Floor plate derived netrin-1 is an instructive long-range guidance cue for commissural axons in the embryonic spinal cord

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Abstract

Gradients of secreted long-range attractant and repellent proteins have been proposed to be critical to guide growing axons to their proper locations during neural development. In the embryonic spinal cord, commissural axons are guided by various extracellular cues as they extend to the ventral midline. A major midline cue, netrin-1, is expressed by cells in the ventricular zone and floor plate and is essential for commissural axon guidance *in vivo*. Recent studies in embryonic mouse spinal cord and hindbrain have shown that netrin-1 expressed by ventricular zone progenitor cells is necessary for commissural axons to reach the midline. While netrin-1 is essential for commissural axon extension, it is not clear to what extent netrin-1 protein functions as a long-range versus short-range attractant, nor has the functional significance of the distribution of netrin-1 protein as a gradient *in vivo* been identified.

The purpose of this thesis is to determine how the distribution of netrin-1 in the embryonic spinal cord affects commissural axon guidance and identify a specific role for floor plate derived netrin-1. Here, we show a gradient of netrin-1 extending dorsally from the floor plate in early embryonic chick and mouse spinal cord, and demonstrate that a genetic reduction of the amount of netrin-1 expressed in the embryonic mouse spinal cord reduces the steepness of the gradient and results in a graded severity of axon guidance defects at the ventral midline. Further, selective deletion of netrin-1 from the floor plate results in loss of netrin-1 protein within ~200 μm from the midline, and a corresponding flattening of the gradient and altered commissural axon trajectories. In gain-of-function assays, we demonstrate that manipulating the distribution of netrin-1 with ectopic addition of protein to the embryonic spinal cord redirects commissural axon extension. Our findings indicate that netrin-1 secreted by floor plate cells is distributed as a long-range axon guidance cue, and provide strong evidence that the precise

distribution of netrin-1 protein is critical to direct commissural axon extension in the embryonic spinal cord. These studies emphasize the importance of the precise distribution of netrin-1 in the embryonic spinal cord and demonstrate a critical role for floor plate derived netrin-1 in commissural axon guidance.

Résumé

Les gradients de protéines attirantes ou repoussantes sécrétées ont été proposés d'être essentiels à diriger les axones en croissances, afin de permettre celles-ci d'atteindre leurs destinations appropriées pendant le développement neuronal. Dans la moelle épinière embryonnaire, les axones commissuraux sont menées à la ligne médiane ventrale grâce à la diversité de signaux moléculaires extracellulaires retrouvées aux environs. La nétrine-1, une molécule de guidage importante de la ligne médiane, est exprimée par les cellules de la zone ventriculaire et de la plaque du plancher. De plus, celle-ci est essentielle pour le guidage axonal commissural *en vivo*. Des études récentes sur la moelle épinière et le cerveau postérieur de la souris embryonnaire ont démontré que la nétrine-1 exprimée par les cellules progénitrices de la zone ventriculaire est nécessaire pour l'arrivée des axones commissuraux à la ligne médiane. Bien que la nétrine-1 se montre essentielle à l'extension des axones commissuraux, la mesure dans laquelle nétrine-1 exerce son fonctionnement comme signal attractif de longue distance versus à distance courte n'est pas clair. En outre, la signifiance fonctionnelle de la distribution protéique de la nétrine-1 comme gradient *en vivo* n'a pas encore été identifiée.

L'objectif de cette thèse est de déterminer la manière dans laquelle la distribution de la nétrine-1 retrouvée dans la moelle épinière embryonnaire affecte le guidage des axones commissuraux de plus à l'identification d'un rôle spécifique pour la netrine-1 dérivée de la plaque du plancher. Ci-dessous, nous illustrons un gradient de nétrine-1 s'étendant dorsalement de la plaque du plancher dans les embryons de moelle épinière de poussin et de souris. Nous démontrons davantage qu'une réduction génétique quantitative de nétrine-1 exprimée dans la moelle épinière embryonnaire de souris réduit l'inclinaison de la pente du gradient et entraîne des erreurs de guidage des axones à la ligne médiane ventrale avec une sévérité graduelle. De

plus, la délétion sélective génétique de la nétrine-1 de la plaque du plancher entraîne une perte protéique de celle-ci d'environ 200 μm de la ligne médiane et incite une réduction correspondant du gradient et des trajectoires axonales commissuraux modifiées. Dans les expériences de gain de fonction, nous démontrons que la manipulation de la distribution de la nétrine-1 par l'ajout ectopique de protéines à la moelle épinière embryonnaire, redirige l'extension de l'axone commissural. Nos résultats indiquent que la nétrine-1 sécrétée par les cellules de la plaque du plancher est distribuée en tant que signal de guidage axonal à longue distance, et fournissent des preuves qui renforcent l'idée que la distribution précise de la protéine nétrine-1 est essentielle pour diriger l'extension des axones commissuraux dans la moelle épinière embryonnaire. Ces études soulignent l'importance de la distribution précise de la nétrine-1 dans la moelle épinière embryonnaire et démontrent un rôle essentiel pour la netrine-1 dérivée de la plaque du plancher dans le guidage des axones commissuraux.

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Contributions of Authors

Celina Cheung (Kennedy lab, McGill University) – wrote thesis and manuscript, developed rationale, and conducted all experiments and data analysis apart from those mentioned below.

Dr. Timothy Kennedy – supervisor, developed rationale and experimental design, and contributed to and edited manuscript

Karen Lai Wing Sun (Kennedy lab, McGill University) – Figure 5a, b – processing, imaging and analyzing mouse embryos, and figure creation

Stephanie Harris (Kennedy lab, McGill University) – Figure 7b – processing open books and adding netrin-1, imaging, and figure creation

Reesha Raja (Cloutier lab, McGill University) – Figure 3a – processing chick in situ hybridization

Nathalie Marcal (Kennedy lab, McGill University) – lab technician, provided recombinant chick netrin-1 protein

Daryan Chitsaz (Kennedy lab, McGill University) – general experiment advice

Kania lab – incubated chicken eggs and provided dissected chick embryos

Chapter 1: Axon Guidance

Literature Review I

I. Introduction

During embryonic development, axons interact with multiple molecular cues in their environment to form highly reproducible trajectories and find their appropriate targets. Santiago Ramon y Cajal was the first to propose the idea that chemotropism might contribute during neural development – that axons respond to gradients of soluble factors in order to navigate (Tamariz and Varela-Echavarria, 2015). This was later overshadowed by the idea of "contact guidance", proposed by Paul Weiss, that axons are guided in response to mechanical topographic cues on the substrates they are in contact with (Tamariz and Varela-Echavarria, 2015). Roger Sperry then showed that regenerating axons in *Xenopus lavis* followed specific pathways back to their original targets, and provided strong evidence that this was due to the presence of chemical cues (Raper and Mason, 2010). He proposed that axons had chemical tags that would allow them to respond to chemical signals in their environment, and that to reduce the number of cues required they might be presented in the form of a gradient (Tamariz and Varela-Echavarria, 2015). The discovery of the ephrin family of axon guidance cues, which are expressed in a gradient in the optic tectum, later validated his hypothesis (Tamariz and Varela-Echavarria, 2015).

Axon guidance cues are molecules found in the extracellular environment that direct axons along reproducible trajectories to their targets (Martinez and Tran, 2015). The first neurons of the mouse CNS are born around embryonic day (E) 9 (Martynoga et al., 2012). Each neuron then develops a process, known as an axon, that extends through the neuroepithelium. Early pioneer axons form scaffolds for subsequent axons to travel along, and find their targets more efficiently (Raper and Mason, 2010). Axons typically use intermediate targets, which in some cases may be individual guidepost cells, as a means of navigating to their final destination (Raper and Mason,

2010). As axons are extending, guidance cues are already present and pattern the neural epithelium for these axons to interact with (Harris, 1989).

Guidance cues can be classified as either short-range or long-range (Moore, 2006). Short-range cues act in the immediate vicinity of their source whereas long-range cues act at a distance from the cell that secreted the cue (Moore, 2006). For instance, netrin-1 in the embryonic spinal cord is known to have the capacity to travel upwards of 250 μm away from its source (Kennedy et al., 1994). At least three types of mechanisms can contribute to the formation of a gradient by a long-range secreted cue. One mechanism would involve a cue being secreted and remaining soluble as it diffuses away from its source (Kennedy et al., 2006). Alternatively, a secreted cue may become bound to extracellular matrix or cell surfaces (Manitt and Kennedy, 2002). In this case, a gradient may form as a result of such an immobilized cue traveling away from its original source of secretion, diffusing through extracellular spaces, and then being captured, to form a gradient of immobilized protein (Manitt and Kennedy, 2002). Alternatively, following secretion the cue may bind to nearby cells that then either migrate away from the source or move away from the source by subsequent cell division (Manitt and Kennedy, 2002). A combination of long- and short-range cues, acting through both attraction and repulsion, are thought to direct most axons to their ultimate targets (Simpson et al., 2000). Guidance cues can be further classified as permissive or instructive. Permissive cues provide a substrate that promotes axon growth, and although cues may provide different levels of permissivity, they do not direct axon growth.(Moore, 2006) Instructive cues are differentially distributed across a tissue, and the distribution of the cue acts to attract or repel axon extension (Moore, 2006). However, these two principles are not mutually exclusive as an instructive cue can have permissive functions, and it

may be the case that if a permissive cue is distributed as a gradient, it may become instructive (Moore, 2006).

II. The Growth Cone and the Cytoskeleton

Neurons sense the environment through a structure called the "growth cone" at the tip of the axon to navigate through the environment (Moore, 2006). The growth cone has a central and a peripheral domain. The central domain is formed primarily from microtubules, and is relatively stable (Itofusa and Kamiguchi, 2011). The peripheral domain of the growth cone contains actin filaments (F-actin) that form bundles of finger-like projections called filopodia connected with sheet-like actin networks of lamellipodia in between (Coles and Bradke, 2015). This region of the growth cone is typically much more dynamic than the central domain (Gomez and Letourneau, 2014).

The growth cone must be able to react rapidly and effectively to external cues (Gomez and Letourneau, 2014). If the axon encounters an attractive cue, the growth cone will expand, whereas a repulsive cue will cause the growth cone to retract or collapse (Moore, 2006). The response to these cues is achieved through changes to the underlying cytoskeletal structure in the growth cone (Gallo and Letourneau, 2004).

F-actin in the peripheral domain is formed from globular (G-) actin subunits which are connected end to end to form polymers (Alberts, 2015). These subunits have a barbed (+) end and a pointed (-) end, giving the entire structure polarity (Alberts, 2015). F-actin is in a constant state of dynamic instability with G-actin monomers subsequently added to the barbed (+) end, while depolymerization occurs at the pointed (-) end of the filament (Gomez and Letourneau, 2014). At steady state, this results in treadmilling of the actin filaments, where there is a balance of actin monomers being added at the barbed end and loss of monomers from the pointed end (Alberts,

2015). If the filament is not anchored to another protein, the F-actin treadmilling gives the appearance that these filaments are sliding backwards relative to the edge of the cell (Alberts, 2015). In a stalled growth cone, this generates a retrograde flow of F-actin; extension due to the addition of subunits of G-actin to the F-actin at the leading edge is limited by plasma membrane tension which forces the growing filament backwards (Suter and Miller, 2011). At the same time, myosin motors may also pull the actin network backwards (Suter and Miller, 2011). However, when membrane receptors at the tip of the growth cone encounter and bind to an attractive guidance cue, local signalling may increase the rate of actin polymerization and generate attachment sites to F-actin (Gomez and Letourneau, 2014). If the membrane receptor is anchored by binding to an immobilized guidance cue outside the cell, the actin filament then pushes the plasma membrane outward (Dent et al., 2011; Nichol et al., 2016). Such attachment sites may form focal adhesions from which myosin motors can push other bundled actin filaments forward (Nichol et al., 2016).

Guidance cues signal through Rho GTPases to influence the underlying cytoskeletal structure of the growth cone (Samuel and Hynds, 2010). This family of small GTPases are well characterized players in cell polarity and migration (Fukata et al., 2003). They have both an active and inactive form, based on whether they are GTP or GDP bound (Luo, 2000). The switch between these states is mediated by guanine exchange factors (GEFs) and GTPase activating proteins (GAPs), which activate and inactivate Rho GTPases respectively (Luo, 2000). The Rho family members Cdc42 and Rac1 are typically associated with growth cone expansion, while RhoA is associated with collapse (Yuan et al., 2003). Cdc42 is associated with the formation of filopodia, while Rac1 is associated with lamellipodia formation (Alberts, 2015). These will activate actin binding proteins, such as the Arp2/3 complex which promotes formation of branched filament arrays,

extending the actin filament and network (Spillane and Gallo, 2014). On the other hand, RhoA is known to signal through ROCK which increases actin-myosin contractility, thereby causing retraction (Samuel and Hynds, 2010).

Evidence has shown that gradients of Ca^{2+} concentration across the growth cone can cause turning (Akiyama and Kamiguchi, 2015). Calcium released from the endoplasmic reticulum is thought to serve as an attractive signal, while calcium influx from the plasma membrane is repulsive (Akiyama and Kamiguchi, 2015). This can in turn signal to CaMKII and subsequently regulate Rho GTPases (Akiyama and Kamiguchi, 2015). Calcium is also thought to regulate local exocytosis and endocytosis of the plasma membrane at the leading and trailing edge of the growth cone respectively (Tojima et al., 2007). Therefore, spatiotemporal regulation of actin dynamics and membrane trafficking helps mediate growth cone dynamics and turning.

Localized signalling at the trailing edge is also necessary for the growth cone to advance. In migrating cells, extension at the leading edge is carefully coordinated with contraction coupled to deadhesion at the trailing edge (Alberts, 2015). In growth cones, microtubule invasion into the peripheral domain occurs when the retrograde flow of actin is slowed, and begins the process of forming the axon shaft (Zhou and Cohan, 2004). This process consists of three stages: protrusion, engorgement, and consolidation (Suter and Miller, 2011). At the protrusion stage, a corridor is cleared for microtubules to extend into, which occurs in the engorgement stage (Suter and Miller, 2011). This is followed by consolidation, where these microtubules are assembled into bundles, and the actin filaments making up the growth cone are disassembled (Suter and Miller, 2011).

Microtubule dynamics are regulated by microtubule associated proteins (MAPs) and microtubule plus-end tracking proteins (+TIPs) (Liu and Dwyer, 2014). MAPs will bind along the length of

the microtubule, while +TIPs localize to the ends (Liu and Dwyer, 2014). Microtubules are also dynamic polymers, albeit with slower dynamics that makes them more stable than F-actin, and studies have suggested that microtubules and their regulatory proteins also play a role in growth cone turning (Bearce et al., 2015; Mack et al., 2000). Overall, actin and microtubule dynamics cooperate to allow the growth cone to respond to external guidance cues.

III. Axon Guidance Cues and Models of Axon Guidance

a. Netrins

Netrins are extracellular proteins that belong to the laminin superfamily (Fahey and Degnan, 2012). In mammals, netrin-1, netrin-3, and netrin-4 are secreted proteins, while netrin-G1 and netrin-G2 are GPI anchored (Lai Wing Sun et al., 2011). Depending on which receptor they bind to, they can mediate attraction or repulsion of axons. Although first characterized as a guidance cue, netrins also have many other functions both within the CNS and outside of it. They are expressed in the floor plate and ventricular zone, and in the retina, as well as in the heart and many other regions (Kennedy et al., 1994).

The first member of the netrin family, Unc-6, was identified in *C. elegans* (Ishii et al., 1992). Following this, netrin-1 and netrin-2 were purified from embryonic chick, and shown to function as a chemoattractant for embryonic spinal commissural axon outgrowth and turning (Kennedy et al., 1994; Serafini et al., 1994). Netrin-3 was first identified in mouse and during development is highly expressed by dorsal root ganglia neurons, sympathetic neurons, and ventral horn motor neurons (Wang et al., 1999), suggesting that netrin-3 may play a role in axon guidance in the peripheral nervous system (Seaman and Cooper, 2001). Netrin-4 is expressed in the adult rat brain and can bind to the netrin receptors DCC and Unc-5a (Qin et al., 2007; Zhang et al., 2004).

It has been reported to promote neurite outgrowth and play roles in axon guidance as well (Koch et al., 2000; Qin et al., 2007).

Netrin-G1 and netrin-G2 bind to NGL1 and NGL2 respectively, and regulate synapse formation and synaptic plasticity (Matsukawa et al., 2014). They are preferentially expressed in the CNS, with netrin-G1 and netrin-G2 expressed in distinct regions of the CNS (Nakashiba et al., 2002).

b. Slits

Slits are a family of midline repellents expressed in the embryonic spinal cord that prevent axons from recrossing (Kidd et al., 1999). Mammals have 3 slit homologs, and signal through the Roundabout (Robo) family of transmembrane receptor proteins (Blockus and Chedotal, 2016; Brose et al., 1999). In *drosophila*, commissureless (comm) sequesters Robo in an intracellular compartment prior to midline crossing (Keleman et al., 2002; Kidd et al., 1998). Once axons have crossed, Robo is released, preventing axons from recrossing (Blockus and Chedotal, 2016). In mammals, different populations of axons express different combinations of Robo family members, resulting in different lateral positioning in the spinal cord (Rajagopalan et al., 2000). In mammals, studies have suggested that Rig-1, also known as Robo3 functions, somewhat like comm in Drosophila, to suppress Robo1 responsiveness prior to crossing the midline (Sabatier et al., 2004). It was later demonstrated that the Robo3.1 splice isoform is responsible for this silencing, whereas Robo3.2 was involved in slit repulsion postcrossing (Chen et al., 2008). Robo3 has also been shown to bind NELL2, which mediates repulsion from areas such as the motor column of the embryonic spinal cord (Jaworski et al., 2015).

In addition to their function in the spinal cord, slits have been shown to be involved in the development of thalamocortical and callosal projections (Blockus and Chedotal, 2014). Thalamocortical axons express Robos and are repelled by slits, while callosal axons may express

another putative receptor, EVAC1, which may also contribute to Slit-mediated guidance (Jaworski et al., 2015).

c. Morphogens

Sonic hedgehog (SHH), a well-known morphogen that directs the formation and patterning of the neural tube, is also involved in axon guidance (Murdoch and Copp, 2010). It is secreted from the notochord and floor plate during embryonic development, and forms a gradient (Charron et al., 2003). It can elicit commissural axon turning through the Boc receptor, but does not appear to promote outgrowth (Charron et al., 2003; Okada et al., 2006). SHH also influences the trajectories of retinal ganglion cells (RGCs) in the optic tectum (Sanchez-Camacho and Bovolenta, 2008).

Bone morphogenic proteins (BMPs) also influence axon guidance. These are members of the TGF-β superfamily, and canonical BMP signalling will induce transcription in the nucleus and subsequently cell fate specification (Yam and Charron, 2013). In the embryonic spinal cord, BMP7 expressed in the roof plate contributes to repelling pre-crossing commissural axons away from the dorsal midline.(Augsburger et al., 1999) They are secreted as heterodimers with GDF7, and act through the receptor complex BMPRIB:BMPRII (Yam and Charron, 2013).

Wnts are a family of secreted glycoproteins that signal through Frizzled and β-catenin to activate gene transcription and specify cell fate (He et al., 2018). This is known as the canonical pathway of wnt signalling (He et al., 2018). In the embryonic spinal cord, Wnt4 is expressed as a gradient along the antero-posterior axis, and helps guide ascending commissural axons after they cross the ventral midline (Charron and Tessier-Lavigne, 2007). Similarly, Wnt1 and Wnt5a are expressed as a gradient, and repel corticospinal tract axons to direct them along the spinal cord (Charron and Tessier-Lavigne, 2007). This is mediated by the receptor Ryk (Yam and Charron, 2013). On

the other hand, Wnt3 is expressed in the tectum and influences RGC guidance (Charron and Tessier-Lavigne, 2007).

d. Semaphorins

The semaphorin (sema) family of proteins is characterized by the presence of a sema domain at the N-terminal (Alto and Terman, 2017). This domain is also present in their classic receptors, the plexins (Alto and Terman, 2017). Semaphorins cause growth cone collapse by locally disassembling the cytoskeleton and cell adhesion as a result of plexin signalling (Alto and Terman, 2017). Interestingly, the first receptors identified for semaphorins were the neuropilins; however, these appear to be dispensable for axon guidance (Mann et al., 2007). Instead, they function as co-receptors for all class 3 semaphorins, with the exception of sema3E (Alto and Terman, 2017). The first semaphorin identified in vertebrates was sema3A, which was found to induce growth cone collapse of sensory neurons *in vitro* (Mann et al., 2007). Sema3B is expressed in the embryonic floor plate, and has also recently been shown to be taken up by neural progenitor cells from cerebral spinal fluid (Pignata et al., 2016). It is known to regulate midline crossing of commissural axons in the embryonic spinal cord (Pignata et al., 2016). While class 3 semaphorins are secreted, class 4-7 semaphorins are membrane-associated (Yazdani and Terman, 2006). In addition to their role in the nervous system during development and adulthood, semaphorins also have functions in the immune, endocrine, and circulatory systems, amongst others (Alto and Terman, 2017; Pignata et al., 2016). They are widely expressed and play important roles from development extending into maturity (Yazdani and Terman, 2006).

e. Ephrins

Ephrins are membrane-associated proteins capable of both forward and reverse signalling (Xu and Henkemeyer, 2012). They are classified into two groups: class A and class B (Blits-Huizinga

et al., 2004). Ephrin receptors, Ephs, are similarly categorized, with EphA receptors typically binding class A ephrins, and EphB receptors binding class B ephrins (Cramer and Miko, 2016). Although ephrins act as ligands and will initiate signalling downstream of Ephs, they can initiate signalling in the cell they are expressed in as well (Huot, 2004). Signalling in the cell expressing the Eph receptor is termed forward signalling, while signalling in the cell expressing ephrin is known as reverse signalling (Huot, 2004).

A role for ephrins in axon guidance was first described in the optic tectum (Cramer and Miko, 2016). Gradients of ephrin A and Eph A are expressed in the superior colliculus and retinal axons, respectively (Huot, 2004). In this way, axons expressing lower levels of EphA map to regions further along the gradient of ephrin A, as they are repelled to a lesser extent than are axons expressing higher levels of EphA (Huot, 2004). These complementary patterns of expression creates a topographical map along the antero-posterior axis of the optic tectum (Huot, 2004).

Class B ephrins are capable of eliciting commissural growth cone collapse (Kadison et al., 2006). Ephrin B1-3 are expressed in the embryonic spinal cord, with ephrin B3 made by floor plate cells (Kadison et al., 2006). Ephrin B3-mediated forward signalling is necessary for axon guidance at the midline; commissural axons in ephrin B3 and EphB mutant mice exhibit pathfinding errors at the ventral midline (Kadison et al., 2006). Postnatal corticospinal axons expressing EphA4 are also repelled from the midline by ephrin B3.(Yokoyama et al., 2001)

f. Commissural Axon Guidance in the Embryonic Spinal Cord

A classic experimental model to study axon guidance is the mouse embryonic spinal cord, in which a variety of guidance cues have been identified for commissural neurons (Stoeckli, 2018). Commissural neurons are interneurons with axons that cross the midline, and function to transfer

information between the left and right sides of the nervous system (Castellani et al., 2013; Martinez and Tran, 2015). In the spinal cord, there are eight initial subpopulations of dorsal spinal interneurons, of which the dI1 and dI2 populations are known to be commissural (Helms and Johnson, 2003). These are located in the deep dorsal horn, with the roof plate providing factors that are required for their specification (Helms and Johnson, 2003). In mouse, the initial commissural neurons are born at ~E9, and their axons have begun crossing the midline by E10 (Wentworth, 1984). By E19 commissural neurons have become highly heterogenous: including the ventral interneurons, a total of 18 different populations commissural neurons can be distinguished morphologically (Silos-Santiago and Snider, 1992).

As commissural axons begin to extend, they are initially directed by guidance cues secreted by roof plate cells. These are repulsive cues that include BMPs and Draxin (Augsburger et al., 1999). As they extend ventrally, the commissural axon growth cones encounter attractive cues provided by the floor plate (Nawabi and Castellani, 2011). Floor plate-derived cues include netrin-1, SHH and VEGF (Nawabi and Castellani, 2011). At the same time, they are repelled from the motor column by NELL2 (Ducuing et al., 2019). As commissural axons cross the floor plate, they lose their initial attraction to the midline cues, and are instead repelled by slits (Nawabi and Castellani, 2011). There is also evidence that sema3B and SCF also promote the exit of commissural axons from the floor plate (Nawabi and Castellani, 2011). After passing the floor plate, commissural axons then turn longitudinally and travel rostrally (Aviles et al., 2013). Opposing gradients of Wnt and SHH cooperate to attract and repel, respectively, commissural axons along the spinal cord toward the brain (Aviles et al., 2013).

Chapter 2: Netrin-1

Literature Review II

I. Netrin-1

In the late 1980's, the floor plate was shown to secrete a diffusible factor that could elicit outgrowth and alter the direction of commissural axon extension (Tessier-Lavigne et al., 1988). Floor plate explants were shown to elicit axon outgrowth from explants of the dorsal embryonic spinal cord, and could also reorient commissural axon trajectories in intact spinal cords, deflecting them towards an exogenous floor plate explant on their ventral edge up to ~250 um away (Placzek et al., 1990; Tessier-Lavigne et al., 1988). It was later shown that a key floor plate derived protein that promotes outgrowth and turning is netrin-1 (Bin et al., 2015; Kennedy et al., 1994; Serafini et al., 1996).

Netrin-1 was first purified from homogenates of embryonic chick brain through a number of steps, including affinity chromatography on a heparin column (Serafini et al., 1994). These high salt netrin-1 extracts were capable of promoting axon outgrowth, similar to high salt extracts from the floor plate (Serafini et al., 1994). The proteins responsible for these activities were then purified and partial amino acid sequences obtained (Serafini et al., 1994). Cloning the corresponding cDNAs revealed two proteins, netrin-1 and netrin-2, both of which were homologous to UNC-6, a secreted axon guidance protein that had been recently identified in *C. elegans* (Serafini et al., 1994).

Netrin-1 is a secreted extracellular matrix protein that is a member of the laminin superfamily (Lai Wing Sun et al., 2011). Specifically, the amino terminal domain VI, and domain V, composed of EGF-like repeats in the middle of the protein sequence, are named for their homology with the domains found at the amino terminal end of laminins (Serafini et al., 1994). The C-terminal domain of netrins has limited sequence similarity to tissue inhibitors of metalloproteinases (TIMPs) (Finci et al., 2014; Koch et al., 2000). Netrin-1 is expressed in both

the developing and mature CNS, as well as in other tissues in the body (Lai Wing Sun et al., 2011). Aside from its originally described role as an axon guidance molecule, other functions for netrin-1, such as synapse formation and plasticity, regulating cellular adhesion and directing angiogenesis, have also been demonstrated (Lai Wing Sun et al., 2011).

II. Netrin-1 Receptors and Downstream Signalling

The first identified receptor for netrin-1 was deleted in colorectal cancer (DCC), which was originally identified as a candidate tumour suppressor (Fearon et al., 1990; Keino-Masu et al., 1996). It is homologous to UNC-40 in *C. elegans* and frazzled in drosophila (Keino-Masu et al., 1996). DCC primarily mediates chemoattraction, but also contributes to in repulsion in response to netrins (Lai Wing Sun et al., 2011). The main receptors involved in repulsion are the UNC-5 homologue family, which consists of Unc5A, B, C and D in mammals (Lai Wing Sun et al., 2011). Chemorepulsion to netrin-1 may occur with or without co-expression of DCC (Lai Wing Sun et al., 2011). Additionally, neogenin, a member of the DCC family, and DSCAM, which was originally discovered due its role in Down syndrome, are also involved in netrin-1 mediated attraction (Lai Wing Sun et al., 2011).

In chemoattractive responses, netrin-1 binds and dimerizes DCC to activate multiple downstream signalling pathways (Lu et al., 2004). Activation of Fyn and FAK lead to regulation of RhoGTPases (Lai Wing Sun et al., 2011; Lu et al., 2004). Netrin-1 recruits Fyn to the DCC intracellular domain, which is thought to lead to activation of Rac1 and Cdc42 (Meriane et al., 2004; Shekarabi et al., 2005). At the same time, netrin-1 signalling through DCC may result in inhibition of RhoA (Moore et al., 2008). Netrin-1 binding also induces the formation of a DCC-NCK1-FAK signalling complex (Finci et al., 2015; Lai Wing Sun et al., 2011). This complex will regulate SFK signalling, RhoGTPase activity, and release of calcium stores, and structural

changes in F-actin (Lai Wing Sun et al., 2011). In addition, NCK1 functions as an adaptor to recruit PAK1, linking NCK1 to the activation of the RhoGTPases Rac1 and Cdc42(Bagrodia and Cerione, 1999; Shekarabi et al., 2005). Netrin-1 also acts through the MAP kinase pathway, with ERK-1/2 recruited to the DCC intracellular domain (Forcet et al., 2002). This may play a role in the regulation of transcription and protein synthesis (Forcet et al., 2002). Netrin-1 binding DCC also promotes PIP_2 synthesis (Lai Wing Sun et al., 2011; Xie et al., 2006). Phosphorylation of PIP_2 to generate PIP_3 may enhance RhoGTPases binding to their effectors (Di Paolo and De Camilli, 2006). On the other hand, PIP_2 hydrolysis into DAG and IP_3 activates PKC and subsequently triggers Ca^{2+} release from intracellular stores (Logan and Mandato, 2006).

The signalling pathways downstream of UNC-5 homologue family members are less well characterized. Heterodimers of UNC-5 and DCC can recruit Src and FAK upon netrin-1 stimulation, causing UNC-5 to be phosphorylated (Boyer and Gupton, 2018). This phosphorylation is necessary for netrin-1 mediated chemorepulsion to occur (Boyer and Gupton, 2018).

III. Netrin-1 Function

Netrin-1 has important functions inside and outside the nervous system. Similar to its role in axon guidance, netrin-1 guides cell migration. There are multiple examples of this throughout the nervous system. During neural tube formation, netrin-1 signalling through neogenin contributes to neural fold migration in *Xenopus lavis* (Kee et al., 2013). Later in development, netrin-1 repels optic nerve glial precursors at the optic chiasm, as well as oligodendrocyte precursors in the ventral ventricular zone of the spinal cord (Tsai and Miller, 2002). Floor plate netrin-1 is also required for inferior olivary neuron migration, allowing the cell bodies to cross the floor plate (Marcos et al., 2009). During cerebellar development, neurons in the lower rhombic lip are

attracted to netrin-1 while granule cell progenitors in the upper rhombic lip are repelled (Alcantara et al., 2000). In the PNS, netrin-1 also induces Schwann cell migration through p38 MAPK and PI3K-Akt signalling pathways (Lv et al., 2015). Outside the nervous system, netrin-1, in conjunction with Slit, plays a role in cardioblast migration during *drosophila* heart development (Raza and Jacobs, 2016). The two proteins influence leading edge filopodial and lamellipodial activity during cardioblast migration towards the dorsal midline (Raza and Jacobs, 2016).

Roles for netrin-1 in cell adhesion have also been demonstrated. During development of the mammary gland in mouse, netrin-1 is required for cell-cell adhesions, through its receptor neogenin, notably functioning in cap cells at the terminal end buds to adhere them to the prelumenal epithelium beneath (Srinivasan et al., 2003). Evidence has also been provided for interactions between netrin-1 and integrins in the pancreatic epithelium (Yebra et al., 2003). Epithelial Integrins α6β4 and α3β1 have been argued to function as receptors for netrin-1 (Yebra et al., 2003). In the developing pancreas, these integrins colocalize with netrin-1 (Yebra et al., 2003). Further, α6β4 binds to a region of the netrin-1 C-terminus, and is thought to mediate epithelial cell adhesion to netrin-1 (Yebra et al., 2003). α3β1 may also have a role in netrin-1 mediated cell migration (Yebra et al., 2003).

Netrin-1 has an important well documented role in angiogenesis, with current evidence suggesting that in different contexts it may function as a pro- or anti-angiogenic factor (Castets and Mehlen, 2010). Angiogenesis involves the breakdown of the basement membrane followed by migration and proliferation of endothelial cells to form new blood vessels (Tu et al., 2015). Unc5b in endothelial cells interacts with netrin-1 (Tu et al., 2015). One study showed that netrin-1 will cause endothelial filopodia expressing unc5b to retract, while another study demonstrated

that unc5b promoted placental arteriogenesis (Lu et al., 2004; Navankasattusas et al., 2008). Netrin-1 and Unc5b were also implicated in the progression of atherosclerotic plaques by preventing macrophage migration and attracting coronary artery smooth muscle cells (Bongo and Peng, 2014).

Interestingly, its roles in cell migration, adhesion, and angiogenesis suggest a role for netrin-1 in cancer. In fact, it has been implicated in several forms of epithelial cancer, including breast cancer, pancreatic duct adenocarcinoma, as well as cancer in the nervous system, notably glioblastoma and medulloblastoma (Ylivinkka et al., 2016). Levels of netrin-1 protein have been found to be elevated in many cancers, while DCC and UNC-5 homologue expression were decreased in some cancers (Kefeli et al., 2017). Netrins are also argued to have an anti-apoptotic function, along with arguments that they regulate somatic cell reprogramming (Ozmadenci et al., 2015). These findings provide evidence that imbalances between netrin-1 and DCC affect reprogramming efficiency and apoptosis, while cleavage of the DCC intracellular domain has been suggested to induce apoptosis (Ozmadenci et al., 2015). Additionally, reduced levels of DCC are argued to increase reprogramming efficiency (Ozmadenci et al., 2015). In the semicircular canal of the vestibular system, netrin-1 prevents apoptosis (Nishitani et al., 2017). Specifically, when overexpressed, netrin-1 prevents the apoptosis necessary for canal formation (Nishitani et al., 2017). Netrin-1 has also been found to prevent inappropriate lateral branching in the stalk region of the lung epithelium (Liu et al., 2004).

Netrin-1 also exerts important effects on neurons after axon guidance. Netrin-1 increases axon and dendrite arborization (Goldman et al., 2013). In cultures of E16 hamster cortical neurons, netrin-1 elicited DCC-mediated axon branching, suggesting a shift in netrin-1 function from axon guidance to axon branching with age or cell type (Matsumoto and Nagashima, 2017).

Netrin-1 induced release of Ca^{2+} is essential for netrin-1 dependent axon branching, and these Ca^{2+} transients coincide with branch formation (Tang and Kalil, 2005). In the dendrites of tectal neurons, application of netrin-1 causes rapid reorganization of dendritic arbors (Nagel et al., 2015). Dendrites showed more branching, and decreased stability (Nagel et al., 2015). Netrin-1 is also enriched at synapses, and increases the number and strength of excitatory synapses of developing cortical neurons (Goldman et al., 2013). A local source of immobilized netrin-1 recruits synaptic proteins during development, and both netrin-1 and DCC are enriched at mature synapses (Glasgow et al., 2018). Activity-dependent secretion of netrin-1 regulates synaptic transmission and synaptic plasticity in the hippocampus (Glasgow et al., 2018). In this context, netrin-1 promotes the accumulation of GluA1 containing AMPA receptors at the synapse in an NMDAR-independent manner (Glasgow et al., 2018).

Oligodendrocytes also require netrin-1 for normal development and maturation. Netrin-1 serves as a repellent for oligodendrocyte precursor cells (OPCs) in the embryonic spinal cord (Jarjour et al., 2003). In this context, the gradient that attracts commissural axons also repels migrating OPCs away from the midline (Jarjour et al., 2003). Netrin-1 also appears to contribute to OPC migration after these cells reach the nascent white matter (Tsai et al., 2006). OPCs injected into the developing white matter of netrin-1 null mice failed to migrate radially and expand (Tsai et al., 2006). The response to netrin-1 switches as oligodendrocytes mature, with netrin-1 protein promoting process elaboration via a mechanism dependent on netrin-1 inhibiting RhoA in mature oligodendrocytes (Rajasekharan et al., 2010). After differentiation, oligodendrocytes express both netrin-1 and DCC, which promote myelin-like membrane extension (Rajasekharan et al., 2009). This pathway involves Fyn, FAK and N-WASP (Rajasekharan et al., 2009). In the mature

CNS, netrin-1 promotes the maintenance of paranodal junctions by myelinating oligodendrocytes (Jarjour et al., 2008).

Overall, proteins that were initially identified as guidance cues have now been shown to have multiple roles during development. Netrin-1, and many other classic guidance cues, demonstrate widespread functions outside their initially derived roles in axon guidance, extending beyond nervous system development to make many functional contributes in tissues other than the CNS and PNS.

IV. Roles of Netrin-1 in Chemoattraction

Netrin-1 was first characterised for its role guiding commissural axons to the ventral midline of the embryonic spinal cord. *In situ* hybridization analysis revealed that it is expressed by floor plate cells at all axial levels (Kennedy et al., 1994; Serafini et al., 1994). Since the majority of netrin-1 protein initially isolated from chick embryos was associated with cellular membranes in whole brain homogenates, the question of whether netrin-1 could diffuse and act as a long range cue was of particular interest. To test this, Kennedy et al. used COS cells engineered to express and secrete netrin-1, and discovered that netrin-1 was able to effectively elicit axon outgrowth from dorsal spinal cord explants co-cultured in a collagen gel at a distance of up to several hundred microns (Kennedy et al., 1994). Similarly, aggregates of COS cells expressing netrin-1 placed next to an explanted intact spinal cord also reorient commissural axon trajectories, suggesting a role in commissural axon guidance (Kennedy et al., 1994). Further, assays using retinal ganglion cell axons demonstrated that a gradient of netrin-1 released from a micropipette could cause a growth cone to turn up the gradient, and gradients of netrin-1 protein established

using microfluidic chambers or immobilized on a substrate, also reoriented axon extension (Mai et al., 2009; Serafini et al., 1996; Sloan et al., 2015).

Kennedy et al. (2006) reported the distribution of netrin-1 mRNA and protein in the embryonic chick spinal cord (Kennedy et al., 2006). Only floor plate cells at the ventral midline express netrin-1 mRNA in the early embryonic chick spinal cord (Kennedy et al., 2006). In contrast to this restricted distribution of netrin-1 mRNA, netrin-1 protein was detected in a graded distribution across the neuroepithelium, a substantial distance dorsal to the floor plate cells (Kennedy et al., 2006). A similar distribution of netrin-1 protein was detected in the embryonic mouse spinal cord, despite netrin-1 being produced by both floor plate cells and ventricular zone cells (Kennedy et al., 1994; Kennedy et al., 2006).

Recent studies have brought two key questions to the forefront of the field: does netrin-1 function as a long range cue, and, what is the functional contribution of floor plate derived netrin-1 to commissural axon guidance to the ventral midline. Several recent papers have claimed that floor plate-derived netrin-1 is not necessary for commissural axon guidance, and suggest a critical role for ventricular zone-derived netrin-1 functioning as a short range guidance cue (Dominici et al., 2017; Varadarajan et al., 2017). In these studies, they genetically deleted netrin-1 from floor plate cells and from ventricular zone cells in both hindbrain and spinal cord (Dominici et al., 2017; Varadarajan et al., 2017). They demonstrate that ventricular-zone derived netrin-1 is essential for commissural axon guidance to the ventral midline, whereas selective deletion of netrin-1 from the floor plate does not prevent commissural axons from projecting to the ventral midline (Dominici et al., 2017; Varadarajan et al., 2017). These papers argued that floor plate derived netrin-1 does not function as a long range cue (Dominici et al., 2017; Varadarajan et al., 2017).

These specific questions were examined by several studies in the past. Early experiments were carried out using the Danforth's short-tail mouse, a line of mutant mice that lack segments of notochord, and as a result, lack the adjacent sections of the floor plate (Bovolenta and Dodd, 1991). These studies showed that in the absence of a floor plate in a segment of spinal cord, commissural axons still follow a circumferential trajectory along the edge of the spinal cord to the ventral midline (Bovolenta and Dodd, 1991). Having crossed the midline, the axons then inappropriately continue to extend in a dorsal-circumferential direction along the edge of the spinal cord on the contralateral side, or exit the spinal cord (Bovolenta and Dodd, 1991). In contrast, axons within ~120 μm of an intact segment of floor plate reorient their extension and reroute towards that floor plate (Bovolenta and Dodd, 1991). This finding provided early *in vivo* evidence that the floor plate secretes a long-range attractant for commissural axons. A subsequent experiment, performed by Matise et al., examined the effect of genetic removal of the floor plate in $Gli2^{-/-}$ mice, which lack Shh downstream signaling, and therefore floor plate cells are not induced by Shh secreted by the notochord (Matise et al., 1999). Like the Danforth short-tail mouse, commissural axons extend to and cross the ventral midline, however, their trajectories become disorganized in the vicinity of the floor plate and they fail to form a commissure (Matise et al., 1999). Very few axons manage to turn longitudinally (Matise et al., 1999). Interestingly, Shoja-Taheri et al. provided evidence arguing that in mouse hindbrain netrin-1 may not be required for commissural axon guidance to the midline, but is essential for guidance postcrossing (Shoja-Taheri et al., 2015). These findings suggest that different regions of the hindbrain along the rostral-caudal axis may have different requirements for netrin-1. Overall however, the findings emphasize the critical role of the floor plate and netrin-1 produced

by floor plate cells to guide commissural axons in relation to the midline, with evidence for roles before, during and after crossing.

Model systems outside of rodents have presented similar findings. In zebrafish, removal of the floor plate through either genetic manipulation or laser ablation induced commissural pathfinding errors at the ventral midline (Greenspoon et al., 1995). However, in spite of this deletion, some axons still correctly crossed (Greenspoon et al., 1995). Ablation of both the floor plate and notochord increased the number of errors, suggesting that the notochord also contributes to commissural axon guidance, and may secrete a diffusible cue (Greenspoon et al., 1995). Similarly, in *Xenopus lavis* embryos lacking both the notochord and floor plate, axons cross the midline at abnormal angles and make errors when turning to project longitudinally, with many instead turning caudally (Clarke et al., 1991). Similar to what has been described in the Danforth short tail mouse, after crossing the midline, axons continue to grow circumferentially, or exit the spinal cord (Clarke et al., 1991). As such, floor plate-derived netrin-1 appears to be essential for proper midline crossing and subsequent turning to extend longitudinally.

A study in the *drosophila* visual system provides an example of long-range netrin not being required for axon guidance, but instead promoting short-range adhesion (Akin and Zipursky, 2016). This study showed that axons lacking Frazzled, the drosophila orthologue of DCC, successfully reach their ultimate synaptic target layer but do not form stable adhesions (Akin and Zipursky, 2016). Another study carried out in *drosophila* engineered the expression membrane-tethered netrin and demonstrated that it is sufficient to guide growth cones to the midline and form a commissure, again providing evidence that short-range function of netrin is sufficient for midline guidance in this context (Brankatschk and Dickson, 2006). The same paper also

demonstrated that these membrane tethered netrins were incapable of repelling unc-5 expressing axons located further away, demonstrating an essential contribution of long-range secreted netrin protein (Brankatschk and Dickson, 2006). These experiments that genetically altered the distribution of netrin protein, provide strong evidence that netrins function via short- and long-range mechanisms *in vivo* in different circumstances during development of the *Drosophila* nervous system.

Overall, these experiments suggest that, while netrin-1 protein is capable of being secreted, it is not clear to what extent floor plate derived netrin-1 is distributed *in vivo* as a long-range cue, and it is not clear to what extent a long-range distribution of netrin-1 is required for commissural axon guidance. Addressing the functional requirement, floor plate derived netrin-1 was shown in the mouse spinal cord to influence commissural axon trajectories in the ventral spinal cord and impact midline crossing. Wu et al. demonstrated that mice that specifically lack netrin-1 expression only from floor plate cells exhibit altered commissural axon trajectories, with increased lateral deviation from the midline and invasion into the ventral horn motor column (Wu et al., 2019). The laterally displaced commissural axon bundles form a U-shaped trajectory, as opposed to their wild type counterparts which have a more V-shaped trajectory (Wu et al., 2019). These animals also have a reduced commissure thickness (Moreno-Bravo et al., 2019; Wu et al., 2019). Moreno-bravo et al. further provide evidence that commissural axons in the absence of netrin-1 exhibit difficulty exiting the floor plate (Moreno-Bravo et al., 2019). Axons tended to linger in the floor plate, with some axons remaining on the ipsilateral side of the spinal cord (Moreno-Bravo et al., 2019). Other errors included axons making aberrant crossings, with the floor plate entry and exit points not aligning along the longitudinal axis (Moreno-Bravo et al., 2019). Together, these findings suggests a role for the floor plate in commissural axon guidance,

particularly in the ventral spinal cord, directing axons to break away from the lateral edge in the dorsal portion of the spinal cord, and as they cross the midline.

The role of the floor plate and floor plate derived netrin-1 has long been disputed, with recent studies both adding to the controversy and shedding new light on their functions. Here, we set out to examine the role of floor plate derived netrin-1 in the embryonic spinal cord. We examine whether netrin-1 patterns in the spinal cord indicate that it is a travelling as a long range cue. We also further investigate the effects of removing netrin-1 from the floor plate on commissural axon trajectories.

Chapter 3: Floor plate derived netrin-1 is an instructive long-range guidance cue for commissural axons in the embryonic spinal cord

in preparation

Floor plate derived netrin-1 is an instructive long-range guidance cue for commissural axons in the embryonic spinal cord

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ABSTRACT

Gradients of secreted long-range attractants and repellents are proposed to guide growing axons to their targets during development. Netrin-1, a major midline cue that is essential for commissural axon guidance in the embryonic spinal cord, is expressed by cells in the ventral ventricular zone and floor plate. Netrin-1 is essential for commissural axon extension; however, it is not clear to what extent netrin-1 protein functions as a long-range versus short-range attractant, nor has the functional significance of the distribution of netrin-1 protein as a gradient *in vivo* been identified. Here, we address how the distribution of netrin-1 protein in the developing spinal cord influences axon guidance. In early embryonic chick spinal cord, netrin-1 is expressed only by floor plate cells, yet a gradient of netrin-1 protein extends ~100-200 μm dorsal of the floor plate, exemplary of a long-range cue. In the embryonic mouse spinal cord, we show that genetic reduction of the amount of netrin-1 expressed reduces the steepness of the gradient and results in a graded severity of axon guidance defects at the ventral midline. Further, selective deletion of netrin-1 from the floor plate results in loss of netrin-1 protein within ~200 μm from the midline, and within the same distance, remarkable flattening of the gradient and altered commissural axon trajectories. In gain-of-function assays, we demonstrate that manipulating the distribution of netrin-1 with ectopic addition of protein to the embryonic spinal cord redirects commissural axon extension. Our findings indicate that netrin-1 secreted by floor plate cells is distributed as a long-range axon guidance cue, and provide strong evidence that the precise distribution of netrin-1 protein is critical to direct commissural axon extension in the embryonic spinal cord.

INTRODUCTION

Commissural axons in the embryonic spinal cord pioneer a circumferential trajectory to the ventral midline of the neural tube in response to multiple cues (Colamarino and Tessier-Lavigne, 1995). In the late 1980's, the floor plate was shown to secrete a diffusible factor that could elicit outgrowth from explants of dorsal embryonic spinal cord and alter the direction of commissural axon extension over a distance of up to ~ 250 μm (Placzek et al., 1990; Tessier-Lavigne et al., 1988). Netrin-1 was subsequently identified as the floor plate derived protein responsible for this effect on commissural axons (Kennedy et al., 1994; Serafini et al., 1994).

Netrins are a small family of secreted proteins that are members of the larger laminin superfamily. They can act as attractants or repellents, depending in part on the receptors expressed by the responsive cell (Lai Wing Sun et al., 2011). Receptors for netrin-1 in vertebrates include DCC, the DCC homologue neogenin, and the UNC5 homologues, UNC5A-D. DCC is required for chemoattractant responses to netrin-1, while the UNC5 homologues mediate chemorepulsion (Lai Wing Sun et al., 2011). DCC is expressed by commissural neurons, and mice lacking either DCC or netrin-1 exhibit major defects in commissural axon projections, with many axons failing to reach the floor plate at the ventral midline (Bin et al., 2015; Fazeli et al., 1997; Serafini et al., 1996).

In situ hybridization analysis revealed that netrin-1 is expressed by floor plate cells at all axial levels of the spinal column (Kennedy et al., 1994; Serafini et al., 1994). The initial purification of netrin-1 from homogenates of embryonic day 10 chick brains found that although netrin-1 is a secreted protein, the vast majority of netrin-1, over 95%, was present in a high speed pellet containing cell membranes, and that extraction from this pellet required high concentrations of salt, above 1.2 M NaCl. Further, the purification of netrin-1 utilized a number of protein

fractionation steps, including high affinity binding to a heparin column (Serafini et al., 1994), raising the possibility that extracellular netrin-1 may be bound by glycosaminoglycans (GAGs) on cell surfaces or in the extracellular matrix (ECM) (Baker et al., 2006). The tight association of the majority of netrin-1 protein with cellular membranes in whole brain homogenates immediately raised questions related to the capacity of netrin-1 to diffuse within the neural epithelium, its distribution as a short-range or long-range cue, and the possibility that it might function as a soluble or immobilized cue to direct axon extension (Kennedy et al., 1994).

Subsequent studies of netrin function during development now support function for both short-range and a long-range distributions (Brankatschk and Dickson, 2006; Kennedy, 2000; Kennedy et al., 2006; Lai Wing Sun et al., 2011). 'Short-range' refers to factors that remain close to, or attached to, the surface of the cell that secretes the cue. Secreted long-range cues, by contrast, may diffuse and function many cell diameters away from the cellular source that secreted the cue.

To test whether netrin-1 could function as a long range cue, Kennedy et al. (1994) used COS cells engineered to express and secrete ectopic netrin-1, and discovered that netrin-1 was able to effectively elicit axon outgrowth from dorsal spinal cord explants co-cultured in a collagen gel at a distance of up to several hundred microns (Kennedy et al., 1994). Similarly, aggregates of COS cells expressing netrin-1 placed next to an explanted intact spinal cord were demonstrated to reorient commissural axon trajectories, suggesting a role in not only promoting outgrowth, but directing commissural axon guidance (Kennedy et al., 1994). Further, assays using retinal ganglion cell axons demonstrated that a gradient of netrin-1 released from a micropipette could cause growth cone turning up the gradient, and gradients of netrin-1 protein established using microfluidics or immobilized on a substrate, also reoriented axon extension (Mai et al., 2009; Serafini et al., 1996; Sloan et al., 2015).

Kennedy et al. (2006) also reported the distribution of netrin-1 mRNA and protein in the embryonic chick spinal cord. Only floor plate cells at the ventral midline express netrin-1 mRNA in the early embryonic chick spinal cord. In contrast to this restricted distribution of netrin-1 mRNA, netrin-1 protein was detected in a graded distribution across the neuroepithelium, a substantial distance dorsal to the floor plate cells (Kennedy et al., 2006). A similar distribution of netrin-1 protein was detected in the embryonic mouse spinal cord, with netrin-1 being produced by both floor plate cells and ventricular zone cells (Kennedy et al., 1994).

These studies have confirmed that a gradient of netrin-1 has the capacity to direct commissural axon outgrowth *in vitro* and shown that netrin-1 is indispensable for accurate commissural axon outgrowth *in vivo*. It remains unclear, however, whether netrin-1 functions *in vivo* as a permissive cue that promotes axonal growth without imparting directional guidance information, or if it plays a more instructive role by attracting commissural neurons along a defined path.

Several recent papers have claimed that floor plate derived netrin-1 is not necessary for commissural axon guidance to the ventral midline in embryonic mouse, and instead suggest a critical role for ventricular zone derived netrin-1 as a short range guidance cue (Dominici et al., 2017; Varadarajan et al., 2017). Similar to previous studies that examined the effect of removing the floor plate from the embryonic spinal cord (Matise et al., 1999), or in which the entire spinal cord was genetically dorsalized (Bovolenta and Dodd, 1991), more recent papers report that selective genetic deletion of netrin-1 from floor plate cells does not prevent commissural axons from reaching the ventral midline (Dominici et al., 2017; Varadarajan et al., 2017), suggesting that floor plate derived netrin-1 is not essential for commissural axon guidance.

Here, we provide evidence that floor plate-derived netrin-1 is distributed as a long-range cue, and that the precise distribution of netrin-1 protein is essential to its instructive function, directing commissural axon guidance.

METHODS

Mouse Lines

All animal procedures were performed in accordance with the Canadian Council on Animal Care guidelines for the use of animals in research. Mice heterozygous for the β-galactosidase netrin-1 recombinant allele (netrin-1βgeo) (Serafini et al., 1996) were obtained from Marc Tessier-Lavigne (Rockefeller University, NYC, USA). Netrin-1 deleted mice were generated as described (Bin et al., 2015). Both lines were maintained on a C57BL/6 genetic background. SHH:cre mice (#005622, Jackson Laboratories)(Harfe et al., 2004) were crossed with netrin-1 floxed mice. Only males carrying the cre gene were used for breeding. Sprague-Dawley rats were obtained from Charles Rivers Laboratory at various developmental stages (St-Constant, QC, Canada) (vaginal plug = E0). Fertilised chicken eggs (FERME GMS, Saint-Liboire, QC, Canada) were incubated at 38° C and staged as described (Hamburger and Hamilton, 1992).

Embryo Collection and Fixation

Timed pregnancies were set up between mice of the appropriate genotypes and embryos were collected at embryonic day 9.5 (E9.5), E10.5 or E12.5. Heads were kept for genotyping. Each embryo was fixed in 10 ml of Carnoy's Fixative (60% absolute ethanol, 30% chloroform, and 10% acetic acid) for 2h at room temperature on a shaker. Embryos were then transferred to ethanol for two 20 min washes, then cleared in toluene for 1h on the rocker. Embryos were then placed in liquid paraffin and kept in the 60^{o} C oven under vacuum for at least 2h. Embryos were then transferred to fresh paraffin, and placed in the oven under vacuum for another 15-30 min. Embryos were then oriented, and the paraffin allowed to harden in the mould.

Antibodies

The following antibodies were used: rabbit anti-netrin-1 (EPR5428, Abcam #ab126729) , mouse anti-tubulin β3 (Tuj-1) (Biolegend #801201), goat anti TAG-1 (R&D Systems #AF4439), rabbit anti-myc (FITC) (Abcam #ab1263), mouse anti-tetra-His (Qiagen #34670), monoclonal mouse anti-pan-axonal neurofilament (Covance, SMI-312), polyclonal rabbit anti-GAPDH Santa Cruz Biotechnology FL-335). Tyramide Signal Amplification Superboost kit solutions (Thermo Fisher, cat #B40922 and B40913) were also used.

Western Blot and Coomassie blue staining

Recombinant netrin-1 protein was purified from a HEK 293-EBNA cell line engineered to secrete netrin-1. 50 ng of chick recombinant netrin-1 and netrin-2 protein (R&D Systems) were run on 10% SDS-PAGE gels and transferred to a nitrocellulose membrane before being probed with antibodies. Blots were resolved with Immobilon Western Chemiluminescent HRP Substrate (Millipore, MA, USA). SDS-PAGE gels were also stained with Coomassie dye to assess equal loading between lanes.

Proteins were separated by SDS-PAGE and transferred to polyvinyldiene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA). The membranes were probed with primary antibodies overnight. Immunoreactivity was visualized using Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA).

In situ Hybridization

The following solutions were used in these experiments: 20x SSC (3M NaCl, 0.34M sodium citrate, pH 7.0), hybridization solution (5x Denhardt's solution, 250 μg/ml baker's yeast tRNA, 5x SSC,

50% formamide), phosphate buffered saline pH 7.4, buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5), buffer 2 (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5), colour solution (NBT/BCIP mix in buffer 2). All solutions used DEPC-treated ddH_2O.

Sections of Carnoy's fixed embryonic chick spinal cords were incubated in hybridization solution for 2h to overnight at 60^{o}C. The cRNA probe was then added in hybridization solution overnight at 60^{o}C. Slides were then washed using SSC and SSC with formamide. The slides were then washed in buffer 1 and blocked for 1h in 1% blocking reagent in buffer 1. After washing again, the slides were incubated with anti-Digoxigenin-AP in buffer 1 for 1.5 – 3h. The slides were then washed 2 x 15 min in buffer 1, followed by a 5 min wash in buffer 2. The colour reaction was developed for 1 – 48h at room temperature.

Immunohistochemistry

10μm thick sections were dewaxed for 1h or overnight at 60^{o}C, then rehydrated sequentially in 2 x 3 min washes in xylene, 100% ethanol, 95% ethanol, 70% ethanol, and 1x PBS. Slides were then subjected to 20 min of antigen retrieval in boiling 10 mM sodium citrate buffer at pH = 6.0 (Kennedy et al., 2006; Shi et al., 1991), and washed for 2 x 5 min in PBS, before immunohistochemistry was performed.

For regular processing of slides, tissue was blocked in 3% milk + 0.3% triton-x 100 in PBS for 2h at room temperature. Primary antibodies were then added overnight in 3% milk + 0.1% triton-x 100 in PBS and left at 4^{o}C. Slides were then washed in PBS for 3 x 10 min. Secondary antibodies were diluted in 3% milk + 0.1% triton-x 100 in PBS and incubated for 1h at room temperature. Slides were then washed for 3 x 10 min in PBS, rinsed in distilled water, and mounted in fluorogel (Electron Microscopy Sciences).

For tyramide enhanced slides, sections were first quenched with 3% hydrogen peroxide for 1h at room temperature to remove endogenous peroxidase activity before being blocked in either 3% milk + 0.1% triton-x 100 in PBS or 10% goat serum supplied with the tyramide kit. Primary antibodies were added overnight in 3% milk + 0.1% triton-x 100 in PBS at 4^{o}C. Slides were then washed for 3 x 10 min in PBS. Secondary HRP antibodies were added for 1h at room temperature, then washed for 3 x 10 min in PBS. Meanwhile, the tyramide enhancement solution was prepared according to the instructions provided. Approximately 100µl of solution was prepared for each slide, and slides were incubated for 2-10 min. The reaction was then stopped using the provided stop reagent, then rinsed three times in PBS. Slides were either rinsed in distilled water then mounted in fluorogel, or complexed with another antibody. For slides that require labelling with a second primary antibody, the tissue was blocked again with 3% milk + 0.3% triton-x 100 in PBS for 1 hr at room temperature. Primary antibodies were added for 1 hr at room temperature or overnight at 4^{o}C in 3% milk + 0.1% triton-x 100 in PBS. The regular immunohistochemical protocol was then followed.

Microscopy Imaging and Data Analysis

Sections were imaged on a Zeiss Axiovert S100TV using constant exposure and thresholding within datasets. Fluorescence intensity was quantified in Image J (Schneider et al., 2012). Gradients were measured by sampling with 5µm radius circles for E9.5 mice and 10µm radius circles for chick and E10.5 mice along either the edge of the spinal cord or along the commissural axon tract. From these circles, the average fluorescence intensity within the area was calculated.

All ventral commissures were quantified from images of spinal cords taken using a 20X objective lens. Commissural axons were visualized by immunohistochemistry using goat anti-

TAG-1 antibody (R & D Systems) on sections from E10.5 and E12.5 embryos, and mouse anti-pan neurofilament (SMI-312, Covance) on E14.5 sections. The images were first thresholded to obtain binary images of the axonal trajectories. An 18.75μm x 80.21μm box was drawn over the centre of the ventral commissure in each image and the number of pixels above threshold were counted. To account for the variable sizes of the embryos, the number of pixels was normalized to the height of the spinal cords.

Commissural bundle turning was measured at the point of intersection of the bundle with the base of the spinal cord. The distance between the two points of intersection in the cord was measured and normalized to the width of the spinal cord. Turning distance was analysed in GraphPad Prism 6.

Open Book Spinal Cord Assay

Spinal cords were micro dissected from E11/12 rat embryos and embedded in a 3D collagen matrix as described (Moore and Kennedy 2008). Explants were treated with 5μg/mL netrin-1 for 2 days *in vitro*. Controls were left untreated. Spinal cords were then fixed with 4% PFA for 1h on ice, and axon trajectories visualized using immunohistochemistry.

To visualize axons in the open book explants, they were first blocked and permeabilized in 3% heat inactivated horse serum (hiHS) and 1% triton-X 100 for 24h. Explants were then incubated in primary antibody diluted in 1% hiHS and 1% triton-X 100 for 2 days at 4^{o} C. They were then washed multiple times throughout the day and left overnight in PBS. Secondary antibodies diluted in 1% hiHS and 1% triton-X 100 were added for 2 days at 4^{o}C. The explants were washed multiple times throughout the day and left overnight in PBS before they were mounted on slides in fluorogel (Electron Microscopy Services).

RESULTS

Long-range ventral-dorsal distribution of netrin-1 in the embryonic spinal cord?

To investigate the distribution of netrin-1 protein, we first validated the specificity of a commercially available monoclonal antibody, demonstrating selective and specific detection of netrin-1 on western blots of mouse and chick spinal cord homogenates and the absence of non-specific binding on sections of netrin-1-null embryonic mouse spinal cord (Figure 2). (Bin et al., 2015) Using this validated monoclonal antibody, we then examined the distribution of netrin-1 protein in the embryonic spinal cord to determine to what extent netrin-1 protein may be located distant from its cellular source, the defining characteristic of a long-range cue. In mouse, netrin-1 is expressed by floor plate cells at the ventral midline and by ventricular zone cells in an increasing dorsal-ventral gradient of gene expression (Serafini et al., 1996). Thus, in mouse spinal cord, netrin-1 expression by cells dorsal to the floor plate confounds using immunohistochemistry to identify the distribution of netrin-1 protein specifically secreted by the floor plate. In contrast, in embryonic stage 17 chick brachial spinal cord, as the earliest pioneer commissural axons begin to extend, only floor plate cells express netrin-1 (Kennedy et al., 1994; Kennedy et al., 2006). Taking advantage of this restricted expression in embryonic chick, and using the validated netrin-1 specific monoclonal antibody, we detected a graded distribution of netrin-1 protein in the neural epithelium up to 200 μm from the netrin-1 expressing floor plate cells. Quantification revealed graded immunoflorescence intensity along the trajectory of axon extension (Figure 3c). The detected distribution of netrin-1 protein in the embryonic chick spinal cord is exemplary of a long-range cue.

To determine if netrin-1 may be distributed as a long-range cue in mammals, we examined the distribution of netrin-1 protein in E9.5 mouse brachial spinal cord, as commissural axons

extend to the ventral midline (Wentworth, 1984). Measurement of immunohistochemical fluorescence intensity at E9.5 revealed netrin-1 distributed along the edge of the mouse spinal cord similar to sections of stage 17 chick spinal cord, decreasing dorsally with a peak at ~100 μm from the midline (Figure 4).

Steep medial-lateral gradient of netrin-1 at lateral edge of embryonic spinal cord

Commissural axons initially extend ipsilaterally along a narrow corridor adjacent to the lateral edge of the neural epithelium. Examining these circumferential projections relative to the distribution of netrin-1 indicated that growth cones migrate along netrin-1 immunoreactive neural epithelial cells, consistent with past ultrastructural studies of this projection (Bittar, 1998; Holley, 1982; Yaginuma et al., 1991). An aberrant trajectory taken by commissural axons in netrin-1 null embryos is to mis-project radially toward the ventricle, suggesting that netrin-1 directs the axons to project along the edge of the spinal cord. Our findings revealed a steep radial gradient of netrin-1 that peaks at the lateral edge of the neural tube (Figure 4, C), suggesting that this may function to attract the axons radially to channel their extension along the lateral edge of the neural epithelium. Quantification of the relative fluorescence intensity of netrin-1 immunoreactivity, measured from the lateral edge of the spinal cord and extending medially, at 20%, 40%, 60% and 80% of the dorsal-ventral height of the spinal cord revealed a short steep gradient that flattens within 50 μm towards the middle of the spinal cord. The slope of the gradient was roughly similar in the three ventral-most measures, but the apex of netrin-1 intensity detected was greatest in the ventral portion of the spinal cord, superimposing the steep radial gradient on the longer dorsal-ventral circumferential gradient.

Graded netrin-1 gene dosage alters gradient shape and results in graded severity of guidance deficits

Of three identified floor plate derived axonal chemoattractants, Shh, VEGF, and netrin-1 (Bin et al., 2015; Charron et al., 2003; Kennedy et al., 1994; Ruiz de Almodovar et al., 2011; Serafini et al., 1996), only netrin-1 is essential for the vast majority of commissural axons to reach the ventral midline of the neural tube. While netrin-1 is required, the significance of the graded distribution of netrin-1 *in vivo* remains unclear. To address the functional significance of the distribution of netrin-1, and begin to differentiate between the proposed permissive function of netrin-1 as a non directional cue that only promotes axon outgrowth (Dominici et al., 2017; Varadarajan and Butler, 2017; Varadarajan et al., 2017), versus a tropic instructive cue that directs axon migration, we first investigated the impact of genetically manipulating the amount of *netrin-1* expressed on the distribution and function of netrin-1 protein.

We examined two different mouse alleles that disrupt *netrin-1* expression: a line carrying a *netrin-1*-null allele (Bin et al., 2015), and a loss-of-function *netrin-1*$^{\beta geo}$ allele, in which a *β-galactosidase* gene trap insertion disrupts the endogenous *netrin-1* gene (Serafini et al., 1996). While these are both loss-of-function alleles, mice homozygous for the *netrin-1*$^{bgeo/bgeo}$ allele express ~5% of the amount of full-length netrin-1 protein compared to wild-type embryonic littermates, as a result of a low frequency of alternative RNA splicing that removes the inserted transgene to generate an mRNA encoding full length netrin-1 (Bin et al., 2015; Serafini et al., 1996).

To determine how differences in gene dosage may impact the shape of the gradient in the embryonic spinal cord, we measured the fluorescence intensity of netrin-1 immunolabelling in the two mouse lines along the trajectory of commissural axons in sections of wild type and

heterozygote E10.5 spinal cord. Netrin-1 immunoreactivity was not detected in null embryos and was therefore not measured. In heterozygous embryos of both lines, the overall fluorescence intensity was reduced and the netrin-1 gradient more shallow (Figure 5C), indicating that reduced netrin-1 gene dosage reduces gradient slope.

To then assess the functional impact of reduced netrin-1 gene dosage on axon guidance, commissural axons were visualized in sections of E10.5 spinal cord using an antibody against TAG-1 (Dodd et al., 1988). *Netrin-1*$^{bgeo/bgeo}$ and *netrin-1*$^{-/-}$ homozygous embryos were substantially different from wild-type and heterozygous counterparts, with commissural axons initially projecting ventrally, but then stalling or becoming lost and migrating aberrantly into the epithelium in various directions (Figure 5). The thickness of the ventral commissure was substantially reduced in homozygous embryos, of either genotype, indicating that a decreased number of commissural axons reach and cross the midline in the absence of netrin-1. To quantify this, we measured the area of fluorescent TAG-1 immunoreactivity at the ventral midline as a correlate of commissure size, and normalized this to the dorsal-ventral height of the spinal cord to account for any size difference between embryos. Consistent with previous findings, the netrin-1 deficient mice exhibit a considerable reduction in the size of the spinal ventral commissure compared to wild-type and heterozygous littermates (Bin et al., 2015; Serafini et al., 1996; Wu et al., 2019) (Figure 5b). The *netrin-1*$^{-/+}$ heterozygotes also display significant reduction in commissure intensity compared to wild type, and notably is also reduced compared to *netrin-1*$^{bgeo/+}$ (Figure 5b). These findings indicate that a graded alteration in gene dosage changes the amount of netrin-1 protein expressed, and results in a graded severity of commissural axon guidance defects at the ventral midline.

Selective deletion of floor plate netrin-1 alters netrin-1 protein gradient shape

At least two distinct sources of netrin-1 can be described in the embryonic mouse spinal cord: floor plate derived netrin-1 and ventricular zone derived netrin-1. Selective deletion of netrin-1 from floor plate cells results in commissural axons deviating from their normal trajectory within ~200 µm of the floor plate (Wu et al., 2019). Based on these previous findings, we hypothesized that it should be possible to detect an altered distribution of netrin-1 protein, that reflects the ~200 µm distance, following deletion of floor plate netrin-1. To examine the specific contribution of floor plate derived netrin-1, we generated mice expressing cre recombinase regulated by the sonic hedgehog (SHH) promoter, Shh:cre (Harfe et al., 2004), and homozygous for a floxed allele of netrin-1, *Shh:cre/netrin-1*$^{flox/flox}$ (Bin et al., 2015). Cre negative littermates were used as wild type controls. We then measured the fluorescence intensity of netrin-1 immunoreactivity along the outer edge of the spinal cord, and also along the path of commissural axons as they turn away from the lateral edge of the cord and extend along the medial edge of the cluster of motor neurons in the ventral horn. Interestingly, we found that in embryos lacking floor plate netrin-1, commissural axons in the ventral half of the spinal cord were much less obviously marked with netrin-1 immunoflorescence, in spite of still reaching and crossing the midline (Figure 6). This indicates that the majority of the netrin-1 protein that prominently decorates commissural axons as they approach the ventral midline is likely floor plate derived. In wild type embryos, quantification of netrin-1 immunoflorescence along either the edge of the cord, or following the commissural axons, revealed a graded distribution. In contrast, in embryos that selectively lack floor plate derived netrin-1, the distribution of immunofluorescence was substantially reduced for ~200 µm extending away from the floor plate (Figure 6C).

Examining these spinal cords for axon guidance deficits, we confirmed that commissural axon trajectories taken to the midline in floor plate cell conditional netrin-1 knockout embryos differed in the vicinity of the floor plate, appearing more U-shaped, as opposed to the V-shape characteristic of wild type embryos, consistent with a previous study that described this phenotype (Wu et al., 2019) (Fig. 6, B). A Mann-Whitney U-test comparing genotypes revealed a significant difference in the median turning distance from the midline. These findings detect the specific loss of netrin-1 within ~200 μm of the floor plate, along with a deviation in commissural axon guidance exactly within this range. Together, these findings indicate that floor plate derived netrin-1 is distributed and functions as a long-range commissural axon guidance cue over a distance of ~200 μm.

Is netrin-1 a permissive or an instructive cue?

While netrin-1 is required for commissural axons to reach the ventral midline of the embryonic spinal cord, it may be only permissive, promoting axon extension while other cues with tropic function direct axon growth. Alternatively, the specific distribution of netrin-1 may instruct growth cone migration by providing critical spatial cues that direct axon extension. As an initial step to test between permissive versus instructive functions for netrin-1, we carried out gain of function studies that add ectopic netrin-1 to the distribution of endogenous netrin-1. If commissural axons only require extracellular netrin-1 to be present to reach the midline, and directional information is provided by other cues in the spinal cord, then adding to the distribution of netrin-1 should not affect axon guidance, as netrin-1 would only need be present to provide a permissive surface that allows axon extension. Alternatively, if the precise extracellular distribution presents critical spatial information, disrupting this by adding more netrin-1 is predicted to alter the direction of

commissural axon extension, providing evidence that the distribution of netrin-1 protein is instructive and required to guide axon growth. To test between permissive versus instructive functions, explants of E11 rat spinal cord were cultured as open book explants, with the endogenous floor plate intact. In wild type open book spinal cords, commissural axons extend directly toward the floor plate at the midline, following the trajectory taken *in vivo* (Kennedy et al., 1994). To add to the endogenous distribution of netrin-1, open book spinal cords were flooded with an excess of recombinant netrin-1 protein (5 µg/ml), aiming to override the distribution of endogenous netrin-1 protein. Our findings indicate that this did not block the capacity of commissural axons to extend, but resulted in severe disruption of commissural axon guidance, with axons misdirected within the neural epithelium and extensive outgrowth of axons projecting from the explants into the surrounding 3D collagen gel (Figure 7). These findings provide strong evidence that netrin-1 is not merely permissive, allowing the axons to extend with directional information provided by other cues, but instead, indicate that the precise distribution of netrin-1 is critical, with an excess of exogenous netrin-1 triggering exuberant misdirected axon growth.

DISCUSSION

The possibility that secreted chemotropic molecules function as long-range cues to form gradients that direct axon extension *in vivo* was first posed over 100 years ago. Here we detect, quantify, and assess the functional significance of the distribution of netrin-1 protein in embryonic spinal cord.

Distribution of netrin-1 in embryonic mouse and chick spinal cord

Netrin-1 is critical for ipsilateral commissural axon guidance to the ventral midline (Bin et al., 2015; Serafini et al., 1996; Yung et al., 2015). Our findings support the conclusion that netrin-1 is distributed as a long-range cue secreted by floor plate cells, and that gradients of netrin-1 direct ipsilateral axon extension to the midline. Here, we show that, in the embryonic chick spinal cord, where *netrin-1* is expressed solely by floor plate cells, netrin-1 protein is distributed significantly dorsal to the floor plate in the embryonic spinal cord, revealing a long-range distribution. We then quantified the fluorescence intensity of netrin-1 immunolabeling, revealing graded distributions of netrin-1 protein that extends dorsally in both embryonic chick and mouse spinal cords in a similar overall pattern. Further, for the first time, we documented a medial-lateral gradient. In netrin-1 null embryos, many commissural axons turn prematurely and follow an aberrant radial trajectory toward the ventricle. We speculate that this increasing, steep, medial to lateral gradient of netrin-1 protein functions to constrain commissural axons as they extend adjacent to the lateral edge of the neural epithelium, preventing them from straying medially toward the ventricle.

Floor plate expression contributes to the netrin-1 gradient

Is floor plate derived netrin-1 distributed as a long-range cue in embryonic mouse spinal cord? Floor plate specific knockouts of netrin-1 in mouse provide strong evidence that this specific cellular source is responsible for generating a graded long-range distribution of netrin-1 that extends dorsally for ~200 μm from the midline. Notably, our findings indicate that a considerable portion of netrin-1 protein found along the trajectory of commissural axons in embryonic mouse is floor plate-derived, consistent with the particularly high level of *netrin-1* expressed by floor plate cells. Specifically, conditional deletion of netrin-1 expression by floor plate cells greatly reduced the amount of netrin-1 in the path of extending axons and the gradient was severely flattened. We also detected substantially less netrin-1 protein associated with the surface of commissural axons that have crossed the midline. This suggests that floor plate derived netrin-1 bound to the surface of pioneer commissural axons could be used by follower axons as a navigational cue. The striking absence of this staining when netrin-1 was selectively deleted from floor plate cells suggested that loss of floor plate derived netrin-1 may promote defasciculation and increase local guidance errors and misrouting.

Our findings, along with other recent studies (Moreno-Bravo et al., 2019; Wu et al., 2019), identify at least two distinct sources for the netrin-1 distributed in the embryonic mouse spinal cord. A corridor of netrin-1 extends along the lateral edge of the spinal cord that is overlaid by a long-range gradient of netrin-1 secreted by the floor plate. In agreement with Varadarajan et al. (Varadarajan et al., 2017) and Dominici et al. (Dominici et al., 2017), our findings are consistent with the corridor of netrin-1 being generated by expression of netrin-1 by neural progenitors in the ventricular zone, as removing netrin-1 from the floor plate did not affect this relatively dorsal

distribution of netrin-1. In contrast, our findings indicate that the gradient extending ~200 μm from the midline is derived from netrin-1 secreted by the floor plate.

Gradients encode information as a spatial distribution. In the ventral spinal cord, the gradient generated by floor plate derived netrin-1 directs commissural axons to break away from the edge of the spinal cord, navigate past the motor column and reach the ventral midline, in contrast to their relatively simpler previous trajectory along the edge of the spinal cord. Prior studies have demonstrated that commissural axons are capable of following a circumferential trajectory around the spinal cord in the absence of a floor plate (Bovolenta and Dodd, 1991; Matise et al., 1999), and only exhibit noticeable phenotypes at or around the ventral midline. As such, the floor plate directs commissural axons to turn away from the corridor of netrin-1 that extends circumferentially along the edge of the neural tube, and redirects axon extension toward the ventral midline.

Distribution of netrin-1 protein *in vivo* directs axon guidance

Netrin-1 was initially proposed to function in the embryonic spinal cord as an instructive cue that directs axon guidance (Kennedy et al 1994, Serafini et al 1994, Serafini et al 1996, Kennedy et al 2006), but more recently has been argued to have only a permissive function, promoting but not directing the extension of commissural axons (Dominici et al., 2017; Varadarajan et al., 2017). Although our findings indicate that netrin-1 secreted by the floor plate is distributed as a long-range cue that forms a gradient, the distribution of protein is not sufficient to differentiate between instructive and permissive functions *in vivo*. Here, we examined the functional significance of the gradient of netrin-1 by visualizing the distribution of protein in the spinal cord and the corresponding disruption of axonal trajectories when *netrin-1* expression was either genetically

reduced or deleted from specific cell types. *Netrin-1* null mice exhibit severe disruption of commissural axon trajectories in the embryonic spinal cord (Serafini et al 1996, Bin et al 2015). Here, we show that disruption of the distribution of netrin-1 protein and commissural axon guidance is apparent even with reduced levels of netrin-1 protein in *netrin-1* heterozygous embryonic mouse spinal cords. Studying the consequence of netrin-1 gain-of-function, we employed the open book assay, and flooded the neural epithelium with exogenous netrin-1 protein. Here, our aim was to mask any instructive capability of the endogenous netrin-1 by imposing a more uniform distribution throughout. Our findings demonstrate that this results in massive disruption of commissural axon guidance to the ventral midline, indicating that netrin-1 is not simply a permissive cue that must be present for commissural axons to extend, but that the specific extracellular distribution of netrin-1 is an essential instructive component of commissural axon guidance.

Potential significance of the size of the neural tube and length of circumferential trajectory

Netrin-1 secreted by floor plate cells generates a gradient over a distance of ~200 μm in the neural epithelium. In early stages of spinal cord development in mouse, rat and chick, this gradient is capable of extending across almost the entire ventral-dorsal diameter of the spinal cord. We speculate that, for smaller embryos with smaller spinal cords and in smaller regions of the neural tube, floor plate derived netrin-1 may be sufficient for commissural axon extension to the midline. In contrast in larger embryos and larger regions of the neural tube, such as the hindbrain compared to the spinal cord, expression by cells in ventricular zone extends the length of the path along which axons can extend in response to netrin-1.

The dependence of commissural axons on ventricular zone derived netrin-1 and floor plate derived netrin-1 is different between embryonic spinal cord and hindbrain. The substantial size difference between hindbrain and spinal cord is likely critical. The circumferential trajectory taken by pioneer commissural axons to the floor plate in the hindbrain is ~700 µm long (Dominici et al 2017), while in the E9.5 mouse brachial spinal cord it is ~300 µm, and in stage 17 chick brachial spinal cord ~200 µm (Kennedy et al 2006). Along relatively long trajectories, like the hindbrain, Yamauchi et al (2017) and Dominici et al (2017) demonstrated that the dorsal source of ventricular zone derived netrin-1 is essential to initially direct axons ventrally. In contrast, for the relatively small early chick spinal cord, floor plate netrin-1 is likely sufficient for commissural axon guidance. Our findings in the embryonic mouse spinal cord indicate that a gradient of floor plate derived netrin-1 overlaps with ventricular zone derived protein, and once axons come within range of floor plate derived netrin-1, this source of protein contributes to directing axons to the ventral midline.

In an early review, Colamarino and Tessier-Lavigne (Colamarino and Tessier-Lavigne, 1995) argued that "short-range cues may serve primarily to direct growth along the edge of the spinal cord, while at least one role for the [floor plate-derived] chemoattractant may be to direct axons that have to grow to the ventral midline through the cellular environment of the motor column." Current findings continue to support this model. We show that embryos that selectively lack floor plate netrin-1 exhibit abnormal axon guidance phenotypes within 100-200 µm of the ventral midline, at the motor column and commissure. Other recent studies have also shown that removing netrin-1 from the floor plate will displace the commissural axon tract laterally and decrease the size of the commissure (Wu et al., 2019). Commissural axons had difficulty crossing the midline and post-crossing trajectories were altered in the absence of floor plate derived netrin-1 (Moreno-Bravo et al., 2019). This is similar to embryos null for the two other known floor plate

chemoattractants, Shh (Charron et al., 2003) and VEGF (Ruiz de Almodovar et al., 2011) in which some commissural axons do not project directly to the floor plate but splay out as they enter the ventral spinal cord and wander in the motor column, yet ultimately reach and cross the midline at the ventral commissure. Here, we quantified similar disruption of commissural axon trajectories in mice that selectively lack netrin-1 expression by floor plate cells, and correlated these defects with corresponding local change in the distribution of netrin-1 protein.

Our findings provide evidence for the short-range distribution of ventricular zone netrin-1 and long-range distribution of floor plate derived netrin-1 in the embryonic spinal cord. An earlier paper investigated short-range and long-range functions for netrin during Drosophila development. Brankatschk and Dickson (2006) showed that netrin tethered to the membrane of midline cells in a Drosophila embryo is sufficient to rescue axonal midline crossing and short-range midline repulsion. The same paper also reported defects in long-range chemorepellent axon guidance more than ~30-40 μm from the midline source of netrin. Brankatschk and Dickson conclude "most importantly in the context of this work, these data confirm that endogenous netrins can guide axons at both short and long range". The short range function is likely feasible since the midline is almost within filopodial grasp (~20 μm) of a newly formed growth cone at the initiation of axon extension, however the findings also provide a key demonstration that netrin functions as a long-range cue at greater distances from the midline. The molecular mechanisms that determine if secreted netrin-1 becomes distributed at short- or long-range remains to be determined, but the flexibility provided in different developmental contexts, appears to be evolutionarily conserved. Studies currently in preparation in the Kennedy lab provide evidence that proteoglycan binding is critical to determining the distribution of netrin-1 protein in the developing neural epithelium (Harris et al., in preparation).

Model for the functional significance of short- and long-range distributions of netrin-1 in the embryonic mouse spinal cord

Our studies provide support for the following model. We propose that as commissural axons extend circumferentially toward the ventral midline of the mouse neural tube, they encounter a dorsal-ventral increasing gradient of netrin-1 protein generated by two sources: locally-secreted, proteoglycan bound, short-range ventricular zone derived netrin-1 protein, that overlaps with an ~200 – 300 μm gradient of long-range netrin-1 secreted by floor plate cells. Initially the axons are constrained along the lateral edge of the neural epithelium by the increasing ventral-dorsal gradient and the medial-lateral radial gradient of netrin-1. In the embryonic mouse spinal cord, the growth cone of an extending commissural axons first encounters the relatively dorsal source of locally expressed ventricular zone netrin-1. In the absence of this dorsal netrin-1, an axon may deviate from its appropriate dorsal-ventral trajectory and not come within range of floor plate derived netrin-1. This model is consistent with the dorsal source of ventricular zone derived netrin-1 being essential for commissural axons along longer trajectories to the midline, such as in the hindbrain (~700 μm) (Dominici et al., 2017; Yamauchi et al., 2017), while along a shorter circumferential path, such as the embryonic chick or mouse spinal cord (~200-300 μm), the gradient of long-range floor plate derived netrin-1 is sufficiently long to guide axons to the ventral midline. This unified model of netrin function, engaging secreted long-range with local short-range functions, has substantial explanatory potential for how a graded guidance cue directs axon migration in this classic context.

Conclusion

Overall, these studies aimed to determine if netrin-1 is distributed as a gradient and functions as a long-range cue, and to clarify the functional significance of the different sources of netrin-1 in the spinal cord. Ongoing experiments are examining how ectopic expression of netrin-1 in the embryonic spinal cord affects commissural axon guidance.

Figures and Figure Legends

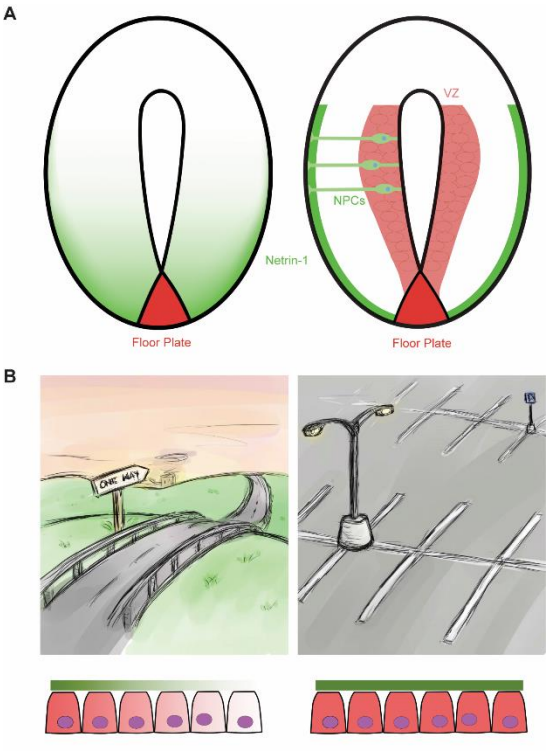

Figure 1. Permissive and Instructive Axon Guidance Cues

(a) Two models, each emphasizing different contributions to the overall distribution of netrin-1 in the embryonic spinal cord are depicted. On the left, netrin-1 secreted by floor plate cells generates a gradient that extends dorsally. On the right, neural progenitor cells (NPCs) in the ventricular zone (VZ) deposit netrin-1 along the edge of the spinal cord. (b) Instructive cues provide directional information, functioning like signs along a road. Permissive cues provide an surface or environment through which axons may extend, analogous to the asphalt paved surface of a road or a parking lot, but do not provide directional information per se. Guidance cues that are instructive are differentially distributed across space, while permissive cues are often evenly distributed.

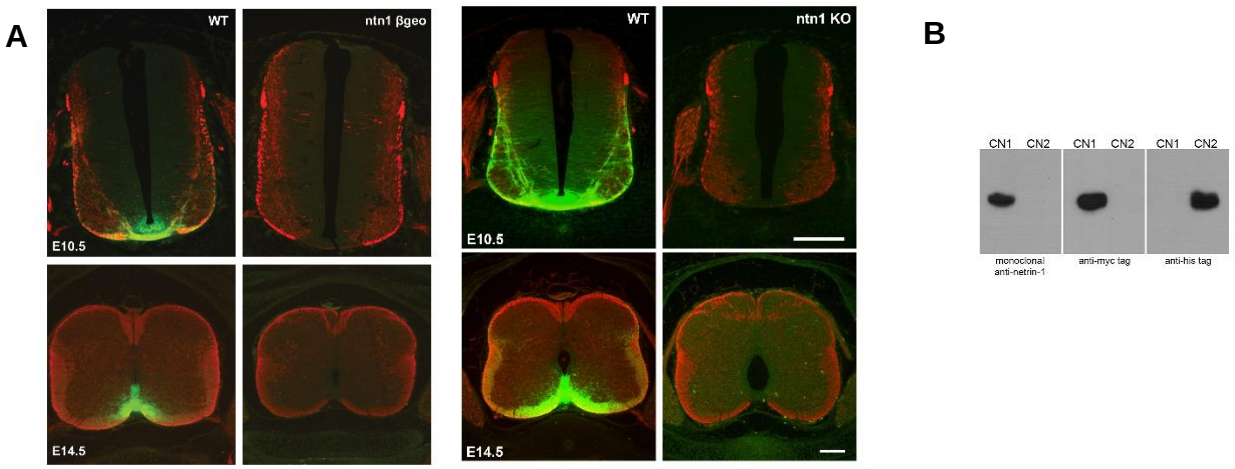

Figure 2. Validation of netrin-1 monoclonal antibody.

(a) Rabbit monoclonal netrin-1 antibody labels wild type embryonic mouse spinal cords at E10.5 and E14.5. No signal was detected in knockouts. Scale bars correspond to 200 μm (b) Western blot of recombinant chick netrin-1 and netrin-2 show netrin-1 antibody is specific for netrin-1 protein.

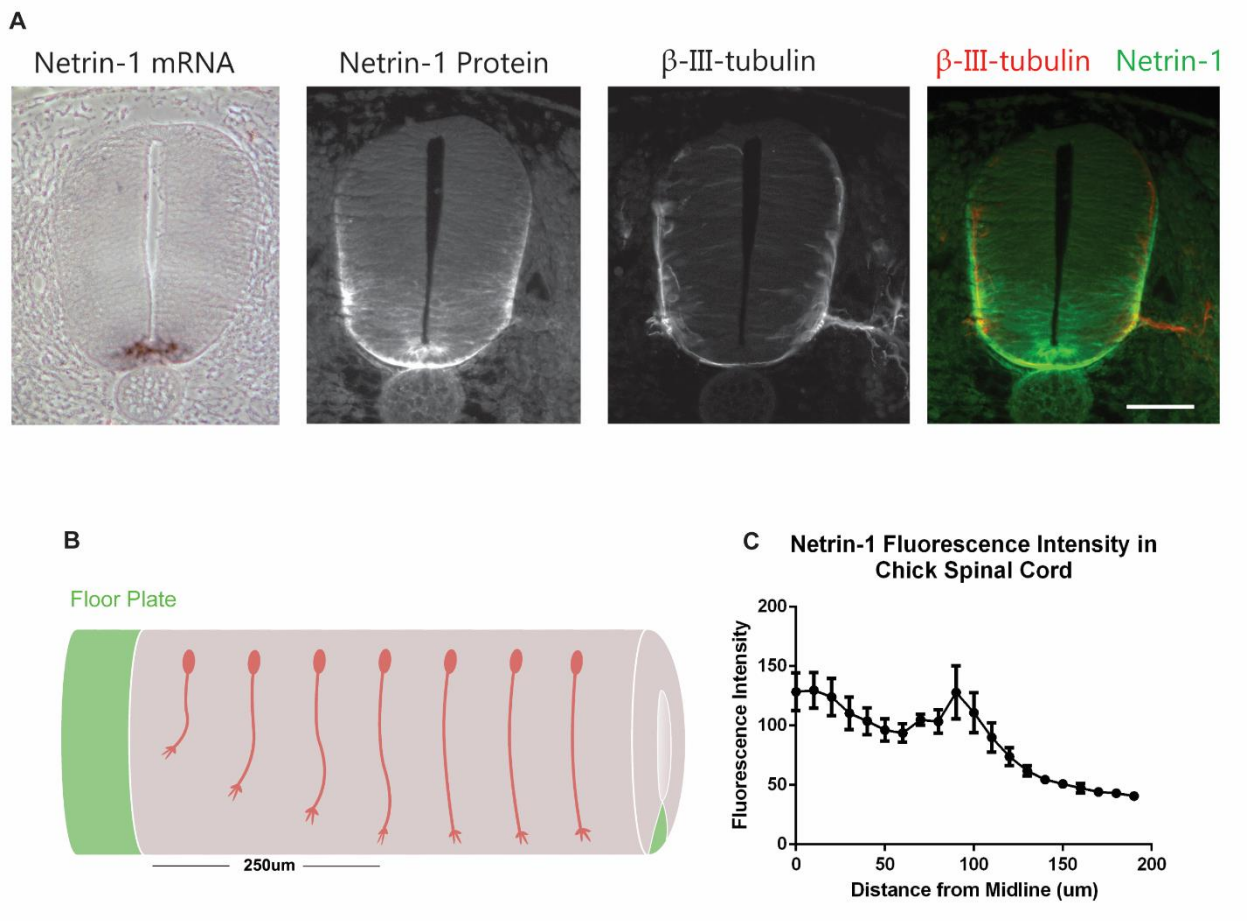

Figure 3. Dorsal-Ventral Gradient of Netrin-1 Protein in the Embryonic Chick Spinal Cord

Fig 3. (a) Stage 16-17 embryonic chick spinal cord expresses netrin-1 mRNA solely in the floor plate. Netrin-1 protein is detected further dorsally in the neuroepithelium. Scale bar is 50μm. (b) Previously, the commissural axon turning assay demonstrated that an ectopic source of netrin-1 will elicit commissural axon growth cone turning up to ~250 μm away within the neural epithelium. (c) Measurements of netrin-1 fluorescence intensity show a graded distribution extending dorsally.

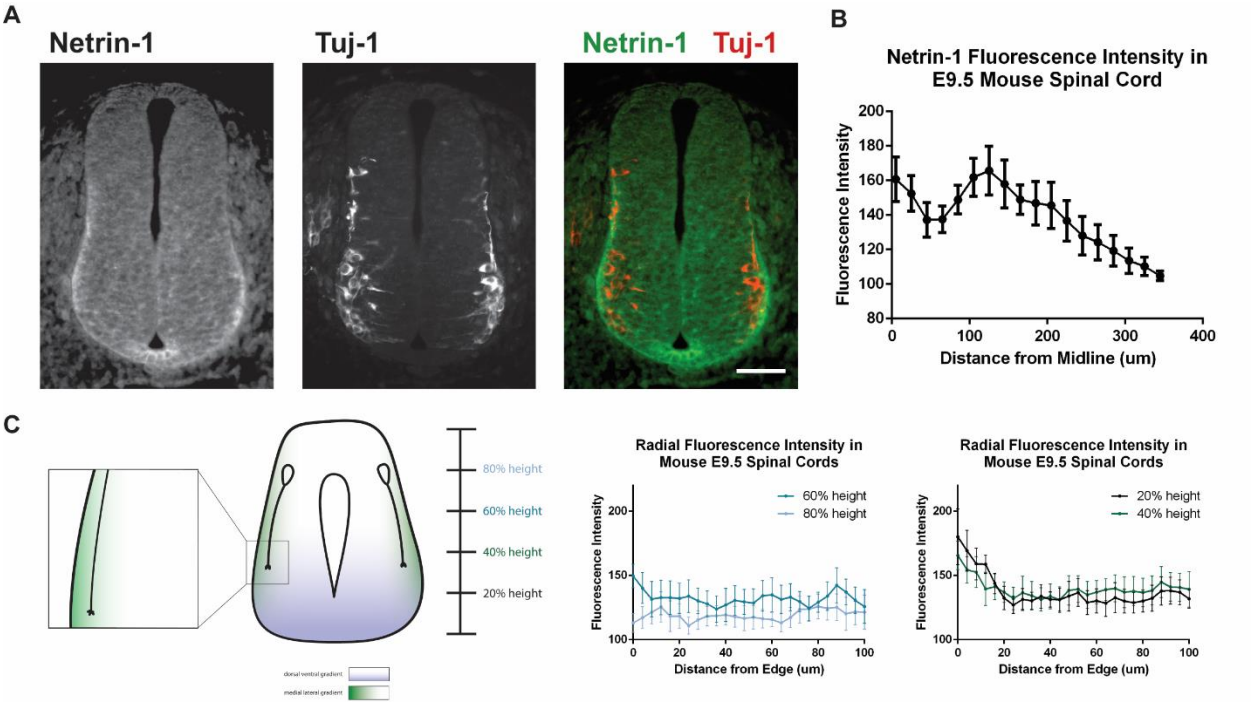

Figure 4. Dorsal-Ventral and Radial Gradients of Netrin-1 in Mouse Spinal Cord

(a) Netrin-1 immunohistochemistry in the embryonic mouse spinal cord reveals a graded distribution extending dorsally. Scale bar is 50 μm. (b) Measures of netrin-1 fluorescence intensity in E9.5 mouse show a similar fluorescence pattern to the chick. (c) Measurements of fluorescence intensity reveals a sharp radial increase in netrin-1 protein toward the lateral edge. This gradient may instruct axons to extend near the edge of the spinal cord. Notably, the radial gradient dissipates as the axons approach the ventral spinal cord and turn medially to bypass motoneurons populating the ventral horn.

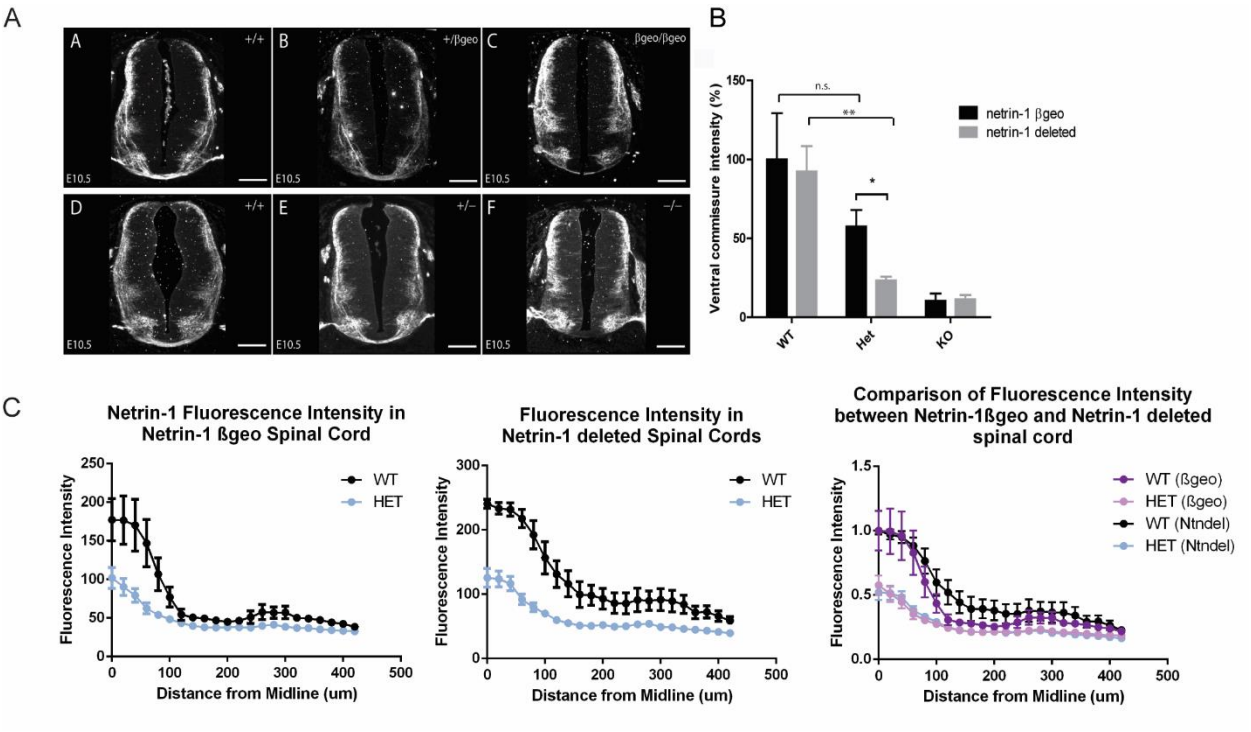

Figure 5. Graded Reduction of Netrin-1 Gene Dosage Reduces Gradient Steepness and Produces Graded Deficits in Commissural Axon Guidance

(a) Immunohistochemistry of Tag-1 on E10.5 netrin-1 βgeo and netrin-1 flox/flox spinal cords to visualize commissural neurons. Scale bars correspond to 100μm. (b) Fluorescence intensity of the ventral commissure of E10.5 embryos were quantified. (* indicates $p < 0.05$, ** indicates $p < 0.01$) (c) Fluorescence intensity measurements in E10.5 mouse also display a gradient extending along the commissural axon tract. This gradient is shallower in heterozygous animals.

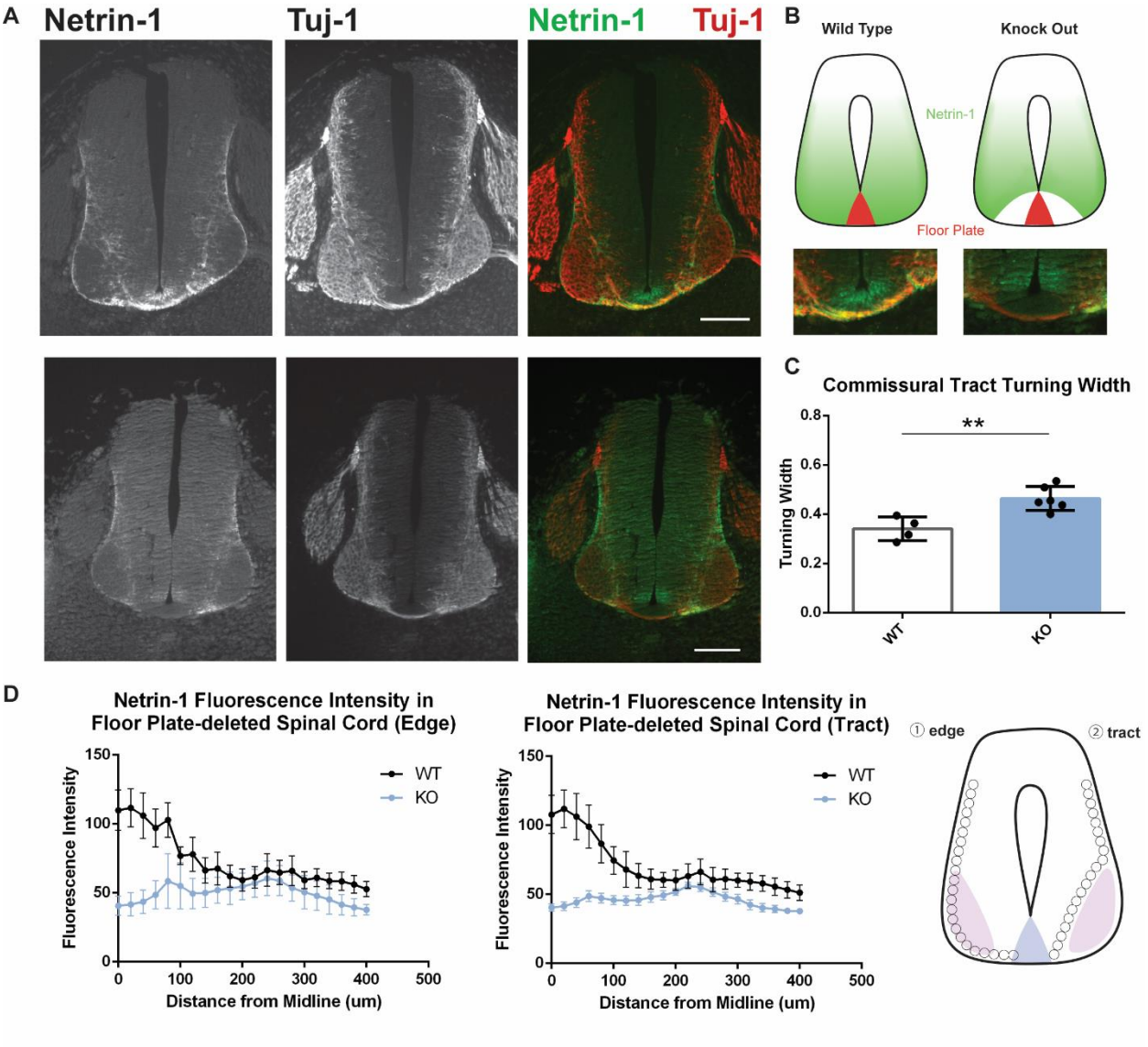

Figure 6. Floor Plate Netrin-1 is a Long-Range Guidance Cue

(a) The distribution of netrin-1 protein on sections embryonic mouse spinal cord that selectively lack netrin-1 expression by floor plate cells exhibit an absence of fluorescence in the vicinity of the floor plate. The exposure time for the image of the floor plate knockout was increased to better reveal the distribution of remaining netrin-1 protein in the neural epithelium. Scale bars correspond to 100 μm. (b) Model of netrin-1 protein distribution in wild type and Shh cre/netrin-1 flox/flox animals. Netrin-1 floor plate knockouts lack netrin-1 protein within ~200 μm of the floor plate.

Zoom in of the floor plate region in both wild type and knockout animals shows the difference in distribution of netrin-1 protein. (c) The commissural axon bundle turns medially significantly further from the midline in floor plate-knockout animals. (** indicates $p < 0.01$) (d) The difference in netrin-1 fluorescence intensity between wild type and knockout animals is apparent in the first 200-250 μm extending away from the midline. Diagram illustrates the paths along which fluorescence intensity was measured.

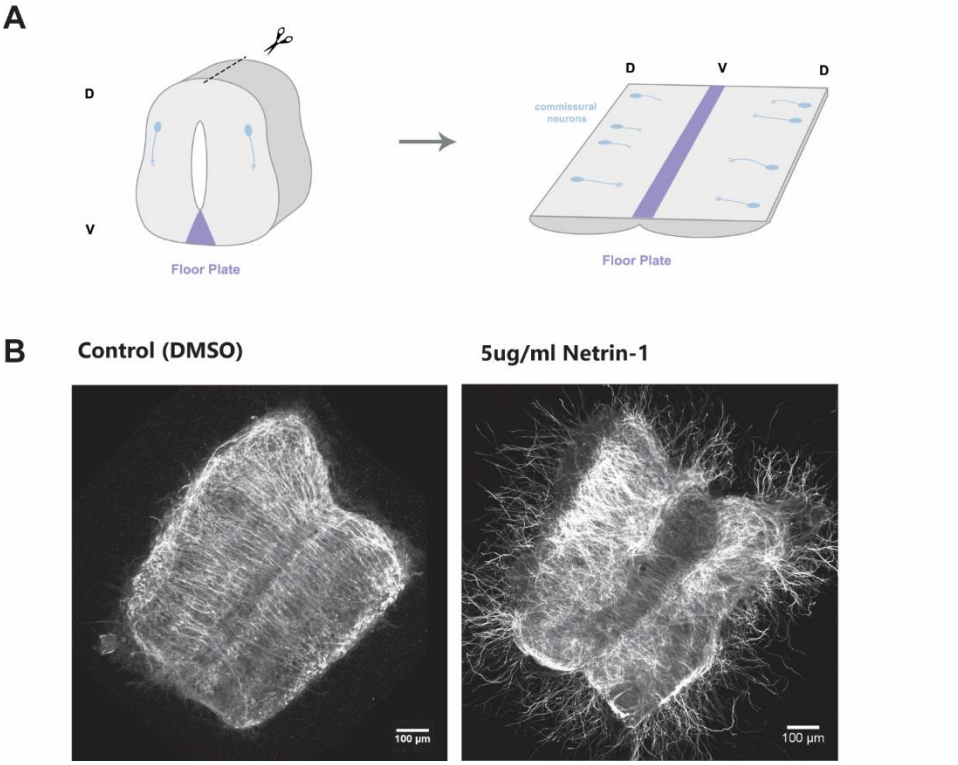

Figure 7. The Distribution of Netrin-1 is Instructive for Commissural Axon Guidance.

(a) Open book preparation of E11 rat spinal cord. Spinal cords are cut along the roof plate and opened similar to an open book. Commissural axons travel towards the center of the sample.

(b) To mask the distribution of endogenous netrin-1 E11 rat spinal cord open book explants were flooded with 5 µg/ml netrin-1 protein and cultured for 2 DIV. Explants with added netrin-1 exhibit highly altered commissural axon trajectories in comparison to control spinal cords. TAG1 immunostaining. Scale bars correspond to 100 µm.

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Chapter 4: General Discussion and Conclusion

Discussion

Netrin-1 was first characterised as a secreted axon chemoattractant protein (Kennedy et al., 1994; Serafini et al., 1994) and later shown to be distributed as a gradient in the embryonic spinal cord (Kennedy et al., 2006). These early studies identified floor plate cells as a potential source of secreted long-range netrin-1 in the embryonic spinal cord. At the same time, these same reports also provided evidence that a substantial amount of netrin-1 is bound to cell surfaces or extracellular matrix and may distributed as a short-range cue. Subsequent studies have sought to answer whether growth cones respond to soluble or bound netrin-1 and address the specific functional contribution of the floor plate to commissural axon guidance in the spinal cord. In 2017, the findings generated by two independent studies raised significant issues that questioned whether netrin-1 might be distributed and function as a long range chemoattractant (Dominici et al., 2017; Varadarajan et al., 2017). Our studies therefore focused on clarifying the controversy surrounding the distribution of netrin-1 in the embryonic spinal cord as a short-range or long-range cue, and its function as a permissive or instructive cue. The current literature is often imprecise when discussing what is meant by long-range and short-range cues, and similarly confused when describing permissive and instructive axon guidance functions. Here, we aimed to provide clear definitions that distinguish between long-range and short-range distributions, and permissive and instructive functions, and in this context, we address the distribution and function of netrin-1.

To determine the importance of netrin-1 in the embryonic spinal cord, the Kennedy lab had previously obtained evidence for a genetic dose dependent effect of netrin-1 on the number of commissural axons crossing the midline (Lai Wing Sun, 2015). Here, we examined how reducing *netrin-1* gene dosage affects the shape of the gradient. These findings, comparing the distribution of netrin-1 protein in null, heterozygous and wild type mouse embryonic spinal cord,

demonstrated a substantial flattening of the gradient within ~150 μm of the ventral midline, compared to more dorsally distributed netrin-1. These findings indicate that having two copies of *netrin-1* is crucial to generate the distribution of netrin-1 protein found in wild type embryos near the floor plate. Additionally, we show the presence of a medial lateral gradient of netrin-1 along the edge of the spinal cord, and we speculate that this radial gradient may contribute to constraining the extension of commissural axons along the outer edge of the neural epithelium.

By creating a mouse line with selective removal of *netrin-1* expression only from floor plate cells, we identified which portion of the gradient this specific source of netrin-1 was responsible for. This mouse line gave us key insights into where floor plate-derived netrin-1 traveled to, showing that it was capable of moving up to ~200 μm away from the midline. This finding provides key evidence that floor plate derived netrin-1 protein is distributed as a long range cue, which is defined as a molecule or cue that is distributed at a distance from its source. Our findings also support the conclusion that floor plate-derived netrin-1 is responsible for the steeper portion of the gradient nearer to the midline. In addition to this steep gradient of floor plate derived netrin-1, our findings also reveal a shallower, longer gradient, that we conclude is derived from ventricular zone netrin-1. We further demonstrate the functional significance of the gradient of floor plate derived netrin-1 by showing disruption of axonal trajectories in animals that selectively lack floor plate derived netrin-1. This is in line with a recent study from the Tessier-Lavigne lab which similarly reported disruption of axonal trajectories and reduced thickness of the ventral commissure in animals that selectively lack floor plate netrin-1 (Wu et al., 2019). These findings support the conclusion that netrin-1 has an instructive function. To address this issue directly, we performed additional experiments.

Using *ex vivo* explants of segments of embryonic spinal cord, we examined the functional significance of the distribution of netrin-1 protein. To mask the distribution of endogenous netrin-1 protein, we flooded embryonic rat spinal cords with ectopic netrin-1. In doing so, the goal of this manipulation was to overwhelm instructive information encoded in the distribution of endogenous netrin-1 derived from the spinal cord. The results of these studies demonstrate that the loss of instructive information has a significant impact on the trajectories taken by extending commissural axons. Our finding supports the conclusion that the distribution of netrin-1 has an instructive function in the embryonic spinal cord.

Overall, our studies dispute recent claims (Dominici et al., 2017; Varadarajan et al., 2017) that floor plate derived netrin-1 does not contribute to commissural axon guidance in the embryonic mouse spinal cord. While the presence of multiple guidance cues provides substantial redundancy as safeguards for axon guidance in the spinal cord (Stoeckli, 2018), our findings indicate that floor plate netrin-1 exerts a readily detectable impact on the trajectory of commissural axons, most notably as they turn away from initial circumferential extension along the edge of the neural tube to then project toward the midline. Our findings corroborate and extend studies by the Tessier-Lavigne lab (Wu et al., 2019), that described a wider turning angle and a thinner commissure in animals lacking floor plate derived-netrin-1. These findings support a model whereby floor plate derived netrin-1 works in conjunction with ventricular zone derived netrin-1, as two partially overlapping gradients, floor plate netrin-1 generating a long-range graded distribution and short-range ventricular zone derived netrin-1 generating a gradient of *netrin-1* expression, to guide commissural axons toward the ventral midline of the embryonic spinal cord.

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