradient in the Dunn chamber depends on the position it occupies along the annular bridge, all axon positions and trajectories were rotated such that the gradient always increased parallel to the y-axis.

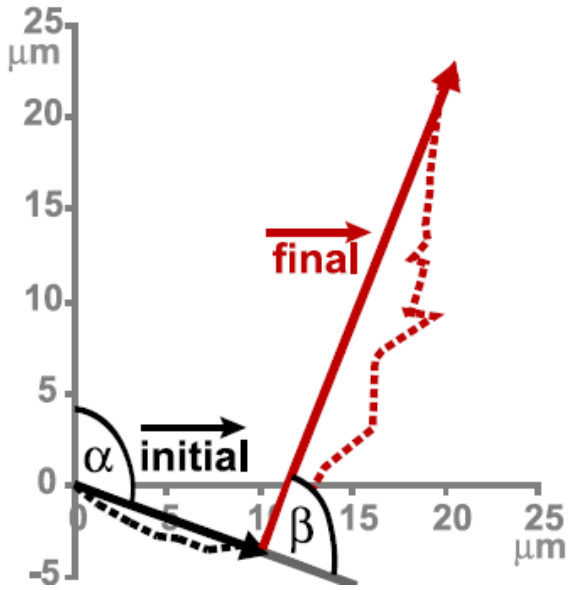

Figure 2.2: Calculation of final turning angle made by growing axon. The initial angle (α) was calculated as the angle between the initial position of the axon and the direction of the gradient, where $0° \leq \alpha \leq 180°$. The angle turned (β) was defined as the angle between the original direction of the axon and a straight line connecting the base of the growth cone from the first to the last time point of the assay period. The angle turned was defined as positive for turns toward the gradient, and negative for turns away from the gradient (Adapted from Yam et al. 2009)

The angle of rotation was calculated from the coordinates of the stage position relative to the center of the Dunn chamber, and the final angle was determined using a dot product based on initial and final angles of turning (Figure 2.2). Illustration of a sample quantification of the angle turned and displacement of the growing axon, along with a demonstration of rotated vs. non-rotated trajectories is provided in Figure 2.3.

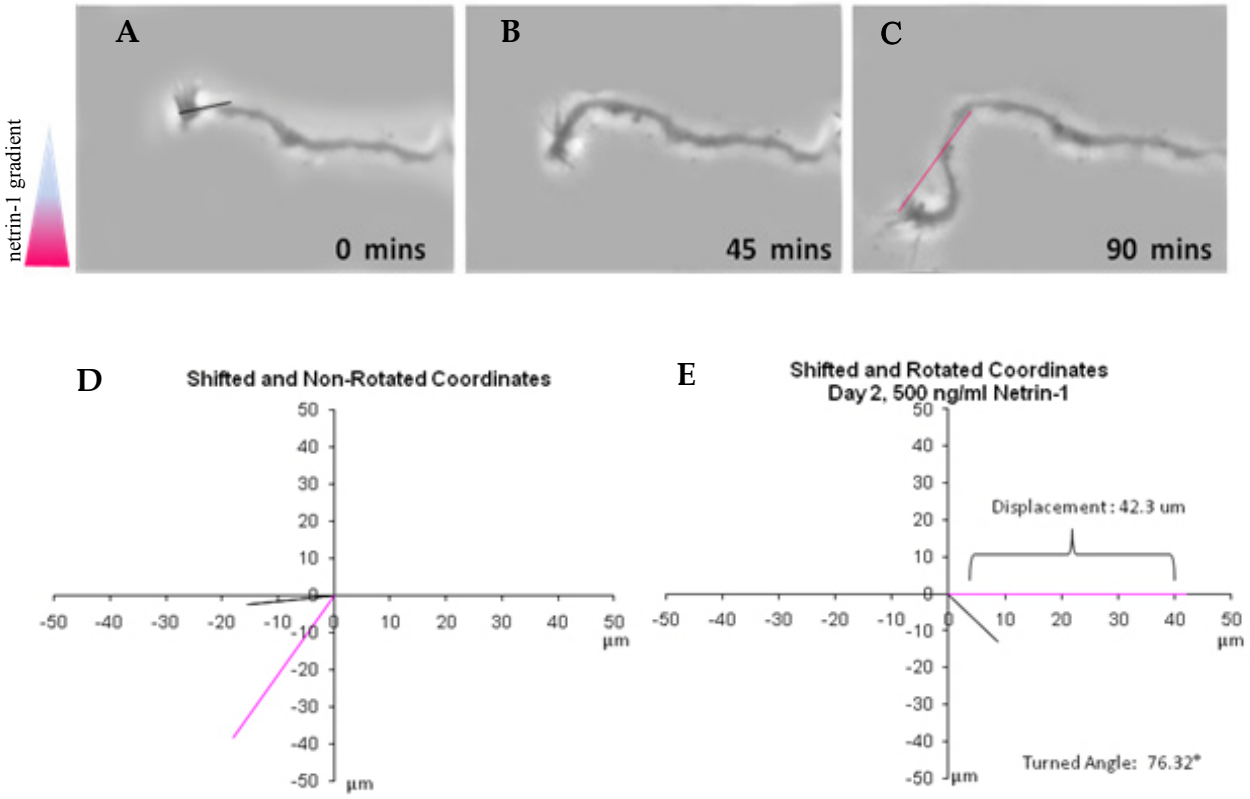

Figure 2.3: Sample calculation of displacement and final turning angle made by a wild type cortical axon. *A)* Growth cone from cortical axon at time 0. 10 µm drawn from base of growth cone to axon body to define the initial trajectory of the axon (represented by a black line) to a gradient of netrin-1 that increases at the bottom of the panel. *B)* Axon turns towards the netrin-1 gradient at approximately 45 minutes, and reaches a final position at *C)* 90 minutes after the concentration gradient had been established. A fuscia line is drawn to represent the axon's final trajectory. *D)* Schematic representation of the initial and final displacements of the growth cone before being translated and rotated relative to the center of the stage. *E)* Shifted and rotated coordinates of axon, with a total displacement of 42.3 µm displacement from origin and an overall positive turning angle of 76.32° (angles not drawn to scale).

RESULTS

Optimizing Conditions for Live Cortical Neuron Imaging

In order to examine axonal turning using a Dunn chamber, certain conditions had to be optimized to ensure maximum motility within the assay. Specifically, it was important to calibrate the concentration of PDL used in order to promote the adherence of cortical neurons to the coverslips. Initially, a concentration of 10ug/mL of PDL was used per coverslip, and removed after 2.5 hours. However, this concentration proved too high, resulting in stagnant axonal processes that appeared stuck to the substrate (Figure 2.4). Thus, a lower concentration of 5 ug/mL was applied for the same amount of time, and this proved far more conducive to axon extension without compromising on the adherence of the cell to the coverslip (Figure 2.5). A still lower concentration of 1 ug/mL was used, also for a 2.5 hour period. However, this concentration was too low and resulted in cell bodies being easily dislodged from the coverslip.

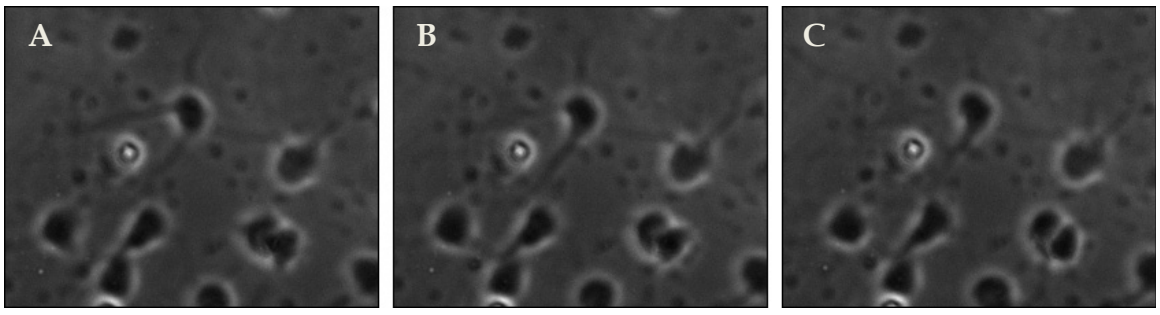

Figure 2.4: Acquired images of cortical neurons grown on a 10 ug/mL concentration of PDL. A) Image acquired at time 0. B) Image acquired after one hour of imaging. C) Image acquired after two hours of imaging elapsed. All images acquired using 20% light intensity with an exposure lasting 450 ms, using 20 X objective lens after 1DIV.

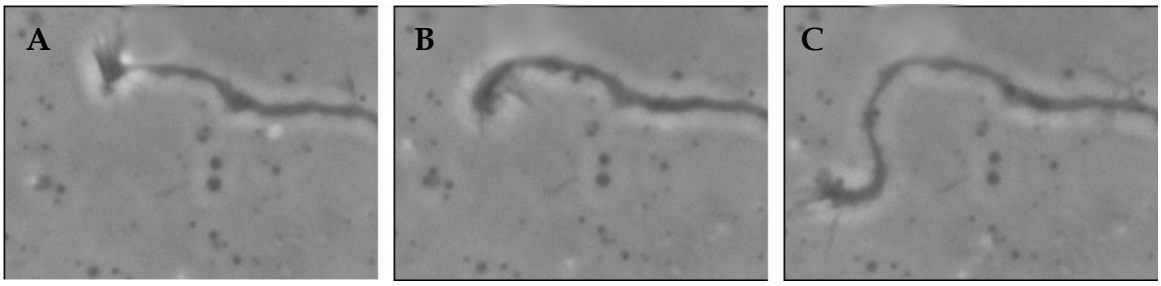

Figure 2.5: Acquired images of cortical neurons grown on a 5 ug/mL concentration of PDL. A) Image acquired at time 0. B) Image acquired after one hour of imaging. C) Image acquired after two hours of imaging elapsed. All images acquired using 20% light intensity with an exposure lasting 450 ms, using 40 X objective lens after 1DIV.

Dunn Chamber Forms a Stable Gradient Suitable for Studying Chemotaxis

In order to effectively study axon turning using the Dunn chamber, the first step was to test the chamber to ensure that a stable and uniform gradient of guidance cue could be established and maintained for at least several hours for live imaging. By adding tetramethylrhodamine-40 kDa dextran to the outer concentric well of the Dunn chamber, a concentration gradient was expected to develop along the annular ridge. Furthermore, since the dextran used is fluorescent, it was possible to observe the formation of the gradient using an epifluorescent inverted microscope. Consistent with the published literature, a gradient was formed within 30 minutes, and remained stable for up to 2 hours (Figure 2.6)

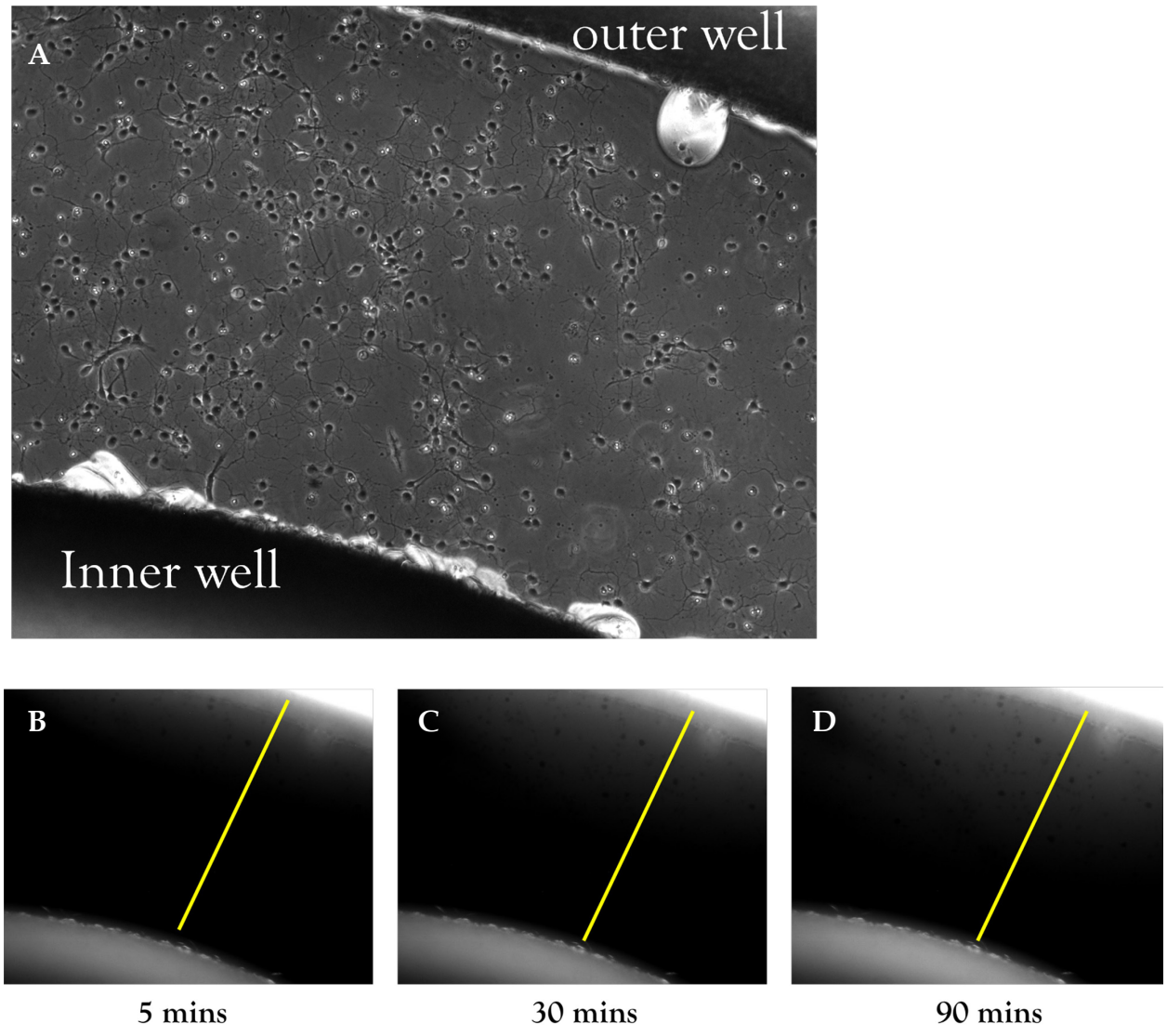

Figure 2.6: Qualitative evidence for the establishment of a fluorescent dextran gradient in the Dunn chamber. *A)* Live image of cortical neurons along the annular bridge to be examined using fluorescence after 1DIV. The inner well contains medium alone; outer well consists of medium + 40 kDa dextran. *B)* After five minutes, a gradient begins to form but is not complete. Note that the outer well in images B-D appears intensely white (strong fluorescence), while the inner well contains no fluorescence. *C)* Gradient stabilizes after 30 minutes and persists until *D)* 90 minutes elapse.

No Turning Observed in WT Cortical Axons in Control Media

As a control, cortical axons obtained from wild type mice were exposed to media only (that is, in the absence of any added guidance cue that would form a concentration gradient). A total of twenty three axons were imaged over five separate experiments. All axons grew and turned stochastically, with an overall mean turning angle (MTA) of 3.24° and a 2.42 standard error of the mean (SEM) (Figure 2.7).

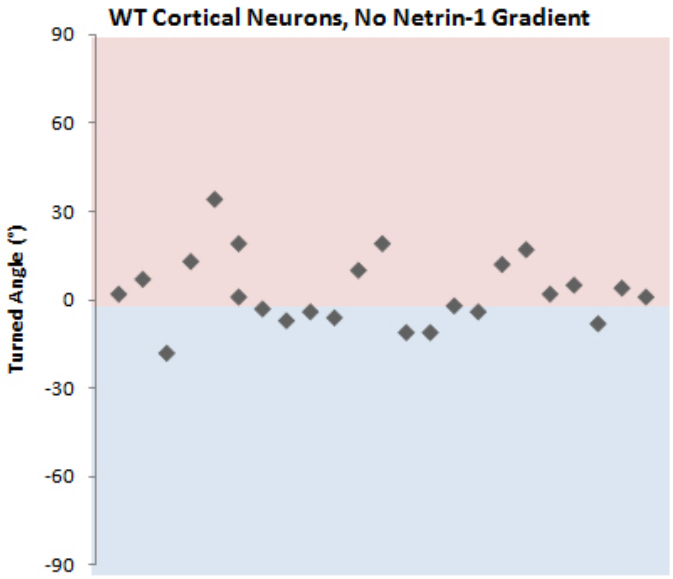

Figure 2.7: Turning angles of twenty-three wild type cortical axons imaged after 1DIV, exposed to a control environment (only conditioned media) and no gradient of netrin-1. The mean turning angle recorded was 3.24° with an SEM of 2.42°. Red represents positive angles greater than 0°, which suggest turning towards the outer well (the source of guidance cue), and angles in the red region, which represents negative angles less than 0°, stand for axons that have turned away from the outer well and towards the inner well.

WT Cortical Axons Turn Toward a Source of Netrin-1

The same Dunn chamber chemotaxis procedure was repeated for cortical axons obtained from wild type E15/16 embryos, except this time, axons were exposed to a gradient of netrin-1. Since all axons being imaged were midway between the source of the gradient and the sink, and since a concentration between 100-200 ng/mL of netrin-1 is typically used in axon guidance assays, 500 ng/mL of netrin-1 were inserted into the outer well to ensure that axons imaged in an intermediate position are exposed to a concentration of netrin-1 estimated to be within the range of 100-300 ng/mL.

Figure 2.8 depicts trajectories for each axon imaged, both before and after rotation. Of 36 axons from six experiments selected for imaging according to the aforementioned criteria, 29 turned positively towards the outer-well (i.e., up the gradient of netrin-1) (Figure 2.9). Of the 7 axons that turned away from the source of netrin-1, only one outlier appears to have turned sharply away. Nevertheless, the MTA of all imaged axons was 19.63°, a significant positive turn ($p = 1.18 \times 10^{-6}$). Therefore, wild type cortical axons are attracted to a gradient of netrin-1.

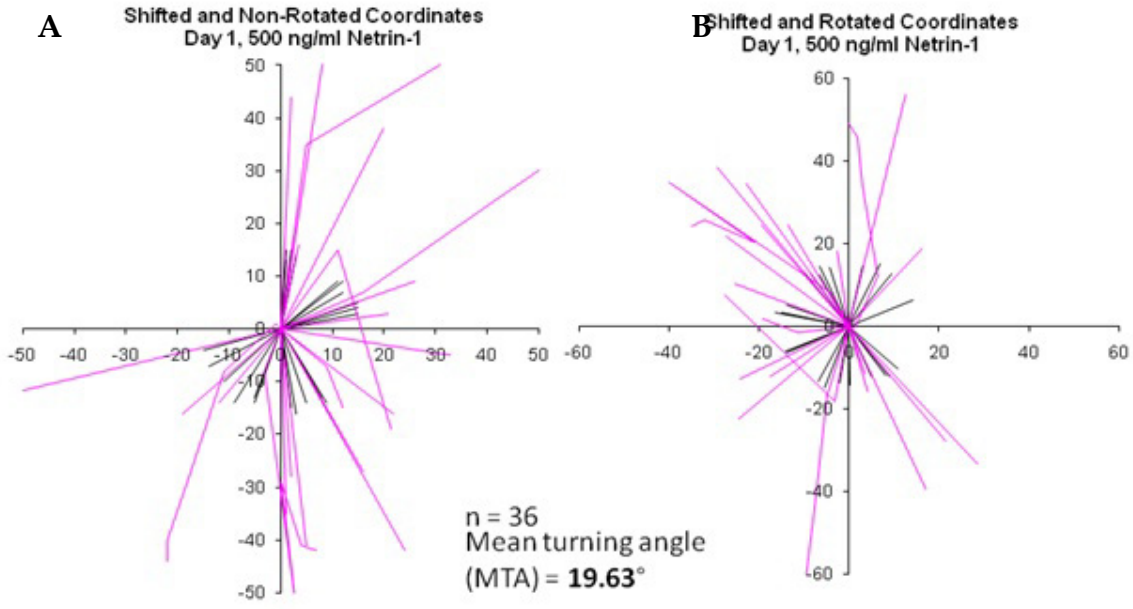

Figure 2.8: Geometric representation of the initial (black lines) and final (fuscia lines) trajectories travelled by each of 36 cortical axons obtained from wild type embryos that were exposed to a gradient of netrin-1. When all trajectories were shifted and rotated such that they are all aligned along the same quantitative plane (panel B), all final trajectories that were in the positive vertical plane represented axons that turned towards the gradient, and all final trajectories that were in the negative vertical axis represented axons that turned away from the gradient. Units along both x and y axes represent displacement of the axons in μm.

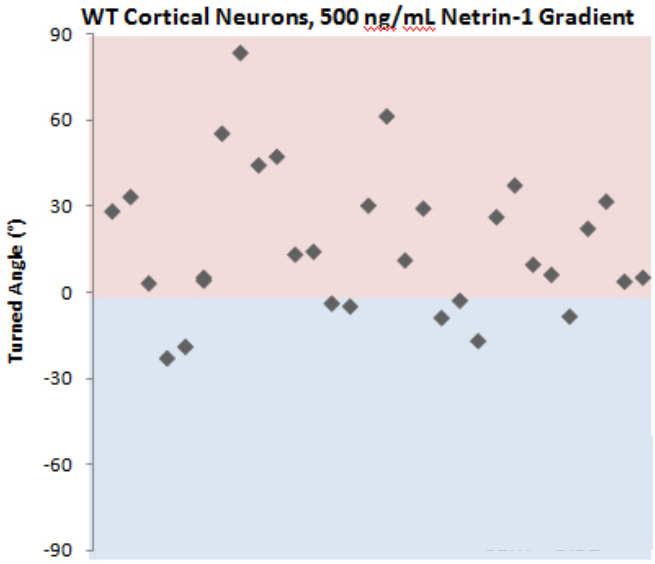

Figure 2.9: Turning angles of thirty-six wild type cortical axons imaged after 1DIV, exposed to a gradient of netrin-1 with a source of 500ng/mL. MTA recorded was 19.63° with a standard error of the mean of 3.32°. Using a Student's t-test to compare the data sets obtained from both experiments using wild-type cortical axons, with or without a gradient of netrin-1, a p-value of 1.18×10^{-6} was obtained, indicating that the differences in these results were statistically significant and did not arise merely due to chance.

No Turning Observed in DCC-/- Cortical Axons in Control Medium

Cortical neurons obtained from age-matched DCC-knockout embryos were next analyzed using the Dunn chamber. As with the wild-type control axons, DCC-knockout axons in this experiment were not presented with netrin-1, and thus, no concentration gradient was formed. As with axons obtained from wild-type embryonic cortical neurons that were not exposed to a gradient of netrin-1, DCC-knockout axons also failed to demonstrate a statistically-significant spontaneous turning response. The MTA calculated for 15 such axons was 6.44°, with a SEM of 3.96°. Using a Student's t-test to compare both wild type and DCC-knockout

embryonic cortical neurons that were not exposed to netrin-1, a p-value of 4.83 x 10^{-1} was obtained, indicating that the difference observed in MTAs between these two datasets was not statistically significant. Indeed, Figure 2.10 illustrates stochastic turning, comparing one axon to another.

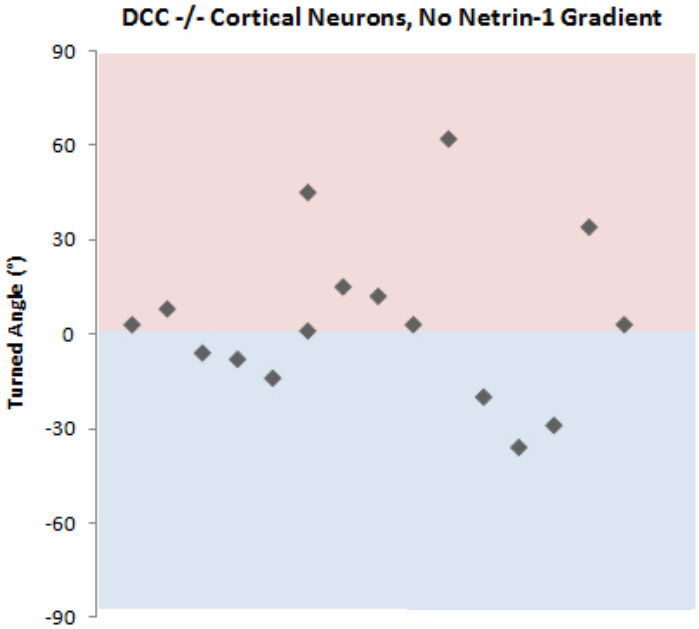

Figure 2.10: Turning angles of fifteen DCC-knockout cortical axons imaged after 1DIV, unexposed to a gradient of netrin-1. MTA recorded was 6.44° with a standard error of the mean of 3.96°. Using a Student's t-test to compare this data set with that obtained from wild-type cortical axons without netrin-1, a p-value of 4.83 x 10^{-1} was obtained, indicating that the differences in these results were statistically insignificant.

DCC-/- Cortical Axons are Repelled by a Gradient of Netrin-1

To test the hypothesis that DCC is not required to mediate netrin-1 induced chemorepulsion, cortical neurons obtained from DCC-knockout mice were exposed to a gradient of netrin-1 and growing axons were analyzed using the Dunn chamber after 1DIV. As with the related wild-type treatment, these axons were exposed to a concentration gradient that peaked at a source of 500 ng/mL.

A total of 19 axons were imaged and their movement was recorded. The MTA calculated for these axons was -11.23°, with a SEM of 4.87°. Compared to the identical treatment for wild-type cortical axons, statistical analysis revealed a significant difference. Using a Student's t-test to compare both data sets, a highly significant p-value of 1.04×10^{-7} was obtained (Figure 2.11).

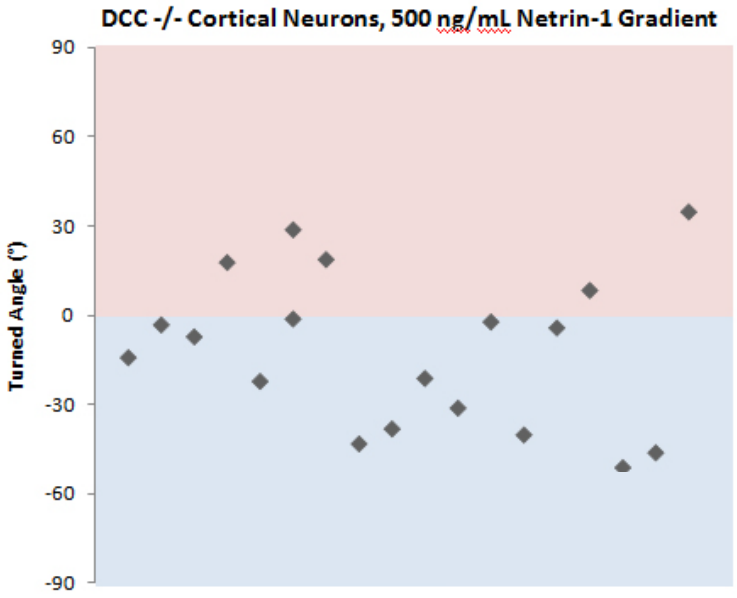

Figure 2.11: Turning angles of eighteen DCC-knockout cortical axons imaged after 1DIV, exposed to a gradient of netrin-1. MTA recorded was 6.44° with a standard error of the mean of 3.96°. Using a Student's t-test to compare this data set with that obtained from wild-type cortical axons without netrin-1, a p-value of 1.04×10^{-7} was obtained, indicating that the differences in these results were statistically significant. However, a more reliable measure of significance is a two-way ANOVA, shown below.

Furthermore, the significance of these results remained when all four conditions were compared against each other using a two-way analysis of variance (ANOVA) (Figure 2.12). Taken together, these results indicate that DCC is not required to mediate netrin-1 induced chemorepulsion.

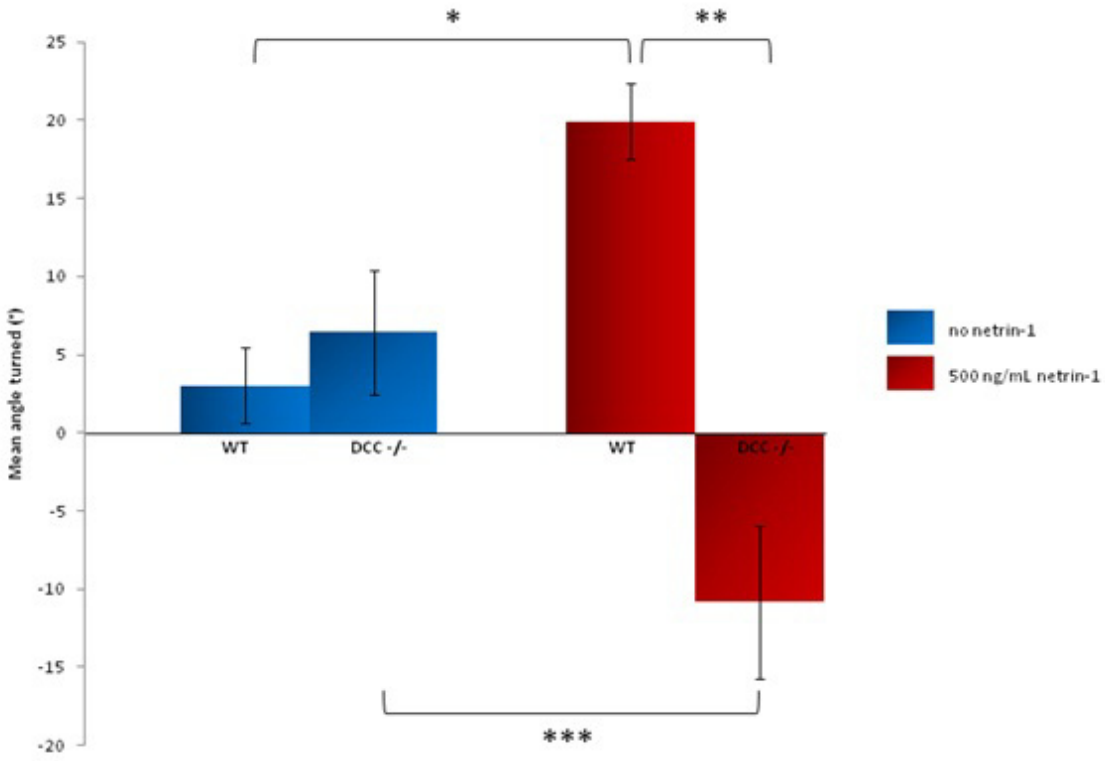

Figure 2.12: Turning responses of axons observed in all treatment conditions. In order to determine whether the differences observed in all four conditions were significant, a two-way ANOVA was applied (given that there were two pairs of different treatment conditions (with and without netrin-1; wild-type and DCC knockout axons). Three significant results emerged. First, the attractive turning response of wild type axons to netrin-1 is significant (* $p = 0.006$). Second, the different turning responses to netrin-1 observed between wild type and DCC-knockout neurons is significant (** $p = 0.001$). Finally, the difference in turning observed in DCC-knockout neurons with or without netrin-1 is significant (*** $p = 0.050$). All results obtained after Tukey correction was applied.

DISCUSSION

Netrin-1 is a chemotropic guidance cue that has the capacity to attract some axons and repel others, depending in part on the receptors engaged. However, the reasons behind these different responses between axons are largely unexplored, and the mechanisms underlying these processes remain unclear. Approximately twelve years ago, Kyonsoo Hong and her colleagues demonstrated that a ligand mediated between the cytoplasmic domains of UNC5 and DCC results in the conversion of netrin-induced chemoattraction to repulsion (Hong et al., 1999). Amongst many intriguing findings, Hong et al. set out to discover whether DCC would be required by UNC5 to mediate repulsion. Since UNC5 is capable of binding netrin on its own, they reasoned it might be plausible for UNC5 to mediate chemorepulsion in the absence of DCC.

To test this hypothesis, they obtained a monoclonal antibody (AF5) that was directed against the extracellular domain of DCC, which would serve to block netrin-induced attraction of *Xenopus* axons (Ming et al., 1997). They found that in the presence of the DCC antibody, netrin-1 had neither an attractive nor a repulsive response – the mean turning angle of axons was close to zero. This led them to conclude that DCC is required to mediate netrin-induced chemorepulsion, in conjunction with UNC5. Since the conclusions reached by these authors and the current findings discussed here are diametrically opposed,

it is important to investigate the inherent differences of both approaches, and attempt to reconcile these seemingly contradictory results.

First, these studies were based on the overexpression of UNC5H2 via ectopic injection of *unc5h2 mRNA* into embryos, allowing spinal neurons to mature and express UNC5H2 on the surface. Second, by injecting an antibody raised against the extracellular domain of DCC, the expected result would be a saturation of all DCC extracellular domains. Although this does not directly prevent netrin-1 from binding to DCC, it could indirectly lead to greater netrin-1 binding to UNC5H2. However, even if this is the case, it is not clear whether the effect of the antibody is to deactivate DCC only, or if it leads to signaling events that also deactivate UNC5 (suggesting that it is some influence of the antibody and not DCC that plays a direct role in deactivating UNC5). Also of note is that a mammalian homologue of UNC5 was expressed in an unnatural environment. Furthermore, only UNC5H2 was expressed in these spinal neurons (as opposed to all four mammalian homologues), which does neither mimics natural conditions for *Xenopus* nor mice (for example, it is known that UNC5H1 is more widely expressed in the mammalian CNS, and perhaps its coexpression with UNC5H2 would have resulted in a significant repellent response. Finally, the turning assay used by Hong et. al has several limitations, including: a) it does not produce a concentration gradient that is stable for many hours; b) it does not

allow for the imaging of multiple axons at once, and as a result; c) it requires a significantly larger number of experiments.

In order directly examine the possibility that UNC5 mediates chemorepulsion independently of DCC, we designed a series of experiments that did not involve the overexpression of any UNC5 homologues. By utilizing cortical neurons obtained from E15/16 DCC-knockout embryos, we ensured that any and all effects of DCC were completely abolished, without introducing any antibodies that may have had additional and unexpected effects of disrupting the function of other endogenous receptors and/or signaling events. Furthermore, by utilizing a turning assay that forms a stable gradient of guidance cue, we avoided any uncertainty about maintaining a consistent concentration of netrin-1 from one axon to another and one experimental set up to another, while having the opportunity to study the turning behavior of several axons simultaneously. Although it is possible that a dose-dependency effect may occur, whereby axons may behave differently depending on whether they are close to the source or the sink of netrin-1, this concern was modestly circumvented by imaging axons that lie along the same circumference. Taken together, these modifications in experimental design allowed us to answer this question more directly and explicitly, and we were surprised to discover that DCC is not absolutely required to mediate netrin-1 induced chemorepulsion.

Although these results confirm that netrin-1 induced repulsive axonal turning can take place in mammalian neurons in the absence of DCC, they do not allow us to conclude that this phenomenon is UNC5-dependent. Importantly, we have not ruled out the participation of another netrin receptor, such as Neogenin, acting either on its own, or as a co-receptor with an UNC5 homologue, in mediating this process. Furthermore, even if this phenomenon is UNC5-dependent, it is unclear whether a single UNC5 receptor is sufficient to mediate this response, or if two receptors homodimerize to transduce the signaling events responsible for repellant turning. Finally, it remains important to delineate the functional differences between the various UNC5 receptors in mammalian neurons, in order to determine whether their ability to mediate repulsion is equivalent or variable. Thus, it would be valuable to systematically knockout members of the UNC5 family, and observe any differences this may cause in terms of affecting chemorepulsion. For example, since UNC5A is highly expressed in cortical neurons, an important experiment would be to study chemotaxis of cortical neurons obtained from UNC5A/DCC double-knockout embryos and see if this leads to an abolishment of turning in the presence of netrin-1.

Nevertheless, the results we have obtained contribute to our developing understanding of netrin-mediated axon guidance in the mammalian central nervous system.

CHAPTER 3:

Investigating a Role for UNC5A in Mossy Fiber Sprouting *in vivo*

INTRODUCTION

Robust axonal sprouting can be induced in an adult mammalian brain by seizure- activity associated with epilepsy, lesion, or simply as a result of aging (Represa and Ben-Ari, 1992; Salin et al., 1995; Esclapez et al., 1999). The sprouting of the axons of the hippocampal dentate granule cells, known as mossy fibers, is of particular interest (Figure 3.0A). In the normal brain, mossy fibers project through the dentate hilus to the CA3 region of the hippocampus (Figure 3.0B) (Blaabjerg and Zimmer, 2007). In the epileptic brain, mossy fibers branch out into axonal collaterals in the hilus of the dentate gyrus. As a result, these collaterals project towards the molecular layer and innervate it, thus, "sprouting" and forming excitatory synapses with granule cells (Buckmaster et al., 2002; Cavazos et al., 2003).

It is believed that mossy fiber sprouting as a result of seizure activity is a consequence of a two-step hyperactivity-induced disruption of axon guidance in neurons (Muramatsu et al., 2010). The first step includes excessive branching of collaterals, followed by the second step of ectopic projection to the molecular layer (Koyama and Ikegaya, 2004)., Koyama et. al suggested that activity-induced brain-derived neurotrophic factor accounts for the sprouting that occurs in the first step (Koyama et al., 2004). To address the second step, they examined the potential involvement of netrin-1. In their study, Muramatsu et al. (2010) reported that enhanced neuronal activity alters netrin-1 cell targeting by

hyperactive axons. Specifically, they postulated that netrin-1 attracts mossy fibers via DCC, while repelling them via cAMP-induced expression of UNC5A, suggesting that *unc5a* knockdown actually rescued this mistargeting (Muramatsu et al., 2010). Another diametrically opposite model postulates that UNC5A serves to restrain mossy fiber sprouting from occurring during development, and therefore, that disruption or loss of UNC5A would lead to aberrant mossy fiber sprouting in the hippocampus as a result of the release of this inhibition**.** In order to determine if UNC5A plays a key role regulating mossy fiber sprouting in vivo, we used histological methods to investigate the consequences of loss of UNC5A function on the projection of mossy fiber axons in the mammalian hippocampus.

UNC5A knockout mice live to adulthood (Williams et al., 2006). Knockouts and wild type littermates were raised to 1 and 4 months of age, and mossy fiber sprouting was then assessed histochemically using the Timm staining method. This histological technique is useful because it precipitates metals in the brain and then detects them using a silver sulfide solution with very high contrast and resolution. Since mossy fibers are very rich in zinc (Sloviter, 1985), this technique is very useful for examining mossy fiber sprouting, both qualitatively and quantitatively.

If the UNC5A knocknout animals exhibited mossy fiber sprouting relative to the wild type animals, these studies would provide evidence obtained *in vivo* that UNC5A expression by neurons in the mature CNS functions to restrain axonal

sprouting and thereby contributes to maintaining normal patterns of synaptic innervation. If no mossy fiber sprouting is observed in UNC5A knockouts compared with wild type littermates, this would suggest that UNC5A is not essential to restrain mossy fiber sprouting at least from the time of development up to four months of age. At this point, it would be useful to investigate the potential role, if any, played by other UNC5 homologues, and whether this role is redundant or novel compared to UNC5A.

The Role of UNC5B-D in Mossy Fiber Sprouting

UNC5A knockout mice continue to express three other UNC5 homologues (UNC5B, C, and D). The precise role and interaction of these receptors is poorly

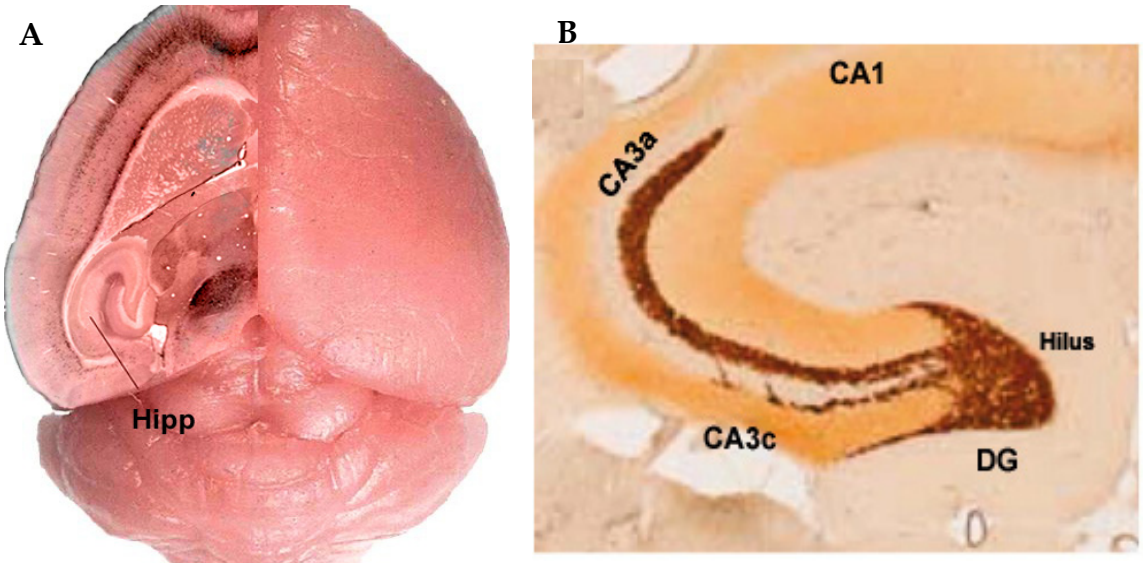

Figure 3.0: A) Axial (horizontal) representation of hippocampus in 4-month old mouse brain. B) Horizontal slice of hippocampus processed using Timm stain. Mossy fibers are richly stained (brown), with cell bodies or axonal bundles originating in the hilus of the dentate gyrus (DG) and projecting onto layer CA3 (divided into proximate CA3c and distal CA3a).

understood, it is plausible that no abnormal mossy fiber sprouting may be detected due to compensation by the other receptors. To address this potential complication, Dr. Dong Han, a post-doctoral fellow in the Kennedy lab, has generated a line of transgenic mice that she named UNC5T, which express a truncated UNC5B receptor chimeric protein composed of the extracellular and transmembrane domains of rat UNC5B, with the intracellular domain of UNC5B replaced by green fluorescent protein. These mice were made using standard transgenesis with random insertion of the sequence encoding UNC5T into the genome. In these mice, cell-type specific expression of UNC5T can be regulated using the Cre-Lox recombination system (Sauer, 1998). The construct generated includes a cyan fluorescent protein (CFP) followed by a stop codon, flanked by two *loxP* sites (Figure 3.1). The sequence encoding the UNC5 extracellular domain (UNCECD) is located downstream of the stop codons in each of the three reading frames. Expression of Cre recombinase causes recombination between the two *loxP* sites, excision of the stop codons, and allows the sequence encoding UNC5ECD-GFP chimera to be transcribed and translated. Cells expressing the chimera can be detected due to the presence of green fluorescent protein (GFP) following recombination. Since UNC5 requires its intracellular domain to transduce the netrin-1 mediated signal, overexpression of UNC5T by these mice is hypothesized to function as a dominant negative competitor for netrin-1 signaling through UNC5 homologues, disrupting the function of all UNC5 homologues expressed in the UNC5T expressing cell.

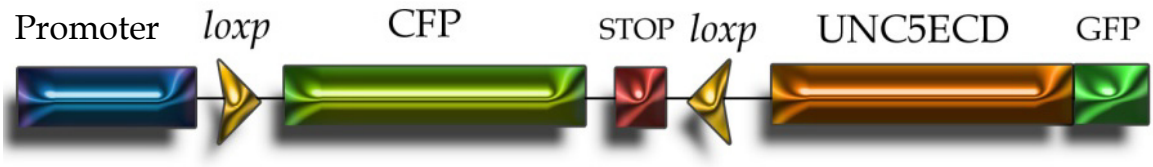

Figure 3.1: Genetic construct for expressing UNC5T. A strong promoter exists upstream of two loxp sites that flank a cyan fluorescent protein and a stop codon. In the absence of Cre recombinase, cyan fluorescent protein is produced. However, when Cre recombinase is expressed, the UNC5ECD is expressed, and can be verified by observing green fluorescence.

MATERIALS & METHODS

Histological Analysis: Timm Staining

All animal work was performed in accordance to the Canadian Council on Animal Care Guidelines. All mice used in this project were born and housed in the Animal Care Facility of the Montreal Neurological Institute. The Timm silver-sulfide staining method takes advantage of mossy fibers being relatively rich in zinc. This technique involves the precipitation of metals by sulfide, followed by deposition of metallic silver on the metal-sulfidegrain (Timm, 1958). In practice, a mouse is perfused transcardially with a buffered solution of sodium sulfide (consisting of sodium sulfide, sodium dihydrogen phosphate and sucrose), followed by cryostat sectioning of axial (horizontal) sections approximately 20 µm thick. The staining is then developed using a solution comprised of silver nitrate, hydroquinone, gum Arabic, and a 2M solution of citrate buffer solution (consisting of citric acid and sodium citrate). Although the Timm stain

indiscriminately detects all insoluble metal sulfides that exist in the brain sections, mossy fibers are extremely rich in zinc, which makes them stain relatively darkly using this method.

The Timm staining technique makes it possible to distinguish metal distributions on a cellular level (Lenglet et al., 1984). It is also worthy to note that this histological method is semiquantitative, since the color intensity of the staining not only depends on the duration of the physical development, but also, on the concentration of the stainable metal (Timm, 1958). To hold the duration of the development constant and thereby make the stain intensity proportional to the concentration of stainable metal, all slices were developed for 40 minutes, which is consistent with the published literature (Li et al., 2004a; Tian et al., 2009). Quantification is typically done by assessing the surface area of stained neurite projections. This is important in order to control for unavoidable qualitative differences in tissue permeability and light contrast, where mossy fiber sprouting may be more subtle in one specimen than another. .

RESULTS

CAMKII Expression Observed

In order to verify that Cre Recombinase is functionally active, Katherine Horn, a Ph.D candidate in the Kennedy lab, crossed T29-1 mice with ROSA26-lacZ mice. In this *CamKIIa-cre* transgenic strain, *cre* is initially restricted to CA1 hippocampal pyramidal neurons, and is first expressed at approximately 2.5 weeks of age (Tsien et al., 1996) . After one month of age, the expression of *cre* is

observed in other regions of the hippocampus, including pyramidal neurons in CA1 and CA3, dentate gyrus granule cells and neurons throughout the neocortex, but not by glial cells (Sonner et al., 2005). By crossing T29-1 mice with ROSA26-lacZ reporter transgenic mice (Soriano, 1999), *cre*-induced recombination takes place and results in cells expressing β-galactosidase (β-gal).

This occurs because ROSA26-lacZ transgenic mice have been engineered to include a reporter gene (lacZ) that is transcriptionally silenced by a floxed stop sequence immediately upstream (Soriano, 1999; Rao and Monks, 2009). However, upon expression of Cre-recombinase in target tissues, the stop sequence is removed by a recombination event, and the β-gal reporter is transcribed. This was histologically confirmed with β-gal staining (Figure 3.2)

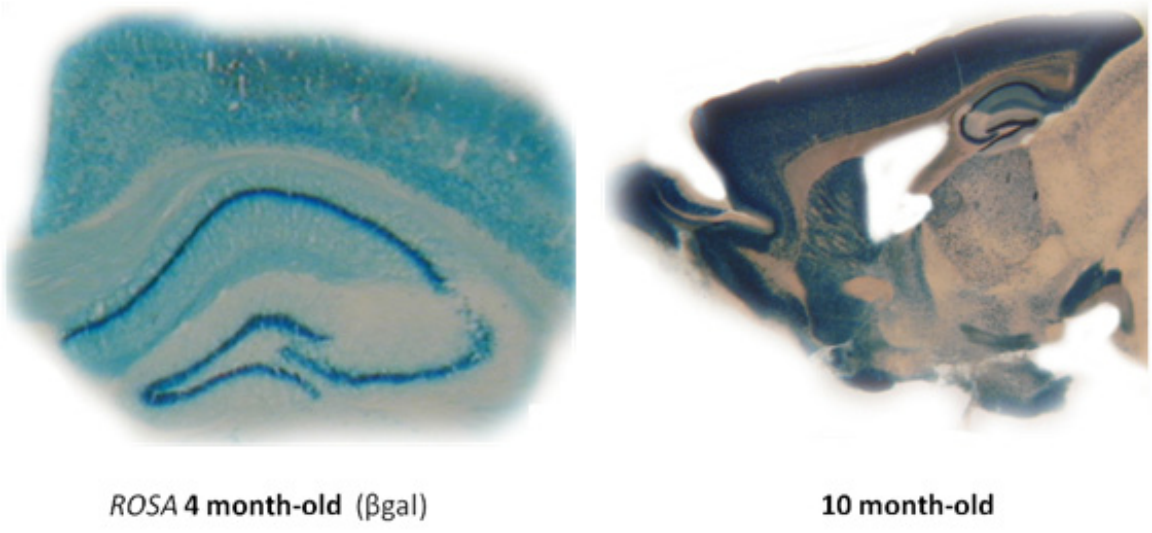

Figure 3.2: β-gal staining of ROSA mice. Courtesy of Katherine Horne.

No Mossy Fiber Sprouting in 4-month-old Wild Type Mice

As a control, hippocampi of 4-month old wild type mice were processed using Timm staining and no mossy fiber sprouting was observed.

Figure 3.3: No mossy fiber sprouting observed in wild type mice 4 months of age (n=3). Three axial sections (one from each mouse) taken from approximately the same point along the dorso-ventral axis are displayed, illustrating appropriate mossy fiber targeting to CA3.

No Mossy Fiber Sprouting in 4-month old UNC5A knockout mice

Next, hippocampi from 4-month old UNC5A knockout mice were processed using Timm staining and no mossy fiber sprouting was observed here as well.

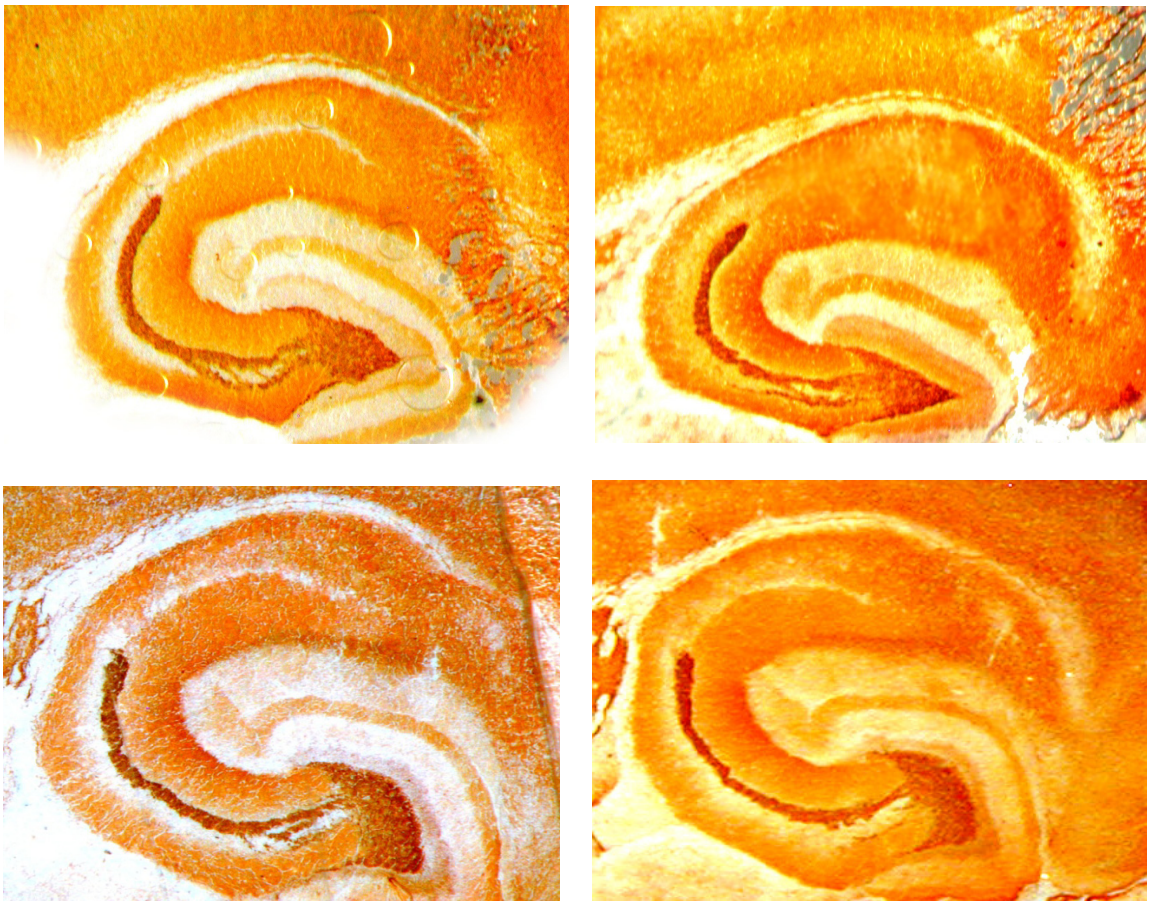

Figure 3.4: No mossy fiber sprouting observed in UNC5A mice 4 months of age (n=4). Four axial sections (one from each mouse) taken approximately along the same dorsoventral axis are displayed, illustrating appropriate mossy fiber targeting to CA3.

No Mossy Fiber Sprouting in 4-month old UNCT knockout mice

Next, hippocampi from 4-month old UNC5T mice were processed using Timm staining and no mossy fiber sprouting was observed.

Figure 3.5: No mossy fiber sprouting observed in UNC5T mice 4 months of age (n=3). Three axial sections (one from each mouse) taken along approximately the same dorsoventral axis are displayed, illustrating appropriate mossy fiber targeting to CA3.

DISCUSSION

There are many factors that lead to mossy fiber sprouting in the hippocampus, including spontaneous seizures (Tian et al., 2009), forced physical exercise (Toscano-Silva et al., 2010), and kindling (Adams et al., 2002). According to Muramatsu et. al (2010), netrin-1 attracts mossy fibers via the DCC receptor, while repelling them via cAMP-induced UNC5A under hyperexcitable conditions, resulting in mossy fiber sprouting. Thus, according to their studies, *Dcc* knockdown does not affect hyperactivity-induced mossy fiber sprouting in slice cultures, whereas *Unc5a* knockdown rescued this mistargetting (Muramatsu et al., 2010).

An easy way to test whether the presence of UNC5A either reduces or promotes mossy fiber sprouting was to examine the effect of the complete loss of UNC5A on the hippocampal projections. Since no mossy fiber sprouting was observed in these mice before 4 months of age, it is reasonable to conclude that UNC5A does not serve to restrain aberrant mossy fiber sprouting that would otherwise take place in its absence, although more quantitative approaches would be useful to confirm this (for example: analysis of the molecular layer of the dentate gyrus, more extensive analysis of mossy fiber projection patterns in CA3, etc). Nevertheless, these results indicate that UNC5A is not essential for the normal projection of axons and synapse formation made by the mossy fibers during development, and that UNC5A is not required for setting up the normal architecture of the hippocampal mossy fiber projections, which appears normal

in the knockouts. However, in order to definitively understand the role played by UNC5A vis-à-vis mossy fiber sprouting, it would be important to compare wildtype and UNC5A mice that have had seizures. This could be done artificially by inducing seizures using kainic acid. Furthermore, in order to test the hypothesis that that continued expression of UNC5A by mature granule cells contributes to maintaining appropriate synaptic architecture by inhibiting sprouting, aged knockouts could be examined - which were developmentally normal when they were young - and see if there is a difference in the sprouting that occurs when they are old.

Although no mossy fiber sprouting was observed in mice overexpressing UNC5T, this result is highly preliminary since the UNC5T mice have not been fully characterized to date. Furthermore, it is not clear whether this construct serves as a dominant negative that simply out-competes endogenous UNC5 homologues for netrin-1, or if it may exert a more broad influence that disrupts other intracellular signaling cascades. Thus, an important step would be to characterize these mice and study the effect that expressing UNC5-TGFP may have on events inside the growing axon.

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