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Functional characterization of *Netrin-1* and its role in CNS-PNS boundary maintenance in the developing vertebrate brainstem

A dissertation presented

by

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to

The Division of Medical Sciences

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Functional characterization of *Netrin-1* and its role in CNS-PNS boundary maintenance in the developing vertebrate brainstem

Abstract

Netrin-1 (Ntn1) has represented the archetype of diffusible guidance cues since its discovery in the 1990s. It is often depicted in a gradient emanating from the floor plate of the embryonic neural tube, through which it instructs ventral commissural axon guidance. However, Ntn1 can also act permissively and over short-range. The molecular basis for Ntn1's functional versatility is unknown for two major reasons. First, because until recently the only available mutant alleles of *Ntn1* were severely hypomorphic, the full extent of Ntn1's functions *in vivo* remained unclear, as the absence of phenotypes could reflect either the activity of alternative ligands or residual Ntn1. Second, there are few systems where Ntn1's permissive and instructive activities can be clearly distinguished.

To address these challenges, I defined the scope of Ntn1's activities *in vivo* using a new *Ntn1* null allele. By analyzing tissues in *Ntn1* null mice that exhibit phenotypic discrepancies with Ntn1 receptor mutants and *Ntn1* hypomorphs, I showed that low levels of Ntn1 account for persistent commissural attraction to the midline in the hypomorphs. In contrast, Ntn1 is not necessarily the dominant ligand for Unc5 family members *in vivo* and may not play a major role in survival or angiogenesis.

Using the *Ntn1* null mice, I also discovered a new local and permissive role for Ntn1 in the vertebrate brainstem. During development, rhombic lip-derived neurons undertake extensive tangential migrations throughout the hindbrain; many migrate near cranial nerves yet remain in

the CNS. I found that Ntn1 accumulates beneath the pial surface separating the CNS from the PNS, with gaps of protein at nerve roots. Loss of *Ntn1* from the sub-pial region causes hindbrain neurons to enter cranial nerves and enter the periphery; conversely, expression of Ntn1 throughout the mutant hindbrain can prevent their departure. We propose that Ntn1 confines rhombic lip-derived neurons by providing a preferred substrate for tangentially migrating neurons in the SPR, preventing their entry into nerve roots. Since Ntn1 plays distinct roles in the confinement and guidance of rhombic lip neurons, this model system provides an opportunity to dissect the mechanism underlying different modes of Ntn1 function.

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— CHAPTER ONE —

Introduction

“The innumerable processes and intercellular connections offered by the adult nervous system can be interpreted as the morphological expression of the infinite routes traced in space by currents of inducing or positive chemotropic substances during the entire developmental period.”

— Santiago Ramón y Cajal

“Not all those who wander are lost.”

— J.R.R. Tolkien

Today, any textbook model or review of axon guidance includes a diagram summarizing the four classes of guidance cues: long-range and short-range chemoattractants and chemorepellents. This model has provided a useful framework with which to begin understanding the logic and molecular basis of axon guidance; however, it has been 20 years since these categories were formally introduced (Tessier-Lavigne and Goodman, 1996). In the interim, research has shown that many guidance cues act over both long- and short-ranges and as an attractant or a repellent depending on the developmental context. Some also have functions beyond the nervous system. With two decades of research to incorporate, it may be time to consider updating these categories, which originated from classic debates about how axons determine their direction of growth.

In the early 20th century, the founder of modern neurobiology Santiago Ramón y Cajal penned the first description of the distal tip of an axon, or growth cone. Noting its appearance as a “club endowed with exquisite chemical sensitivity,” he accurately predicted that these growth cones enabled axons to roam through tissue, interact with distant targets and ultimately establish the elaborate yet precise connections of the mature nervous system. To explain their movement,

Cajal proposed that growing axons orient toward and grow along gradients of diffusible chemoattractants secreted by their final targets. This "neurotropic hypothesis", however, enjoyed but brief acclaim. It was widely assumed that for an axon to follow a diffusible gradient, it must be able to travel through liquid medium. When axon outgrowth was elicited only in the presence of a solid substrate (Harrison, 1914), contact guidance—the idea that growing axons are oriented by physical patterns in the substrate such as tiny grooves (Weiss, 1934)—became the dominant theory in axon guidance for the next 70 years.

Over time, contact guidance absorbed parts of the neurotropic hypothesis. Physical patterns in the substrate grew to encompass substrate-bound molecules with the discovery of basement membrane (BM) molecules such as laminin and type IV collagen and their ability to orient neurite outgrowth (Ebendal, 1976). Haptotaxis, or movement along a gradient of adhesion (Carter, 1967), explained why neurites preferentially grew along coated substrates to which they adhered to more strongly (Gundersen, 1987; Letourneau, 1975). These adaptations culminated with Sperry's chemoaffinity hypothesis. He proposed that nerve cells and fibers carry chemical identification tags that are selectively attractive to specific populations of growing axons, thereby dictating their trajectory. He was nevertheless careful to mention that while some of these tags may be organized in a gradient, this occurred "without the 'distance action' imputed in some definitions of chemotaxis" (Sperry, 1963).

Almost a full century after Cajal, Lumsden and Davies provided the first modern evidence for the neurotropic hypothesis. Using a newly developed collagen gel matrix co-culture system they showed that in the peripheral nervous system, specific populations of neurons responded to chemoattractants secreted from cells in the target region, but not the path (Lumsden and Davies, 1986), just as Cajal had predicted. Shortly thereafter, Tessier-Lavigne *et al.* used a

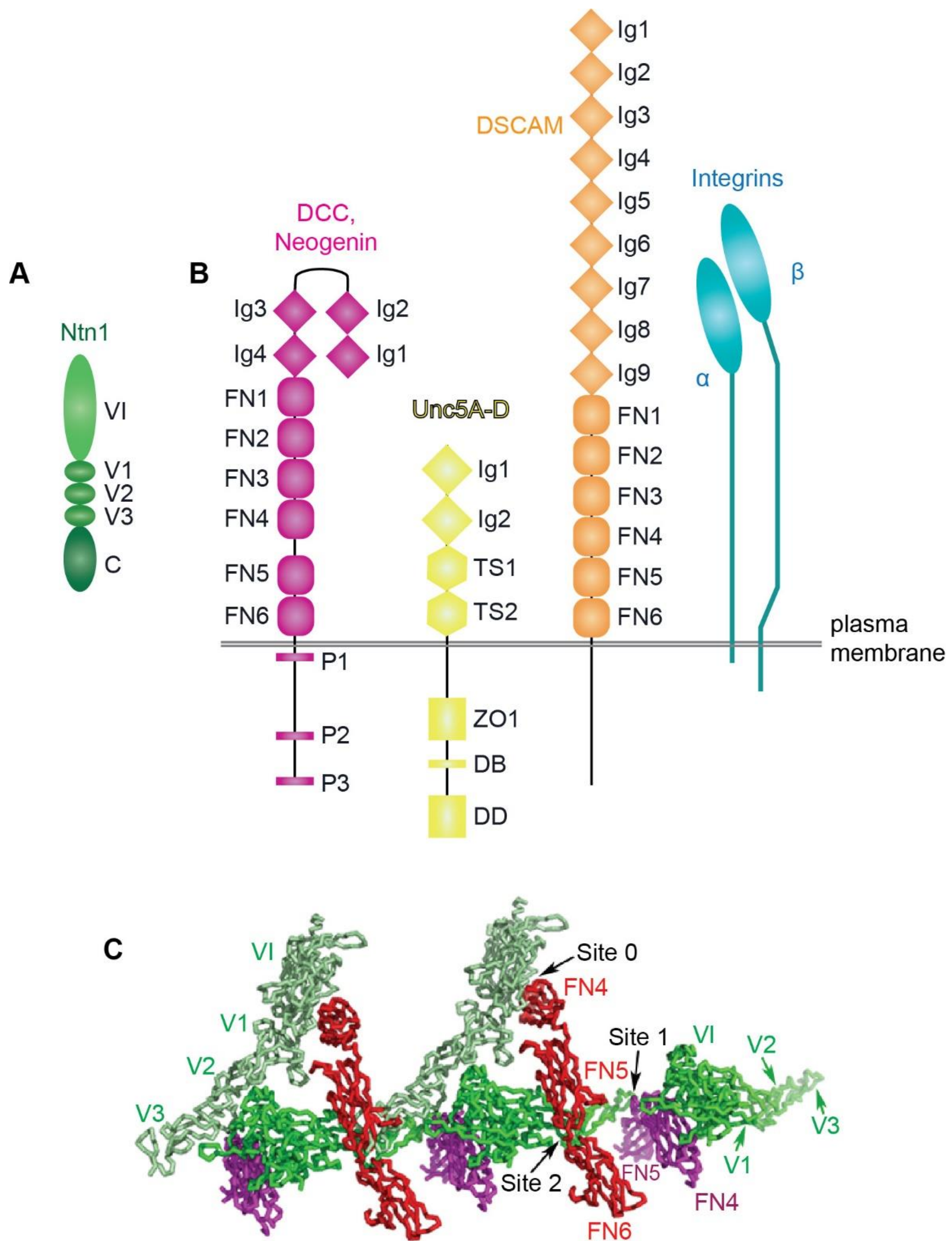
similar co-culture system to demonstrate that intermediate targets in the CNS also support chemoattraction from 100-400 μm away (Tessier-Lavigne et al., 1988). While these studies bolstered support for Cajal's neurotropic hypothesis, it did not become more widely accepted until the discovery of the first vertebrate axon guidance cue, Netrin-1 (Ntn1), which was thought to act in an instructive gradient. Subsequent studies within and beyond the nervous system have revealed a remarkable diversity in both the cellular processes that Ntn1 regulates and its mode of activity. This chapter provides an overview of Ntn1's functions, highlighting its context-dependent effects and the need to uncover the molecular basis for its versatility.

Netrin protein family and structure

As members of the laminin superfamily, the N-terminal domains of all Netrins (Ntns) are homologous to those of laminin, sharing a signal peptide followed by domain VI and three epidermal growth factor (EGF) repeats, which make up domain V (Ishii et al., 1992) (Figure 1A). Ntns have been detected in invertebrates and vertebrates through all levels of bilateria, including the sea anemone *N. vectensis,* one of the earliest examples of a bilaterally symmetric organism (Matus et al., 2006). Homologs of Ntn1 have been reported in *C. elegans* (UNC-6) (Ishii et al., 1992), *D. melanogaster* (NetA and NetB) (Harris et al., 1996; Mitchell et al., 1996), *D. rerio* (Lauderdale et al., 1997; Strähle et al., 1997), *X. laevis* (de la Torre et al., 1997)*, G. gallus* (Kennedy et al., 1994; Serafini et al., 1994)*,* and *M. musculus* (Serafini et al., 1996). There are five secreted Ntns in vertebrates, in addition to NtnG1 and 2 (also called laminet-1 and -2), which are tethered to the plasma membrane by C-terminal glycosylphosphatidyl (GPI) tails (Nakashiba et al., 2000, 2002; Yin et al., 2002). Mammals express Ntn1 (Serafini et al., 1996), Ntn3-5 (Wang et al., 1999; Yamagishi et al., 2015; Yin et al., 2000), and both tethered Ntns

Figure 1. Structure of Ntn1 and its receptors. (A) Ntn1 protein is ~600 amino acids in length. Its N-terminus consists of domain VI and the three EGF-repeats of domain V, which are also found in the N-terminus of laminins. Ntn1's C-terminus does not share homology with laminins. (B) Ntn1's receptors include the orthologs DCC/Neogenin (pink), members of the Unc5 family (yellow), DSCAM (orange), and integrins (blue). DSCAM's intracellular domain is unlabeled because there are no identifiable motifs (Yamakawa et al., 1998), and for simplicity, only the α and β subunits of integrins are shown. Ig, immunoglobulin domain; FN, fibronectin type III domain, TS, thrombospondin type I domain; P1-3, conserved regions of DCC's cytoplasmic tail; ZO1, zona occludens-1 domain; DB, DCC-binding domain; DD, death domain. (C) When all three Ntn1-DCC binding sites are occupied (sites 0, 1, and 2), Ntn1 (green) and DCC (red and purple) can oligomerize and form a cluster of ligand-receptor complexes. The crystal structure was adapted from (Finci et al., 2015) with permission.

Figure 1 (Continued)



(Nakashiba et al., 2000, 2002; Yin et al., 2002). Ntn2 is exclusive to zebrafish (Park et al., 2005) and chickens (Serafini et al., 1994), though Ntn2 and 3 were recently proposed to be the same gene (Friocourt et al., 2017). The N-terminal domains of Ntn1-3 share greater homology with the gamma subunit of laminin (Serafini et al., 1994; Wang et al., 1999), whereas those of Ntn4 (Koch et al., 2000; Yin et al., 2000) and NtnG1-2 (Nakashiba et al., 2000, 2002; Yin et al., 2002) are more similar to laminin's beta subunit. Only Ntn4 can bind to laminins, which polymerize with one another, and this occurs via a site in the N-terminus that is not conserved in Ntn1 (Reuten et al., 2016; Schneiders et al., 2007).

The C-terminus of Ntn1 bears homology to domains found in several proteins with diverse functions, including complement proteins, secreted frizzled-related proteins, type I C-proteinase enhancer proteins (PCOLCEs), and tissue inhibitors of metalloproteinases (TIMPs) (Bányai and Patthy, 1999). However, its function remains unclear, as it does not appear to be required for axon guidance (Lim and Wadsworth, 2002; Lim et al., 1999). Because the C-terminus contains many basic amino acids and can bind to heparin (Kappler et al., 2000), it is predicted to permit interactions with cell surfaces covered in charged sugars associated with proteoglycans. In summary, Ntn1 is a laminin-like molecule that is associated with cell surfaces, but it is distinguishable from laminins due to its charged C-terminus.

In an open relationship: Ntn1's receptors

Ntn1 is a highly promiscuous molecule, interacting with a plethora of receptors. With the exception of integrins, all Ntn1 receptors are single-pass type I transmembrane proteins in the immunoglobulin (Ig) family, including UNC-40 and UNC-5, which were the first two receptors

identified along with UNC-6 in a *C. elegans* screen for axon guidance molecules (Hedgecock et al., 1990).

UNC-40, or its fly and vertebrate homologs Frazzled (Fra) (Kolodziej et al., 1996) and Deleted in Colorectal Carcinoma (DCC) (Keino-Masu et al., 1996) respectively, comprises four Ig domains, six fibronectin (FN) type III domains, a transmembrane domain, and a large cytoplasmic tail that contains three conserved motifs called P1-3 (Chan et al., 1996; Kolodziej et al., 1996) (Figure 1B). DCC mediates both chemoattractive and chemorepulsive responses to Ntn1, and Ntn1-DCC interactions take place over three sites. Site 1 involves Ntn1's EGF-3 domain and DCC's FN5 domain. Site 2 involves Ntn1's EGF-1/2 domains and the FN5-6 domain of DCC. These sites are non-overlapping, allowing a single Ntn1 molecule to bind to two DCC receptors (Finci et al., 2014; Xu et al., 2014). An additional interaction at site 0 between Ntn1's domain VI and DCC's FN4 enables individual signaling units to cluster (Finci et al., 2014; Xu et al., 2014) (Figure 1C). By oligomerizing DCC at the cell surface, Ntn1 brings DCC's unstructured cytoplasmic tails into close proximity with each another. The dimerization of DCC's P3 domains (Stein et al., 2001) recruits Focal Adhesion Kinase (FAK) to the cell membrane (Xu et al., 2018), where PIP2 and Src family kinases can complete its activation (Goñi et al., 2014). The activation of both FAK and Src are required for chemoattractive responses to Ntn1 (Li et al., 2004; Meriane et al., 2004; Ren et al., 2004) and ultimately trigger cytoskeleton remodeling, thereby mediating guidance and migratory behaviors in response to Ntn1. Unlike site 0 and 1, site 2 on Ntn1 is not specific for DCC because it does not involve direct protein-protein interactions with DCC. This means that any receptor with an interface complementary to Ntn1's could replace DCC (Finci et al., 2014), changing the composition of the Ntn1-receptor complex and, as a consequence, downstream signaling and function.

UNC-5 (Leung-Hagesteijn et al., 1992) and its homologs are one set of alternate Ntn1 co-receptors. One fly (Keleman and Dickson, 2001) and four vertebrate homologs of UNC-5 (Unc5A-D) have been identified (Ackerman et al., 1997; Leonardo et al., 1997), each of which harbor two Ig domains, two thromospondin-1 (TSP1) domains, a transmembrane domain, and a cytoplasmic tail that includes a zona occludens-1 domain, a DCC-binding (DB) domain, and a death domain (Ackerman et al., 1997; Hofmann and Tschopp, 1995; Hong et al., 1999; Leonardo et al., 1997; Schultz et al., 1998) (Figure 1B). Loss of *Unc5* or its homologs in worms (Hedgecock et al., 1990) and mice (Burgess et al., 2006) impairs dorsal migrations, suggestive of a role in Ntn1-mediated repulsion. Unc5 typically relies on both DCC and Ntn1 to signal (Colavita and Culotti, 1998; Hong et al., 1999). In the absence of Ntn1, Unc5 and DCC do not interact (Hong et al., 1999). By binding to DCC and the Ig domains of Unc5 (Geisbrecht et al., 2003; Grandin et al., 2016), Ntn1 allows their cytoplasmic tails to interact at the P1 and DB domains respectively, thereby forming a signaling heterodimer unit and converting an attractive response to a repulsive one (Hong et al., 1999). In some cases, signaling by Unc5 alone is sufficient to induce a repulsive response. For example, pan-neuronal expression of *Unc5* prevents commissural axons from crossing the midline in the absence of *fra* (Keleman and Dickson, 2001), and UNC5C+ medial lateral motor column axons are still repelled by Ntn1 in the absence of *DCC* (Poliak et al., 2015). Src family kinases are required for Ntn1-Unc5-mediated chemorepulsion in either case (Lee et al., 2005).

Curiously, despite the conserved role of Ntn1, *DCC* is not present in all species, having been lost in galliformes (Friocourt et al., 2017; Patthey et al., 2017), the avian group that includes the chicken (*Gallus gallus*). With ~50% amino acid identity, the DCC ortholog Neogenin is highly structurally similar (Figure 1B), sharing Ntn1 binding sites 0 and 1 (Xu et al.,

2014), and it acts in place of *DCC* in chick commissural axon guidance (Phan et al., 2011). Neogenin also collaborates with DCC to mediate chemoattractive responses to Ntn1 in the mouse neural tube (Xu et al., 2014), where presumably it can act either as a homodimer or a heterodimer with DCC.

Other proposed Ntn1 receptors act independently of DCC or DCC-like receptors. Down Syndrome Cell Adhesion Molecule (DSCAM) (Figure 1B) is required for commissural axon guidance in *Drosophila* (Andrews et al., 2008) and chickens (Liu et al., 2009) independently of DCC (Ly et al., 2008). However, loss of *DSCAM* does not affect commissural axon guidance in mice (Palmesino et al., 2012), raising the possibility that it may act as a Ntn1 receptor only in certain species or conditions. The C-terminus of Ntn1 can also bind to integrins $\alpha6\beta4$ and $\alpha3\beta1$ (Stanco et al., 2009; Yebra et al., 2003) (Figure 1B). Additional receptors likely remain to be found, particularly since known receptors do not account for all of Ntn1's functions (Nishitani et al., 2017). Another member of the Ig superfamily, CD146, was in fact recently identified as a receptor mediating Ntn1's pro-angiogenic effects (Tu et al., 2015). Still, the diversity of Ntn1's functions cannot be explained by its numerous receptors, as many are used across multiple contexts.

Ntn1 functions: the long and short of it

<u>Free-range: Ntn1's long-range functions</u>

As the first diffusible axon guidance cue discovered, Ntn1 is best known for its role in long-range chemoattraction and chemorepulsion. The founding member of the Netrin family, UNC-6, was uncovered in a *C. elegans* screen for axon guidance cues, and loss of *unc-6* impaired both ventral and dorsal circumferential guidance (Hedgecock et al., 1990), indicating

that UNC-6 can act as both a chemoattractant and a chemorepellent. Due to its structural similarities with laminin, UNC-6 was originally thought to mediate guidance by providing "a favored substrate for pioneering growth cones" (Ishii et al., 1992). Circumferential or commissural axon growth in vertebrates, however, appeared to be independent of path-derived cues. In embryonic mice and rats, commissural neurons reside in the dorsal neural tube and initially project axons along the lateral edge of the neural tube. At the motor columns, the axons adopt a ventromedial trajectory to reach the floor plate, an intermediate target at the ventral midline, where they cross and turn rostrally. Floor plate explants elicited two types of responses from dorsal spinal cord explants. When cultured opposite the ventral edge of the latter, floor plate explants induced rampant commissural outgrowth from up to 400 µm away (Tessier-Lavigne et al., 1988). When floor plate explants were placed up to 220 µm away from the lateral edges of dorsal spinal cord explants, commissural axons turned toward the floor plate instead of projecting ventrally (Placzek et al., 1990; Tessier-Lavigne et al., 1988). Ventral spinal cord explants also promoted commissural axon growth and turning, but the effect was relatively weak and limited to a distance of 10 µm (Placzek et al., 1990). Together, these results suggested that the floor plate secretes a potent chemoattractant that diffuses to the ventral spinal cord in limited amounts.

A single, highly conserved protein accounted for the dual activities of the floor plate *in vitro* and *in vivo*. The chicken homolog of UNC-6, chicken Ntn1 (cNtn1) is expressed at the floor plate and capable of both inducing and orienting commissural axon growth *in vitro*. cNtn2, which was purified at the same time as cNtn1, displayed the same activities but is expressed in the ventricular zone (Kennedy et al., 1994; Serafini et al., 1994). Reducing or eliminating *Ntn1* and its homologs in flies (Brankatschk and Dickson, 2006; Harris et al., 1996; Mitchell et al.,

1996) and zebrafish (Lauderdale et al., 1997) prevented commissural axons from reaching and crossing the midline, and hypomorphic *Ntn1* mice (*Ntn1$^{trap/trap}$*) created by gene-trapping technology (Serafini et al., 1996; Skarnes et al., 1995) exhibited a similar phenotype in the embryonic neural tube. A small subset of commissural axons, however, still reached the midline. This led to speculation that either residual Ntn1 or redundant cues mediate commissural axon guidance, or a subset of commissural axons do not require Ntn1 to cross (Charron et al., 2003; Ruiz de Almodovar et al., 2011; Serafini et al., 1996) (addressed in **Chapter 2**). Other neuronal populations that migrate or project to the ventral midline also displayed pathfinding defects. *Ntn1$^{trap/trap}$* animals are missing the corpus callosum, the anterior and hippocampal commissures (Serafini et al., 1996), pontine nuclei (Serafini et al., 1996; Yee et al., 1999), and the ventral acoustic stria (Howell et al., 2007) and have diminished inferior olivary nuclei and lateral reticular nuclei (Bloch-Gallego et al., 1999; Marcos et al., 2009). Since both floor plate explants and Ntn1-secreting cells attracted migrating precerebellar neurons and their processes *ex vivo* (Alcantara et al., 2000; Diego et al., 2002; Yee et al., 1999), it was predicted that, as in commissural axon guidance, a floor plate-derived gradient of Ntn1 instructed the ventral migration of these neurons. Altogether, these studies demonstrated the importance of floor plate-derived Ntn1 in long-range chemoattraction and nervous system development.

Ntn1 also retains its chemoattractive functions beyond the floor plate. As examples, lot cells, a population of cortical neurons that line the presumptive lateral olfactory tract, are attracted to Ntn1 in the olfactory tubercle (Kawasaki et al., 2006), and gonadotropin-releasing hormone neurons depend on Ntn1 to migrate to the basal forebrain (Murakami et al., 2010; Schwarting et al., 2004). Ntn1 expressed in the ganglionic eminence, an intermediate stop for corticothalamic and thalamocortical axons, promotes and orients the growth of cortical efferent

axons *ex vivo* (Metin et al., 1997; Richards et al., 1997) and organizes the topography of thalamocortical axons through a combination of attraction and repulsion (Powell et al., 2008).

Indeed, consistent with defects in dorsal migration in *unc-6* worm mutants (Hedgecock et al., 1990), Ntn1 can also act as a long-range chemorepellent. Floor plate explants and cells secreting Ntn1 deflected or suppressed axon outgrowth from cranial motor neuron explants, such as those containing trochlear motor neurons, which project dorsally away from the floor plate (Colamarino and Tessier-Lavigne, 1995; Shirasaki et al., 1996; Varela-Echavarría et al., 1997). Oligodendrocyte precursors (OPCs) cultured with Ntn1 display decreased surface area and process length and number, as if retracting from a repulsive cue (Jarjour et al., 2003). Complementarily, when Ntn1 levels are reduced *in vivo*, OPCs originating from the ventricular zone fail to disperse throughout the spinal cord, instead accumulating ventrally (Jarjour et al., 2003), or in the case of injected OPCs, remaining near the injection site (Tsai et al., 2006). Ntn1 expressed in the striatal ventricular zone also repels late-born striatal neurons into the post-mitotic region *ex vivo* (Hamasaki et al., 2001).

As a result of all of these findings, Ntn1 was embraced as the archetype of diffusible guidance cues. Its long-range chemoattractive functions are summarized in textbook figures illustrating commissural axons growing up a floor plate-derived gradient of Ntn1 (Figure 2A). However, these models overlook some key results. First, *in vivo,* Ntn1 expression is not a point source. Although cNtn1 is restricted to the floor plate, cNtn2—which also evokes commissural axon outgrowth—is expressed throughout the ventricular zone (Kennedy et al., 1994). The combined expression pattern of the two cNtns is similar to Ntn1 expression in the mouse (Serafini et al., 1996). It has also proved difficult to detect a smooth gradient of Ntn1 extending throughout the ventral half of the spinal cord *in vivo.* Instead, Ntn1 immunoreactivity is enriched

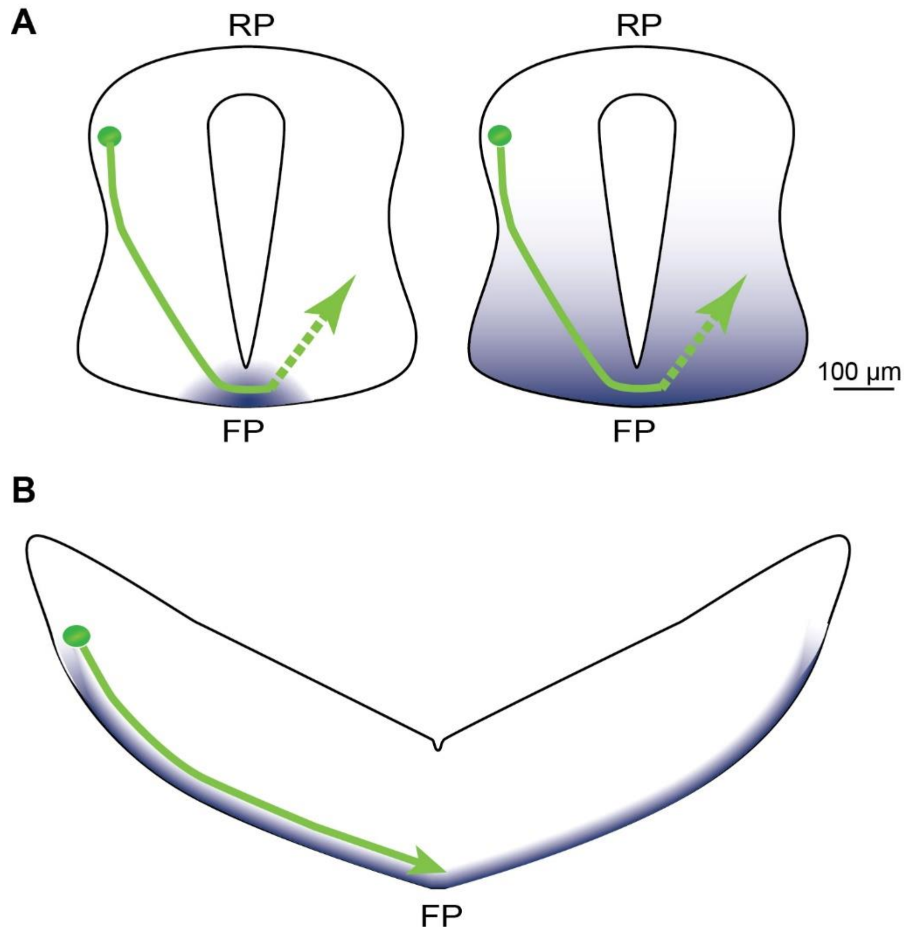


Figure 2. Popular models of Ntn1-mediated chemoattraction to the ventral midline. (A-B) The trajectories of commissural neurons (green) are shown relative to Ntn1 (purple) in the developing spinal cord (A) and hindbrain (B). (A) Classic illustrations of Ntn1-mediated long-range attraction in the developing spinal cord depict commissural axons traveling up a gradient of Ntn1 protein at the floor plate (left) or extending throughout the ventral half of the spinal cord (right). Neither model reflects the distribution of Ntn1 detected via immunohistochemistry. (B) Current models favor Ntn1 as a preferred substrate. Unlike commissural neurons in the spinal cord, commissural axons in the hindbrain travel along the Ntn1-enriched sub-pial surface for the entirety of their journey to the floor plate. FP, floor plate; RP, roof plate.

in the sub-pial surface, though gradients of protein extending 250 μm have been found on commissural axons and along their trajectory in the neuroepithelium (Dominici et al., 2017; Kennedy et al., 2006; MacLennan et al., 1997; Varadarajan et al., 2017; Yamauchi et al., 2017). In a similar vein, the membrane fraction, but not the soluble fraction, of floor plate homogenates elicited commissural axon outgrowth from dorsal spinal cord (Serafini et al., 1994). While these results do not rule out the possibility of a free-standing diffusible gradient of Ntn1, they suggest that within the embryonic spinal cord, Ntn1 may act in a substrate-bound gradient.

These discrepancies ultimately led to recent genetic studies that questioned the role of floor plate-derived Ntn1 in commissural axon guidance. In both the hindbrain and the spinal cord, commissural axon guidance remained grossly intact after deleting *Ntn1* from the floor plate. In contrast, eliminating *Ntn1* from the ventricular zone prevented commissural axons from orienting and growing toward the ventral midline, with few axons reaching the floor plate (Dominici et al., 2017; Varadarajan et al., 2017; Yamauchi et al., 2017). Consequently, these studies concluded that floor plate-derived Ntn1, or a long-range gradient of Ntn1, is dispensable for commissural axon guidance. Since loss of *Ntn1* only from the dorsal ventricular zone impaired commissural axon guidance (Varadarajan et al., 2017; Yamauchi et al., 2017) and since the deletion of *Ntn1* from the VZ selectively eliminated Ntn1 protein from the pial surface (Dominici et al., 2017; Varadarajan et al., 2017), these studies instead proposed that Ntn1 primarily acts locally as a growth substrate for commissural axons (Figure 2B), in agreement with the discoverers of UNC-6 (Ishii et al., 1992).

There have been many other hints that a long-range instructive role is not representative of Ntn1's functions. The expression of UNC-6 by guidepost and pioneering neurons in *C. elegans*, for example, suggested that Ntn1 might delineate a favorable path for follower axons

(Wadsworth et al., 1996). At the *Drosophila* midline, tethered Ntns rescued defects in commissural axon guidance and prevented commissural crossing when *Unc5* was expressed in all neurons (Brankatschk and Dickson, 2006), indicating that both attractive and repulsive functions for Ntn1 are active at short-range. In fact, since Ntn1's discovery, many studies have revealed a remarkable versatility in both the cellular processes that Ntn1 regulates and its mode of activity. Because canonical models of Ntn1 function in axon guidance have emphasized its instructive activity in a gradient, Ntn1's roles in these other contexts stand out for their local and/or permissive functions.

<u>Locally sourced: Ntn1's short-range functions</u>

The functional consequences of local Ntn1 signaling are both highly diverse and context-dependent. One of the first pieces of evidence to suggest that there are roles for Ntn1 apart from long-range chemoattraction or repulsion came from a study of retinal ganglion cell (RGC) axon guidance. In the murine retina, *Ntn1*is expressed by glial cells in the optic disc surrounding the optic nerve, through which RGC axons exit to enter the brain. In *Ntn1*$^{trap/trap}$ animals, DCC+ RGC axons reach the optic disc but splay out and stall, resulting in optic nerve hypoplasia. Given that Ntn1 protein accumulation was restricted to the optic disc and because RGC axons were still able to navigate to the exit, Ntn1 is likely acting locally to funnel axons into the nerve (Deiner et al., 1997). In this case, Ntn1's short-range function depends on its localization at its source. This is the basis of Ntn1's many other short-range functions. For instance, Ntn1 secreted by the lateral habenula is required to permit the entry of DCC+ dopaminergic neurons that otherwise accumulate at its border (Schmidt et al., 2014). In worms and flies, local UNC-6 and Ntn production respectively promote synaptogenesis by facilitating target recognition (Park et al.,

2011; Winberg et al., 1998) and specifying sites of presynaptic assembly (Colón-Ramos et al., 2007; Poon et al., 2008).

Short-range functions of Ntn1 also emerge when Ntn1 is trafficked away from its source, giving rise to a discrete region of highly concentrated Ntn1 protein. Ntn1 frequently accumulates at the BM from which it directs tissue morphogenesis. During vulval development in the worm, a specialized uterine cell, an anchor cell, crosses the BM separating the uterus and the vulva to connect the two reproductive organs and permit egg-laying. The accumulation of UNC-6—derived from the ventral nerve cord—at the BM recruits and stabilizes clusters of UNC-40 at the ventral edge of the anchor cell, thereby driving polarization and the formation of an invasive membrane (Ziel et al., 2009). Similarly, in the developing lung, proximal epithelial cells deposit Ntn1 and Ntn4 into the overlying BM, which likely signals through DCC and Unc5B to restrict ectopic budding and limit morphogenesis to the tips of the growing lung (Liu et al., 2004). While it is unclear how Ntn1 localizes to the BM, its high affinity for type IV collagen (Yebra et al., 2003), a major component of the extracellular matrix, raises the possibility that Ntn1 may accumulate there while diffusing through extracellular space.

Ntn1's distribution is also actively controlled by its receptors. PVD dendritic tiling requires UNC-6 secreted from the ventral nerve cord, but this is independent of a gradient since delocalized expression of UNC-6 rescued dendritic self-avoidance in *unc-6* null worms. Instead, UNC-40 captures UNC-6 and presents it to UNC-5 to mediate contact-dependent repulsion between individual PVD dendrites, thereby converting a diffusible cue into a local signal (Smith et al., 2012). Fra can also capture and redistribute Ntns. In the fly ventral nerve cord, Fra generates a NetAB-rich region that dictates the guidance of adjacent pioneering dMP2 axons (Hiramoto et al., 2000). Likewise, in the visual system, the local release and capture of Ntns by

L3 growth cones and Fra+ neurons respectively concentrates NetAB in layer M3 of the medulla, where Fra+ R8 photoreceptor axons terminate (Timofeev et al., 2012). Thus, multiple mechanisms to sequester Ntn1 exist across different species, further underscoring the importance of and diversity in Ntn1's short-range functions *in vivo*.

Proceed: Ntn1's permissive functions

Some of Ntn1's functions also stand out for their permissive nature. Because a Ntn1 gradient can direct axon outgrowth, Ntn1 is typically thought of as an instructive cue, but it can also enable axonal growth or cell movement without providing directional information, thereby acting as a permissive cue. For example, NetAB maintains, but does not direct, R8 axons in M3 (Akin and Zipursky, 2016). In the developing mammary gland, Ntn1-Neogenin interactions prevent the detachment of the proliferating cap cell layer from the adjacent prelumenal cell layer (Srinivasan et al., 2003); likewise, Fra is required for adhesion between cardioblasts during *Drosophila* vessel morphogenesis (Macabenta et al., 2013). The accumulation of Ntn1 in the BM also provides an adhesive substrate that facilitates migration of epithelial cells (Yebra et al., 2003). Additionally, altering Ntn1 levels modulates process extension and myelin-like sheet formation in DCC+ oligodendrocytes in the spinal cord (Rajasekharan et al., 2009), as well as axon branching and the assembly and stabilizing of presynaptic sites *in vitro* (Dent et al., 2004; Goldman et al., 2013; Tang and Kalil, 2005) and *in vivo* (Manitt et al., 2009). Thus, Ntn1's permissive roles regulate many aspects of development, though it primarily mediates adhesion.

Importantly, none of Ntn1's modes of behavior are mutually exclusive. At the fly midline, NetAB act over both long- and short-range to mediate repulsion in different neuronal populations (Brankatschk and Dickson, 2006; Keleman and Dickson, 2001). In worms, UNC-6

plays dual functions in the development of a single neuron. HSN neurons polarize ventrally, which restricts subsequent neurite formation to the ventral side and ensures axon outgrowth in the correct direction. In the absence of *unc-6,* HSN neurons fail to form a leading edge, forming neurites in all directions prior to axon specification (Adler et al., 2006). Expressing *unc-6* throughout the animal using a heat-shock promoter induced HSN asymmetry without rescuing axon guidance, which likely depends on a gradient of UNC-6 from the ventral nerve cord (Adler et al., 2006). In summary, while Ntn1 enables HSN neuron polarization as a permissive cue, Ntn1 directs ventral HSN axon guidance as an instructive cue. Developmental context therefore plays a direct role in sculpting the functional outcome to Ntn1 signaling.

A triple-threat: Ntn1's multi-functionality in cancer

Given the critical role Ntn1 plays in the development of many organ systems, it is no surprise that Ntn1 signaling also contributes to disease. Ntn1 and its receptors have been implicated in many cancers; in fact, DCC was originally identified as a tumor suppressor gene in colorectal cancer. Many cancers downregulate DCC and Unc5 family members (reviewed in Arakawa, 2004); conversely, increased Ntn1 expression has been detected in brain, breast, colorectal, liver, lung, and pancreatic cancers (reviewed in Ylivinkka et al., 2016). In addition to promoting cancer cell proliferation (Chen et al., 2017; Delloye-Bourgeois et al., 2012; Huang et al., 2014; Lee et al., 2007; Qi et al., 2015; Wang et al., 2009; Yin et al., 2017), Ntn1 is associated with greater invasiveness and metastatic capability (reviewed in Ylivinkka et al., 2016).

Ntn1 may also confer a survival advantage for cancer cells. As dependence receptors, DCC and Unc5 receptors can activate caspase-3 and induce apoptosis in the absence of Ntn1. Ntn1 binding blocks initiation of the apoptotic pathway and activates an anti-apoptotic signal

instead (Ma et al., 2010). Consistent with this model, Ntn1 knock-down led to increased cell death *in vivo* (Fitamant et al., 2008) and *in vitro* (Delloye-Bourgeois et al., 2009, 2009), whereas overexpression *in vivo* inhibited apoptosis and enhanced tumor growth (Mazelin et al., 2004). As with many of its other functions, Ntn1's role in survival varies with context and species: although NetB is required for the survival of commissural neurons in the fly (Newquist et al., 2013) and overexpression of Ntn1 inhibits apoptosis in the chick otic vesicle (Nishitani et al., 2017), *Ntn1$^{trap/trap}$* mice do not display increased cell death (Williams et al., 2006).

Cancer cells may also benefit from Ntn1's Unc5B-dependent pro-angiogenic roles. *Ntn1* and *Unc5b* knockdown, for example, prevented the formation of a major longitudinal vessel in zebrafish (Navankasattusas et al., 2008; Wilson et al., 2006). Injections of Ntn1 or Ntn1 function-blocking antibodies increased and reduced blood vessel density in the mouse brain respectively (Cayre et al., 2013), and local delivery of Ntn1 restored blood flow following hindleg ischemia, concomitant with increased capillary density (Wilson et al., 2006). In addition to signaling directly to endothelial cells, Ntn1 released by tumor cells could engage a pro-angiogenic program in the surrounding immune cells (Binet et al., 2013). Conversely, Ntn1-Unc5B signaling can also inhibit vessel sprouting in mice (Lu et al., 2004), chick (Bouvrée et al., 2008), and zebrafish (Lu et al., 2004). The pro- or anti-angiogenic state of Ntn1 may depend on its concentration (Yang et al., 2007), its survival effect on endothelial cells (Castets et al., 2009), and context, since responses to Ntn1 vary by vascular bed.

These examples showcase Ntn1's remarkable versatility and suggest that its long-range instructive role in the developing nervous system may be the exception rather than the rule. They also raise an important question: how does a single molecule mediate such divergent functions?

A model that offers a unifying explanation for all of Ntn1's functions may prove difficult to test, particularly in axon guidance—the ideal model system would offer a read-out for Ntn1's instructive versus permissive functions, but the absence of a permissive or instructive attractive cue can both result in axon stalling.

To address this impasse, my thesis defines the full extent of Ntn1's functions *in vivo* using a newly generated mouse that is completely null for *Ntn1* (**Chapter 2**). Using this null mouse, I identify a local and permissive role for Ntn1 in the maintenance of the CNS-PNS boundary in the vertebrate brainstem that can be clearly distinguished from its effects in guidance (**Chapter 3**). In the future, this model system offers an opportunity to investigate how a single population of neurons switches between permissive and instructive responses to Ntn1.

— CHAPTER TWO —

Phenotypic analysis of mice lacking Netrin-1

Andrea R. Yung, Allison M. Nishitani, and Lisa V. Goodrich.

A.M.N created the floxed and null alleles of Ntn1 (Figure 3), and A.R.Y performed and analyzed all subsequent experiments. This chapter was reproduced with permission from *Development* 142 (2015), pp. 3686-91, doi: 10.1242/dev.128942.

Introduction

Netrin-1 (Ntn1) is a secreted molecule in the laminin superfamily (Ishii et al., 1992) best known for its role in axon guidance (Serafini et al., 1996), with additional roles in adhesion (Srinivasan et al., 2003; Yebra et al., 2003), angiogenesis (Lu et al., 2004), and survival (Mazelin et al., 2004). To mediate these diverse functions, Ntn1 signals through multiple receptors, including Deleted in Colorectal Cancer (DCC) (Fazeli et al., 1997), Neogenin (Srinivasan et al., 2003), Unc5 family members (Leonardo et al., 1997), and integrins (Yebra et al., 2003). However, as shown by RT-PCR and *in situ* hybridization, the most commonly studied *Ntn1* mutant is a severe hypomorph that does not exhibit all of the phenotypes predicted by *in vitro* assays and phenotypic analyses of Ntn1 receptor mutants (Lu et al., 2004; Serafini et al., 1996; Williams et al., 2006). Another gene trap allele is also available, but likely to suffer the same issues as the original line (Salminen et al., 2000). Thus, even after twenty years of active research, it is unclear whether the absence of predicted defects is due to redundant cues or residual Ntn1, raising questions about Ntn1's full contributions to development *in vivo*.

To resolve lingering questions regarding the broad functions of Ntn1 *in vivo*, we created a null allele of *Ntn1*. Phenotypic analysis of this improved mouse model confirmed a primary role for Ntn1 during midline guidance of commissural axons, but not for all Unc5-mediated effects on repulsion, neuronal survival, or blood vessel branching.

Experimental Procedures

Animals. The *Ntn1* gene trap line was previously reported (Serafini et al., 1996) and has been backcrossed to C57Bl6 animals for >10 generations. Noon on the day of the plug was considered embryonic day 0.5 (E0.5). All animal work was conducted in compliance with protocols approved by the Institutional Animal Care and Use Committee at Harvard Medical School.

Immunoblotting. E11.5 heads were lysed in 50 mM Tris pH7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1X Pefabloc SC PLUS protease inhibitor (Roche). Primary antibodies used include goat anti-Boc (1:1000, R&D), goat anti-DCC (1:1000, Santa Cruz), rabbit anti-Flk1 (1:2000, Cell Signaling), goat anti-Neogenin1 (1:1000, R&D) and rat anti-Ntn1 (1:500, R&D). Each blot was performed twice using independent lysates.

In situ *hybridization.* *In situ* hybridization on 20 μm frozen sections as described (Lu et al., 2011).

Immunochemistry. For sections, embryos were fixed in 4% paraformaldehyde (PFA)/PBS at 4°C overnight, cryoprotected in sucrose, embedded in Neg50, and cryosectioned at 12 or 20 μm. Primary antibodies used were mouse anti-Islet1/2 (1:100, DSHB), anti-neurofilament (1:1000, DSHB), goat anti-Robo3 (1:100, R&D), and goat anti-TAG1 (1:1000, R&D).

For wholemounts, embryos were fixed in 4% PFA/PBS at 4°C overnight, dehydrated in methanol, incubated in Dent's bleach and fixative, and rehydrated in PBS. Samples were blocked overnight (20% DMSO, 5% normal goat serum, 0.3% Triton-X, and 0.025% sodium azide in PBS), incubated with mouse anti-neurofilament for 5 days at 4°C, washed in blocking solution, and incubated with secondary antibody at room temperature for 2 days. Embryos were cleared in

BABB. Staining of open-book spinal cord samples followed the same protocol, but after fixation the tissue was placed directly in blocking solution.

Spinal cord sections and wholemount embryos were imaged on an Olympus FV1000 confocal microscope using 10X, 0.40 numerical aperture (NA) or 20X, 0.75 NA dry objectives and with optimal step sizes in the *z*-axis (1.16 µm steps for 20X and 4.27 µm steps for 10X). Open book wholemounts were imaged on a Leica SP8 X confocal microscope with a 20X, 0.70 NA objective. Quantification was performed using ImageJ, where coverage denotes the percentage of a standardized area covered by either Robo3+ or PECAM+ pixels; two independent measurements were taken per open book. To measure the distance between the trochlear nucleus and the midline, a straight line was drawn from the center of the nucleus to the IVth ventricle. All statistical analyses were performed with Prism 4 (GraphPad software); all data are presented as means±S.D.

Results and Discussion

Wild-type Ntn1 protein persists in Ntn1$^{trap/trap}$ *mice, but is absent from* Ntn1$^{-/-}$ *mice*

Western blot analysis of E11.5 head lysate with an antibody targeted to Ntn1 domain VI (Bin et al., 2013) revealed that residual wild-type protein (~75 kDa) persists in *Ntn1* gene trap (*Ntn1*$^{trap/trap}$; n=2) animals, confirming this allele is hypomorphic. To generate a null mouse, we inserted *loxP* sites around the second exon of *Ntn1*, which encodes the start codon and most of the N-terminus, which when fused to Fc is sufficient for axon outgrowth *in vitro* (Keino-Masu et al., 1996; Lim and Wadsworth, 2002). We crossed this floxed *Ntn1* allele to the germline-specific Cre line *EIIa*^{Cre} to delete exon 2 from subsequent generations (Figure 3A). In contrast to gene trap mutants, no Ntn1 protein was detected in *Ntn1*$^{-/-}$ animals (Figure 3B; n=4), which die neonatally without any gross malformations: E18.5 null embryos were present in Mendelian ratios (33/121 embryos), but no *Ntn1*$^{-/-}$ pups (out of 51) were observed at P5.

Ntn1 is the major cue for midline attraction

As a chemoattractant, Ntn1 acts through DCC and Neogenin (Xu et al., 2014) to promote the growth and guidance of dorsally located commissural neurons toward the ventral floor plate (Serafini et al., 1996). Although many commissural axons mis-project to the ventricular zone and the motor columns in *Ntn1* hypomorphs, a subset of axons still orient toward and reach the floor plate. These observations led many groups to look for additional floor plate-derived cues, resulting in the discovery that VEGF (Ruiz de Almodovar et al., 2011) and Sonic Hedgehog (Shh) (Charron et al., 2003) also function as chemoattractants. Unfortunately, the persistence of Ntn1 in *Ntn1*$^{trap/trap}$ mice makes it difficult to distinguish the contributions of these cues from those of Ntn1 during nervous system wiring.

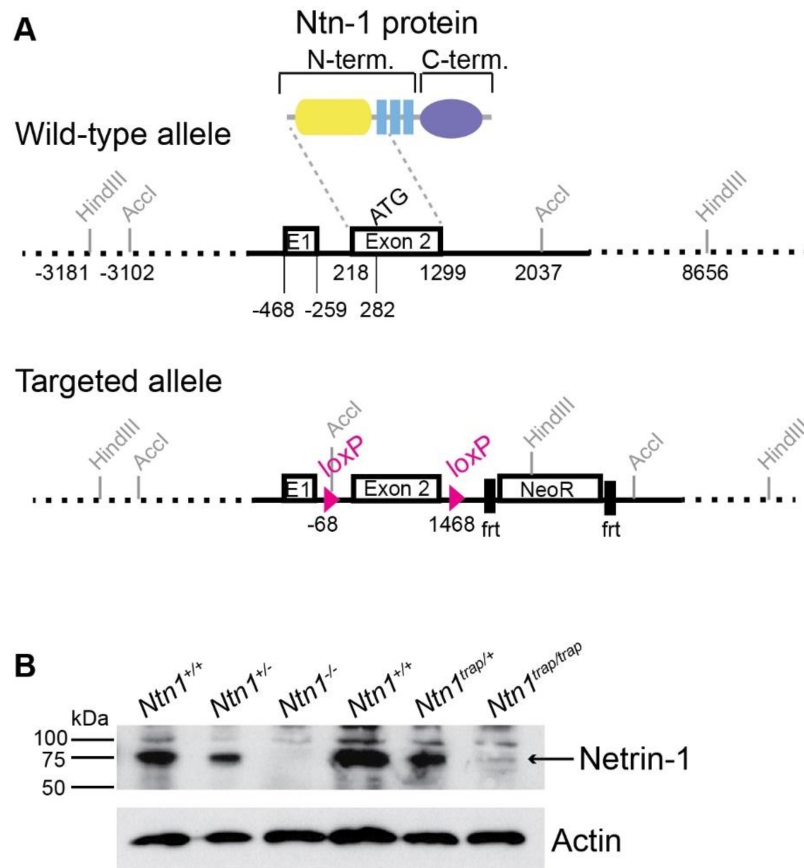


Figure 3. Generation of the *Ntn1* null mouse. (A) Map of the wild-type and floxed Ntn1 loci with GenBank annotations. loxP sites flank exon 2; its protein product (yellow, domain VI; blue, domain V) is delineated by dashed lines. (B) Western blots of E11.5 head lysate show residual protein in *Ntn1*$^{trap/trap}$ mutants but no detectable protein in the newly generated *Ntn1*$^{-/-}$ mutants. Loading controls (actin) were obtained from a shorter exposure of the same gel.

To assess the extent of Ntn1-independent commissural axon guidance, we stained E11.5 *Ntn1*$^{-/-}$ spinal cord sections for commissural markers TAG-1 and Robo3 (Sabatier et al., 2004; Serafini et al., 1996). In wild-type embryos, fasciculated axons travel along the lateral edge of the neural tube, turn ventromedially at the motor columns, and cross the floor plate (Figure 4A; n=3). Since no differences were observed between wild-type and heterozygous animals, both genotypes were used as controls. In *Ntn1*$^{trap/trap}$ mutants, some axons still arrive at the floor plate with normal trajectories (Figure 4B; n=2), consistent with Serafini *et al*, 1996. In comparison, *Ntn1*$^{-/-}$ mutants display defasciculated TAG-1 and Robo3-positive axons that project toward the ventricular zone, into the motor columns, or even dorsally (Figure 4C; n=6). Very few axons appear to cross the midline. By contrast, the gross organization of the spinal cord was normal, with sensory and motor axons growing through expected entry and exit points (Figure 4D-F; n=4).

To quantify the extent of crossing in *Ntn1*$^{-/-}$ animals, we stained for Robo3+ commissural axons at the floor plate in open book preparations of E11.5 spinal cords (Figure 4H-K) and calculated the ratio of ventral to adjacent dorsal areas covered by Robo3+ axons in a ~500 µm segment of the cervical-thoracic spinal cord. Both *Ntn1*$^{trap/trap}$ and *Ntn1*$^{-/-}$ mutants displayed highly disorganized commissural axons that were often oriented away from the midline. However, the degree of crossing was significantly decreased in *Ntn1*$^{-/-}$ embryos (n=4) compared to *Ntn1*$^{trap/trap}$ (p<0.0001; n=5) and *Ntn1*$^{+/-}$ animals (p=0.0007; n=3; Mann-Whitney test).

To investigate whether the enhanced strength of the commissural phenotype observed in *Ntn1*$^{-/-}$ animals might be secondary to changes in the availability of other chemoattractants, we examined the expression of other floorplate-derived cues and their receptors in *Ntn1* mutants. We found that floor plate identity is preserved, as revealed by *in situ* hybridization for *Shh*, a floor

Figure 4. The *Ntn1*$^{trap/trap}$ commissural phenotype is enhanced in *Ntn1*$^{-/-}$ mutants. (A-C″) Low (A-C, A"-C") and high (A'-C') magnification views of E11.5 spinal cord sections stained for TAG-1 and Robo3 reveal that fewer commissural axons (arrowheads) cross the midline in *Ntn1*$^{-/-}$ animals (C) compared with controls (A) and *Ntn1*$^{trap/trap}$ hypomorphs (B), with some axons projecting dorsally (arrow). (D-F) Neurofilament (NF) stains show grossly normal organization of the spinal cord in E11.5 null mutants. (G-J) Robo3 staining of open-book preparations of E11.5 spinal cords (G) show that fewer axons cross the midline (dashed lines) in *Ntn1*$^{trap/trap}$ animals (I) compared with controls (H). This phenotype is more severe in *Ntn1*$^{-/-}$ animals (J). (K) Quantification of the midline crossing phenotypes illustrated in (H-J). Yellow boxes in (H) indicate the dorsal and ventral areas quantified. RP, roof plate; FP, floor plate; D, dorsal; V, ventral. ****P<0.0001, ***P<0.005; Mann-Whitney test. Error bars indicate s.d.

Figure 4 (Continued)

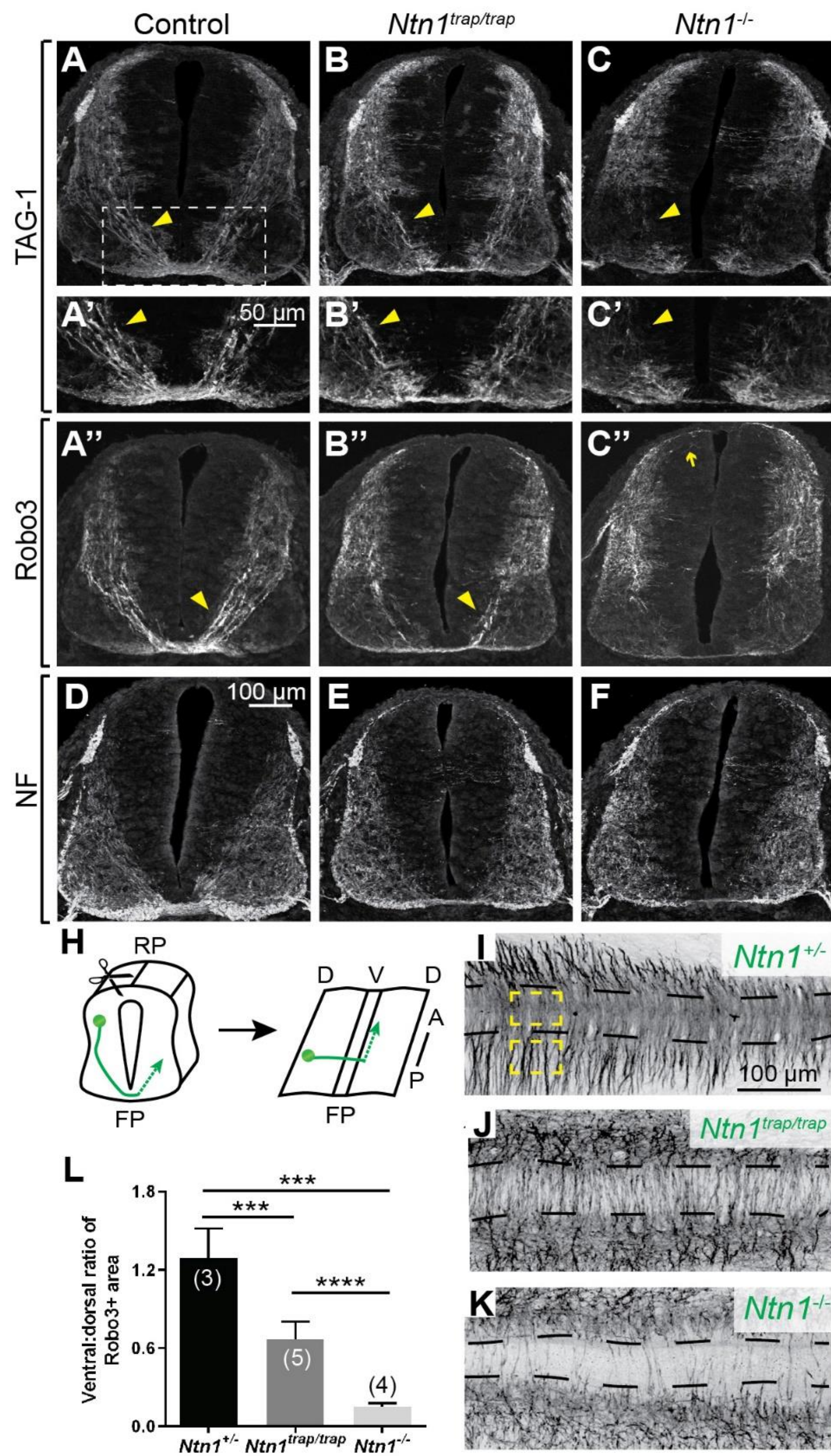


plate marker and a short-range chemoattractant for commissural axons (Charron et al., 2003); *VEGF,* a chemoattractant (Ruiz de Almodovar et al., 2011); and chemorepellents *Slit1* and *Slit2* (Long et al., 2004) (Figure 5A-H; n=3 null embryos). Moreover, a combination of Western blotting (n=3 E11.5 heads per genotype), immunostaining (n=3 animals per genotype) and *in situ* hybridization (n=2 animals per genotype) confirmed that the Shh receptor Boc (Okada et al., 2006), VEGF receptor Flk1 (Ruiz de Almodovar et al., 2011), and DCC and Neogenin are still present (Figure 5I-K), so the enhanced phenotype should not be due to lack of responsiveness. On the contrary, DCC ($p<0.05$) and Neogenin levels ($p<0.05$, Student's t-test) were significantly increased in *Ntn1* mutants (Figure 5I), similar to observations from an independent null strain (Bin et al., 2015), implying that Ntn1 regulates the availability of its own receptors.

Together, these data suggest that although other cues and their receptors are present, they may not compensate for the absence of Ntn1. Low levels of Ntn1 in the hypomorph are sufficient to attract many commissural axons to the midline, consistent with reports that fewer than five Ntn1 molecules can induce growth (Pinato et al., 2012). In Ntn1's absence, some axons still grow ventrally, likely by chance, at which point attractants such as VEGF and Shh may guide them across the midline. Indeed, Shh can induce turning but not outgrowth, and mice lacking Shh or VEGF activity show relatively subtle guidance phenotypes (Charron et al., 2003; Ruiz de Almodovar et al., 2011). Thus, whereas Ntn1 guides over long distances to establish the overall pathway, other cues act at close range to fine-tune axon trajectories within this framework.

Trochlear nerve projections remain intact in* Ntn1$^{-/-}$ *mice

The striking contrast in the severity of the *Ntn1*$^{trap/trap}$ versus *Ntn1*$^{-/-}$ commissural phenotype highlights the potent attractive capabilities of Ntn1 even at reduced levels. This raised

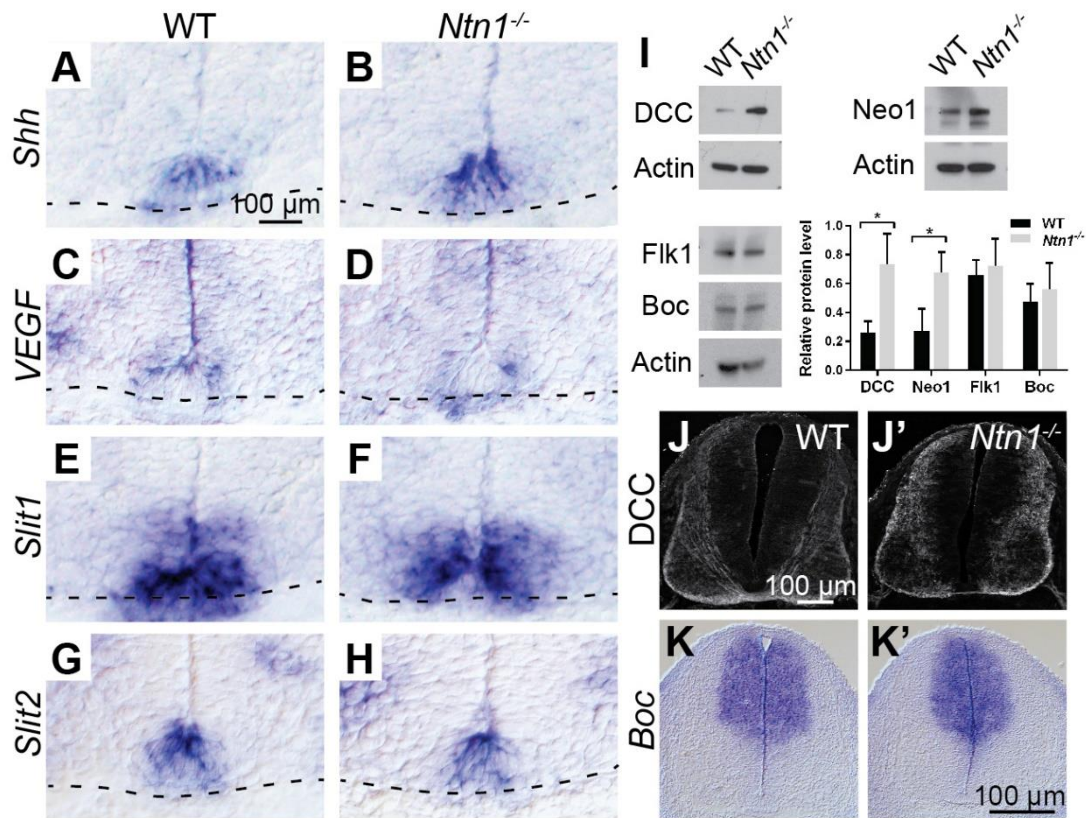


Figure 5. ***Ntn1*** $^{-/-}$ **mutants maintain expression of other guidance cues and their receptors.** (A-H) *In situ* hybridization of E11.5 spinal cord sections; dashed lines indicate the ventral edge. *Shh* (A, B), *Vegf* (C, D), *Slit1* (E, F) and *Slit2* (G, H) are expressed at the floor plate of mutant animals, as in wild-type (WT) controls. (I) Quantified western blots for DCC, Neogenin, Flk1 and Boc show similar, or upregulated, levels of these receptors in wild-type and null animals. *P<0.05; Student's t-test. Error bars indicate s.d. (J-K′) Immunostaining (J, J′) and *in situ* hybridization (K, K′) confirm that *DCC* and *Boc* expression is preserved in E11.5 mutant spinal cords.

the possibility that low levels of Ntn1 remaining in hypomorphs may have obscured other guidance defects *in vivo*. In addition to acting as an attractant, Ntn1 has been proposed to repel trochlear motor neurons, which reside in the ventral hindbrain and project axons dorsally, away from the floorplate. However, although Ntn1 repels trochlear axons from hindbrain explants *in vitro* and loss of Ntn1 receptor *Unc5c* causes guidance defects in the trochlear nerve *in vivo*, no defects in the trochlear nerve have been observed in *Ntn1*$^{trap/trap}$ animals (Burgess et al., 2006; Colamarino and Tessier-Lavigne, 1995; Serafini et al., 1996; Varela-Echavarría et al., 1997).

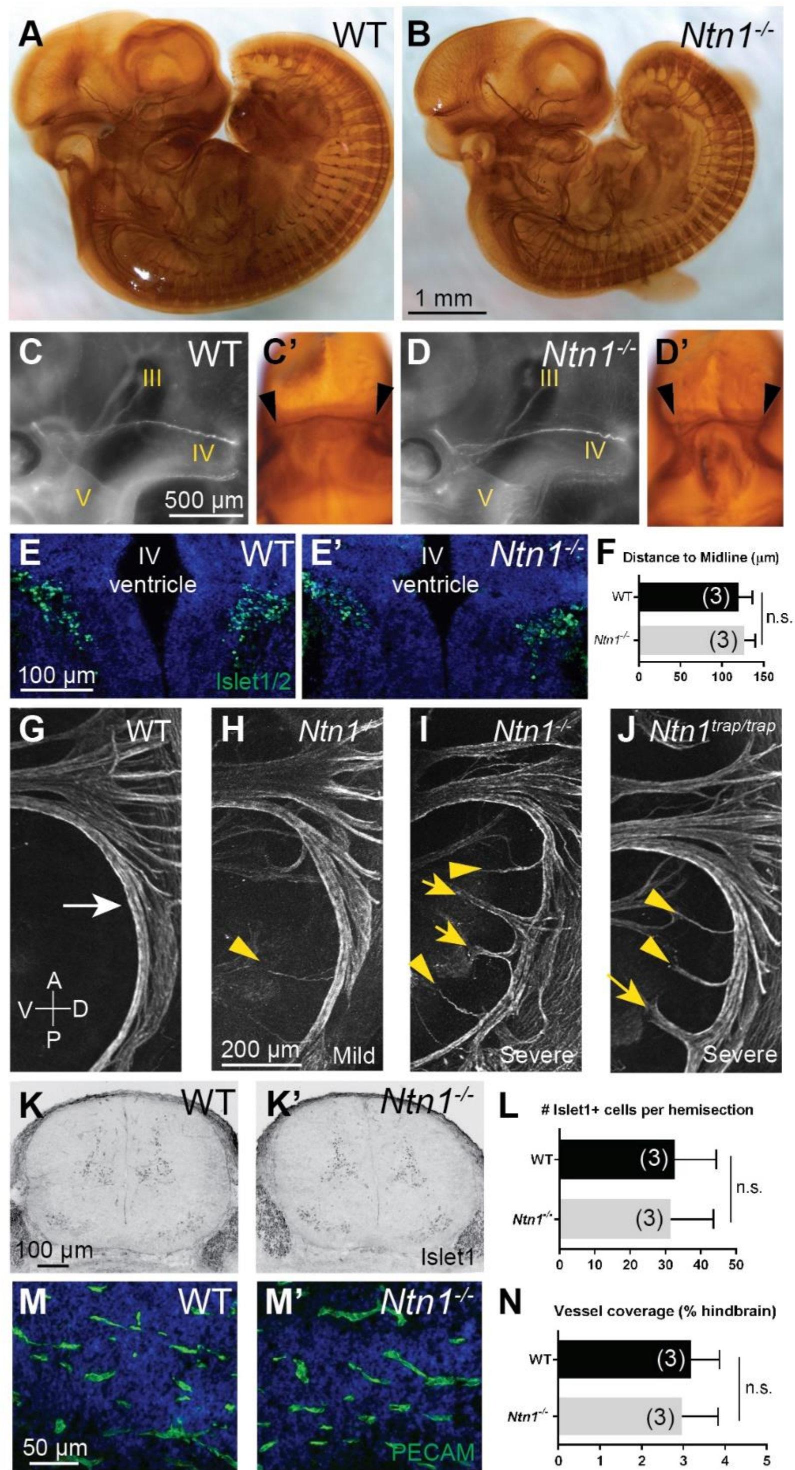
To determine if residual Ntn1 obscured a role in trochlear pathfinding, we visualized peripheral axons in E11.5 *Ntn1*$^{-/-}$ embryos by performing wholemount neurofilament stains. The overall pattern of sensory and motor projections appeared unchanged (Figure 6A, B), and we detected no qualitative differences in the presence, trajectory, or dorsal decussation of the trochlear nerve in null mutants (Figure 6C, D; n=4 wild-type, 8 *Ntn1*$^{-/-}$ embryos). Additionally, the position of the trochlear nuclei relative to the midline, as shown by Islet1/2 immunostaining at E12.5, was similar across controls and mutants (Figure 6E, F; n=3 animals per genotype; p=0.474, Mann-Whitney test). These data suggest that Ntn1 does not act as the major repulsive cue for trochlear axons and that other ligands are responsible for Unc5C-dependent growth and guidance of the trochlear nerve.

Absence of other Unc5-mediated phenotypes in **Ntn1**$^{-/-}$ *mice*

Given the discordance between the *Ntn1*$^{-/-}$ and *Unc5c*$^{-/-}$ trochlear nerve phenotypes, we wondered whether other Unc5-mediated activities might also be independent of Ntn1. To investigate this possibility, we first examined projections from spinal accessory motor neurons (SACMNs), which showed variable defects in dorsal migration and guidance in *Ntn1* hypomorphs and in mice mutant for DCC or Unc5C (Dillon et al., 2005, 2007). SACMN neurons

Figure 6. ***Ntn1*$^{-/-}$ mutants do not display many known Unc5 receptor mutant phenotypes.** (A-D') Colorimetric and fluorescent wholemount neurofilament stains show comparable sensory and motor projections in wild-type (A) and Ntn1 null (B) E11.5 embryos, including normal trochlear (IV) nerve trajectories (C, D) with an intact dorsal decussation (C', D'; arrowheads indicate trochlear nerve on either side). Cranial nerves III, IV and V are indicated. (E-F) Islet1/2-positive trochlear nuclei retain their normal position relative to the midline in mutant E12.5 coronal hindbrain sections, as quantified in F. (G-J) SACMN axons normally form a smooth, hook-shaped nerve (white arrow), but both null (H, I) and hypomorphic (J) mutants show variable defects, ranging from a few axons (yellow arrowheads) to whole bundles of axons (yellow arrows) wandering away from the nerve at many positions. (K-L) Islet1 immunostains show no change in the number of motor neurons in the spinal cord of E13.5 wild-type and null animals, as quantified in L. (M-N) Immunostaining for PECAM in E12.5 hindbrain sections show no change in blood vessel coverage, as quantified in N. n.s., not significant (F, P=0.474; L, P=0.445; N, P=0.480; Mann–Whitney test). Error bars indicate s.d.

Figure 6 (Continued)



are ventrally located in the cervical spinal cord and extend axons dorsally along the lateral edge of the spinal cord before exiting and turning rostrally to form a longitudinal, hook-shaped nerve (Figure 6G). All mutants (n=13 nerves) displayed spinal accessory nerve abnormalities in at least one side of the body, but the phenotype remained variable. In mildly affected nerves, a few axons wander ventrally (Figure 6H), while in more severe cases, entire segments of the nerve branch ventrally (Figure 6I), similar to what occurs in severely affected gene trap mutants (Figure 6J). Thus, residual Ntn1 does not explain the partial penetrance of the SACMN guidance defect in *Ntn1* hypomorphs, indicating that closer comparison of DCC and Unc5C-dependent SACMN populations is warranted.

Intriguingly, in *Unc5a*$^{-/-}$ mutants, these same neurons are less susceptible to cell death, hinting that Ntn1 might also function as a survival cue (Williams et al., 2006). However, *in vivo* evidence for this model is lacking, as SACMN number was unchanged in *Ntn1*$^{trap/trap}$ embryos, perhaps due to its hypomorphic nature. To resolve this issue, we stained E13.5 cervical spinal cord sections and saw no significant difference (Figure 6K, L; p=0.4448, Mann-Whitney test) in the number of Islet1-positive SACMN cells per hemisection in serial sections between wild-type (n=8) and null (n=8) embryos, suggesting that Unc5A-mediated survival of SACMNs occurs through another ligand. Consistent with this interpretation, cell death also appeared unaffected in a different *Ntn1*$^{-/-}$ strain (Bin et al., 2015).

Given the mis-match in receptor and ligand neural phenotypes *in vivo,* we sought to clarify whether Unc5 receptors also act independently of Ntn1 during blood vessel development. Although there are no obvious vascular malformations in *Ntn1* hypomorphs, *Unc5b* mutants die at E12.5 with excessive blood vessel branching in numerous regions, including the hindbrain (Lu et al., 2004). Despite complete loss of Ntn1, there were no obvious differences (Figure 6M, N;

p=0.4799, Mann-Whitney test) in blood vessel coverage in wild-type (n=3) versus null (n=3) animals, as assessed by staining E12.5 hindbrain sections for the vascular marker PECAM. Together with lack of trochlear and SACMN survival phenotypes, these data imply that other ligands may be more important for the activation of Unc5 receptors in many contexts.

A growing body of literature has shown that members of the fibronectin and leucine-rich transmembrane protein family mediate repulsion through Unc5 receptors in multiple systems (Yamagishi et al., 2011). Similarly, Draxin, an axon guidance molecule expressed in the dorsal neural tube, acts on the same neurons that respond to Ntn1 and can bind to both DCC and Unc5 family members (Islam et al., 2009). It is tempting to speculate that these molecules could contribute to trochlear nerve guidance, though compensation by other Netrin family members might also play a role. The increasing number of ligands for Ntn1 receptors ultimately contributes to the diversity of molecular cues available to direct brain wiring and invites broader consideration about how receptors integrate signals from multiple ligands during circuit formation and other developmental processes.

It is somewhat surprising that complete loss of *Ntn1* did not uncover obvious novel phenotypes, given its broad expression and demonstrated potency. Evidence for additional roles may emerge on different genetic backgrounds, since lethality is earlier in another $Ntn1^{-/-}$ strain (Bin et al., 2015). Indeed, the enhanced commissural phenotype highlights the need to re-examine other tissues where $Ntn1^{trap/trap}$ animals do not phenocopy receptor mutants. This *Ntn1* floxed allele provides an ideal tool to study other developmental functions within and beyond the nervous system, particularly in postnatal and adult animals, as illustrated by a recent study of sympathetic arterial innervation (Brunet et al., 2014).

— CHAPTER THREE —

Netrin-1 confines rhombic lip-derived neurons to the central nervous system

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A.R.Y. designed, performed, and analyzed all experiments and wrote the manuscript together with L.V.G. N.R.D. discovered the phenotype. This chapter was published in *Cell Reports* 22 (2018), pp. 1666-80.

Introduction

A basic organizing principle of the nervous system is the segregation of the peripheral and central nervous systems, which are anatomically and functionally distinct yet linked by nerves. This is particularly apparent in the vertebrate brainstem, which houses ten cranial nerves as well as a constellation of nuclei that govern functions critical to life, from motor coordination to auditory processing (Farago et al., 2006; Wang et al., 2005). Many of these nuclei are comprised of neurons originating from the rhombic lip, a transient strip of proliferating neuroepithelium lining the fourth ventricle during development (Ray and Dymecki, 2009). The formation of hindbrain nuclei therefore depends on the successful tangential migration of newborn neurons from the rhombic lip to their final destinations. This route is unusually long and complex, especially since the surface of the hindbrain is broken by multiple cranial nerve roots that the rhombic lip derivatives must ignore. Although several guidance cues play critical roles sculpting the trajectory of tangentially migrating neurons *in vivo* (Kratochwil et al., 2017), nothing is known about the molecular mechanisms that confine these neurons to the central nervous system (CNS), despite opportunities to deviate into the periphery.

Pontine neurons (PNs) traverse one of the longest migratory routes in the hindbrain, ultimately settling at the midline to supply excitatory mossy fiber input to the cerebellum (Kratochwil et al., 2017). PNs originate from the rhombic lip in rhombomeres (r)6 – 8 from embryonic day (E)12.5 – 16.5. They extend long leading processes (Ono and Kawamura, 1990; Yee et al., 1999) as they migrate beneath the pial surface, maneuvering between the trigeminal (V^{th}), facial (VIIth) and vestibulocochlear ($VIII^{th}$) nerve roots and arriving at the ventral midline of r3 – 4 several days later (Nichols and Bruce, 2006). This navigation depends on the activity of several guidance cues, including Slits, which are secreted by the facial motor nucleus to prevent

premature ventral migration (Geisen et al., 2008), and meninges-derived chemokine SDF-1, which keeps PNs from migrating into the neuroepithelium (Zhu et al., 2009). What prevents PNs from escaping in the opposite direction, into the periphery, is unknown.

One of the first guidance cues implicated in PN migration is the classic chemoattractant Netrin-1 (Ntn1). PNs are highly sensitive to Ntn1 and can migrate toward a source of Ntn1 over unusually long distances *in vitro* (Yee et al., 1999); *in vivo,* the pontine nuclei are missing in mice severely hypomorphic for *Ntn1* (Serafini et al., 1996; Yee et al., 1999). These data were originally interpreted to indicate that a floor plate-derived gradient of Ntn1 guides PNs to the midline during the final leg of their migration (Zelina et al., 2014), much as Ntn1 was proposed to guide commissural axon growth in the developing spinal cord (Kennedy et al., 2006; Serafini et al., 1996). However, *Ntn1* is also expressed in the ventricular zone (Kennedy et al., 1994; Serafini et al., 1994), and this source is required for proper commissure formation (Charron et al., 2003). The protein itself is deposited in the sub-pial region (SPR) adjacent to the basement membrane (BM) surrounding the neural tube (Kennedy et al., 2006; MacLennan et al., 1997; Varadarajan et al., 2017). Recent genetic studies have underscored the importance of SPR-localized Ntn1 for commissural axon (Dominici et al., 2017; Varadarajan et al., 2017; Yamauchi et al., 2017), consistent with documented functions for Ntn1 in the BM of other tissues (Liu et al., 2004; Srinivasan et al., 2003; Yebra et al., 2003). This suggests that additional roles for locally-produced Ntn1 in the developing nervous system likely remain to be found, particularly in cases such as PN migration, where neurons migrate along the pial surface rather than through the neuroepithelium. Indeed, the complete range of Ntn1's effects on PN migration is still unclear, due both to the hypomorphic nature of the original allele and the lack of information about the ultimate fate of PNs.

Here, we show that Ntn1 functions as a permissive cue to confine PNs to the CNS. We propose that Ntn1 in the sub-pial region provides a corridor for migrating rhombic lip-derived neurons, allowing them to distinguish the appropriate migratory substrate and avoid opportunities to migrate instead into the periphery. These findings introduce another local function for Ntn1 and establish an additional molecular explanation for how CNS-PNS segregation is achieved in the brainstem, a hub for CNS-PNS interactions that are vital for life.

Experimental Procedures

Animal Models. The following mouse strains were used and genotyped as described previously: *$Ntn1^{fl/fl}$, $Ntn1^{+/-}$* (Yung et al., 2015), *$Ntn1^{CE/+}$* (Nishitani et al., 2017), *$DCC^{+/-}$; $Neo1^{Gt/+}$* (Fazeli et al., 1997; Leighton et al., 2001; Xu et al., 2014), *$Neo1^{-/-}$ and $Neo1^{fl/fl}$* (Kam et al., 2016), *$Atoh1^{CreERT2}$* (Machold and Fishell, 2005), *$Six3^{Cre}$* (Furuta et al., 2000), *$Nestin^{Cre}$* (Tronche et al., 1999), *$MafB^{GFP}$* (Moriguchi et al., 2006), *Ai14* Cre-dependent *tdTomato* (Madisen et al., 2010). Mice were maintained on a C57Bl6 background. Noon on the day of the plug was considered embryonic day (E) 0.5. Tamoxifen (Sigma Aldrich) injections were carried out at 20 mg/mL in sunflower oil at 1 mg per 10 g of bodyweight. Since all experiments were performed on embryonic mice, whole litters, which include both male and female mice, were used for experiments. We did not detect any sex-based differences in our phenotype. The ages used in each experiment are included in the relevant text, figures, and Figure legends. Experiments were performed with the observer blind to genotype, though the ensuing image analyses was not due to the obvious nature of the phenotypes. All animal experiments were approved by the Institutional Animal Use Care Committee at Harvard Medical School.

Immunohistochemistry. Embryos were fixed in 4% paraformaldehyde (PFA)/PBS at 4°C overnight, cryoprotected in sucrose, frozen in NEG-50 (Thermo-Scientific), and sectioned at 12 to 16 µm. Sections were blocked in 3% BSA and incubated in the following primary antibodies at 4°C overnight: 1:500 goat anti-DCC (Santa Cruz), 1:750 rabbit anti-laminin (Sigma), 1:500 rabbit anti-MafB, 1:250 rat anti-myc (Santa Cruz), 1:500 goat anti-Netrin-1 (R&D), 1:400 goat anti-Neogenin (R&D), 1:400 rabbit anti-Pax6 , 1:100 mouse anti-RC2 (DSHB), 1:100 goat anti-TAG1 (R&D), and 1:1000 mouse anti-Tuj1 (Covance). For antigen retrieval, the sections were treated with boiling 10 mM sodium citrate, pH 7.0, for 20 minutes prior to blocking. Species-

specific secondary antibodies conjugated to Alexa-Fluor fluorophores from Jackson ImmunoResearch or Invitrogen were used afterward.

Whole-mount brains were dissected and fixed in 4% PFA/PBS at 4°C overnight and blocked in 10% normal donkey serum (NDS) and 1% Triton-X in PBS at 4°C overnight. After incubating in primary antibody for 3 nights, the brains were incubated in HRP-conjugated secondary antibodies. Detection was performed using a DAB substrate.

In situ *hybridization.* Standard *in situ* hybridization was performed as described on 12 µm sections (Lu et al., 2011). The *Egr2* probe was provided by Advanced Cell Diagnostics (ACD, Hayward, CA) for use with their RNAscope Fluorescent Multiplex Kit. Tissue sections were rinsed with PBS to wash off residual Neg-50 and treated with protease III (ACD) before following the manufacturer's protocol.

Transmission electron microscopy (TEM). Embryos were collected and rinsed in 0.1 M sodium cacodylate buffer before drop-fixing in a modified Karnovsky fixative (2.5% paraformaldehyde, 5% glutaraldehyde, and 0.06% picric acid in 0.2 M cacodylate buffer) (Ito and Karnovsky, 1968) for 3-5 days at 4˚C. Whole fixed embryos were then embedded in epon resin, and ultrathin sections of 80 nm were collected on copper grids and counter-stained with Reynold's lead citrate (0.2% lead citrate).

Imaging. Images were collected on an Olympus VS120 slide scanner at 10X and 20X. Higher power images were taken with an Olympus Fluoview 1200 at 40X or a Leica SP8 confocal microscope at 25X or 40X. A 1200EX electron microscope (JEOL) equipped with a 2k CCD digital camera (AMT) captured all TEM images. Images were processed using ImageJ (NIH) and Adobe Photoshop.

Image Quantification. To quantify the number of Pax6+ nuclei in the cochlea, we counted the number of labeled cells in the base and middle turns of the cochlea in the earliest section where all three turns of the cochlea are first visible. To perform the same analyses in the VIIth nerve root, we only used sections where the nerve could be seen exiting the hindbrain to be confident of the anatomy. We counted all cells present in a 110 x 218 pixel box over this initial segment of the VIIth nerve.

The area covered by laminin was measured by centering a 561 x 386 pixel box over the BM adjacent to the AES, with the top of the box meeting the dorsal tip of the BM where it stops to permit the entry of the VIIIth nerve. After thresholding, the area covered by laminin within the box was calculated.

Ntn1 intensity was measured using confocal images at the floor plate or at the lateral edges of the hindbrain. The z-stack was summed and converted to an 8-bit image, and a 158 μm^2 circle was placed at the floor plate or at the basement membrane immediately ventral to the VIIIth nerve root. If a blood vessel was present, the circle was placed at the next available area. Each data point in the figure represents the mean intensity found over the area of the circle. All image analyses were performed using ImageJ (NIH).

Statistical Analysis. All statistical comparisons were done using Prism software (GraphPad, La Jolla, CA) and presented as mean ± S.D. Statistical significance was determined by a Student's t-test if comparing between two groups. If more than two groups were being considered, a one-way ANOVA was performed with Tukey's multiple comparisons test. In cases of the latter, the multiplicity adjusted *p*-values were included in the figures and the *p*-value of the ANOVA was reported in the figure legends. Sample size for all experiments was determined empirically based

on standards in the field. Specific details for each experiment are included in the text (*n* values and their meaning) or in the Figure legends (statistical tests used).

Results

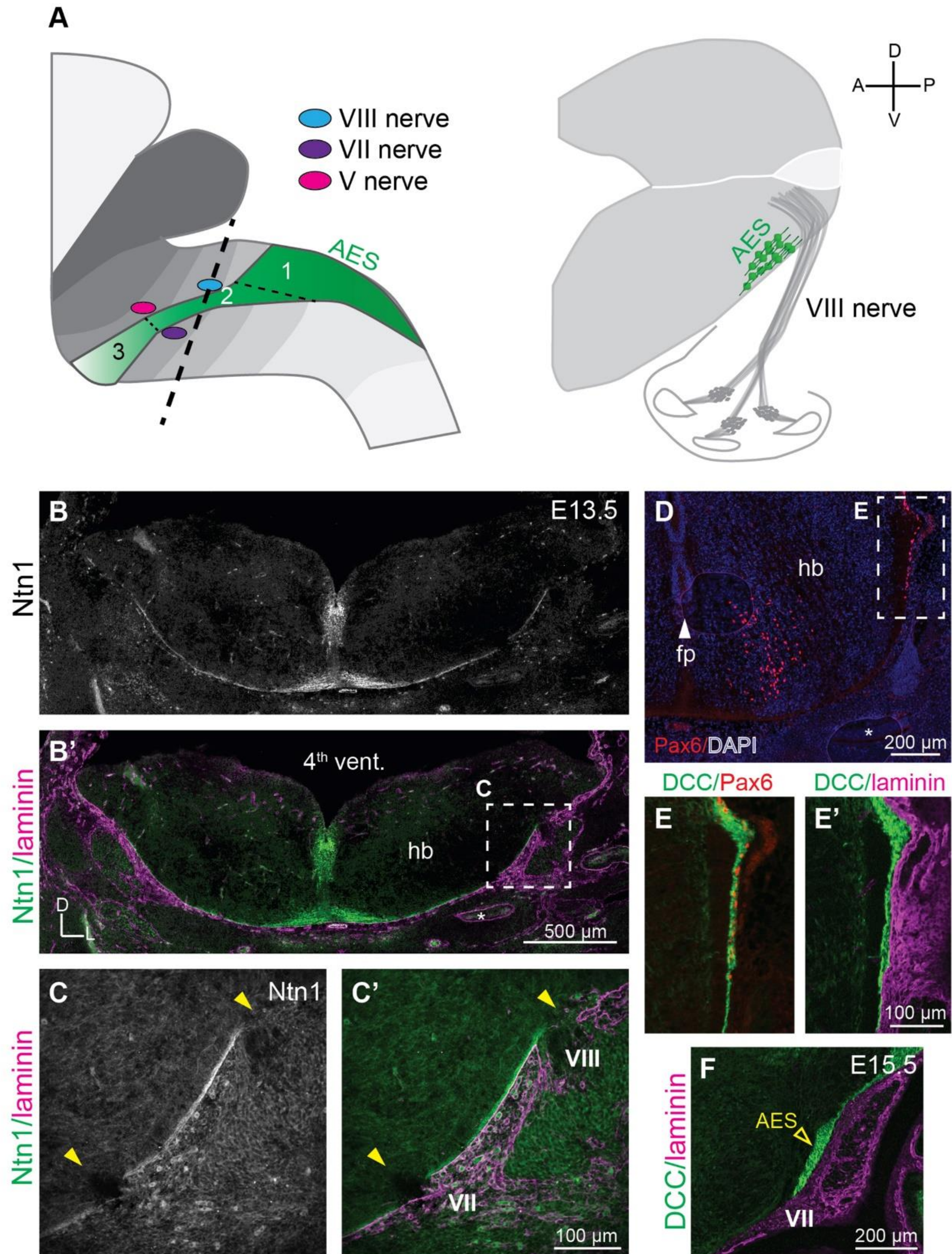
Ntn1 protein is enriched in the sub-pial region in the developing hindbrain

Migrating pontine neurons (PNs) follow a stereotyped pathway to the ventral midline that can be divided into three phases: 1) a short ventral migration marking the departure from the rhombic lip, 2) a relatively straight rostral migration between and past the vestibulocochlear (VIIIth), facial (VIIth), and trigeminal (Vth) nerves respectively, and 3) a final ventral turn before resting at the midline (Figure 8A) (Geisen et al., 2008; Nichols and Bruce, 2006). The entire migration takes place in the space beneath the pia, which we call the sub-pial region (SPR). While the molecules that define many aspects of this complex migratory route have been identified, the cues that instruct PNs to stay in the SPR and avoid cranial nerve roots remain unknown. During the peak of migration at E15.5, PNs traverse hundreds of microns as they move past the cranial nerves. Throughout this journey, PNs express the Ntn1 receptor Deleted in Colorectal Carcinoma (DCC) and are exposed to Ntn1 produced in the floor plate (FP) and ventricular zone (Yee et al., 1999). When *Ntn1* levels are reduced, rare spinal cord interneuron axons can be found in dorsal root ganglia (Laumonnerie et al., 2014), hinting at a role in setting the CNS-PNS boundary. We therefore hypothesized that Ntn1 not only guides PNs to the midline, but also keeps them contained within the CNS.

Since Ntn1 is a potent secreted cue, we examined the localization of Ntn1 relative to its sources in the hindbrain at the onset of PN migration at E13.5 (Diego et al., 2002; Yee et al., 1999). Immunostaining revealed that Ntn1 protein is widely distributed (Figure 7B-B'; n=2 animals), accumulating in the FP, on commissural axons, and in the SPR in the vicinity of the laminin-positive pial basement membrane (BM), as described (Dominici et al., 2017; Kennedy et al., 2006; MacLennan et al., 1997; Varadarajan et al., 2017). Notably, Ntn1 is absent from nerve roots, where cranial nerves project into or out of the CNS via gaps in the pial BM at stereotyped

Figure 7. Ntn1 protein is enriched in the sub-pial region (SPR) in the developing hindbrain. (A) Schematic depicting the three phases of PN migration (green) across multiple rhombomere segments (shaded in gray) and a view of the AES in an E15.5 transverse section. Thick dashed line indicates plane of section. D, dorsal; A, anterior; P, posterior; V, ventral. (B – F) Immunostains of transverse embryonic head sections. At E13.5, low-power (B, B') and high- (C, C') power images show strong Ntn1 staining at the FP, on crossing commissural axons at the midline, and in the sub-pial region (SPR) adjacent to the laminin-positive pial basement membrane (magenta). Curiously, Ntn1 appears to be absent from nerve roots (yellow arrowheads). Low (D) and high (E, E') magnification images show ventrally migrating pontine neurons in the SPR (D – E', E13.5), even as they avoid cranial nerve roots later in their migration (F, E15.5). Pontine neurons express Pax6 (red, D-E) and DCC (green, E-F). AES, anterior extramural stream; fp, floor plate; hb, hindbrain; 4th vent., fourth ventricle; VII, facial nerve; VIII, vestibulocochlear nerve.

Figure 7 (Continued)



locations (Figure 7C-C'; n=2 animals). Thus, Ntn1 protein is present where PNs migrate, but not at sites they avoid.

To determine if migrating PNs might encounter and respond to Ntn1 in the SPR, we stained for PN markers Pax6 and DCC at E13.5, when PNs have begun exiting the rhombic lip, and at E15.5, when they are passing by cranial nerves. At E13.5, Pax6+/DCC+ cells cluster beneath the pial BM (Figure 7D-E'; n=4 animals) and maintain this position as they navigate near the facial (VII) and vestibulocochlear (VIII) nerves at E15.5 (Figure 7F). Since Ntn1 is enriched in the SPR, migrating DCC+ PNs likely encounter Ntn1 from early on. Given the conspicuous absence of Ntn1 at nerve roots—which migrating PNs avoid—this pattern of distribution suggests that Ntn1 may contribute to the confinement of tangentially migrating neurons in the hindbrain by providing an attractive substrate.

Loss of* Ntn1 *causes PNs to exit the hindbrain and enter the periphery

Earlier analyses of hypomorphic *Ntn1* animals (*Ntn1$^{trap/trap}$*) suggested that Ntn1 mediates the final ventral migration to the midline, as PNs complete most of the first and second phases of their migration (Zelina et al., 2014). However, phenotypic analyses of *Ntn1* null animals (*Ntn1$^{-/-}$*) showed that residual Ntn1 in hypomorphs masks the full extent of its role in guidance (Bin et al., 2015; Yung et al., 2015). We posited that complete loss of Ntn1 might reveal additional functions earlier in migration, particularly since PNs are exposed to Ntn1 far before their final ventral turn.

To visualize PN distribution, we collected transverse sections of embryonic *Ntn1$^{-/-}$* heads spanning the anterior extramural stream (AES) through which PNs travel. In E15.5 control animals (n=4), the AES is identifiable as a dense stripe of Pax6+ nuclei and DCC+ processes

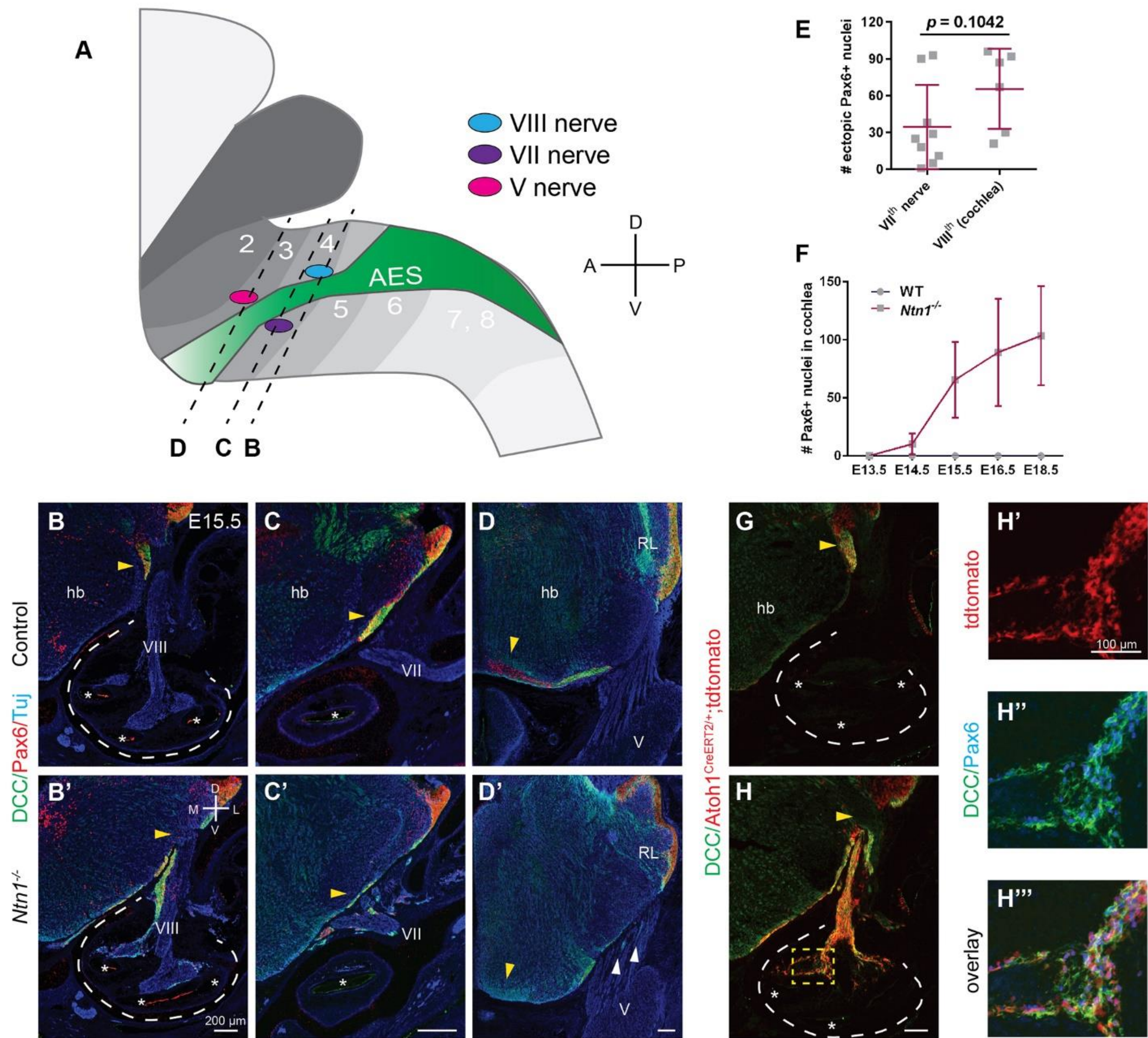
traveling beneath the pial surface (Figure 8B–D), giving rise to the pontine nuclei at the midline, which first appear at late E14.5 (Nichols and Bruce, 2006). In contrast, in *Ntn1*$^{-/-}$ animals (n=6), the AES was missing, and there were ectopic streams of Pax6+ and DCC+ neurons immediately ventral to the stereotyped location of the AES, as if the PNs had been diverted into the periphery (Figure 8B', C'). Ectopic Pax6+ nuclei and a few DCC+ processes were rarely found in the trigeminal (V) ganglion (Figure 8D'). However, the facial and vestibulocochlear nerves contained similar numbers of ectopic nuclei (Figure 8E; n=6 nerves each). We focused on the vestibulocochlear (VIII) nerve as a site of exit, as it is the first nerve root migrating PNs pass and because the cochlea is an enclosed, easily recognizable landmark. These findings suggest that, in addition to mediating the final ventral turn to the midline, Ntn1 acts earlier to prevent PNs from migrating along cranial nerves and into the periphery.

To confirm the origin and identity of these neurons, we genetically labeled PNs by providing tamoxifen to E13.5 *Atoh1*^{CreERT2}*;Ai14* crossed onto the *Ntn1* null background. tdTomato+ cells also expressed both Pax6 and DCC (Figure 8G-H'''; n=3 animals), demonstrating that the ectopic neurons in the cochlea derive from the rhombic lip. Likewise, in *Ntn1* mutants, Pax6+ neurons first reach the cochlea at E13.5 and increase in number steadily up until E15.5 (Figure 8F, n=6 cochleae per time point), matching the timing of PN production and migration. These neurons accumulate mostly in the base and middle turns of the cochlea, which lie closest to the hindbrain. Taken together, these data demonstrate that the Pax6+ neurons invading *Ntn1*$^{-/-}$ cochleae are *bona fide* PNs.

Despite their ectopic location, the misrouted PNs survived and differentiated within the cochlea. At E18.5, 103.5 ± 42.6 (S.D.) Pax6+ PNs were present in the cochlea, but instead of integrating into the spiral ganglion, the PNs, which express low levels of Gata3, formed a rind

Figure 8. Pontine neurons (PNs) exit the CNS along cranial nerves in the absence of *Ntn1*. See also Figures S1-2. (A) Schematic of the PN migratory route (anterior extramural stream, green) across multiple rhombomeres (numbered, shaded in gray) and relative to cranial nerve roots. (B-D') E15.5 transverse head sections immunostained for DCC and Pax6 to label migrating PNs (yellow arrowheads), which normally travel rostrally beneath the pial surface toward the midline at three rostrocaudal levels (B, C, and D), as indicated by the dashed lines in (A). A mix of WT and *Ntn1*$^{+/-}$ tissue are shown as controls. In *Ntn1*$^{-/-}$ animals, the AES is missing. PNs instead diverge into the VIIIth (B') and VIIth (C') nerves. Rare ectopic processes are present in the Vth nerve (D', white arrowheads). (E, F) Quantification of the number of Pax6+ neurons in the VIIth and VIIIth nerves (E, mean ± S.D., Student's t-test) and in the base and middle turns of control and *Ntn1*$^{-/-}$ cochlear sections over time (F, mean ± S.D). (G-H''') Low- (G, H) and high-power (H-H''') images of fate-mapped PNs in the cochlea which were labeled with tdTomato (G, H, H'), DCC (G, H, H''), and Pax6 (H'') in *Atoh1*$^{CreERT2/+}$*; Ai14; Ntn*$^{+/-}$ (G) and *Ntn*$^{-/-}$ (H-H''') embryos exposed to tamoxifen at E13.5. A merged image is shown in (H''') Dotted lines indicate the cochlea; Roman numerals indicate cranial nerves. Hb, hindbrain; *, cochlear duct.

Figure 8 (Continued)



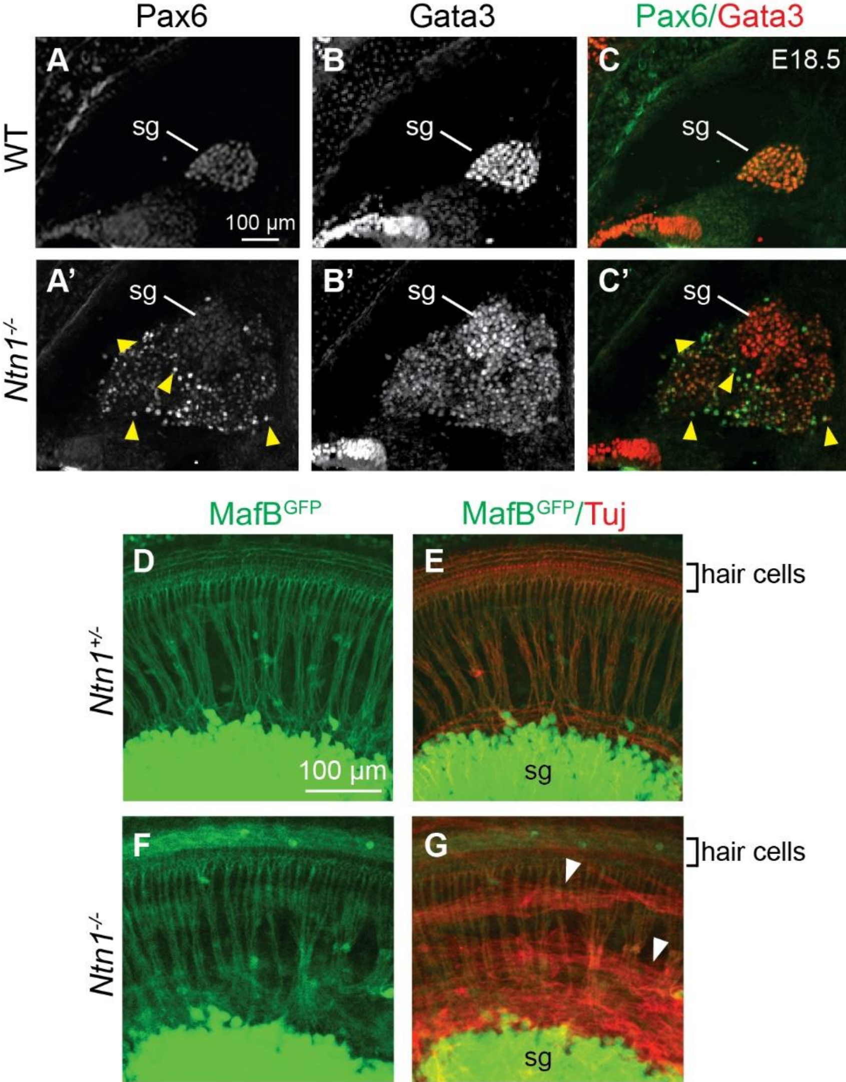
around the Gata3-high spiral ganglion neurons (SGNs; Figure 9A-C'; n=3 *Ntn1*$^{-/-}$). The gross organization of the spiral ganglion was remarkably normal, as visualized by crossing a *MafB*^{GFP} allele (Moriguchi et al., 2006) into the *Ntn1*$^{-/-}$ background. As in control embryos, GFP+ SGNs extended orderly bundles of radial fibers toward the hair cells in mutants (Figure 9D, F; n=3 *Ntn1*$^{-/-}$), beneath an overlying swath of processes from GFP-/Tuj+ PNs (Figure 9E, G). Since *Ntn1* mutants die at birth, we could not examine the fate of ectopic PNs in adults. Nonetheless, these data show that PNs thrive in the cochlea but stay segregated from the SGNs.

Multiple populations of neurons escape the CNS in* Ntn1 *mutants

In addition to PNs, many other rhombic lip derivatives migrate through the SPR, including neurons of the cochlear nucleus, inferior olive, and external cuneate nucleus (Machold and Fishell, 2005; Nichols and Bruce, 2006; Wang et al., 2005). As these neurons also respond to Ntn1 (Alcantara et al., 2000; Bloch-Gallego et al., 1999; Causeret et al., 2002; Howell et al., 2007; Ma et al., 2014; Marcos et al., 2009), we wondered if Ntn1's role in confinement extends to other rhombic lip derivatives. Since *Ntn1* is expressed in the neural tube as early as E9.5 (Kennedy et al., 1994; Serafini et al., 1994; Yee et al., 1999), we examined the trajectory of some of the earliest-born rhombic lip-derived neurons in the CNS by injecting tamoxifen at E9.5 into *Atoh1*^{CreERT2}*;Ai14* mice crossed to the *Ntn1* null background. As expected, many commissural neurons were labeled, shown by the presence of tdTomato+ processes crossing the midline at E11.5 (Figure S1A'-A'''; n=2 controls). In *Ntn1*$^{-/-}$ animals, tdTomato+ neurons resided in more dorsal locations, and the commissure failed to form. Additionally, many labeled cell bodies and processes were found outside the hindbrain near and along cranial nerve roots (Figure S1B'-B'''; n=3 mutants). As in older animals, the tdTomato+ cells escaped through the Vth, VIIth, and VIIIth

Figure 9. Ectopic neurons do not integrate into the spiral ganglion. (A-C') E18.5 transverse sections of the base of the cochlea immunostained for Pax6 (A, A') and Gata3 (B, B'). Only mutant cochleae contain Pax6+ neurons (yellow arrowheads), which form a rind around spiral ganglion neurons (SGNs) that express higher levels of Gata3 (C'). (D-G) Whole mount immunostains of E18.5 cochleae from control and *Ntn1*$^{-/-}$ embryos also harboring a *MafB*^{GFP} allele, which is expressed in SGNs. GFP+ SGN processes (green, D-G) form bundles extending radially to hair cells in both *Ntn1*$^{+/-}$ (D, E) and mutant mice (F, G). In addition, *Ntn1*$^{-/-}$ cochleae contain Tuj+ PNs that extend GFP- processes longitudinally over the SGNs and their radial fibers (G; white arrowheads). Merged images shown in (E) and (G). Sg, spiral ganglion.

Figure 9 (Continued)



nerves, raising the possibility that later-born neurons such as PNs depend on these existing ectopic tracts to exit the CNS. However, we found that within the VIIIth nerve, PNs were not physically associated with any other ectopic DCC+ axons and were consistently present independent of other ectopic projections, such as those from the earlier-born neurons in the ventral cochlear nucleus (Figure S2). These observations argue that the departure of PNs is not secondary to earlier phenotypes. Thus, multiple populations of neurons exit the CNS in the absence of *Ntn1*, indicating that Ntn1 plays a general role in establishing the CNS-PNS boundary.

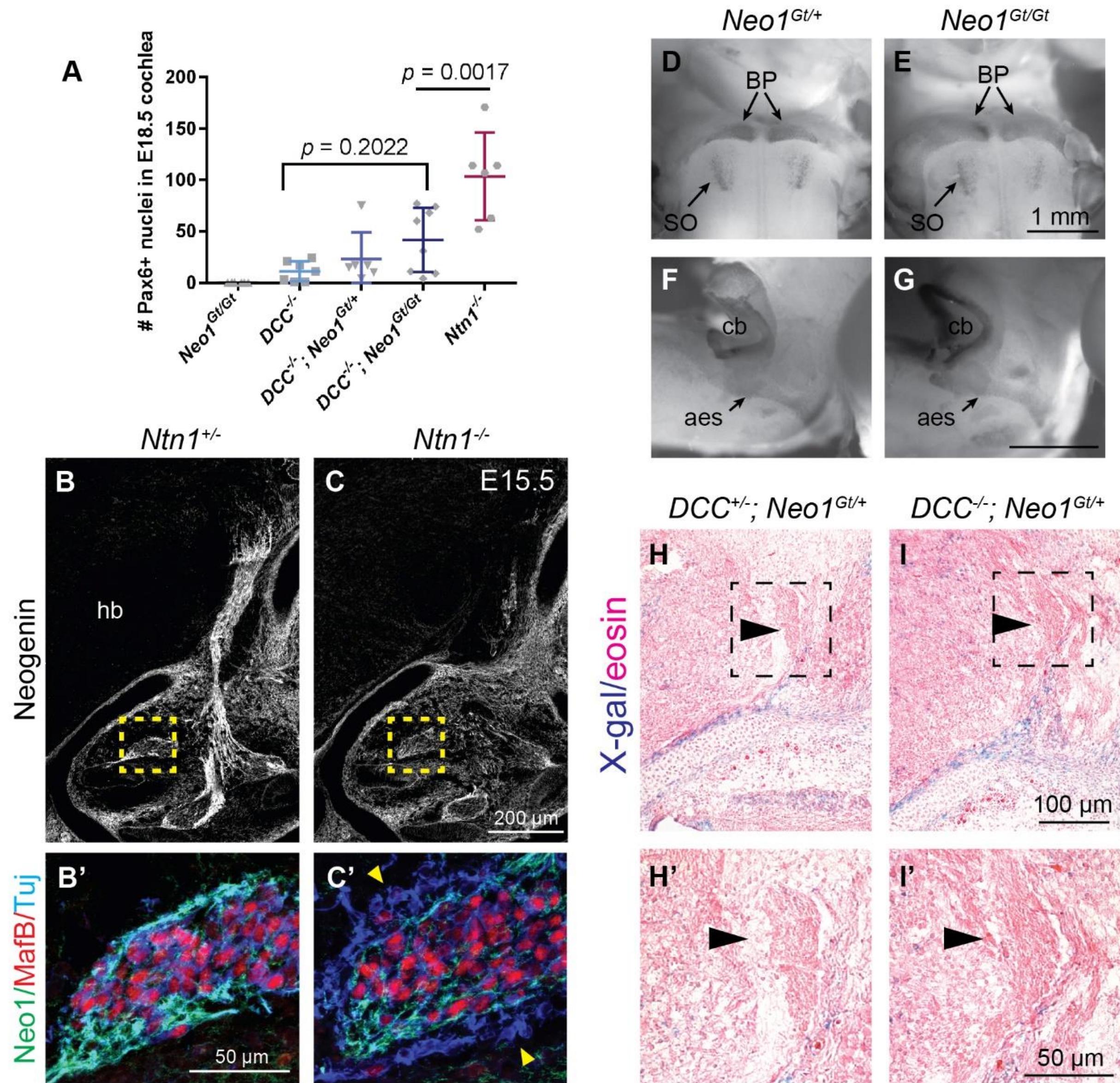
DCC is also required for PN confinement

Since PNs express DCC and fail to reach the midline in *DCC*$^{-/-}$ animals (Fazeli et al., 1997; Yee et al., 1999), we hypothesized that Ntn1-DCC signaling underlies their confinement. Consistent with this idea, *DCC*$^{-/-}$ animals contain ectopic neurons in the cochlea that were assumed to be displaced spiral ganglion neurons (Kim et al., 2016). We found not only that ectopic neurons in *DCC*$^{-/-}$ cochlea express Pax6, indicative of PN identity instead, but that Pax6+ neurons are also present elsewhere in the periphery such as in the VIIth and VIIIth nerve, demonstrating that DCC enables Ntn1-mediated confinement. However, there were not as many neurons in *DCC*$^{-/-}$ cochleae as in *Ntn1* mutants (Figure 10A, n≥6 cochlea per genotype), hinting that another receptor also contributes.

Ntn1 signals through many receptors, including Unc5 family members, DSCAM, integrins, and the DCC ortholog Neogenin (reviewed in Lai Wing Sun et al., 2011). We predicted that Neogenin might influence Ntn1-mediated confinement, since DCC and Neogenin collaborate to mediate midline crossing in commissural neurons (Xu et al., 2014). As in previous

Figure 10. *DCC$^{-/-}$; Neo1$^{Gt/Gt}$* double mutants phenocopy *Ntn1$^{-/-}$* mutants, but Neogenin acts non-cell-autonomously. See also Figure S3 & S7. (A) The number of Pax6+ PNs found in the base and middle turns of the cochlea in E18.5 *DCC* and *Neo1* gene trap (*Neo1^{Gt}*) single and double mutants (mean ± S.D, $p < 0.0001$, F = 14.33; DF = 28; one-way ANOVA, Tukey's multiple comparisons test). (B-C') Immunostained E15.5 transverse sections show that Neogenin is expressed strongly in MafB+ SGNs and in the surrounding mesenchyme in control (B) and *Ntn1$^{-/-}$* tissue (C). High-power images of the boxed areas show that MafB+ SGNs normally express Neogenin (B'), but ectopic MafB- PNs in the ear do not (C', yellow arrowheads). (D-G) Ventral (D, E) and sagittal (F, G) views of heterozygous (D, F) and homozygous (E, G) *Neo1* E15.5 brains immunostained for Pax6. Rostral is up in (D, E) and to the right in (F, G). (H-I') X-gal reactions (blue) in eosin-stained tissue from E15.5 control and DCC$^{-/-}$ embryos carrying the *Neo1^{Gt}* allele, which drives expression of β-galactosidase in Neogenin+ cells. No signal is detected in the AES (black arrowheads) in controls or *DCC$^{-/-}$* animals, shown at low (H, I) and high (H', I') magnification. AES, anterior extramural stream; BP, basilar pons; cb, cerebellum; hb, hindbrain; SO, superior olive.

Figure 10 (Continued)



reports (Brugeaud et al., 2014; Fitzgerald et al., 2007; Heuvel et al., 2013), we observed low levels of Neogenin throughout the E15.5 hindbrain (Figure 10B, B'; n=2 *Ntn1*$^{+/-}$), with stronger expression in the surrounding mesenchyme and SGNs. We did not detect Neogenin in migrating PNs and found no obvious qualitative differences in the size or location of the pontine nuclei or the trajectory of the AES in either *Neo1* hypomorphs (*Neo1*$^{Gt/Gt}$) (Bae et al., 2009; Leighton et al., 2001) (Figure 10D-G; n=3 *Neo1*$^{Gt/Gt}$) or null animals (Kam et al., 2016) (Figure S3A-B; n=3 *Neo1*$^{-/-}$). Ectopic PNs in the cochleae also did not express Neogenin (Figure 10C, C'; n=3 *Ntn1*$^{-/-}$), suggesting that Neogenin is not required for PN migration or confinement. Nonetheless, increasing numbers of Pax6+ neurons accumulated in the cochlea as more copies of *Neo1* were lost in the *DCC* null background (Figure 10A). Thus, Neogenin affects Ntn1-mediated PN confinement when DCC is absent, but not when DCC is present.

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Neogenin functions non-cell-autonomously in PN migration

A redundant function for Neogenin within PNs would provide the simplest explanation for why *DCC*$^{-/-}$*; Neo1*$^{Gt/Gt}$ double mutants, but not *DCC*$^{-/-}$ or *Neo1*$^{Gt/Gt}$ single mutants, more closely mimic the phenotype in *Ntn1*$^{-/-}$ animals. To test if PNs upregulate Neogenin in the absence of DCC, we crossed the *Neo1*$^{Gt/+}$ allele into the *DCC*$^{-/-}$ background. This allowed us to assay β-galactosidase activity as a proxy for *Neo1* expression, which is more sensitive than immunostaining. As expected, β-galactosidase reaction product was present in SGNs and the surrounding mesenchyme at E15.5, but not in the AES in either control or mutants (Figure 10H-I'; n=3 control, 4 *DCC-/-*). Thus, Neogenin is unlikely to compensate for DCC in migrating PNs.

To be sure that early or low levels of Neogenin in rhombic lip-derived neurons do not explain the stronger phenotype in *DCC*$^{-/-}$*; Neo1*$^{Gt/Gt}$ double mutants, we used *Wnt1*^{Cre} and a

floxed *Neo1* allele (Kam et al., 2016) to remove Neogenin from *Wnt1*+ rhombic lip precursors in a *DCC* null background. Deletion of *Neo1* from early PNs did not enhance the *DCC* phenotype (Figure S3C-D; n=3 conditional mutants), making it highly unlikely that Neogenin and DCC function redundantly in PNs. Additionally, though more PNs migrated all the way into the cochlea in $DCC^{-/-}$; $Neo1^{Gt/Gt}$ double mutants than in $DCC^{-/-}$ single mutants, similar numbers entered the nerve roots in E15.5 animals of both genotypes. Ntn1 therefore appears to act largely through DCC to prevent PNs from crossing the CNS-PNS boundary, but may influence their subsequent behavior through Neogenin expressed in other tissues.

$Ntn1^{-/-}$ *mutants retain boundary cap cells at nerve roots*

Our results show that in addition to its canonical role as a chemoattractant, Ntn1 contributes to CNS-PNS segregation, raising the question of how Ntn1 mediates this function. Many cellular structures contribute to the compartmentalization of the CNS. Neural crest-derived boundary cap cells (BCCs), for example, reside at all spinal nerve roots, and loss of BCCs or the cues they secrete results in the ectopic migration of motor neurons into the ventral root (Bron et al., 2007; Garrett et al., 2016; Mauti et al., 2007; Vermeren et al., 2003). The role of BCCs in the hindbrain is less well understood, though they reside at the trigeminal and facial nerve roots in mice (Garrett et al., 2016) and chicks (Niederländer and Lumsden, 1996). Loss of *Ntn1* could alter the position of BCCs at cranial nerve roots, in turn permitting the departure of CNS neurons along nerves.

To test this possibility, we used RNAscope to detect *Egr2*, one of the only markers for BCCs (Vermeren et al., 2003), and counterstained for laminin to assess the distribution of BCCs at the Vth, VIIth, and $VIII^{th}$ nerves. At E11.5, when DCC+ processes have already entered the

periphery (Figure S1), *Egr2*+ BCCs were observed at nerve entry and exit sites in both controls and mutants (Figure 11A-B"; n=3 animals per genotype). This result is not unexpected given that, based on the number of ectopic CNS neurons in the periphery, the *Ntn1*$^{-/-}$ hindbrain phenotype is much more severe than what was previously described in animals lacking BCCs (Vermeren et al., 2003). These data show that Ntn1 maintains the CNS-PNS divide through mechanisms distinct from those of BCCs.

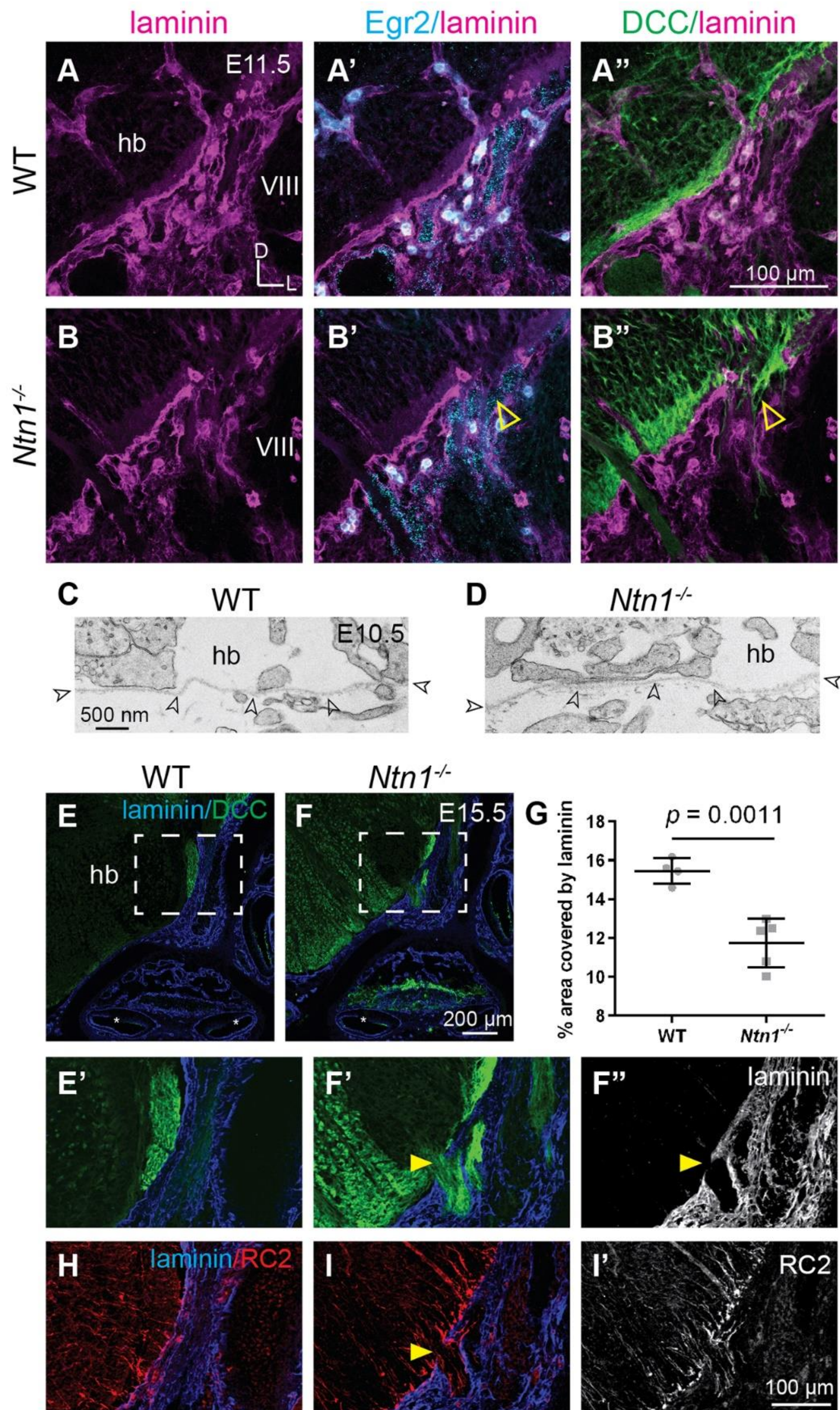
Ectopic neurons exit the CNS independent of defects in BM organization

In addition to BCCs, an effective CNS-PNS border depends on BM integrity, which is maintained in part by radial glial endfeet lining the pial surface. Deletion of BM components or detachment of radial glial endfeet from the pial surface causes BM rupture, defects in neuronal migration, extrusion of cortical neurons into the subarachnoid space, and ectopic migration of spinal cord motor neurons into the ventral root (Beggs et al., 2003; Halfter et al., 2002; Lee and Song, 2013; Moore et al., 2002; Nakagawa et al., 2015; Satz et al., 2010). Since Netrins affect BM integrity in some tissues (Abraira et al., 2008; Liu et al., 2004; Srinivasan et al., 2003; Yebra et al., 2003; Ziel et al., 2009), we wondered if loss of *Ntn1* might cause defects in the pial BM or in the organization of the radial glial endfeet, thereby enabling PN exodus.

To assess BM integrity prior to the earliest signs of the phenotype, we performed transmission EM of E10.5 control and *Ntn1*$^{-/-}$ animals. At this age, the BM looks like a thin, diffuse rope surrounding the hindbrain, and we were able to follow the BM from the ventral edge of the VIIIth nerve root to the midline. The appearance of the BM was highly variable, altering in thickness, smoothness, and curvature with no discernable pattern in wild-type and null animals (Figure 11C, D; n=3 WT, 4 *Ntn1*$^{-/-}$). In rare cases, we observed what appeared to be ectopic

Figure 11. Neurons exit the CNS independent of defects in boundary cap cells, radial glial endfeet, and the basement membrane. (A-B") High-power images of E11.5 transverse head sections near the VIIIth nerve root show that Egr2+ boundary cap cells (BCCs) (blue) are present at gaps in laminin (magenta) in both WT (A-A") and *Ntn1*$^{-/-}$ (B-B") animals. In *Ntn1*$^{-/-}$ mutants, ectopic DCC+ processes exit the CNS despite the presence of BCCs (hollow yellow arrowheads). (C-D) TEM images of the basement membrane (BM, hollow black arrowheads) surrounding the WT (C) and *Ntn1*$^{-/-}$ (D) hindbrain. (E-G) Immunostains of transverse sections from WT (E) and *Ntn1*$^{-/-}$ (F) E15.5 embryos. Low (E, F) and high (E-F") power images of laminin (blue) and DCC (green) show an ectopic break in the BM (yellow arrowhead, F', F") in *Ntn1* mutants, quantified in (G) (mean ± S.D., Student's t-test). (H-I') Stains on the same control (H) and mutant (I, I') sections for RC2, a radial glia marker, show that the radial glia endfeet (red) remain attached to the laminin-positive BM (blue), even extending together with PN processes through breaks in the laminin (yellow arrowhead), shown also in a single-channel image for RC2 in (I'). Hb, hindbrain; VIII, vestibulocochlear nerve; WT, wild-type.

Figure 11 (Continued)



processes reaching into the periphery, yet the surrounding BM still did not look diminished in a way that would *a priori* enable neurons to exit.

Several days later, the BM in *Ntn1*$^{-/-}$ mutants still looked intact overall, as assessed by laminin staining. However, small ectopic breaks were consistently observed near the AES and the vestibulocochlear nerve (Figure 11E-F”; n=3 *Ntn1*$^{-/-}$), where ectopic DCC+ processes protrude, resulting in a significant decrease in the area covered by laminin adjacent to the AES (Figure 11G; n=4 control, 5 *Ntn1*$^{-/-}$ ears). These breaks appeared independent of impaired radial glia architecture, whose RC2/Nestin+ endfeet remained attached to the pial surface in E15.5 mutants, as in controls (Figure 11H-I; n=2 control, 3 *Ntn1*$^{-/-}$). Moreover, at the sites of BM breaks, the radial glial endfeet projected further without showing obvious changes in their morphology or organization (Figure 11I’). Since BM integrity is normal at E10.5, with no apparent changes in radial glia organization at E15.5, it is unlikely that Ntn1 is required for BM integrity *per se*, consistent with the fact that Ntn1 has no effects on laminin assembly *in vitro* (Schneiders et al., 2007). Altogether, the lack of defects in key cell types that contribute to CNS integrity indicate that Ntn1 in the SPR acts directly on migrating neurons to keep them in the CNS.

SPR-localized Ntn1 produced by hindbrain progenitors is required for confinement

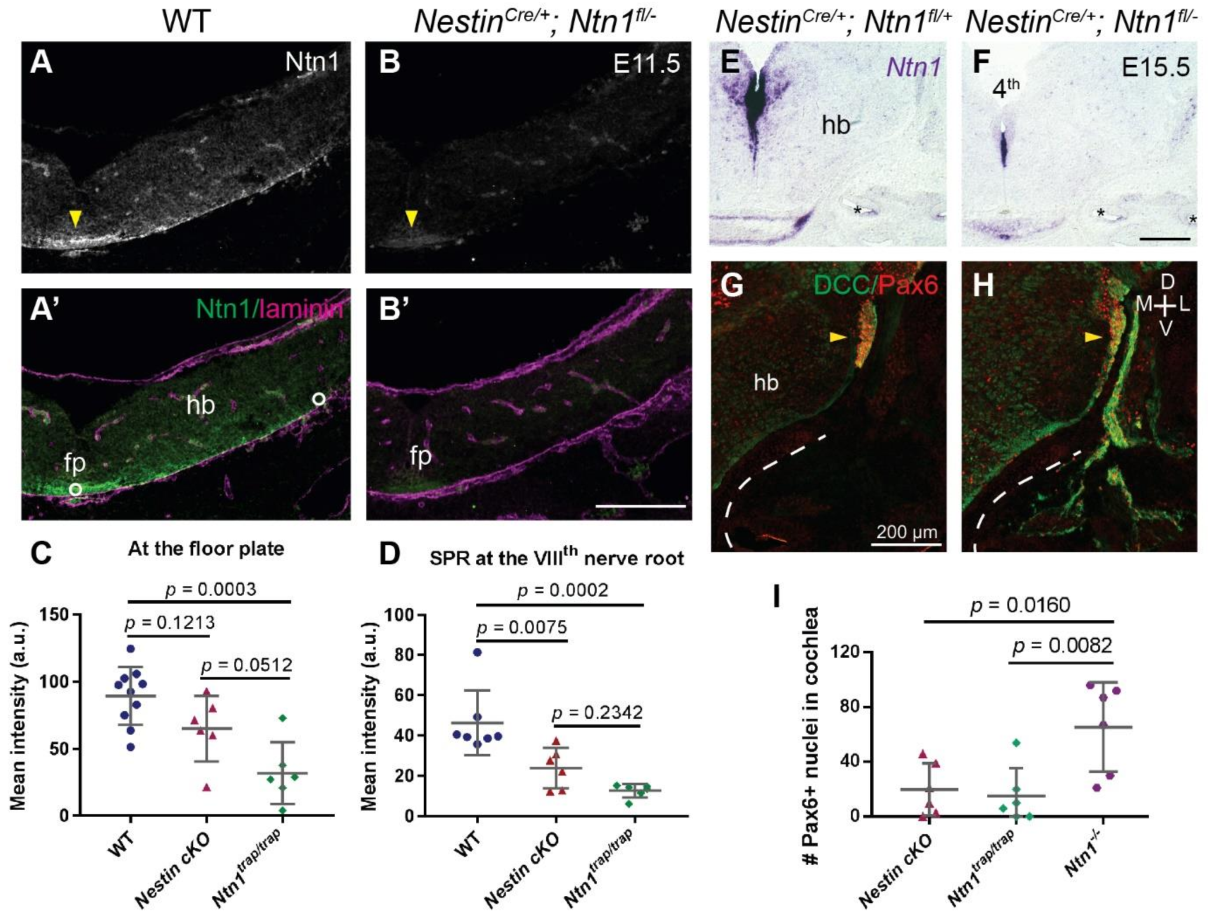
Our results contrast with previous studies that reported no phenotypes in the spiral ganglion of *Ntn1* hypomorphs (Howell et al., 2007; Kim et al., 2016). These differences could be attributed to the hypomorphic nature of the *Ntn1*$^{trap/trap}$ mice, which show a weaker phenotype: many PNs get close to their final destination (Figure S4D, D’) and there are significantly fewer Pax6+ cells in E15.5 *Ntn1*$^{trap/trap}$ cochleae (Figure S4B-C’; n=6 cochleae). The number of ectopic neurons remained unchanged at E18.5 (Figure S4E; n=6 cochleae), further indicating that unlike

null animals, the phenotype does not worsen as later-born PNs migrate out. Despite this difference, the pontine nuclei are absent in *Ntn1$^{trap/trap}$* animals, indicating that confinement and guidance are differentially affected by the loss of Ntn1, possibly due to differences in the availability or localization of Ntn1 *in vivo*.

In the developing hindbrain, Ntn1 is present both in the FP and in the SPR (Figure 7) (Dominici et al., 2017; MacLennan et al., 1997). We wondered whether the role in confinement might be attributed specifically to Ntn1 in the SPR, which is primarily supplied by progenitors in the ventricular zone (Dominici et al., 2017; Varadarajan et al., 2017; Yamauchi et al., 2017). Using *Nestin$^{Cre/+}$* (Zimmerman et al., 1994) and a floxed allele of *Ntn1*, we significantly reduced Ntn1 in the SPR of *Nestin$^{Cre/+}$*; *Ntn1$^{fl/-}$* (*Nestin* cKO) animals (Figure 12A–D; n=2 control and 3 *Nestin* cKO). Despite the presence of residual *Ntn1* protein (Figure 12C, D) and transcript (Figure 12E, F) at the FP, PNs migrated ectopically into the ear (Figure 12G, H; n=2 control and 3 *Nestin* cKO), partially phenocopying *Ntn1$^{-/-}$* animals and fully phenocopying the hypomorphs (Figure 12I; n=6 ears per genotype), which showed a similar distribution of Ntn1 protein, i.e. a severe decrease in the SPR (Figure 12D) with residual Ntn1 present at the midline (Figure 12C, S4; $n \geq 3$ animals per genotype). These results support two conclusions. First, Ntn1 derived from the ventricular zone—which provides most of the Ntn1 in the SPR—ensures the compartmentalization of the CNS and PNS. Second, residual Ntn1 in hypomorphs and from the FP of *Nestin* cKO animals is sufficient to reduce the departure of CNS neurons into the periphery, but not to guide them reliably to the midline, as the pontine nuclei are missing in the hypomorph (Serafini et al., 1996; Yee et al., 1999).

Figure 12. Ntn1 in the SPR, but not the floor plate, is required for PN confinement. See also Figure S4. (A-B') Immunostaining for Ntn1 shows depletion from the SPR of E11.5 *Nestin* cKO animals (B, B') compared to controls (A, A'), with maintained expression in the FP (arrowheads). Ntn1 intensity was measured at the FP or in the SPR (white circles in A'), quantified in (C) and (D) respectively (mean ± S.D.) (C: $F = 12.06$; $DF = 19$, $p = 0.0004$; D: $F = 14.73$, $DF = 16$; $p = 0.0002$; one-way ANOVA with Tukey's multiple comparisons test). (E-F) *In situ* hybridization for *Ntn1* further illustrates that relative to $Nestin^{Cre/+}$; $Ntn1^{fl/+}$ animals (E), *Ntn1* is selectively reduced in the ventricular zone of E15.5 *Nestin* cKO embryos (F). (G-I) DCC (green) and Pax6 (red) immunostains on E15.5 transverse head sections. *Nestin* cKO animals (H) retain the AES (yellow arrowheads), but it is smaller and deformed, and there are many Pax6+ nuclei in the cochlea, quantified in (I, mean ± S.D., $F = 7.542$; $DF = 15$, $p = 0.0054$; one-way ANOVA with Tukey's multiple comparisons). Refer to Figure S4 for raw data for the gene trap allele. Dotted lines indicate the outline of the cochlea. Fp, floor plate; hb, hindbrain; 4th, fourth ventricle; *, cochlear duct.

Figure 12 (Continued)



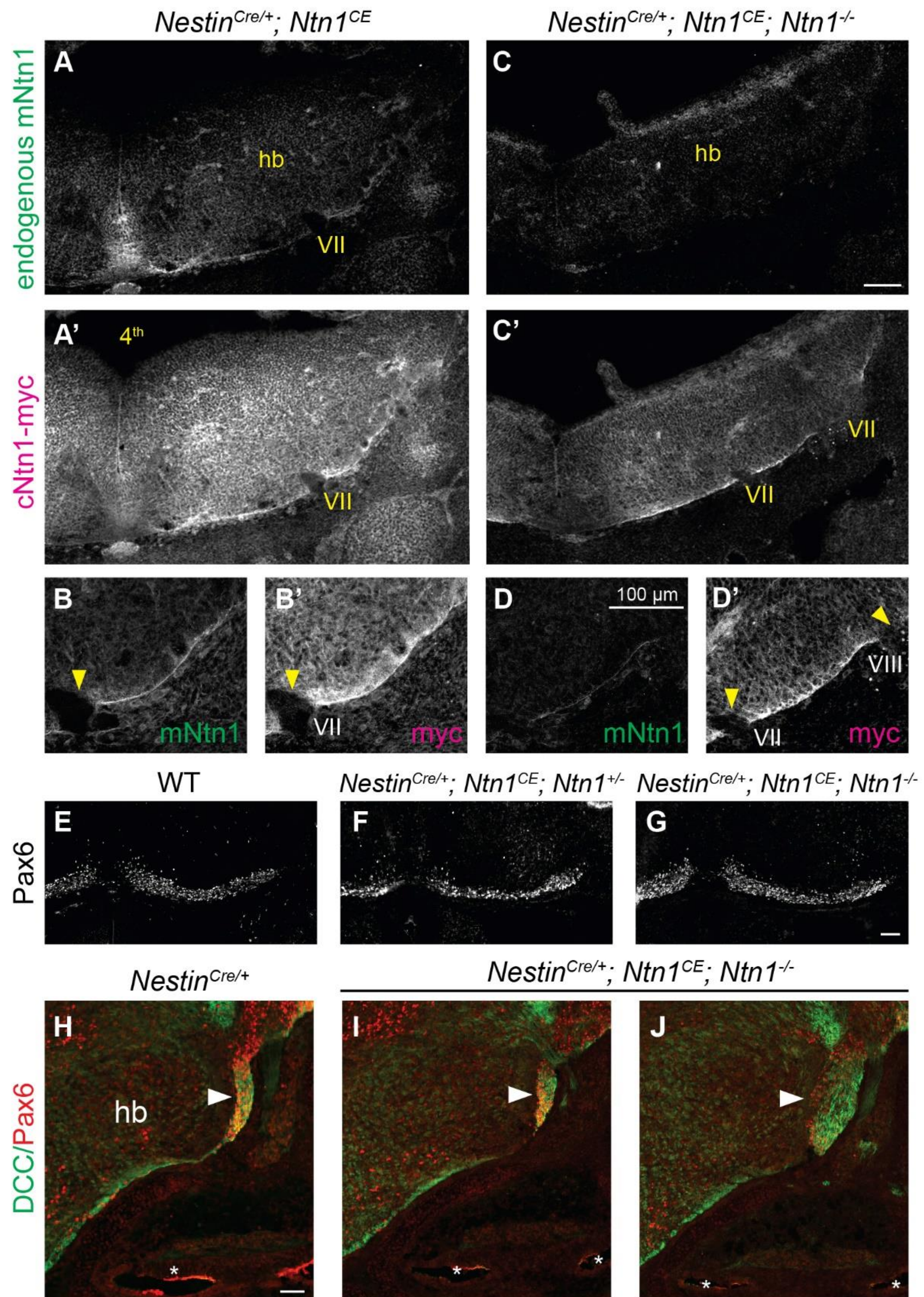
Overexpression of* Ntn1 *throughout the CNS rescues CNS-PNS boundary integrity

Our results raise the possibility that Ntn1 serves dual functions in the developing hindbrain, both securing the CNS-PNS boundary and attracting PNs to the ventral midline. In this model, the low levels of Ntn1 that persist in *Ntn1* hypomorphs may be sufficient to establish a partially functional boundary, but not to mediate guidance to the midline, thereby explaining phenotypic differences between the hypomorphic and null mutants. Thus, Ntn1 could act instructively in a gradient to direct PNs to the midline and permissively in the SPR to keep them in the CNS.

To disambiguate these two possible functions, we disrupted Ntn1's role as a guidance cue by altering its pattern of distribution using a Cre-dependent *Ntn1* conditional expressor ($Ntn1^{CE/+}$), which produces a myc-tagged chick Ntn1 (cNtn1) protein with the same biological activity as mouse Ntn1 (Serafini et al., 1994). E11.5 $Nestin^{Cre/+}$; $Ntn1^{CE/+}$ animals showed widespread expression of cNtn1-myc throughout the hindbrain, overlaid on top of endogenous mNtn1 protein in the FP and SPR (Figure 13A-B'; see also Figure S5; n=4 $Nestin^{Cre/+}$; $Ntn1^{CE/+}$). Despite this clear change in Ntn1 protein distribution, PN migration appeared qualitatively normal: PNs reached the midline (Figure 13E, F), and no ectopic neurons were observed in the periphery (data not shown). To determine if the lack of a phenotype reflected a dominant role for endogenous mNtn1, we crossed the $Nestin^{Cre/+}$; $Ntn1^{CE/+}$ animals onto the $Ntn1^{-/-}$background. Both *cNtn1* transcript (Figure S6; n=3 $Nestin^{Cre/+}$; $Ntn1^{CE/+}$; $Ntn1^{-/-}$) and protein (Figure 13C-D'; n=2 $Nestin^{Cre/+}$; $Ntn1^{CE/+}$; $Ntn1^{-/-}$) were present throughout the hindbrain, with cNtn1-myc enriched in the SPR but reduced at the FP. Thus, we significantly altered Ntn1 localization, thereby distorting any directional information that might be encoded in a gradient, while maintaining a rich source of Ntn1 in the SPR.

Figure 13. Broadly expressing *cNtn1* in the hindbrain can rescue confinement defects in *Ntn1*$^{-/-}$ animals. See also Figures S5-6. (A-D') Transverse sections through E11.5 conditional expressor tissue immunostained for mNtn1 (A-D) and cNtn1-myc (A'-D'). cNtn1-myc is broadly distributed throughout the hindbrain (A'), overlaid on top of endogenous Ntn1 protein (A). Whereas mNtn1 is enriched at the floor plate, cNtn1 is relatively reduced, but both are enriched in the SPR (B-B'). Low-power images (C, C') show that a similar distribution of cNtn1 persists in the null background, where despite the absence of mNtn1 (C), cNtn1 is present throughout the hindbrain with less in the FP (C'). High-power images (D, D') show that without mNtn1 (D), cNtn1 is the only Ntn1 enriched in the SPR (D'). In all cases, note the absence of any Ntn1 near nerve entry roots (yellow arrowheads). (E-J) E15.5 transverse sections immunostained for Pax6 (red) and DCC (green). Single-channel images of Pax6 (E-G) show PNs accumulating at the midline of all conditional expressors, in both *Ntn1*$^{+/-}$ (F) and *Ntn1*$^{-/-}$ (G) backgrounds. Conditional expression of cNtn1-myc rescued confinement in some *Ntn1*$^{-/-}$ animals (I), as shown by a qualitatively normal AES (white arrowheads). In others, we observed a partial rescue in the form of a misshapen AES and a cluster of ectopic neurons in the nerve (J). Control sections at the midline (E) and AES (H) are provided for comparison. Hb, hindbrain; 4th, fourth ventricle; Roman numerals indicate cranial nerves; *, cochlear duct.

Figure 13 (Continued)



Despite the drastic change in Ntn1 distribution, PN migration was surprisingly normal in all *Nestin*$^{Cre/+}$; *Ntn1*$^{CE/+}$; *Ntn1*$^{-/-}$ embryos (n=4), as evidenced by the presence of both a well-defined AES and PNs at the midline where the pontine nuclei are normally found (Figure 13G). Although some PNs reached the midline in all animals, the extent of rescue varied, even between the two sides of the animal. We observed a complete rescue in 4 out of 8 cases (two per embryo), as defined by a qualitatively normal AES and no PNs detected in the periphery (Figure 13H, I). In the other cases, the AES was misshapen and sometimes accompanied by clusters of Pax6+ neurons in the proximal segment of the vestibulocochlear nerve or sparse Pax6+ neurons in other cranial nerves (Figure 13J). Although PNs appear to be resistant to major disruptions in the pattern of *Ntn1* expression, these occasional errors may reflect some requirement for the wild-type pattern of *Ntn1* expression. Alternatively, the degree of rescue may be sensitive to slight variations in the timing or efficiency of *Nestin*^{Cre}-mediated recombination. Importantly, none of the embryos contained Pax6+ neurons in the cochlea. Thus, broad expression of Ntn1 is sufficient to restrict PNs from migrating into the periphery, consistent with the model that Ntn1 acts locally to provide a preferred substrate for neuronal migration, thereby keeping neurons confined to the CNS.

Discussion

In the developing hindbrain, rhombic lip-derived neurons migrate long distances to form brainstem nuclei amidst a crowded network of nerves linking the CNS and PNS. We show here that SPR-localized Ntn1 maintains the CNS-PNS divide by preventing these highly motile neurons from straying into cranial nerves and entering the periphery. Our findings point to a model in which Ntn1 in the SPR acts as a preferred substrate for migrating neurons, thereby keeping them away from nerve roots devoid of Ntn1 (Figure 14). Like flags marking a hiking trail, Ntn1 facilitates the successful migration of rhombic lip-derived neurons by establishing a preferred corridor for growth. Without this corridor, the neurons wander off-trail, losing track of and failing to reach their destination.

In support of the idea that Ntn1 acts in the SPR to keep migrating neurons on track, migrating PNs express the Ntn1 receptor DCC and respond to Ntn1 *in vitro* (Yee et al., 1999). Ntn1 protein is also notably enriched in the SPR but absent at cranial nerve roots, which rhombic lip derivatives avoid. Thus, DCC+ PNs may so prefer the Ntn1-rich environment surrounding the nerve roots that they reliably migrate around them, with the Ntn1-negative gap discouraging their entry. In agreement with this interpretation, the amount of Ntn1 in the SPR correlates with the strength of the confinement phenotype. For instance, using *Nestin*^{Cre} to selectively reduce Ntn1 in the SPR but not the floor plate (FP) caused many PNs to enter the periphery. More strikingly, no ectopic PNs were observed in the cochlea when *Nestin*^{Cre} was used to restore cNtn1-myc only to the SPR in the null background, where Ntn1 is never produced by the FP and the broad ectopic distribution of Ntn1 throughout the hindbrain obscures any positional information normally encoded by localized Ntn1. Thus, the pattern of Ntn1 expression does not seem to

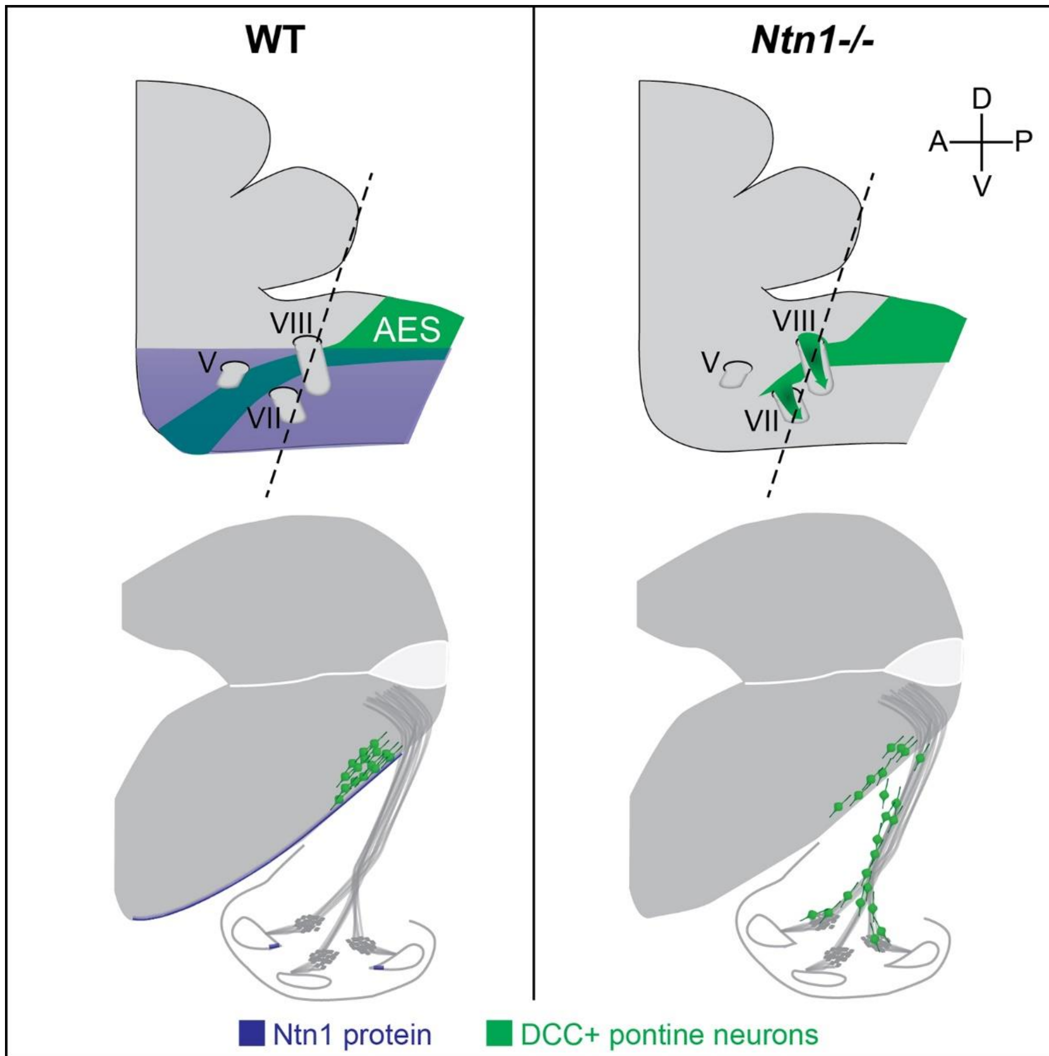


Figure 14. A model for Ntn1's role in confinement. Schematic of PN migration in a E15.5 wholemount hindbrain (top) or a transverse section (bottom). Dashed lines indicate the plane of section. (A) In a WT animal, Ntn1 protein is enriched in the SPR but absent from cranial nerve roots. DCC+ neurons migrating beneath the pial surface encounter Ntn1 as a substrate, which they prefer to the Ntn1-negative cranial nerve roots. (B) In the absence of Ntn1, there is no molecular distinction between the SPR and cranial nerve roots, and migrating neurons cannot distinguish between the two. As a result, when they encounter cranial nerves, they will leave the CNS or stay at random.

matter for the confinement of migrating neurons, so long as Ntn1 protein accumulates in the SPR.

Although a direct role for Ntn1 seems most likely, indirect effects might also contribute to the overall phenotype. For example, the departure of PNs could be facilitated by the presence of errant axons from earlier-born neurons that breached the CNS-PNS border. However, such a mechanism is unlikely to account for the entire phenotype, as both neuronal cell bodies and processes have already entered the periphery at E11.5 (arrows, Figure S1B"), the earliest we can detect a phenotype. Likewise, PNs appear segregated from other DCC+ ectopic axons in the VIIIth nerve, and these ectopias can arise independently (Figure S2). Thus, the departure of neurons does not seem to depend *a priori* on the presence of a pre-existing ectopic axon tract. It is of course possible that PNs occasionally migrate along earlier-born ectopic processes as they escape the CNS, similar to the fasciculation of PN leading processes within the normal AES (Ono and Kawamura, 1990) and of later-born axons that follow pioneer axons toward their targets. However, this would not rule out or diminish the role of Ntn1 in the confinement of rhombic lip-derived neurons overall.

In another scenario, SPR-localized Ntn1 could promote or maintain a physically sound CNS-PNS boundary, in addition to affecting neuronal migration. While we cannot rule out subtle changes, the overall organization of the CNS-PNS boundary appeared intact in *Ntn1*$^{-/-}$ animals. Boundary cap cells were present at nerve roots, and there were no obvious changes in the integrity of the BM surrounding the hindbrain, consistent with the fact that Ntn1 has no effect on BM assembly *in vitro* (Schneiders et al., 2007). These data suggest that Ntn1 acts directly on PNs to corral them within the SPR, thereby preventing them from leaving the CNS altogether. Our findings add to a growing body of evidence supporting a permissive role for Ntn1 (Dominici

et al., 2017; Varadarajan et al., 2017; Yamauchi et al., 2017) and expand the repertoire of Ntn1 functions in the developing nervous system.

Distinct functions for Ntn1 in confinement along the rostrocaudal axis

Compared to other aspects of neural development, little is known about the initiation or maintenance of the CNS-PNS boundary, which selectively permits the passage of neural processes – but not cell bodies – into peripheral nerves. Studies in the spinal cord have highlighted the importance of BCCs (Vermeren et al., 2003) and chemorepellents such as Ntn5 (Garrett et al., 2016), Sema3B, Sema3G, and Sema6A (Bron et al., 2007; Mauti et al., 2007) in retaining motor neuron cell bodies inside the CNS, even as they extend their axons out to the periphery. In stark contrast, confinement phenotypes have not been reported in the hindbrain, although BCCs express similar repellents at cranial nerve roots. For example, we found no evidence of ectopic Pax6+ neurons in the cochlea of *Ntn5*$^{-/-}$ mice (data not shown), despite the presence of ectopic motor neurons in the spinal cord (Garrett et al., 2016). This discrepancy underscores two points. First, the molecular mechanisms that define the CNS-PNS boundary in the vertebrate brainstem remain unknown, and second, the hindbrain and the spinal cord may have evolved unique ways of maintaining the CNS-PNS boundary.

Indeed, our work illustrates that the same molecule may have distinct functions in hindbrain versus spinal cord confinement. In the spinal cord, Ntn1 plays a relatively limited role, preventing the axons of a single population of neurons from straying into the periphery. Notably, the cell bodies do not follow in *Ntn1* mutants (Laumonnerie et al., 2014). Thus, in this context, the misrouting of CNS axons into the PNS is much like other axon guidance phenotypes. In contrast, in the hindbrain, Ntn1 signaling appears to play an integral role in defining the CNS-PNS boundary, as evidenced by both the sheer number of neurons exiting the CNS, and most

importantly, the departure of cell bodies, which an effective CNS-PNS boundary absolutely forbids. These differences in Ntn1 function may reflect the distinct developmental demands of the two brain regions: whereas migration is limited in the spinal cord, there is extensive migration of multiple populations of neurons over long distances and past multiple nerve roots in the hindbrain. As such, having a centrally derived cue play a weightier role in confinement may offer the greater fidelity and robustness needed for rhombic lip derivatives to complete their migratory routes successfully. It remains to be seen whether another centrally derived cue might play a more prominent role in confinement in the spinal cord, particularly since most motor neurons stay within the CNS even when all BCCs are ablated (Vermeren et al., 2003).

Cell- and non-cell-autonomous functions for multiple Ntn1 receptors in confinement

Our findings provide additional evidence for Ntn1's multi-functionality, which likely depends on its diverse repertoire of receptors. PNs express receptors mediating both attraction, such as DCC, and repulsion, such as Unc5B/C. However, Ntn1-mediated confinement does not seem to depend on repulsion, since PNs remain within the CNS in *Unc5b* and *Unc5c* mutants (Di Meglio et al., 2013; Kim and Ackerman, 2011). Moreover, both HoxB4+ (Unc5B-low) and HoxB4- (Unc5B-high) PNs (Di Meglio et al., 2013) escape into the periphery in *Ntn1* hypomorphs (Figure S7), indicating that differential responsiveness to Ntn1 cannot account for the partial phenotype. Thus, Unc5B/C appear to influence only later stages of Ntn1-mediated PN migration, much as Unc5A/C position commissural neuron cell bodies in the spinal cord but are not required for confinement of their axons (Laumonnerie et al., 2014).

In contrast, as an obligate receptor expressed on commissural axons and PNs, DCC mediates confinement in both the spinal cord and hindbrain. We also discovered a surprising role

for Neogenin *in trans*, as *Neogenin* is neither expressed nor required in migrating PNs, though it is present at low levels throughout the neuroepithelium and at higher levels on cranial nerves and in the surrounding mesenchyme. These data suggest that complete containment depends both on Ntn1-DCC signaling within PNs, but also on Ntn1-receptor interactions in the environment. Since Ntn1-Neogenin interactions mediate adhesion in the developing mammary gland (Srinivasan et al., 2003), similar interactions in the BM around the VIIIth nerve could prevent movement into the nerve root, providing an additional safeguard for CNS-PNS segregation. However, any effects of Ntn1 signaling on the structural integrity of the CNS-PNS border are likely to be subtle, as the BM did not appear strikingly different in *Ntn1*$^{-/-}$ mutants by EM or immunostaining. Moreover, PNs stay confined to the CNS in *ISPD* mutants (data not shown), which have fragmented BMs (Wright et al., 2012). Hence, disrupting boundaries alone is not sufficient to induce the departure of CNS neurons, indicating that Ntn1 plays an active signaling role across multiple cell types in confining migrating neurons to the CNS.

Finding unity in Ntn1's diverse functions: a role for locally-produced Ntn1 in neural development

In addition to being the archetype of diffusible guidance cues, much of Ntn1's prominence can be attributed to its versatility. Beyond its role in axon guidance, cell migration, and confinement, Ntn1 modulates angiogenesis and tissue morphogenesis, cell adhesion, synapse formation, and cell survival in cancer (reviewed in Cirulli and Yebra, 2007; Lai Wing Sun et al., 2011). Historically, a large emphasis has been placed on the division between long- and short-range functions, which are categorized based on where Ntn1 acts relative to the source of its expression. For instance, textbook models of Ntn1 as a long-range attractant depict commissural

axons navigating along an increasing gradient of FP-derived Ntn1 in the spinal cord. The situation *in vivo*, however, is more complicated. Although Ntn1 is distributed in a gradient in the spinal cord (Kennedy et al., 2006) and can act over a distance *in vitro* (Kennedy et al., 1994; Yee et al., 1999), it was purified as a heparin-binding protein (Serafini et al., 1994) and found to interact with BM components, including type IV collagen and heparin sulfate proteoglycans (Geisbrecht et al., 2003; Geisen et al., 2008; Kappler et al., 2000). This had raised the possibility that it might function at both short- and long-range (Serafini et al., 1994; Kennedy et al., 1994), and a local role in guidance was soon demonstrated at the optic nerve head (Deiner et al., 1997). Short-range functions have also been demonstrated during tissue morphogenesis, such as BM breakdown in the inner ear (Nishitani et al., 2017; Salminen et al., 2000) or adhesion between two cell layers in the mammary gland (Srinivasan et al., 2003).

Our findings add to a growing body of work that suggest that many of Ntn1's other functions in the nervous system may be grounded in local signaling, a shared mechanism that may provide a foundation for its diverse roles. Membrane-tethered versions of Ntn, for example, can rescue guidance defects in the *Drosophila* nerve cord and visual system that were previously ascribed to soluble Ntn (Brankatschk and Dickson, 2006; Timofeev et al., 2012). More recently, several groups have demonstrated that commissural guidance depends on ventricular zone-derived Ntn1 accumulating in the SPR and along the commissural axons (Dominici et al., 2017; Varadarajan et al., 2017; Yamauchi et al., 2017), expanding on related observations (Charron et al., 2003; Kennedy et al., 2006). We have similarly revealed a role for SPR-localized Ntn1 in cellular confinement, providing an alternative explanation for both the reduced number of PNs in $Ntn1^{trap/trap}$ mice, which was thought to reflect Ntn1's tropic and trophic roles (Yee et al., 1999), and the presence of ectopic neurons in $DCC^{-/-}$ cochleae, which was attributed to a mis-

positioning of spiral ganglion neurons (Kim et al., 2016). Thus, across multiple species and in multiple regions of the nervous system, Ntn1 appears to act locally to mediate its purported long-range functions, blurring the distinction between its long- and short-range activities.

Given the clear importance of Ntn1 for nervous system wiring, the possibility that Ntn1 may not act as a long-range instructive cue for pontine neurons raises the question of where the directional information comes from. One idea is that a gradient of Ntn1 activity is achieved through interactions with other cues in the environment. Indeed, every confirmed Ntn1 receptor also interacts with additional ligands (Ahmed et al., 2011; Karaulanov et al., 2009; Rajagopalan et al., 2004; Yamagishi et al., 2011), raising the possibility that Ntn1 is a crucial collaborator for many guidance pathways, perhaps mediating short-range interactions that are necessary for axons to grow reliably toward other ligands. This could occur either directly, i.e. by binding to the same receptors, or indirectly, i.e. by attaching migrating neurons to the BM, where they may be steered by other cues such as Slits. This may explain Ntn1's ability to augment the effect of other guidance molecules synergistically (Morales and Kania, 2016). Thus, even twenty years after its discovery, Ntn1 continues to inform new models for how the complex networks of the nervous system are constructed reliably and accurately using relatively few guidance cues.

— CHAPTER FOUR —

Concluding remarks and future directions

Within the past year, multiple papers (Dominici et al., 2017; Moreno-Bravo et al., 2018; Varadarajan et al., 2017; Yamauchi et al., 2017; Yung et al., 2018) have revealed a local role for Ntn1 in model systems where Ntn1 was previously thought to function as a long-range chemoattractant. Though it may appear as if the field is moving to a unified view of Ntn1 as a short-range cue, we are still far from decisively ruling out all long-range functions for Ntn1 in vertebrate development. Part of the difficulty in drawing a firm conclusion about Ntn1's functions lies in our poor understanding of how Ntn1 behaves as a molecule *in vivo.* How efficiently does Ntn1 diffuse, and how is its movement affected by cell density, components of the extracellular matrix, and even other axon guidance cues? More specifically, in the hindbrain, is Ntn1 actively trafficked away from nerve roots, or does the absence of a BM allow it to diffuse to the adjacent SPR? The answers to these questions have a direct impact on our interpretation of genetic experiments. For example, hindbrain commissural axons reached the midline in spite of a gap in *Ntn1* expression adjacent to the floor plate (Yamauchi et al., 2017). Yamauchi et al. interpreted the absence of a phenotype as evidence of local Ntn1 diffusion, but if Ntn1 is a poor diffusible molecule *in vivo,* then the data instead point toward the presence of a redundant instructive cue. As with most either-or biological questions, Ntn1 likely can act at both long- and short-range depending on context, which is provided by the extracellular milieu in the immediate vicinity of Ntn1 protein.

The truth is out there: regulators of Ntn1 diffusion in extracellular space

Extracellular components are well-known to regulate both the distribution and function of almost all axon guidance cues (reviewed in Van Vactor et al., 2006), including Ntn1. In particular, heparin sulfate proteoglycans (HSPGs), which are membrane-tethered or secreted

proteins that are decorated with glycosaminoglycan heparan sulfate chains, have been heavily implicated in Ntn1 function. Ntn1 can bind to heparin and heparin sulfates (Geisbrecht et al., 2003; Kappler et al., 2000; Shipp and Hsieh-Wilson, 2007), and sulfate ions facilitate Ntn1-DCC interactions at site 2 (Finci et al., 2014). Loss of *Ext1,* an enzyme that synthesizes heparin sulfate chains, reduces the number of commissural axons at the floor plate and prevents them from responding to Ntn1 *ex vivo* (Matsumoto et al., 2007)*,* and in worms, the HSPGs glypican (Blanchette et al., 2015) and perlecan (Yang et al., 2014) also modulate UNC-6 signaling. Thus, HSPGs play an important role in Ntn1 signaling, though it is unclear how different HSPG isoforms lead to different outcomes of Ntn1 activity and what the precise mechanism(s) of HSPG-Ntn1-DCC interactions are.

One way in which extracellular components could contribute to Ntn1 signaling is by altering Ntn1 localization. The affinity of HSPG isoforms for ligands and receptors varies with the pattern of protein modification; indeed, Ntn1 preferentially binds to heparins with sulfates at certain positions *in vitro* (Shipp and Hsieh-Wilson, 2007). Distinct HSPG isoforms are also frequently cell-specific (Van Vactor et al., 2006). With up to 10^{36} possible isoforms of HSPGs, a combinatorial code of HSPGs could control the spread of Ntn1in a highly context-dependent and tissue-specific manner. For example, Sulf1, an enzyme that removes sulfates from a specific location within heparin sulfate chains, is expressed at the floor plate of the *Xenopus* neural tube where it promotes the ventral accumulation of Shh (Ramsbottom et al., 2014). In other words, due to the presence of a unique HSPG, Shh is less mobile at the floor plate than it is in the rest of the neural tube, resulting in a sharper gradient. One could imagine that by modifying the isoforms of HSPGs—or other BM and extracellular matrix components—along the dorsoventral axis of the spinal cord, a similar mechanism could sculpt locally deposited Ntn1 into an

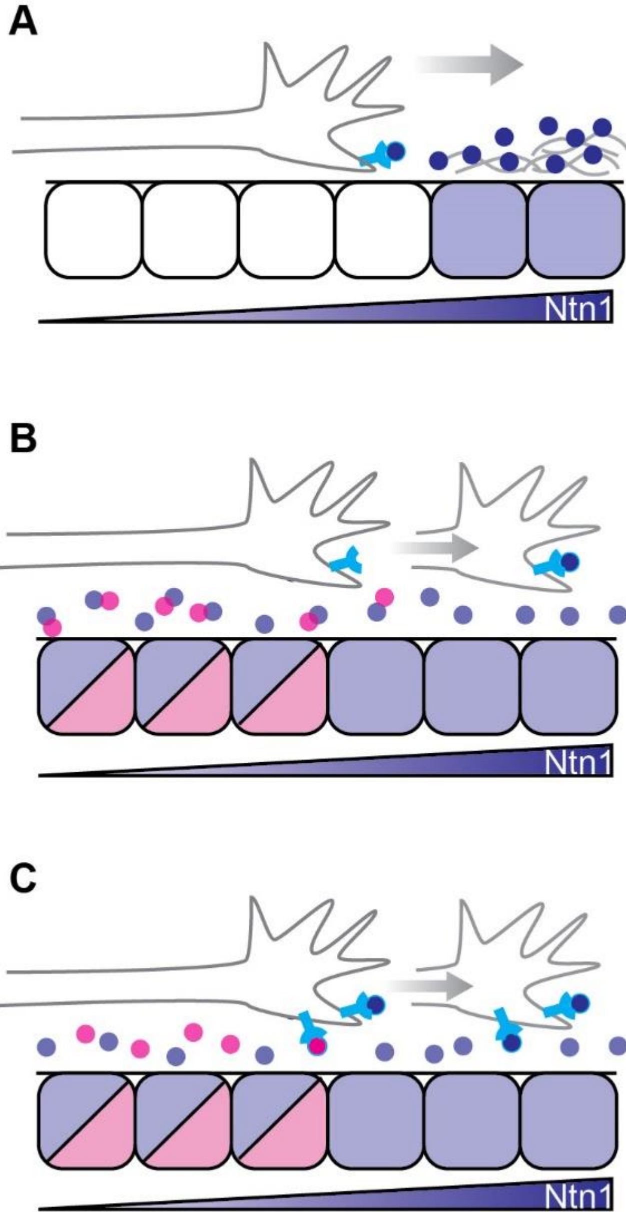
instructive gradient along the sub-pial region (SPR), blurring the boundaries between its purported short- versus long-range mechanisms of function (Figure 15A).

As of yet, HSPGs have not been shown to affect Ntn1 localization, but immunostaining for Ntn1 in *Ext1* mutants, which cannot synthesize heparin sulfate chains, could reveal whether the gross distribution of Ntn1 is dependent on the presence of HSPGs at all. Ntn1 localization may also depend on other extracellular components such as dystroglycan, a glycoprotein required for the proper distribution of Slits in the embryonic spinal cord (Wright et al., 2012). In mutant *Large^{myd}* mice where dystroglycan is abnormally modified, PNs stall in locations similar to those in *Ntn1^{traptrap}* mice (Qu et al., 2006), raising the possibility that dystroglycan is required for Ntn1 distribution. If there is a detectable difference in Ntn1 localization in the hindbrains of *Large^{myd}* mice, this mutant also offers an opportunity to glean insight into how Ntn1's roles in confinement vs. guidance may depend on its localization. Lastly, rather than identifying how single components of the extracellular matrix contribute to the Ntn1 distribution, we can assess the diffusive capabilities of Ntn1 in different tissues *in vivo* by adapting studies of morphogen gradients (Müller et al., 2012). We could start simply by asking what the diffusible range of Ntn1 is in the zebrafish neural tube, which is amenable to live imaging. We can acutely express Ntn1-GFP throughout the CNS, photobleach volumes at different locations such as the floor plate, SPR, and neuroepithelium, and assess how long it takes for the bleached areas to recover. The rate of recovery would reflect Ntn1-GFP mobility in different regions of the neural tube. These experiments would identify where a Ntn1 gradient could form and provide a framework for the extent to which a source of Ntn1 could influence nearby cells.

Even if Ntn1 protein is not distributed in a gradient, variations in the extracellular components present could nevertheless generate a gradient of Ntn1 activity. In addition to

Figure 15. Shaping Ntn1 gradients. (A-C) A growth cone expressing DCC (light blue) migrates over cells expressing Ntn1 (blue rectangles) or a combination of Ntn1 and another guidance cue (blue and pink rectangles). All axon guidance cues are deposited in the immediate overlying extracellular space, and a graded arrow indicates the direction of growth. In (A), two cells each produce 5 molecules of Ntn1 (blue dots). However, Ntn1 produced by the left cell diffuses farther due to a less densely packed extracellular matrix (grey lines), generating a gradient of Ntn1 protein despite its uniform expression. Consequently, the growth cone grows to the right, up the gradient of Ntn1 protein. A gradient of Ntn1 protein can also arise from interactions with other guidance cues (pink dots). In (B) and (C), three cells on the left deposit both another guidance cue and Ntn1, which is produced by all cells. Because this additional guidance molecule can bind to Ntn1 and prevent its interaction with DCC (B), a gradient of Ntn1 protein arises due to differences in its availability. Alternatively, this other guidance cue can bind to DCC and block its interactions with Ntn1 (C), generating a gradient of Ntn1 activity based on the availability of DCC. In both cases, the growth cone is driven to the right, up the perceived Ntn1 gradient.

Figure 15 (Continued)



HSPGs, other morphogens and axon guidance cues also occupy the extracellular space. Draxin, an axon guidance molecule that is unrelated to other known attractants and repellents, binds to site 1 on Ntn1 with an affinity comparable to DCC (Gao et al., 2015; Liu et al., 2018). Since it is expressed in the roof plate and accumulates in the SPR in the dorsal half of the chicken and mouse neural tube, it has been proposed to act as a Ntn1 sink (Gao et al., 2015), sequestering Ntn1 away from DCC and sharpening the gradient of available Ntn1 (Figure 15B). Conversely, Cerebellin 4, is expressed in the spinal cord motor column where it can compete with Ntn1 to bind DCC's FN4-6 domains (Haddick et al., 2014). By occupying DCC receptors, Cerebellin 4 effectively sharpens the Ntn1 gradient in the ventral spinal cord as well (Figure 15C). Ultimately, identifying the plethora of extracellular factors that modulate Ntn1 distribution and activity and understanding how they interact with Ntn1 will shed light on the basis of Ntn1's long- and short-range actions *in vivo,* which likely exist along the continuum of Ntn1's diffusive capabilities.

Functional versatility from the integration of extracellular ligands

In addition to regulating a gradient of Ntn1 protein or activity, extracellular components directly contribute to Ntn1's functional versatility. Many other axon guidance cues can feed into Ntn1 activity via receptor cross-talk, thereby diversifying responses to Ntn1 in a context-dependent manner. There are several ways in which cross-talk occurs (reviewed in Morales and Kania). In some cases, the convergence of two pathways results in a synergistic effect. Commissural axons, for example, are attracted to a combined gradient of Ntn1 and Shh, though shallow gradients of either cue alone do not elicit a response *in vitro* (Sloan et al., 2015). Synergy can also occur in Ntn1-mediated repulsion, as evidenced by the enhanced repulsive response of medial spinal lateral motor column (LMC) axons to sub-repellent concentrations of

ephrinB2 and Ntn1, and in signal integration, such as the heightened responses lateral LMC axons display to both sub-threshold concentrations of ephrinA5, a repellent, and Ntn1, an attractant (Poliak et al., 2015).

In other cases, exposure to additional guidance cues determines Ntn1 responsiveness. The presence of Slit1 enables thalamocortical axons to respond to Ntn1 *in vitro* (Dupin et al., 2015) and *in vivo* (Bielle et al., 2011), and FLRT3-Robo1 *cis* interactions in turn permit Slit1-induced Ntn1 attraction (Leyva-Díaz et al., 2014). Interestingly, different types of cross-talk can occur between the same pathways within the same tissue. In the mammary gland, adhesion between the cap and prelumenal cell layers in the terminal end bud is mediated by Ntn1 and Slit2 signaling in parallel, whereas adhesion between ductal epithelial cell layers is mediated by Ntn1 and Slit2 signaling in synergy (Strickland et al., 2006). These examples highlight the importance of cellular context in Ntn1 signaling, where the functional output of Ntn1 depends on the integration of all extracellular elements, from BM and extracellular matrix components to other cues in the immediate extracellular vicinity.

Ntn1's own receptors could serve as one site of integration. All of Ntn1's receptors, including the canonical Ntn1 receptors DCC/Neogenin and Unc5 family members, associate with additional ligands. Repulsive Guidance Molecules (RGMs) bind to the FN5-6 domains of Neogenin (Bell et al., 2013); FLRTs, members of the fibronectin leucine-rich repeat transmembrane protein family, bind the first Ig domain in Unc5 family members (Karaulanov et al., 2009; Söllner and Wright, 2009; Yamagishi et al., 2011); and Draxin binds the N-terminal Ig domains of DCC in addition to interacting with Ntn1 (Ahmed et al., 2011; Liu et al., 2018). Given its affinity for Ntn1 and as the only alternate ligand that does not compete with Ntn1 for receptor binding, Draxin is uniquely positioned to cross-link Ntn1-DCC complexes across axons,

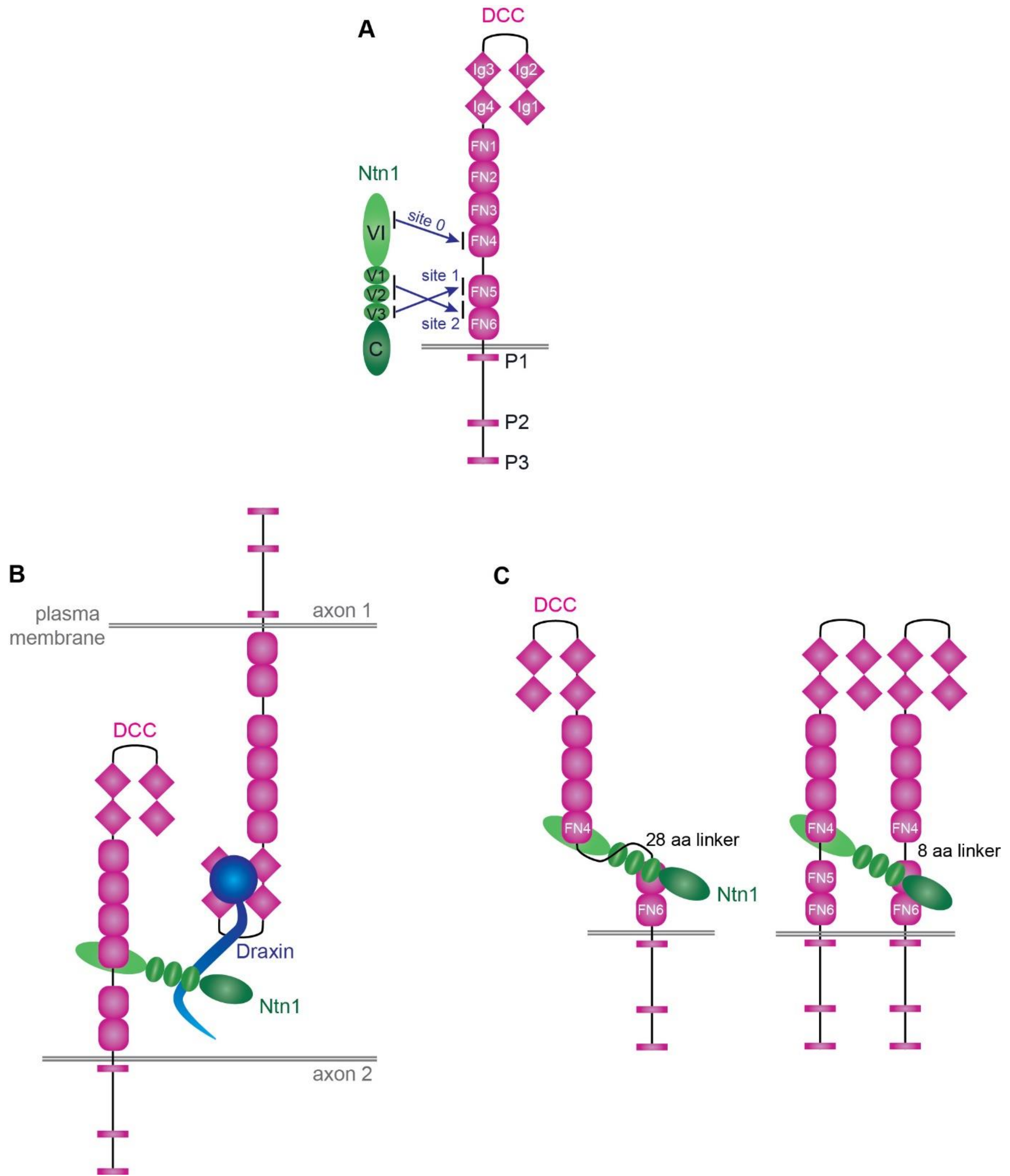
thereby marrying guidance with fasciculation and adhesion (Liu et al., 2018) (Figure 16A, B). This model is consistent with defasciculated commissural axon bundles observed in *Draxin*$^{-/-}$ spinal cords (Islam et al., 2009). Although no phenotypes in PN migration have been reported in *Draxin*$^{-/-}$ mice (Riyadh et al., 2014), PNs strongly express Draxin (Islam et al., 2009) and migrate collectively in a packed stream of neurons. These observations raise the possibility that PNs lacking a Draxin-Ntn1-DCC complex are less capable of maintaining cell-cell contacts, which may facilitate their departure of the AES, and consequently, the CNS in *Ntn1*$^{-/-}$ embryos (Yung et al., 2018). To test this hypothesis, we could use *in utero* electroporations to simultaneously knock down endogenous DCC and introduce a DCC unable to bind to Draxin in migrating PNs. A positive result indicates that DCC integrates Draxin and Ntn1 signaling to mediate PN migration. Furthermore, depending on whether electroporated PNs exit the CNS and/or display defects in their organization and migration, Draxin signaling may distinguish Ntn1's role in confinement and guidance and point to a role in diversifying Ntn1's functions.

Is it time for cake yet

Just as the distribution of the ligand is important to its signaling, the arrangement of Ntn1 receptors at the cell surface could also contribute to Ntn1 activity. Changes in receptor density are predicted to alter a cell's overall sensitivity to a ligand (Caré and Soula, 2011), and in Eph-ephrin signaling, the cellular response is dictated by the scale and organization of Eph clusters (Kania and Klein, 2016). While hetero- and homodimers of Ntn1 receptors are sufficient to initiate signaling (Stein et al., 2001), DCC is often found in clusters (Gopal et al., 2016; Wang et al., 2014), and recent structural data suggest that Ntn1-DCC complexes likely oligomerize as well (Finci et al., 2014; Xu et al., 2014). The functional significance of DCC clustering is

Figure 16. DCC-mediated variations in Ntn1 binding and signaling. (A-C) Ntn1 (green)-DCC (pink) interactions vary with the presence of other guidance cues (B) and the length of the FN4-5 linker (C). (A) Ntn1 and DCC interactions normally occur over three sites (Finci et al., 2014; Xu et al., 2014). The corresponding sites on the ligand and the receptor are linked by blue arrows. (B) Draxin (navy) can bind to the horse-shoe-shaped Ig domains of DCC (pink) (Liu et al., 2018) and to Ntn1's V3 domain (Gao et al., 2015), allowing it to bridge Ntn1 and DCC *in trans*. Draxin-Ntn1 interactions also prevent Ntn1's V3 domain from interacting with DCC. (C) Alternative splicing generates isoforms of DCC that include either a 28 amino acid (aa) linker (left) or a 8 aa linker (right) between FN4-5 that are predicted to bind Ntn1 in different conformations. The length of the 28 aa linker allows one DCC receptor to interact with Ntn1 over two sites, site 0 and site 1 (left, Finci et al., 2017). An additional receptor (not depicted) can interact with the same Ntn1 molecule at site 2 and permit oligomerization of Ntn1 and DCC. The shorter DCC (right) can only interact with Ntn1 one site at a time (Xu et al., 2014).

Figure 16 (Continued)



unclear, yet these data suggest that the density and pattern of DCC localization before and after Ntn1 binding may influence Ntn1's function. Consistent with this hypothesis, the signaling pathways used and the outcome of Ntn1 receptor activation depends on the location of DCC and Neogenin relative to lipid rafts (Furne et al., 2006; Guirland et al., 2004; Hérincs et al., 2005; Tassew et al., 2014), regions in a cell's plasma membrane with elevated cholesterol that are enriched with receptors and signaling molecules. Some of Ntn1's receptors can also recruit additional co-receptors via their cytoplasmic domains, which would further diversify the possible outcomes of Ntn1 signaling. For example, DCC recruits Robo3 via its P3 domain to mediate the ventral migration of precerebellar neurons (Zelina et al., 2014).

A final source of variability in Ntn1 function lies in the structures of its own receptors. DCC and Neogenin are alternatively spliced between FN4-FN5, resulting in isoforms with either an 8 amino acid (DCC_{short}) or 28 amino acid (DCC_{long}) linker between FN4-5 (Keeling et al., 1997; Reale et al., 1994). Though both bind to Ntn1 with similar affinities, DCC_{short} and DCC_{long} are predicted to adopt different conformations upon binding (Finci et al., 2017; Xu et al., 2014) (Figure 16A, C), which may mediate divergent responses to Ntn1. Consistent with that prediction, E19.5-10.5 embryos predominantly express DCC_{short} but shift to DCC_{long} at E10.5-11.5 (Cooper et al., 1995), and DCC_{short} cannot compensate for a reduction in DCC_{long} in dorsal spinal cord explants (Leggere et al., 2016). Whereas loss of *DCC* and decreased DCC_{long} both reduced the width of the ventral commissure to a similar extent, commissural axons appeared defasciculated and invaded the motor column only in *DCC* null animals (Leggere et al., 2016). While the functional distinction(s) between $\mathrm{DCC}_{\mathrm{long}}$ and $\mathrm{DCC}_{\mathrm{short}}$ remain unclear, these results suggest that the two isoforms play complementary roles, with $\mathrm{DCC}_{\mathrm{long}}$ mediating guidance and $\mathrm{DCC}_{\mathrm{short}}$ mediating the adhesion and fasciculation of axons.

To define the roles of each isoform, we can take advantage of two new techniques. BaseScope, a variant of RNAscope that uses short (~50 bp) probes, allows us to detect *DCC*$_{long}$ versus *DCC*$_{short}$ transcripts and characterize their expression pattern. RT-PCR of dorsal spinal cords suggests that some cells, such as commissural neurons, may express both isoforms (Leggere et al., 2016), but another possibility is that distinct cell types may express a single *DCC* isoform. For example, the mammary gland and other non-neuronal tissues may selectively express *DCC/Neogenin*$_{short}$, which may explain why those cells respond to Ntn1 primarily as an adhesive cue. Though this result does not definitively rule out a role for DCC$_{long}$ in adhesion, it is consistent with isoform-specific functions and suggests that different DCC isoforms may bias the cellular response to Ntn1, thereby explaining some of the diversity in the functional outcomes of Ntn1-DCC signaling.

We can also use CRISPR to generate isoform-specific knock-outs and define the functional space of each isoform of DCC. DCC alternative splicing does not occur around a whole exon; instead, the isoforms result from alternative splice sites located at the beginning of exon 17. Consequently, it is easier to delete *DCC*$_{long}$ by introducing a frameshift mutation in the alternatively spliced segment. Based on their expression pattern and the phenotypic differences between *DCC* and *DCC*$_{long}$ null mice, we can identify roles specific to DCC$_{long}$ and DCC$_{short}$ across different tissues. For example, *DCC*$_{long}$ null mice will phenocopy *DCC* null mice only if *DCC*$_{short}$ is not expressed, and a partial phenocopy indicates a role for DCC$_{short}$. One caveat is that defects stemming from loss of *DCC*$_{long}$ may obscure DCC$_{short}$ functions. For example, in the spinal cord, complete loss of *DCC*$_{long}$ may exacerbate the commissural axon guidance defect seen in mice where *DCC*$_{long}$ is reduced. The enhanced guidance defect could secondarily cause defasciculation even if it is primarily mediated by DCC$_{short}$. To more directly interrogate DCC$_{short}$

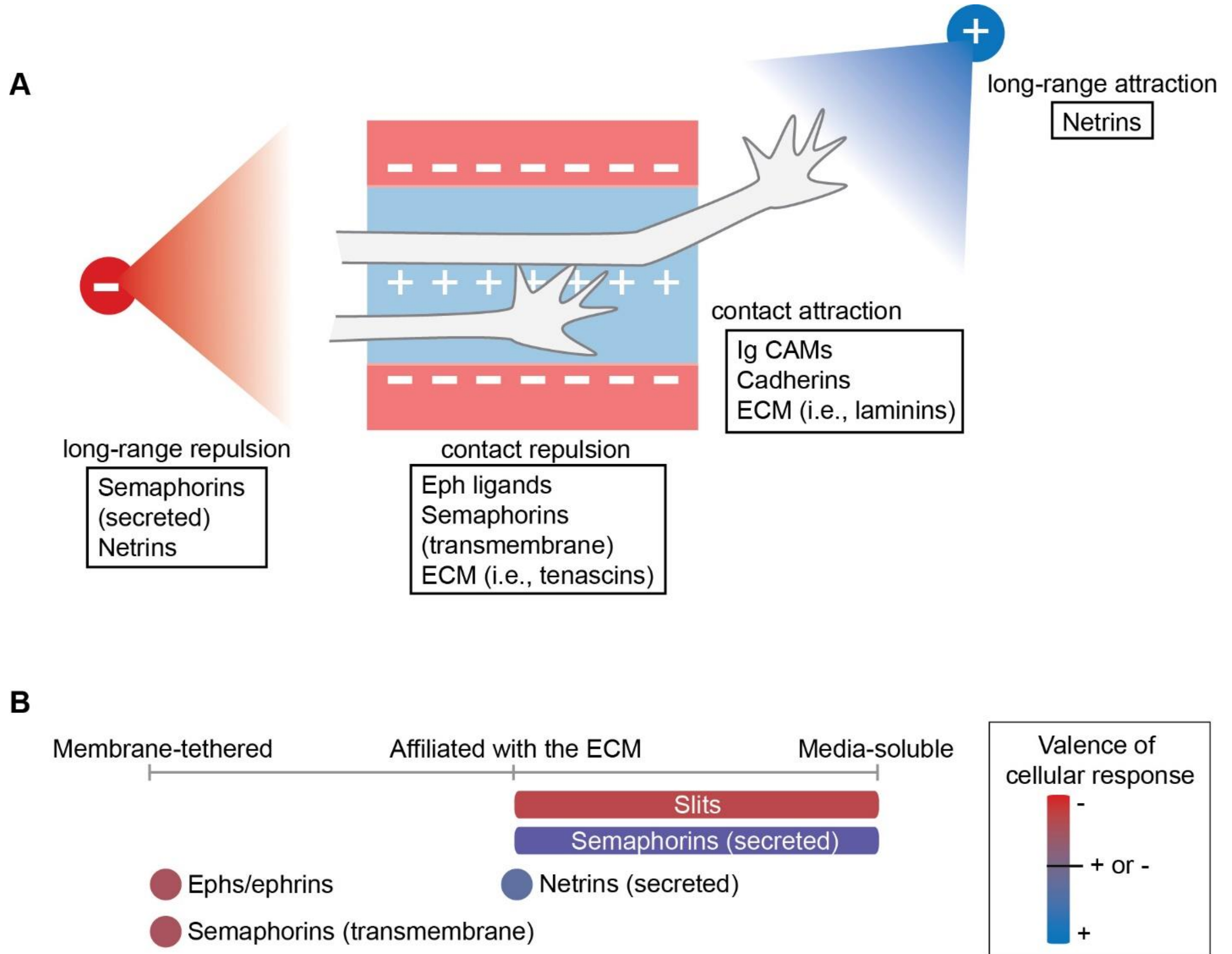
functions in commissural neurons and other accessible cell types, we can knock down DCC_{short} *in vivo* by designing and electroporating shRNA specific to DCC_{short} by targeting the alternatively spliced exon 16-17 junction of its transcript. Ultimately, defining the functional scope of $\mathrm{DCC_{long}}$ and $\mathrm{DCC_{short}}$ would impart a fuller understanding of the molecular basis of Ntn1's versatility and shed light on the relationship between the conformational changes induced by Ntn1-DCC binding and the choice of signaling pathways downstream. In time, we may be able to develop a DCC-based combinatorial code of Ntn1 functions that may allow us to predict how a cell responds to Ntn1 if it is expressing DCC_{long}, DCC_{short}, or some combination of the two receptor isoforms.

While it is unclear how all of these sources of variation feed into Ntn1's roles, the flexibility embedded in the interactions between Ntn1 and its receptors— alternate ligands, the contribution of extracellular components, cross-talk with other guidance pathways, and the range of receptors available and their distribution—likely plays a major role in generating its functional versatility. Since many of Ntn1's divergent functions use the same receptors, it seems probable that its long- and short-range and instructive versus permissive roles exist along a continuum that is fine-tuned by additional extracellular signals that provide environmental context to Ntn1 signaling. Many other axon guidance cues also exhibit dual behaviors and may integrate signals from other extracellular cues, too. Secreted semaphorins, for example, are soluble in media or affiliated with cell membranes (Bagnard et al., 1998), pointing to both long- and short-range functions. Like Ntn1, it can act as an attractant or repellent and has multiple functions in the in the nervous system and beyond (Bagnard et al., 1998; reviewed in Roth et al., 2009).

Rather than trying to fit Ntn1 and other guidance cues into four stringent classes of axon guidance molecules, I propose updating the classic framework (Figure 17A) to better reflect the functional versatility of these ligands that is now known. Any new representation of axon guidance cues (Figure 17B) should communicate the range of cellular responses a ligand can elicit and where it can be found *in vivo,* which directly informs the physical limits of its activity. For example, membrane-bound cues such as transmembrane Semaphorins can only mediate contact-dependent interactions between cells, whereas Ntn1's affiliation with the ECM implies that it has a more flexible range of activity, as extracellular matrix components will regulate its diffusive capability. Moreover, by focusing on where a ligand resides instead of the distance over which it acts, we can motivate research that may inform our understanding of Ntn1 as a molecule, which is required to generate a model of Ntn1 signaling that explains all of its functions in development and disease. Just as current debates about Ntn1 function reflect old arguments for and against neurotropism and contact guidance, we might recall that "[it is an] error to make a hard distinction between chemical and mechanical guidance of nerve fiber growth because at short range they boil down to the same thing: the physiochemical interaction between the nerve fiber and its environment" (Jacobson, 1978).

Figure 17. Updating classic models of axon guidance. (A) Over 20 years ago, four classes of axon guidance cues—long-range chemoattractants and chemorepellents and contact attractants and repellents—were proposed to mediate axon guidance. While it is true that attraction and repulsion at long- and short-ranges can explain much of a growth cone's behavior, the functional flexibility in many axon guidance cues and their roles beyond the nervous system suggest that these stringent classes should be updated to better reflect the broad functions of axon guidance cues *in vivo.* (B) This new graphical summary of axon guidance cues incorporates recent data both on where these ligands are localized (Bagnard et al., 1998; Brose et al., 1999; Varela-Echavarría et al., 1997) and on their effects on target cells within and beyond the nervous system (Brose and Tessier-Lavigne, 2000; Kania and Klein, 2016; Roth et al., 2009). Cues that elicit a predominantly positive response (i.e., attraction, adhesion) are depicted in a bluer hue; complementarily, cues that induce predominantly negative responses are tinted red. In some cases, both positive and negative responses can result, indicated by purple. Thus, at a glance, the reader can intuit how these ligands behave and what their functional space is, independent of the model system. (A) was adapted from Tessier-Lavigne and Goodman, 1996, and the original examples of each axon guidance category are provided as further historical perspective on how views in the axon guidance field have changed.

Figure 17 (Continued)



— APPENDIX —

Supplemental figures

Figure S1. Earlier born rhombic lip neurons exit the CNS at cranial nerve roots in the absence of *Ntn1*. Related to Figure 8. (A-B''') Immunostains of E11.5 transverse head sections from animals that were injected with tamoxifen at E9.5. Low power images of laminin (blue) and tdTomato (red) show that in the absence of Ntn1, commissural neurons fail to form a ventral commissure (white arrowhead) and are located more dorsally (A, B). In control animals (A-A'''), tdTomato+ neurons do not take advantage of weaker areas of BM integrity near nerve entry zones (yellow asterisks, A'-A'''). In contrast, in mutants (B-B'''), a number of processes and cell bodies are observed migrating through the BM (yellow arrows), generating *de novo* breaks in laminin (yellow asterisks). In some cases, neurons traverse the BM before a clear break is observed (green asterisk, B'-B'''). Hb, hindbrain; VIII, vestibulocochlear nerve.

Figure S1 (Continued)

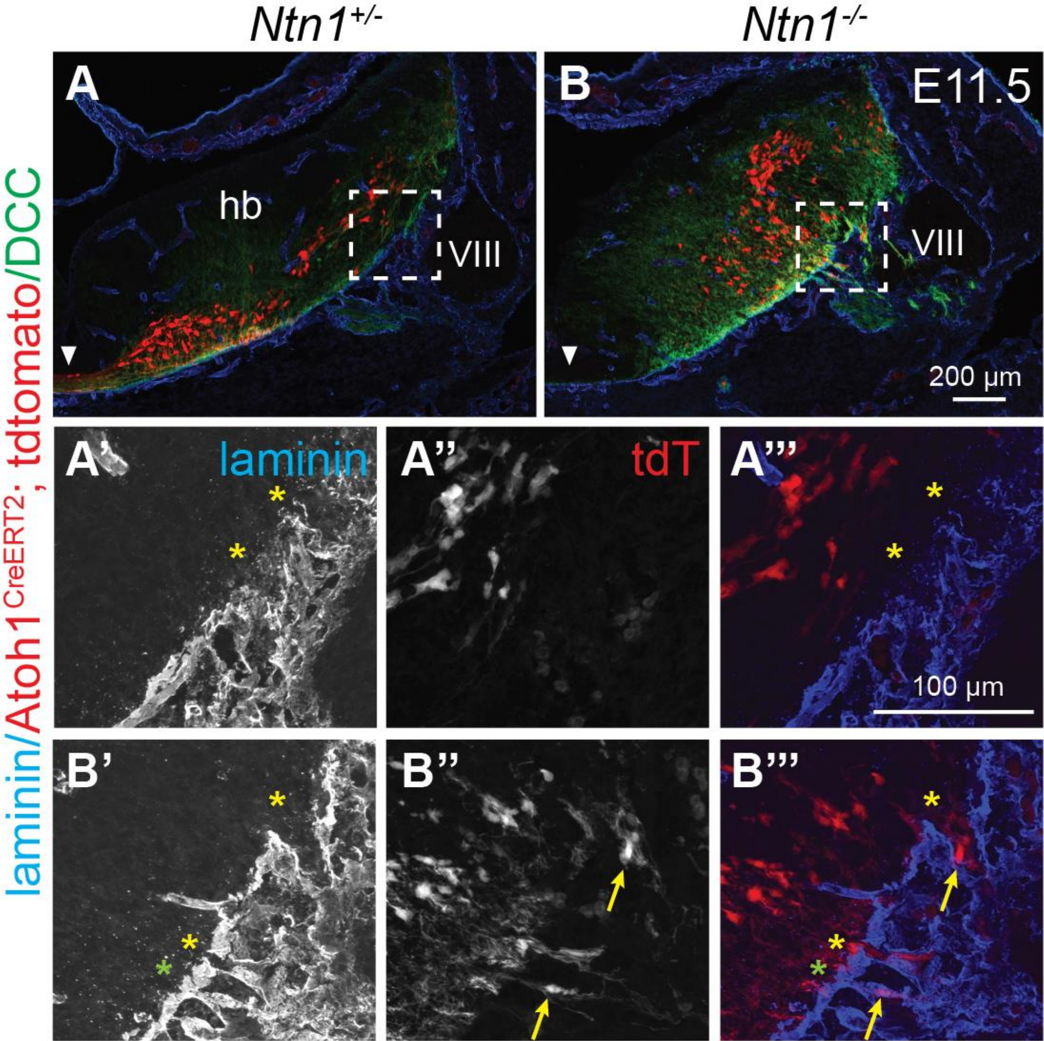


Figure S2. PNs exit the CNS independently of other populations of neurons. Related to Figure 8. (A-E) E15.5 transverse sections of the embryonic head immunostained for DCC (green, A, C-E) and Pax6 (red, A, C-E) or for MafB (B), a marker of earlier born ventral cochlear nucleus (VCN) neurons (Howell *et al.,* 2007). Comparisons between anatomically similar WT sections (A, B) reveal that the MafB+ VCN (*; outlined in B) lies ventromedial to the DCC/Pax6+ secondary rhombic lip (RL) and dorsal to the DCC/Pax6+ AES (filled arrowhead). In 5 out of the 9 *Ntn1*$^{-/-}$ embryos examined, DCC+ VCN axons appear confined to the CNS (C, hollow arrowhead) as in WT animals (A, hollow arrowhead). In the remaining 4/9 animals, VCN axons were seen projecting along the outside of the VIIIth nerve to varying degrees (D, E; arrows). However, in all cases, AES neurons exited the CNS and followed a path that was distinct from the occassional ectopic VCN axons, which departed via the more medial aspect of the VIIIth nerve. AES, anterior extramural stream; RL, secondary rhombic lip; sg, spiral ganglion; *, VCN.

Figure S2 (Continued)

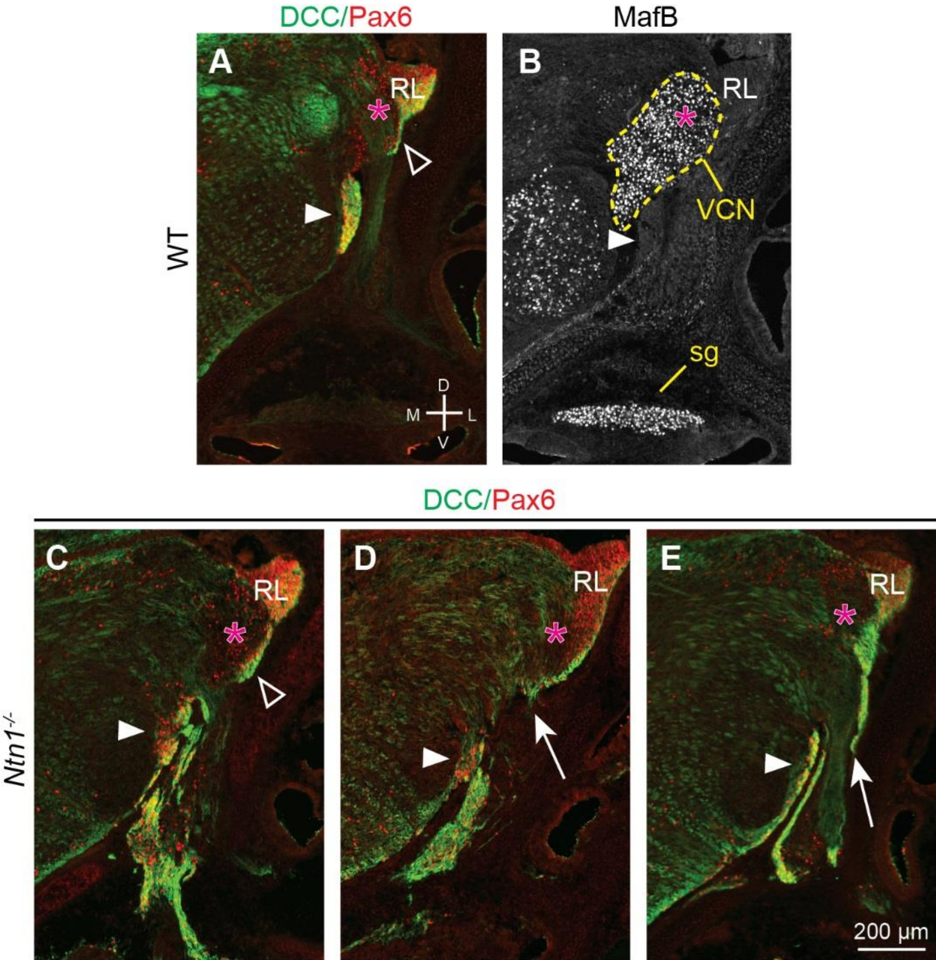


Figure S3. PNs migrate normally in complete *Neo1* null mutants, and loss of *Neo1* selectively from the rhombic lip does not enhance CNS departure in *DCC*$^{-/-}$ animals. Related to Figure 10. (A-D) Immunostains in E15.5 receptor mutants. The AES (yellow arrowhead), indicated by Pax6 (red) and DCC (green, A-B) immunoreactivity, looks grossly normal in complete *Neo1* nulls (A, B). Loss of *Neo1* specifically in rhombic lip precursors (D) did not greatly enhance the *DCC* single mutant phenotype (C). In both cases the AES is semi-intact, and there are a smattering of neurons residing outside of the CNS. Many of these Pax6+ neurons were still located within the vestibulocochlear nerve and had not yet traveled to the cochlea proper. Hb, hindbrain; *, cochlear duct.

Figure S3 (Continued)

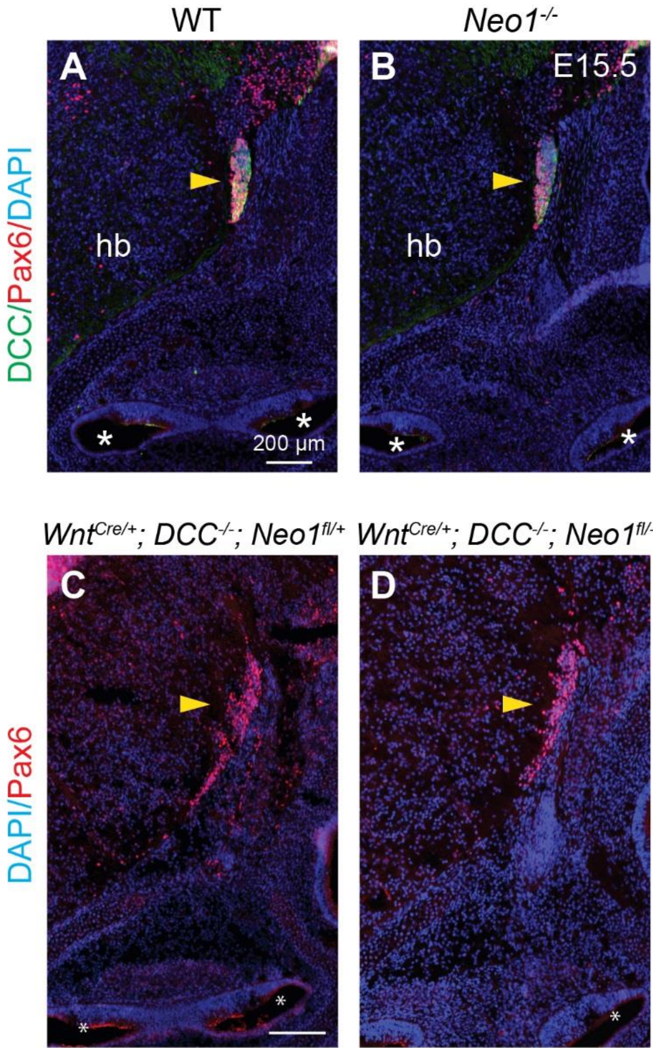
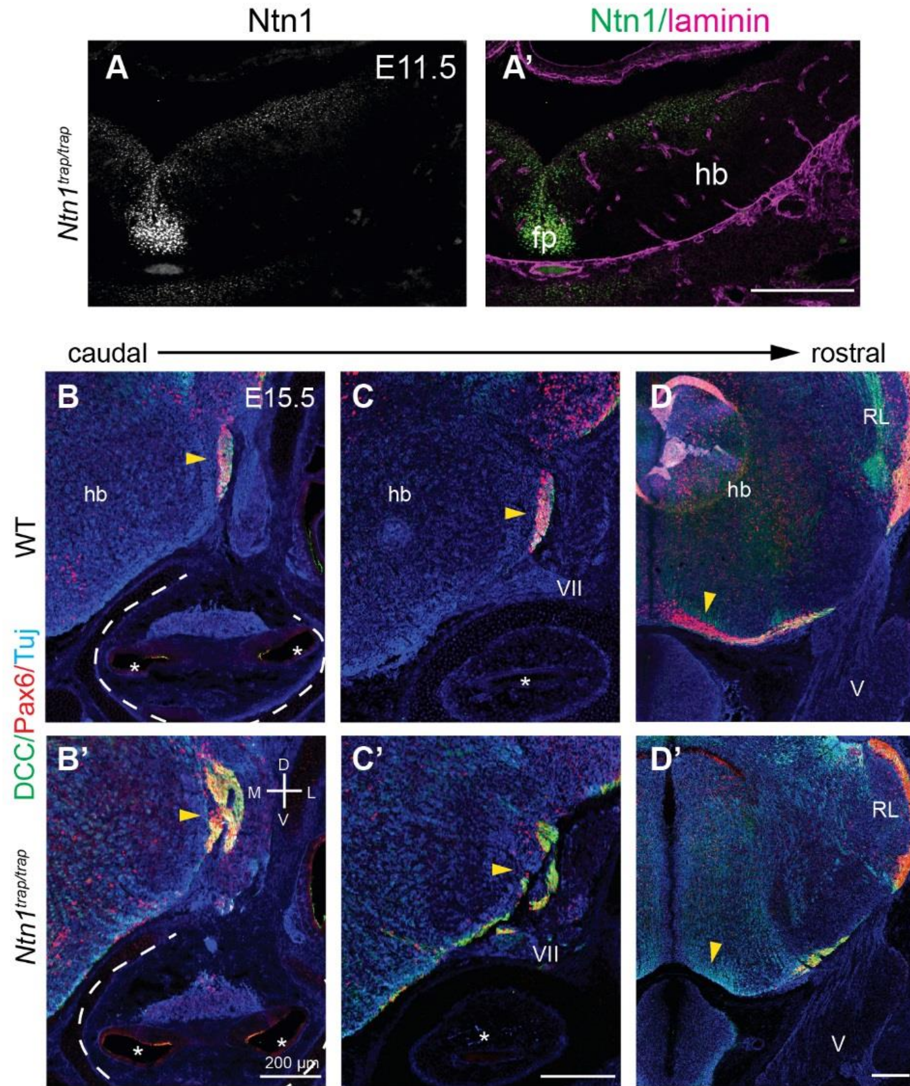
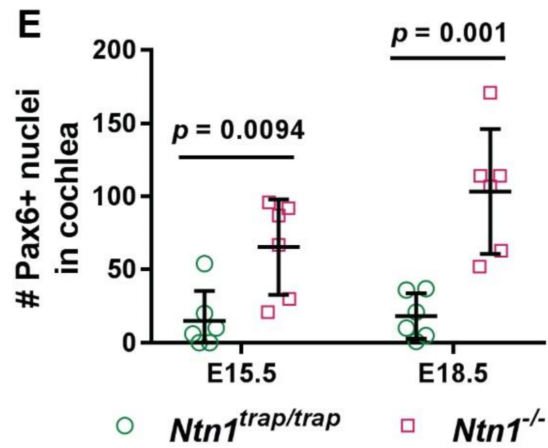


Figure S4. Fewer PNs exit the CNS in hypomorphic *Ntn1* mutants. Related to Figure 12. (A-D') Immunostains of transverse sections of *Ntn1*$^{trap/trap}$ embryonic heads. (A-A') Ntn1 (green) and laminin (magenta) immunostaining at E11.5 shows an absence of Ntn1 at the SPR and puncta reflecting trapped Ntn1 fusion protein at the floor plate and ventricular zone. (B-D') Immunostaining for Tuj (blue), Pax6 (red) and DCC (green) at E15.5 reveal ectopic PNs in hypomorphs (B'-D'). Compared to the intact AES along the sub-pial region in WT animals (B-D, yellow arrowheads), the stream of migrating PNs appears disrupted in *Ntn1*$^{trap/trap}$ animals, with some departing into the periphery at the level of the VIIIth (B') and VIIth (C') nerve roots. Many PNs get close to the midline (D'), which is rarely observed in complete nulls (see Fig. 2), but the pontine nuclei still fail to form. (E) Quantification of ectopic Pax6+ neurons in the base and middle turns of the cochlea in hypomorphic vs. complete null mice (mean ± S.D., Student's t-test). Fp, floor plate; hb, hindbrain; V, trigeminal nerve; VII, facial nerve.

Figure S4 (Continued)





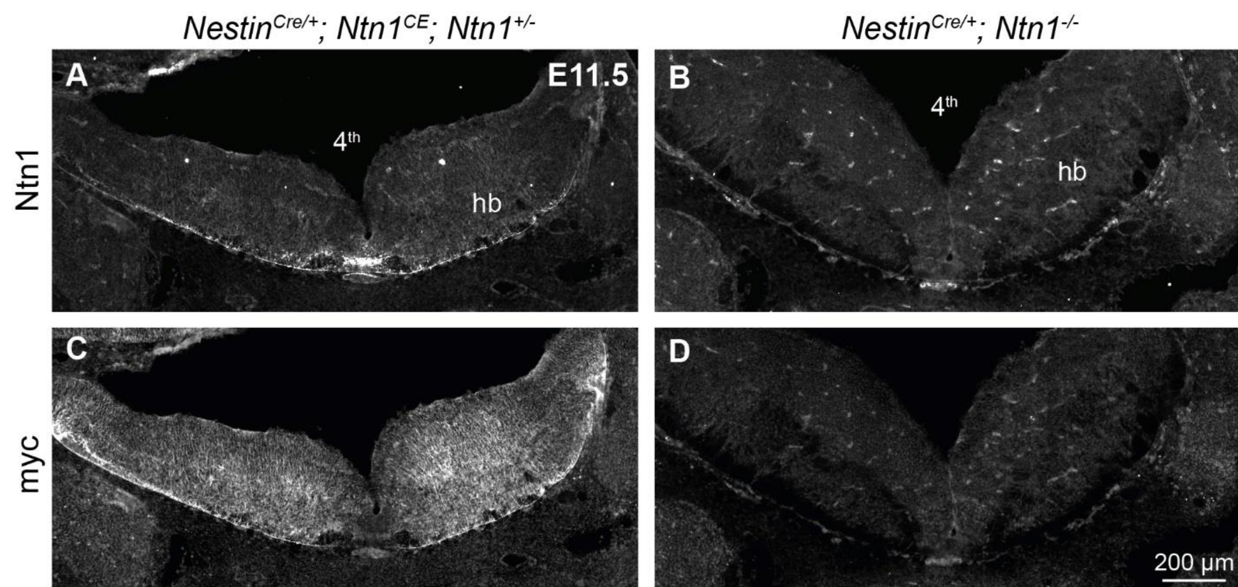


Figure S5. Ntn1 and myc antibodies are epitope-specific. Related to Figure 13. (A-D) Immunostains of transverse sections of E11.5 embryonic heads. (A-B) Ntn1 immunostaining is eliminated in *Ntn1*$^{-/-}$ animals. (C-D) Myc immunostaining is only present in the presence of Cre and the *Ntn1* conditional expressor allele. 4th, fourth ventricle; hb, hindbrain.

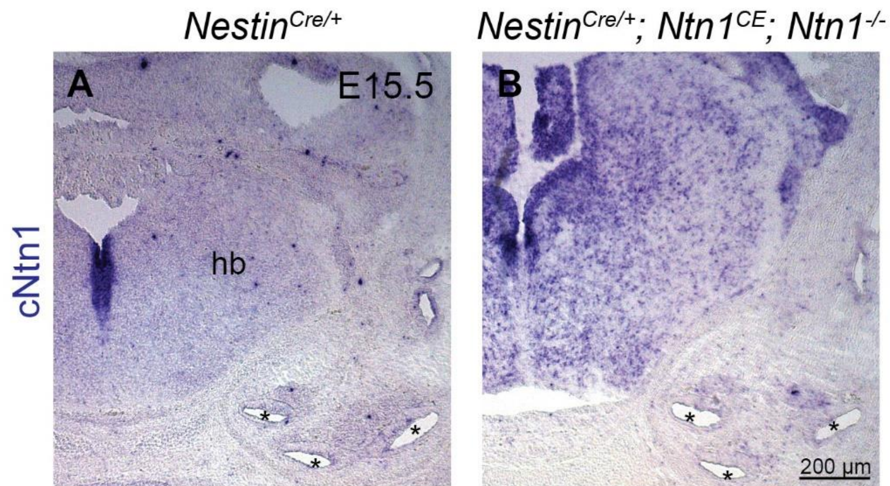


Figure S6. ***Nestin^{Cre}*** **drives broad** ***cNtn1*** **expression in the hindbrain. Related to Figure 13.**

(A-B) *In situ* hybridization for *cNtn1* shows cross-reactivity with endogenous *mNtn1* at the midline (A); *cNtn1* expression expands throughout the rest of the hindbrain in the presence of the conditional allele (B). Hb, hindbrain; *, cochlear ducts.

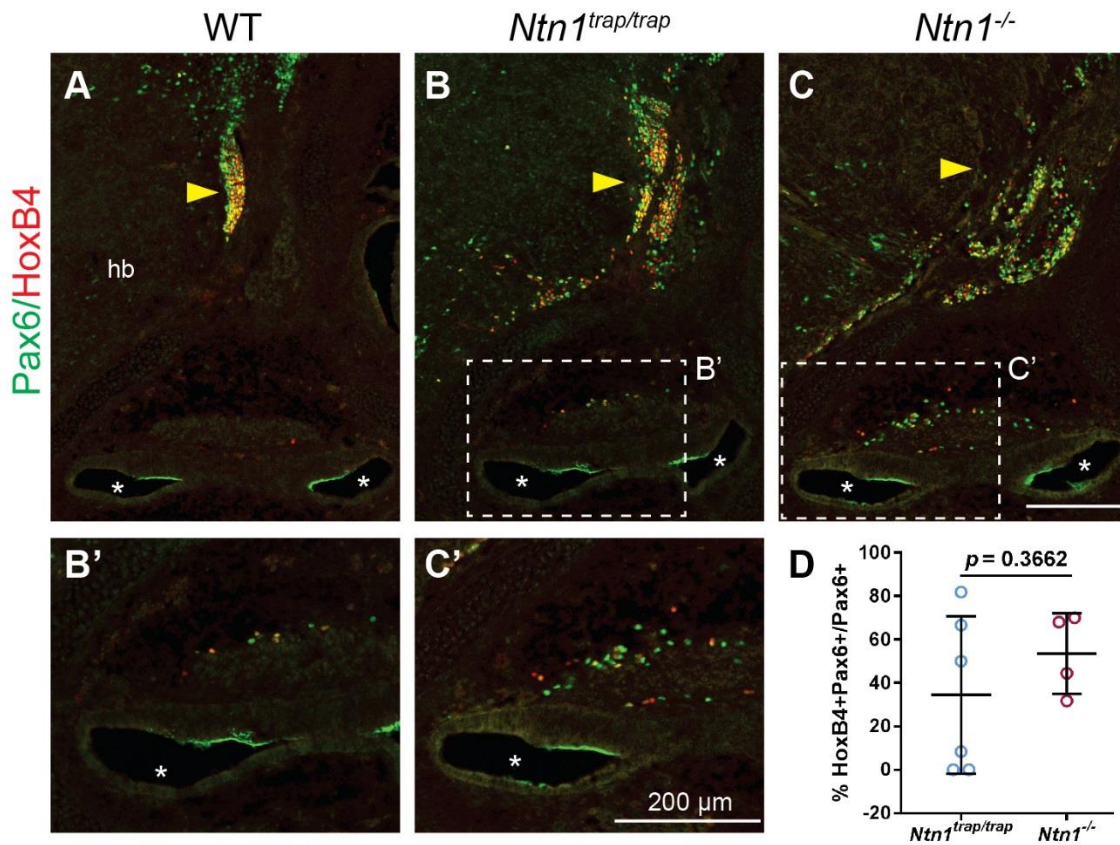


Figure S7: PN subsets do not preferentially exit the CNS in *Ntn1* mutants. Related to Figure 10. (A-C) E15.5 transverse embryonic head sections immunostained for Pax6 (green), which labels all PNs, and HoxB4 (red), which labels subsets of PNs in a dorsoventral gradient (dorsal low, ventral high) (di Meglio et al., 2013). When compared to WT animals (A), *Ntn1* hypomorphs appear to retain this gradient of expression in remnants of the AES (B), indicating that the identity of PN subsets are preserved. We find that both HoxB4+ and HoxB4- subsets of PNs exit the CNS when Ntn1 levels are reduced (B) or eliminated (C), and they appear in the base of the cochlea at similar rates (B', C'), quantified in (D) (mean ± S.D., Student's t-test). Hb, hindbrain; *, cochlear duct.

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