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Getting axons onto the right path: the role of transcription factors in axon guidance

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The normal function of the nervous system requires that the constituent neurons are precisely 'wired together'. During embryogenesis, each neuron extends an axonal process, which can navigate a considerable distance to its target. Although a number of the receptors and guidance signals that direct axonal growth have been identified, less is known about the transcription factors that regulate the expression of these molecules within the neuron and its environment. This review examines recent studies in vertebrates and *Drosophila* that address the identity of the transcription factors that either control the repertoire of guidance receptors and signals that permits an axon to take a particular trajectory or act themselves as novel extracellular guidance factors.

Introduction

The diverse functions of the nervous system, from cognition to movement, are possible because neurons rapidly and accurately communicate with their targets using precisely ordered neuronal networks. These networks arise during embryonic development, when an intricate but unerring pattern of axonal connections is generated between neurons and their synaptic partners. This complex circuitry is established when an axon extends away from its neuronal cell body and navigates through the diverse embryonic environment towards its synaptic target. To complete its trajectory, the growth cone at the tip of the extending axon must be able to distinguish between multiple signals within this complex environment and then adapt its response to these signals over time. A current goal within the axon guidance field is to understand the balance between the information provided to the neuron at its time of birth and the information gained from the environment as the axon travels along its route. Is the complete repertoire of receptors required by a growth cone to navigate its entire pathway determined intrinsically during neurogenesis, or are additional receptors activated as the axon grows? Are early guidance decisions dependent on transcription, and later outgrowth decisions made without recourse to the nucleus using post-transcriptional mechanisms? If post-transcriptional mechanisms are involved, do earlier-acting transcription factors dictate how these mechanisms are subsequently used by the growth cone? Recent work, discussed below, has identified key roles for transcription factors in determining the initial pathway selected by an axon and in influencing later axon-pathway choices as the axons extend towards their target. Transcription factors have also been found to regulate the expression of appropriate extrinsic cues necessary for accurate axon guidance.

Here, we review the amount of information that appears to be encoded by transcription factors within the neuron when it makes a final fate choice and how far that information shapes the pathway taken by an axon. We further discuss the role of transcription factors in providing both intrinsic and extrinsic information to an axon during its trajectory.

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The molecular basis of axon guidance

The axon is guided along its pathway by the growth cone, a structure at the leading edge of the axon. The growth cone selects the direction of extension by detecting and processing molecular guidance cues presented by intermediate targets in the extracellular environment. Guidance signals include the patterned expression of attractant and repellent molecules (Fig. 1) within the substrate, and the graded expression of diffusible molecules secreted by distant targets (see Box 1 for a list of the main guidance cues). An axon projects in a series of steps towards the synaptic target by correctly interpreting these guidance cues to make the appropriate pattern of extensions and turns to navigate along its particular trajectory.

Guidance cues were at first thought to act as only repellents or attractants, but not both; however, they have subsequently been shown to be bifunctional (i.e. one guidance cue can be either attractive or repulsive, depending on the status of the receiving neuron) (Dickson, 2002). First, different receptor complexes can provide alternative responses to the same cue (Yu and Bargmann, 2001). For example, Netrin1 has two receptors: DCC is thought to mediate the attractive response of an axon to Netrin1, whereas Unc5H mediates the repellent response (Chan et al., 1996; Hamelin et al., 1993; Hong et al., 1999; Keino-Masu et al., 1996; Kolodziej et al., 1996). Second, the attractive response of an axon to Netrin1 can be switched to a repulsive one, or vice versa, by modulating the levels of cytosolic cyclic AMP (cAMP) in the growth cone in vitro (Song et al., 1997). This paradigm is generally true for axon guidance cues in vitro – the response of a given guidance cue can be reversed by altering the status of intracellular cAMP or cyclic GMP (cGMP) (Song et al., 1998). Thus, the nature of the response of an axon to a particular guidance signal may depend on the receptors present in the growth cone and/or on the recent history of second-messenger activation in the growth cone.

Transcription factors as early intrinsic regulators of pathway choice

The subset of transcription factors expressed in neurons is crucial for not only neural identity, but also for the next step of neuron differentiation – the extension of axons into the neuroepithelium. This observation has been best illustrated by studies in both vertebrates and invertebrates, which have examined how motoneurons (MNs) are directed to extend towards their particular target muscle.

Motor neuron circuitry

Studies of the guidance of MNs to either the abdominal wall musculature in *Drosophila* or the vertebrate limb have revealed some parallels between these two systems, in both the general organization of MNs and in the types of molecules that direct circuit formation.

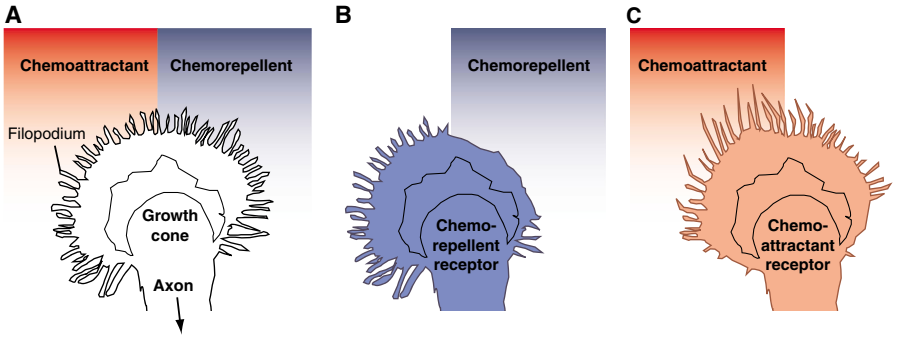

Fig. 1. General mechanisms of axon guidance. The response of a growth cone to signals in the environment depends on the complement of receptors it expresses. (**A**) The growth cone will be unresponsive to external guidance cues if it does not contain the relevant receptors to perceive a gradient of either a chemoattractant (red) or of a chemorepellent (blue). (**B**) If the growth cone expresses the appropriate chemorepellent receptor (light blue), the activation of this receptor will result in the local depolymerisation of the actin cytoskeleton, such that the growth cone reorients away from the repellent. (**C**) Alternatively, activation of a chemoattractant receptor (red) in the growth cone results in the stabilization and extension of filopodia, such that the growth cone extends towards the attractant. Schematic of growth cone modified with permission from Forscher and Smith (Forscher and Smith, 1988).

In *Drosophila*, there are 36 MNs per abdominal hemisegment, the simple half-segment unit of the insect nervous system that is identical on the left and right sides of the *Drosophila* abdomen. These MNs can be grouped by the route that they take and the muscle field that they innervate. Distinct classes of MNs extend axons into either the intersegmental nerve (ISN), segmental nerve (SN) or transverse nerve. These classes can be subdivided further by their target area: motor axons that extend along the ISN can innervate the dorsal (ISN^D), lateral (ISN^L) or ventral (ISNb and ISNd) muscle groups, whereas motor axons that follow the SN innervate lateral (SNa) or ventral (SNc) muscle groups (Landgraf and Thor, 2006) (Fig. 2A).

In the vertebrate spinal cord, MNs are clustered such that the position of the cell body predicts its axon trajectory and synaptic target (Tanabe and Jessell, 1996). The somatic MNs in the vertebrate spinal cord are arranged in columns that project along common nerve pathways [e.g. axons from the medial motor column (MMC) extend to axial muscles, whereas axons from the lateral motor column (LMC), present only at brachial and lumbar limb levels, extend to the muscles of the limb]. These columns are subdivided further into divisions of neurons that take different pathways once they reach the target region [e.g. the LMC is divided into a medial (m) division that innervates the ventral region of the limb, whereas MNs in the lateral (l) division of the LMC innervate dorsal limb]. Finally, these divisions are organized into MN pools that innervate individual muscle groups (Fig. 2B). The identity and axon trajectory pattern of each of these groups can be defined, to a greater or lesser extent, by their expression of individual, or combinations of, transcription factors. Studies over the last couple of years have begun to assign the selection of an axon pathway to the activity of certain transcription factors and, in some cases, identify possible candidates for the axon-guidance effectors regulated by these factors.

Hox genes control the selectivity of MN innervation

Vertebrate somatic MNs differ from their *Drosophila* counterparts in that the position of their cell bodies is highly predictive of their axonal projection pattern. Thus, the Hox genes that control cellular identity along the rostral-caudal axis are good candidates to stand at the top of the hierarchies of transcription factor activity that specify MN identity. And, in fact, Hox genes do appear to determine the columnar identity of vertebrate MNs (e.g. *Hoxa6* and *Hoxc6* activity specifies brachial LMC identity and is necessary to direct LMC axons into the chick limb; Fig. 2B) (Dasen et al., 2003). The loss of constitutive Hox gene activity results in axon projection defects; but, are the Hox genes direct activators of axon-pathway-choice effectors? Recently, it has been shown that the particular repertoire of Hox molecules, the so-called ‘Hox code’, expressed by MNs plays a role in both establishing pool identity in the LMC and directing axonal connectivity (Dasen et al., 2005). This result is exemplified by the study of Hox5 and Hoxc8: *Hox5* is persistently expressed by MNs that innervate the scapulohumeralis posterior (Sca) muscle, whereas *Hoxc8*-positive MNs innervate the pectoralis (Pec), anterior latissimus dorsi (ALD) and flexor carpi ulnaris (FCU) muscles (Fig. 2B). RNAi knockdown of *Hoxc8* from caudal LMC neurons in chick embryos results in the caudal expansion of *Hox5* expression, so that these neurons extend axons to the Sca muscle. The exact muscle target innervated by the $Hoxc8^+$ population of MNs depends on the activities of Hox4 and Hoxc6. Thus, MNs expressing *Hoxc8* and *Hox4* innervate the FCU, whereas those that express *Hoxc8*, *Hox4* and *Hox6c* innervate the Pec and ALD muscles. Changing the Hox code for these pools causes the MNs to innervate the predicted inappropriate targets (Dasen et al., 2005). However, it appears unlikely that the Hox proteins are themselves directly regulating the downstream axon guidance effectors. Rather, their expression pattern and any manipulation thereof correlates with the pattern of expression of downstream transcription factors [e.g. the MNs that innervate the Sca muscle express runt related transcription factor 1 (*Runx1*), while the FCU MNs express the POU domain transcription factor *Pou3f1* (previously known as *Scip*), suggesting that it is these molecules that help direct connectivity. Whether these molecules directly activate the expression of specific axon guidance receptors or effectors is not yet fully characterized, but the relative levels of Runx proteins are known to direct the laminar termination of sensory axons (see below) (Chen et al., 2006a).

In *Drosophila*, the somatic MNs are generated from several different neuroblasts rather than from a restricted set of neural progenitor cells, as in vertebrates, and are also not limited to a particular location in the nerve cord (Schmid et al., 1999). Yet, a number of the MN determinates that appear to play an early role in the specification of MN identity and are also necessary for MN axons to exit the CNS and follow their particular nerve routes (Fig. 2A). The homeodomain protein Nkx6 and the zinc-finger transcription factor Zfh1 are both necessary in order for ventrally projecting MNs (vMNs) (e.g. ISNb) to leave the CNS (Broihier et

al., 2004; Layden et al., 2006) (Fig. 2). The loss of activity of these proteins does not appear to affect other parameters of MN identity, but does restrict their outgrowth potential. It is presumed that these molecules have common downstream targets within the vMNs that are necessary for them to exit the CNS. It is likely that their action on outgrowth may be indirect and involve downstream regulators, such as Islet and Lim3 (see below), although, in a subset of vMNs, one potential axon guidance target of Nkx6 has been identified as the cell adhesion molecule Fasciclin III (Broihier et al., 2004). For dorsally projecting MNs, it appears that the homeodomain-containing molecule Even-skipped (Eve) plays a major role in directing their outgrowth (Landgraf et al., 1999). Eve may do this by suppressing the expression of the ventral determinates *Hb9* and *islet*, activating the Netrin receptor Unc5 (Labrador et al., 2005) and regulating the activity of the cell adhesion molecule Fasciclin II, which mediates intra-axonal adhesion within the ISN (Fujioka et al., 2003; Landgraf et al., 1999; Sanchez-Soriano and Prokop, 2005). Unc5 perceives Netrin as a repellent, and this activity is important for the appropriate projection of ISNb axons to the dorsal muscles (Keleman and Dickson, 2001; Labrador et al., 2005). Unc5 is expressed by the MNs that express Eve, and misexpression of Eve in ventrally projecting neurons drives the expression of *Unc5* and directs their axons dorsally. The misexpression of Eve does not alter cell fate, but rather it defines the axonal trajectory of the MNs, suggesting that the Eve transcription factor may directly regulate *Unc5*. However, this may not be true for all Eve-positive MNs, because recent evidence has suggested that the position of Eve within the genetic cascade that directs the outgrowth of individual neurons may vary between individual neurons (Fujioka et al., 2003; Garces and Thor, 2006).

LIM domain transcription factors dictate MN-pathway choices

The groups of vMNs in *Drosophila* or in the vertebrate LMC are divided into two major subtypes. In *Drosophila*, the division is between those that extend along the ISNb or ISNd pathways, whereas, in vertebrates, the division is between those that extend to dorsal or ventral limb muscles. The extension of MNs along particular axon-outgrowth pathways is determined by their expression of distinct combinations of LIM-homeodomain proteins. This observation has led to the idea that a combinatorial 'code' of LIM proteins specifies MN diversity (Tsuchida et al., 1994). The profile of LIM homeodomain proteins expressed post-mitotically is thought to confer particular classes of vMNs with the ability to select specific axon pathways, and thereby the topographic organization of motor projections within their particular domain.

This paradigm is conserved for both *Drosophila* and vertebrates. In the chick and rodent spinal cord, neurons in the lateral (l) part of the LMC extend to dorsal muscles, whereas those with a medial (m) position extend to the ventral muscles (Fig. 2B). Both sets of LMC neurons initially express *Islet1* (*Isl1*, also known as *tailup*), however the expression of this gene is only maintained in LMC(m). LMC(l) neurons subsequently express *Lim1*, which then represses *Isl1* expression in these neurons (Kania and Jessell, 2003). The activity of Lim1 determines the ability of the LMC(l) neurons to select a dorsal trajectory. The loss of *Lim1* does not appear to affect the fate of these neurons nor the initial stages of axon extension out of the spinal cord. However, in the absence of Lim1, LMC(l) axons do not select their normal dorsal trajectory but rather extend into ventral regions of the mouse limb (Kania et al., 2000). Conversely, ectopic expression of *Lim1* in chick LMC neurons is sufficient to direct LMC(m) axons into the dorsal limb (Kania and Jessell, 2003).

Box 1. Key families of guidance cues and their receptors

In recent years, significant advances have been made in the identification of the ligands and receptors that dictate and detect the trajectory taken by an individual axon. Guidance receptors act either through the activation of second messenger systems to direct local rearrangement of the cytoskeleton to promote growth towards or away from the target or by mediating differential adhesion. Diffusible ligand/receptor pairs include members of the Netrin/DCC, Slit/Robo, and Semaphorin/Plexin/Neuropilin families. Membrane-bound ligand/receptor pairs, which require contact between growth cone and substrate to mediate signaling, include members of the Semaphorin family, the ephrins/EphR and members of the diverse families of cellular adhesion molecules (CAMs). Recently, the repertoire of potential guidance factors has been expanded to include morphogens – growth factors that act earlier during development to specify cell fate. To date, morphogens shown to also have axon guidance activity include members of the bone morphogenetic protein (Bmp), hedgehog (Hh), Wnt and fibroblast growth factor (Fgf) families (Charron and Tessier-Lavigne, 2005). It remains unclear whether the same receptors that mediate the induction of cellular fate also transduce axon guidance activity.

Similarly, the transient expression of two LIM-homeodomain factors, *Lhx3* (also known as *Lim3*) and *Lhx4* (*Lim4*), in the mouse MMC is crucially required for the ability of ventral MNs to extend axons out of the ventral root (Sharma et al., 1998). In mouse embryos that lack both *Lhx3* and *Lhx4*, the ventral root is absent and a more dorsal fascicle, the spinal accessory fascicle, is enlarged. This phenotype results from ventral MNs switching their subtype identity to that of dorsal MNs. Thus, the ventral MNs now extend axons more dorsally out of the spinal cord in the appropriate manner for their new identity. Conversely, the misexpression of *Lhx3* in dorsal-exiting MNs was sufficient to direct their axonal projections out of the ventral root (Sharma et al., 1998). A candidate downstream effector of Lhx3 is fibroblast growth factor receptor 1 (FGFR1), which is expressed in the MMC and is necessary for their normal guidance (Shirasaki et al., 2006).

Lim3, the *Drosophila* homologue of *Lhx3/4*, also functions as a binary switch to control the trajectory of different classes of motor axons (Thor et al., 1999). *Lim3* is expressed in a subset of neurons per hemisegment of the ventral nerve cord. This subset includes MNs that additionally co-express *islet* – the *Drosophila* homologue of *Isl1* and *Isl2* – and project axons into ISNb. *islet* is also expressed by MNs that extend axons into ISNd, the other ventral branch of the ISN. *Lim3*-mutant *Drosophila* embryos show no gross abnormalities in embryonic axonal organization, suggesting that Lim3 does not have a role in early patterning. However, there are specific defects in the trajectories of the ISNb MNs (Thor et al., 1999). In wild-type embryos, $Lim3^+$ $Islet^+$ MNs in ISNb innervate a subset of muscles different from the ones innervated by $Lim3^-$, $Islet^+$ MNs in ISNd (Fig. 2A). However, in *lim3*-mutant embryos, ISNb-specific muscles were abnormally innervated and, concomitantly, the ISNd branch was thicker, indicating that ISNd-specific muscles were being ectopically innervated (Thor et al., 1999). Thus, in the absence of *lim3*, ISNb MNs appear to switch their identity to that of $Islet^+$ MNs and innervate the ISNd target area. This model was tested further by misexpressing *lim3* in the majority of MNs. Under these conditions, significant increases were observed in the number of processes in the ISNb branch at the expense of the ISNd branch, which, in some hemisegments, was completely absent (Thor et al., 1999). Backfilling from the ISNb-innervated muscles demonstrated that

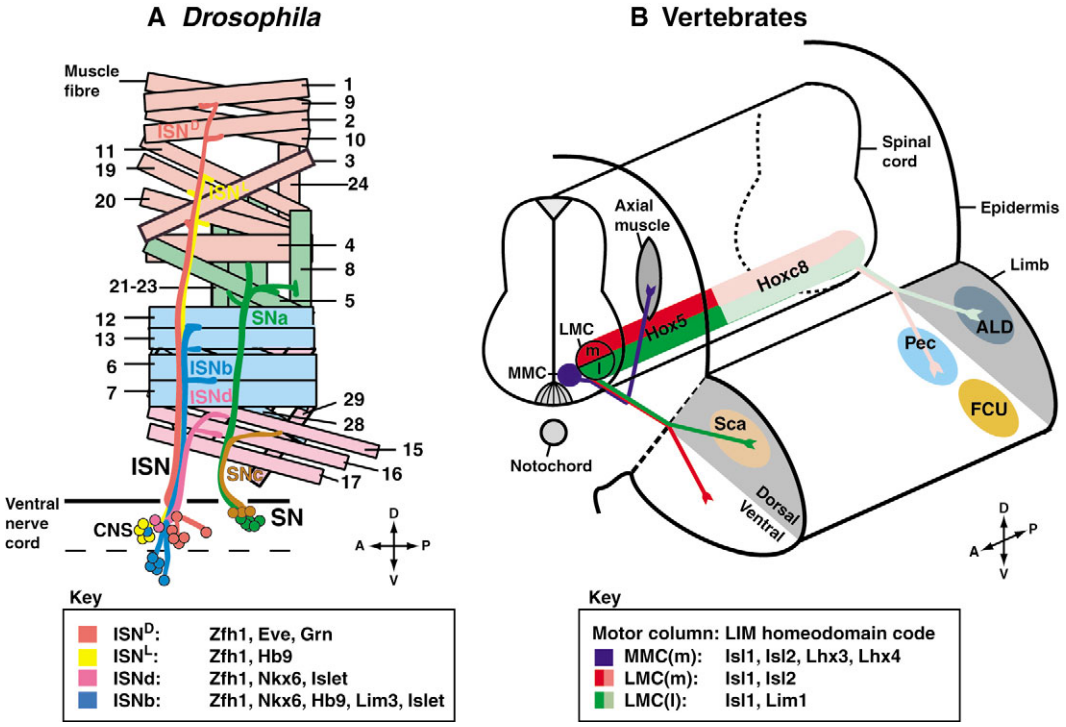

Fig. 2. Combinatorial action of LIM-homeodomain and Hox transcription factors dictate *Drosophila* and vertebrate motor axon guidance. Motor neurons (MNs) in *Drosophila* and vertebrates can be identified by the routes that they take and the muscle fields that they innervate. (**A**) In *Drosophila*, most MNs exit from the ventral nerve cord along two major nerve routes, the segmental nerve (SN) and intersegmental nerve (ISN), from which they defasciculate to innervate discreet populations of muscles (represented by numbers 1-29). The MNs express different combinations of transcription factors that appear to dictate which muscle fields they innervate, as shown in the key. (**B**) In vertebrate spinal cord, somatic MNs are arranged in columns that project to common targets and can be distinguished by the combinatorial expression of LIM-homeodomain transcription factors (see key). The medial motor column (MMC; blue) projects axons to axial muscle, whereas, at the brachial and lumbar levels, the lateral motor column (LMC; red and green) projects to the limb. On reaching the limb, the LMC subdivides such that the medial (m) division (red) projects to the ventral limb, whereas the lateral (l) division (green) projects to the Scapulohumeralis (Sca) muscle of the dorsal limb. These divisions are further subdivided into pools of MNs that innervate particular muscle groups. At brachial levels, the LMC is subdivided by the expression of *Hox5* and *Hoxc8*, which appear to control the projection pattern of LMC axons into distinct motor pools in the Pec (pectoralis), anterior latimuss dorsi (ALD) and flexor carpi ulnaris (FCU) muscles. (A) Modified with permission from Landgraf and Thor (Landgraf and Thor, 2006) and (B) modified with permission from Kania et al. (Kania et al., 2000).

this target was being innervated by normal ISNb MNs and by MNs whose position was consistent with that of ISNd MNs. Thus, ISNd MNs forced to express Lim3 are routed to the same path as ISNb axons.

LIM proteins may directly regulate vertebrate EphRs

Taken together, these studies suggest that the combinatorial role of LIM-homeodomain proteins in establishing neural subtypes and directing axon connectivity is an evolutionarily conserved mechanism. Complexes of LIM proteins dictate the nature of axon trajectories, presumably by regulating the expression of specific genes involved in interpreting either attractive or repulsive signals from intermediate or synaptic targets. Although most downstream LIM-protein targets remain unknown, a candidate downstream axon-guidance effector has been identified for Lim1 (Kania and Jessell, 2003) – the receptor tyrosine kinase, EphA4. EphA4 is a member of the Eph-receptors family, which, together with their membrane-bound ligands, the ephrins, have been implicated as guidance signals for many classes of axons. EphA4 is present at much higher levels on Lim1$^+$ LMC(l) axons when they make their choice to enter the dorsal region of the limb (Eberhart et al., 2002). This increased EphA4 expression on LMC(l) compared to LMC(m) neurons could direct LMC(l) axons away from the repellent ligand ephrinA5, which is enriched in the ventral limb mesenchyme. Increasing the levels of Lim1 in chick LMC neurons increases their expression of EphA4, whereas knockdown of *Lim1* results in lower EphA4 levels in the affected neurons. Thus, it appears that Lim1 directs the trajectory of LMC(l) neurons by activating the expression of EphA4 (Kania and Jessell, 2003). Whether members of the Eph-ephrin signalling pathway also regulate pathway choice by MNs in *Drosophila* is not clear; however, it has emerged that Islet (also known as Tailup) and Lim3 might regulate the expression of an immunoglobulin-containing cell-adhesion molecule from the Beaten-path (Beat) family – Beat-Ic (Certel and Thor, 2004) – and that the ability of the LIM molecules to regulate downstream effectors in particular subclasses of MN also requires interactions with the POU transcription factor, Drifter (also known as Vvl) (Certel and Thor, 2004).

POU-domain transcription factors in retinal and olfactory axon guidance

Within the visual system, retinal ganglion cells (RGCs) navigate from the retina to their target in the superior colliculus. Many of the well-known axon-guidance molecules are known to mediate this migration (Oster and Sretavan, 2003). Although it is not clear how all of these components are regulated, an important role has emerged for the POU-

domain transcription factor Brn3.2 (also known as Brn3b and Pou4f2) in the specification of RGC axon pathfinding (Erkman et al., 2000; Wang et al., 2000). In *Brn3.2*$^{-/-}$ mice, few RGC axons are able to leave the retina and enter the optic nerve despite there being no apparent defects in the generation or identity of RGCs. DiI tracing suggests that the *Brn3.2*$^{-/-}$ axons exhibit pathfinding defects, with many of them failing to navigate towards the optic chiasm. Several molecules have been identified as downstream targets for Brn3.2, including Neuritin and abLIM, an actin binding protein (Erkman et al., 2000; Mu et al., 2004). The expression of dominant-negative forms of abLIM produces similar pathfinding abnormalities, suggesting that it is a likely downstream axon-guidance effector of Brn3.2 (Erkman et al., 2000), although it has yet to be shown that abLIM is a direct target of Brn3.2.

A further role for POU-domain transcription factors in the regulation of connectivity has been identified in the wiring of *Drosophila* olfactory projection neurons (Komiyama et al., 2003). The projection neurons are the second order neurons of the fly olfactory system that extend dendrites to olfactory glomeruli and axons to a higher centre, and are thus equivalent to the mitral/tufted cells in the vertebrate olfactory bulb. The targeted loss of the POU domain transcription factor *acj6* (abnormal chemosensory jump) in these neurons causes axon and dendritic targeting errors without affecting their fate (Komiyama et al., 2003). In particular, *acj6*$^{-/-}$ DL1 projection neurons were unable to extend a dorsal axonal branch into the lateral horn, a structure analogous to the vertebrate primary olfactory cortex. Both Acj6 and Drifter are also necessary for dendritic targeting of the projection neurons to their glomeruli in the antennal lobe, and misexpression of these molecules disrupts dendritic targeting (Komiyama et al., 2003). Although the targets of these molecules are unknown, it appears that they play a role in translating lineage information into neurite targeting.

Runx: specifying the laminar termination pattern of sensory afferents

The Runx family of transcription factors has been implicated in specifying patterns of axon outgrowth for vertebrate sensory spinal afferents in the dorsal root ganglion (DRG). Distinct subclasses of sensory neurons encode different information from the periphery and can be distinguished by a variety of markers, including the expression of neurotrophic receptors (Mu et al., 1993). Thus, temperature sensitivity and pain are conveyed by the TrkA$^+$ and Ret$^+$ nociceptive neurons, touch by the TrkB$^+$ mechanoreceptors, and muscle stretch and tension by the Type Ia, Type Ib and Type II TrkC$^+$ proprioceptive neurons. These subclasses of neurons can also be distinguished by the termination points of their axons along the dorsal ventral (DV) axis of the spinal cord (Brown, 1981). The afferents bringing in cutaneous information terminate in different laminae in the dorsal spinal cord. Type Ib proprioceptors terminate in the intermediate spinal cord, whereas Type Ia and Type II afferents project to ventral regions of the spinal cord. Both how the differential cellular identity and the projection pattern of these neurons is established has remained unclear. These questions have been addressed by three recent studies examining the role of the Runx family in the development of DRG sensory neurons (Chen et al., 2006a; Chen et al., 2006b; Kramer et al., 2006). Previous work had shown that Runx1 is expressed at early stages in TrkA$^+$ nociceptors, and at later stages in the Ret$^+$ population of nociceptors, whereas Runx3 is restricted to TrkC$^+$ proprioceptors (Inoue et al., 2002; Levanon et al., 2002). Using complementary gain- and loss-of-function approaches in mouse and chick, the Arber, Jessell and Ma laboratories have shown that Runx1 and Runx3 have a crucial role in dictating the identity and axonal trajectories of particular classes of DRG neurons (Chen et al., 2006a; Chen et al., 2006b; Kramer et al., 2006).

In the absence of Runx1, the Ret$^+$ population of nociceptors transforms into TrkA$^+$ nociceptors, and their axon trajectories correspondingly terminate in laminae I and laminae IIo, the relevant lamina for TrkA$^+$ axons (Chen et al., 2006b). This alteration in trajectory is mirrored by a profound behavioural defect: the mice do not respond to chronic neuropathic pain, although they can sense mechanical (inflammatory) pain. This result suggests that Runx1 is a crucial switch between the Ret$^+$ and TrkA$^+$ classes of nociceptors. By contrast, altering the levels of *Runx3* affects the expression of *TrkB* (also known as *Ntrk2*), suggesting that Runx3 acts to repress *TrkB* expression in TrkC$^+$ proprioceptors (Chen et al., 2006a; Chen et al., 2006b; Kramer et al., 2006). However, the over-expression of *Runx3* does not appear to result in a clear-cut transformation of cellular identity: DRG neurons forced to express *Runx3* by in ovo electroporation can nonetheless continue to express *TrkA* (also known as *Ntrk1*). However, these Runx3$^+$;TrkA$^+$ neurons exhibit a dramatic alteration in the end point of their axonal trajectory in the spinal cord (Chen et al., 2006a). Instead of projecting to the dorsal laminae, as is characteristic of nociceptors, they terminate ventrally, as do Type Ia or Type II proprioceptive afferents. Moreover, an acute reduction in *Runx3* levels in DRG neurons by RNAi results in the targeting of presumptive proprioceptors to the laminae of the chick dorsal horn (Chen et al., 2006a). Intriguingly, a more moderate reduction in *Runx3* levels produces a different result: the axons of presumptive proprioceptor neurons now terminate in the intermediate region of the spinal cord, as is characteristic of Type Ib afferents. Together, these results suggest that the graded activity of Runx3 might determine the pattern of sensory afferent innervation of the spinal cord. In the absence of Runx3 activity, cutaneous afferents innervate the dorsal horn, whereas low Runx3 activity in Type Ib proprioceptors results in their termination in the intermediate spinal cord, and high Runx3 activity directs Type Ia and Type II proprioceptors to terminate in the ventral spinal cord (Chen et al., 2006a). The mechanism by which the graded activity of Runx3 is interpreted to result in the relevant guidance choice remains unclear. However, these guidance choices may be independent of the decisions that dictate cellular fate. This mechanism may also be evolutionarily conserved between vertebrates and invertebrates: misexpression of Runt in the outer photoreceptor neurons of the compound eye in *Drosophila* results in the inappropriate targeting of axons to the medulla instead of the lamina (Kaminker et al., 2002).

The Lola transcription factor regulates Robo in *Drosophila*

Within the CNS, interneurons are directed whether to extend an axon across the midline. Ipsilaterally projecting axons never cross the midline, whereas contralaterally projecting axons cross the midline only once. This choice is determined by the sensitivity of axons to the midline repellent, Slit (Kidd et al., 1999). In *Drosophila,* Roundabout (Robo), the receptor for Slit, is upregulated in contralateral axons only after they cross the midline, whereas ipsilateral axons express Robo continuously (Kidd et al., 1998b). Robo is prevented from reaching the cell surface of contralateral axons prior to crossing the midline by Commissureless (Comm) (Keleman et al., 2002; Keleman et al., 2005; Kidd et al., 1998a; Myat et al., 2002). *comm* is transcribed only in the contralateral axons and little is known about the transcription factors that regulate this expression, although Engrailed has been reported to bind within the *comm* transcription unit (Solano et al., 2003). The initial activation of *robo* transcription is, however, dependent on the transcription factor Lola (longitudinals

lacking) (Crowner et al., 2002). In the absence of Lola, ipsilaterally projecting CNS axons project inappropriately across the midline with no observable changes in cellular fate in the associated neurons. Gene expression studies have shown that the levels of both Slit and Robo are reduced in *lola*$^{-/-}$ embryos, suggesting that the axon-guidance defects result from the ability of Lola to regulate the transcription of both *robo* and *slit* (Crowner et al., 2002). How might Lola regulate both of these genes? Intriguingly, alternative splicing of the *lola* gene generates 19 distinct isoforms that are expressed in distinct cell types and appear to regulate different axon-guidance decisions (Goeke et al., 2003).

Transcription factors and the regulation of extrinsic guidance cues

A number of the molecular cues that direct axon guidance have been identified, yet it remains unclear how the expression of these guidance cues is regulated such that they are present at the right time and place during embryogenesis. Some studies have thus focused on the transcription factors that do not act within neurons but are required within the environment to activate or regulate the expression of extrinsic guidance cues.

Transcription factors that regulate guidance signals in the developing vertebrate eye

In the developing eye in vertebrates, one of the earliest guidance events is the targeting of retinal axons from the ganglion cell layer to the optic stalk. RGCs first extend axons into the optic fibre layer, where the axons then project towards the central optic disc, becoming increasingly fasciculated in the process. This process is determined in part by a member of the Slit family, Slit1, which is selectively expressed in a subset of cells in the ganglion cell layer (Erskine et al., 2000). *Slit1*-expressing cells appear to act as positive intermediate targets that guide retinal ganglion axons into and within the optic fibre layer. The expression of *Slit1* is regulated by Irx4, a member of the Iroquois family of homeobox genes that is present in a subset of cells, not overlapping those expressing *Slit1*, in the ganglion cell layer (Jin et al., 2003). Misexpression of *Irx4* specifically reduces *Slit1* expression and results in axon fasciculation defects. RGC axons avoid the regions that have no or low *Slit1* expression and become dramatically over fasciculated. These results indicate that Irx4 negatively regulates Slit1 expression (Jin et al., 2003). Once RGC axons have left the retina, they make a choice at the optic chiasm whether to extend contralaterally to the opposite

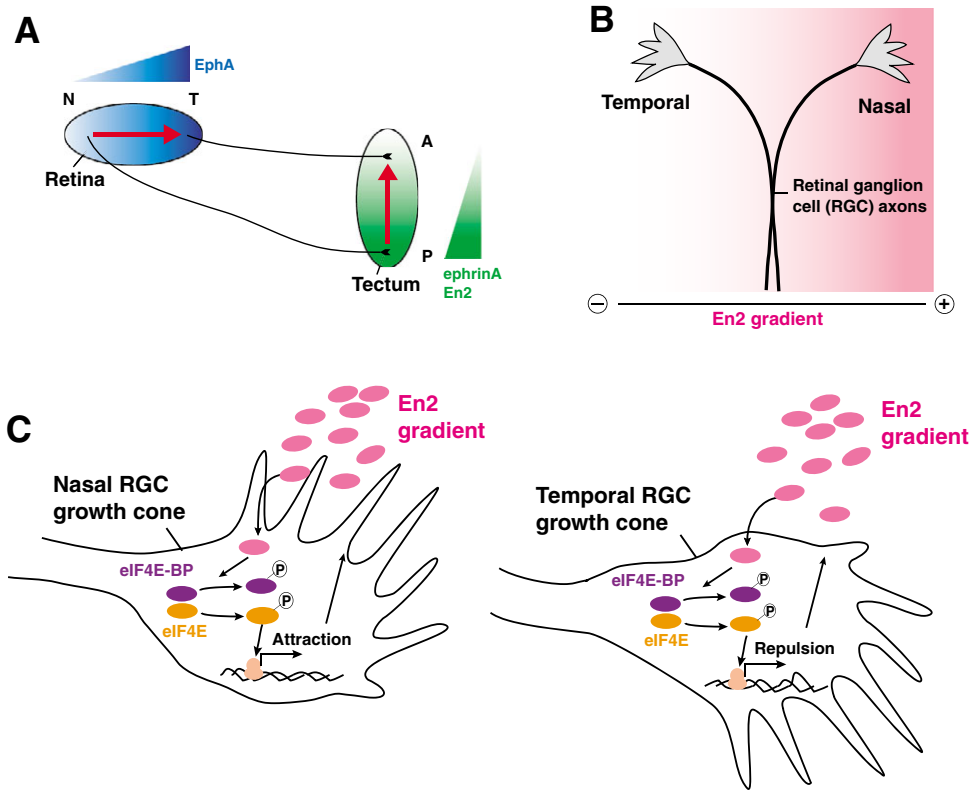

Fig. 3. Retinotectal axon guidance requires the graded expression of receptors and ligands, as well as a possible paracrine role for a transcription factor. (**A**) Retinal ganglion cells (RGCs) project axons in an orderly manner from the retina to the tectum in order to ensure that an image (red arrow) perceived in the retina is precisely represented in the tectum. Axons from the temporal (T) region of the retina project to the anterior (A) region of the tectum, whereas axons from the nasal (N) region extend to posterior (P) tectum. Formation of this precise retinotopic map relies on the graded activity of several molecules, including the EphA receptor tyrosine kinases, their ephrin ligands and the transcription factor En-2. RGCs with high EphA-receptor levels are repelled by ephrin and navigate to the tectal region that has lower levels of the ligand. RGCs with lower receptor levels can extend into regions of the tectum with higher levels of ephrin. En-2 levels in the tectum also influence map formation, with temporal axons avoiding posterior regions of the tectum that have higher levels of En-2. (**B**) En-2 can act as a soluble molecule to differentially influence the outgrowth of temporal and nasal RGCs. In an in vitro turning assay, nasal axons are attracted to a source of En-2(+), whereas temporal axons are repelled by high levels of En-2. (**C**) En-2 enters the temporal and nasal growth cones where it stimulates eIF4E and eIF4E-BP phosphorylation. Phosphorylated eIF4E is believed to trigger the translation of different proteins in nasal and temporal axons to generate an attractive or repulsive response, respectively.

side of the brain or remain ipsilateral. The expression of two transcription factors, *Zic2* and *Foxd1*, is restricted to those RGCs that take an ipsilateral trajectory, suggesting that these factors may control the activity of guidance receptors, such as EphB1, necessary to mediate this choice (Herrera et al., 2003; Herrera et al., 2004; Pak et al., 2004). A LIM-homeodomain protein, Islet2 (Isl2), may regulate the restriction of Zic2 to ipsilaterally projecting RGC neurons. Isl2 is present only in contralaterally projecting RGC neurons and represses the expression of *Zic2* in this population of RGCs (Pak et al., 2004).

During the development of the visual system, a precise map of the visual field is projected into the brain. To produce this map, RGC axons project in an orderly manner to the optic tectum, which lies within the brain (Fig. 3A). Temporal RGC axons project to anterior regions of the tectum, whereas nasal RGC axons project to its posterior regions. These migrations occur in such a way that there is a one-to-one correspondence between the point of origin of the RGC on the retina to its termination site on the tectum. Many studies have suggested that this retinotopic map is generated by the gradient of Eph and/or ephrins, both on RGC axons and in the tectum (McLaughlin and O'Leary, 2005). EphrinA ligands are graded along the anterior-posterior axis of the tectum, whereas the RGC axons contain graded levels of EphA receptors. RGC axons with the highest level of EphA receptors are repelled by ephrinA and thus project to anterior regions of the tectum that express the least amount of ephrin ligand (Fig. 3A). However, it is still not well understood how the expression domains of the Eph receptors and/or ephrins are established.

During the early development of the chick eye, a winged helix transcription factor, *CBF1* (Takahashi et al., 2003), and two homeobox-containing genes, *SOHo1* and *GH6* (Schulte and Cepko, 2000), are expressed in the nasal retina, in an opposing gradient to that of EphA3. Misexpression of *CBF1*, *SOHo1* or *GH6* in the developing retina has demonstrated that these genes can repress EphA3 expression selectively in the retina (Schulte and Cepko, 2000; Takahashi et al., 2003). The repression of EphA3 throughout the retina results in alterations to the retinotopic map: RGC axons from the temporal region of the retina that normally express EphA3 now project aberrantly. It remains unknown whether EphA3 is a direct target of SOHo1 and GH6 regulation or if the loss of *SOHo6* or *GH6* permits the ectopic expression of *EphA3*. The projection pattern of neurons along the dorsal-ventral axis of the retina, the axis orthogonal to the nasal-temporal axis, may be established in both chick and rodents by the combinatorial action of Vax2, a homeobox gene expressed in the ventral retina (Barbieri et al., 1999; Schulte et al., 1999), and Tbx5, a T-box transcription factor present in the dorsal retina (Koshiba-Takeuchi et al., 2000). The gain or loss of Vax2 function results in the altered expression of the ventrally located EphRs, *EphB2* and *EphB3* (Mui et al., 2002; Schulte et al., 1999), whereas the misexpression of *Tbx5* results in dorsalized retinal cells and the expansion of *ephrinB1* and *ephrinB2* expression (Koshiba-Takeuchi et al., 2000). In both cases, altering the distribution of Vax2 and Tbx5 results in RGC axons projecting aberrantly (Koshiba-Takeuchi et al., 2000; Mui et al., 2002; Schulte et al., 1999).

Regulation of axon-guidance signals by LIM-domain proteins

As discussed above, LIM-domain transcription factors have crucial roles in directing the cellular fate of neurons, thereby determining whether they express the particular complement of receptors to respond to certain axon-guidance cues. As we discuss below, LIM-domain family members also regulate axon guidance events by patterning the environment in which axons project.

Several major axon pathways cross the midline of the vertebrate forebrain during development. These pathways include the post-optic commissure (POC) – which is formed from neurons in the lateral diencephalon extending across the midline – and axons from the RGCs – which project across the midline to form the optic nerve and chiasm. These trajectories are disrupted in *belladonna* (*bel*)-mutant zebrafish: axons from both the POC and RGCs fail to cross the midline (Seth et al., 2006). The *bel* gene has been recently cloned and was found to encode Lhx2, a member of the LIM-domain family of transcription factors. In the zebrafish, *lhx2* is expressed regionally throughout the brain, and *bel* mutants were found to have subtle forebrain-patterning defects (Seth et al., 2006). These results suggested that *bel(lhx2)* might specify the regions of the diencephalon that present axon-guidance cues necessary for retinal and commissural axon outgrowth. Consistent with this model, in *bel* mutants, the midline glial cells that provide the cellular substrate for the retinal and commissural axons are disorganized, and the expression of key axon-guidance signals, including Sema3d, Netrin1a, Slit2 and EphB2, are specifically altered in the pre-optic area of the diencephalon (Seth et al., 2006). It remains to be determined how Lhx2 regulates the expression of these axon-guidance signals and how alterations in their expression patterns produce such a specific guidance defect.

A further role of LIM-domain transcription factors in the regulation of the expression of extrinsic guidance cues has been shown in the developing vertebrate limb. As discussed previously, the embryonic limb is innervated by the motor axons of the LMC (see Fig. 2B). This projection pattern is controlled in a coordinated manner by the respective expression of Lim1 and/or Isl1 in LMC(l) and/or LMC(m) neurons (see above), and *Lmx1b*, which is expressed in the dorsal limb mesenchyme and delineates the position of the LMC branch point (Kania et al., 2000). In an elegant series of studies, Lim1 and Lmx1b have been shown to regulate the two sides of an interaction between the ephrinA:EphA effectors (Kania and Jessell, 2003). Thus, Lim1 upregulates the expression of *EphA4*, resulting in EphA4 being present primarily in LMC(l) axons, whereas Lmx1b represses the expression of *ephrinA5* from the dorsal half of the limb. In *Lmx1b* mutants, expression of ephrinA5 is detectable throughout the developing limb, and axons from the LMC(l) motor column randomly innervate either side of the limb. These results suggest that the downregulation of ephrinA5 by Lmxb1 prevents the EphA4^{+} LMC(l) axons from entering the ventral limb.

Is axon trajectory specified by transcription factors acting solely in the nucleus?

Classical experiments have shown that, when the growth cone is isolated from its cell body, it can continue to extend and make simple pathway choices (Harris et al., 1987; Shaw and Bray, 1977), suggesting that new nuclear information is not necessary for outgrowth or single pathway choices. More recent data have revealed that growth-cone-turning decisions do not require transcription, but do require protein synthesis and degradation, indicating that local changes in protein levels within the growth cone dictate pathway choice (Campbell and Holt, 2001; Leung et al., 2006; Wu et al., 2005). The history of the growth cone and/or axon also affects how the axon perceives cues in its environment (e.g. axons crossing the midline of the CNS ignore rostral/caudal cues and are insensitive to Slit prior to reaching the midline). However, once across the midline, they become sensitive to Slit (Garbe and Bashaw, 2004; Tear, 1999). These switches in sensitivity can involve the local translation of stored mRNAs, trafficking of receptors to the cell

surface or changes in intracellular concentration of cAMPs or cGMPs (Brittis et al., 2002; Garbe and Bashaw, 2004; Keleman et al., 2002; Song et al., 1998).

Do the early-acting transcription factors install all the components necessary for the complete navigation properties of a neuron? Because the number of transcription factors known to direct axon guidance is still small, it is difficult to answer this question completely. In *Drosophila*, manipulation of *eve* expression can cause the complete reprogramming of axon growth. Eve is required for ISN^D MNs to extend to dorsal muscles and, in its absence, these axons extend to ventral muscles. By contrast, misexpression of *eve* in all MNs diverts ventrally directed ISNb motorneurons to the dorsal muscle field (Fujioka et al., 2003; Landgraf et al., 1999). These results suggest that Eve provides the information to extend dorsally. However, Eve does not direct all aspects of dorsal MN growth, because the redirected neurons are unable to recognize and innervate dorsal muscles (Landgraf et al., 1999). In vertebrates, loss of Lim1 from LMC(l) blocks the ability of LMC(l) axons to select a dorsal trajectory once within the limb. Misexpression of *Lim1* does not affect the distal extension of LMC axons, but it does cause the inappropriate selection of a dorsal trajectory (Kania and Jessell, 2003). Thus, in both these cases, perhaps not surprisingly, a single transcription factor is not responsible for determining the entire trajectory of the motor neurons, but provides information to the neuron to allow it to recognize cues that direct part of the pathway. Lim1 seems to supply information that is needed later in the trajectory, whereas Eve appears to provide the information, such as the activation of Unc5 (Labrador et al., 2005), that directs axons distally but does not determine target choice. As with Lim1, Runx activity in the sensory neurons is not required for the early extension of axons: in the absence of Runx3 activity, DRG axons still reach the spinal cord (Chen et al., 2006b; Kramer et al., 2006). Thus, Runx3 may provide the information that directs axons to their correct target region. Lim1 is thought to activate the expression of EphA4, a receptor required later in the trajectory (Kania and Jessell, 2003). It is not known whether Runx similarly activates the expression of a receptor that is used later in axon growth or whether it primes the axon with the cellular components that can be used later in a post-transcriptional mechanism. Further research to reveal the specific downstream targets of these transcription factors will hopefully provide answers to these questions.

A novel transcription factor role as an extrinsic cue

A non-traditional role for the En-2 (also known as En2)-homeodomain transcription factor as a diffusible extrinsic signal that directs axon guidance has been suggested recently (Brunet et al., 2005). In the tectum En2 is expressed in a gradient from high in the posterior to low in the anterior, where it may play a classical role in regulating ephrinA levels (Fig. 3). However, Brunet et al. (Brunet et al., 2005) have demonstrated that exogenously applied En-2 can act as a bifunctional guidance cue that attracts nasal and repels temporal *Xenopus* RGC axons in vitro (Fig. 3B). This result suggests that a graded activity of secreted En-2 could pattern RGC outgrowth in vivo, and is consistent with previous experiments showing that nasal RGC axons are attracted to ectopic patches of En-2 expression in vivo, whereas temporal axons avoid these areas (Friedman and O'Leary, 1996). In vitro, En-2 directly enters the RGC growth cone to elicit a turning response within 20 minutes. This response can be blocked by translational, but not transcriptional, inhibitors, indicating that En-2 initiates new protein synthesis in the RGC growth cones from existing RNAs (Fig. 3C). En-2 stimulates the phosphorylation of both eIF4E and of its regulatory binding partner eIF4E-BP in a manner similar to that seen in growth cones after their exposure to Netrin-1 and Sema3A, which also rapidly activate local translation within the growth cone to elicit turning (Campbell and Holt, 2001). The phosphorylation of eIF4E-BP causes its dissociation from eIF4E and allows the initiation of translation (Fig. 3C). Thus, En-2 appears to act via components of the translation machinery to stimulate the local translation of proteins that affect growth cone turning.

How En-2 might simultaneously attract nasal growth cones and repel temporal growth cones, or indeed whether En-2 acts non-autonomously in vivo, is unclear. Nasal and temporal growth cones might be primed with differing En-2-responsive mRNA populations or different growth cones might respond differently to the proteins synthesized in response to En-2. Both these scenarios require that the different RGCs are in some way pre-determined, perhaps by an earlier-acting transcription factor, to respond differently to the same En-2 cue. There is also little evidence that En-2 is secreted in the tectum. Previous experiments trying to manipulate the En-2 gradient have infected the chick tectum with retroviruses that encode En-2, and the protein does not appear to extend beyond the infected cells (Friedman and O'Leary, 1996). Nonetheless, should homeodomain transcription factors be able to act non-autonomously as signal molecules, this would be an elegant method by which extending axons could receive information about their spatial position as they migrate towards, or finally recognize, their specific target. It is clear that axons require information from their environment to regulate axonal responsiveness to new incoming cues as they extend into new territories (Garbe and Bashaw, 2004; Stoeckli and Landmesser, 1998), and abundant evidence reveals that these regulative signals use post-transcriptional mechanisms (Campbell and Holt, 2001; Leung et al., 2006; Wu et al., 2005). It is therefore of great interest to find a novel paracrine activity for a transcription factor that plays a role in instructing this axon-guidance property.

Conclusions

Over recent years, numerous transcription factors have been characterised that either specify neuronal fate or pattern the environment through which axons extend. It is now crucial to close the gap and identify the most-downstream-acting transcription factors that directly regulate axon-guidance effectors. Such studies will tell us how much guidance information is provided to the neuron as it begins its extension and will clarify the ability of axons to identify and respond to guidance cues during both the early and later stages of its trajectory. Axons can adapt their responsiveness to environmental signals by either assembling receptors into different complexes or by adjusting how they respond to the signal from an activated receptor. However, the extent to which this ability is encoded in the neuron by early-acting transcription factors remains unresolved. The fact that transcription factors themselves might also act as secreted cues that communicate information between neurons and their targets widens the possible influence of these molecules. Identifying the transcription factors, or their combinations, that direct axon guidance opens up the possibility of using array technologies to identify their targets and to provide us with an overview of the molecules activated in particular neurons. The continued investigation of these factors will hopefully lead us to understand how an axon starts on the correct path and is directed so precisely to its specific target.

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