CHAPTER FIVE

Dorsal commissural axon guidance in the developing spinal cord

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Abstract

Commissural axons have been a key model system for identifying axon guidance signals in vertebrates. This review summarizes the current thinking about the molecular and cellular mechanisms that establish a specific commissural neural circuit: the dI1 neurons in the developing spinal cord. We assess the contribution of long- and short-range

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signaling while sequentially following the developmental timeline from the birth of dI1 neurons, to the extension of commissural axons first circumferentially and then contralaterally into the ventral funiculus.

1. Introduction

Our ability to think, feel and perform complex tasks depends on neurons making the correct connections with their target cells. The task of precisely wiring the nervous system begins during embryonic development when neurons extend axons into the embryonic environment, to navigate a specific path toward their synaptic targets. Errors in this process can lead to devastating deficits in functions as diverse as cognition, respiration, movement and speech. The process of axon guidance has been studied for over 100 years, beginning with Ramón y Cajal's seminal observation that the direction of axonal growth is mediated by the growth cone, a specialized, transient structure at the tip of the axon (Ramón y Cajal, 1995; Sotelo, 2002). Cajal described growth cones for axons projecting along early trajectories in the chicken embryonic spinal cord. He was thus also the first to note the importance of commissural axons, which project contralaterally, across midline structures. Commissural axons are a key organizing feature of the central nervous system (CNS) (Comer, Alvarez, Butler, & Kaltschmidt, 2019), with the capacity to carry and integrate information across the nervous system. While they arise from multiple populations of interneurons in the spinal cord and brain (Wentworth, 1984; Yaginuma, Homma, Kunzi, & Oppenheim, 1991), this review will focus specifically on the cellular and molecular mechanisms that specify and guide commissural axons extending from the dorsal interneuron (dI) 1 population of spinal neurons. dI1s mediate proprioception, our usually unconscious ability to sense the position and movement of our bodies in space (Yuengert et al., 2015).

dI1 commissural axons have been a key model system for identifying the molecular repertoire of signals that direct growth cones into their correct trajectory (Charron & Tessier-Lavigne, 2005; Chedotal, 2019; Ducuing, Gardette, Pignata, Tauszig-Delamasure, & Castellani, 2019). Guidance cues are generally classified as acting as either attractants or repellants, with short-range or long-range activities depending on the range over which they elicit a response (Dickson, 2002; Tessier-Lavigne & Goodman, 1996). Thus, long-range cues are usually diffusible, acting over hundreds of microns, while short-range cues are often

contact-dependent, either membrane-bound or tethered to the extracellular matrix (ECM), with effects limited to a range of 1–2 cell diameters (Tessier-Lavigne & Goodman, 1996). While most studies have focused on the role of long-range cues guiding dI1 axons, interest has more recently shifted to considering the importance of local signals. This review will weigh the contribution of long- and short-range signaling while sequentially following the dI1 population over the developmental period where neural progenitor cells (NPCs) are specified as dI1s, and dI1 axons are directed into their trajectory, first circumferentially toward ventral midline, and then longitudinally in the spinocerebellar tract.

2. Specification of the dI1 population of dorsal spinal neurons

2.1 Cellular organization of the embryonic spinal cord

Dorsal spinal commissural neurons first arise in the neural tube, an early embryonic structure that is generated when the neural plate folds to form a cylindrical tube. Initially only a single cell thick, the neural tube is a pseudostratified epithelium comprised of rapidly dividing neural progenitor cells (NPCs). The early neural tube consists of two compartments: a medial ventricular zone (VZ), containing the nuclei of the NPCs and a more lateral marginal layer, containing the processes of the NPCs (Altman & Bayer, 1984; Butler & Bronner, 2015). These processes are radial, spanning the VZ with an apical attachment, on the lumenal side of the neuroepithelium, and a basal "endfeet" attachment on the basement membrane, which is also known as the pial surface (Fig. 1A). This architecture permits the cell bodies of the NPCs to migrate back and forth along a lateral-medial axis within the VZ, as a function of the cell cycle, with mitosis taking place on the apical surface. NPCs continue dividing until they are ready to differentiate into mature neuronal subtypes. Newly formed post-mitotic neurons lose their apical-basal attachments and migrate laterally out of the VZ to form a third layer, the mantle layer. This layer will ultimately become the gray matter of the adult spinal cord. As development proceeds, the marginal layer expands to contain the fiber tracts, eventually becoming the white matter of the spinal cord.

As NPCs divide, they are subdivided into discrete progenitor (p) domains, defined by combinatorial codes of transcription factors (Fig. 1B, C) (Briscoe, Pierani, Jessell, & Ericson, 2000; Jessell, 2000; Shirasaki & Pfaff, 2002). The identity of these progenitor domains is dependent on both intrinsic signaling

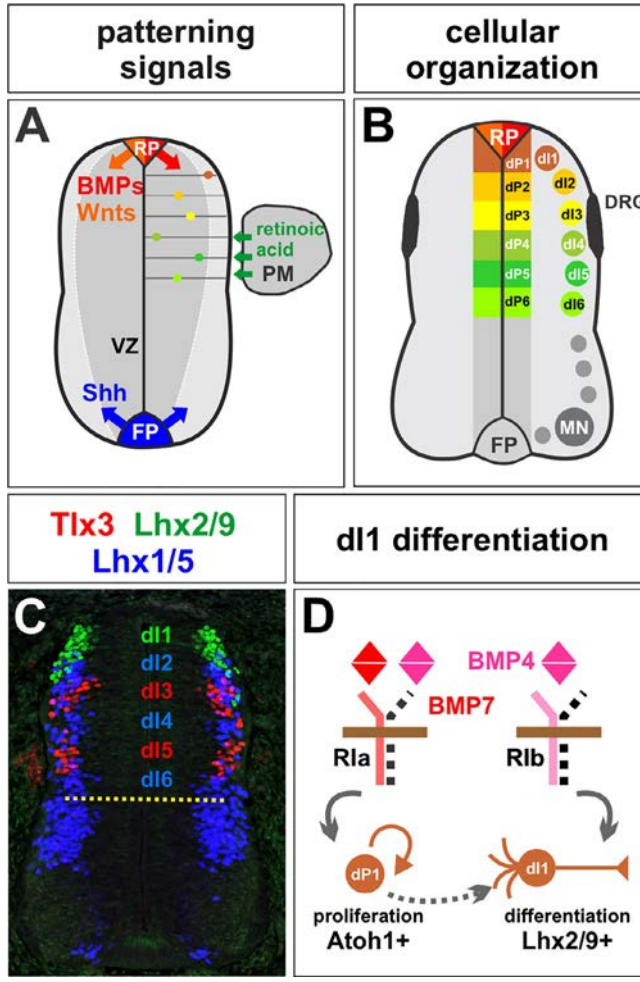

Fig. 1 Organization and specification of dI1s in the spinal cord. (A, B) Six domains of dorsal progenitor neurons (dP1–dP6) arise in the ventricular zone (VZ) in response to BMP/Wnt signals from the roof plate (RP) and retinoic acid (RA) from paraxial mesoderm (PM). This process results in six distinct classes of post-mitotic dorsal neurons (dI1-dI6). The ventral spinal cord is patterned by a gradient of Shh from the floor plate FP. (C) Dorsal interneurons can be distinguished by their expression of distinct complements of transcription factors (Andrews et al., 2017). (D) Model for the specification of dI1s. Both BMP4 and BMP7 can promote Atoh1^{+} dP1 patterning through Bmprla or Bmprlb (chicken), but only BMP4 directs progenitors to differentiate as Lhx2^{+} dI1s through Bmprlb (mouse and chicken) (Andrews et al., 2017).

from midline structures as well as extrinsic signaling from surrounding tissue, including the notochord and the paraxial mesoderm (Butler & Bronner, 2015). Briefly, ventral identity is specified by a putative gradient of sonic hedgehog (Shh) emanating from the floor plate (FP) at the ventral midline. Shh acts as a morphogen, with the concentration and duration of Shh signaling being decoded by signaling machinery in the primary cilia of NPCs on the apical side of the VZ (Briscoe & Ericson, 2001; Dessaud et al., 2007; Kong et al., 2015; Sasai & Briscoe, 2012). Sharp boundaries between the progenitor domains are cemented by cross-repressive interactions between the transcription factors that define domain identity (Alaynick, Jessell, & Pfaff, 2011; Briscoe et al., 2000). This process results in the specification of at least five progenitor domains in the ventral spinal cord: p0-3 and pMN, which give rise to the ventral interneurons and motor neurons (MNs) respectively. Similarly, the dorsal spinal cord contains at least six classes of dorsal interneurons, dI1-dI6, which are derived from six dP populations, dP1-dP6 (Fig. 1B, C). The identity and proliferative capacity of the dorsal-most NPCs (dP1-3P3) depends on signals from the roof plate (RP) at the dorsal midline (Lee, Dietrich, & Jessell, 2000), which include members of the bone morphogenetic protein (Bmp) and Wnt families (Andrews, Kong, Novitch, & Butler, 2019; Liem, Tremml, & Jessell, 1997). The more ventral-dorsal NPCs (dP4-dP6) arise independently from signals from the RP; their identity may be dependent on signals from the adjacent paraxial mesoderm (Le Dreau & Marti, 2013). This organization of the spinal cord results in the different laminae of the spinal cord containing neurons segregated according to their distinct physiological properties and functions (Rexed, 1954). In general, cells associated with control of motor functions are located in or adjacent to the ventral horns whereas cells mediating sensory activities are present within the dorsal horn.

2.2 Molecular specification of dI1 commissural neurons

The focus of this review is to assess the mechanisms that direct the formation of the dI1 commissural circuit. This process begins when the dorsal-most NPCs flanking the RP sequentially differentiate into dP1s and then dI1s. Multiple Wnts have a role in this process, including Wnt1 and Wnt3a, which are required for the formation of both the dI1s and dI2s (Muroyama, Fujihara, Ikeya, Kondoh, & Takada, 2002). Wnts have been proposed to act as mitogens that regulate the size of dI populations (Megason & McMahon, 2002), although their exact role remains unclear.

Bmp signaling is also required for the specification of the dI1 population (Lee et al., 2000; Lee, Mendelsohn, & Jessell, 1998; Wine-Lee et al., 2004). Multiple Bmps, including Bmp4, Bmp5, Bmp6, Bmp7 and Growth/Differentiation Factor (Gdf) 7 are secreted by the RP (Butler & Dodd, 2003; Liem et al., 1997) and are sufficient to promote some dI1 formation (Andrews et al., 2017; Gupta et al., 2018; Lee et al., 1998; Liem. et al., 1997). Bmps have been proposed to act collectively as spatial (Lee & Jessell, 1999) and/or temporal (Tozer, Le Dreau, Marti, & Briscoe, 2013) morphogen gradient(s), to specify dorsal cell fates by analogy with the models of Shh patterning in the ventral spinal cord (Briscoe & Ericson, 2001; Dessaud et al., 2007). However, it has alternatively hypothesized that different Bmps have specific effects on the induction of particular dorsal neural fates (Andrews et al., 2019; Le Dreau et al., 2012; Lee & Jessell, 1999).

Recent studies have distinguished between these models by methodically assessing the ability of RP-derived Bmps to direct dorsal spinal fates by either manipulating *Bmp* expression in chicken embryos *in vivo*, or culturing mouse (m) embryonic stem cells (ESCs) with different concentrations of recombinant Bmp proteins *in vitro* (Andrews et al., 2017). These studies unambiguously demonstrated that Bmps do not act as concentration-dependent morphogens. While altering the level of Bmps changed the effectiveness of NPC responses, i.e. the number of cells that were directed to a specific fate, the dose-dependent changes in dI identity predicted by the spatial or temporal morphogen models were not observed. Rather, each Bmp showed both distinct and reiterative activities directing RP and dI1-dI3 fates (Andrews et al., 2017; Lee & Jessell, 1999). dI1s appear to be most effectively specified by Bmp4 (Andrews et al., 2017; Duval et al., 2019) which acts reiteratively to first promote dP1 proliferation, and then direct these dP1s to differentiate into dI1 neurons (Fig. 1D). Bmps mediate their signal-specific activities by activating distinct type Bmp receptors (Bmprs) in dPs to permit differential progression through the cell cycle (Andrews et al., 2017; Panchision et al., 2001; Timmer, Wang, & Niswander, 2002; Yamauchi, Phan, & Butler, 2008). Activated type I Bmprs phosphorylate and thereby activate the R-Smads, the canonical intracellular mediators of Bmp signaling (Shi & Massague, 2003). Smad1 and Smad5 are the only R-Smads present in the developing spinal cord (Hazen et al., 2012) and it remains unresolved whether both Smad1 and Smad5 (Le Dreau et al., 2012) or Smad5 alone (Hazen et al., 2012) is critical for dI1 fate specification.

As in the ventral spinal cord, the consequence of dorsal signaling is to regulate codes of homeodomain and bHLH transcription factors that first

define, discrete progenitor domains and then distinct classes of post-mitotic neurons (Andrews et al., 2019; Helms & Johnson, 2003; Zhuang & Sockanathan, 2006). The dorsal tube is demarcated by the expression of Pax3 (Goulding, Chalepakis, Deutsch, Erselius, & Gruss, 1991) and Pax7 (Jostes, Walther, & Gruss, 1990), which are the earliest known general markers of dorsal spinal identity. Work *in vitro* has suggested that the upregulation of Pax3/7 in dorsal tissue may depend on retinoic acid (RA) signaling from the paraxial mesoderm (Gupta, Kawaguchi, Mandric, Castellanos, & Butler, 2020). Subsequent Bmp signaling from the dorsal midline results in the cells immediately flanking the RP activating Atoh1 (Helms & Johnson, 1998), a bHLH transcription factor that is both necessary and sufficient for dP1 identity (Gowan et al., 2001). Atoh1 in turn upregulates a transcriptional cassette mediated by Lhx2 (Lh2a) and Lhx9 (Lh2b), which establishes the intraspinal trajectory of commissural neurons. Lhx2 and Lhx9 are initially coexpressed by all new-born dI1s as they start to extend axons (Lee et al., 1998). dI1s also concomitantly migrate ventrally within the mantle layer; on reaching the deep dorsal horn, they segregate into two subpopulations that differentially express either Lhx2 or Lhx9 (Lee et al., 1998; Wilson, Shafer, Lee, & Dodd, 2008). These subpopulations have distinct axial positions and axonal trajectories. The $Lhx2^{+}$ population (dI1c), has a medial position and projects axon contralaterally across the FP whereas the $Lhx9^{+}$ population (dI1i) has a more lateral position and projects axons ipsilaterally within the ventral funiculus (Avraham et al., 2009; Phan, Hazen, Frendo, Jia, & Butler, 2010; Wilson et al., 2008).

3. Guidance of dI1 axons away from the dorsal midline

The first decision made by all newly formed spinal neurons in the mantle zone, is to extend a growth cone into the marginal zone in a specific direction. Depending on their complement of guidance receptors, growth cones interpret signaling information in the neuroepithelial environment to guide them into their initial trajectory. Remarkably, all spinal commissural neurons initially extend axons ventrally thereby establishing one of the organizing architectural principles of the spinal cord: that contralaterally projecting axons only cross at the ventral midline (Altman & Bayer, 1984; Ramón y Cajal, 1995; Wentworth, 1984). The mechanisms that direct commissural axons ventrally have been most extensively assessed for the dI1 population, because it was one of the first vertebrate trajectories to be distinguishable by immunohistochemistry. dI1 axons were initially

detected using antibodies against the glycoprotein, Tag1, which label the dorsal-most subset of commissural axons in their transverse trajectory in the spinal cord, i.e. as they extend up to and across the FP (Dodd, Morton, Karagogeos, Yamamoto, & Jessell, 1988). More recently, antibodies against robo3 have been shown to generally label all dorsal commissural axons (Sabatier et al., 2004; Tulloch, Teo, Carvajal, Tessier-Lavigne, & Jaworski, 2019), while dI1 axons can be unambiguously identified using a genetically encoded marker: the Atoh1::tau*gfp* transgenic mouse line (Hazen et al., 2012; Tran et al., 2013). dI1s are born relatively early, ~E10.5 in the mouse rostral spinal cord (Hazen et al., 2012), arising immediately flanking the RP. In addition to instructing cell fate specification, the RP is also thought to be a key source of axon guidance cues for dI1 axons. There are two complementary mechanistic possibilities: first, the RP is itself intrinsically repulsive and does not support axon growth, and second, the RP secretes a diffusible repellent that orients axons away it, thereby directing dI1 axons to grow ventrally. Supporting the latter model, *in vitro* tissue culture "reorientation" assays demonstrated that an ectopically placed RP explant is sufficient to reorient $Tag1^+$ axons away from the RP, as they extend within an explant of rat dorsal spinal cord (Augsburger, Schuchardt, Hoskins, Dodd, & Butler, 1999).

Multiple factors have been identified as RP repellents. Keratin sulfate, a sulfated proteoglycan specifically localized to the RP, is the key candidate for a short-range signal that locally renders the RP repulsive to axon growth (Snow et al., 1990b). *In vitro* stripe assays have shown that sulfated proteoglycans can repel elongating neurites in a concentration-dependent manner (Snow et al., 1990a). Both members of the Bmp family (Augsburger et al., 1999), and draxin, a novel secreted molecule (Islam et al., 2009), have been proposed to act as long-range guidance repellents for dI1 axons. In both cases, these factors were good candidates based on their localized presence in the RP, and their observed activities in *in vitro* assays. First, Bmps can phenocopy the ability of an ectopic RP to reorient rat $Tag1^+$ commissural axons *in vitro* (Augsburger et al., 1999); a heterodimer of Bmp7 and Gdf7 was subsequently observed to have the most potent reorienting activity (Butler & Dodd, 2003). These studies were amongst the first in the field to demonstrate that instructive growth factors can also act as axon guidance signals, with the striking implication that the Bmp family reiteratively specifies different cellular processes for dI1s, as they mature during development. Similarly, draxin was identified by its ability to prevent the outgrowth of $Tag1^+$ axons extending from a chicken dorsal spinal cord explant (Islam et al., 2009).

While both Bmps and draxin have dramatic *in vitro* activities consistent with a role mediating a long-range repellent, both factors have relatively modest loss of function phenotypes in mouse embryonic spinal cords *in vivo* (Butler & Dodd, 2003; Islam et al., 2009). It thus remains largely unresolved whether the polarizing activities observed *in vitro*, are required to guide dI1 axons away from the dorsal midline *in vivo*. Moreover, subsequent genetic studies assessing whether type I Bmp receptors (BmprI) have reiterative roles directing the formation of dI1 circuits (Yamauchi et al., 2008) did not robustly observe the randomized growth predicted for the loss of a chemorepellent. Similarly, the Smad family are not required for the putative reorienting activities of the Bmps *in vitro* (Hazen et al., 2012; Perron & Dodd, 2011). Rather, the most profound phenotypes observed *in vivo* after modulating the activity of the Bmp signaling pathway, were alterations in the rate at which dI1 axons grew. Specifically, decreasing signaling through the Bmp pathway accelerated dI1 axon growth (Phan et al., 2010; Yamauchi, Varadarajan, Li, & Butler, 2013), while slowed/stalled dI1 axon growth was observed when Bmp signaling was increased (Phan et al., 2010; Yamauchi et al., 2008). Together, these studies suggest that a key *in vivo* role of the Bmps is to set the rate at which dI1 axons grow through the dorsal spinal cord toward the FP. These studies thereby identified a novel "temporal" mode by which the process of axon guidance can be regulated (Phan et al., 2010). In contrast to the well described "spatial" cues which regulate the direction of axon growth, temporal guidance signals regulate rate at which axons grow toward their targets thereby ensuring that axons encounter subsequent spatial signals at a particular time in development. This process permits neural circuits to develop in concert with the rest of the developing embryo. If axons grow at the wrong speed, this process can become uncoupled; for example, accelerated dI1 axons make guidance errors on encountering the FP too early in development (Phan et al., 2010; Phan & Butler, 2013).

The temporal guidance activity of the Bmps appears to be mediated through Lim kinase (Limk) 1, a non-canonical effector of Bmp signaling that regulates actin dynamics (Wen et al., 2007). Limk1 is a direct inhibitor of cofilin, and they act together to control the rate of actin polymerization or "treadmilling". In the current model, the rate which dI1 axons first extend away from the RP is controlled by the Bmps binding to a heterodimeric complex of BmprIb (Yamauchi et al., 2013) and BmprII, which releases Limk1 into the cytosol (Lee-Hoeflich et al., 2004). Limk1 then phosphorylates and inactivates cofilin, which inhibits the depolymerization of actin

(Meberg & Bamburg, 2000) thereby slowing the rate of dI1 axons as they extend through the dorsal spinal cord.

4. Guidance of dI1 axons around the ventricular zone

The transverse path of dI1 axons in the developing spinal cord follows a circumferential dorsal to ventral route toward the FP at the ventral midline. Previous studies suggested this trajectory could be achieved mechanistically by a "push" from the RP and a "pull" from the FP (Fig. 2A) (Kaprielian, Imondi, & Runko, 2000). However, dI1 axons extend precisely around the border of VZ and avoid numerous potential exit points for pioneering axons, including the dorsal root entry zone (DREZ) and the lateral and motor exit points (Laumonnerie, Da Silva, Kania, & Wilson, 2014). Such complex pathfinding is likely to require instructive cues in addition to a simple combination of push/pull signaling. Supporting this hypothesis, netrin1 has been shown to play a crucial role both guiding commissural axons around the VZ and maintaining the CNS-PNS boundary (Laumonnerie, Tong, Alstermark, & Wilson, 2015; Moreno-Bravo, Roig Puiggros, Mehlen, & Chedotal, 2019; Varadarajan & Butler, 2017; Varadarajan et al., 2017). The netrins are a family of laminin-like proteins that were first characterized, in a seminal breakthrough in the early 1990s, as the prototypical vertebrate axon guidance cue (Kennedy, Serafini, de la Torre, & Tessier-Lavigne, 1994; Serafini et al., 1994). Netrin1 (from the Sanskrit *netr*, meaning "one who guides") was identified as a candidate for the FP attractant, whose existence was first hypothesized by Ramón y Cajal in 1890 (de Castro, López-Mascaraque, & De Carlos, 2007); see Section 5 for more details. Netrin1 mediates its activity through two classes of receptors: Deleted in Colon Cancer (Dcc) and the Unc5 family, a

ll members of the immunoglobulin (Ig) domain super family (Engelkamp, 2002; Keino-Masu et al., 1996; Leonardo et al., 1997; Moore, Tessier-Lavigne, & Kennedy, 2007). Dcc is thought to mediate the attractive properties of netrin1 (Fazeli et al., 1997), while the members of the Unc5 family, Unc5A-Unc5D, can mediate the repulsive properties of netrin1 (Dillon et al., 2007; Engelkamp, 2002; Leonardo et al., 1997; Williams et al., 2006).

Netrin1 has a complex distribution pattern in the mouse spinal cord: it is transcribed by both NPCs and the FP cells, with a sharp expression boundary in the dorsal spinal cord at the level of the DREZ (Fig. 2B, C). However, netrin1 protein does not remain in the VZ, rather it appears to be transported

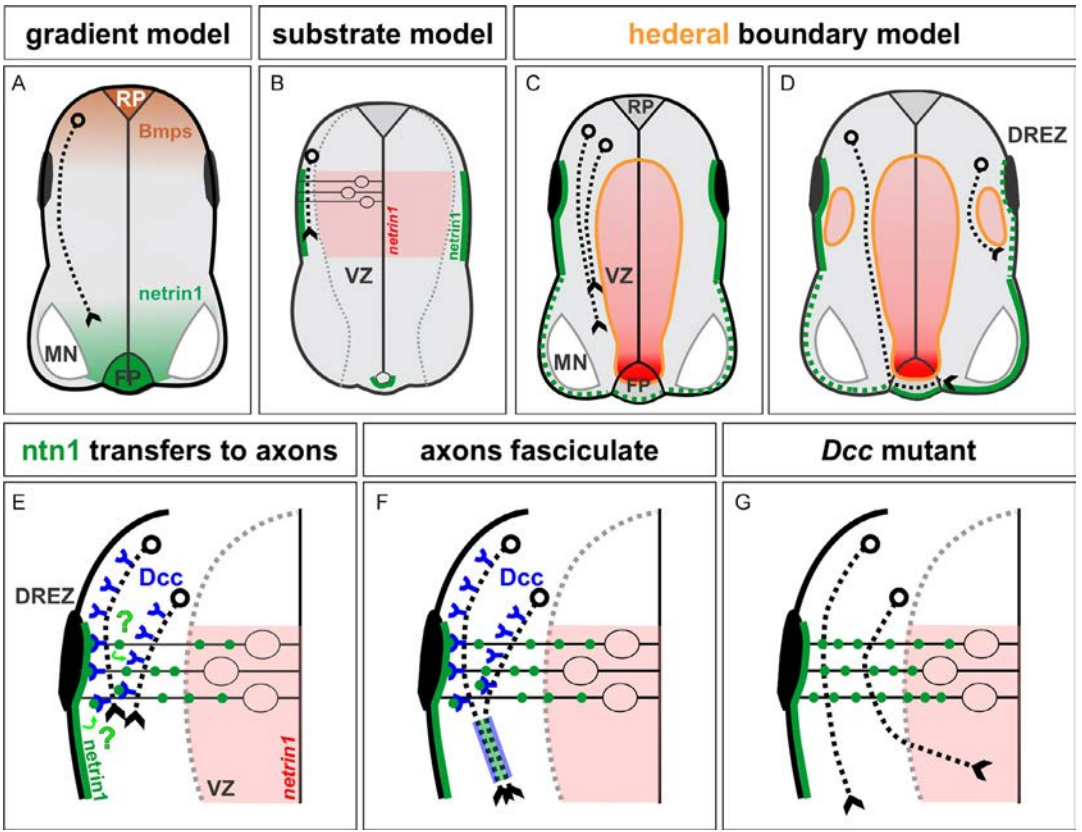

Fig. 2 See figure legend on opposite page.

along the radial processes of the NPCs (Fig. 2B, E). Netrin1 is then deposited on the pial surface, via the glial endfeet, and accumulates on commissural axons (Fig. 3A) (Dominici et al., 2017; Varadarajan et al., 2017). Pial-netrin1 deposition starts as early as E9.5 (Varadarajan & Butler, 2017), immediately before the pioneering dI1 axons commence their trajectory toward the FP (Fig. 2B).

4.1 Netrin1 prevents dI1 axons from innervating the VZ

As pioneering dI1 axons extend ventrally, they grow around the edge of the VZ, without invading it. This behavior is not unique to dI1 axons, rather all spinal axons avoid growing VZ, thereby establishing another architectural organizing principle of the spinal cord. Recent studies have shown that this remarkably homogenous behavior is mediated by NPC-derived netrin1. In the complete absence of netrin1, spinal axons, most notably the robo3^{+} commissural axons, are profusely defasciculated and extend randomly, including into the VZ (Fig. 3B) (Varadarajan et al., 2017; Wu et al., 2019). This effect is

Fig. 2 Models for the long- and short-range activities of netrin1. (A) In the long-range gradient model, the RP-derived Bmps act as a chemorepellent "pushing" commissural axons away from the dorsal midline, while FP-derived netrin1 and Shh function as chemoattractants "pulling" commissural axons to the ventral midline. (B) In the short-range model, pial-netrin1 first orients early born commissural axons to extend ventrally. NPCs transcribe *netrin1* (red) and then deposit netrin1 protein (green) on the pial surface, where it may act as a growth substrate to promote axon extension (Varadarajan et al., 2017). (C) As development progresses, pre-crossing commissural axons extend into the ventral spinal cord, and no longer grow adjacent to pial-netrin1 (dotted green line). Rather, they project precisely around a "hederal" boundary of *netrin1* expressing NPCs (orange line). We have proposed that the netrin1 hederal boundary promotes directed axon fasciculation while preventing innervation of *netrin1* expressing cells. This activity permits commissural axons to grow around the VZ. (D) Commissural axons extend across the FP in a highly fasciculated bundle within a narrow corridor bounded by hederal-*netrin1* and pial-netrin1. Post-crossing commissural axons then turn rostrally to extend in the ventral funiculus, again growing adjacent to a pial-netrin1 substrate (solid green line). Concomitantly, a domain of *netrin1* expressing cells (red) emerges adjacent to the DREZ, which continue to sculpt axonal trajectories within the spinal cord. (E–G) We propose that the netrin1 produced by neural progenitors is transported to the pial surface in their progenitor endfeet, and then transfers from this pial-substrate to Dcc^{+} commissural axons (E) (Varadarajan et al., 2017; Varadarajan & Butler, 2017). Dcc and netrin1 then interact *in cis* to promote the selective fasciculation and growth commissural axons around *netrin1* expressing NPCs (F). In absence of Dcc, netrin1 does accumulate on the pial surface, but does not transfer to commissural axons. These axons fail to fasciculate and grow randomly, including into the VZ (G).

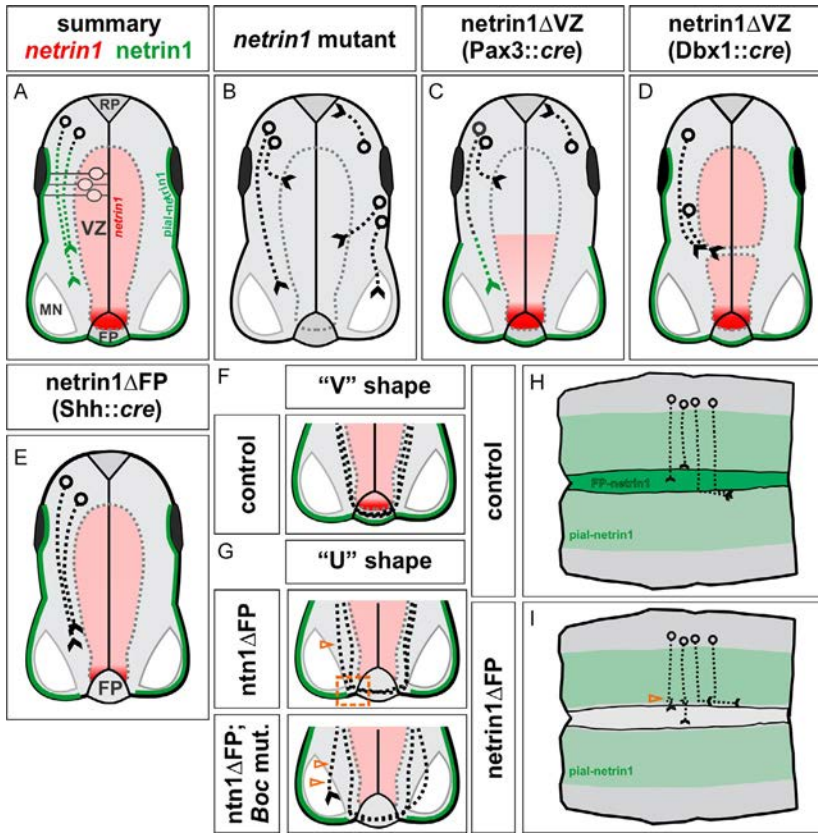

Fig. 3 Short- and long-range phenotypes observed in the absence of NPC- or FP-derived netrin1. (A) Summary of the distribution of netrin1 transcript (red) and protein (green). NPCs and FP cells express *netrin1*, while netrin1 protein is present on the pial surface and on commissural axons. (B) In *netrin1* loss of function mutants, spinal axons are highly defasciculated, extending into the VZ, and wandering across the motor column. (C, D) *Short range phenotypes for NPC-derived netrin1*: When *netrin1* expression is removed from either a large (C) or small (D) number of NPCs using conditional genetic approaches (C = Pax3::*cre* driver; D = Dbx1::*cre* driver) (Varadarajan et al., 2017), axons defasciculate specifically and locally in the region where netrin1 activity is absent. In particular, the introduction of ectopic *netrin1* on::off boundaries (D) locally reshapes axon trajectories in a manner consistent with the hederal boundary model. *Long-range phenotypes for NPC-derived netrin1*: none observed. (E–I) *Short-range phenotypes for FP-derived netrin1*: In netrin1ΔFP mice (Shh::*cre* driver), commissural axons project ventrally toward the FP (E) in a manner largely indistinguishable from controls. However, errors occur ~10 μm from the FP (yellow box, G), when axons reach the "off" edge of the ventral domain pial-netrin1, where they locally defasciculate (arrowheads, I). Axons start to cross the FP from this "off" edge, resulting in a laterally displaced "U" shaped trajectory (G), distinct from the "V" shape observed in controls (F). Control

(Continued)

phenocopied by the conditional removal of netrin1 from either the Pax3 domain (Fig. 3C) (Varadarajan et al., 2017; Yamauchi et al., 2017), i.e. all dorsal NPCs (netrin1 Δ dVZ), or the Dbx1 domain, i.e. a specific subpopulation of NPCs (Fig. 3D). In netrin1 Δ dVZ spinal cords, randomized axon growth is only observed in the dorsal spinal cord, where deposition of pial-netrin has been disrupted (Fig. 3C). This effect is very short-range: when netrin1 is removed from $Dbx1^+$ NPCs, axons only invade the narrow channel in the VZ where netrin1 has been depleted (Varadarajan et al., 2017) (Fig. 3D). Together, these studies identified that NPC-derived netrin1 establishes a boundary, that promotes the growth of fasciculated commissural axons around the VZ. This boundary activity of NPC-netrin1 is mediated by the Dcc receptor. In the absence of Dcc, netrin1 is still present at the pial surface but does not accumulate on commissural axons, which then grow in a profoundly defasciculated manner (Varadarajan et al., 2017) (Fig. 2F, G). Thus axonal-netrin1 appears to be key to establishing the VZ boundary.

4.2 Netrin1 supplies a "hederal" boundary

How mechanistically is the NPC-derived boundary of netrin1 established? The deposition of netrin1 on the pial surface suggests the formation of a local, attractive growth substrate in the dorsal spinal cord. And indeed, in the earliest stages of axogenesis, dorsal pial-netrin1 may act by haptotaxis, the directed growth of cells along an adhesive surface (Carter, 1965), instructing pioneering axons to grow ventrally, immediately adjacent to the $netrin1^+$ substrate (Fig. 2B). However, pial-netrin1 does not subsequently shape the transverse trajectory of commissural axons in the ventral

Fig. 3—Cont'd commissural axons then turn rostrally to project longitudinally beside the FP in the ventral funiculus (H). Most axons correctly make the rostral turn in netrin1ΔFP mice, however, we (S.A. and S.J.B., personal communication) and others (Moreno-Bravo et al., 2019) have observed that a subset of commissural axons fail to cross the FP, and turn ipsilaterally (I), resulting in a thinning of the FP commissure (Wu et al., 2019). It remains unresolved whether this ipsilateral turn is the result of axons following an ectopic hederal boundary created by the specific loss of netrin1 in the FP (I), or a direct requirement for FP-netrin1 in axon crossing. *Long-range phenotypes for FP-derived netrin1*: While we have not observed this phenotype, other reports (Wu et al., 2019) have shown that commissural axons are modestly defasciculated in the ventral spinal cord in the absence of FP-derived netrin1 (yellow arrowheads, G) i.e. ~100μm from the FP. This phenotype is significantly enhanced when netrin1ΔFP is combined with a *Boc* mutation (G), the non-canonical receptor that mediates the axon guidance activities of Shh (Wu et al., 2019).

spinal cord, rather commissural axons grow to precisely follow the boundary of the *netrin1*$^{+}$ VZ (Fig. 2C, D). Multiple conditional manipulations of *netrin1* expression (Varadarajan & Butler, 2017; Varadarajan et al., 2017) have shown that commissural axons will deviate from their trajectories to extend around ectopic borders of *netrin1*-expressing cells, as if these cells are supplying a boundary repellent (Fig. 3C, D, I).

We have proposed the hederal boundary model to reconcile these apparently distinct activities (Varadarajan & Butler, 2017) (Fig. 2C, D). This model is drawn from the analogy of a wall supporting a growing hedera (ivy plant); the wall provides a substrate for ivy to grow along, while preventing the ingrowth of the ivy into the wall. We propose that the hederal boundary is mediated by the ability of netrin1 to locally transfer, or be trafficked, from NPCs, to the pial surface and then onto Dcc^{+} axons (Fig. 2E, F). Axonal-netrin1 can both promote the directed fasciculated growth of commissural axons and prevent them from growing on *netrin1*-expressing cells in the VZ. Thus, early extending Dcc^{+} commissural axons are first oriented by the dorsal boundary of pial-netrin1 in the spinal cord. Commissural axons then accumulate netrin1; axonal-netrin1 acts to fasciculate axons together, and direct them around the *netrin1* expressing VZ. This model is supported by the observations that pial-netrin is not sufficient to guide *Dcc*$^{-}$ commissural axons (Fig. 2G) and that occasional axons growing aberrantly in the VZ in controls, have never accumulated netrin1 (Varadarajan et al., 2017).

The mechanism by which netrin1 becomes sequestered on axons is unresolved. Draxin may have a role in this process: the crystal structure of draxin either alone or in a netrin1-Dcc complex has shown that netrin1 and Dcc can bind to draxin on adjacent sites (Liu et al., 2018). Moreover, the sequestration of draxin results in the same pattern of randomized axon growth observed in *netrin1* and *Dcc* mutant spinal cords (Islam et al., 2009). Thus, draxin may bind to both molecules to facilitate the binding of netrin1 to Dcc^{+} axons, to promote fasciculation and directed growth.

Finally, the hederal activity of netrin1 appears to act over a very short-range. It remains unresolved whether netrin1 is always substrate bound, or whether there is some limited diffusion from the radial fibers (question marks, Fig. 2E). However, it is notable that there is no protein accumulation on the pial surface in the dorsal-most spinal cord, i.e. where NPCs are not expressing *netrin1*, suggesting very limited diffusivity, if any. Further studies examining the activities of membrane-bound forms of netrin1 *in vivo* are necessary to answer these questions.

4.3 Netrin1 maintains the CNS-PNS boundary

As dI1 axons begin their journey away from the RP, they must also avoid exiting the dorsal spinal cord from the DREZ, the entry point into the CNS for peripheral dorsal root ganglion (DRG) axons. The DRG begin innervating the spinal cord through the DREZ at E13.5 in mice (Yoshida, Han, Mendelsohn, & Jessell, 2006). Netrin1 plays a role preventing both commissural axons from leaving the spinal cord and sensory axons entering the spinal cord inappropriately. In *netrin1/Dcc* mutants, commissural axons aberrantly exit the spinal cord and grow into the periphery (Laumonnerie et al., 2014). The transient expression of netrin1 at E12.5 in a domain adjacent to the DREZ (Masuda et al., 2008; Watanabe et al., 2006), acts as a repellent boundary that prevents DRG axons from prematurely entering the spinal cord (Masuda et al., 2008; Varadarajan & Butler, 2017; Yoshida et al., 2006). Recent studies have suggested a role for cues emanating from the spinal meninges, which include the pial-substrate. Studies using dorsal spinal explants have shown that commissural axons are repelled by spinal meninges tissue while motor and sensory axons are attracted by meninges-derived cues (Suter, DeLoughery, & Jaworski, 2017). Is the meninges-bound cue that prevents commissural axons from leaving the spinal cord mediated by netrin1? The addition of netrin1 to the explant cultures complicates this interpretation, however there is support for this hypothesis from *in vivo* studies. In the absence of pial-netrin1, commissural axons, as well as precerebellar neurons in the hindbrain and pontine neurons, erroneously cross the CNS/PNS boundary and are no longer confined to the CNS (Dominici et al., 2017; Laumonnerie et al., 2014; Moreno-Bravo et al., 2018; Varadarajan et al., 2017; Yung et al., 2018).

5. Guidance of commissural axons toward and across the FP

On entering the ventral spinal cord, commissural axons detach from the circumferential edge of the spinal cord, to continue growing around the VZ, and toward the FP at the ventral midline. As commissural axons enter the ventral spinal cord, they become more progressively fasciculated until they project across the FP in very tightly bundled fascicle. The passage of commissural axons toward and across the FP has been the focus of intensive study, with multiple long- and short-range cues identified.

5.1 Long-range guidance signals from the FP

Netrin1 was initially identified as a compelling candidate for the "pull" attractant acting from the FP to draw commissural axons to the ventral midline. *Netrin1* is expressed at highest levels by FP cells (Kennedy et al., 1994), and COS cell aggregates expressing *netrin1* can mimic the ability of the FP to promote the fasciculated growth of commissural axons toward them (Kennedy et al., 1994; Placzek, Tessier-Lavigne, Yamada, Dodd, & Jessell, 1990; Serafini et al., 1994). Loss of function studies initially revealed that mice mutant for *netrin1* or *Dcc* exhibit a transient stall in Tag1^{+} commissural axon growth, with axons failing to reach the FP (Fazeli et al., 1997; Serafini et al., 1996). Together, these studies suggested the model that FP-derived netrin1 is a long-range cue that acts by chemotaxis to guide commissural axons to the ventral midline (Fig. 2A).

However, more recent studies have cast doubt on this model. Studies first *in vitro* (Moore, Biais, & Sheetz, 2009) and then *in vivo* in Drosophila (Akin & Zipursky, 2016; Brankatschk & Dickson, 2006; Timofeev, Joly, Hadjieconomou, & Salecker, 2012) demonstrated that tethered netrin1 can effectively function as a guidance cue. Most recently, NPC-derived netrin1 has been shown to direct fasciculated commissural axon growth, while specifically depleting FP-derived netrin1 (netrin1ΔFP) results in surprisingly minimal effects on the passage of robo3^{+} commissural axons toward the FP (Fig. 3E) (Dominici et al., 2017; Varadarajan et al., 2017; Wu et al., 2019). Studies further characterizing netrin1ΔFP embryos have found local defects (boxed region, Fig. 3F, G, see figure legend for details); in particular, some commissural axons now grow ipsilaterally immediately adjacent to the netrin1-depleted FP (Fig. 3H, I) (Moreno-Bravo et al., 2019). This phenotype is consistent with either a local role for FP-derived netrin1 promoting contralateral growth, or the secondary consequence of introducing a longitudinal hederal boundary, given that the netrin1ΔFP manipulation introduces an ectopic on:off *netrin* boundary along the edge of the FP (Fig. 3H, I).

Other candidates for the "pull" long-range attractant include the morphogen sonic hedgehog (Shh) (Charron, Stein, Jeong, McMahon, & Tessier-Lavigne, 2003), and vascular endothelial growth factor (Vegf) (Ruiz de Almodovar et al., 2011) which are both secreted by the FP. Localized sources of both Shh and Vegf can reorient commissural axons toward them (Charron et al., 2003; Ruiz de Almodovar et al., 2011), as has been observed for FP explants (Kennedy et al., 1994; Placzek et al.,

1990; Serafini et al., 1994) . Shh appears to act through a complex of smoothened (Charron et al., 2003) and brother of Cdo (Boc) (Okada et al., 2006). In the absence of these receptors, commissural axons defasciculate, and extend into the motor column. A similar phenotype is observed when Vegf, or its receptor fetal liver kinase 1 (Flk1), is specifically depleted from the FP or commissural neurons, respectively (Ruiz de Almodovar et al., 2011).

Together, these studies suggest that long-range activities supplied by FP-derived Shh and Vegf are necessary to direct fasciculated axon growth in the ventral spinal cord. Supporting this hypothesis, synergistic guidance interactions between Shh and netrin1 have been identified. These interactions were first observed *in vitro*: use of microfluidic guidance assays revealed that commissural growth cones can sense a shallow combined Shh/netrin1 gradient, but are nonresponsive to an equivalently shallow gradient of Shh or netrin1 alone (Sloan, Qasaimeh, Juncker, Yam, & Charron, 2015). This activity was recently observed *in vivo*: the combined loss of Boc and netrin1 ΔFP results in significantly more defasciculation of robo3^{+} axons into the motor column than is observed for the loss of Boc or netrin1 ΔFP alone (Fig. 3F, G) (Wu et al., 2019). Thus, long-range signaling from the FP may be necessary for the progressive fasciculation of commissural axon bundles as they project to the FP.

5.2 Short-range axon guidance cues in the FP

As commissural growth cones reach the ventral midline, their extension slows to navigate the FP (Bak & Fraser, 2003), which is the source of many short-range guidance cues (Table 1). Commissural axons then extend contralaterally across the FP, and turn rostrally, to project longitudinally in the ventral funiculus. In mouse embryos that lack a FP, commissural axons project relatively normally to the ventral midline, but make errors navigating the FP, including inappropriately accumulating in the midline and turning randomly, both rostrally and caudally, after crossing the FP (Kadison & Kaprielian, 2004; Kadison, Murakami, Matise, & Kaprielian, 2006; Matise, Epstein, Park, Platt, & Joyner, 1998; Matise, Lustig, Sakurai, Grumet, & Joyner, 1999). These phenotypes are consistent with key role(s) for short-range signaling from the FP.

Multiple members of the Ig domain superfamily, including Tag1, L1 and Nrcam, appear to mediate contact-dependent interactions between commissural growth cones/axons and FP cells. Nrcam is expressed by FP cells

Table 1 Summary of molecules present in the FP that have been proposed to act as short-range commissural axon guidance cues.

Name	Model	Proposed function	References
Ngcam-related CAM (Nrcam)	Mouse, chicken	Promotes growth of commissural axons across the FP	Stoeckli and Landmesser (1995)
Slits	Mouse, chicken	Repels post-crossing commissural axons from the FP	Brose et al. (1999), Long et al. (2004)
Class 3 semaphorins	Mouse, chicken	Repels post-crossing commissural axons from the FP	Nawabi et al. (2010), Zou et al. (2000)
Fspondin	Rat, chicken	Promotes growth of commissural axons across the FP	Burstyn-Cohen et al. (1999)
Neuropilin2	Mouse	Promotes growth of commissural axons across the FP	Hernandez-Enriquez et al. (2015)
P84	Mouse	Role unresolved, may serve as a substrate for axon outgrowth.	Chuang and Lagenaur (1990)
Neurite outgrowth inhibitor (nogo) b (reticulon4b)	Mouse	Repels post-crossing commissural axons from the FP	Wang et al. (2017)
Dystroglycan	Mouse	Repels post-crossing commissural axons from the FP	Wright et al. (2012)
Nectin3	Mouse	Promotes for correct axon fasciculation, and rostral turn for post-crossing axons	Okabe et al. (2004)
MAM domain-containing glycosylphosphatidylinositol anchor (Mdga) 1	Chicken	Function has not been characterized	Fujimura, Iwashita, Matsuzaki, and Yamamoto (2006)
Mdga2	Chicken	Promotes rostral turn for post-crossing axons	Joset et al. (2011)

Continued

Table 1 Summary of molecules present in the FP that have been proposed to act as short-range commissural axon guidance cues.—cont'd

Name	Model	Proposed function	References
Synaptic cell adhesion molecules (Syncam)/nectin-like molecules (Necls)	Chicken	Promotes growth of commissural axons across the FP and rostral turn for post-crossing axons	Niederkofler, Baeriswyl, Ott, and Stoeckli (2010)
Plexina2	Chicken	Promotes growth of commissural axons across the FP and rostral turn for post-crossing axons	Andermatt et al. (2014)

(Stoeckli & Landmesser, 1995), while Tag1 (known as axonin1 as in chicken) (Dodd et al., 1988; Stoeckli et al., 1989), and L1 (known as Ngcam in chicken) (Krushel, Prieto, Cunningham, & Edelman, 1993; Moscoso & Sanes, 1995) have complementary distributions on commissural axons. Thus, Tag1 is expressed on commissural axons prior to crossing the FP, while L1 is present on post-crossing commissural axons growing longitudinally in the ventral funiculus (Dodd et al., 1988). This distribution suggested that Tag1/L1 controls the response of commissural axons to signals in the FP, such as Nrcam (Stoeckli & Landmesser, 1995). Together, these cues may interact to direct commissural axons across the FP and into their contralateral rostral turn, by preventing them from turning prematurely along an ipsilateral trajectory. *In vitro* studies have provided support for this model (Fitzli et al., 2000), while the loss of Tag1 or Nrcam *in vivo* results in some commissural axons failing to cross the midline and growing along the ipsilateral FP border (Stoeckli & Landmesser, 1995; Stoeckli, Sonderegger, Pollerberg, & Landmesser, 1997). These adhesion molecules may also regulate commissural axon fasciculation across the FP. In Tag1, L1 and Nrcam mutants, some commissural axons locally defasciculate as they approach the midline (Stoeckli & Landmesser, 1995).

Extracellular matrix (ECM) proteins have also been implicated in regulating the passage of commissural axons across the FP. These signals include netrin1, as discussed in Section 5.1 and F-spondin. F-spondin is produced by the FP and localizes to the basal lamina immediately underlying FP (Klar, Baldassare, & Jessell, 1992). F-spondin can promote outgrowth of

commissural axons *in vitro,* while *in vivo* perturbations result in axon defasculation and turning defects (Burstyn-Cohen et al., 1999).

5.3 Robo/slit signaling changes commissural axon responsiveness

The slit/robo pathway is the most well studied, short-range molecular mechanism that regulates whether axons do, or do not, cross a midline structure. First identified in Drosophila, the slit glycoproteins mediate axon repulsion by binding to the roundabout (robo) receptors, also members of the Ig domain superfamily (Kidd et al., 1998; Nusslein-Volhard, Wieschaus, & Kluding, 1984; Seeger, Tear, Ferres-Marco, & Goodman, 1993; Tear, Seeger, & Goodman, 1993). Classic studies have led to a model in which axon responsiveness to a slit^{+} midline is regulated by the presence or absence of robo receptors. Thus, robo^{+} axons grow ipsilaterally to avoid the slit^{+} midline, while robo^{-} axons grow contralaterally across a slit^{+} midline, because they are unable to detect the slit repellent. If contralaterally projecting axons subsequently become robo^{+}, this mechanism then prevents them from re-crossing the slit^{+} midline.

Much of this evidence for this model has come from Drosophila studies (Blockus & Chedotal, 2014; Comer et al., 2019; Evans & Bashaw, 2010). Here, we will briefly review the role of the slit/robo pathway directing commissural axons in the spinal cord. In vertebrates, there are three homologues of the Drosophila robo1 gene - robo1, robo2, and robo3/Rig1 - which are present on commissural axons (Kidd et al., 1998; Sundaresan et al., 1998; Yuan et al., 1999) and three members of the slit family - slit1, slit2, and slit3 - that are secreted by the FP (Brose et al., 1999). Robo1 and robo2 are present at higher levels on the post-crossing segment of commissural axons (Long et al., 2004; Mambetisaeva, Andrews, Camurri, Annan, & Sundaresan, 2005; Sabatier et al., 2004). They are thus candidates for the repellent receptors that identify the slit1/2/3^{+} FP as being repulsive, thereby preventing commissural axons from re-crossing the FP. *In vitro* studies have shown that the vertebrate slit proteins can bind robo1 and robo2 to mediate a repulsive interaction (Brose et al., 1999; Li et al., 1999). Genetic *in vivo* studies are more complex: slit triple knockouts exhibit the re-crossing and axon stalling phenotypes predicted for the loss of a repellent at the FP (Long et al., 2004). However, while robo1/2 double mutants display similar phenotypes, they are less severe than the slit triple mutants suggesting the existence of additional slit receptors (Jaworski, Long, & Tessier-Lavigne, 2010).

In contrast, robo3 is present on the pre-crossing segments of commissural axons and has been proposed to prevent pre-crossing commissural axons from responding prematurely to the slit^{+} midline (Sabatier et al., 2004). In the absence of robo3, commissural axons reach the FP, but then dramatically fail to cross it, rather turning ipsilaterally to grow alongside it, as if they are now responsive to slit signaling (Sabatier et al., 2004). The mechanism by which robo3 suppresses the response of commissural axons to slit remains unresolved. Robo3 has been proposed to function by interfering with the robo1/2 complex. However, neither robo1 nor robo2 are upregulated in pre-crossing axons in *robo3* mutants, suggesting that robo3 does not regulate *robo1/2* expression (Sabatier et al., 2004). Robo3 also does not have a high affinity for slit proteins, making it unlikely that robo3 competes with robo1/2 for slit binding (Mambetisaeva et al., 2005; Zelina et al., 2014). *In vitro* studies have suggested that robo3 can promote robo1/2 degradation (Li et al., 2014). Robo3 has also been proposed to prevent axons from invading the motor column, by acting through a novel secreted ligand, neural epidermal growth factor-like-like 2 (nell2) (Jaworski et al., 2015; Pak et al., 2020). Nell2 is present in the spinal cord motor column and central canal. While single mutants in *nell2* have no commissural axon guidance phenotype, adding a *robo3*$^{+/-}$ transheterozygous mutation, i.e. *nell2*$^{-/-}$ *robo3*$^{+/-}$, results in aberrant axon growth in the motor column, consistent with a role for this pathway mediating a repellent interaction in the motor column (Jaworski et al., 2015).

The distribution of robo3 in pre-crossing commissural axons is regulated by the transcriptional programs that define neuronal subtype that were discussed in Section 2. Lhx2, the key transcription factor directing dI1c neural identity, binds to the control region of the *robo3* gene. In *Lhx2/9* double mutants, *robo3* expression is lost from dI1c axons, which then fail to cross the midline. Together, these findings suggest that Lhx2 directly upregulates robo3 in the contralateraly-projecting dI1 axon population (Wilson et al., 2008). What downregulates robo3 in post-crossing axons? One candidate from cancer studies is miR-383, a microRNA that suppresses *robo3* expression with a distribution that is inversely correlated with robo3 (Han et al., 2015).

What regulates the distribution of robo1/2 on commissural axons? Multiple pathways have been shown to alter robo1/2 cell surface levels. The WAGR syndrome gene, proline-rich and gla domain 4 (Prrg4) can prevent robo1 from being trafficked to the cell surface *in vitro* (Justice, Barnum, & Kidd, 2017), while Rab guanine nucleotide dissociation

inhibitor (GDI), ubiquitin-specific peptidase 33 (Usp33), and calsyntenin1 can stabilize or promote robo1 surface localization (Alther, Domanitskaya, & Stoeckli, 2016; Philipp et al., 2012; Yuasa-Kawada, Kinoshita-Kawada, Wu, Rao, & Wu, 2009). Nedd4-family interacting proteins, Ndfip1 and Ndfip2, have been shown to recruit the Nedd4 E3 ubiquitin ligases to target robo1 to endosomes for degradation by ubiquitylation. Loss of *Ndfip1/2 in vivo* results in increased *robo1* expression on commissural axons and reduced midline crossing (Gorla et al., 2019). The distribution of robo1/2 may also be controlled at the expression level. miR-92 can suppress the translation of robo1 protein in the spinal cord *in vivo* (Yang et al., 2018). Finally, intriguing recent studies have identified that the neuro-oncological ventral antigen (Nova) splicing factors can produce robo1/robo2 isoforms with different efficacies as repellents. Through the alternative splicing of a microexon, Nova1/2 may regulate the production of these isoforms at different times in development to control the timing by which commissural axons approach and cross the midline (Johnson, Junge, & Chen, 2019)

6. Commissural axons exit the FP and turn rostrally into the ventral funiculus

On exiting the FP, dI1 commissural axons sharply orthogonally to travel immediately beside the border of the FP (Bovolenta & Dodd, 1990). After extending for a short distance, dI1 axons fan out over an arcuate trajectory to extend longitudinally along the ventral funiculus toward the brain (Avraham et al., 2009; Kadison & Kaprielian, 2004). This trajectory is bounded along the dorsal-ventral axis, preventing commissural axons from extending back into the dorsal spinal cord on the contralateral side.

6.1 Semaphorin signaling mediates commissural axon exit from the FP

Commissural axons may be directed to exit from the spinal cord by the class3 semaphorins, present in the FP. Class3 semaphorins signal through a complex of neuropilin2 (Nrp2) and its co-receptor plexina1 (Pignata, Ducuing, & Castellani, 2016). While plexina1 is present specifically on commissural axons, Nrp2 is found on both axons and in the FP (Hernandez-Enriquez et al., 2015; Nawabi et al., 2010). *Nrp2* deficient mice exhibit a variety of pathfinding defects, including commissural axons stalling within the FP and growing aberrantly in their longitudinal trajectory along the contralateral FP border (Tran et al., 2013; Zou, Stoeckli, Chen, & Tessier-Lavigne, 2000). How is a

premature response to FP-derived semaphorins prevented? *In vitro* studies using spinal cord explants, have shown that pre-crossing commissural axons are repelled by sema3b. However, this sensitivity to sema3b repulsive signaling is inhibited by FP-derived Npn2. One possibility is that FP-derived Nrp2 sequesters semaphorins, preventing repulsion of pre-crossing axons (Hernandez-Enriquez et al., 2015). It remains unresolved how this sequestration is lifted to permit post-crossing commissural axons to detect and then be repelled by FP-derived sema3b.

6.2 Long-range cues in the FP direct the rostral turn

Two long-range signaling pathways, both present in the FP, have been proposed to mediate the ability of post-crossing commissural axons to turn rostrally. A rostral-low, caudal-high Shh gradient has been shown to act as a repulsive signal (Bourikas et al., 2005; Yam et al., 2012), while a rostral-high, caudal-low Wnt gradient, can attract commissural axons rostrally (Aviles & Stoeckli, 2016; Lyuksyutova et al., 2003). The hedgehog-interacting protein (Hip) mediates the repulsive response to Shh (Bourikas et al., 2005). Disrupting this interaction *in vivo* in chicken embryos leads to some commissural axons projecting both rostrally and caudally. Thus, the initial attractive response of pre-crossing commissural axons to Shh appears to be modulated to repulsion in post-crossing axons. This modulation may be mediated by the 14-3-3 family of adaptor proteins. In both *in vivo* and *in vitro* studies in rodents, increasing the levels of 14-3-3 proteins switches axonal attraction to Shh to repulsion. Conversely, the loss of 14-3-3 proteins changes the response of commissural axons to Shh from repulsive to attractive (Yam et al., 2012).

The Wnt protein gradient is mediated in mouse embryos by Wnt4, acting through the frizzled3 receptor (Lyuksyutova et al., 2003). In the absence of frizzled3, commissural axons turn randomly after crossing the midline. Graded Wnt levels have not been identified in chicken embryos, rather graded Wnt5a and Wnt7a activity may be produced a rostral-high, caudal-low gradient of a Wnt antagonist: secreted frizzled-related protein (Sfrp1) (Domanitskaya et al., 2010). The gradient of Sfrp1 is established by the Shh gradient at the FP. Thus, in chicken, Shh controls the trajectory of post-crossing commissural axons both directly (Bourikas et al., 2005) and indirectly, by regulating Wnt activity (Domanitskaya et al., 2010).

6.3 Bounding the longitudinal commissural trajectory to the ventral funiculus

Finally, the Eph/ephrin signaling pathway has a key role creating the barrier that constrains post-crossing commissural axons to the ventral funiculus (Flanagan & Vanderhaeghen, 1998; Wilkinson, 2000). Many classic studies have identified the Eph/ephrin pathway as mediating short-range repulsive axon guidance decisions. In the mouse and chicken, ephrinB ligands are expressed in the dorsal spinal cord and FP, while the ephrin receptors, including EphB1 and EphA2, are expressed on distal segments of post-crossing commissural axons (Brittis, Lu, & Flanagan, 2002; Imondi, Wideman, & Kaprielian, 2000). Upregulation of Eph receptors, specifically EphA2, in post-crossing axons seems to be regulated by localized protein synthesis and expression (Brittis et al., 2002). Commissural axons turn at a boundary of ephrinB expression and project longitudinally (Imondi, 2000). Disturbing endogenous EphB-ephrinB interactions in cultured spinal cord explants results in growth of commissural axons into dorsal regions of the spinal cord where ephrinB is normally expressed (Imondi & Kaprielian, 2001).

7. Conclusions and speculations

This review has traced the current thinking about the molecular and cellular mechanisms that establish the early stages of dorsal commissural neural circuit, from the birth of dI1 neurons, to the mechanisms that direct their commissural axons first circumferentially and then contralaterally into the ventral funiculus. This is a very well-studied pathway, with clear, yet still puzzling, themes emerging. First, while the commissural trajectory itself is relatively simple: axons extend toward and across a midline, a multitude signals have been implicated in the guidance of this trajectory, with multiple signals often identified for each putative choice point. Why is it so complicated? Potential explanations include that many genes with overlapping redundancies are required to establish this trajectory with absolutely fidelity, or - less interestingly - that some of the identified factors are spurious, because this axonal pathway is easy to perturb through genetic manipulation. However, it is also notable that only a few of the genes required for formation of this neural circuit in vertebrates have highly penetrant, highly expressive loss of function phenotypes. For example, commissural axons are completely defasciculated in the absence of the *netrin1/Dcc* pathway, and

they all project ipsilaterally in the absence of *robo3*. Thus, an alternative possibility is that only a few of the identified factors are "architects", critically required to establish the dI1 axonal circuit, while the other signals represent "sculptors", refining or modulating the response to the architect factors. Supporting this model, it is notable that commissural axons continually grow in a netrin1^{+} environment. We have suggested that pre-crossing axons extend around a "hederal" boundary of *netrin1*-expressing cells, including both NPCs and the FP; while post-crossing axons grow longitudinally within the pial-netrin1 substrate in the ventral spinal cord (Fig. 3H) (Varadarajan & Butler, 2017). It remains unresolved whether additional signals regulate these responses to netrin1, permitting pre-crossing commissural axons to avoid, or be unresponsive to, ventral pial-netrin1, while post-crossing commissural axons again use it as a growth substrate.

A second theme in this review, is a reassessment of the importance of long-range signaling. While *bona fide* long-range signals remain (Shh, Bmps), the key signaling pathways required to guide commissural axons appear to act principally by short-range mechanisms. What is their general cellular purpose? Clues may come from the involvement of the large number of Ig domain superfamily receptors, including Tag1, Dcc, the Unc5 and robo families, L1, Nrcam and Boc, and their predominant loss-of-function phenotype: modest to severe axon defasciculation. Thus, the critical "architect" activities of netrin1/robo3 may be to define the commissural axon trajectory as series of local fasciculation decisions to direct axons (1) ventrally and (2) ipsilaterally versus contralaterally. This model stands in contrast to the prevailing long-range model, whereby axons grow in discrete steps from one long-range directional guidepost signal to the next. In the short-range model, the "sculpting" activities could include mediating defasciculation and/or the strength of fasciculation as axons progressively bundle together to cross the FP, as well as directing the topographic position of axons within fascicles. This reassessment opens up many new avenues for mechanistic exploration and may explain why relatively little progress has been made using putatively long-range axon guidance cues as regenerative factors. Understanding how guidance signals act locally to direct fasciculation, may have more mechanistic significance for repairing damaged or diseased nerve tracts.

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