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REVIEW

Understanding axon guidance: are we nearly there yet?

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ABSTRACT

During nervous system development, neurons extend axons to reach their targets and form functional circuits. The faulty assembly or disintegration of such circuits results in disorders of the nervous system. Thus, understanding the molecular mechanisms that guide axons and lead to neural circuit formation is of interest not only to developmental neuroscientists but also for a better comprehension of neural disorders. Recent studies have demonstrated how crosstalk between different families of guidance receptors can regulate axonal navigation at choice points, and how changes in growth cone behaviour at intermediate targets require changes in the surface expression of receptors. These changes can be achieved by a variety of mechanisms, including transcription, translation, protein-protein interactions, and the specific trafficking of proteins and mRNAs. Here, I review these axon guidance mechanisms, highlighting the most recent advances in the field that challenge the textbook model of axon guidance.

KEY WORDS: Axon guidance, Guidance cues, Neural development, Local translation, MicroRNA, Vesicular transport

Introduction

Neural circuits are the basis of neural function in health and disease, and the faulty assembly or disintegration of these circuits can result in disorders of the nervous system. For example, neurodevelopmental disorders, such as intellectual disability, autism spectrum disorders and schizophrenia, have all been linked to aberrant development of neural circuits. In addition, neurodegenerative disorders, such as Alzheimer's or Parkinson's disease, are caused by the disintegration of neural circuits. Genome-wide association studies of familial and spontaneous forms of developmental and degenerative disorders have identified genes involved in the formation of neural circuits (Antonell et al., 2013; Bossers et al., 2009; Gilman et al., 2012; Iossifov et al., 2014; Pinto et al., 2014). Thus, a molecular understanding of the different steps contributing to neural circuit formation is crucial not only for providing insight into neural development but also for a better comprehension of the pathology of neural disorders.

During the development of neural circuits, axons navigate through pre-existing tissues to find their target cells, where they then form synapses. However, what sounds like a relatively simple process turns out to be a major problem, given that the human brain consists of about 80 billion neurons. How can each one of them send its axon to the proper target cell? This was a question raised by Ramón y Cajal more than 100 years ago, long before a molecular basis could even be imagined. Today, a large number of axon guidance cues and receptors have been identified and we can now study the molecular basis of neural circuit formation. These studies have converged to our current understanding of axon guidance mechanisms. In short, these studies have revealed that axons express guidance receptors on their elongating tip (the 'growth cone') and navigate to their targets by integrating attractive and repulsive guidance information present in their environment.

Axon guidance molecules can be subdivided into attractive and repulsive cues that act either over long distances or locally, in a contact-dependent manner. Cooperation between long- and short-range guidance cues is required for the navigation of growing axons to their target cells. Because of the long distances that need to be covered, axons also use intermediate targets on the way to their final target. These intermediate targets provide important guidance information and prepare axons for the next stage of their journey (Squarzoni et al., 2015; de Ramon Francàs et al., 2017). However, axonal navigation at these choice points remains poorly understood. Obviously, axons are attracted to these intermediate targets but, rather than staying there to form synapses, axons continue with or without delay along their trajectory to the final target. This requires a switch in responsiveness of the growth cone from attraction to repulsion. This switch must be timed precisely, as premature switching would prevent contact with the intermediate target, whereas delayed switching would cause stops at choice points. Such stops are seen at some, but not all intermediate targets. For instance, no stop is seen at the floor plate, the ventral midline of the spinal cord, whereas axonal lingering has been observed at the base of the limb (Wang and Scott, 2000; Huber et al., 2005). In all cases, a switch from attraction to repulsion is required for axons to move on. This switch can, in theory, be achieved by a change of guidance cues at the intermediate target, or a change of guidance receptors on the growth cones. At the choice points at which no axonal pauses are observed, a change in the expression of guidance cues in the intermediate target seems unlikely, as this would mean that all axons have to travel in synchrony. Even a minor change in growth speed would result in the inability of slower axons to contact and pass the intermediate target. Furthermore, it would be very difficult to synchronize axonal navigation between different subpopulations of neurons using the same choice point but taking different routes. For this reason, it seems more straight-forward to change the expression of surface receptors on the growth cones. In fact, this is what has been observed in several cases of axonal navigation of a choice point.

So how many of these guidance cues and receptors do we need to explain the formation of neural circuits? One set for each class of neurons? Can different classes of neurons share axon guidance cues for common parts of the pathway? Despite the fact that axon guidance cues are shared by various classes of neurons, although sometimes with different results depending on the receptors expressed, the answer is that we need a large number of axon guidance molecules and receptors. However, compared with the complexity of neural circuits, and in view of the enormous number of different populations of axons that make their way to specific targets, the number of guidance molecules is surprisingly small

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(Pasterkamp and Kolodkin, 2013; Kolodkin and Pasterkamp, 2013). This would still be true even if many more axon guidance molecules and receptors were identified. However, this is not expected as very few new guidance cues have been discovered over the last decade or so. Draxin, a secreted protein that has no homology with other previously identified guidance cues, was identified as a repellent for axons in the developing brain and spinal cord (Islam et al., 2009; Shinmyo et al., 2015; Ahmed et al., 2011), and a guidance function was recently demonstrated for phosphatidyl-β-D-glucoside and its derivative lyso-phosphatidyl-β-D-glucoside (LysoPtdGlc) expressed on glial cells in the dorsal spinal cord (Guy et al., 2015).

The current focus has, therefore, clearly shifted away from the discovery of new guidance cues to deciphering the regulatory mechanisms underlying the crosstalk between different families of guidance cues and their receptors. In this Review, I summarize recent findings on the interaction between different families of guidance receptors during axonal navigation, focussing on the axon guidance mechanisms at play in the developing spinal cord and within the brain. I also highlight recent studies that question and modify our textbook model of axon guidance.

A richness of signals: redundancy of guidance information ensures correct navigation within the spinal cord

The largest variety of axon guidance cues has been identified for commissural axons in the developing spinal cord. In particular, the dorsal-most class of commissural neurons – the dI1 neurons – have been studied extensively with respect to their choice to send axons ventrally towards the floor plate, their intermediate target (for recent reviews, see de Ramon Francàs et al., 2017; Pignata et al., 2016). These axons cross the midline and turn rostrally upon exiting the floor plate on the contralateral side (Fig. 1). For this particular population of axons, guidance cues and receptors controlling all stages of this navigation process have been identified and include long-range attractants and repellents, as well as short-range guidance cues that mediate navigation of the floor plate and turning into the longitudinal axis.

Long-range guidance

Netrin 1 was the first long-range guidance cue identified for dI1 commissural axons (Kennedy et al., 1994). Netrin produced by the floor plate was thought to attract commissural axons by forming a gradient, with highest concentration next to the floor plate. However, this model has recently been questioned (see Box 1) by findings that netrin from the floor plate cannot be the main driver because it is dispensable for axonal guidance to the floor plate; instead, netrin derived from precursor cells along the central canal was found to be required (Dominici et al., 2017; Varadarajan et al., 2017; Fig. 1). The chemoattractive effect of netrin is supported by that of Shh, which is expressed by the floor plate (Charron et al., 2003), although the effect of Shh is weaker than that of netrin as it can only be detected in embryos deficient for netrin signalling. In addition to netrin and Shh, vascular endothelial growth factor (VEGF) acts as an attractant to guide commissural axons towards the floor plate (Ruiz de Almodóvar et al., 2011). Furthermore, dI1 axons are not only attracted towards the ventral midline but are also repelled from the dorsal spinal cord by the long-range repellents BMP7 and Draxin (Fig. 1), which are derived from the roof plate (Augsburger et al., 1999; Butler and Dodd, 2003; Islam et al., 2009).

Short-range guidance

When axons arrive at the floor plate, short-range guidance cues take over. Axons have been shown to enter the floor plate as a result of interactions between axonal contactin 2 (also known as axonin) and neuronal cell adhesion molecule (NrCAM) in the floor plate (Stoeckli and Landmesser, 1995; summarized by De Ramon Francàs et al., 2017). Given all this attraction, it was not clear why axons would cross the midline and exit the floor plate at all. An explanation was found with the discovery of repulsive molecules associated with the midline. Based on initial screens in the fly (Kidd et al., 1998, 1999), midline-associated, repulsive molecules, the Slits, together with their receptors, the Roundabout (Robo)

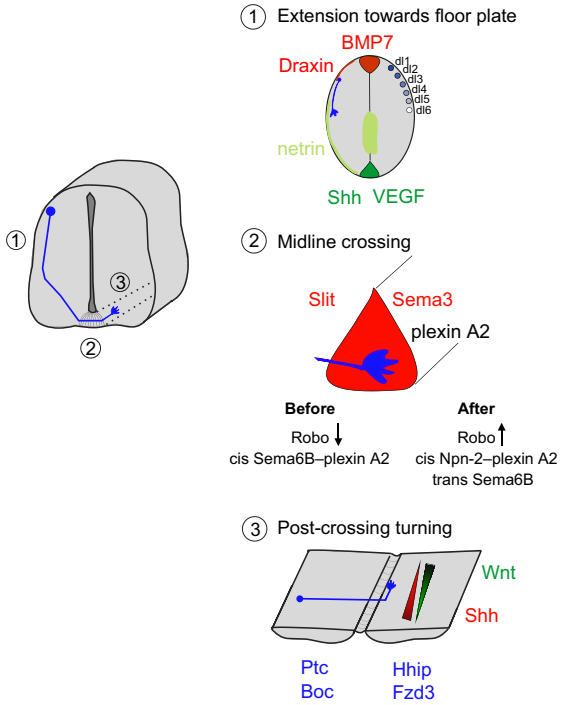

Fig. 1. Axon guidance within the spinal cord. The schematic on the left indicates the three stages of commissural axon pathfinding: (1) extension towards the floor plate; (2) midline crossing; and (3) post-crossing turning. The molecular mechanisms involved in the three steps are summarized on the right. Note that the molecular mechanisms used by different populations of neurons may be similar but have been studied in most detail in the dI1 population. At early stages (1), commissural axons (blue) are subdivided into six dorsal subpopulations (dI1-dI6) and extend axons towards the floor plate. They are guided to the floor plate, their intermediate target, by repellents derived from the roof plate (red; BMP7 and Draxin). In addition, the floor plate expresses the long-range attractants netrin, Shh and VEGF, although recent studies demonstrate that netrin derived from the floor plate (dark green), but not the precursor cells in the ventral ventricular zone (light green), is dispensable for commissural axon growth to the floor plate (see Box 1). Once dI1 growth cones have reached the floor plate (2), they enter it as a result of growth cone-expressed Cntn2 interacting with NrCAM in the floor plate (not shown). Upregulation of Robo1 then ensures floor-plate crossing, and axons are expelled from the floor plate by Slit-Robo1 interactions. In addition, the cis interaction between plexin A2 and Sema6B on pre-crossing axons prevents responsiveness to repulsive class-3 semaphorins (Sema3) expressed by the floor plate; this cis interaction no longer exists on post-crossing axons, where plexin A2 now forms a complex with neuropilin 2 (Npn-2), thus making post-crossing axons sensitive to repulsion by Sema3. Shh also contributes to the induction of Npn-2-mediated responsiveness to class-3 semaphorins (not shown). Upon reaching the contralateral floor-plate border (3), axons turn rostrally in response to opposing gradients of Wnt (which has an attractive effect) and Shh (which has a repulsive effect). Shh acts as attractant for pre-crossing axons expressing the receptors Ptc (patched) and Boc, and induces the expression of Hhip, its receptor, on post-crossing axons. In addition to its direct repulsive effect on post-crossing axons, Shh modulates Wnt activity by shaping the Wnt activity gradient in an Sfrp-dependent manner and by regulating Fzd3 surface expression in a Shisa2-dependent manner.

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Box 1. The role of the long-range chemoattractant netrin revisited

The role of netrin as a guidance cue for commissural axons extending towards the floor plate was described more than 20 years ago (Kennedy et al., 1994; Serafini et al., 1996). The initially characterized mouse line was not a full knockout but rather expressed hypomorphic levels of netrin, but the role of netrin has been confirmed more recently in a new complete knockout mouse line (Yung et al., 2015; Bin et al., 2015). These studies showed that the complete absence of netrin prevented axonal midline crossing in the spinal cord despite the presence of Shh and VEGF (see main text). However, netrin's role in midline crossing is not restricted to the spinal cord, as effects on commissure formation have also been reported for the corpus callosum in the netrin hypomorphic mouse (Fothergill et al., 2014). Similarly, netrin has been shown to be an important contributor to post-crossing axon guidance in the hindbrain, but not for midline crossing (Shoja-Taheri et al., 2015). A big surprise in the axon guidance field was the recent finding that netrin released by the floor plate is not the major chemoattractant for dI1 axons to their intermediate target. In fact, two laboratories independently reported that netrin expression in floor plate cells is not necessary for dI1 axons to grow to their intermediate target (Dominici et al., 2017; Varadarajan et al., 2017). Instead, netrin expressed in spinal cord precursor cells was found to be required and to make dI1 axons grow ventrally in a Dcc-dependent manner. These new findings, termed the 'growth substrate model', revise the canonical textbook model of netrin as a long-range attractant derived from the floor plate, the intermediate target of commissural axons (Kennedy et al., 1994; Bin et al., 2015; Yung et al., 2015).

receptors, were identified in vertebrates (Brose et al., 1999; Long et al., 2004). Subsequent studies revealed that the timing of Robo surface expression regulates the responsiveness of commissural axons to Slits and ensures that pre-crossing axons do not respond, whereas axons in the floor plate switch their responsiveness and are thus expelled from the floor plate (Keleman et al., 2002, 2005; Philipp et al., 2012; Fig. 1). The mechanisms controlling Robo expression have been studied in different species and seem to diverge, although the common finding is that the regulation happens at the post-translational level (summarized by Blockus and Chédotal, 2016).

Slits are not the only repellents associated with the floor plate. Class-3 semaphorins also repel post-crossing axons without affecting pre-crossing axons (Zou et al., 2000; Nawabi et al., 2010; Fig. 1). Interestingly, the timing of sensitivity to class-3 semaphorins [semaphorin 3B (Sema3B) and Sema3F] also seems to be regulated at the post-translational level (Nawabi et al., 2010; Charoy et al., 2012). For instance, plexin A1, one component of the receptor for Sema3B, together with neuropilin 2 (Npn-2; Nrp2 – Mouse Genome Informatics), is degraded by calpain in pre-crossing axons. When axons approach the floor plate, calpain is inactivated by NrCAM. Thus, floor-plate contact and calpain inactivation results in stabilization of plexin A1 on the growth cone surface and repulsion of post-crossing axons by Sema3B/F. In addition to calpain, Shh has been shown to modulate the responsiveness of post-crossing axons to Sema3B/F (Parra and Zou, 2010).

Additional short-range axon guidance cues for dI1 navigation at the midline have been identified that affect midline crossing or turning into the longitudinal axis, or both. For example, midline crossing is normal but turning is affected after perturbation of SynCAMs (also known as nectin-like molecules; Niederkofler et al., 2010) or MDGA2 (Joset et al., 2011). Both floor-plate crossing and rostral turning of dI1 axons are affected after silencing Sema6B and plexin A2 in axons or plexin A2 in the floor plate (Andermatt et al., 2014).

Guidance along the rostro-caudal axis

Following midline crossing, axonal navigation along the rostro-caudal axis is directed by morphogen gradients (Fig. 1). Wnts have been shown to attract post-crossing commissural axons rostrally by forming a rostral^{high}-caudal^{low} gradient at the floor plate (Lyuksyutova et al., 2003; Avilés and Stoeckli, 2016). At the same time, post-crossing axons are repelled by Shh, which forms a rostral^{low}-caudal^{high} gradient along the floor plate (Bourikas et al., 2005; Wilson and Stoeckli, 2013). In contrast to the attractive effect of Shh on pre-crossing axons, which is mediated by non-canonical Shh signalling mediated by Src family kinases (Yam et al., 2009), Shh acts as a repellent for post-crossing axons expressing Hhip (Hedgehog-interacting protein) as a receptor (Bourikas et al., 2005). Interestingly, Shh itself induces the change in receptor expression by binding to glypican 1 on pre-crossing axons, resulting in transcriptional regulation and expression of Hhip (Wilson and Stoeckli, 2013).

The functions of Shh and Wnt signalling during the rostral turning of post-crossing axons are not independent of each other (Domanitskaya et al., 2010). The graded expression of Shh has been shown to affect Wnt signalling via secreted frizzled-related proteins (Sfrps), which are endogenous Wnt antagonists that soak up Wnts and prevent them from interacting with frizzled 3 (Fzd3), the Wnt receptor expressed by commissural axons. Thus, high levels of Shh trigger high levels of Sfrps in the caudal spinal cord, thereby reducing attraction by Wnt, whereas the absence of Sfrp in the more rostral spinal cord allows for high Wnt attraction. Recently, another link between Shh and Wnt signalling has been demonstrated (Onishi and Zou, 2017). Shh was shown to regulate Fzd3 surface expression via Shisa2, which prevents Fzd3 modification and transport from the Golgi to the plasma membrane (Onishi and Zou, 2017). Shh signalling keeps Shisa2 levels low, thus allowing Fzd3 processing and insertion into the growth cone membrane. For this to be compatible with the observed expression of Fzd3 and the regulation of responsiveness of post- versus pre-crossing axons, only canonical Shh signalling but not Src family kinase-mediated Shh signalling is expected to regulate Shisa2.

Fzd3 expression is also regulated by calsyntenin 1, which is a type I transmembrane protein that belongs to the cadherin superfamily of proteins. The calsyntenin 1-dependent regulation of vesicle trafficking was identified as a mechanism to keep Fzd3 off the growth cone membrane in pre-crossing axons (Alther et al., 2016). Calsyntenin 1 also regulates Robo1 surface expression, suggesting that both Slit sensitivity and sensitivity to Wnts are regulated by trafficking rather than transcription or translation. This is in contrast to sensitivity to Shh; the difference in responsiveness between pre- and post-crossing axons involves transcriptional regulation of Shh receptors on post-crossing axons (Bourikas et al., 2005; Wilson and Stoeckli, 2013). Irrespective of the underlying mechanism, both the response to midline repellents and the sensitivity to morphogen gradients need to be timed precisely, as only post- but not pre-crossing axons are allowed to respond, or need to respond in a different manner, respectively.

Axon guidance mechanisms in the brain

The surprisingly large number of axon guidance cues and receptors required for midline crossing and rostral turning of dI1 commissural axons raises the question of how the navigation of more complex circuits in the brain are regulated. Are similar guidance mechanisms involved? Based on axon guidance studies looking at thalamocortical connectivity (Garel and López-Bendito, 2014; Gezelius and López-Bendito, 2017), corpus callosum formation

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(Blockus and Chédotal, 2014; Lindwall et al., 2007), visual system wiring (Herrera et al., 2018; Erskine and Herrera, 2014), and the wiring of midbrain and brainstem neurons (Brignani and Pasterkamp, 2017; Fenstermaker et al., 2010), it does appear that the mechanisms and molecules identified in the spinal cord are conserved in the brain (Chédotal and Richards, 2010), and there is no evidence for many more or completely different axon guidance cues and receptors in the brain compared with the spinal cord (Fig. 2). For example, Slit/Robo signalling controls formation of the corpus callosum and other commissures in the brain (Fothergill et al., 2014; Unni et al., 2012; López-Bendito et al., 2007). Class-3 semaphorins and neuropilins are involved in the regulation of midline crossing at the chiasm (Erskine et al., 2011) and the corpus callosum (Piper et al., 2009). Netrin and Slit gradients sort out thalamocortical connections (Leyva-Díaz et al., 2014; Bielle et al., 2011), and membrane-bound class-6 semaphorins, plexins and cell adhesion molecules of the immunoglobulin superfamily (IgSF-CAMs) regulate axon pathfinding and targeting in the visual system (Kuwajima et al., 2012; Bruce et al., 2017). Morphogens also contribute to axonal navigation in the brainstem (Fenstermaker et al., 2010). All these studies confirm that the same molecular mechanisms are involved in the establishment of neural circuits throughout the nervous system. In turn, this means that the regulation of the different signalling pathways and the temporal and spatial coordination of the different guidance cues and receptors at choice points are likely to be even more complex in the brain.

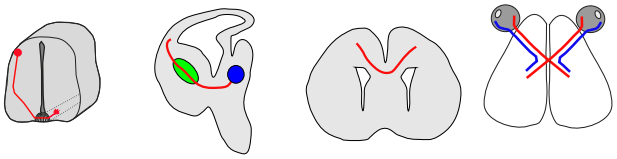
Crosstalk between different families of guidance cues

As highlighted above, midline crossing by commissural axons in the spinal cord and brain involves Slits and their Robo receptors (Blockus and Chédotal, 2016) as well as Sema3B and Npn-2 (Pignata et al., 2016). This suggests that there may be some level of crosstalk and/or cooperation between these ligand-receptor sets. Indeed, recent studies have identified a number of links between these and other guidance cues during axonal navigation (see Table 1).

Robo receptors act to expel axons from their intermediate target, the CNS midline, which expresses Slits. The Class-3 semaphorin Sema3B, which is expressed by the floor plate and mediates its repulsive effect via a receptor complex formed by Npn-2 and plexin A1, is also required for midline crossing (Nawabi et al., 2010; Parra and Zou, 2010; Charoy et al., 2012). Recent studies have identified a link between these two repulsive signalling pathways: plexin A1 was shown to also bind to Slit (Delloye-Bourgeois et al., 2015). A detailed analysis revealed that the N-terminal fragment of Slit (Slit-N) binds to Robo1 and Robo2, whereas the C-terminal fragment of Slit (Slit-C) binds to plexin A1. The proteolytic cleavage of Slit was further analysed in flies (Alavi et al., 2016), where Dscam1 was identified as an additional Slit receptor when interacting in cis with the receptor tyrosine phosphatase RPTP69D (Dascenco et al., 2015). This study demonstrated that Robo1 prefers full-length Slit as a ligand in the absence of Dscam, but the cis complex between Dscam1 and Robo1 binds N-Slit in preference.

Another cis-interacting modulator of Robo1 is FLRT3 (fibronectin and leucine-rich transmembrane protein 3) (Leyva-Díaz et al., 2014). Thalamocortical axons have been shown to be deflected along the rostro-caudal axis depending on the expression of FLRT3. For example, the co-expression of FLRT3 and Robo1 in rostral thalamocortical axons increases the levels of the netrin receptor Dcc (see Box 2) on their growth cones in a protein kinase A-dependent manner. Therefore, these axons are attracted rostrally by parallel gradients of Slit1 and netrin 1 (Bielle et al., 2011). In contrast, intermediate thalamocortical axons that express the same level of Robo1, but no FLRT3, express low levels or no Dcc and therefore are not attracted by netrin (Leyva-Díaz et al., 2014). The observed difference in axonal behaviour in the presence of either netrin or Slit, in comparison with the combination of the two cues, has been corroborated *in vitro* (Dupin et al., 2015).

Further support for a link between Robo-Slit and netrin-Dcc signalling has been provided by a recent study of the divergent Robo receptor Robo3 (Zelina et al., 2014; see Box 3). This study showed that the presence of netrin results in the phosphorylation of Robo3, which in turn is required for axonal growth to the floor plate. Netrin was found not to bind to Robo3, however, but rather to Dcc, which interacts with Robo3 in cis. Interestingly, the role of Robo3 differs between mammals and non-mammalian vertebrates (Zelina et al.,



Guidance cue/ receptor	dI1 commissural axons	Thalamo-cortical axons	Corpus callosum	Optic chiasm
Netrin/Dcc	+	+	+	+
Slit/Robo	+	+	+	+
Sema3/neuropilin	+	+	+	+
Sema6/plexin	+	+	?	+
Ephrin/Eph	+	+	+	+
Wnt/Frz3 or Ryk	+	+	+	+
Draxin/Dcc	+	+	+	?

Fig. 2. Axon guidance within the brain. The guidance cues that are involved in the navigation of dI1 commissural axons are conserved; they are used during the projection of thalamocortical axons from the different nuclei of the thalamus (blue) through corridor cells (green) to the cortex, and by cortical neurons forming the corpus callosum. Similarly, these guidance cues are involved in the wiring of the visual system and the formation of the optic chiasm. References not mentioned in the main text for cortical axons: Hutchins et al., 2011; Keeble et al., 2006; for visual system: Plump et al., 2002; Sánchez-Camacho and Bovolenta, 2008; Plachez et al., 2008; Wang et al., 2016; Fabre et al., 2010.

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Table 1. Crosstalk between different axon guidance pathways

Cues/receptors	Crosstalk	References
Ligands binding to different receptors (canonical receptor in parentheses)		
Netrin (Dcc)	Dcc and Draxin compete for netrin binding	Gao et al., 2015; Ahmed et al., 2011
Slit (Robo)	Plexin A1 identified as alternative Slit receptor	Delloye-Bourgeois et al., 2015
	Cis binding with RPTP69D turns DsCAM into Slit receptor	Alavi et al., 2016
Crosstalk between different axon guidance cues		
Netrin and Shh	In flies, Hh and Netrin attract axons to the midline	Ricolo et al., 2015; Garbe et al., 2007
	In vertebrates, synergism through Src-family kinase activity	Sloan et al., 2015
Netrin and Slit	Combination of Slit and netrin attract thalamocortical axons	Bielle et al., 2011
Shh and Wnt	Shh regulates Wnt signalling via Sfrp and Shisa2	Domanitskaya et al., 2010; Onishi and Zou, 2017
Shh	Shh induces sensitivity to class-3 semaphorins	Parra and Zou, 2010
Sfrp	Sfrp-mediated regulation of ADAM activity modulates CAM-CAM interaction	Marcos et al., 2015
Crosstalk between guidance receptors		
Robo3 and Dcc	Robo3 modulates netrin-mediated attraction by cis interaction with Dcc	Zelina et al., 2014
NrCAM and plexin A1	Upregulation of plexin A1 confers sensitivity to Sema3 repulsion	Charoy et al., 2012; Nawabi et al., 2010
FLRT and Robo	Cis binding of FLRT to Robo upregulates Dcc surface expression and thus modulates attraction	Leyva-Díaz et al., 2014; Bielle et al., 2011
NrCAM and plexin A1	Complex between Sema6D/NrCAM/plexin A1 promotes midline crossing at chiasm	Kuwajima et al., 2012

2014; see Friocourt and Chédotal, 2017 for a detailed review on the changes in Robo3 function during evolution).

Cooperation of guidance cues has also been identified for netrin and Shh in vertebrates (Sloan et al., 2015), and for Netrin and Frazzled with the planar cell polarity pathway component Flamingo (Starry night – FlyBase; known as Celsr3 in mouse) in flies (Organisti et al., 2015). Netrin was also found to synergize with ephrin in the activation of Src family kinase signalling during muscle innervation by motoneurons (Poliak et al., 2015). Crosstalk between Shh and Sema3 signalling during midline crossing has also been reported (Parra and Zou, 2010). In particular, it was shown that exposure of pre-crossing commissural axons to Shh made them sensitive to the repellent effect of the class-3 semaphorins Sema3B and Sema3F, thus explaining the repulsive effect on post-crossing axons observed *in vitro* and *in vivo* (Zou et al., 2000).

Overall, these findings highlight that a great deal of cooperation and crosstalk occurs between various guidance cues and receptors. These studies also demonstrate how important it is to take the complexity of the developing nervous system into account; *in vitro* experiments looking at a single molecule are likely to miss important aspects of axonal navigation that are influenced by crosstalk between different molecules.

Box 2. Netrin receptors

Dcc was identified as the receptor mediating the long-range attractive response to netrin in the spinal cord (Keino-Masu et al., 1996) and in the brain (Leyva-Díaz et al., 2014; Fothergill et al., 2014). However, Dcc has also been shown to mediate an inhibitory response to the long-range repellent Draxin (Ahmed et al., 2011), and it was thus suggested that Dscam might function as an alternative receptor for the attractive effect of netrin 1 (Andrews et al., 2008; Ly et al., 2008; Liu et al., 2009). However, these findings were questioned in a recent study demonstrating that Dscam knockout mice do not have any pathfinding errors in the spinal cord (Palmesino et al., 2012). Furthermore, the netrin-dependent exit of retinal ganglion cell axons from the eye was not affected by the absence of Dscam, although axon fasciculation and their growth from the chiasm to the target was perturbed due to aberrant growth speed (Bruce et al., 2017). Thus, Dcc appears to be the major netrin receptor, although the related molecule neogenin was identified as an alternative receptor for netrin's attractive effect based on experiments in birds and structural analyses (Friocourt and Chédotal, 2017).

The regulation of axon guidance receptors at choice points

After reaching a choice point, growth cones need to change their surface receptors to be prepared for the next stage of their trajectory. Such changes are possible by different mechanisms. In theory, changes can be at the transcriptional, the translational, or the protein/post-translational level. As I summarize below, studies have demonstrated that all of these possibilities appear to be used by navigating axons (Fig. 3).

Changes in transcription

Analyses of temporal and spatial expression patterns demonstrate that initiation of the expression of guidance receptors very often coincides with the arrival of axons at a choice point (for example, Andermatt et al., 2014; Wilson and Stoeckli, 2013). The signals triggering this change in transcription, however, are mostly unknown (Fig. 3A). One possibility is a time-dependent mechanism – an intrinsic timer that changes the gene expression programme of a neuron depending on its age. This would mean that the growth cone changes its surface receptors independently of its environment and its position along the trajectory. Therefore, changes in growth speed would interfere with axon guidance as the encountered guidance cues and their receptors would be out of synchrony. Evidence for an effect of growth speed, and an internal timing mechanism of gene expression has been found in the visual system for responsiveness to netrin (Shewan et al., 2002; Bruce et al., 2017) and Sema3A (Baudet et al., 2012), and in commissural axons of the spinal cord (Phan et al., 2010). Although it is unclear how time is counted by a neuron, a mechanism that has been suggested involves accumulation of the 14-3-3 adaptor proteins (Kaplan et al., 2014; Yam et al., 2012). High levels of 14-3-3 were suggested to change the attractive effect of Shh into repulsion. On its own, however, the accumulation of an adaptor protein may not be a solid regulator of axonal behaviour. A more robust mechanism that has been proposed involves a contact-dependent switch: the contact between a growth cone and its intermediate target could induce a transcriptional change in the neuron and thus result in the insertion of guidance receptors in a precisely timed manner. This has been

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Box 3. Robo3

In mouse, Robo3 has been shown to affect midline crossing of dI1 commissural axons in the spinal cord (Sabatier et al., 2004). Initially, Robo3 was thought to inhibit Slit interactions with Robo1 and Robo2 by binding to Robo1/2 in cis and thereby preventing their interaction with Slit (Chen et al., 2008). One model suggests that Robo3 exists as two splice variants with different expression patterns. In this model, Robo3.1 is thought to bind to Robo1 on pre-crossing axons and prevent repulsive interactions between Robo1 and Slit, whereas this interaction is thought to be possible for post-crossing axons because they express Robo3.2 rather than Robo3.1. Although it is unclear how the change in splice isoforms is regulated, it has been shown that axonal contact with the floor plate triggers local translation of Robo3.2 in growth cones, whereas nonsense-mediated mRNA decay was suggested as a mechanism of keeping Robo3.2 transcripts low (Colak et al., 2013).

demonstrated for the switch in responsiveness to Shh based on *in vivo* experiments. Shh itself was found to trigger the expression of Hhip, its receptor on post-crossing axons, in a glypican 1-dependent manner (Bourikas et al., 2005; Wilson and Stoeckli, 2013), providing an alternative or additional mechanism to the 14-3-3-dependent switch mentioned above. In flies, the Netrin receptor Frazzled was shown to be cleaved by γ-secretase (Neuhaus-Follini and Bashaw, 2015). The released intracellular domain is then transferred to the nucleus to induce the expression of Commissureless, which in turn regulates the expression of Robo on post-crossing axons at the post-translational level (see below) and, thus, controls sensitivity to Slit during midline crossing (Keleman et al., 2002, 2005).

Changes in translation

Regulation of the surface expression of guidance receptors is not necessarily dependent on changes in gene transcription; changes in translation can also play a key role (Fig. 3A). Indeed, a recent study identified the RNA-binding protein Hermes as a regulator of Neuropilin 1 translation and hence axon sorting in the zebrafish optic tract (Hörnberg et al., 2016). It had also previously been demonstrated that the sensitivity of retinal ganglion cell axons to the repellent Sema3a is regulated by translational control of Neuropilin 1 expression in a microRNA (miRNA)-dependent manner (Baudet et al., 2012). A role for miRNAs in axonal connectivity in the visual system was suggested previously by the perturbation of the miRNA processing pathway component Dicer1 (Pinter and Hindges, 2010). Since then, miRNAs have been shown to not only regulate the expression of guidance receptors in the cell body but also regulate protein synthesis locally in the growth cone (reviewed by Rajman and Schratt, 2017). For example, Slit2-mediated control of growth cone behaviour was recently shown to involve miRNA-mediated transcriptional regulation of cofilin-1, a protein that modulates actin dynamics (Bellon et al., 2017).

The importance of local translation in growth cones was in fact reported many years ago (Campbell and Holt, 2001). However, local protein synthesis was initially shown for β-actin or proteins that regulate cytoskeletal dynamics and, therefore, growth cone behaviour in response to guidance cues rather than for the regulation of the expression of guidance receptors (summarized by Jung et al., 2012). In recent years, however, a number of guidance cues and receptors have been shown to be translated locally within growth cones. For example, netrin was shown to regulate the local translation of its receptor Dscam in growth cones (Jain and Welshhans, 2016) and to affect Dcc-mediated translation (Tcherkezian et al., 2010). Local synthesis in growth cones at the spinal cord midline has also been described for EphA2 (Brittis et al., 2002).

The local translation of proteins in growth cones depends on transport of mRNAs into the distal axon (Fig. 3B). Therefore, evidence for local translation is also provided by the requirement of RNA-binding proteins in axonal navigation (Hörnberg and Holt, 2013; Jung et al., 2012). For instance, the RNA-binding protein ZBP1 (zip-code protein 1) has been implicated in attractive growth cone turning (Donnelly et al., 2011) via regulation of β-actin synthesis (Welshhans and Bassell, 2011) and, more recently, the attractive effect of Shh on pre-crossing axons was shown to affect the dynamics of the cytoskeleton via ZBP1-mediated translation of β-actin in the growth cone (Lepelletier et al., 2017). IMP2 (Igf2bp2), another RNA-binding protein, was also shown to be required for local regulation of Robo1 and Robo2 (Preitner et al., 2016).

Regulation at the post-translational level

The surface expression of active guidance receptors has also been shown to be regulated by specific trafficking and processing, and by interactions with other proteins (Fig. 3C). The specific delivery of guidance receptors can thus be used to regulate responsiveness to a particular guidance cue in a temporal manner. For instance, the insertion of Robo1 into the growth cone surface during midline crossing was shown to depend on vesicular trafficking regulated by calsyntenin 1 and Rab GDP dissociation inhibitor (RabGDI) (Alther et al., 2016; Philipp et al., 2012). Calsyntenin 1, but not RabGDI, is also required for the regulation of frizzled 3-mediated post-crossing commissural axon guidance. The surface insertion of Robo3 receptors in flies is increased by co-expression of the receptor protein tyrosine kinase RPTP69D (Oliva et al., 2016), and Dcc levels on retinal ganglion cell axons are controlled by the ESCRT-II (endosomal sorting complex required for transport) complex, an essential regulator of cargo sorting (Konopacki et al., 2016).

In addition to regulated membrane insertion of guidance receptors, the opposite – the selective removal or shedding of guidance receptors – has also been found (Fig. 3C). The shedding of guidance receptors by ADAM proteases has been implicated in L1CAM and N-cadherin (cadherin 2) function in mouse retinal ganglion cells (Marcos et al., 2015) and the selective sensitivity of nociceptive compared with proprioceptive sensory afferents to Sema3A (Pond et al., 2002; Romi et al., 2014). Regulation of ADAM-mediated receptor shedding has been characterized in particular detail in the CNS and involves leucine-rich repeats and immunoglobulin-like domains 2 (Lrig2; van Erp et al., 2015), which prevents ADAM-dependent shedding by binding to neogenin and thus regulates responsiveness to RGMa, a repulsive ligand.

Interactions between guidance receptors in the plane of the membrane (cis interactions) can also modulate their activity at choice points (Fig. 3C). The best-studied example of this, which involves cis interaction-dependent attenuation of signalling, occurs during the innervation of the tectum by retinal ganglion cell axons. In this context, repulsive signalling is fine-tuned by cis interactions between EphA receptors and ephrin A ligands on the growth cone surface (Suetterlin and Drescher, 2014; Fiederling et al., 2017). Similarly, shedding of the EphA4 extracellular domain as an alternative regulatory mechanism to cis attenuation is required for proper limb innervation (Gatto et al., 2014), although in this case the regulatory mechanisms has not been characterized.

Fine-tuning of axonal responsiveness can also be achieved via cis interactions of guidance receptors that modulate the binding affinity to molecules on other membranes (trans-interactions). An example

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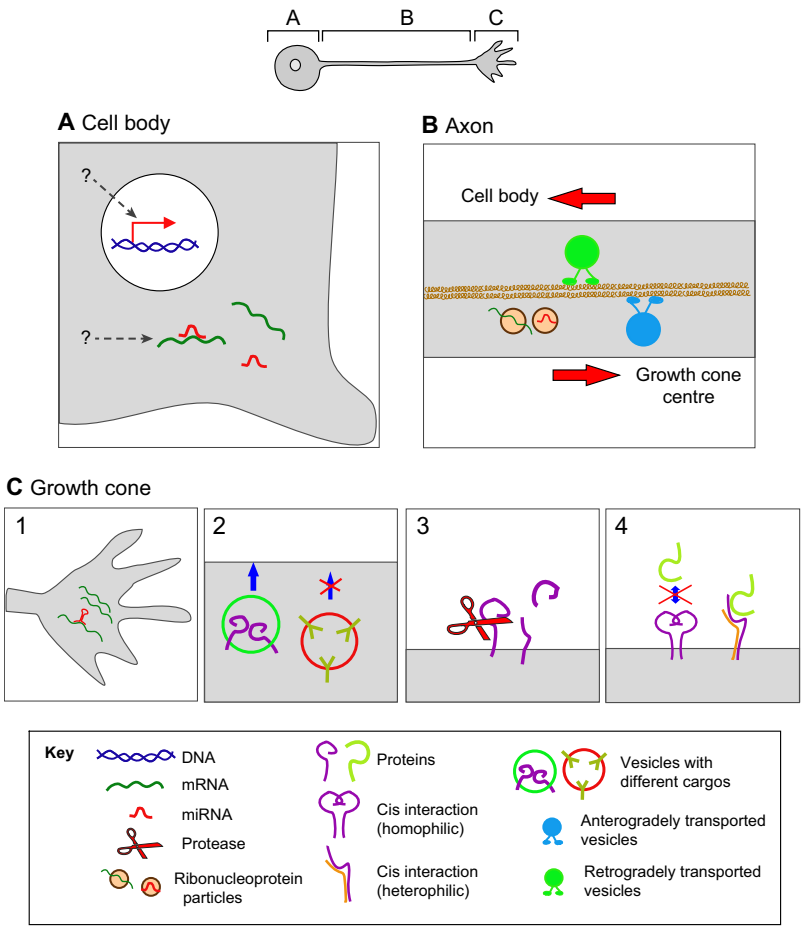

Fig. 3. Axon guidance at choice points. (A-C) Regulation of the surface expression of guidance receptors on growth cones is achieved by precisely orchestrated processes. When a growth cone reaches a choice point, or intermediate target, it needs to change the guidance receptors expressed on its surface in order to change its responsiveness from attraction to repulsion and to be prepared for the next stage of its journey. Changes can be made at different levels, including within the cell body (A), the axon (B) or the growth cone (C). (A) Changes can occur at the level of gene transcription in the nucleus, followed by mRNA translation in the cell soma. Translation can be further regulated by miRNAs, thus the presence of an mRNA does not necessarily predict protein synthesis. (B) Proteins synthesized in the cell body are transported through the axon to the growth cone by anterograde vesicle transport mediated by kinesin motors. Conversely, retrograde vesicular transport mediated by dynein motors is responsible for signal transport from the growth cone to the cell body. (C) mRNAs and miRNAs can be transported to the growth cone to modulate local translation (1). Proteins synthesized in the cell body can also be transported and inserted into the plasma membrane in a precisely controlled manner, because of the selection of specific vesicles by cargo adaptor proteins. These vesicles can then fuse upon a specific trigger derived from growth cone-target interaction (2). Protein levels on growth cones can also be controlled by specific proteases, which in turn can be regulated specifically by cell-cell interactions (3). Finally, interactions between guidance receptors and guidance cues can be prevented or modulated by cis interactions between molecules in the plane of the growth cone membrane (4). These interactions can be between two identical (homophilic) or two different (heterophilic) proteins. Depending on the interaction partner in cis, the affinity for trans interaction partners is set differently.

of this type of regulation was found for class-6 semaphorins and plexin A receptors. A role for class-6 semaphorins was initially described for the formation of thalamocortical connections (Leighton et al., 2001). Although they are transmembrane proteins, they were initially considered to act as ligands for plexin A receptors. Today, however, it is clear that class-6 semaphorins have dual functions, acting as ligands in some contexts but also as receptors in others (Jongbloets and Pasterkamp, 2014; Pasterkamp, 2012). Sema6A, for instance, acts as a receptor in boundary cap cell precursors (Mauti et al., 2007; Bron et al., 2007), and Sema6B acts as a receptor for plexin A2 and contributes to midline crossing and post-crossing commissural axon guidance in the spinal cord (Andermatt et al., 2014). Several studies have shown that the signalling activities and interactions of class-6 semaphorins are regulated by a complex combination of cis- and trans-interactions with plexin A receptors (Haklai-Topper et al., 2010; Andermatt et al., 2014; Perez-Branguli et al., 2016). A role for membrane-bound Semaphorin 1a has also been shown for midline crossing in invertebrates, although in this case the effect was found to be independent of Plexin binding (Hernandez-Fleming et al., 2017).

The cis interaction-mediated regulation of signalling downstream of guidance receptors has been identified as an important mechanism of axon pathfinding in many contexts. Initially, the importance of cis interactions in the modulation of trans interactions was shown for cell adhesion molecules of the immunoglobulin superfamily of cell adhesion molecules (IgSF-CAMs; Kunz et al., 1996; Kunz et al., 2002). More recent studies have confirmed this for commissural axons (Niederkofler et al., 2010) and sensory afferent navigation into the spinal cord (Frei et al., 2014). Crosstalk between IgSF-CAMs and neuropilins has also been shown to fine-tune growth cone behaviour (Castellani et al., 2000, 2002; Law et al., 2008), and interactions between IgSF-CAMs, class-6 semaphorins and plexin A1 control midline crossing at the chiasm (Kuwajima et al., 2012).

Perspectives: so, are we nearly there yet?

As I have highlighted here, our knowledge of neural circuit formation in the brain is still very much in its infancy. We can infer molecular mechanisms from what we have learned in one system to another but there is still not a single population of axons for which we have a complete understanding of the molecular mechanisms of navigation to the final target. So, we are clearly not there yet! A major challenge remains the characterization of the precise temporal regulation of guidance signals and the interactions between different signalling pathways that cooperate to guide axons to their intermediate, and ultimately final, targets. Axon guidance studies in a variety of organisms clearly indicate that the regulation of axon guidance signalling involves all possible mechanisms of regulation: transcriptional and translational control, trafficking of specific vesicles, and changes in protein-protein interactions as well as protein stability. Furthermore, the link between the interactions of guidance receptors and their ligands with the observed behaviour of growth cones is still missing. We also only have a very superficial understanding of the association between surface receptors and the regulation of cytoskeletal dynamics responsible for steering growth cones (Gomez and Letourneau, 2014). Similarly, our knowledge on specific intra-axonal trafficking of signals is poor. To make the next step in our understanding of axon guidance, it will be important to

keep complexity in mind. Classical loss-of-function approaches might not reveal the complex interaction between guidance cues and their different receptors. Precise temporal control of experiments during embryonic development is difficult in mammals. Therefore, it will be important to make use of diverse animal models, each with its strengths and weaknesses. A particular challenge will be the visualization of the functional link between surface receptors and the cytoskeleton. This is becoming easier to do *in vitro* thanks to high-resolution imaging techniques, but *in vitro* experiments will not allow for the analysis of axon guidance as they will never be able to mimic the complexity of cell-cell interactions in the developing tissue. It is thus clear that understanding axon guidance remains a challenge, and that a multifaceted, multidisciplinary approach will be required to understand not only how a single axon finds its target but how billions of axons manage to do so.

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