Title
Netrin1 in spinal cord development: More than an axon guidance molecule

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Publication Date
2023

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Netrin1 in Spinal Cord Development:

More Than an Axon Guidance Molecule

A dissertation submitted in partial satisfaction of the requirements for the degree

Doctor of Philosophy in Molecular Biology

by

Sandy Alvarez

2023

ABSTRACT OF THE DISSERTATION

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Doctor of Philosophy in Molecular Biology

University of California, Los Angeles, 2023

Professor Samantha J Butler, Chair

Neural circuit development is dependent on the precise patterning of the spinal cord and the specific wiring of axons. Netrin1 has been historically studied for its axon guidance properties. However, studies have found netrin1 to be involved in other physiological functions, including suppressing Bone Morphogenic Protein (BMP) signaling. During development, BMP signaling patterns the dorsal spinal cord, setting the framework for the formation of neural circuits. This finding postulates netrin1 as a molecule that regulates multiple processes in the spinal cord: 1. As a potential modulator of spinal cord patterning and 2. As a mediator of neural circuit formation. In this thesis, I summarize findings from studies focused on investigating these distinct roles of netrin1 in spinal cord development.

First, we assessed the role of nertin1 in the patterning of the spinal cord. We found that ectopic expression of netrin1 reduces the number of dorsal interneurons (dI) in the spinal cord and inhibits the differentiation of mouse ESCs into dorsal dIs. In contrast, the loss of netrin1 *in vivo* increases BMP-dependent dorsal progenitors (dP). Furthermore, we find that netrin1 modulates BMP signaling by affecting levels of pSmad1/5/8, the effectors of BMP signaling, as well as the Ids, BMP downstream target genes. Together, these findings suggest a new role for netrin1 in the developing spinal cord, modulating BMP signaling.

Then, I assessed netrin1 in the context of axon guidance. I aimed to assess if cleavage of ventricular zone (VZ)-derived netrin1 could facilitate the transfer of netrin1 to the pial surface where it acts as an axon guidance cue. I used epitope-specific immunohistochemistry to characterize the distribution of netrin1 protein and observed that the antibodies decorate different spinal cord regions. I tracked myc-tagged-netrin1 and observed multiple myc-tagged fragments in the lysates. Additionally, I observed that the electroporation of c-terminally tagged netrin1-myc results in efficient localization of netrin1 and myc to the pial surface. This preliminary data supports the hypothesis that netrin1 cleavage products have the capacity to differentially localize within the spinal cord. Together, these studies advanced our understanding of nerin1-mediated activities in the developing spinal cord.

The dissertation of Sandy Alvarez is approved.

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2023

Dedication

This work is dedicated to my mom, Gloria - I hope I make you proud, Mom. To my siblings: Jafer, Jorge, Lucy, Diego, and Daniel, who have offered support, in many ways, and encouragement when I've needed it throughout my studies, thank you.

This is a dream come true and I am so grateful to have had this opportunity.

Dedicacion

Este trabajo está dedicado a mi mamá, Gloria - espero hacerte sentir orgullosa, mamá. A mis hermanos: Jafer, Jorge, Lucy, Diego y Daniel, que me han ofrecido apoyo, de muchas maneras, y aliento cuando lo he necesitado a lo largo de mis estudios, gracias.

Este es un sueño hecho realidad y estoy muy agradecida por la oportunidad.

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Acknowledgments

I would like to thank Dr. Samantha Butler for allowing me to do my thesis work in her lab, for being a great mentor, for her patience, and for creating a place for me to grow as a scientist and as a person. It's been a great experience. I would also like to thank Dr. Ben Novitch for giving me valuable feedback on my work during my time in the lab and my doctoral committee for helping guide my projects throughout my time as a Ph.D. student. I would like to thank my colleagues in both the Butler and Novitch labs, past and present, for their help, feedback, and kindness over the years.

I want to acknowledge those who came before me, for opening doors of opportunity for women and minorities in science. First a big thanks to the Bridges to the Baccalaureate program at Pierce Community College and UCLA, which brought me to UCLA in 2012, as a community college student, for my first research experience and introduced me to scientific research. I acknowledge Dr. Diana Azurdia who contributed to those efforts. A big thanks to the minority science programs (MSP) at UC Irvine-specifically Dr. Luis Mota-Bravo and Dr. Marlene Cruz who guided me on my early journey to graduate school, thanks to the MORE programs at CSULA and to Dr. Edith Porter, for being a mentor throughout my graduate school journey. I would like to thank my multiple funding sources that supported the completion of my thesis work: UCLA Eugene V. Cota-Robles Fellowship, T32 Cell, and Molecular Training Grant at UCLA, UCLA Whitcome Predoctoral Fellowship**,** UCLA Hilliard Neurobiology Grant**,** and the NIH F31 Pre-Doctoral Ruth L. Kirschstein National Research Service Award GM007185 to SA, and the NINDS/NIH (R01 NS107509), the Albon Scholars Program, Rose Hills Foundation, and Eleanor I. Leslie Chair in Pioneering Brain Research to SJB.

Chapter 1 includes material, with permission, from a published review:

Alvarez S, Varadarajan SG, Butler SJ. Dorsal commissural axon guidance in the developing spinal cord. Curr Top Dev Biol. 2021; 142:197-231. doi: 10.1016/bs.ctdb.2020.10.009. Epub 2020 Nov 19. PMID: 33706918; PMCID: PMC9109821. We thank Orkun Akin, Greg Bashaw, James Briscoe, Lorenzo del Castillo, Bennett Novitch, Marc Tessier-Lavigne, and Larry Zipursky for discussions.

Chapter 2 is a manuscript that is in preparation:

Alvarez, S, Gupta, S, Honeychurch, K, Mercado-Ayon, Y, Butler, SJ. Netrin1 patterns the dorsal spinal cord through modulation of BMP signaling. In preparation. We thank Dr. Lisa Goodrich for the netrin1 knockout line and Thomas Muller for the antibodies. We thank members of the Butler and Novitch labs for their discussion and comments.

Chapter 3 summarizes preliminary unpublished data from a project aimed at investigating the existence of netrin1 isoforms in the spinal cord. We thank the UCI transgenic mouse core for their help in creating the myc-tagged netrin1 mouse lines.

Chapter 4 summarizes this thesis and provides concluding statements.

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Alvarez, Sandy, Supraja G. Varadarajan, and Samantha J. Butler. Dorsal Commissural axon guidance in the developing spinal cord. Current Topics in Developmental Biology: Neural Development and Disease. 2020. https://doi.org/10.1016/bs.ctdb.2020.10.009

Comer, John D., **Sandy Alvarez**, Samantha J. Butler and Julia A. Kaltschmidt. Commissural Axon Guidance in the developing spinal cord: from Cajal to the present day. Neural Development. 2019; 14(9). https://doi.org/10.1186/s13064-019-0133-1

Selected Conference Posters

Sandy Alvarez, Sandeep Gupta, Kaitlyn Honeychurch, Yesica Mercado- Ayon and Samantha J. Butler. "Netrin patterns the dorsal spinal cord through modulation of BMP signaling". Society for Developmental

Biology 82nd Annual Meeting, Chicago, IL. July 20-23, 2023. Best Graduate Student Presentation Honorable Mention.

Sandy Alvarez, Nary Choe, and Gui-Feng Zhang. "Development of an in vitro cell-based assay to characterize the recycling of therapeutic IgGs". Eli Lilly and Company LatinX Science Day poster symposium, Lilly Biotechnology Center, San Diego, CA. November 09, 2022.

Sandy Alvarez and Samantha Butler. "Investigating the existence of netrin isoforms in the developing spinal cord". Molecular Biology Interdepartmental Ph.D. program Virtual Retreat. September 14, 2020.

Seminar Presentations

Sandy Alvarez. "Development of an in vitro cell-based assay to characterize the recycling of therapeutic IgGs. Eli Lilly and Company, Lilly Biotechnology Center, San Diego, CA. December 08, 2022.

Sandy Alvarez. "Investigating the mechanisms underlying netrin1-mediated axon guidance in the developing embryonic spinal cord". California State University, Los Angeles (CSULA) Biology Seminar Series. Zoom Seminar. April 30th, 2020.

Sandy Alvarez "Investigating netrin1-mediated commissural axon guidance in the developing spinal cord" UCLA Molecular Biology Interdepartmental Ph.D. program Student Seminar Series. March 20, 2019

Honors and Awards

National Institutes of Health/ National Institute of Neurological Disorders and Stroke/F31 Ruth L. Kirschstein National Research Service Award	2020-2022
Whitcome Pre-doctoral Fellowship in Molecular Biology	2020-2021
Jessamine K. Hilliard Neurobiology Graduate Student Grant	2020
NIH T32 Ruth L. Kirschstein National Research Service Award-	2018-2020
UCLA Eugene V. Cota-Robles Graduate Fellowship	2016-2020

Chapter 1 - Introduction

The formation of neuronal circuits is critical for normal nervous system function. Failure to make appropriate connections can result in errors in processing sensory stimuli, movement disorders, and intellectual disabilities. Neural circuits are generated when growth cones at the tips of axons use molecular cues in the environment to guide axon extension[6,7]. The formation of neural circuits begins in early embryonic development and involves the cooperation of multiple signaling centers and factors to ensure the successful development of the nervous system.

Organization of the spinal cord

Dorsal spinal commissural neurons first arise in the neural tube, an early embryonic structure that is generated when the neural plate folds to form a cylindrical tube. Initially only a single cell thick, the neural tube is a pseudostratified epithelium comprised of rapidly dividing neural progenitor cells (NPCs). The early neural tube consists of two compartments: a medial ventricular zone (VZ), containing the nuclei of the NPCs, and a more lateral marginal layer, containing the processes of the NPCs[8,9]. These processes are radial, spanning the VZ with an apical attachment, on the luminal side of the neuroepithelium, and a basal "endfeet" attachment on the basement membrane, which is also known as the pial surface (Figure 1-1A). This architecture permits the cell bodies of the NPCs to migrate back and forth along a lateral-medial axis within the VZ, as a function of the cell cycle, with mitosis taking place on the apical surface. NPCs continue dividing until they are ready to differentiate into mature neuronal subtypes. Newly formed post-mitotic neurons lose their apical-basal attachments and migrate laterally out of the VZ to form a third layer, the mantle layer. As development proceeds, the marginal layer expands to contain the fiber tracts, eventually becoming the white matter of the spinal cord.

Cellular organization of the spinal cord

As NPCs divide, they are subdivided into discrete progenitor (p) domains, defined by combinatorial codes

of transcription factors (Figure 1-1B, C). The identity of these progenitor domains is dependent on both intrinsic signaling from midline structures as well as extrinsic signaling from surrounding tissue, including the notochord and the paraxial mesoderm[9]. Briefly, ventral identity is specified by a gradient of sonic hedgehog (Shh) emanating from the floor plate (FP) at the ventral midline. Shh acts as a morphogen, with the concentration and duration of Shh signaling being decoded by signaling machinery in the primary cilia of NPCs on the apical side of the VZ[10-13]. Sharp boundaries between the progenitor domains are cemented by cross-repressive interactions between the transcription factors that define domain identity[14,15]. This process results in the specification of at least five progenitor domains in the ventral spinal cord: p0-3 and pMN, which give rise to the ventral interneurons and motor neurons (MNs) respectively. Similarly, the dorsal spinal cord contains at least six classes of dorsal interneurons, dI1- dI6, which are derived from six dP populations, dP1-dP6 (Figure 1-1B, C). The identity and proliferative capacity of the dorsal-most NPCs (dP1-3P3) depends on signals from the roof plate (RP) at the dorsal midline[16], which include members of the bone morphogenetic protein (BMP) and Wnt families[17,18]. The more ventral-dorsal NPCs (dP4-dP6) arise independently from signals from the RP; their identity may be dependent on signals from the adjacent paraxial mesoderm[19]. This organization of the spinal cord results in the different spinal cord laminae containing neurons segregated according to their distinct physiological properties and functions. In general, cells associated with control of motor functions are in or adjacent to the ventral horns whereas cells mediating sensory activities are present within the dorsal horn.

BMP specification of the spinal cord

Bmp signaling is required for the specification of dorsal interneuron populations[16,20,21]. Multiple BMPs, including BMP4, BMP5, BMP6, BMP7, and Growth/ Differentiation Factor (Gdf) 7 are secreted by the RP[18,22] and are sufficient to promote some dI1 formation[1,18,20,23]. BMPs have been proposed to act collectively as spatial[24] and/or temporal[25] morphogen gradient(s), to specify dorsal cell fates by analogy with the models of Shh patterning in the ventral spinal cord[10,13]. However, it has alternatively been

hypothesized that different BMPs have specific effects on the induction of dorsal neural fates[17,19,24]. Recent studies have distinguished between these models by methodically assessing the ability of RP-derived BMPs to direct dorsal spinal fates by either manipulating BMP expression in chicken embryos in vivo or culturing mouse (m) embryonic stem cells (ESCs) with different concentrations of recombinant Bmp proteins in vitro[1]. These studies unambiguously demonstrated that BMPs do not act as concentration-dependent morphogens. While altering the level of BMPs changed the effectiveness of NPC responses, i.e. the number of cells that were directed to a specific fate, the dose-dependent changes in dI identity predicted by the spatial or temporal morphogen models were not observed. Rather, each BMP showed both distinct and reiterative activities directing RP and dI1-dI3 fates[1,24]. dI1s appear to be most effectively specified by Bmp4[1,26] which acts reiteratively to first promote dP1 proliferation, and then direct these dP1s to differentiate into dI1 neurons (Figure 1-1D). BMPs mediate their signal-specific activities by activating distinct type BMP receptors (Bmprs) in dPs to permit differential progression through the cell cycle[1,27-29]. Activated type I Bmprs phosphorylate and thereby activate the R-Smads, the canonical intracellular mediators of Bmp signaling[30]. Smad1 and Smad5 are the only R-Smads present in the developing spinal cord[31] and it remains unresolved whether both Smad1 and Smad5[19,32] or Smad5 alone[31] is critical for dI1 fate specification. As in the ventral spinal cord, the consequence of dorsal signaling is to regulate codes of homeodomain and bHLH transcription factors that first define, discrete progenitor domains and then distinct classes of post-mitotic neurons[17,33,34]. The dorsal tube is demarcated by the expression of Pax3[35] and Pax7[36], which are the earliest known general markers of dorsal spinal identity. Work in vitro has suggested that the upregulation of Pax3/7 in dorsal tissue may depend on retinoic acid (RA) signaling from the paraxial mesoderm[37]. Subsequent BMP signaling from the dorsal midline results in the cells immediately flanking the RP activating Atoh1[38], a bHLH transcription factor that is both necessary and sufficient for dP1 identity[39]. Atoh1 in turn upregulates a transcriptional cassette mediated by Lhx2 (Lh2a) and Lhx9 (Lh2b), which establishes the intraspinal trajectory of commissural neurons. Lhx2 and Lhx9 are initially co-expressed by all new-born dI1s as they start to extend axons[20].

dI1s also concomitantly migrate ventrally within the mantle layer; on reaching the deep dorsal horn, they segregate into two subpopulations that differentially express either Lhx2 or Lhx9[20,40]. These subpopulations have distinct axial positions and axonal trajectories. The Lhx2+ population (dI1c), has a medial position and projects axons contralaterally across the FP whereas the Lhx9+ population (dI1i) has a more lateral position and projects axons ipsilaterally within the ventral funiculus[40-42].

Formation of neural circuits

The first decision made by all newly formed spinal neurons in the mantle zone is to extend a growth cone into the marginal zone in a specific direction. Depending on their complement of guidance receptors, growth cones interpret signaling information in the neuroepithelial environment to guide them into their initial trajectory. All spinal commissural neurons initially extend axons ventrally thereby establishing one of the organizing architectural principles of the spinal cord: that contralaterally projecting axons only cross at the ventral midline[8,43]. The mechanisms that direct commissural axons ventrally have been most extensively assessed for the dI1 population, because it was one of the first vertebrate trajectories to be distinguishable by immunohistochemistry. dI1 axons were initially detected using antibodies against the glycoprotein, Tag1, which labels the dorsal-most subset of commissural axons in their transverse trajectory in the spinal cord, i.e. as they extend up to and across the FP[44]. More recently, antibodies against robo3 have been shown to generally label all dorsal commissural axons[45,46], while dI1 axons can be unambiguously identified using a genetically encoded marker: the Atoh1::taugfp transgenic mouse line[31,47]. dI1s are born relatively early, E10.5 in the mouse rostral spinal cord[31], arising immediately from flanking the RP. In addition to instructing cell fate specification, the RP is also thought to be a key source of axon guidance cues for dI1 axons. There are two complementary mechanistic possibilities: first, the RP is itself intrinsically repulsive and does not support axon growth, and second, the RP secretes a diffusible repellent that orients axons away, thereby directing dI1 axons to grow ventrally. Supporting the latter model, in vitro tissue culture "reorientation" assays demonstrated that an ectopically placed RP explant is

sufficient to reorient Tag1+ axons away from the RP, as they extend within an explant of rat dorsal spinal cord[48].

Netrin1 as an axon guidance molecule

The transverse path of dI1 axons in the developing spinal cord follows a circumferential dorsal-to-ventral route toward the FP at the ventral midline. Previous studies suggested this trajectory could be achieved mechanistically by a "push" from the RP and a "pull" from the FP[49] (Figure. 1-2A). However, dI1 axons extend precisely around the border of VZ and avoid numerous potential exit points for pioneering axons, including the dorsal root entry zone (DREZ) and the lateral and motor exit points[50]. Such complex pathfinding is likely to require instructive cues in addition to a simple combination of push/pull signaling. Supporting this hypothesis, netrin1 has been shown to play a crucial role in both guiding commissural axons around the VZ and maintaining the CNS-PNS boundary[2,3,27,51].

The netrins are a family of laminin-like proteins that were first characterized in the early 1990s as the prototypical vertebrate axon guidance cue[52,53]. Netrin1 (from the Sanskrit netr, meaning "one who guides") was identified as a candidate for the FP attractant, whose existence was first hypothesized by Ramon y Cajal in 1890[54]. Netrin1 mediates its activity through two classes of receptors: Deleted in Colon Cancer (Dcc) and the Unc5 family, all members of the immunoglobulin (Ig) domain superfamily[55-58]. Dcc is thought to mediate the attractive properties of netrin1[59], while the members of the Unc5 family, Unc5A-Unc5D, can mediate the repulsive properties of netrin1[55,57,60,61]. Netrin1 has a complex distribution pattern in the mouse spinal cord: it is transcribed by both NPCs and the FP cells, with a sharp expression boundary in the dorsal spinal cord at the level of the DREZ (Figure 1-2B, C). However, netrin1 protein does not remain in the VZ, rather it appears to be transported along the radial processes of the NPCs (Figure 1-2B, E). Netrin1 is then deposited on the pial surface, via the glial endfeet, and accumulates on commissural axons[2,62] (Figure 1-3A). Pial-netrin1 deposition starts as early as E9.5[4], immediately before the pioneering dI1 axons commence their trajectory toward the FP (Figure 1-2B).

Netrin1 prevents dI1 axons from innervating the VZ

As pioneering dI1 axons extend ventrally, they grow around the edge of the VZ, without invading it. This behavior is not unique to dI1 axons; all spinal axons avoid growing VZ, thereby establishing another architectural organizing principle of the spinal cord. Recent studies have shown that this remarkably homogenous behavior is mediated by NPC-derived netrin1. In the complete absence of netrin1, spinal axons, most notably the robo3^{+} commissural axons, are profusely defasciculated and extend randomly, including into the VZ[2,5] (Figure 1-3B). This effect is phenocopied by the conditional removal of netrin1 from either the Pax3 domain[2,63] (Figure 1-3C), i.e. all dorsal NPCs (netrin1△dVZ), or the Dbx1 domain, i.e. a specific subpopulation of NPCs (Figure 1-3D). In netrin1△dVZ spinal cords, randomized axon growth is only observed in the dorsal spinal cord, where deposition of pial-netrin has been disrupted (Figure 1-3C). This effect is very short-range: when netrin1 is removed from Dbx1+ NPCs, axons only invade the narrow channel in the VZ where netrin1 has been depleted[2] (Figure 1-3D). Together, these studies identified that NPC-derived netrin1 establishes a boundary, that promotes the growth of fasciculated commissural axons around the VZ. This boundary activity of NPC-netrin1 is mediated by the Dcc receptor. In the absence of Dcc, netrin1 is still present at the pial surface but does not accumulate on commissural axons, which then grow in a profoundly de-fasciculated manner[2] (Figure 1-2F, G). Thus axonal-netrin1 appears to be key to establishing the VZ boundary.

Netrin1 supplies a "hederal" boundary

How mechanistically is the NPC-derived boundary of netrin1 established? The deposition of netrin1 on the pial surface suggests the formation of a local, attractive growth substrate in the dorsal spinal cord. Indeed, in the earliest stages of axogenesis, dorsal pial-netrin1 may act by haptotaxis, the directed growth of cells along an adhesive surface[64], instructing pioneering axons to grow ventrally, immediately adjacent to the netrin1^{+} substrate (Figure 1-2B). However, pial-netrin1 does not subsequently shape the transverse trajectory of commissural axons in the ventral spinal cord, rather commissural axons grow to precisely

follow the boundary of the netrin1^{+} VZ (Figure 1-2C, D). Multiple conditional manipulations of netrin1 expression[2,4] have shown that commissural axons will deviate from their trajectories to extend around ectopic borders of netrin1-expressing cells as if these cells are supplying a boundary repellent (Figure 1-3C, D, I). We have proposed the hederal boundary model to reconcile these distinct activities (Figure 1-2C, D). This model is drawn from the analogy of a wall supporting a growing hedera (ivy plant); the wall provides a substrate for ivy to grow along while preventing the ingrowth of the ivy into the wall. We propose that the hederal boundary is mediated by the ability of netrin1 to locally transfer, or be trafficked, from NPCs to the pial surface and then onto Dcc+ axons (Figure 1-2E, F). Axonal-netrin1 can both promote the directed fasciculated growth of commissural axons and prevent them from growing on netrin1-expressing cells in the VZ. Thus, early extending Dcc^{+} commissural axons are first oriented by the dorsal boundary of pial-netrin1 in the spinal cord. Commissural axons then accumulate netrin1; axonal-netrin1 acts to fasciculate axons together and direct them around the netrin1 expressing VZ. This model is supported by the observations that pial-netrin is not sufficient to guide Dcc- commissural axons (Figure 1-2G) and that occasional axons growing aberrantly in the VZ in controls have never accumulated netrin1[2]. The mechanism by which netrin1 becomes sequestered on axons is unresolved. Draxin may have a role in this process: the crystal structure of draxin either alone or in a netrin1-Dcc complex has shown that netrin1 and Dcc can bind to draxin on adjacent sites. Moreover, the sequestration of draxin results in the same pattern of randomized axon growth observed in netrin1 and Dcc mutant spinal cords[65]. Thus, draxin may bind to both molecules to facilitate the binding of netrin1 to Dcc^{+} axons, to promote fasciculation and directed growth. Finally, the hederal activity of netrin1 appears to act over a very short range. It remains unresolved whether netrin1 is always substrate-bound, or whether there is some limited diffusion from the radial fibers (question marks, Figure 1-2E). However, it is notable that there is no protein accumulation on the pial surface in the dorsal-most spinal cord, i.e., where NPCs do not express netrin1, suggesting very limited diffusivity, if any. Further studies examining the activities of membrane-bound forms of netrin1 in vivo are necessary to answer these questions.

Netrin1 maintains the CNS-PNS boundary

As dI1 axons begin their journey away from the RP, they must also avoid exiting the dorsal spinal cord from the DREZ, the entry point into the CNS for peripheral dorsal root ganglion (DRG) axons. The DRG begins innervating the spinal cord through the DREZ at E13.5 in mice[66]. Netrin1 plays a role in preventing both commissural axons from leaving the spinal cord and sensory axons from entering the spinal cord inappropriately. In netrin1/Dcc mutants, commissural axons aberrantly exit the spinal cord and grow into the periphery[50]. The transient expression of netrin1 at E12.5 in a domain adjacent to the DREZ[67,68], acts as a repellent boundary that prevents DRG axons from prematurely entering the spinal cord[4,66,67]. Recent studies have suggested a role for cues emanating from the spinal meninges, which include the pial-substrate. Studies using dorsal spinal explants have shown that commissural axons are repelled by spinal meninges tissue while motor and sensory axons are attracted by meninges-derived cues[69]. Is the meninges-bound cue that prevents commissural axons from leaving the spinal cord mediated by netrin1? The addition of netrin1 to the explant cultures complicates this interpretation, however, there is support for this hypothesis from in vivo studies. In the absence of pial-netrin1, commissural axons, as well as pre-cerebellar neurons in the hindbrain and pontine neurons, erroneously cross the CNS/PNS boundary and are no longer confined to the CNS[2,3,50,62,70].

Guidance of commissural axons toward and across the FP

On entering the ventral spinal cord, commissural axons detach from the circumferential edge of the spinal cord, to continue growing around the VZ, and toward the FP at the ventral midline. As commissural axons enter the ventral spinal cord, they become more progressively fasciculated until they project across the FP in very tightly bundled fascicle. Netrin1 was initially identified as a compelling candidate for the "pull" attractant acting from the FP to draw commissural axons to the ventral midline. Netrin1 is expressed at highest levels by FP cells[52], and COS cell aggregates expressing netrin1 can mimic the ability of the FP to promote the fasciculated growth of commissural axons toward them[52,53,71]. Loss of

function studies initially revealed that mice mutant for netrin1 or Dcc exhibit a transient stall in Tag1$^+$ commissural axon growth, with axons failing to reach the FP[53,59,72]. Together, these studies suggested the model that FP-derived netrin1 is a long-range cue that acts by chemotaxis to guide commissural axons to the ventral midline (Figure 1-2A). However, more recent studies have cast doubt on this model. Studies first in vitro[73] and then in vivo in Drosophila[74-76] demonstrated that tethered netrin1 can effectively function as a guidance cue. Most recently, NPC-derived netrin1 has been shown to direct fasciculated commissural axon growth, while specifically depleting FP-derived netrin1 (netrin1△FP) results in surprisingly minimal effects on the passage of robo3+ commissural axons toward the FP[2,5,62] (Figure 1-3E) . Studies further characterizing netrin1△FP embryos have found local defects (boxed region, Figure 1-3F, G, see figure legend for details); in particular, some commissural axons now grow ipsilaterally immediately adjacent to the netrin1-depleted FP[3] (Figure 1-3H, I). This phenotype is consistent with either a local role for FP-derived netrin1 promoting contralateral growth, or the secondary consequence of introducing a longitudinal hederal boundary, given that the netrin1△FP manipulation introduces an ectopic on: off netrin boundary along the edge of the FP (Figure 1-3H, I). Other candidates for the "pull" long-range attractant include the morphogen sonic hedgehog (Shh), and vascular endothelial growth factor (Vegf)[77] which are both secreted by the FP. Localized sources of both Shh and Vegf can reorient commissural axons toward them[77,78], as has been observed for FP explants[52,53,79]. Shh appears to act through a complex of smoothened[78] and brother of Cdo (Boc)[80]. In the absence of these receptors, commissural axons defasciculate, and extend into the motor column. A similar phenotype is observed when Vegf, or its receptor fetal liver kinase 1 (Flk1), is specifically depleted from the FP or commissural neurons, respectively[77]. Together, these studies suggest that long-range activities supplied by FP-derived Shh and Vegf are necessary to direct fasciculated axon growth in the ventral spinal cord. Supporting this hypothesis, synergistic guidance interactions between Shh and netrin1 have been identified. These interactions were first observed in vitro: use of microfluidic guidance assays revealed that commissural growth cones can sense a shallow combined Shh/netrin1 gradient but are nonresponsive to an equivalently

shallow gradient of Shh or netrin1 alone[81]. This activity was recently observed in vivo: the combined loss of Boc and netrin1△FP results in significantly more defasciculation of robo3+ axons into the motor column than is observed for the loss of Boc or netrin1△FP alone[5] (Figure 1-3F, G). Thus, long-range signaling from the FP may be necessary for the progressive fasciculation of commissural axon bundles as they project to the FP.

Functions of netrin1 beyond axon guidance

Many studies have focused on the role of netrin1 directing neural circuit formation in the developing spinal cord, where it was initially identified[52,53]. However, netrin1 is widely expressed in both the developing and adult nervous systems, including in the forebrain, optic disc, cerebellum, and spinal cord[52,82-84], as well as in various tissues outside of the nervous system, including the lung, pancreas, mammary glands, intestine, and developing heart[85-89]. The netrin family has subsequently been shown to play many important roles in developmental and physiological processes beyond axon guidance. Netrin1 is involved in the progression of many types of cancers[90-94], diabetes[95], and inflammatory bowel diseases[96]. Netrin1 has also been shown to direct cellular differentiation across organ systems. In the skeletal system, netrin1 mediates bone remodeling by suppressing osteoclast differentiation and promoting osteoblast differentiation[97]. Netrin1 also plays a role in the morphogenesis and differentiation of the mouse mammary glands[98] and can induce neuroectodermal-like differentiation of human EC cells[99].

Netrin1: a novel regulator of BMP signaling?

More recently, netrin1 has been shown to suppress the BMP signaling pathway in different cell types *in vitro*[100]. This finding is significant as BMP signaling is critical for many aspects of development, both netrin1 and members of the Bmp family are widely expressed, with the BMPs also having reiterative roles in cell growth, differentiation, migration, apoptosis, and homeostasis in the developing embryo and adult[101]. In mouse models, loss of BMP2[102] or BMP4[103] results in embryonic lethality, and BMP1[104], and BMP7[105] knockouts die shortly after birth. Loss of BMP12/GDF7 results in loss of interneuron

subclasses[20]. BMP7 deletion results in reduced cortical thickening and impaired neurogenesis[106]. BMP4 deficient mice do not have differentiation of mesoderm, suggesting that BMP4 is essential for developmental processes as early as gastrulation[107].

Regulation of BMP signaling has been previously characterized and it is known that BMP signaling is regulated by extracellular, intracellular, and membrane modulators[108]. Extracellular modulators act as agonists or antagonists of BMP signaling and these include the CAN (Cerberus and DAN) family of proteins, Twisted gastrulation, Chordin, Noggin and Crossveinless[109]. Intracellular regulators of BMP signaling include microRNAs, I-SMADS, phosphatases such as PP1 and PP2A that dephosphorylate the receptor and R-Smad, and FK506-binding protein 1A (FKBP1A or FKBP12) that binds the GS domain of type I receptors to inhibit receptor internalization[110]. Co-receptors, including Endoglin, in the plasma membrane that interact with type I and type II receptors further regulate BMP signaling[111]. The recent finding that netrin1 suppresses BMP signaling in vitro postulates netrin1 as a novel modulator of BMP signaling but the significance of this finding in vivo has yet to be described.

Summary

The canonical role of netrin1 in the embryonic spinal cord as an axon guidance molecule mediating the formation of neural circuits has been widely studied. The recent discovery that netrin1 acts as a short-range guidance cue to direct commissural axons toward the floor plate, has opened avenues for further exploration. One of the many questions that remain to be explored is how does netrin1 get from the ventricular zone to the pial surface where it guides axons towards the ventral midline. Additionally, the recent discovery that netrin1 suppresses BMP signaling in vitro, postulates netrin1 as a modulator of spinal cord patterning.

In Chapter 2, we assessed the role of netrin1 in the process of spinal cord patterning through modulation of BMP signaling using both gain- and loss-of-function approaches to assess whether netrin1 restricts BMP activity to the dorsal spinal cord. Supporting this hypothesis, the ectopic expression of netrin1 *in*

vivo leads to reduced number of dorsal interneurons and *in vitro* inhibits differentiation of mouse ESCs into dorsal dIs. By contrast, the loss of netrin1 *in vivo* increases BMP-dependent dorsal progenitors. Furthermore, we find that netrin1 modulates levels of pSmad1/5/8, the effectors of BMP signaling as well as the Ids, Bmp-signaling downstream target genes involved in inhibition of differentiation.

In Chapter 3, I revisit netrin1 in the context of axon guidance. Ventricular zone (VZ) derived netrin1 is transferred along radial processes to the pial surface where axons use it as a surface to help guide themselves to the floor plate. The mechanism by which netrin is transported to the pial remains unknown. Previous studies have suggested that cleavage of netrin1 into distinct isoforms could facilitate transfer of netrin1[112,113]. To assess whether different isoforms of netrin1 exist in the spinal cord, I used epitope specific immunohistochemistry to characterize distribution of netrin1 protein and observed that the antibodies decorate different regions of the spinal cord. I tracked myc-tagged-netrin1 *in vitro* and observed multiple fragments containing myc in the lysates. Additionally, I observed that the electroporation of c-terminally tagged netrin1-myc results in efficient localization of netrin1 and myc to the pial surface. This preliminary data supports the hypothesis that netrin1 can be cleaved and that cleavage products have the capacity to differentially localize within the spinal cord.

Figures and Tables

Figure 1-1. Organization and specification of dI1s in the spinal cord.

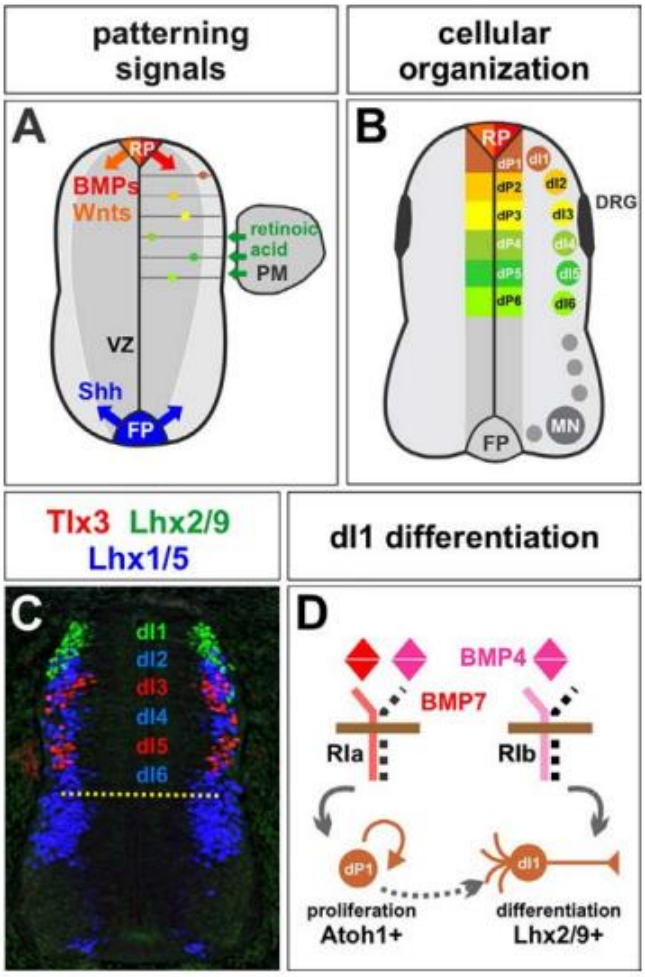

Figure 1-1. Organization and specification of dI1s in the spinal cord. (A, B) Six domains of dorsal progenitor neurons (dP1–dP6) arise in the ventricular zone (VZ) in response to Bmp/Wnt signals from the roof plate (RP) and retinoic acid (RA) from paraxial mesoderm (PM). This process results in six distinct classes of post-mitotic dorsal neurons (dI1-dI6). (C) Dorsal interneurons can be distinguished by their expression of distinct transcription factors[1]. (D) Model for the specification of dI1s. Both BMP4 and BMP7 can promote Atoh1+ dP1 patterning through BmprIa or BmprIb (chicken), but only BMP4 directs progenitors to differentiate as Lhx2+ dI1s through BmprIb (mouse and chicken).

Figure 1-2. Models for the long- and short-range activities of netrin1.

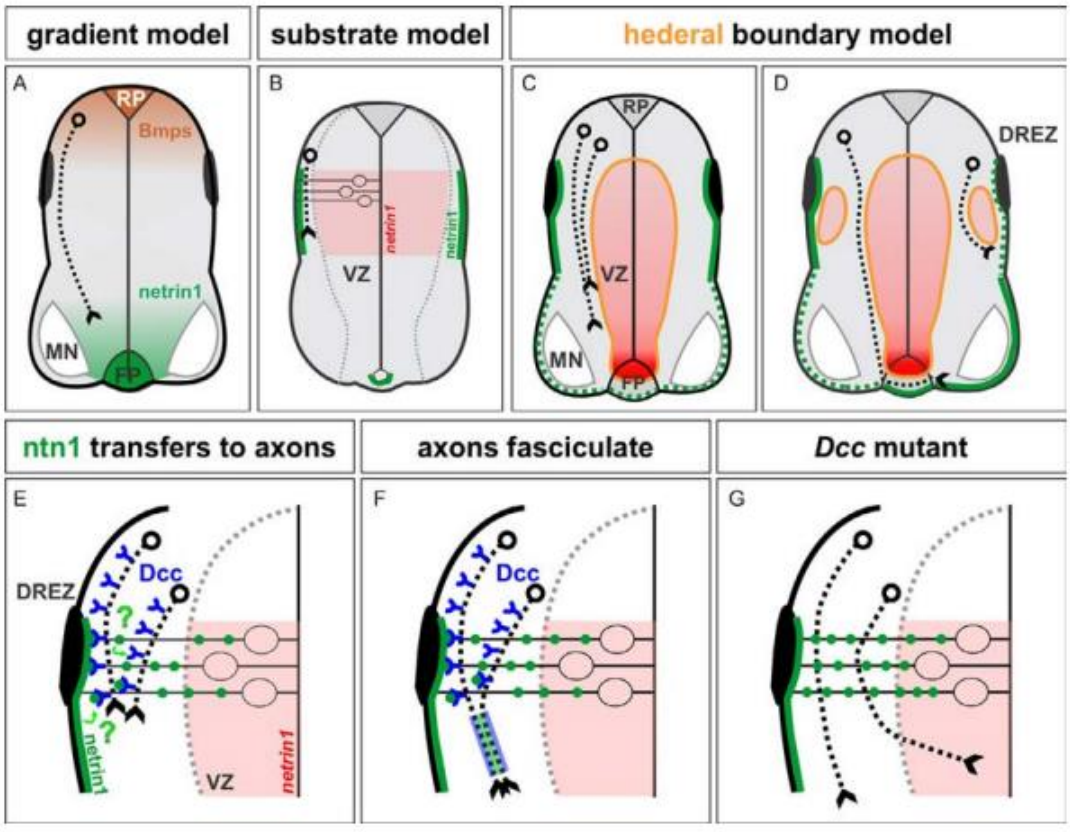

Figure 1-2. Models for the long- and short-range activities of netrin1. (A) In the long-range gradient model, the RP-derived Bmps act as a chemorepellent "pushing" commissural axons away from the dorsal midline, while FP-derived netrin1 and Shh function as chemoattractants "pulling" commissural axons to the ventral midline. (B) In the short-range model, pial-netrin1 first orients early born commissural axons to extend ventrally. NPCs transcribe netrin1 (red) and then deposit netrin1 protein (green) on the pial surface, where it may act as a growth substrate to promote axon extension[2]. (C) As development progresses, pre-crossing commissural axons extend into the ventral spinal cord, and no longer grow adjacent to pial-netrin1.

Figure 1-2 Continued

(dotted green line). Rather, they project precisely around a "hederal" boundary of netrin1 expressing NPCs (orange line). We have proposed that the netrin1 hederal boundary promotes directed axon fasciculation while preventing innervation of netrin1 expressing cells. This activity permits commissural axons to grow around the VZ. (D) Commissural axons extend across the FP in a highly fasciculated bundle within a narrow corridor bounded by hederal-netrin1 and pial-netrin1. Post-crossing commissural axons then turn rostrally to extend in the ventral funiculus, again growing adjacent to a pial-netrin1 substrate (solid green line). Concomitantly, a domain of netrin1 expressing cells (red) emerges adjacent to the DREZ, which continue to sculpt axonal trajectories within the spinal cord. (E–G) We propose that the netrin1 produced by neural progenitors is transported to the pial surface in their progenitor endfeet, and then transfers from this pial-substrate to Dcc+ commissural axons[2,4] (E). Dcc and netrin1 then interact in cis to promote the selective fasciculation and growth commissural axons around netrin1 expressing NPCs (F). In absence of Dcc, netrin1 does accumulate on the pial surface, but does not transfer to commissural axons. These axons fail to fasciculate and grow randomly, including into the VZ (G).

Figure 1-3. Short- and long-range phenotypes observed in the absence of NPC- or FP-derived netrin1.

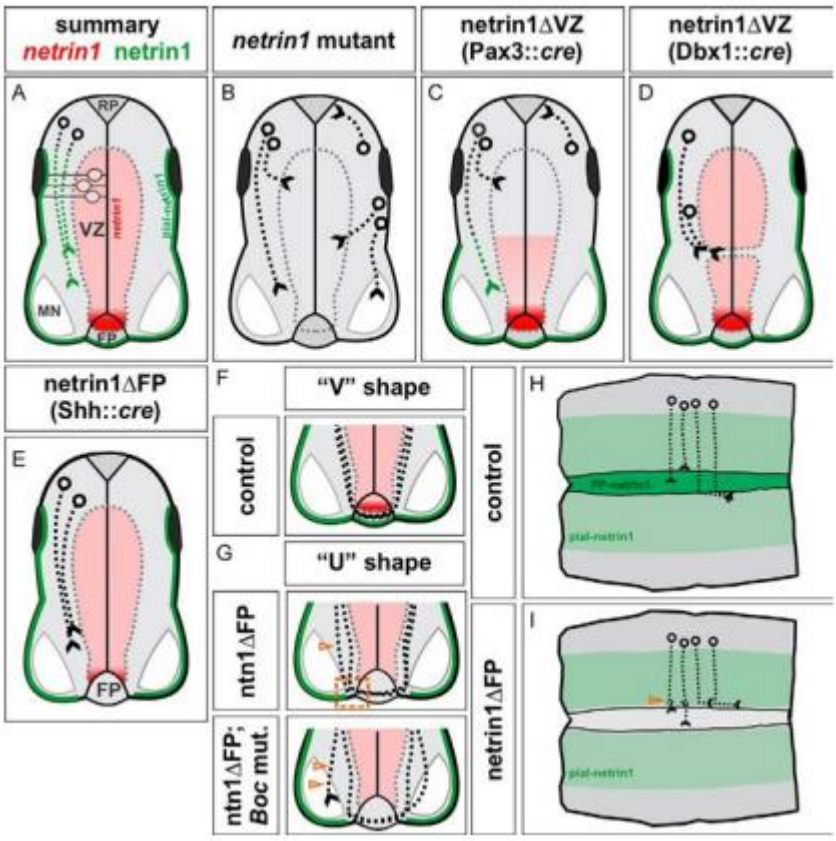

Figure 1-3. Short- and long-range phenotypes observed in the absence of NPC- or FP-derived netrin1. (A) Summary of the distribution of netrin1 transcript (red) and protein (green). NPCs and FP cells express netrin1, while netrin1 protein is present on the pial surface and on commissural axons. (B) In netrin1 loss of function mutants, spinal axons are highly defasciculated, extending into the VZ, and wandering across the motor column. (C, D) Short range phenotypes for NPC-derived netrin1: When netrin1 expression is removed from either a large (C) or small (D) number of NPCs using conditional genetic approaches (C=Pax3::cre driver; D=Dbx1::cre driver)[2] , Axons defasciculate specifically and locally in the region where netrin1 activity is absent. In particular, the introduction of ectopic netrin1 on::off boundaries (D) locally reshapes axon trajectories in a manner consistent with the hederal boundary model.

Figure 1-3 Continued

Long-range phenotypes for NPC-derived netrin1: none observed. (E–I) Short-range phenotypes for FP-derived netrin1: In netrin1ΔFP mice (Shh::cre driver), commissural axons project ventrally toward the FP (E) in a manner largely indistinguishable from controls. However, errors occur 10μm from the FP (yellow box, G), when axons reach the "off" edge of the ventral domain pial-netrin1, where they locally defasciculate (arrowheads, I). Axons start to cross the FP from this "off" edge, resulting in a laterally displaced "U" shaped trajectory (G), distinct from the "V" shape observed in controls (F). Control commissural axons then turn rostrally to project longitudinally beside the FP in the ventral funiculus (H). Most axons correctly make the rostral turn in netrin1ΔFP mice, however, we (S.A. and S.J.B., personal communication) and others[3] have observed that a subset of commissural axons fail to cross the FP, and turn ipsilaterally (I), resulting in a thinning of the FP commissure[5]. It remains unresolved whether this ipsilateral turn is the result of axons following an ectopic hederal boundary created by the specific loss of netrin1 in the FP (I), or a direct requirement for FP-netrin1 in axon crossing. Long-range phenotypes for FP-derived netrin1: While we have not observed this phenotype, other reports[5] have shown that commissural axons are modestly defasciculated in the ventral spinal cord in the absence of FP-derived netrin1 (yellow arrowheads, G) i.e. 100μm from the FP. This phenotype is significantly enhanced when netrin1ΔFP is combined with a Boc mutation (G), the non-canonical receptor that mediates the axon guidance activities of Shh[5].

Chapter 2- Netrin1 patterns the dorsal spinal cord through modulation of BMP signaling.

Abstract: We have identified an unexpected role for netrin1 as a suppressor of bone morphogenetic protein (BMP) signaling in the developing dorsal spinal cord. Using a combination of gain- and loss-of-function approaches in chicken, embryonic stem cell (ESC) and mouse models, we have observed that manipulating the level of netrin1 specifically alters the patterning of the BMP-dependent dorsal interneurons (dIs), dI1-dI3. Altered netrin1 levels also change BMP signaling activity, as measured by bioinformatics, and monitoring phosophoSmad1/5/8 activation, the canonical intermediate of BMP signaling, and Id levels, a known Bmp target. Together, these studies support the hypothesis that netrin1 acts from the intermediate spinal cord to regionally confine BMP signaling to the dorsal spinal cord. Thus, netrin1 has reiterative activities shaping dorsal spinal circuits, first by regulating cell fate decisions and then acting as a guidance cue to direct axon extension.

Introduction: Netrin1 is a laminin-like protein that was first characterized for its axon guidance activities during embryonic development[52,53]. Netrin1 is widely expressed in both the developing and adult nervous systems, including in the forebrain, optic disc, cerebellum, and spinal cord[52,82-84], as well as in various tissues outside of the nervous system, including the lung, pancreas, mammary glands, intestine, and developing heart[85-89].

Many studies have focused on the role of netrin1 directing neural circuit formation in the developing spinal cord, where it was initially identified[52,53]. Spinal neurons arise at stereotyped positions along the dorsal-ventral axis, such that the dorsal spinal cord is comprised of at least six populations of dorsal interneurons (dIs), dI1-dI6, that are derived from six dorsal progenitor (dP) domains, dP1-dP6[17,114]. This pattern is generated by multiple signals, which collectively act on proliferating neural progenitor cells (NPCs) in the ventricular zone (VZ)[17,114]. In the dorsal spinal cord, these signals include multiple members of bone morphogenetic protein (BMP) family, which are secreted from the roof plate (RP) at the dorsal midline of the spinal cord[18,22]. BMPs are sufficient for the specification of the RP itself, as well as

the dI1s, dI2s and dI3s[1,16,21,115,116]. The BMPs activate distinct type I Bmp receptors[1,27], which in turn phosphorylate the receptor-regulated (R)-Smads, Smad1/5/8, the intracellular mediators of Bmp signaling[117], to direct dPs to differentiate into post-mitotic dIs[118]. Additional signals include retinoic acid (RA), present in the surrounding paraxial mesoderm and important for patterning and neuralization[119-121]. Immediately after neurogenesis, dIs start to extend axons towards their synaptic targets[7]. Netrin1 acts to direct dI1 (commissural) axons towards the floor plate (FP), at ventral midline of the spinal cord[52]. While *netrin1* is expressed by both NPCs in the intermediate VZ, and the FP in the mouse spinal cord, recent studies have suggested that it is the NPC-derived netrin1 that is most critical for mediating axon guidance events[2,4,63,122]. NPC-derived netrin1 is thought to be trafficked along the radial processes of the NPCs until it is deposited on the pial surface, where it forms an adhesive growth substrate that locally orients dI1 axon extension[122-124].

The netrin family has subsequently been shown to play many critical roles in developmental and physiological processes beyond axon guidance. Netrin1 is involved in the progression of cancers[90-94], diabetes[95] and inflammatory bowel diseases[96]. Netrin1 has also been shown to direct cellular differentiation across organ systems. In the skeletal system, netrin1 mediates bone remodeling by suppressing osteoclast differentiation and promoting osteoblast differentiation[97]. Netrin1 also plays a role in the morphogenesis and differentiation of the mouse mammary glands[98] and can induce neuroectodermal-like differentiation of human EC cells [99]. However, no role for netrin1 directing cell fate in the developing spinal cord has been described.

Here, we provide the first evidence that netrin1 can regulate cell fate specification in the dorsal spinal cord. Since netrin1 has been previously shown to suppress the BMP signaling pathway in different cell types *in vitro*[100], we sought to investigate the relationship between netrin1 and BMP signaling in the patterning of the embryonic spinal cord. These studies support the hypothesis that netrin1 acts from the intermediate spinal cord to regionally limit the extent of BMP signaling to the dorsal spinal cord. Using a combination of bioinformatics with gain and loss of function approaches in chicken, mouse and stem cell

models, we have found that modulating the level of netrin1 has profound effects on the number of the BMP-dependent dIs, i.e., dI1-dI3s. Netrin1 appears to mediate its effects through the BMP pathway, given that changes in dI number were accompanied by alterations in the levels of both phosopho (p) Smad1/5/8 and *Id* expression. The Id family are key downstream mediators of BMP signaling[125], that modulate the activities of the proneural basic helix-loop-helix (bHLH) proteins[126-128] to prevent exit from the cell cycle[129,130]. Thus, activation of Ids can result in NPCs being held in a proliferative state.

Together, these findings suggest a new role for netrin1 in the developing spinal cord, modulating BMP signaling to fine tune neural patterning. Thus, netrin1 has an earlier role than previously realized, with reiterative activities shaping dorsal spinal circuits, first by regulating cell fate decisions and then acting as a guidance cue to direct axon extension. Both netrin1 and members of the BMP family are widely expressed, with the BMPs also having reiterative roles in cell growth, differentiation, migration, apoptosis, and homeostasis in the developing embryo and adult[131-133]. These studies then also open the possibility that netrin1 can modulate BMP-dependent processes in other organs.

Results

Netrin1 misexpression does not perturb the architecture of the spinal cord

To investigate whether netrin1 has effects in the spinal cord distinct from its role mediating axon guidance, we examined the consequence of ectopically expressing netrin1 in the chicken spinal cord. Two members of the netrin family, netrin1 and netrin2, are present in the embryonic chicken spinal cord with distinct distributions at different stages (Figure 2-1A-L)[52,134]. At Hamburger Hamilton (HH) stage 18, when dI fate specification commences[1], netrin1 is present in the ventral spinal cord (Figure 2-1A, B), while netrin2 is present everywhere except the most dorsal and ventral regions (arrowheads, Figure 2-1F). By HH stage 24, when axogenesis is ongoing, *netrin1* is specifically expressed in the FP (arrowhead, Figure 2-1I), while the domain of netrin2 has contracted to a region in the intermediate spinal cord that spans from immediately below the dorsal root entry zone to just above the motor column (arrowheads,

Figure 2-1K, L). In both cases, the distribution of *netrin* mRNA is largely distinct from the distribution of netrin protein. This is most evident for netrin2: *netrin2* mRNA is present in the ventricular zone (VZ), while netrin2 protein accumulates on the pial surface immediately adjacent to its expression domain, similar to what has been observed for netrin1 in mouse [2].

We first assessed whether the misexpression of netrin1 altered the general structure of the chicken spinal cord. A range of concentrations (50ng, 500ng, 1µg) of C-terminally myc-tagged netrin1 (netrin1-myc) were electroporated into the HH stage 14 spinal cord under the control of the ubiquitously expressed CAG enhancer[135], and the consequences examined two days later, at HH stage 24/25. CAG::*gfp* was concomitantly electroporated in all experiments, to both serve as a control (Figure 2-1M-Q), and indicate the extent of electroporation. Initially, both chicken and mouse netrin1 were used in these experiments, which are ~90% similar at the amino acid level. However, since their activities were found to be very similar (data not shown), we proceeded with mouse netrin1, which could be additionally identified as a distinct signal using species specific antibodies. While the GFP fluorophore had no effect on the distribution of the endogenous chicken netrin1 (arrow, Figure 2-1M, O), the misexpression of netrin1-myc resulted in both myc and netrin1 being targeted to the pial surface (arrows, Figure 2-1A-U, 2-1Y-AA). Increasing concentration of electroporated netrin1-myc increased the amount of pial-myc (compare arrows, Figure 2-1T and 2-1Z). However, even at the highest levels of exogenous netrin1-myc, there was no effect on the integrity of the laminin$^+$ basal membrane (Figure 2-1V, 1W, 1BB, 1CC), or the nestin1$^+$ radial processes of the neural progenitor cells (NPCs; Figure 2-1X, 1DD), compared to control electroporations (Figure 2-1P-R). Thus, misexpression of netrin1 does not change the overall architecture of the spinal cord.

Netrin1 overexpression in chicken embryos results in a dose dependent reduction in dIs

Although the general architecture of the spinal cord was not affected, we did observe an apparent reduction in the size of the spinal cord after electroporation with netrin1-myc. To further assess this

phenotype, we quantified the area bounded by either the $Sox2^+$ NPCs or $p27^+$ post mitotic neurons in a control (Figure 2-2A-C, N) versus netrin1-myc (Figure 2-2D-F, N) electroporation. In the control condition, the electroporated vs. non-electroporated sides of the embryo were statistically indistinguishable for both $Sox2^+$ NPCs ($p>0.65$, Student's *t*-test) or $p27^+$ neurons ($p>0.60$). In contrast, there was a ~25% reduction in the total area bounded by $Sox2^+$ NPCs and ~33% decrease in area of the $p27^+$ neurons after netrin1-myc was ectopically expressed (Figure 2-2N). This reduction was seen with all concentrations of netrin1, suggesting that netrin1 can potently affect the number of neurons that arise in the spinal cord.

While the size of the entire spinal cord was reduced, the effect of netrin1 on the dorsal spinal cord was both more pronounced and observed at lower concentrations (Figure 2-2O). To further assess the consequence of netrin1 misexpression on specific dorsal identities, we used a well described panel of antibodies against transcription factors that distinguish specific dI fates[136], to monitor the numbers of $Lhx2^+$ dI1s, $Lhx1/5^+$ $Pax2^-$ dI2s, $Isl1^+$ dI3 and $Lhx1/5^+$ $Pax2^+$ dI4s. We observed that the BMP-dependent populations[1], i.e., dI1, dI2 and dI3, are all significantly reduced after netrin1-myc electroporation compared to the GFP control. These reductions were concentration-dependent, such that more dI1/dI2/dI3s were lost, as the amount of netrin1-myc increased. ~75% of dI1s and ~50% of dI2/dI3s were ablated at the highest concentration of netrin1-myc tested (Figure 2-2R). In contrast, the dI4s, a BMP-independent population, was less profoundly affected. The lowest level of netrin1-myc did not significantly affect the numbers of dI4s, rather dI4s were only lost as the concentration of netrin1-myc increased (Figure 2-2R). The loss of neurons observed after electroporation with high levels of netrin1-myc may be a consequence of cell death, since the number of $caspase^+$ cells significantly increased, as the concentration of netrin1-myc increased.

Addition of netrin1 to mESCs blocks their ability to acquire dI1/dI3 fates

We next assessed the activity of netrin-myc in stem cell model that recapitulates the early events that direct cell fate in the developing spinal cord[121]. In brief, bFGF/Wnt signaling directs mouse embryonic stem cells (mESCs) to the bipotential neuromesodermal progenitor (NMP) fate, that is a critical intermediate for the cells of the caudal neural tube[137] (Figure 2-3A). Our recent work has shown that addition of retinoic acid (RA), from day 3-5, directs NMPs to a caudal dorsal progenitor (dP) fate, specifically that of the intermediate neural tube, ultimately resulting in the specification of dI4, dI5 and dI6[121,138]. The sequential addition of BMP4 from day 4-5 further dorsalizes the NMPs into the dPs that specify the dI1, dI2 and dI3 fates. Thus, the RA±BMP4 directed differentiation protocols provide an additional model to investigate the mechanisms that drive dorsal spinal cord development.

We assessed the effect of adding exogenous netrin1 to mESC-derived NMPs in the RA±BMP4 protocols at three different timepoints: concomitantly with BMP4 from day 4-5 (condition 1); immediately after BMP4 treatment from day 5-6 (condition 2); and finally, for an extended period after BMP4 treatment from day 5-9 (condition 3) (Figure 2-3A). Additionally, two concentrations of netrin1 were used: 0.125 μg/ml (low) and 0.5 μg/ml (high). The cultures were then assessed for the specification of the dorsal-most dIs at day 9, using qPCR analyses (Figure 2-3B-D). The addition of netrin1 with, or immediately after, BMP4 treatment had no apparent effect on the identity of the cultures (Figure 2-3B, C). We also found no effect on *Foxd3* expression in any of the conditions, i.e., dI2s continue to assume their fate in the presence of netrin1. However, prolonged treatment with 0.5 μg/ml netrin1 in the RA+BMP4 protocol significantly reduced the expression of *Lhx2* and *Lhx9*, both markers of dI1s, and there is a trend ($p<0.07$) towards the loss of *Isl1*, a dI3 marker. Thus, the extended treatment of stem-cell derived NMPs with high levels of netrin1 is sufficient to prevent some dorsalization. This result, coupled with the observation that netrin1 misexpression in the chicken spinal cord most effectively suppresses the BMP-dependent dIs, suggests that netrin1 is counteracting the activities of the BMPs.

The loss of netrin1 increases the size and number of the dorsal most spinal progenitors

We next assessed the effect of removing netrin signaling on dorsal spinal fate specification. For these experiments, we examined mice deficient for *netrin1*; there is no netrin2 homolog in mammals, and the distribution of mouse netrin1 is the composite of the chicken netrin1 and netrin2 expression pattern (Figure 2-4A-G)[72,124,139]. We analyzed a null allele for netrin1 (*ntn1*$^{-/-}$)[140] (Figure 2-4); this mice line has been analyzed for axon guidance defects in the developing spinal cord but has not been carefully evaluated for changes in dorsal cell fate.

We focused our analysis at embryonic (E) day 11.5, the time when dorsal fate specification is robustly ongoing in the spinal cord. We first analyzed whether the dorsal progenitor (dP) domains were affected by the loss of *netrin1*. We observed a marked expansion of the dorsal most Atoh1^{+} dP1 domain which flanks the RP. The domain is almost 2-fold larger in size ($p<0.0001$ significantly different compared to littermate control) and there is a ~25% increase in the number of Atoh1^{+} dP1s ($p<0.045$; Figure 2-4A, D, M). Similarly, the area of the dP2 domain (region bounded by the Atoh1^{+} and Ascl1^{+} domains) was increased by 60%. We also found a ~40% increase in the area ($p<0.0001$) demarked by Ascl1^{+} cells, which form the dP3-dP5 domain (Figure 2-4A, D, M). In contrast, we observed no significant difference in the number of Olig2^{+} cells in control and mutant sections ($p>0.25$; Figure 2-4B, E, M), suggesting that there was no effect on the size of motor neuron progenitor domain (pMN). Together, these results suggest that the loss of *netrin1* results in an increase in neural progenitors, specifically in the dorsal most spinal cord. This increase in dPs did not apparently stem from an increase in the rate of cell division, since there was no significant change in the number of pH3^{+} cells in mitosis ($p>0.5$; Figure 2-4C, F, G), or altered patterns of cell death ($p>0.055$; Figure 2-4H).

We next assessed whether the increased number of dorsal progenitors influenced the number of post-mitotic dIs. To our surprise, we found that there was a ~30% decrease in the number of Lhx2^{+} dI1s ($p<0.0001$; Figure 2-4I, K, N), Foxd3^{+} dI2s ($p<0.017$; Figure 2-4I, K, N) and Isl1^{+} Tlx3^{+} dI3s ($p<0.0001$)

(Figure 2-4J, L, N). In contrast, there was no significant difference in the more intermediate dIs, i.e., the $Pax2^+$ dI4s ($p>0.67$), $Tlx3^+$ $Isl1^-$ dI5s ($p>0.19$) and $Pax2^+$ dI6s/v0/v1 (dI6+; $p>0.48$), or the $Isl1^+$ MNs ($p>0.36$). Thus, the loss of netrin1 appears to specifically affect the development of the BMP-dependent dorsal most dIs and does not affect the BMP-independent intermediate dIs, and ventral spinal cord. This result is consistent with the hypothesis that netrin1 has an additional activity, acting to regulate the activity of BMPs in the dorsal spinal cord.

RNA sequencing reveals netrin1 alters dP gene expression in vitro

We next assessed how netrin1 affects the transcriptomic profiles of cells undergoing neural differentiation using the RA±BMP4 directed differentiation protocol described previously. Two concentrations of netrin1 recombinant protein (0.125μg/ml (low) and 0.5μg/ml (high) were added to the RA±BMP4 protocol at two different timepoints: alongside with BMP4 from day 4-5 (condition 1), and immediately after BMP4 treatment from day 5-6 (condition 2) (Figure 2-5A). Samples were collected and Bulk RNA sequencing was performed to assess for alterations in gene expression (Figure 2-5B, C). The addition of netrin1 on Day 4, both high and low, in combination with BMP4, resulted in no significant differences in gene expression when compared to RA or RA+BMP4 controls (Figure 2-5B). By contrast, the addition of netrin1 to cells that had fully undergone the transition from NMPs to a caudal dorsal progenitor (dP) fate, after day 5, caused changes in the transcriptomic profile of cells when compared to RA or RA + BMP4 controls (Green bars indicate upregulation of genes, red bars indicate down regulation) (Figure 2-5C). To gain further insight to the potential pathways being affected by netrin1 addition, we next conducted a Gene Ontology analysis of data collected from cells treated with low amounts of netrin1 (0.125 μg/mL) in Condition 2. Among the gene modules that were downregulated we found those associated with cell-cell adhesion, brain development, regulation of BMP signaling pathways and SMAD protein signal transduction (Figure 2-5D). The downregulation of BMP signaling and SMAD protein, a BMP signaling mediator, signal transduction is consistent with the hypothesis that netrin1 acts to regulate the activity of BMPs in the dorsal spinal cord.

The gain or loss of netrin1 activity alters the level of Bmp signaling

Finally, to further examine the model the netrin1 is BMP inhibitor, we directly assessed whether modulating the level of netrin1 affects BMP signaling in context of two cellular models. We first examined the effect of netrin1 on phosphorylated (p) Smad1/5/8 levels in COS cells. BMP signaling is canonically mediated by the phosphorylation of the Smad second messenger complex. COS cells can endogenously transduce BMP signaling; when treated with BMP4 for an hour, the level of pSmad1/5/8 robustly increases (Figures 2-6G, K). However, if 0.5μg/mL netrin1 is added concomitantly with BMP4, pSmad1/5/8 levels decrease by ~60%. This is a dose-dependent response: halving the amount of netrin1 diminishes this response, while 0.125μg/mL netrin1 treatment has no significant effect on Smad1/5/8 activation (Figures 2-6G, K).

We then assessed whether the gain or loss of netrin1 activity can alter the level of BMP signaling in the developing spinal cord. BMPs act from the roof plate (RP) at the dorsal midline to pattern the surrounding tissue[7,9]. BMP signaling can be visualized as a graded pSmad1/5/8/ signal flanking the RP (Figure 2-6A, E). Electroporation of mouse netrin1 into the chicken spinal cord suppresses this signal, again in a dose dependent manner. Thus, there is a >50% inhibition of pSmad activation at high (1μg) levels of netrin1 (arrows, Figure 2-6D, K), while lower (500ng, 50ng) levels of netrin1 suppress pSmad activation by 25% (arrows, Figure 2-6B, C, K). In contrast, we observed that the area of pSmad activation is expanded by ~40% in spinal cords taken from netrin1 mutant mice, compared to littermate controls (Figure 2-6E, F, I). Thus, the level of netrin1 has inverse reciprocal effect on the activation of BMP signaling, consistent with the model that it acts directly as a BMP inhibitor.

The loss of netrin1 activity alters the level of Id signaling

To further determine the effect of loss of netrin1 on BMP signaling, we analyzed the expression of downstream BMP signaling genes, Id1 and Id3. We screened both control (Figure 2-7A, D) and *netrin1*$^{-/-}$ (Figure 2-7B, E) E11.5 mouse spinal cords for *d1* (Figure 2-7A, B) and *Id3* (Figure 2-7D, E) expression

using in situ hybridization. Our preliminary results suggest a slight increase in the expression of both *Id1*(Figure 2-7C) and *Id3* (Figure 2-7F) in the dorsal spinal cord, indicative of an increase in BMP signaling. Id, inhibitors of differentiation, genes are thought to maintain progenitors in an undifferentiated state, consistent with our observation that loss of netrin1 results in more dPs, but not more dIs (Figure 2-4) and suggesting that netrin1 limits the number of post mitotic neurons by suppressing the transition from progenitor to differentiated neuron.

Discussion

Netrin1 restricts BMP signaling to regulate spinal cord neural patterning.

In this study we have assessed whether netrin1 limits BMP signaling to the dorsal spinal cord using both gain and loss-of-function model systems. Recent studies have shown that netrin1 suppresses BMP signaling in vitro[100] however this interaction has not been examined in vivo. Here we have shown that netrin1 modulates BMP signaling both in vivo and in vitro. Netrin1 overexpression in chicken embryos mediates a dose dependent reduction in the number of dIs. This effect is most robust in the dorsal-most populations with higher concentrations of netrin1 also affecting the ventral dI population (Figure 2-2R). We find that this reduction is not due to cell death as even low levels of netrin1 (50 ngs/uL) induces a significant reduction of dI1-dI3s but does not lead to significant cell death (Figure 2-2Q). In a mouse embryonic stem cell model of dI differentiation[141], we found that addition of netrin1 blocks the differentiation of dPs into neurons as indicated by the significant reduction of post-mitotic dorsal markers (Figure 2-3B, C). In contrast, the loss of netrin1 *in vivo*, increases the number and size of BMP-dependent dorsal progenitors (dP) domains dP1 and dP3-dP5 (Figure 2-4M). However, these appear to have stalled in their development because we observed a reduced number of dI1s, dI2 and dI3s (Figure 2-4N). Interestingly assessment of levels of BMP downstream target genes, Ids, inhibitors of differentiation[125], are increased in the absence of netrin1 (Figure 2-7E, F), suggesting loss of netrin1 stalls neurons in their progenitor state. In Figure 2-3, we observed that continuous and sustained addition of netrin1 inhibited differentiation to the dorsal fate when netrin1 was added to the culture after the cells had been patterned into the dP fate. These findings suggest that netrin1 is important for the developmental transition from the progenitor to differentiated state. In further assessing BMP signaling, we observed that netrin1 misexpression decreases levels of pSmad 1/5/8 (Figure 2-6H), the effectors of BMP signaling, while their expression is expanded in in netrin1 null mice (Figure 2-6I). We propose that netrin1 acts as a barrier to constrain the influence of BMP signaling to the dorsal most region (Figure 2-6L). Cumulatively our data suggests that netrin1 modulates the transitionary period of dPs to neurons, as a mechanism to fine tune

neural patterning. Netrin1 as a modulator of BMP signaling in the dorsal spinal cord is a novel role for netrin1, a molecule whose role in the spinal cord has been historically as an axon guidance cue.

Assessing mechanisms of action: Physical Interaction

The proximity of the netrin1 domain in the intermediate spinal cord, in a domain that immediately borders the dI1-3 (Figure 2-6L), postulated the possibility that netrin1 modulates BMP signaling through a direct physical interaction between the BMP4 ligand and netrin1. As historically has been described, BMP ligands can be physically sequestered by extracellular antagonists such as Gremlin and Noggin[142,143]. The BMP ligand is sequestered by the extracellular molecules, acting as a sink, preventing the ligand from binding to the BMP receptor[142]. Due to the proximity of the two domains (Figure 2-6L), we hypothesized a direct interaction between the two. We conducted multiple attempts to find an interaction, starting with computational modeling and kinetic binding assays and co immunoprecipitation after transfection of COS7 cells with BMP4-HISK tagged and Mouse Netrin1-cMyc encoding constructs (Data not shown). We found no indication of a direct interaction between the two proteins. Furthermore, we addressed the possibility of a direct interaction between BMP4 and netrin1, in our mESC differentiation culture. We tested this by adding netrin1 in combination with BMP4, at the exact same time, before the cells had been patterned into the dP fate. This combined addition had no effect on the differentiation capacity of the mESCs, the cells were able to differentiate into their dI fates normally. This suggest that 1) the effect of netrin1 on the cells is not due to a direct interaction between BMP4 and netrin1, 2) the effect of netrin1 on BMP4 signaling occurs at the dP state, e.g., the cells must be dorsalized, as dPs to be affected by netrin1, potentially keeping the cells in a progenitor state.

Do canonical netrin1 receptors play a role in the netrin1 mediated suppression of BMP signaling?

Netrin1 binds to a multitude of receptors including DCC and Neogenin1, both localized in the dorsal spinal cord. We sought to find if either the Dcc or Neogenin1 receptor played a role in BMP modulation by netrin1 by probing for the presence of these in Cos7 cells. In our *in vitro* COS7 studies, we observed

that addition of netrin1 in BMP4 stimulated COS7 suppresses levels of pSmad1/5/8 in a dose dependent manner (Figure 2-6G, K). However, we did not observe either receptor expressed in COS7 cells when we probed through western blot, suggesting that netrin1 mediated suppression of BMP signaling is not regulated through interaction with a canonical netrin1 receptor. In our studies the mechanism by which netrin1 suppresses BMP signaling remains unresolved however there are many avenues left to explore. Other possible areas to explore would be to exam if netrin1 directly binds BMP receptors or if netrin1 binds to or prevents pSMAD1/5/8 from translocating and thus preventing transcription of BMP signaling effectors.

Broader Implications

BMP signaling is abundant throughout all organ systems, and it is critical for development across species[19,97,132,133,144]. Netrin1 has historically been studies for its axon guidance properties in the nervous system[52,53,72] however, numerous studies have shown it plays a critical role in other contexts, including that in the development of other organ systems such as the kidney, the lungs, and mammary glands as well as in the progression of diseases like cancer and diabetes[93,94,96,112,145-147]. The interaction between netrin1 and BMP signaling that we characterized in these studies postulate that this signaling axis might be critical for other developmental and disease processes. The interaction between netrin1 and BMP is potentially a fine-tuning mechanism that allows for specification and topographic regulation of structures. These findings 1) Identify a novel function for netrin1 in the developing spinal cord 2) highlight the need to visit other BMP/netrin1 signaling centers.

Materials and Methods

Chicken Embryos, Mice Generation, and Culture of COS-7 and mESC cell lines

Fertile Leghorn eggs (CJ Eggs, Sylmar CA) were incubated for 60 hrs until the embryos developed to HH stages 14–15. The spinal cord was electroporated and then allowed to develop for 48 hrs until HH stages 24–26 at which time the tissue was analyzed.

The netrin1 knockout line was developed in the Lisa Goodrich lab at Harvard and characterized[140]. Embryos were collected from timed matings. The presence of a vaginal plug was considered embryonic day E0.5. Heads were used to isolate the DNA and were amplified by PCR to identify the genotypes of each embryo. All animal procedures were carried out in accordance with University of California Los Angeles IACUC guidelines.

COS-7 cells (ATCC CRL-1651) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Sweden AB, Stockholm, Sweden) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Waltham, Massachusetts) and Penicillin-Streptomycin-Glutamine (100X) (Gibco, Fisher Scientific, Gothenburg, Sweden).

Mouse embryonic stem cell culture

The mouse ESC line MM13 was routinely maintained in ES cell media with LIF on mitotically inactive MEFs. Before differentiation, ESC colonies were dissociated, plated on gelatin-coated plates, and allowed to proliferate for 1–2 days. To initiate differentiation, cells were plated on 0.1% gelatin-coated 24-well CellBIND dishes (Corning) with N2/B27 medium containing 10ng/ml basic fibroblast growth factor (bFGF) (Thermo Fisher Cat#PHG0023). On day 1, small colonies of cells can be observed attached to the bottom of the wells. On day 2, cells were supplemented with 10ng/ml bFGF and 5μM CHIR99021(Tocris Cat#4423) for 24 h to induce a neuromesodermal identity[137]. On day 3, cells were directed towards a spinal lineage by exposing them to 100nM Retinoic Acid (RA) (Sigma Alrdich Cat#R2625) for 24 hours,

followed by 100nM RA + 10ng/ml BMP4 (Thermo Fisher Cat# PHC9534) to induce dorsal spinal cord identity[121]. To evaluate the effects of Netrin on dorsal interneuron (dI) differentiation, two concentrations of mouse recombinant Netrin 1 (high - 0.5 µg/ml, low - 0.125 µg/ml) (R&D Cat no 1109-N1-025) were added in three different timelines (conditions 1, 2, 3).

For condition 1, Netrin1 was added with RA + BMP4 between day 4 and day 5, (1-day Netrin1 exposure). For condition 2, Netrin1 was added between day 5 and day 6, providing a 1-day Netrin exposure after the patterning by RA+BMP4. For condition 3, Netrin1 was added every other day between day 5 to day 9, leading to an extended 4-day Netrin1 exposure. Terminal differentiation was induced by replacing the growth factor containing media with basic N2/B27 medium at day 5, and cultures were allowed to differentiate until day 9. At the end of the differentiation, the cultures were lysed in buffer RLT (Qiagen) and RNA was purified using RNAeasy kit (Qiagen) for preparing cDNA for the quantitative reverse transcriptase PCR analysis.

In ovo electroporation of chicken embryos

Mouse *netrin1* (Addgene #71978) was amplified using PCR and fused on the c terminal end to a c-myc tag (EQKLISEEDL) and then subcloned in front of the CAG enhancer in the CAGGS vector. The CAG enhancer is comprised of a CMV enhancer and chicken β-actin promoter. Fertile Leghorn eggs (CJ Eggs, Sylmar CA) were incubated for 60 hr until the embryos developed to HH stages 14–15. The spinal cord was electroporated and then allowed to develop for 48 hr until HH stages 24–26. The following constructs were used CMV:GFP, CAG::*Net1-myc* (50 ng/µl, 500 ng/µl, or 1000 ng/µl). In all cases, the presence of GFP demonstrates the electroporation efficiency. To alter the concentration of *Netrin* expression: the CAG::*Net-myc* expression vector was diluted with the PCAGEN vector, to hold the DNA concentration constant at 2000 ng/µl across experiments.

Tissue processing

Spinal cords were fixed using 4% paraformaldehyde for 2 h at 4 °C. After fixation, the tissue was cryoprotected in a 30% sucrose solution overnight, following which the tissue was mounted in optimal cutting temperature (OCT) and cryosectioned at 20μm. Sections were collected on slides and processed for immunohistochemistry.

Immunohistochemistry

Chicken embryonic spinal cords and mouse embryonic spinal cords were thin sectioned to yield 20 μm sections. Antibodies against proteins were used for immunostaining and can be found in Table 2-1. Species appropriate Cyanine 3, 5 and Fluorescein conjugated secondary antibodies were used (Jackson ImmunoResearch Laboratories). Images were collected on Carl Zeiss LSM700 confocal microscopes.

In situ hybridization

In situ hybridizations were performed on chicken (HH stage 18–25) and mouse (11.5) embryonic spinal cords. 3'UTR probes were designed using http://primer3plus.com and verified for specificity to the gene of interest using http://www.ncbi.nlm.nih.gov/tools/primer-blast/.

Chicken and mouse primer sequences used to make in situ hybridization probes can be found in Table 2-1. Probes were made using a DIG RNA labeling kit (Roche, Indiananapolis, Indiana). Images were collected on a Carl Zeiss AxioImager M2 fluorescence microscope with an Apotome attachment.

Western blot analysis

COS7 cells were seeded in 12-well or 24 well plates the day before stimulation with netrin1 and BMP4. On the day of stimulation, the cells were starved in FBS-free media for an hour prior to stimulation with 5ng/ml BMP4 and 0.5 μg/ml, 0.25 μg/ml or 0.125 μg/ml netrin1. After one hour of stimulation, the cells were washed with PBS and lysed with RIPA lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics Scandinavia AB, Bromma, Sweden) and the phosphatase inhibitor PhosSTOP (Roche Diagnostics Scandinavia AB). The cell lysates were kept on ice for 30 min and centrifuged at 20,800×g

for 10 min at 4 °C. The clear supernatant was subjected to SDS-PAGE using 10% Tris-Glycine SDS gels followed by transfer onto PVDF membranes (Millipore Sigma, MA, USA). The membranes were blocked using Non-fat dry milk (Bio-Rad Laboratories AB, Solna, Sweden). The blocked membranes were then incubated with the primary antibodies at 4C overnight. Thereafter, the membranes were washed three times with TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20), incubated secondary antibodies for an hour at room temperature and then washed again three times with TBST. The fluorescent bands were analyzed using the Pierce Femto Chemiluminescence system on the Azure Imager.

Reverse transcriptase PCR analysis:

RNA was extracted from at least two independent differentiations using the RNeasy mini purification kit (QIAGEN, catalog no. 74104). cDNA was synthesized using Superscript IV (Thermo Fisher Scientific) using oligodT as primers to convert only mRNAs into cDNAs. RT-qPCR was always performed in triplicate using SYBR Green Master Mix (Roche) on a Roche RT-qPCR machine using gene-specific primers (Table 2-1). The Ct values for each gene were calculated by averaging three technical replicates from independent differentiations for each condition. Expression of the target gene was normalized with the expression of glyceraldehyde-3-phosphaste dehydrogenase (GAPDH), and fold change was calculated using the $2^{-\Delta\Delta Ct}$ method. The variation in fold change in expression is represented in ±SEM (standard error of mean).

Quantification

For the electroporation experiments, the non-electroporated side of the spinal cord was used as the negative control in cell-counting experiments. Thus, the cell number on the electroporated side was normalized against a GFP control electroporation performed at the same time. For the quantification of pSmad1/5/8 intensity, control and experimental embryos were stained on the same slides. The staining intensity and area were quantified using the ImageJ software. Biological replicates: 1–2 chicken embryos

per experimental condition were collected within an experiment. Each electroporation experiment was repeated at least three times. Technical replicates:>10 or more sections per embryo were analyzed.

For the transgenic mice experiments, cell counts, area and stain intensities were normalized to the littermate controls. Biological replicates: 1–2 embryos per experimental condition were collected within an experiment. Embryos were collected from at least three timed pregnancies. All analyses was done using littermate controls. Cell counts were done blindly. Technical replicates:>10 or more sections per embryo were analyzed.

For cell culture experiments, experiments were performed at least 3 independent times. All values were normalized to internal controls.

Statistics

Data are represented as mean ± SEM (standard error of the mean). Tests for statistical significance were performed using Prism software (version 9). Values of $p < 0.05$ were considered significant in all cases.

Figures and Tables

Figure 2-1. Overexpression of netrin1 does not affect the integrity of the developing spinal cord

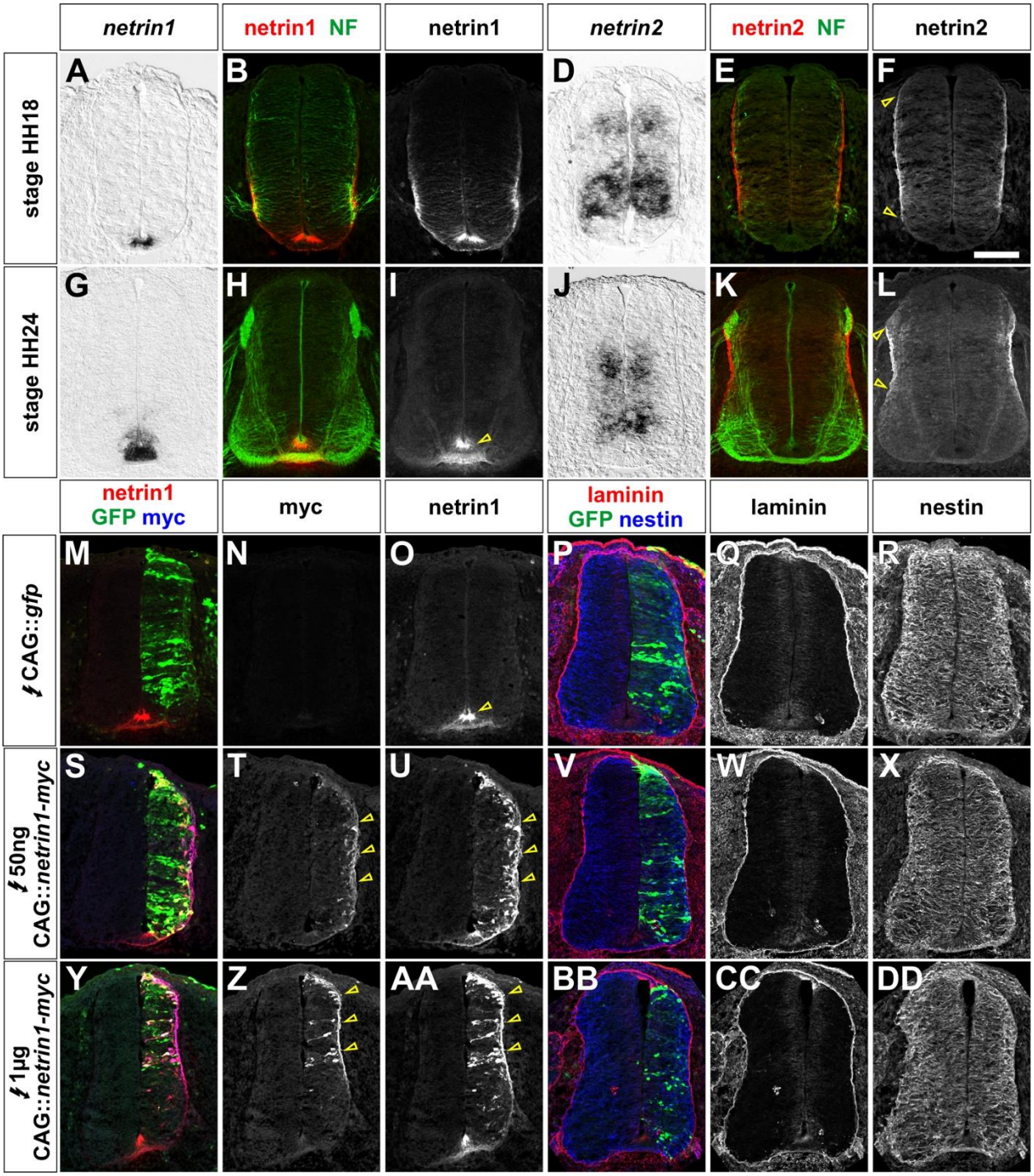

Figure 2-1. Overexpression of netrin1 does not affect the integrity of the developing spinal cord (A-L) Distribution of netrin1 (A-C, G-I) and netrin2 in (D-F, J-L) in thoracic sections of the HH18 (A-F) and HH24 (G-L) spinal cord. *Netrin1* mRNA is expressed in the apical FP (A, G), while netrin1 protein (red, B, H) decorates the apical-most and basal FP (arrowhead, C, I), where it is co-incident with the NF^+ axons crossing the FP (H). *Netrin2* mRNA is expressed in the intermediate VZ (D, J), while netrin2 protein (red, E, K) decorates pial surface in the intermediate spinal cord (arrowheads, F, L), immediately adjacent to NF^+ axons (green) extending ventrally in the dorsal spinal cord (L). (M-R) Electroporation of a control fluorophore, GFP (green, M, P), expressed from a ubiquitously expressed CAG enhancer, does not affect the distribution of endogenous netrin1 (red, M; arrowhead, O), or the integrity of the spinal cord as assessed by antibodies against laminin (red, P, Q) and nestin (blue, P, R). (S-DD) In contrast, electroporation of a low (50ng, S-R) or high (1µg, Y-DD) concentration of netrin1-myc construct, results in the ectopic netrin1 (red, S, Y, U, AA) and myc (blue, S, Y, T, Z) decorating the pial surface of the spinal cord (arrowheads, T, U, Z, AA). However, there is no effect on the distribution of either laminin (red, V, W, BB, CC) or nestin (blue, V, X, BB, DD).

Figure 2-2. Overexpression of netrin1 in chicken embryos results in the loss of dorsal interneurons

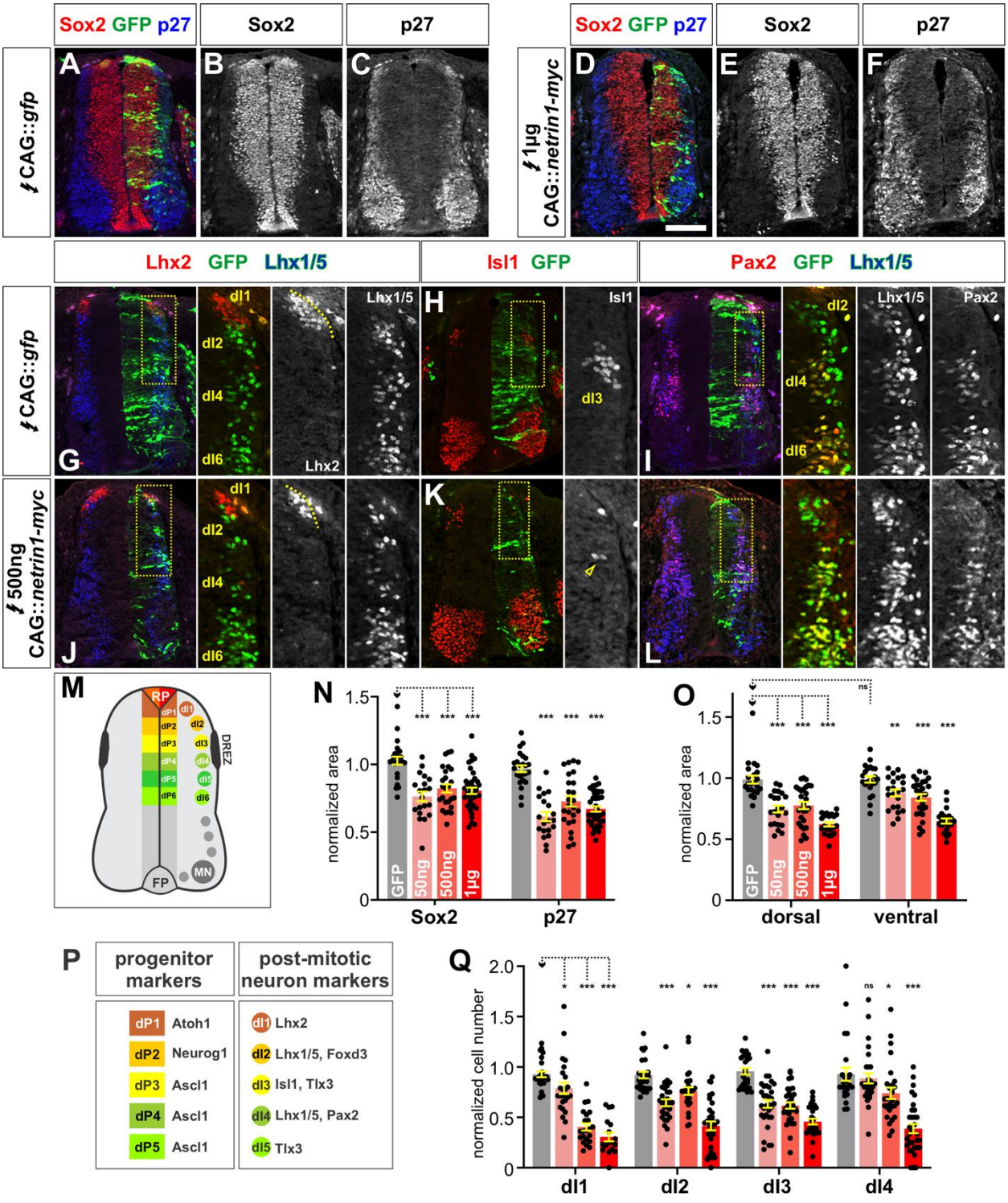

Figure 2-2. Overexpression of netrin1 in chicken embryos results in the loss of dorsal interneurons.

(A-L) Chicken spinal cords were electroporated at HH stage 14 with *Gfp* (A-C, G-I) or different concentrations of *netrin1* (50ng, 500ng, 1µg) (D-F, J-L) under the control of the CAG enhancer and incubated until HH stage 24. Thoracic transverse sections were labeled with antibodies against Sox2 (red, A, B, D, E), p27 (blue, A, C, D, F), Lhx2 (red, G, J), Isl (red, H, K), Lhx1/5 (blue/green G, I, J, L) and Pax2 (red, I, L). The dotted box (G-L) indicated the magnified region in the adjacent panel(s).

(M) Schematic transverse section of the spinal cord, showing the position of the dorsal progenitor (dP) domains, and post-mitotic dorsal interneurons (dIs).

(N) Ectopic *Gfp* expression had no significant effect on the general specification of the spinal cord. In contrast, at all concentrations of netrin1 tested, the area occupied by $Sox2^+$ or $p27^+$ cells was significantly reduced. n= >20 sections, from 4 embryos (GFP, 50ng, 500ng, 1µg netrin), Student's *t*-test.

(O) Ectopic netrin1 results in preferential loss of the dorsal area of the spinal cord. Higher concentrations of netrin1 lead to reduction of ventral area of the spinal cord.

(P) The different classes of dIs can be identified by specific combinations of transcription factors.

(Q) Ectopic *Gfp* expression also had no significant effect on dI specification. However, all concentrations of netrin1 tested were able to significantly reduce the number of $Lhx2^+$ dI1s, $Lhx1/5^+$ $Pax2^-$ dI2s and $Isl1^+$ dI3s in a dose dependent manner. In contrast, only the higher concentrations of netrin1 reduced the number of $Lhx1/5^+$ $Pax2^+$ dI4s. n= >20 sections, from 6 embryos (GFP, 50ng, 500ng, 1µg netrin), One way ANOVA.

Probability of similarity between control and experimental groups: *= $p < 0.05$, *** $p<0.0005$.

Figure 2-3. Addition of netrin1 blocks dorsalization in mESC stem cell model of dI differentiation.

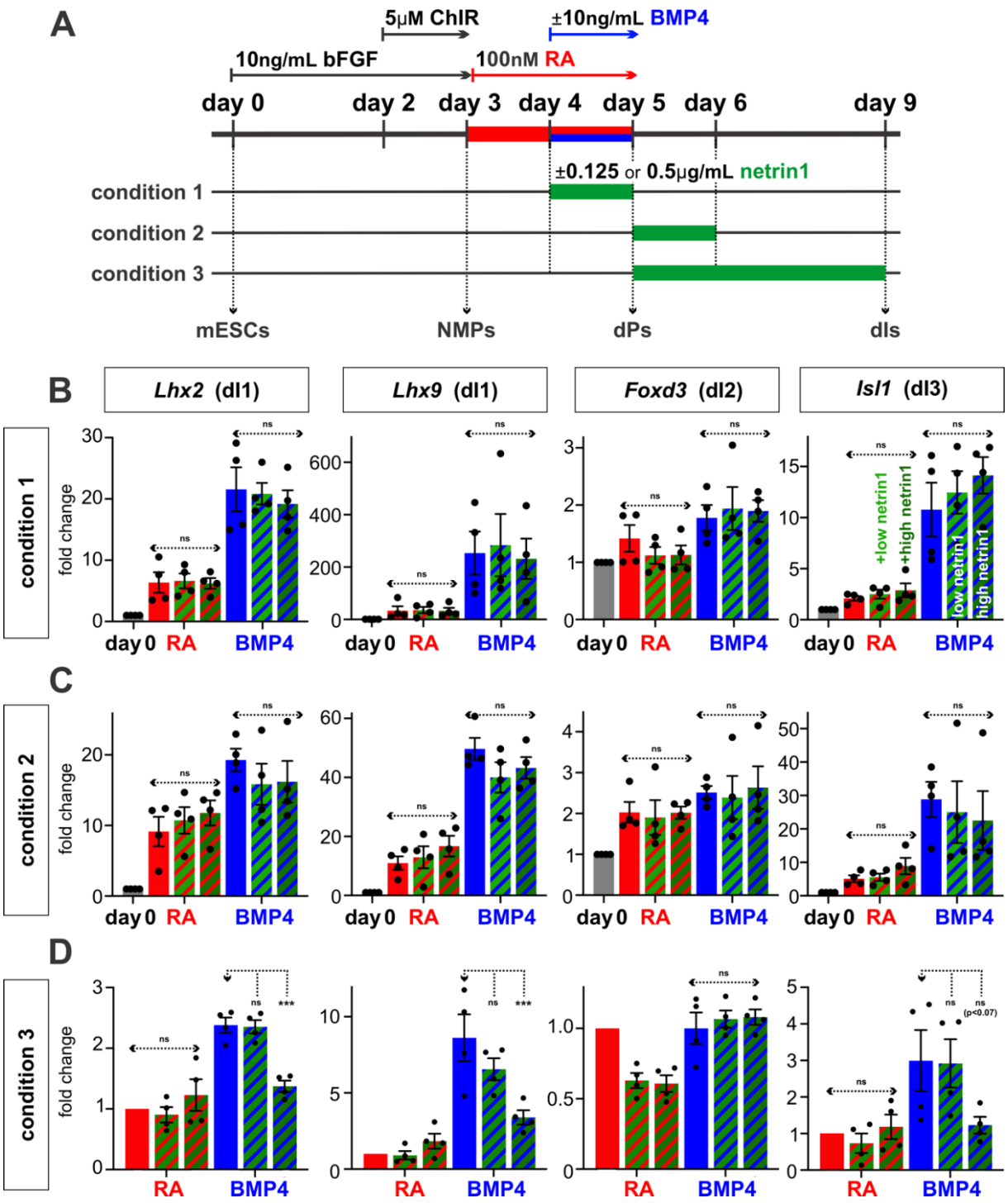

Figure 2-3. Addition of netrin1 blocks dorsalization in mESC stem cell model of dI differentiation.

(A) Two concentrations of Netrin1 recombinant protein (0.125μg/ml (low) and 0.5μg/ml (high) were added to the RA±BMP4 protocol at three different timepoints: alongside with BMP4 from day 4-5 (condition 1); immediately after BMP4 treatment from day 5-6 (condition 2), and for an extended period after BMP4 treatment from day 5-9 (condition 3). qPCR was used to assess alterations in gene expression.

(B, C) The addition of netrin1 in condition 1 and 2 had no significant effect on the expression of *Lhx2* and *Lhx9* (dI1), *Foxd3* (dI2) or *Isl1* (dI3) compared to RA or RA+BMP4 controls.

(D) Prolonged treatment with 0.5μg/ml netrin1 in the RA+BMP4 protocol significantly reduced the expression of the dI1 markers, and there is a trend ($p<0.07$) towards the loss of dI3 marker *Isl1*.

Probability of similarity between control and experimental groups: *= $p < 0.05$

Figure 2-4. Netrin1 is required to limit the number of the dorsal most NPCs

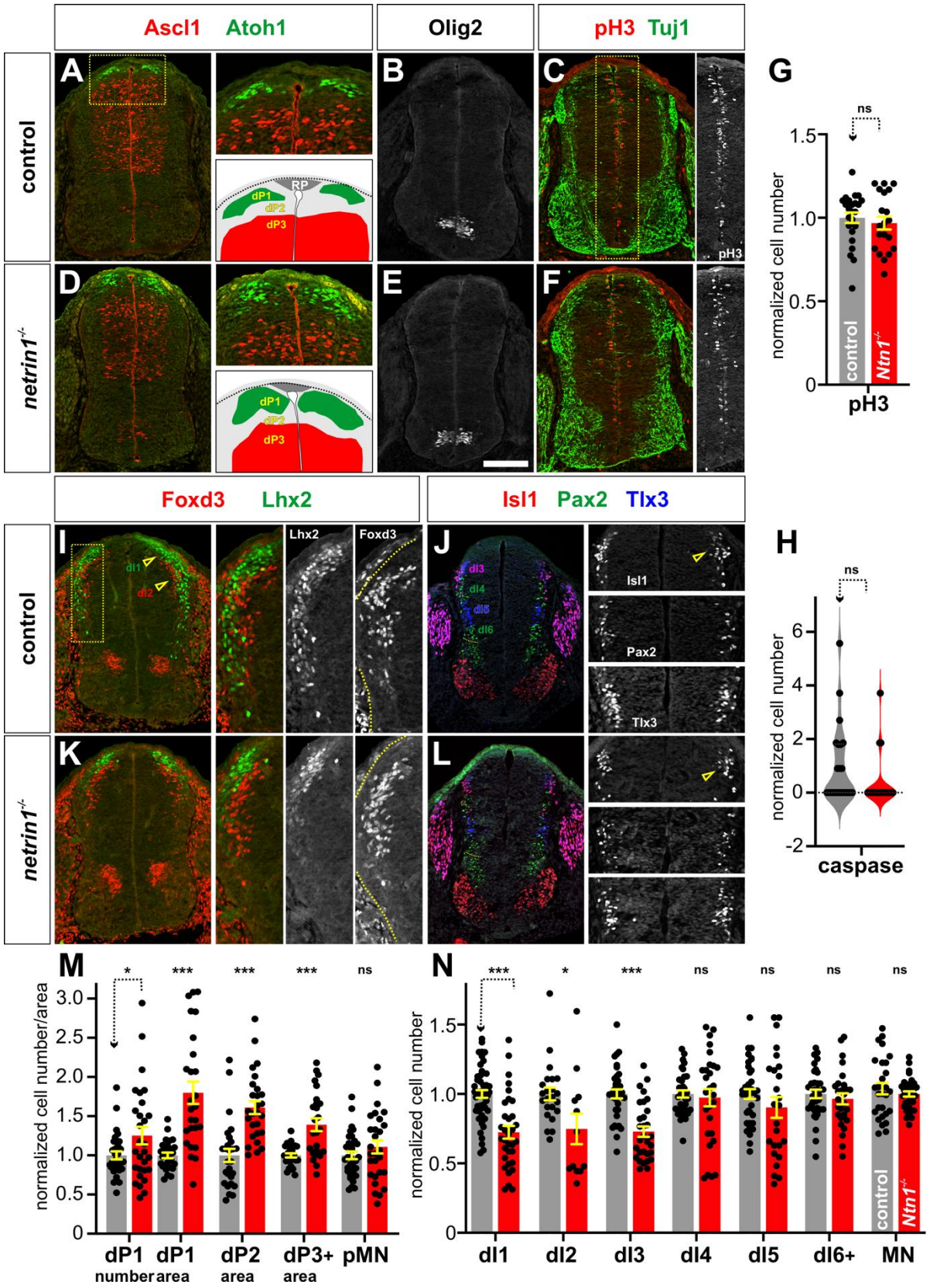

Figure 2-4. Netrin1 is required to limit the number of the dorsal most NPCs

(A-F) Thoracic transverse spinal cord sections from either control (A-C) or *netrin1*$^{-/-}$ (D-F) E11.5 mouse spinal cords were labeled with antibodies against Ascl1 (A, D; red; dP3-dP5), Atohl1 (A, D; green, dP1), Olig2 (B, E; pMN), phospho-histoneH3 (C, F; red; cells in mitosis (M) phase), and Tuj1 (C, F; green; neurites). The dotted box (A, C, D, F) indicates the magnified region in the adjacent panel(s).

(G, H) There was no significant difference in the number of cells either in M-phase (G, $p>0.50$; n= > 20 sections, from 3 control and 3 *netrin1*$^{-/-}$ embryos) or that were caspase^{+} i.e. dying (H, $p>0.28$; n= >13 sections, from 3 control and 3 *netrin1*$^{-/-}$ embryos) between control and *netrin1*$^{-/-}$ spinal cords.

(I-L) To assess for changes in the number of post mitotic dIs, thoracic transverse spinal cord sections from either control (I, J) or *netrin1*$^{-/-}$ (K, L) E11.5 mouse spinal cords were labeled with antibodies against Lhx2 (I, K; red; dI1), Foxd3 (I, K; green; dI2), Isl (J, L; red, dI3, MNs), Pax2 (J, L; green; dI4, dI6, v0), and Tlx3 (J, L; blue; dI3, dI5). The dotted box (I, K, J, L) indicates the magnified region in the adjacent panel(s).

(M) Loss of netrin1 resulted in a 25% increase in the number of Atoh1^{+} dP1s and an almost 2-fold increase in the area occupied by the dP1s. Similarly, the area of the dP2 domain (region bounded by the Atoh1^{+} and Ascl1^{+} domains) was increased by 60%, and the Ascl1^{+} dP3-dP5 domain was increased by 40% (n= > 26 sections, from 4 embryos).

(N) This increased number of progenitors did not result in a loss of dIs. Rather there was a ~30% decrease specifically in the number of dI1, dI2 and dI3s (n= > 20 sections, from 4 embryos), but not in the intermediate dorsal populations or the ventral motor neurons (MNs).

Probability of similarity between control and experimental groups: *= $p < 0.05$, ** $p<0.005$, *** $p<0.0005$; Student's *t*-test.

Figure 2-5: RNA sequencing reveals netrin1 alters dP gene expression in vitro

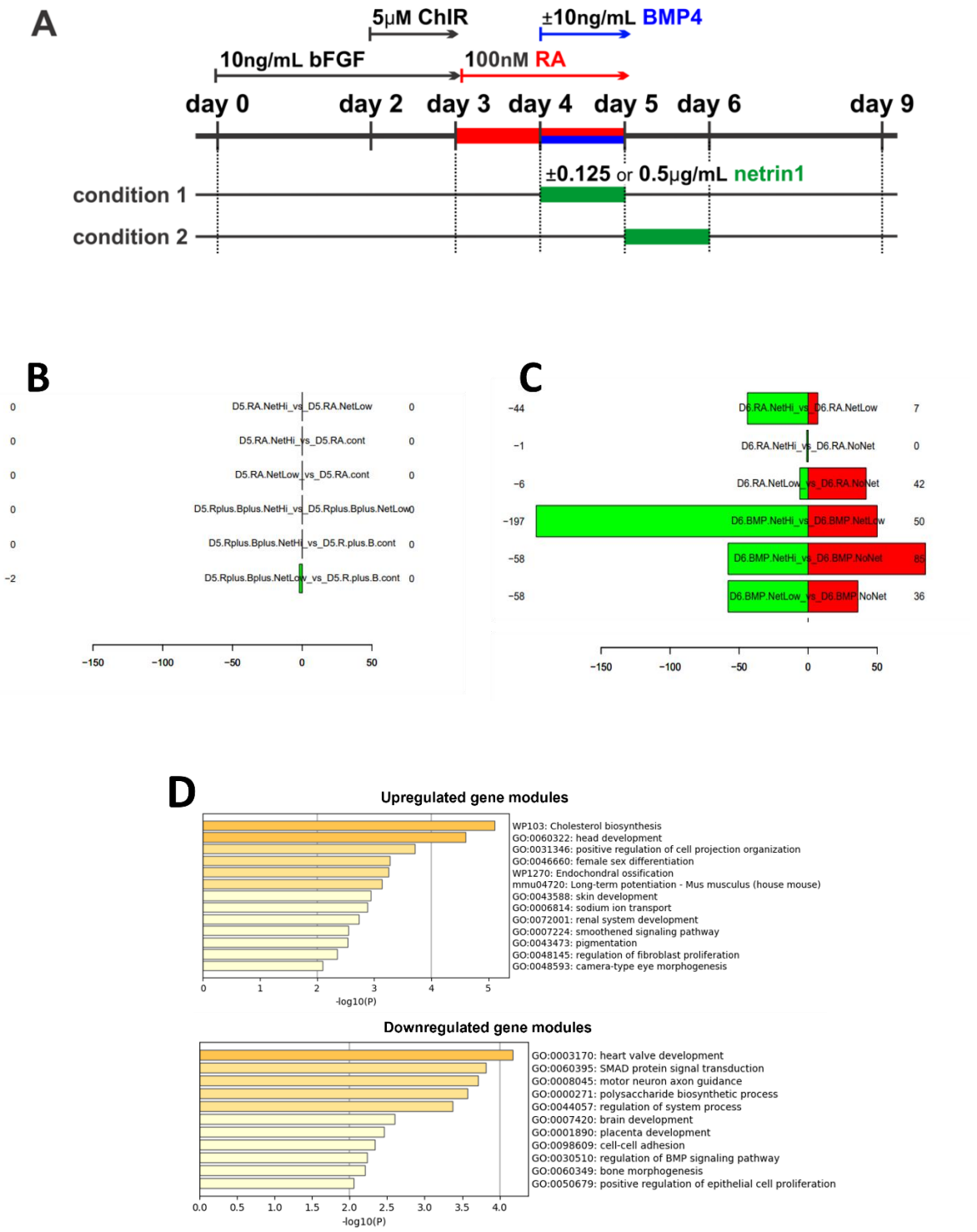

Figure 2-5. RNA sequencing reveals netrin1 alters dP gene expression in vitro

(A) Two concentrations of netrin1 recombinant protein (0.125μg/ml (low) and 0.5μg/ml (high) were added to the RA±BMP4 protocol at three different timepoints: alongside with BMP4 from day 4-5 (condition 1), and immediately after BMP4 treatment from day 5-6 (condition 2). Samples were collected and Bulk RNA sequencing was performed to assess for alterations in gene expression.

(B, C) The addition of netrin1 on Day 4, both high and low, in combination with BMP4, resulted in no differences in gene expression in condition 1 compared to RA or RA+BMP4 controls (B). By the contrary, addition of netrin1 to cells that had already become dorsal progenitors, after day 5, caused changes in the transcriptomic profile of cells treated with netrin1 compared to RA or RA + BMP4 controls (Green bars indicate upregulation of genes, red bars indicate down regulation) (C)

(D) A Gene Ontology analysis of data collected from cells treated with low amounts of netrin1(0.125 μg/mL) in Condition 2 describes the gene modules upregulated and down regulated in treated cells. Downregulated gene modules include regulation of BMP signaling pathways and SMAD protein signal transduction.

Figure 2-6. Netrin1 modulates the level of BMP signaling both *in vivo* and *in vitro*

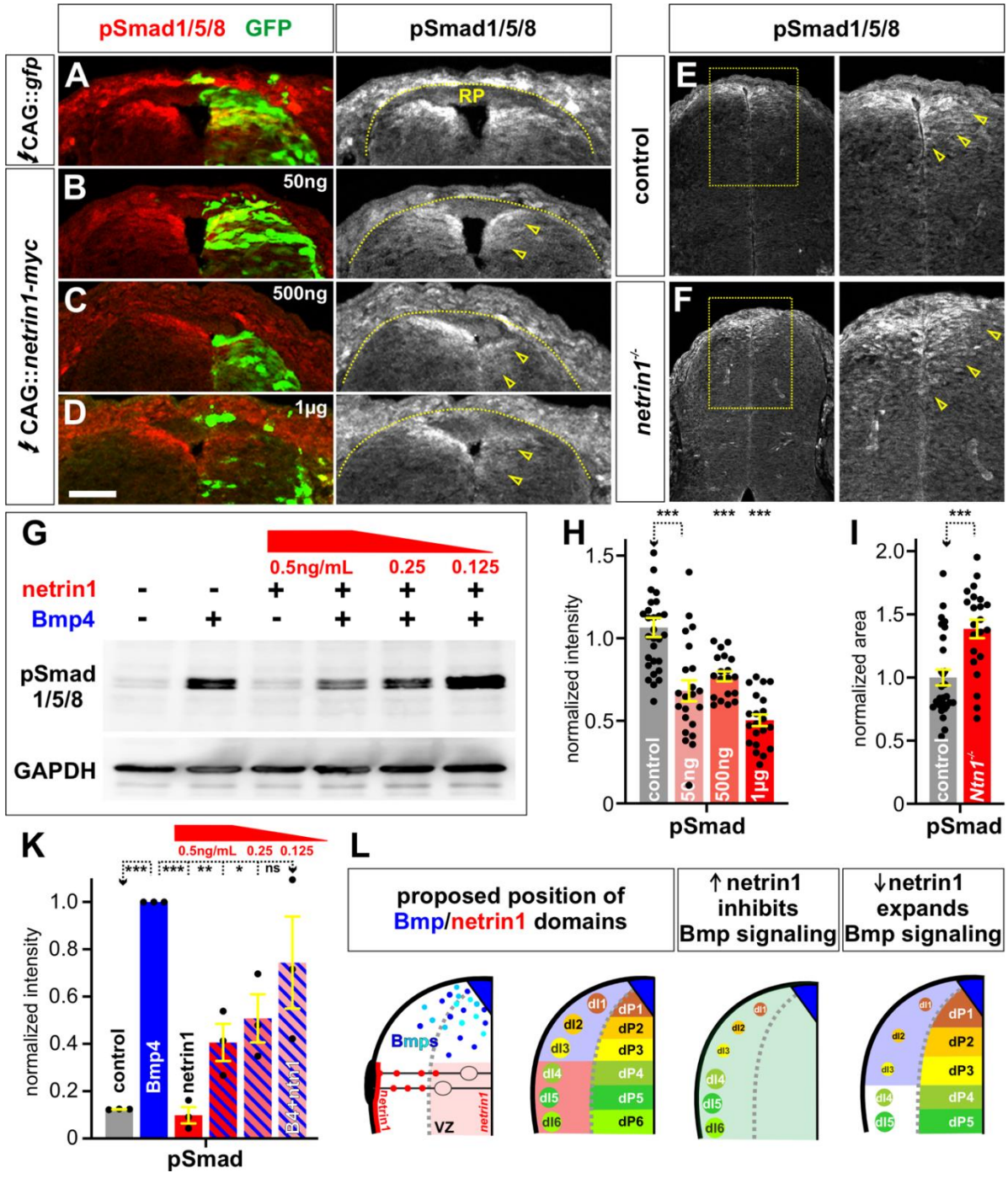

Figure 2-6. Netrin1 modulates the level of Bmp signaling both *in vivo* and *in vitro*

(A-D, H) Chicken spinal cords were electroporated at HH stage 14 with *Gfp* (A) or different concentrations of *netrin1* (50ng, 500ng, 1µg) (B-D) under the control of the CAG enhancer and incubated until HH stage 24/25. Thoracic transverse sections were labeled with antibodies against pSmad1/5/8 (red). Ectopic netrin1 in the dorsal most spinal cord resulted in a ~30-50% decrease in the levels of Smad1/5/8 compared to a control GFP electroporation.

(E, F, I) Thoracic transverse spinal cord sections from either control (A-C) or *netrin1*$^{-/-}$ (D-F) E11.5 mouse spinal cords were labeled with antibodies against pSmad1/5/8. The loss of netrin1 resulted in a ~40% larger Smad^{+} area (I), suggesting Bmp signaling had been increased.

(G, K) The interaction between netrin1 and Bmp4 was further assessed in a western analysis, using GAPDH levels as a loading control. Treating COS cells with Bmp4 resulted in the robust activation of pSmad1/5/8; while treatment with netrin1 alone had no effect on Smad activation above control levels. However, if netrin1 is added together with Bmp4, there is a decrease in Smad activation in a dose dependent manner. The highest level of netrin1 (0.5µg/mL) resulted in a ~60% decrease in the level of pSmad1/5/8, suggesting that Bmp signaling had been suppressed.

(L) Model for the biological significance of the netrin1/Bmp interaction. Multiple Bmps are secreted from the roof plate where they act to pattern the surrounding tissue into the dorsal progenitor domains (dP1-dP3). Netrin1 acts as a boundary, coincident with the DREZ, to limit Bmp signaling spreading into the intermediate spinal cord. Supporting this model, the dorsal-most dIs (dI1-dI3) are preferentially lost when netrin1 is expressed dorsally. In contrast, dP1-dP3 domains expand in the absence of netrin1.

Probability of similarity between control and experimental groups: *= $p < 0.05$, ** $p<0.005$, *** $p<0.0005$; Student's *t*-test.

Figure 2-7. *Ids* expression is increased after the loss of *netrin1*

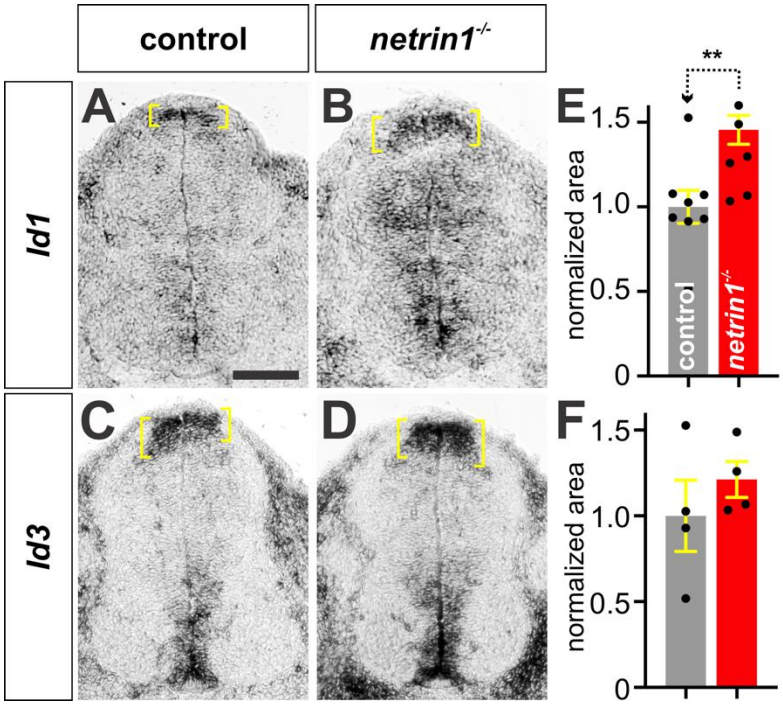

Figure 2-7: *Ids* levels are affected by loss of *netrin1*.

(A-D) Control (A, D) or *netrin1*$^{-/-}$ (B, E) E11.5 mouse spinal cords were assessed for *d1* (A, B) and *Id3* (D, E) expression.

(E, F) There is a an ~50% increase in the domain of *Id1* expression (E) with trend toward increased *Id3* expression (F) in *netrin1*$^{-/-}$ dorsal spinal cord compared to control littermates, consistent with increased BMP signaling. (*Id1*, n= > 7 sections, from 2 embryos, *Id3*, n=4, 1 embryo)

(G) Id genes are thought to maintain progenitors in an undifferentiated state, consistent with the observation that loss of netrin1 results in more dPs, but not more dIs.

Probability of similarity between control and experimental groups: ** $p<0.005$, Student's *t*-test.

Table 2-1 Antibodies and Oligonucleotides

REAGENT	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti Neurofilament	Cell Signaling Technology	Cat#C28E10; RRID: AB_10828120
Mouse anti Tubulin β 3 (TUBB3)/Tuj1	BioLegend	Cat#801202; RRID:AB_10063408
Rabbit anti laminin	Abcam	Cat#11575; RRID: AB_298179
Mouse anti chicken transitin/ nestin	Developmental Studies Hybridoma bank	Cat#EAP3; RRID: AB_2282449
Mouse anti myc tag	Abcam	Cat#ab32; RRID: AB_303599
Mouse anti GFP	Invitrogen	Cat#A-11120
Goat anti mouse netrin1	R&D Systems	Cat#AF1109; RRID: AB_2298775
Goat anti chicken netrin1	R&D Systems	Cat#AF128; RRID:AB_354716
Goat anti chicken netrin2	R&D Systems	Cat#AF127; RR1D: AB_2154709
Goat anti SOX2	Santa Cruz Biotechnology	Cat# sc-17320;RRID:AB_2286684
Mouse anti P27	BD Biosciences	Cat#610241; RRID:AB_397636
Rabbit anti cleaved caspase 3	BD Biosciences	Cat#559565; RRID:AB_397274
Rabbit anti phospho Histone H3	Cell signaling Technology	Cat#9701; RRID:AB_33153
Goat anti LHX2	Santa Cruz Biotechnology	Cat#Sc-19344; RRID:AB_2135660
Rabbit anti LHX2	A gift from Tom Jessell; Liem et al, 1997[18]	N/A

Mouse anti LHX1/5	Developmental Studies Hybridoma bank	Cat#4F2;RRID:AB_531784
Guinea Pig anti TLX3	Gift from Thomas Muller; (Muller et al., 2005)	N/A
Mouse anti ISL1/2	Developmental Studies Hybridoma bank	Cat#39.4DS
Goat anti ISL1/2	R&D Systems	Cat#AF1837, RRID:AB_2126324
Rabbit anti Pax2	Invitrogen	Cat#71-6000
Guinea Pig anti Olig2	Novitch et al., 2001[148]	N/A
Rabbit anti Math1/Atoh1	Helms & Johnson, 1998[38]	N/A
Goat anti ASCL1/MASH1	R&D Systems	Cat#AF2567; RRID:AB_2059505
Guinea Pig anti FOXD3 sera	Gift from Thomas Muller; (Muller et al., 2005)	N/A
Rabbit anti GAPDH	Protientech	Cat# 10494-1-AP; RRID:AB_2263076
Rabbit anti Phospho-Smad1 (Ser463/465)/ Smad5 (Ser463/465)/ Smad9 (Ser465/467) (D5B10)	Cell Signaling Technology	Cat#11971; RRID:AB_2797785
Oligonucleotides		
In situ probe for Chicken netrin1: Forward 5'- GACATCCACATCCTGAAAGCGGA Reverse with T7 sequence attached: GACTAA TACGACTCACTATAGGGTTTCCCCTTC CATCCCTCAA	This Paper	N/A
In situ probe for Chicken netrin2: Forward 5'- GACTTTCTTGTGCAGCAGAGACG-3' Reverse with T3 sequence attached 5'- GACATTAACCCTCACTAAAGGGACTCT CC TCTCTTCCTGCCAC	This Paper	N/A
In situ probe for Mouse Id1: Forward 5'- TCAGGAGGCAAGAAGAAAAA-3' Reverse with T3 sequence attached 5'- GAGATTAACCCTCACTAAAGGGAAGA AATCCGAGAAGCACGAA-3'	This Paper	N/A

In situ probe for Mouse Id3: Forward 5'- GACTCTGGGACCCTCTCTCC-3' Reverse with T3 sequence attached 5'- GAGATTAACCCTCACTAAAGGGATAAT CAGGGCAGCAGAGCTT- 3'	This Paper	N/A
qPCR primer for Lhx2: Forward 5'- CAGCTTGCGCAAAAGACC- 3' reverse: 5'- TAAAAGGTTGCGCCTGAACT-3'	This Paper	N/A
qPCR primer for Lhx9: Forward 5'- CAGGCCTGACCAAAAGAGTT-3' Reverse 5'- TGCCGTCAGCTTTATCAACA-3'	This Paper	N/A
qPCR primer for FOXD3: Forward 5'- CCCCAACACTGACCAACAG- 3' Reverse 5'- GTTTGCTCCGCCAGCTTA-3'	This Paper	N/A
qPCR primer for ISL1: Forward 5'- AGGACAAGAAACGCAGCATC-3' Reverse 5'- TTCCTGTCATCCCCTGGATA- 3'	This Paper	N/A
qPCR primer for GAPDH: Forward 5'- GGCCTTCCGTGTTCCTAC-3' Reverse 5'- TGTCATCATACTTGGCAGGTT-3'	This Paper	N/A

Chapter 3 - Assessing the existence of netrin1 isoforms in the developing spinal cord.

Abstract: Our lab has shown that netrin1 decorates the radial process of the NPCs that bridge the cells of the ventricular zone to the pial surface. Additionally, netrin1 is also observed on the end feet of the nestin^{+} radial process, at the location where these contact the laminin^{+} pial surface. These observations suggest that NPCs make netrin1, it is then anterogradely transported and deposited along the pial surface of the spinal cord[2,62]. Here netrin1 forms an adhesive substrate that positions and promotes commissural axon outgrowth. The mechanisms that allow netrin1 transport within the spinal cord remain unresolved. Previous studies in the visual system suggest that netrin1 can be cleaved into isoform fragments, each with different spatial and biological characteristics[113]. Thus, post-translational modification of netrin1 might facilitate the trafficking of netrin1 from the VZ to the pial surface. In this chapter we summarize our unpublished work aimed to assess if different netrin1 isoforms exist in the spinal cord, and if these are differentially localized to the pial surface of the spinal cord. Our preliminary data suggests that netrin1 isoforms exist in the spinal cord, and that netrin1 is differentially cleaved to allow the protein to localize to the pial surface of the spinal cord.

Introduction:

Netrin1 Domains and Function

The netrin1 protein is encoded by the NTN1 gene located on chromosome 11 in the mouse and 17, in the human genome. The full transcript consists of 7 exons and is translated into a 604 amino acid long protein. Netrin1 is a member of the laminin superfamily of heterotrimeric proteins and is composed of three domains: N-terminal also called the VI domain, followed by domain V, which is composed of three epidermal growth factor (EGF) repeats (Figure 3-1)[149]. Domains VI and V bind to the Deleted in Colorectal Cancer (DCC) and UNC-5 families of netrin 1 receptors[56,57]. The remaining C domain of netrin 1, also known as the netrin-like (NTR) domain binds to heparin with high affinity and may facilitate presenting secreted netrins on cell surfaces or retaining them in extra cellular matrix (ECM)[150].

The NTR domain also shows sequence similarity with secreted Frizzled-related proteins that are involved in axon guidance, with the type I procollagen C-proteinase enhancer proteins (PCOLCE), which are metalloproteinase inhibitors, and in tissue inhibitors of metalloproteinases[151]. The functional significance of the NTR module is poorly understood but structural and functional data suggest an inhibitory activity towards proteinases[152]. The C terminal domain of netrin1 is not required for binding to DCC or the UNC-5 homologs[56,57].

Netrin Isoforms and Disease

Isoforms allow for greater biodiversity of protein encoding genes. Isoforms can result from several modifications including alternative RNA splicing, truncation, modifications to the glycosylation patterns[153]. Multiple isoforms of netrin family members, across species, have become identified (Table 3-1) and increasingly implicated in the progression of diseases like cancer[112], multiple sclerosis[71], and diabetic retinopathy[113], however, a role for netrin isoforms in neural development has yet to be elucidated.

A truncated form of netrin1 has been associated with poor patient survival in cancer patients. A truncated isoform is produced by an alternative internal promotor resulting in an alternative start site and netrin1 lacking the first part of the N-terminal VI domain. This isoform has been shown to localize to the nucleolus. There it interacts with nucleolar proteins and ribosomal DNA promotor and enhances tumor proliferation[112].

In multiple sclerosis patients (MS), a truncation isoform resulting in a VI-V fragment has been associated with a poor prognosis. MS is characterized by demyelinated axons and by impaired remyelination processes[154]. Myelin is made by oligodendrocytes and oligodendrocyte precursors cells are known to play an important role in the remyelination process after injury[155]. The VI-V fragment was found in the white matter of multiple sclerosis patient. In an *in vitro* axon guidance assay, the VI-V domain did not have chemoattractant capabilities, however when placed in the assay with full length netrin1 protein, axon guidance activity decreased. VI-V fragment was capable of binding to the netrin1 receptor but not capable

of eliciting an axon guidance phenotype. This led to the hypothesis that the presence of VI-V fragments in lesion sites lead to limitations in oligodendrocyte precursor migration to the site of the lesion, leading to deficits in remyelination[71]

In patients with diabetic retinopathy (DR), fragments of the VI-V domains have been found to contribute to heightened vascular permeability leading to edema, contributing to blindness. Fragments of the VI-V domains were found in both vitreous humor of diabetic patients and in retina of a murine model of diabetes. Further studies identified that netrin1 is metabolized into a bioactive fragment by the collagenase matrix metalloprotease 9 (MMP-9), which is increased in patients with diabetic macular edema. Suggesting MMP-9 may facilitate release of netrin-1 fragments from the extracellular matrix and facilitate diffusion of the fragment[113].

Matrix Metalloprotease Proteins in Nervous System Development

MMPs are best known for their ability to cleave components of the extracellular matrix (ECM) including other proteinases, proteinase inhibitors, chemotactic molecules, cell surface receptors, cell-cell adhesion molecules[156,157]. In the spinal cord, MMPs have been implicated in the nervous system development, modulating activities such as axon attractive and repellent activity by modulating the ECM to allow axonal trajectories[158-160]. Miloudi and colleagues found evidence to suggest that nertrin1 is cleaved by MMPs into an isoform facilitating the diffusing of the protein to mediate a unique biological activity[113]. Netrin1 is abundantly associated with the ECM of the developing tissue[88,161]. Using in silico modeling, we were able to map the predicted cleavage sites of 3 MMP family members, MMP2, MMP3 and MMP9 on mouse netrin1 (Figure 3-2) and found that netrin1 is indeed a broadly potential candidate for cleavage by the MMP family of proteases.

Netrin 1 appears to be transported from the VZ to form a netrin1^{+} substrate on the pial surface but the mechanisms that allow netrin1 to be transported remain unknown. Previous studies in the visual system suggest that netrin1 can be cleaved into unique isoform fragments, facilitating the diffusion of the

protein[113]. Thus, cleavage of netrin1 by MMPs might facilitate the trafficking of netrin1 from the VZ to the pial surface. Isoforms of netrin family members, across species, have become increasingly implicated in the progression of disease however, no developmental association has been elucidated yet. In this chapter we use *in vivo* and *in vitro* approaches to assess the existence of truncated netrin1 isoforms in the developing spinal cord.

Results

Immunohistochemical assessment of netrin1 isoforms in the spinal cord

To assess whether different isoforms of netrin1 exist in the spinal cord, we stained spinal cord tissue with a 1) "full length" polyclonal antibody that recognizes antigens within 22-603 amino acids in the mouse netrin1 protein, and 2) a "fragment" polyclonal antibody that recognizes an antigen within 300-400 amino acids. In transverse sections of E14 rat spinal cord, we observed that the antibodies decorate different regions of the spinal cord. The antibody against full length netrin1 predominantly decorates the FP, pial surface and commissural axons (Figure 3-3), as previously described[6,19]. The antibody against fragment netrin1 predominantly labels puncta within the VZ (Figure 3-3C). The distinction in staining patterns can be most fully appreciated in open book preparations (Figure 3-3D and 3-3E). These antibodies showed different staining patterns, suggesting that netrin1 isoforms are present in two distinct regions of the spinal cord, the VZ and pial surface.

Tracking tagged-netrin1 in COS cells in vitro

To investigate whether mouse netrin1 can be cleaved as proposed in the visual system[11], we used standard molecular methods to epitope tag mouse netrin1 with c-myc in two locations 1) after the signal sequence on the N-terminal end: sigmyc-netrin1, and 2) the C-terminal end: netrin1-myc (Figure 3-4). The tagged proteins were cloned into expression vectors under the control of a CAG enhancer for ubiquitous expression. We transfected these constructs into COS7 cells, lysed the cells after 48 hours, and ran the samples on a 10% polyacrylamide gel under reducing conditions. We then performed a western analysis

of the collected lysate and probed for c-myc (Figure 3-5). Multiple fragments containing c-myc were observed in the lysates collected from samples transfected with tagged-netrin1, (lane 1 and 2, Figure 3-5). In parallel, we performed a western analysis of E11.5 mouse spinal cord lysate, probed with an anti-netrin1 antibody. We observed both the expected netrin1 band at around 75kDa, in addition to multiple bands of lower and higher molecular weight (Figure 3-5A). This data further supports the hypothesis that mouse netrin1 isoforms exist in the developing spinal cord and suggests that these fragments are a result of cleavage.

Tracking tagged-netrin1 in chicken embryos in vivo

To further investigate the possibility that netrin1 isoforms can localize to different regions of the spinal cord, we assessed the localization of differentially tagged-netrin1 through immunohistochemistry. Chicken embryos are an excellent model organism to study developmental processes because they are easy to manipulate, however, a complication in this system is there are two netrins, netrin1 and netrin 2, in the chick spinal cord. Netrin1 is expressed in the FP while netrin2 is present in the ventral and intermediate VZ[52] However, this complication is also an opportunity, permitting us to study the trafficking of mouse netrin1 in the VZ, where chicken netrin1 is not present. We electroporated the tagged netrin1 constructs into Hamilton Hamburger (HH) stage 14 chicken embryos, the time point immediately before the onset of axiogenesis in the spinal cord. The electroporated embryos were returned to a 37°C incubator to allow for continued development. The embryos were then collected 48h after electroporation, at stage HH24, the time point at which axons would have reached the FP. The embryos were fixed in paraformaldehyde, sectioned into thin microtome sections, and stained with antibodies against c-myc and mouse netrin1 (Figure 3-6). We observed that the electroporation of netrin1-myc results in efficient localization of both netrin1 and myc to the pial surface of the spinal cord (arrows, Figure. 3-6C.). In contrast, electroporation of sigmyc-netrin1 results in lower levels of netrin1 and myc on the pial surface of spinal cord (arrows, Figure 3-6C). This data further supports the hypothesis that netrin1 can be cleaved and that cleavage products have the capacity to differentially localize within the spinal cord.

Furthermore, this result suggests that a C-terminal netrin1 isoform is the key molecular species that establishes the netrin1 substrate on the pial to guide the ventrally directed growth of axons.

Generation of Epitope specific antibodies against fragments of netin1

To assess whether fragments of netrin1 differentially localize to distinct regions of the spinal cord, we partnered with Covance to generate a series of polyclonal antibodies that target epitopes at the N terminus (amino acid, 79-91, Host: Rat), middle hinge region (amino acid 389-401, Host: Guinea Pig) and C terminus (583-600; Host: Rabbit) of mouse netrin1. Each animal project had multiple bleed sessions and each batch was screened for reactivity (not shown), overall, the only antibody that gave a promising and consistent signal was the one raised against rat and targeting the N-terminal end of the netrin1 protein, AA 79-91. Ell.5 mouse spinal cord was stained with antibodies against full netrin1 and customized epitope specific antibody targeting AA 79-91 of mouse netrin1. Initial assessment of the Covance antibody shows that the probe stains the radial processes of the spinal cord that span from the ventricular zone to the pial surface (Figure 3-7). The full length netrin1 stains the pial surface of the spinal cord and the floor plate as expected. We observed no overlap between the 2 signals.

Myc-tagged netrin1 transgenic mouse line

To investigate whether the netrin protein is cleaved allowing the resulting fragments to localize to different regions of the spinal cord *in vivo*, we worked with the University of California, Irvine transgenic facility, to generate mouse lines where myc is inserted at either the N-terminus or C-terminus end of netrin1 protein, using CRISPR/Cas9 genome editing of the endogenous netrin locus. Using immunohistochemistry, we assessed the location of the inserted myc tag by staining with antibodies against myc. In the N-terminal tag mouse lines we see that the myc tag localizes to both the pial surface as well as the floor plate (Figure 3-8C, D), reflecting the pattern of expression of endogenous netrin1 as has been previously described[2]. We observe no myc signal in the WT, no insert control mouse (Figure 3-8A, B). Additionally, we see myc tag on the commissural axons along the floor plate of the spinal cord in

the mutant, when stained with Robo3 (Figure 3-8C). The presence of netrin1 on the axons crossing the floor plate has been previously observed as well[2,4]. This data suggests that we have generated a myc tagged netrin1 mouse line that successfully reflects the expression pattern of the endogenous netrin1 protein. To compare if the myc tag from N-terminal tagged versus C-terminal tagged netrin1 localized to different locations within the spinal cord, we also examined the C-terminal myc-tagged netrin1 mouse line. Using Robo3 as a marker for commissural axons and an antibody against myc, we observe that myc tag localized to the pial surface as well as the floor plate (Figure 3-9C, D). There was no obvious difference in the staining pattern of myc when comparing the N-terminal tagged netrin mouse line versus the C-terminal tagged netrin1 mouse line. Further analysis using modified immunohistochemistry techniques or more sensitive biochemical methods are necessary to further characterize these mouse lines.

Discussion and Conclusions

The objective of the studies summarized in this chapter was to elucidate if netrin1 isoforms exist in the embryonic spinal cord. Using both *in vivo* and *in vitro* techniques, we accumulated data that suggests the existence of netrin1 isoforms in the embryonic spinal cord. Electroporation of tag netrin1 constructs in the chicken spinal cord resulted in preferential localization of the C-terminal tagged netrin1 myc tag to the pial surface of the spinal cord. Expression of the myc tag colocalized to expression of mouse netrin, when presence was probed with a mouse netrin1 specific antibody. Attempts to further characterize the distribution of netrin1 fragments *in vivo* have remained inconclusive. The epitope specific polyclonal antibodies generated in collaboration with Covance yielded limited results. The rat anti mouse netrin1 against the AA 79-91 yielded an interesting result. The probe preferentially localized to the radial processes of the spinal cord. Previous publications have shown that netrin1 localizes to the nestin expressing radial process that link the apical to basal regions[2,62]. Further work is needed to validate the specificity of the antibody but given the association of netrin1 with radial processes, it is possible that a processed form of netrin1 is being recognized by the antibody. Similarly, we collaborated with UC Irvine to develop N and C-terminal netrin1 tagged mice. Our early characterization of the mouse lines shows

that the myc tag localizes to both the pial and FP of the spinal cord, in a manner that reflects the endogenous netrin1 staining pattern. We don't observe any obvious differences in myc distribution however, antibody staining techniques we used might not be sensitive enough to unravel any differences. Further testing should be considered such as the use of antigen retrieval to unmask any potentially masked epitopes, milder detergents might by useful to prevent loss of signal. Biochemical analysis of the mouse lines might yield interesting results. Using techniques such as SDS PAGE might reveal fragments of netrin1 when probing with myc.

The observation that netrin1 decorates the radial process and the pial surface of the spinal cord raises the possibility that netrin1 is transported along radial processes to the pial. The pial-associated netrin1 forms an adhesive substrate that promotes commissural axon outgrowth towards the midline through haptotaxis. The mechanisms that allow netrin1 transport within the spinal cord remain unresolved. However, we proposed that netrin1 could be processed into isoforms, to facilitate this transport. Our data postulates the existence of netrin1 isoforms in the spinal cord. In summary, NPCs of the VZ make netrin and then netrin1 must be cleaved into isoforms to be transported. Together, these two processes allow the formation of the netrin1^{+} substrate that encourages oriented growth of axonal trajectories. Understanding the mechanisms by which NPCs lay down tracks of netrin1 could provide insight into how best to use netrin1 to encourage regeneration of axon tracts and provide a better understanding of the basis of neurodevelopmental disorders. Any of the information gained through these studies will be informative given the novelty of the haptotactic model of axon guidance.

Materials and Methods

Generation of c-myc-tagged netrin1 constructs and in ovo electroporation of chicken embryos

Mouse *netrin1* (Addgene #71978) was amplified using PCR and fused on either the C-terminal end or N-terminal end, after the signal sequence, to a myc tag (EQKLISEEDL) and then subcloned in front of the CAG enhancer in the CAGGS vector. The CAG enhancer is comprised of a CMV enhancer and chicken β-actin promoter. Fertile Leghorn eggs (CJ Eggs, Sylmar CA) were incubated for 60 hr until the embryos developed to HH stages 14–15. The spinal cord was electroporated and then allowed to develop for 48 hr until HH stages 24–26. The following constructs were used CMV: GFP, C-terminus or N-terminus myc tagged netrin1 constructs: CAG:*Net1-myc* (1000 ng/µl). In all cases, the presence of GFP demonstrates electroporation efficiency.

Tissue processing

Spinal cords were fixed using 4% paraformaldehyde for 2 h at 4 °C. For cross sections: After fixation, the tissue was cryoprotected in a 30% sucrose solution overnight, following which the tissue was mounted in optimal cutting temperature (OCT) and cryosectioned at 20µm. Sections were collected on slides and processed for immunohistochemistry. For open book preps: After fixation, preps were washed with PBS and processed for immunohistochemistry.

Immunohistochemistry

Chicken embryonic spinal cords, rate and mouse embryonic spinal cords were sectioned to yield 20 µm sections. The following were used for immunostaining; Goat anti-mouse netrin1 (1:500; R&D Systems, Cat# #AF1109), Goat anti-human Robo3 (1:250; R&D Systems, Cat#AF3076), Chicken anti fragmented mouse netrin1 (AA 300-400) (1:100; Novus, Cat# NB100-1605), Mouse anti-GPP (1:1000; Invitrogen, Cat#A-11120), mouse anti c-myc (1:1000; Abcam Cat#ab32), Rat anti-mouse netrin1 AA 79-91(1:100, Covance Custom Antibody), Species appropriate Cyanine 3, 5 and Fluorescein conjugated secondary

antibodies were used (Jackson ImmunoResearch Laboratories). Images were collected on Carl Zeiss LSM700 confocal microscopes.

Western blot analysis

COS-7 cells (ATCC CRL-1651) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich Sweden AB, Stockholm, Sweden) supplemented with 10% fetal bovine serum (FBS) (Fisher Scientific, Waltham, Massachusetts) and Penicillin-Streptomycin-Glutamine (100X) (Gibco, Fisher Scientific, Gothenburg, Sweden).

COS7 cells were seeded in 12-well the day before transfection. On the day of transfection, cells were washed with DMEM media. Cells were transfected with 1 µg of c-myc tagged netrin1 encoding plasmids, sigmyc-netrin1 and netrin1-myc, using Lipofectamine 2000. After 48 hrs, the cells were washed with PBS and lysed with RIPA lysis buffer containing a protease inhibitor cocktail (Roche Diagnostics Scandinavia AB, Bromma, Sweden) and the phosphatase inhibitor PhosSTOP (Roche Diagnostics Scandinavia AB). The cell lysates were kept on ice for 30 min and centrifuged at 20,800×g for 10 min at 4 °C. The clear supernatant was subjected to SDS-PAGE using 10% Tris-Glycine SDS gels followed by transfer onto PVDF membranes (Millipore Sigma, MA, USA). The membranes were blocked using Non-fat dry milk (Bio-Rad Laboratories AB, Solna, Sweden). The blocked membranes were then incubated with the primary antibodies at 4C overnight. Thereafter, the membranes were washed three times with TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween 20), incubated secondary antibodies for an hour at room temperature and then washed again three times with TBST. The fluorescent bands were analyzed using the Pierce Femto Chemiluminescence system on the Azure Imager.

Myc-tagged netrin1 transgenic mouse line.

Working with the University of California, Irvine transgenic facility, we generated multiple mouse lines in the B6J background, where myc is inserted at either the N-terminus (Chromosome 11, exon 2) or C-terminus, (Chromosome 11, exon 7), end of netrin1 protein, using CRISPR/Cas9 genome editing of the

endogenous netrin locus. Timed plugs were collected et E11.5. Genotype was performed using the following primers: C terminus tag- Forward: CCACTTGTTTGCTTCCTCACAG, Reverse: CCAAGTCCAAGGCTGCCAG. N terminus tag- Forward CGGGGCACTTGCCCGCCACTCCACCGAG, Reverse TGCTGGACACGCGCACGTCCTTG

Generation of Epitope specific antibodies against fragments of netin1

Working with Covance, we generated a series of polyclonal antibodies that target epitopes at the N terminus (amino acid, 79-91: VVSERGEERLRSC; Host: Rat), middle hinge region (amino acid 389-401: RDMGKPITHRKAC; Host: Guinea Pig) and C terminus (583-600: CDTWARRLRKFQQREKKGK; Host: Rabbit) of mouse netrin1.

Figures and Tables

Figure 3-1. Domains of the netrin1 protein.

Figure 3-1. Domains of the netrin1 protein. Netrin1 is composed of three domains: N-terminal also called the VI domain, followed by domain V, which is composed of three epidermal growth factor (EGF) repeats[149]. Domains VI and V bind to the Deleted in Colorectal Cancer (DCC) and UNC-5 families of netrin 1 receptors[56,57,59]. The remaining C domain of netrin 1, also known as the netrin-like (NTR) domain binds to heparin extra cellular matrix[151,152].

Table 3-1. Isoforms of netrin family members.

Protein	Isoform Type	Specifics of Modification	Phenotype/ Biological Activity	Reference
Human netrin1 Mouse netrin1 Chick netrin 1 Chick netrin 2	PTM	Cleavage site immediately after the signal peptide (N terminus)	Signal peptide localizes the protein to the ER (cleaved) > Golgi > further modified for secretion (N-glyc)	Protein Data Bank
Mouse netrin1 (enucleated eyes) Human netrin1 (vitreous fluid) "synthetic" netrin1	PTM	Netrin1 is a substrate for metalloprotease MMP9 leading to cleavage and fragmentation of netrin1 into many fragments: Major: VI-V	The VI-V fragment provokes vascular permeability in diabetics	Miloudi *et al*., 2016
Human netrin1 Mouse netrin1 Chick netrin 1 Chick netrin 2	Co-translational/PTM	4 predicted N-glycosylation sites	Associated with membrane bound and secreted proteins	Protein Data Bank
Human netrin1 (*in vitro)*	Splice Variant- alternative internal promoter	Truncated netrin1	Missing N terminus, and some of VI. C terminus localizes to the nucleolus where it promotes tumor cell proliferation and growth	Delloye-Bourgeois *et al*., 2012
Human netrin1	Splice Variant- Computationally Predicted	Truncated protein, missing both N and C terminal domains- protein reflects only part of EGF2	Predicted to be very soluble due to lack of binding sites that mediate interaction with ECM proteins	UniProt Protein Data Base, *Nucleic Acids Research* (2017) 45: D158–D169

Table 3-1. Isoforms of netrin family members. Netrin isoforms exist across species, chicken and mouse. Modifications occur at the transcript and protein level. Modifications include changes to glycosylation patterns, truncations resulting from alternative splicing as well as cleavage by components of the extra cellular matrix[112,113].

Figure 3-2. Netrin1 has multiple MMP cleavage recognition sites.

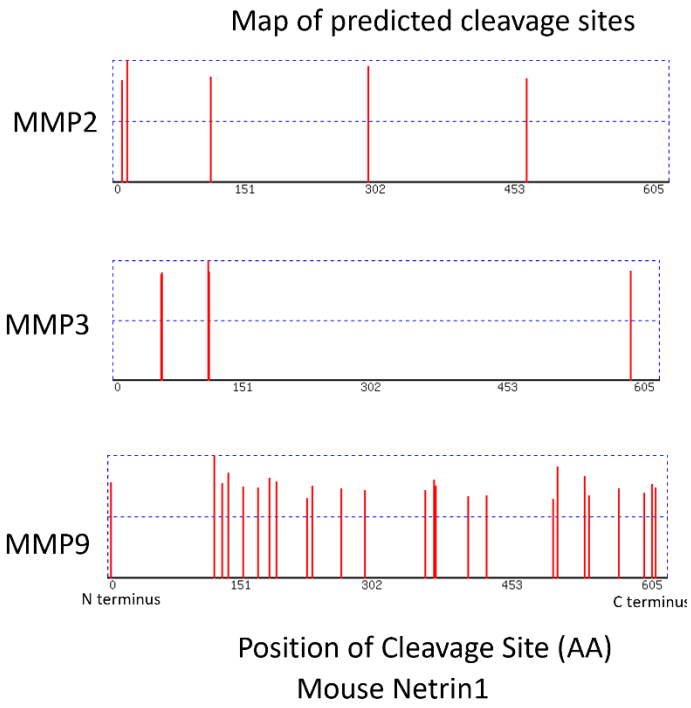

Figure 3-2. Netrin1 has multiple MMP cleavage recognition sites. Using in silico prediction software we were able to confirm that netrin1 is a a candidate substrate for the MMP family members MMP2, MMP3 and MMP9. The latter has the highest amount of cleavage recognition sites along the N to C terminal end of the protein. Source: https://prosper.erc.monash.edu.au/home.html

Figure 3-3. Differential binding of netrin1 antibodies.

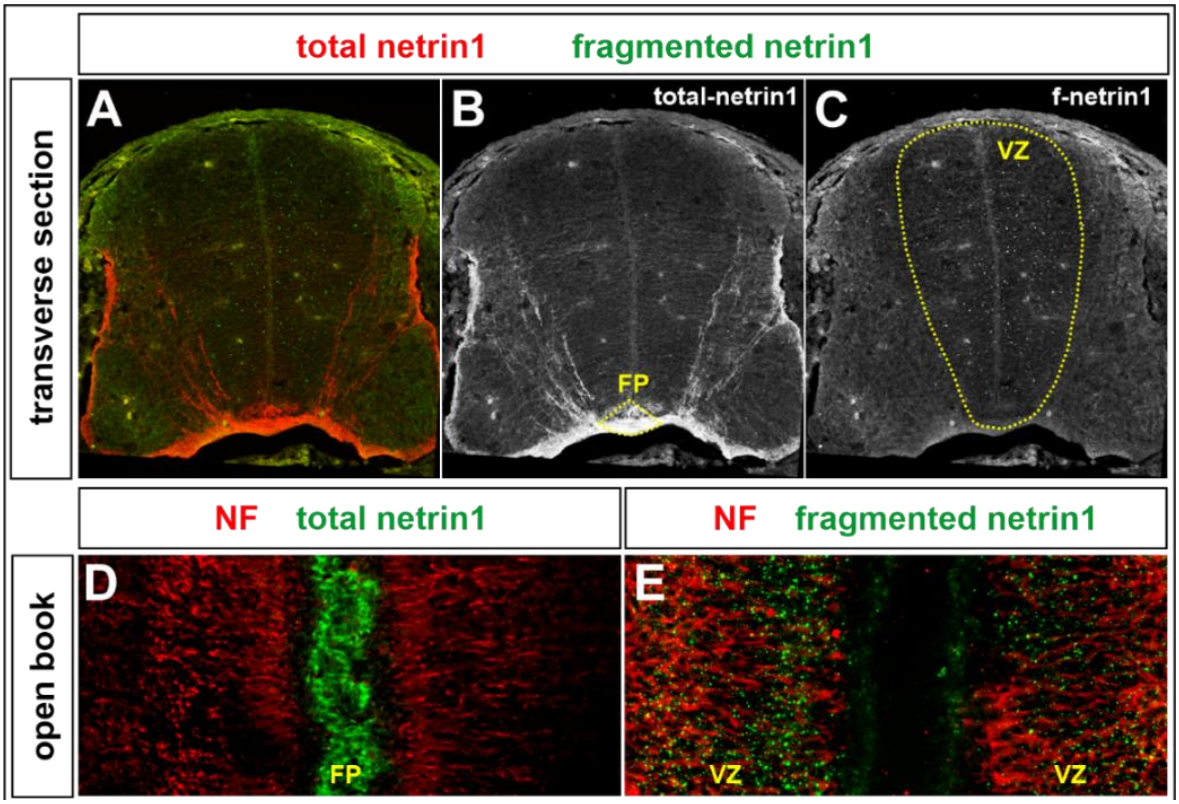

Figure 3-3. Differential binding of netrin1 antibodies. (A-C) Antibodies against full length netrin decorate both the pial surface and the commissural axons. Antibodies against fragmented netrin1 (f-netrin1) label the ventricular zone (VZ, dotted region, C). (D, E) The distinction can be seen in open book preparations. Anti total netrin1is present at higher levels in the floor plate (FP, dotted region, B), anti-fragmented netrin1is present in the VZ, above the NF axons.

Figure 3-4. Constructs encoding for myc tagged mouse netrin1.

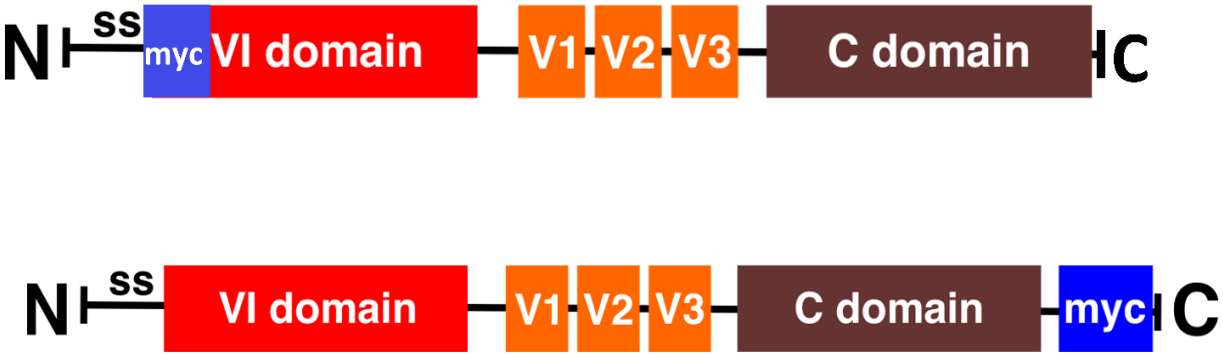

Figure 3-4. Constructs encoding for myc tagged mouse netrin1. Mouse netrin1 was tagged with a myc tag on either the n terminal end (top, myc-netrin1) of the protein or the c terminal end of the protein (bottom, netrin1-myc). The n terminal myc tagged was placed after the signal sequence (ss) to prevent from it being cleaved during the process of secretion.

Figure 3-5. Western blot analysis of netrin1 transfected cells.

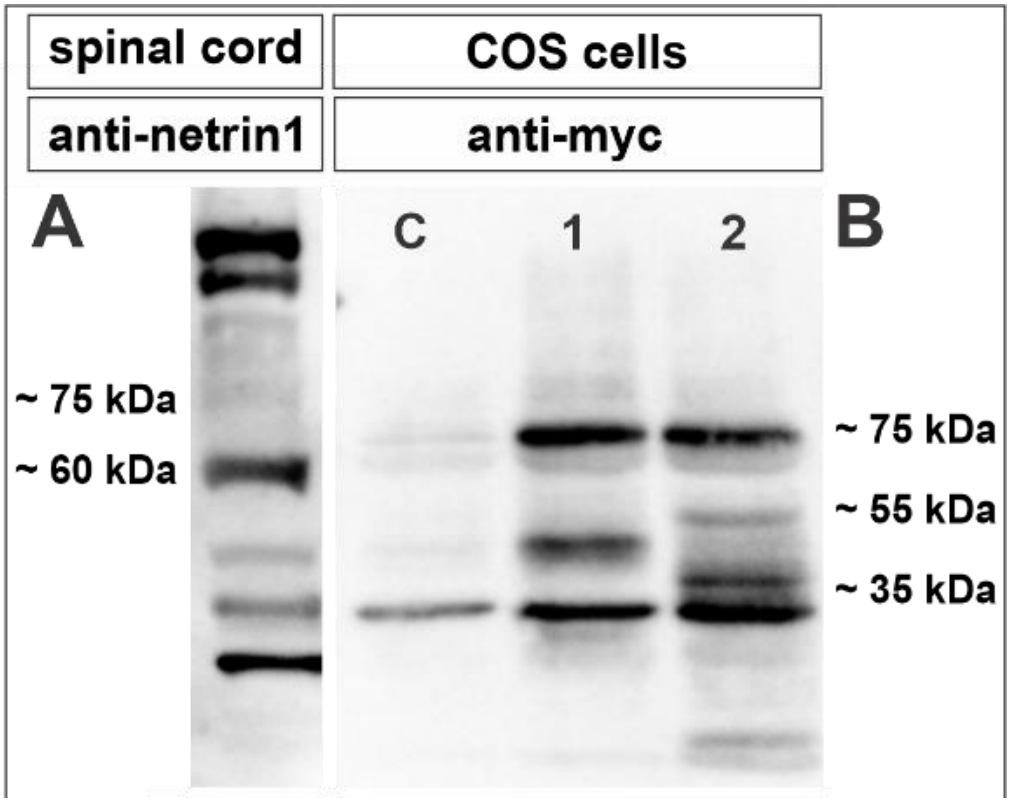

Figure 3-5. Western analysis of netrin1 transfected cells. Multiple bands are observed in protein lysates of (A) WT Ell.5 mice spinal cord and (B) COS7 cells transfected with vector control (C) sigmyc-netrin1 (1), or netrin1-myc (2). The predicted size of netrin1 is ~75 kDa.

Figure 3-6. Tagged netrin1 in chicken electroporations.

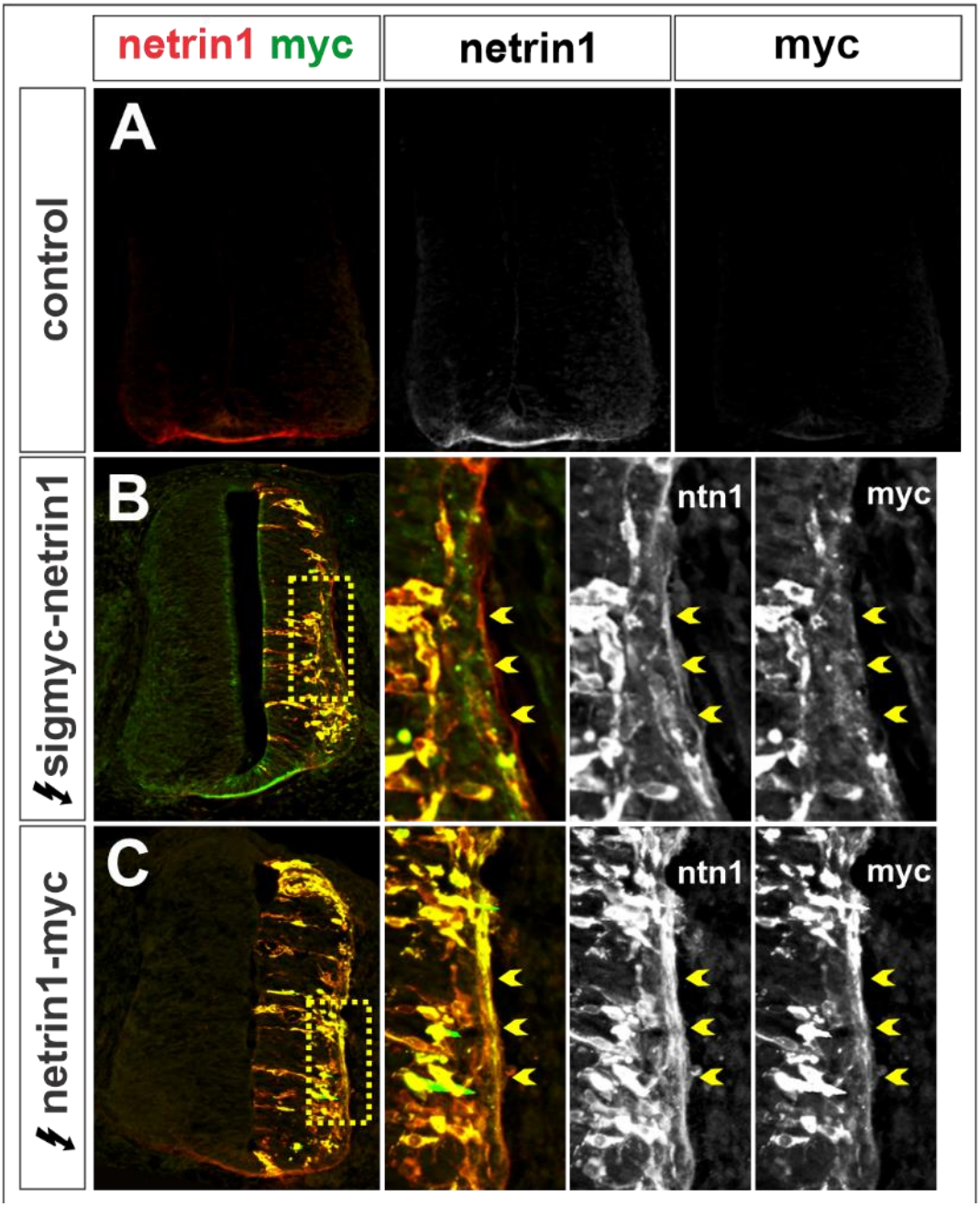

Figure 3-6. Tagged netrin1 electroporation. (A) Chicken netrin1 is normally present at the FP. (B) Electroporation of sigmyc-netrin1 results in low levels of netrin1, but not myc at the pial surface (arrows). (C) In contrast, electroporation of netrin1-myc results in high levels of both netrin1 and myc at the pial surface (arrows).

Figure 3-7. Custom netrin1 epitope-specific antibodies stain the spinal cord.

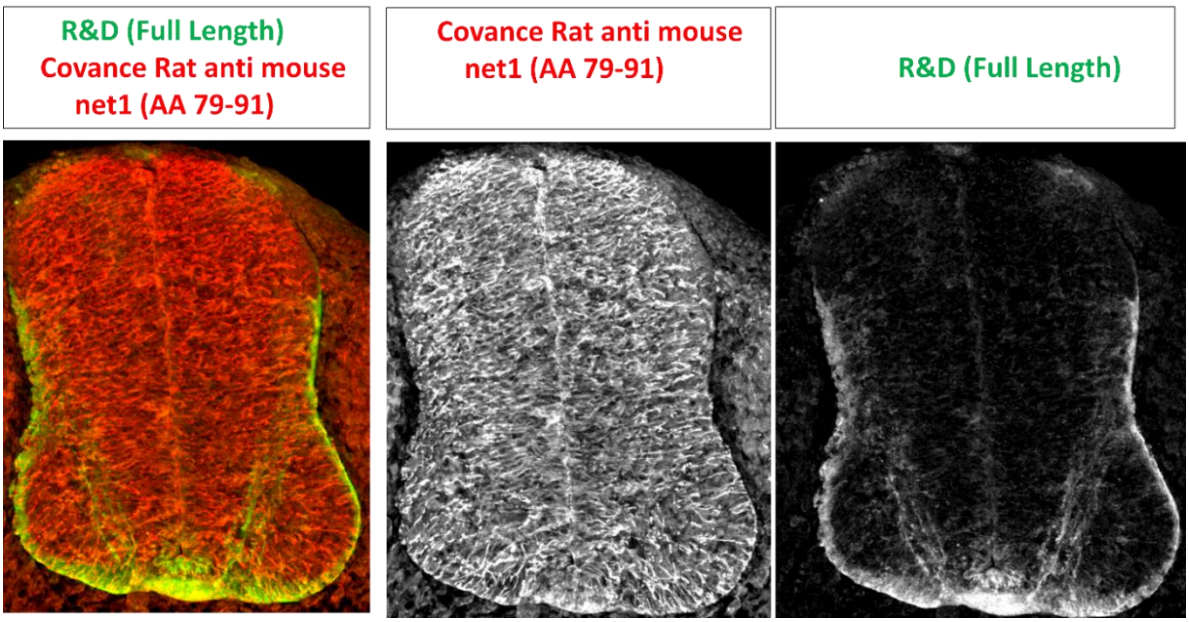

Figure 3-7. Custom epitope specific antibodies stain the spinal cord. Ell.5 mouse spinal cord was stained with antibodies against full netrin1 and customized epitope specific antibody targeting AA 79-91 of mouse netrin1. Initial assessment of the Covance antibody shows that the probe stains the radial processes of the spinal cord that span from the ventricular zone to the pial surface. The full length netrin1 stains the pial surface of the spinal cord and the floor plate.

Figure 3-8. Characterization of N-terminus myc-tagged netrin1 mouse line.

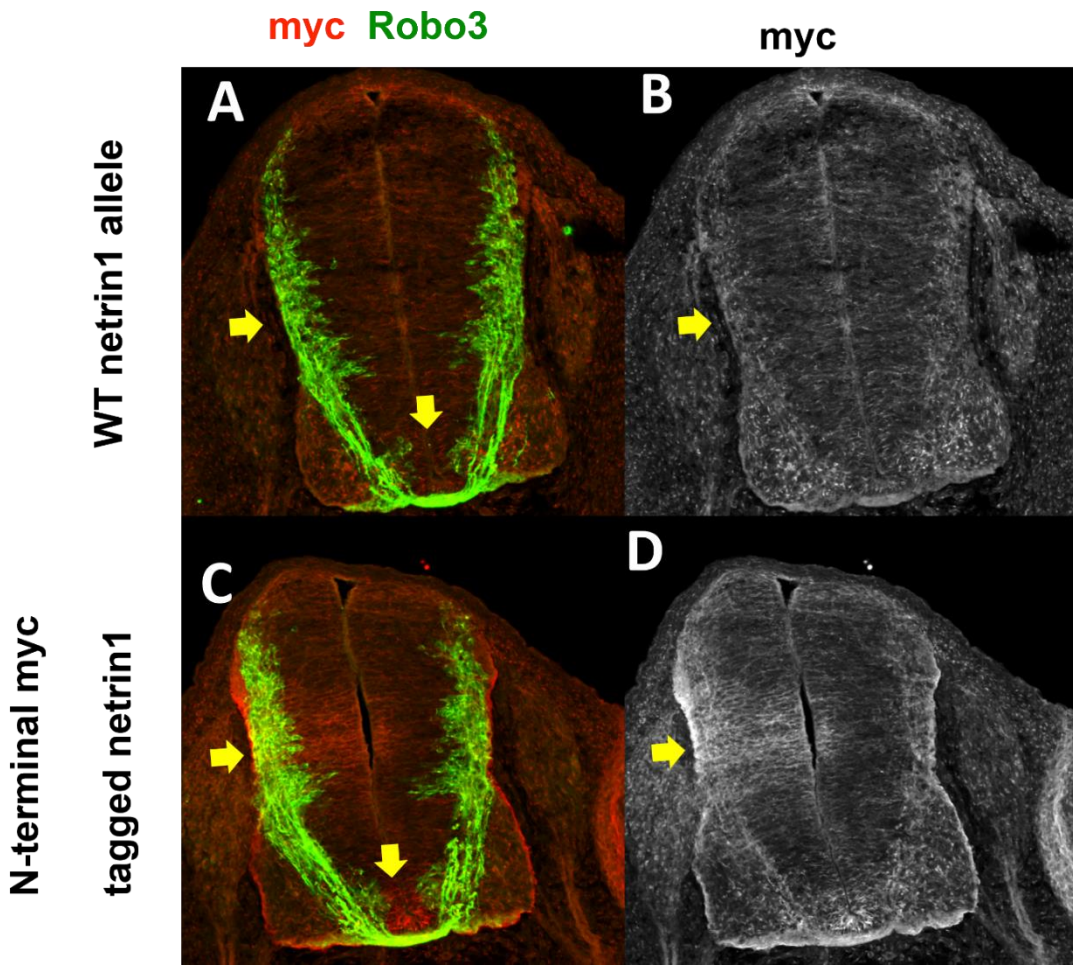

Figure 3-8. Characterization of N-terminus myc-tagged netrin1 mouse line. (C, D) Ell.5 spinal cords of mice with a myc tag inserted to the n terminal end of endogenous netrin1, or (A, B) littermate controls, were stained with antibodies against netrin1 and myc tag. (C, D) In transgenic mice, the myc tag efficiently localizes to both the pial surface of the spinal cord as well as the floorplate, confirming efficiently integration of the myc tag into the endogenous netrin1 loci. (A, B), this pattern of myc expression is not observed in the littermate controls.

Figure 3-9. Characterization of C-terminus myc-tagged netrin1 mouse line.

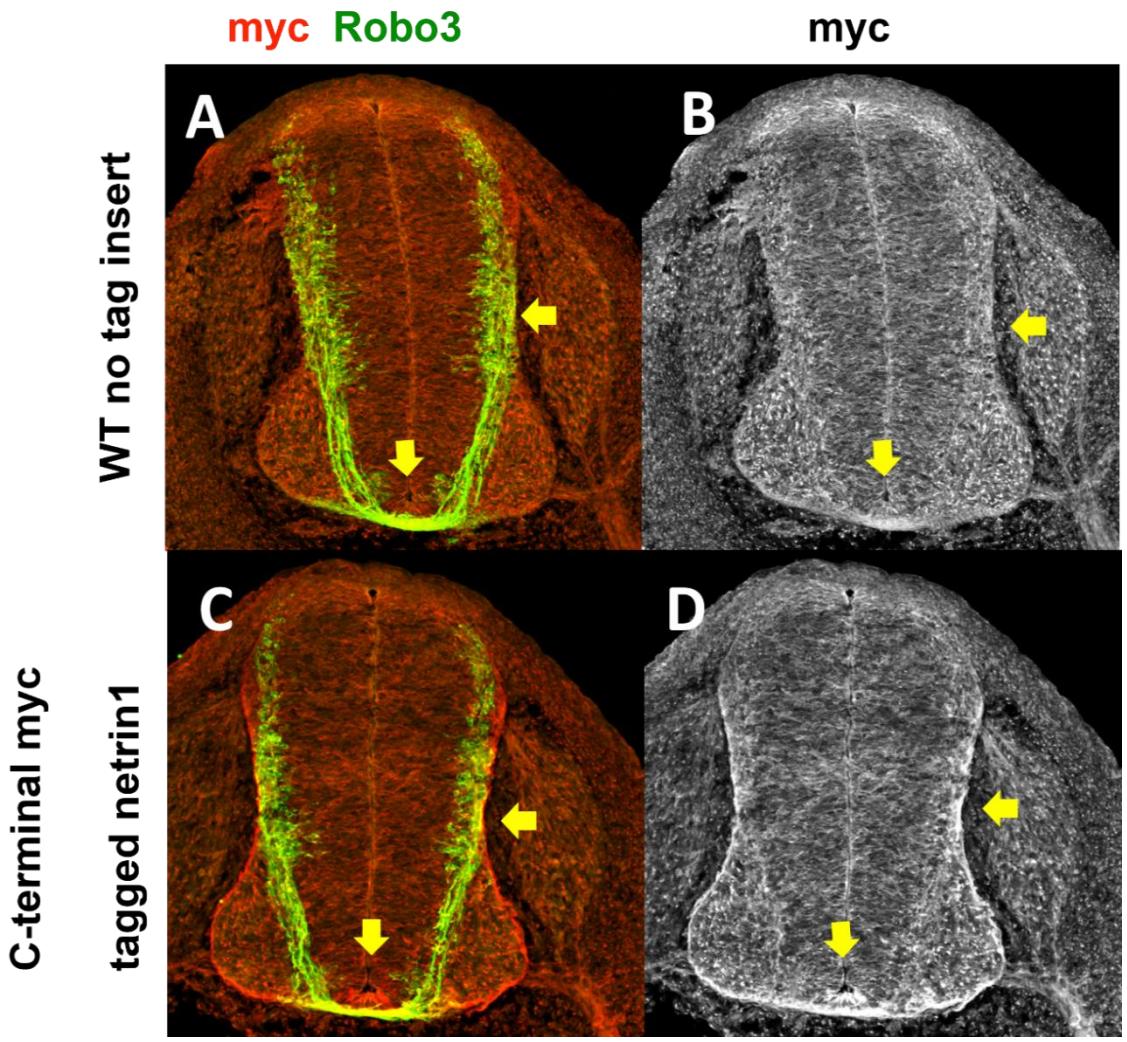

Figure 3-9. Characterization of C-terminus myc-tagged netrin1 mouse line. (C, D) Ell.5 spinal cords of mice with a myc tag inserted to the C terminal end of endogenous netrin1, or (A, B) littermate controls, were stained with antibodies against netrin1 and myc tag. (C, D) In transgenic mice, the myc tag localizes to the pial surface and floor plate (arrows) of the spinal cord, mimicking the pattern of endogenous netrin1 protein expression, (A, B), this pattern of myc expression is not observed in the littermate controls.

Chapter 4 - Conclusions

The axon guidance properties of netrin1 were first identified in chicken embyros[52,53,72]. With the advent of modern molecular techniques and the availability of reagents, including genetic tools, the mechanisms by which netrin1 mediates axon guidance have been redefined[2,4,62,63], and novel roles for netrin1 have been elucidated[90,94,99,100,112,145,146].

The recent discovery that netrin1 suppresses BMP signaling *in vitro* opens the door to the possibility that in addition to acting as a guidance cue, netrin1 may also be involved in patterning the dorsal spinal cord given the critical role BMP signaling plays in spinal cord patterning[1,17]. This would mean that netrin1 plays a role in spinal cord development at an embryonic stage much earlier than that of the onset of axogenesis, E11.5. Both netrin1 and BMPs are highly expressed in the developing spinal cord, in a domain directly adjacent to each other, thus the BMP suppressive activity of netri1 also proposes the possibility that netrin1 directly limits the range of influence of BMP signaling (Figure 3-6L).

Recent work from the Butler laboratory[6] and other groups[7,8] have shown that NPC-derived netrin1 is responsible for guiding fasciculated axon extension towards the spinal cord midline. However, many questions remain, one of which is: how does VZ-derived netrin1 get to the pial surface to allow the formation of the sticky substrate on the pial surface of the spinal cord that is important for the formation of neural circuits?

In Chapter 2, we assessed the role of netrin1 in the process of spinal cord patterning through modulation of BMP signaling. We used both gain- and loss-of-function approaches to assess whether netrin1 restricts BMP activity to the dorsal spinal cord. Supporting this hypothesis, we show that the ectopic expression of netrin1 *in vivo* leads to reduced number of dorsal interneurons when electroporated into the chicken spinal cord (Figure 2-2). Furthermore, addition of netrn1 *in vitro* inhibits differentiation of mouse ESCs into dorsal dIs (Figure 2-3). By contrast, the loss of netrin1 *in vivo* increases BMP-dependent dorsal

progenitors, however, the number of post-mitotic neurons decreases (Figure 2-4). Our data suggests that progenitors remain in their undifferentiated state. The exact mechanism by which this stunted development happens, has yet to be elucidated. However, we find that netrin1 modulates levels of pSmad1/5/8 (Figure 2-6), the effectors of BMP signaling as well as the Ids, BMP-signaling downstream target genes involved in inhibition of differentiation (Figure 2-7). These findings identify a novel role for netrin1 as a modulator of BMP signaling in the developing nervous system.

The interaction between netrin1 and BMP signaling that we have characterized in Chapter 2 of this thesis postulates that this signaling axis might be critical for other developmental and disease processes. The interaction between netrin1 and BMP is potentially a fine-tuning mechanism that allows for specification and regulation of structures during development and highlights the need to visit other BMP/netrin1 signaling centers.

In Chapter 3 of this thesis, I assessed the mode by which NPC-derived *netrin1* give rise to a netrin1^{+} substrate on the pial surface of the spinal cord. Previous studies have suggested that cleavage of netrin1 into distinct isoforms could facilitate transfer of netrin1. Therefore, post-translational modification of netrin1 might facilitate the trafficking of netrin1 from the VZ to the pial surface. To assess whether different isoforms of netrin1 exist in the spinal cord, I used epitope specific immunohistochemistry to characterize distribution of netrin1 protein and observed that the antibodies decorate different regions of the spinal cord (Figure 3-3). I tracked myc-tagged-netrin1 *in vitro* and observed multiple fragments containing myc in the lysates (Figure 3-5). Additionally, I observed that the electroporation of C-terminally tagged netrin1-myc results in efficient localization of netrin1 and myc to the pial surface (Figure 3-6). These findings suggest that the C terminal end of netrin1 might localize to the pial surface. This preliminary data supports the hypothesis that netrin1 can be cleaved and that cleavage products have the capacity to differentially localize within the spinal cord.

Further work is required to understand the significance of the potential existence of netrin1 isoforms in the spinal cord. Further examination of the netrin1 fragments using mass spectrometry could help identify

the amino acid sequence of these protein fragments observed in Cos cells (Figure 3-5) and in lysates of chicken electroporation (Figure 3-6). The myc-tagged mouse lines presented in Figure 3-8 and 3-9 could be further examined for the existence of netrin1 fragments. This would provide further information on the region of the peptide important for formation of the pial netrin1 substrate. In summary, in these studies I examined the molecular mechanism that establishes the formation of a netrin1^{+} growth substrate in the spinal cord, which will shed direct light on the best way to deploy netrin1 for regeneration.

Significance

Failure to have an appropriate organization of neuronal cells in the spinal cord and the appropriate circuitry connections during development can result in movement disorders, an inability to process sensory cues, and altered intellectual development. Cell patterning and subsequent circuitry formation is dependent on signaling centers and the ability of axons to navigate through the embryonic environment using molecular cues[14]. Understanding the mechanism(s) by which these signaling centers and axon guidance signals direct cell patterning and axonal growth is key to 1) understanding the basis of neurodevelopmental disorders, and 2) applying these activities to facilitate repair and regeneration of damaged neural circuits.

In conclusion, this thesis provides important insights into the mechanisms by which netrin1 mediates functions in the developing spinal cord. Now that netrin1 has been shown to be involved in many processes in the vertebrate spinal cord, it would be very exciting to reassess the role of netrin1 in many systems, especially in therapeutic strategies.

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