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Review

Molecular mechanisms of axon guidance

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Abstract

In order to form a functional nervous system, neurones extend axons, often over long distances, to reach their targets. This process is controlled by extracellular receptors and their ligands, several families of which have been identified. These proteins may act to either repel or attract growth cones and a given receptor may transduce either type of signal, depending on the cellular context. In addition to these archetypal axon guidance molecules, it is becoming apparent that molecules previously known for their role in patterning can also direct axonal outgrowth. The growth cone receptors do not act in isolation and combine with members of the same or other families to produce a graded response or even a complete reversal in its polarity. These signals can be further combined and/or modulated by processing of the molecule both directly at the cell surface and by the network of intracellular signalling pathways which are activated. The result is a sophisticated and dynamic set of cues that enable a growth cone to successfully navigate to its destination, modulating its response to changing environmental cues along its pathway.

Keywords: Axon guidance; Growth cone; Signalling; Ephrin; Semaphorin; Slit; Netrin; Morphogen; Second messenger

Introduction

A little over a hundred years ago, the pioneering neuroanatomist Ramón y Cajal, looking at a histochemical section, observed club-shaped structures at the end of processes emanating from nerve cells. He named them 'growth cones' and made the remarkably prescient observation that these might somehow burrow through the embryo, enabling nerves to connect with distant targets. The motility of growth cones was demonstrated a couple of decades later by Harrison, who grew frog neurones in lymph clots. For a detailed account of the early history of growth cone study, see Gordon-Weeks (2000). Despite advances in culturing neurones, the question remained as to how growth cones could be guided in vivo. In 1963, Sperry proposed a chemoaffinity hypothesis, which has become the basis for many subsequent models of axon guidance. He suggested that growth cones carried molecular tags to direct them to their destinations by responding to gradients of guidance cues, growing up an attractive one or down a repulsive one (Sperry, 1963). The advent of precise methods to label neuronal pathways using vital dyes allowed the mapping of neuronal circuits, and the trajectories taken by individual axons could be traced. Axonal processes were thus revealed to make abrupt changes in direction and to possess remarkable capacities for error correction (Guthrie and Lumsden, 1992; Harris, 1986; Lance-Jones and Landmesser, 1981). Many of the early candidates for axon guidance molecules, such as integrins, fasciclin and neural cell adhesion molecules (NCAMs), generally act in a permissive manner by providing a substrate that promotes outgrowth rather than by actively inducing growth cone turning (Lilienbaum et al., 1995). However, in the late 1980s and early 1990s, a series of genetic and biochemical screens identified proteins acting in an instructive manner which can actively attract or repel axons, and it is on these that this review will principally focus. Outgrowth is controlled by the concerted action upon the growth cone of attractive and repulsive cues working in a contact-dependent fashion or at a distance via secreted factors (Fig. 1). Recent data have revealed that, in certain contexts, molecules regarded as archetypal chemorepellents act attractively and vice versa. In addition to receiving inward signals, the growth cone can also initiate them itself and convey these outwards: it is not simply a passive receptor of instructions. These features, combined with alternative mRNA splicing and post-translational modifications of receptors and their ligands, result in a myriad of subtly

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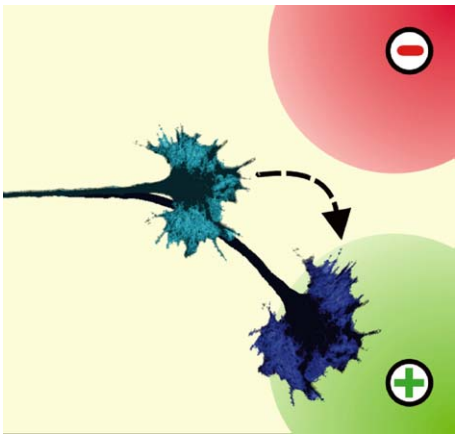

Fig. 1. When a growth cone (light blue) encounters guidance molecules, it extends away from chemorepellents (red) and towards chemoattractants (green). The net effect is to cause a turning of the growth cone (dark blue).

different signals that can be employed to ensure the precise wiring of the nervous system. The resultant molecular cues do not act in isolation but influence each other through interactions at the cell membrane and complicated networks of intracellular signalling cascades.

Fishing for guidance molecules

The following description of four well-characterised families of axon guidance molecule will, in addition to outlining their modes of action, demonstrate the variety of techniques that have been used to identify and characterise them.

Ephrin/Eph

Since the time of Sperry, it had been known that a topographic representation of the chick retina exists in the tectum, mapping the visual field onto a defined neural field. In terms of neuronal projections, this means that retinal ganglion cells (RGCs) from the nasal retina form synapses in the posterior tectum whereas temporal RGCs terminate in the anterior tectum (Fig. 2). Such a stereotyped linkage formed the basis for many theoretical and empirical investigations of axon guidance (Gierer, 1983; Sperry, 1963). In co-culture systems, retinal axons collapse in the presence of membranes derived from the inappropriate half of the tectum, a functional specificity strikingly demonstrated by the stripe assay (Walter et al., 1987). Retinal explants were placed across a series of parallel stripes of anterior and posterior tectal membranes, and emerging axons were thus confronted with the choice of which one to grow along. Temporal axons exhibited a definite preference to grow on the anterior membranes, their natural substrate, and this selectivity diminished as progressively more posterior tectal membranes were encountered. Furthermore, this effect is lost after treatment of the membranes with phosphatidylinositol-specific phospholipase C (PI-PLC), implying that the molecule responsible is linked to the membrane by a glycosylphosphatidylinositol (GPI) anchor. This protein was isolated by comparing the spots present on two-dimensional electrophoresis gels derived from specific regions of the tectum before and after PI-PLC treatment (Drescher et al., 1995). EphrinA5, as it is now known, is indeed expressed in an increasing anteroposterior gradient across the tectum, as is the related gene EphrinA2 (Monschau et al., 1997). Ectopic expression of EphrinA2 in the anterior tectum causes temporal axons to avoid this area (Nakamoto et al., 1996). Conversely, the removal of EphrinA5 in knockout mice leads to temporal axons overshooting into posterior regions (Frisen et al., 1998). EphrinAs are also required for patterning eye-specific projections to the appropriate layers of the lateral geniculate nucleus (LGN) in both mice and ferrets (Huberman et al., 2005; Pfeiffenberger et al., 2005). This is a striking demonstration of the same axon guidance molecules projecting RGC axons to a topographic map in one locality, the superior colliculus, and to discrete layers in another, the LGN. Both Ephrins and their partners, the Eph receptors, are divided into A and B families. EphrinAs have a GPI anchor, whereas EphrinBs are linked to the cell by a transmembrane domain. The EphA and EphB proteins are receptor tyrosine kinases, named based on their preferential binding to the EphrinA and EphrinB family respectively (Pasquale, 2005). Whereas gradients of EphA and EphrinA determine topographic mapping along the anteroposterior tectal axis, EphB and EphrinB gradients control the dorsoventral projection pattern, even acting as chemoattractants via their effect on topographic branching (Hindges et al., 2002; Mann et al., 2002).

The EphB2 null mouse has a diminished anterior commissure; however, this tract is normal in mice in which the EphB kinase domain has been replaced by β-galactosidase,

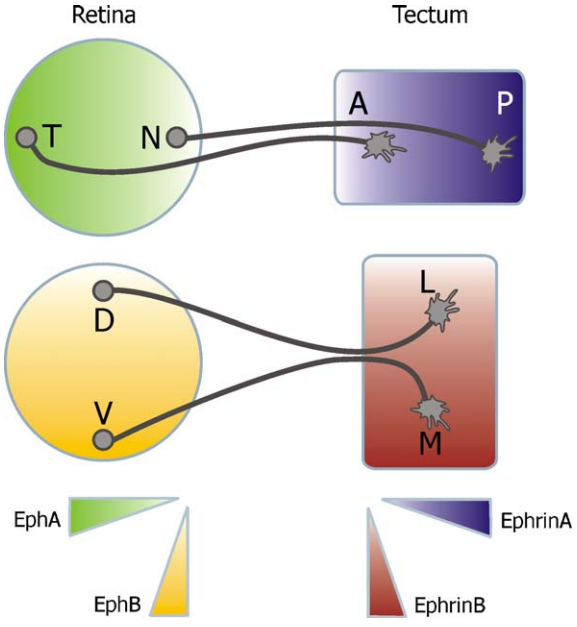

Fig. 2. Topographic maps are set up by opposing gradients of Eph receptors and their Ephrin ligands. Axons from the temporal (T) retina express high levels of EphA (green) and are repelled by the high levels of EphrinA (blue) in the posterior (P) tectum and terminate anteriorly (A). Nasal (N) retinal axons extend into the posterior tectum. However, ventral (V) axons expressing high levels of EphB (yellow) are attracted to the high EphrinB (red) levels in the medial tectum. Dorsal (D) axons terminate in the lateral (L) tectum.

suggesting that signalling via the receptor is not required for guidance within this tract. The anterior commissure axons express high levels of EphrinB1, whilst EphB receptors are found along the pathway, suggesting that in this case Ephrin molecules function as the receptors (Henkemeyer et al., 1996). Subsequently, it was discovered that upon binding to EphB2 the EphrinB intracellular domain is phosphorylated on tyrosine residues, thereby allowing bi-directional signalling (Bruckner et al., 1997; Holland et al., 1996). In this way, growth cones can potentially influence their environment rather than simply responding passively. This could be particularly important in the case of intraneuronal interactions to regulate fasciculation or competition for synaptic targets. EphrinA proteins can also 'reverse signal' (Rashid et al., 2005), an initially surprising observation given that they have a GPI anchor instead of an intracellular domain. The most likely explanation is that they bind a co-receptor via a *cis* interaction which then transduces the signal (Davy and Robbins, 2000; Davy et al., 1999; Huai and Drescher, 2001).

Semaphorins

Semaphorins are a large family of signalling proteins, both secreted and membrane-bound, which, like Ephrins, are required for the development of many organs not just the nervous system (Raper, 2000). They are divided into eight classes of which the most intensively studied in terms of axon guidance are the class 3 secreted Semaphorins (Sema3A–3G). They were identified as a group of molecules with growth-cone-collapsing properties, hence the chick orthologues were originally called collapsins (Luo et al., 1993, 1995). This ability to induce collapse was identified through the use of the eponymous 'collapse assay' in which cultured neurones are exposed to a putative repellent factor. The degree to which it induces stalling or collapse of growth cones can then be readily monitored over time, as can their recovery after its removal (Raper and Kapfhammer, 1990). In a more subtle extension of the technique, localised application of a collapsing factor drives turning of a growth cone away from the source (Fan and Raper, 1995). The repulsive activity of Semaphorins acts upon a variety of neuronal types in vitro, including motor, sensory, olfactory and hippocampal neurones (Chedotal et al., 1998; Kobayashi et al., 1997; Koppel et al., 1997; Luo et al., 1995; Messersmith et al., 1995; Varela-Echavarria et al., 1997). The receptors first identified for the class 3 Semaphorins were the Neuropilins, Npn1 and Npn2. The transmembrane protein Npn1 forms a homodimeric receptor complex for Sema3A (He and Tessier-Lavigne, 1997; Kolodkin et al., 1997), Npn2 does likewise to bind Sema3F but they combine in a heterodimer to bind Sema3C (Chen et al., 1997, 1998; Giger et al., 1998). The significance of these receptor–ligand pairings is underscored by the effect of removing individual genes. Mice lacking *Npn1* have a similar phenotype to those lacking *Sema3A*, namely, marked defasciculation of nerve bundles and aberrant projections of sensory nerves and specific cranial motor nerves (Kitsukawa et al., 1997; Taniguchi et al., 1997). Likewise, mice with *Npn2* or *Sema3F* removed have matching defects both in nerves spared in *Npn1/Sema3A* knockouts such as the oculomotor and trochlear nerves and also some nerves in common, such as the trigeminal and facial nerves (Chen et al., 2000; Giger et al., 2000; Luo et al., 1993; Sahay et al., 2003). The affinity of individual Semaphorins for the two Neuropilins varies with promiscuous and competitive binding occurring, and the complete receptor preferences of all seven ligands are not clear (Nakamura et al., 2000; Takahashi et al., 1998).

Recent evidence implies that Neuropilins are actually a later evolutionary recruit to a signalling complex originating between Semaphorins and the Plexin family. In *Drosophila*, Plexins act as Semaphorin receptors (Winberg et al., 1998), and this prompted a search for vertebrate Plexins. More than ten have been discovered and grouped into four sub-families (Fujisawa, 2004; Tamagnone et al., 1999). These do not bind to class 3 Semaphorins but interact strongly with the transmembrane class 4 and GPI-anchored class 7 Semaphorins. Conversely, classes 4 and 7 do not bind Neuropilins. However, the extracellular domain of Plexins contains a 'Sema' homology region, and this moiety associates with Neuropilins to form a functional receptor complex (Rohm et al., 2000; Takahashi et al., 1999; Tamagnone et al., 1999). Clearly, the permutations of Neuropilin and Plexin interactions potentially permit a range of Semaphorin binding affinities and subsequent responses. As if this were not complicated enough, there are other members of the receptor complex. L1 is a transmembrane cell adhesion molecule required for neural development (Kamiguchi et al., 1998) which forms a stable complex with Npn1 (Castellani et al., 2000). Soluble L1 protein blocks the collapsing effect of Sema3A, but not Sema3B, on the growth cones of cortical neurones or dorsal root ganglia (DRG) and can even provoke chemoattraction. Neurones from L1-deficient mice are unresponsive to either a repulsive or attractive effect of Sema3A. Thus, L1 appears to be a key factor both for transducing the signal and determining the nature of the response (Castellani et al., 2000). In the case of Sema3B and Sema3F binding to Npn2, a different member of the L1 family, NrCAM, is a component of the receptor complex (Falk et al., 2005).

Netrin

In organisms with a bilateral nervous system, it is essential that there is communication between the two halves. This link is provided by axon tracts known as commissures which cross the midline at defined points. How this crossing is regulated is one of the key questions in neurobiology. Firstly, there must be segregation of those axons destined to cross and form contralateral projections from those which should proceed ipsilaterally. After the contralateral axons have been induced to turn towards the midline and cross it, they must subsequently be repulsed to resume their journey on the other side. It transpires that precisely regulated expression of both attractive and repulsive factors and their receptors is required to mediate the formation of commissures.

Vertebrate Netrins were discovered in the search for a chemoattractant factor that had been demonstrated in vitro to emanate from the floor plate and attract commissural axons

(Placzek et al., 1990; Tessier-Lavigne et al., 1988). These processes originate from cells in the dorsal spinal cord and grow ventrally along its lateral edge. They turn through the motor column towards the floor plate and cross the midline before turning through 90° to course longitudinally. Chick brain extracts were screened for their ability to promote axonal outgrowth followed by an epic process of biochemical fractionation to purify the active proteins, namely, Netrin-1 and 2 (Kennedy et al., 1994; Serafini et al., 1994). In order to demonstrate chemoattraction and distinguish it from a general growth-promoting effect, heterologous cells secreting netrin are placed at a defined distance from an explant containing the neurones under study. In the presence of a chemoattractant, axons can be seen to turn towards the source rather than simply increasing in length (Kennedy et al., 1994). Netrin-1 and 2 have high sequence similarity to the UNC-6 protein that had been discovered independently in the nematode worm *Caenorhabditis elegans*, as being required for axon guidance (Ishii et al., 1992). Two other *C. elegans* genes, *unc-5* and *unc-40*, are required along with *unc-6* for cell movement along the dorsoventral axis (Hedgecock et al., 1990). The vertebrate homologue of UNC-40, DCC (Deleted in Colorectal Cancer), functions as a Netrin receptor and is required to mediate the attractant effects of Netrin (Keino-Masu et al., 1996). Complementary studies on *Drosophila* Netrins (Harris et al., 1996; Mitchell et al., 1996) and the DCC-related protein Frazzled (Kolodziej et al., 1996) have demonstrated the evolutionary conservation of this system. Despite being identified initially as a chemoattractant, Netrin can also act as a repellent (Fig. 3) for trochlear motor neurones (Colamarino and Tessier-Lavigne, 1995) and other dorsally projecting hindbrain motor neurone types (Varela-Echavarria et al., 1997). Whereas UNC-40 and its homologues are required for attraction towards a Netrin source, UNC-5 and its three mammalian homologues are necessary for the repulsive effect of Netrin (Ackerman et al., 1997; Hamelin et al., 1993; Leonardo et al., 1997). In *Drosophila* motor neurones, UNC-5

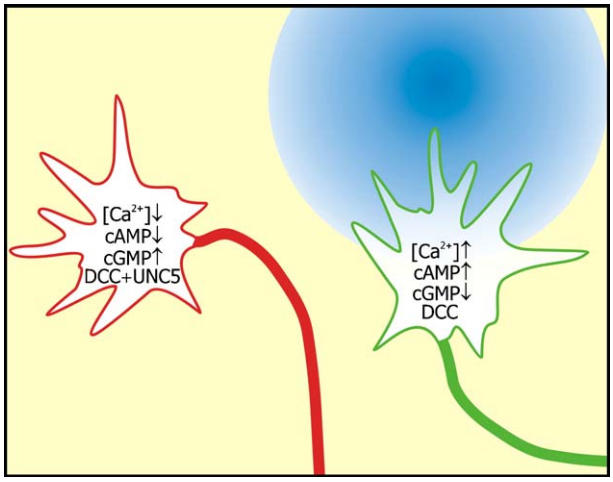

Fig. 3. Netrin (blue) can act as a chemorepellent or attractant. Expression of the DCC receptor, high intracellular calcium concentration ($[Ca^{2+}]_i$) or a high cAMP:cGMP ratio lead to attraction (green). Expression of DCC in conjunction with UNC5, low ($[Ca^{2+}]_i$) or low cAMP:cGMP cause repulsion (red).

functions alone (Keleman and Dickson, 2001) but in a *Xenopus* spinal neurone model system Netrin-mediated repulsion results when UNC-5 and DCC cytoplasmic domains associate, initiated by the binding of Netrin (Hong et al., 1999). Rather than reflecting a competition, the pair of receptors acts like a molecular switch. In other vertebrate neuronal types, it is not clear whether UNC-5 or an UNC-5/DCC complex mediates repulsion.

Robo/Slit

The identification of the Robo receptors and their Slit ligands is testament to the key role genetic screens have played in the elucidation of axon guidance molecules. The fruit fly *Drosophila* has been a powerful tool for developmental neurobiologists; the *Drosophila* central nervous system has a characteristic ladder-like pattern of commissures which can be rapidly screened for defects and the mutated genes responsible identified. A Netrin-based system similar to that in vertebrates attracts commissural axons to the midline, whilst the chemorepellent Slit, produced at the midline, binds to Roundabout (Robo) receptors on non-crossing and crossed axons, ensuring that they are expelled from the midline. In *robo* mutants, commissural axons are insensitive to midline repulsion and so cross and re-cross several times (Seeger et al., 1993). The source of the repellent to which Robo responds is midline glia secreting the Slit protein (Rothberg et al., 1990). In *slit* mutants, rather than crossing the midline, axons never leave it and coalesce into a single longitudinal bundle (Kidd et al., 1999). Thus, a basic model emerged in which axons expressing Robo are repelled by Slit at the midline. Robo protein occurs at high levels on the small proportion of longitudinal axons which form ipsilateral projections. On the commissural axons, Robo is initially expressed at low levels, and they are thus insensitive to repulsion at the midline. Once they have crossed, Robo protein is upregulated to prevent them re-crossing (Kidd et al., 1998). *Commissureless* (*Comm*) too is expressed by crossing axons and by midline glia. It clears Robo from the cell surface and thus prevents it from signalling, although the mechanism by which it does this has not been completely resolved (Georgiou and Tear, 2002; Keleman et al., 2002, 2005; Tear et al., 1996). In *comm* mutant embryos, the commissural axons fail to cross because Robo is never removed. Comm downregulates Robo and abrogates the sensitivity to Slit, allowing crossing to occur (Fig. 4).

This model was soon refined by the discovery of two other *Drosophila* Robo isoforms, Robo2 and 3 (Rajagopalan et al., 2000b; Simpson et al., 2000b), which, like Robo1, are expressed on the longitudinal tracts (Rajagopalan et al., 2000a; Simpson et al., 2000b). Combinatorial expression of the different Robos defines three lateral zones: Robo1 across the whole longitudinal tract; Robo3 in the middle and outer zone; Robo2 only in the outer zone, furthest from the midline (Rajagopalan et al., 2000b; Simpson et al., 2000a).

The general structure of Slits and their action upon commissural axons are largely conserved, and three vertebrate

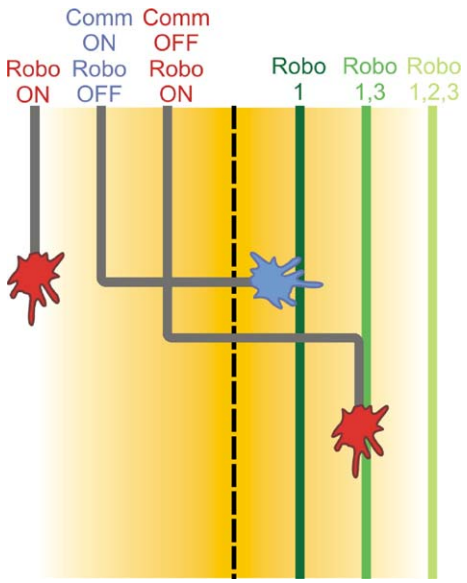

Fig. 4. Left-hand side: growth cones of longitudinal axons expressing Robo (red) are repelled by a gradient of Slit (yellow shading) produced at the midline (dashed). Comm expression (blue) downregulates Robo and axons cross. After crossing, Comm is turned off and Robo returns to the growth cone. Right-hand side: the complement of Robo receptors on a longitudinal axon determines its sensitivity to the Slit gradient and its lateral position.

Slits and Robos have been identified (Brose et al., 1999; Itoh et al., 1998; Long et al., 2004). Whether the same combinatorial Robo code also operates in vertebrates is not clear. One notable difference is that mice lacking Slit1 and Slit2 display midline defects in major forebrain tracts and at the optic chiasm, yet spinal commissural axons appear unaffected (Bagri et al., 2002; Plump et al., 2002). This is despite Robo1 and 2 also being conserved in both structure and expression pattern (Kidd et al., 1998; Long et al., 2004). Only in triple knockouts missing all three Slits do commissural axons linger at the midline (Long et al., 2004). In the vertebrate forebrain, Slit2 also guides axons before they have crossed the midline. Therefore, in this system, Slit has a different role whereby its repulsive action maintains axons within a defined channel (Shu et al., 2003). The groundwork provided by the fly model has also been extended in vertebrates by the finding that, after crossing, the commissural axons become additionally responsive to Semas which act in conjunction with Slit to increase the repulsion away from the midline (Zou et al., 2000). An interesting role has been proposed for the more divergent, third vertebrate Robo, named Rig1 (Yuan et al., 1999). Like the other two Robos, Rig1 binds Slit and is produced by commissural axons with the striking difference that it is expressed at high levels before midline crossing and is downregulated afterwards (Sabatier et al., 2004). Furthermore, in *Rig1* knockout mice, commissural axons fail to cross the midline. This observation, combined with in vitro data, suggests that Rig1 masks the action of Slit upon the other Robo receptors, preventing them from being prematurely repelled by the midline. The surprising conclusion is therefore that its overall effect is actually similar to Comm (Sabatier et al., 2004).

Following the exposure of commissural axons to Slit ligand, the cytoplasmic domain of DCC may interact with that of Robo resulting in a silencing of Netrin-mediated chemoattraction (Stein and Tessier-Lavigne, 2001). This coordination would explain how longitudinal axons can be drawn in by Netrin to enter commissures but do not subsequently re-cross in response to continued attraction. After crossing, the upregulation of Robo serves a dual purpose: to resensitise axons to Slit whilst the Robo–DCC interaction prevents a tug-of-war with Netrin (Fig. 5). Thus, the response to Netrin and Slit can be modulated cell-autonomously by changing the availability and/or interactions of their receptors. This allows a context-dependent reaction to a given guidance molecule and more subtle modification of cues, beyond simply repulsion or attraction. The implications of this and the mechanisms by which it is achieved are examined in a later section.

New tricks for old dogs

Several years have passed since the characterisation of the four families described above. As the human and mouse genomes are annotated in ever greater detail, we will soon have a definitive list, and there will be no more fishing trips for novel molecules. Researchers would be left to explain how millions of axons form precise and intricate connections, directed by only hundreds of genes. One solution is to consider the involvement of molecules traditionally associated with other neuronal functions or even other organs. The reverse certainly occurs since, for example, Slits, Semaphorins, Ephrins and Netrin are all required for vasculogenesis (Eichmann et al., 2005). There is

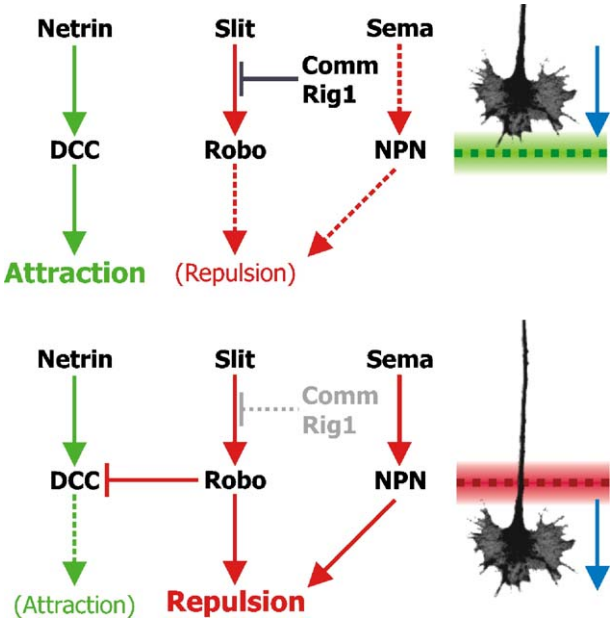

Fig. 5. Different receptors combine to coordinate midline crossing. As a growth cone approaches the midline (top half), netrin binds to DCC to attract the growth cone (gray). Comm (in *Drosophila*) and Rig1 (in vertebrates) block the action of Robo and inhibit the repulsive action of Slit. The midline is a net attractant (green dashes). After crossing (bottom half), Comm/Rig1 is downregulated, relieving the block on Robo which leads to repulsion by Slit. Robo also inhibits DCC and the attraction by netrin. In addition, growth cones become sensitive to chemorepellent Semaphorin. The midline becomes repulsive (red dashes) and pushes the growth cone away.

now strong evidence that factors characterised elsewhere within the fields of developmental biology and neuroscience, involved in embryonic patterning and signal transduction, are also key regulators of growth cone behaviour. The redeployment of molecules to execute different roles at distinct stages in the development, maturation and function of the nervous system opens up new avenues to generate the immense complexity required.

Morphogens

Morphogens, which were characterised based on their effects on early patterning, are being increasingly implicated in axon guidance. The Wnts are one such family of secreted patterning molecules; two of their features that are particularly interesting with respect to axon guidance are their expression in anteroposterior and dorsoventral gradients in the neural tube and their putative role in synaptogenesis (Ciani and Salinas, 2005; Hall et al., 2000). This suggests that they play successive roles: first, to provide positional information to axons and then to regulate the transition to a functional nervous system. The *Drosophila derailed* (*Drl*) gene encodes a receptor tyrosine kinase which can bind Wnt5 and when mutated causes aberrant selection of anterior and posterior commissures (Bonkowsky et al., 1999). Loss of *Wnt5* similarly causes midline defects, independently of the canonical Wnt receptor Frizzled (Yoshikawa et al., 2003). Unlike the Robo/Slit system, the vertebrate data have not provided smooth evolutionary continuation. Evidence from an in vitro system and knockout mice suggests that vertebrate Wnts are involved in rostral pathfinding of commissural axons by acting as chemoattractants via the Frizzled receptor (Lyuksyutova et al., 2003). Several Wnt genes are expressed in an anteroposterior gradient along the cervical and thoracic spinal cord of mice. They act upon the vertebrate Drl homologue, the Ryk receptor, expressed by axons of the corticospinal tract to direct its longitudinal formation via repulsion (Liu et al., 2005). Thus, they can determine anteroposterior distinctions at a segmental level but also guide major projections along the body axis.

Sonic hedgehog (Shh) is secreted by the floor plate in a gradient which directs the specification of neuronal class in the ventral spinal cord (Jessell, 2000). A combination of explant studies and growth cone turning assays has been used to provide compelling evidence that Shh attracts commissural axons to the midline (Charron et al., 2003). In the best traditions of a bifunctional axon guidance molecule, Shh has also been shown to repel spinal commissural axons (Bourikas et al., 2005) and RGCs (Trousse et al., 2001). How Wnt-mediated attraction and Shh-mediated repulsion combine to guide commissural axons rostrally has yet to be resolved.

On the other side of the spinal cord, bone morphogenetic proteins (BMPs) pattern dorsal neurones (Jessell, 2000). BMPs may also have a role in repelling commissural axons away from the dorsal midline, initiating their journey towards the floor plate before attraction to Netrin begins (Augsburger et al., 1999). Thus, it seems that the dorsoventral signals provided by BMPs and Shh to define the spinal neurone pattern are recycled to direct axonal outgrowth. It is possible that this is a relatively recent evolutionary twist, hence the discrepancy between the *Drosophila* and vertebrate data. Guidance dictated by morphogens may refine and elaborate a fundamental map laid down by archetypal axon guidance molecules or act over different scales. The involvement of morphogens in navigation is certainly an interesting development and one that will become clearer now that their ability to guide axons can be distinguished from secondary effects on the specification of surrounding tissue.

Calcium and cyclic nucleotide signalling

The growth cone turning assay is a means to assess the attractive and repulsive responses of isolated growth cones to specific molecules. Factors under investigation are pipetted onto one side of an individual growth cone to produce a chemical gradient and the resultant axonal turning angle is measured. Although there are limitations in terms of accurately reproducing physiological parameters, it has nevertheless proved extremely informative, particularly for the measurement of intracellular events accompanying turning responses to guidance molecules (Henley and Poo, 2004). Furthermore, it has also revealed sensitivity to agents previously considered to transmit electrical rather than navigational signals. The neurotransmitter acetylcholine attracts growth cones (Zheng et al., 1994), whereas γ-amino butyric acid (GABA) can cause attraction through $GABA_A$ receptors or repulsion through $GABA_B$ receptors (Xiang et al., 2002). Neurotransmitters control the flux of ions across the membrane and thus the electrical excitability of the cell. One of the key ions involved is calcium which is also used widely as an intracellular second messenger in many signalling cascades. Cytoplasmic calcium levels are controlled by ion channels in the membrane acting in concert with intracellular stores. Both calcium-specific channels and transient receptor potential (TRP) channels, which allow a range of cations to pass, can trigger growth cone turning (Li et al., 2005; Nishiyama et al., 2003; Shim et al., 2005; Wang and Poo, 2005). The details of how neurotransmitters operate as guidance molecules in vivo are unclear: how is a directional signal maintained without disrupting electrical transmission or vice versa? Would growth cones be confused by autocrine effects? The modulation of calcium levels influences signalling through established guidance receptors, for instance, changing calcium influx can convert Netrin-mediated attraction to repulsion (Hong et al., 2000). This is partly determined by TRP channels (Wang and Poo, 2005), as is the turning response to brain-derived neurotrophic factor (Li et al., 2005). Advances in imaging using fluorescent dyes that indicate calcium levels will hopefully illuminate the mechanisms by which transient changes in cytoplasmic ion concentrations are linked to growth cone behaviour (Henley and Poo, 2004).

Cyclic nucleotides are second messengers, like calcium, used to trigger signalling cascades within the cell. Their levels are controlled enzymatically by the conversion of ATP and GTP into cyclic AMP (cAMP) and cyclic GMP (cGMP) respectively, thereby altering many cellular functions. In the case of growth

cones, the two molecules may act independently, for instance, the level of cAMP in *Xenopus* spinal neurones determines the polarity of response to specific neurotrophins (Song et al., 1997). Under other conditions, behaviour is dependent upon the ratio of the intracellular concentrations of cAMP and cGMP. Semaphorins are typically chemorepellent molecules for *Xenopus* spinal neurones, but the repulsion can be converted to attraction in a turning assay by altering the intracellular levels of cyclic nucleotides (Nishiyama et al., 2003; Song et al., 1998). This is supported by a study on cortical neurones in which asymmetric distribution of guanylate cyclase leads to attraction or repulsion, of dendrites and axons, respectively, by Sema3A (Polleux et al., 2000). The ratio of cAMP to cGMP also determines the polarity of response to Netrin (Fig. 3). In fact, a link between both messenger systems has been demonstrated in response to Netrin, namely, modulation of the activity of calcium channels by cyclic nucleotide signalling (Nishiyama et al., 2003).

Where is axon guidance heading?

Identifying families of guidance molecules and their members is only the beginning, without understanding how the different cues interact, it is little more than a glorified form of archiving. How are their signals integrated and segregated within a growth cone? What is their combinatorial effect? How can they be modulated to produce graded rather than binary responses?

Combining and modulating axon guidance receptors

Studying how receptors act in concert, either cooperatively or antagonistically, is a daunting task with a myriad of combinations to test, but progress is being made. Netrin is often the starting point because it is the best characterised chemoattractant, and it is easier to weigh up competing attractive and repulsive agents than two repulsive ones. There are three main ways in which the actions of guidance molecules may be combined. Firstly, the net sensitivity to two different factors can be used to sort different classes of neuronal projection. In the hindbrain, many dorsally projecting motor neurones are repelled by both Netrin-1 and Sema3A, whereas those that project ventrally are insensitive to Netrin-1 but repelled by Sema3A (Varela-Echavarria et al., 1997). Secondly, coordinating two factors with opposing actions to direct axons towards the same destination can have a 'stick and carrot' effect, producing a stronger impulse than either alone. An example of this occurs in the diencephalon where repulsive cues from Sema3F team up with an attractive source of Netrin-1 to funnel habenular axons along a neuromere boundary (Funato et al., 2000). Thirdly, competition between two signals can attenuate or block a response. In *C. elegans*, the attractive effect of Netrin is inhibited by a receptor protein tyrosine phosphatase (RPTP; Chang et al., 2004). RPTPs are an enigmatic group of axon guidance molecules whose phosphatase activity potentially serves as a counter to the kinase activity of Ephs, however, the ligands for most of them await identification (Ensslen-Craig and Brady-Kalnay, 2004).

Permissive and instructive cues can also act in combination, although the boundary between the two mechanisms is often blurred. This ambiguity is exemplified by the capacity of laminin, a growth-promoting extracellular matrix component, to convert Netrin-mediated attractive instruction to repulsion (Hopker et al., 1999). Conversely, after activation by Slit, Robo inhibits the capacity of N-cadherin to promote adhesion (Rhee et al., 2002).

Combining two signals is essentially a means to modulate the response to one, or both, of them. A molecule may actually have no effect in isolation but instead serve to block or enhance signalling of other receptors. Stromal-cell-derived factor 1 (SDF-1) is a chemokine and attracts leucocytes, but it also repels *Xenopus* spinal growth cones in a turning assay (Xiang et al., 2002). However, a study using cultured chick RGCs and DRG suggested that SDF-1 abrogates the repulsive effect of Slit-2 and Sema3A but has no effect by itself (Chalasani et al., 2003).

Alternatively, the modulating protein may interact with the extracellular portion of the receptor to attenuate or potentiate ligand binding. Heparan sulphate proteoglycans (HSPGs) are a diverse group of glycoproteins that interact with and modulate Slits, Netrins and Ephrins. However, given the enormous biochemical variability of HSPGs, the problem is in determining how they do this, whether by regulating ligand diffusion, receptor localisation or binding. These possibilities and their biochemistry are discussed in more detail elsewhere (Lee and Chien, 2004).

In the most permanent case of modulation, the receptor may be physically altered by alternative splicing at the mRNA level or even proteolytic digestion of the receptor at the cell surface. Sema3A is digested by the furin protease into different lengths, both within the cell and after secretion, to modulate its repulsive activity (Adams et al., 1997). The *Drosophila* homologue of Down Syndrome cell adhesion molecule (DSCAM) exists in multiple forms with a conserved overall architecture containing variable domains. In fact, 38,000 isoforms can theoretically be generated (Schmucker et al., 2000). How many of these are functionally distinct is still open to question, but it seems that homophilic interactions between matching isoforms may be the key (Wojtowicz et al., 2004). Rather than necessarily eliciting thousands of separate responses, the production of a multitude of molecular variants, each differing slightly, allows graded transition in behaviour (Lipscombe, 2005).

In the face of all the signals impinging on a growth cone, there remains a huge gap to fill before it is fully understood how receptors trigger the appropriate signalling cascade to elicit specific cytoskeletal changes and a turning response. In short, an intricate, multi-layered communication network is necessary to receive an extracellular signal, process it and then produce the appropriate motion of the growth cone: cascades of chemical second messengers; regulation of the phosphorylation state – and hence activity – of receptors and effector proteins; alterations to the cytoskeleton. The numerous proteins involved in these stages, and their extensive crosstalk is beyond the scope

of this review but comprehensively addressed elsewhere (Guan and Rao, 2003).

Considering three dimensions

A growth cone has to extend through a continuously changing environment as the rest of the embryo develops around it. A major task will be to understand how landmark choice points are recognised and the coordination of these with the development of the surrounding tissue. There is growing evidence for a change in sensitivity to different guidance cues during the course of an axon's maturation (Shirasaki et al., 1998; van Horck et al., 2004). Huge advances in our understanding of axon guidance have arisen from the detailed characterisation of isolated regions of the nervous system. The next step is to understand how these combine to produce the nervous system as a whole. Common mechanisms, such as midline chemoattraction and repulsion, have been found to act throughout much of the brain (Shirasaki et al., 1995; Tamada et al., 1995).

In the periphery, it is relatively easy to imagine how a growth cone stays on course by responding to local corridors of repulsion and to attractive cues from its target. Yet, even in the best-studied systems, such as motor innervation of the limbs, current understanding extends only as far as the specification of motor pools and the broad dorsoventral bifurcation of the initial projection (Shirasaki and Pfaff, 2002). How transcriptional events impinge upon axon guidance molecules to produce the observed topography to individual muscles is only slowly emerging (Kania and Jessell, 2003). In addition to directing growth cones through cell-autonomous, intrinsic control of gene expression, transcription factors may actually be secreted and form attractive or repulsive gradients. The Engrailed transcription factor has long been implicated in the establishment of retinotectal mapping. It was thought likely that this would be through regulation of the expression of axon guidance receptors, for instance, the Ephrins described above (Retaux et al., 1996). Striking recent data obtained using *Xenopus* RGCs show that an exogenously applied gradient of Engrailed-2 (En2) repels temporal axons and attracts nasal ones (Brunet et al., 2005). En2 is taken up by the growth cone where it influences local protein synthesis to produce its turning effect. Protein synthesis is known to occur in the growth cone independently of the cell body and thereby regulate sensitivity to guidance cues (Piper et al., 2005). The mechanisms by which these transcriptional and translational events correlate with growth cone navigation remain, for the most part, unknown. However, these new findings for the action of En2 give a tantalising glimpse into a direct link between an extracellular signal and changes in gene expression. It will be fascinating to see how many more transcription factors can act in this way. This may prove to be a key mechanism by which the transcriptional profile of a growth cone can be altered as it proceeds along its pathway, providing the necessary changes in responsiveness to sequential cues.

How then does an axon navigate through the brain where thousands upon thousands of other growth cones are growing in different directions, seeking other neurones? Many cell adhesion molecules promote the association of axons into fascicles following paths laid down by pioneer axons. How the axon achieves the correct balance between fasciculation and responding to guidance cues is poorly understood. Growth cones must not only recognise their specific target but be able to alter their mutual interactions and defasciculate from the main nerve (Van Vactor, 1998). These stop signals are just as important as those that enable the axon to reach its destination. Chemotropic agents are often secreted by growth cones themselves, and, in this way, a bundle of axons may regulate their mutual adhesiveness. By providing positive cues on their surface, pioneer axons can lay down a scaffold for their successors to follow. For instance, RPTPδ, in addition to acting as a chemoattractant, increases adhesion through homophilic binding (Sun et al., 2000; Wang and Bixby, 1999). Conversely, expression of a chemorepellent by a sub-population of axons within a growing nerve could force the defasciculation of their neighbours and create branches. These ideas are often difficult to test: in vitro, as emphasised already, it is not easy to distinguish between permissive and instructive effects; in vivo, multiple factors contribute to the overall level of adhesion and so genetic screens can easily overlook the involvement of individual factors due to partial redundancy.

Within the growth cone itself, the various signalling pathways must be coordinated spatially, genes expressed at appropriate times and their products trafficked to the correct part of the growth cone. Modern imaging techniques allow real-time visualisation of behaviours at ever smaller scales. Molecules can be tagged with fluorescent markers and tracked throughout the growth cone, revealing their patterns of membrane localisation, segregation and endocytosis (Castellani et al., 2004; Tani et al., 2005). Advanced technologies such as fluorescent resonance energy transfer (FRET) reveal transient molecular interactions undetectable by other means (Nakamura et al., 2005).

The Nobel Laureate Peter Medawar wrote: *scientific research [is] a steering process, a means by which we find our way about, and try to make sense of, a bewildering and complex world* (Medawar, 1979). In developmental neurobiology, this applies equally well to the research subjects themselves, the exploratory axons in their complex world.

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