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Axon guidance at the central nervous system midline

G. Tear

Molecular Neurobiology Group, 4th Floor, Hodgkin Building, Guy's Hospital Campus, King's College, London Bridge, London SE1 9RT (UK), Fax + 44 171 403 8883

Abstract. A key feature of the central nervous system of most higher organisms is their bilateral symmetry about the midline. The specialised cells that lie at the midline have an essential role in regulating the axon guidance decisions of both neurons that project axons across the midline and those that project on one side. The midline cells produce both attractive and repellent short- and long-range signals to guide axonal growth. The axons themselves express specific receptors that can be dynamically regulated in response to midline-derived signals. In this way, axons extend toward or away from the midline and those that do cross change their behaviour to respond to longitudinal signals on the contralateral side.

Key words. Midline; floor plate; axon; guidance; repulsion; attraction; *Drosophila*; vertebrate; embryo.

Introduction

In the developing nervous system, axons extend long distances along stereotypical pathways to reach their final target. To generate this precise pattern of connections, axons must be able to select their own specific trajectory. To do this, each axon must integrate the many guidance cues in its environment and react in a specific fashion to recognise its particular pathway. Individual axons must be able to interpret these cues in distinct ways since they often respond differentially when in the same environment. One place where axons make selective responses is at the midline of the central nervous system (CNS).

The CNS of most organisms is bilaterally symmetric about the midline. Within the ventral nerve or spinal cord, the axonal pathways generally form an orthogonal structure. Axons extend rostrally or caudally to form the longitudinal tracts or across the midline to form the commissural axon tracts that join the two sides. The majority of CNS axons take a contralateral projection and grow across the midline in one of the commissural tracts before turning into a longitudinal pathway (fig. 1). However, a smaller population of CNS axons extend in a longitudinal tract without crossing the midline and form an ipsilateral projection. Hence CNS axons must make a choice between crossing the midline or remaining on one side. The axons that do cross must make a further choice to turn rostrally or caudally after crossing the midline. This represents a change in the behaviour of these axons because they must turn in response to longitudinal cues that they had previously ignored on their original side. Simultaneously, these axons appear to adapt their responsiveness to the midline cues as they rarely recross. Correlating with these changes in behaviour, the axons regulate their expression of cell surface glycoproteins [1–3].

It is becoming clear in a number of species that the cells that lie at the CNS midline play an essential role in providing cues that enable axons to make appropriate guidance decisions at the midline [4–6]. The midline cells provide attractive and inhibitory guidance cues that act locally or are secreted to guide axons either towards or away from the midline. The midline cells also provide cues that ensure axons navigate correctly near the midline, allow axons across the midline and ensure these axons make the appropriate pathway choices once across the midline.

Midline cells form a distinct population in the CNS

The cells that lie at the midline of the CNS in vertebrates and invertebrates are morphologically distinct from their neighbours. In *Drosophila*, the distinctive

mesectodermal cells lie at the midline. These cells are identifiable prior to gastrulation when they begin to express their determinant *single-minded* [7]. They originate as a single column of cells between the prospective mesodermal and neural cells. Upon invagination of the mesoderm, these cells move to the ventral midline to form a double column of cells that interdigitate creating a single column of cells that separates the two halves of the CNS. These cells differentiate into various cell types including two or three pairs of glia, the MP1 neurons and the VUM neurons [8, 9].

In rodents and chicks, the commissural axons extend to and across the floor plate cells that lie at the ventral midline of the neural tube. The floor plate is composed of columnar ependymal cells that differentiate early in the development of the neural tube. In the rat, the floor plate is about 15–20 cells wide that can also be identified by their expression of specific molecules [10–12]. In the zebrafish, a single column of large cells appears morphologically distinct and provides the midline floor plate structure [13].

The midline cells are established early during CNS development and exist as a separate population at the centre of the ventral nerve cord. This position and its differentiation prior to the extension of the first axonal growth cones makes it a prime early cellular target.

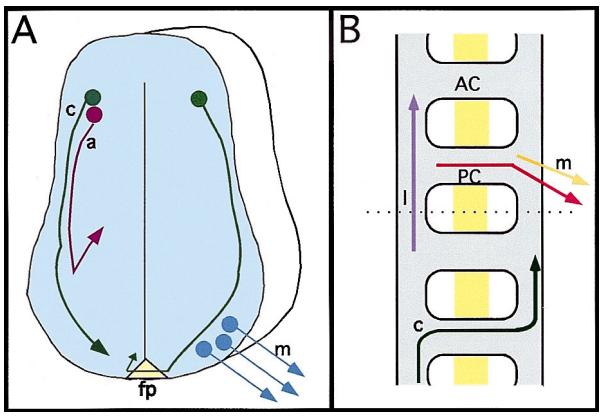

Figure 1. Neuronal populations in the early CNS. The CNS includes a variety of neuronal types that project axons along different trajectories with respect to the midline cells. (*A*) In the vertebrate embryo, commissural axons (c) grow to the floor plate (fp) and cross the midline before turning to project longitudinally. In the chick, these axons are known to turn either rostrally or caudally whilst those in the rodent have only been identified to turn rostrally [5]. Axons of the ipsilaterally projecting association neurons (a) also project ventrally but turn to project longitudinally prior to reaching the floor plate. Motor neurons (m) have their cell bodies close to the midline but project away from the ventral midline to exit the CNS. (*B*) Similarly in the *Drosophila* embryo, axons project towards or away from the midline forming an orthogonal structure. In each segment of the *Drosophila* CNS, commissural axons (c) can be identified that extend to the midline in either the anterior (AC) or posterior (PC) commissure before turning to join the longitudinal tracts, e.g. SP1. A minority of longitudinal axons (l) follow an ipsilateral projection, often close to the midline, but never cross, e.g. pCC. *Drosophila* motor neurons (m) can cross the midline before exiting the CNS on the contralateral side, e.g. RP3, or exit on their own side, e.g. aCC. In both vertebrates and invertebrates commissural axons rarely re-cross the midline. Yellow shading indicates floor plate (*A*) or midline (*B*) cells. Dotted line in (*B*) indicates the segment border.

Cells at the midline are essential for axon guidance

Investigation of nervous system development after depletion of midline cells has revealed their importance for the development of CNS axon pathways. Midline cells can be specifically removed from the embryo by experimental manipulation such as laser ablation or the use of genetic mutations that disrupt midline cell fates. The absence of midline cells results in a number of significant axon guidance errors in many species, suggesting they provide essential axon guidance functions.

The *Drosophila* ventral nerve cord consists of a repeated series of neuromeres. Within each neuromere, axons cross the midline in either the anterior or posterior commissural tract (figs 1, 2A). The first commissural axons to cross the midline migrate towards the anterior glia and the anteriormost VUM neurons to form the posterior commissure. The axons of the anterior commissure extend to a position immediately anterior to where the posterior commissure axons cross the midline. Finally, midline glia and lateral neuronal cell bodies migrate between the two tracts to separate the two commissures [8]. Mutations in a number of genes affect the development of midline cell fate or differentiation [8, 14] and result in a failure of axons to extend correctly at the midline. The most severe phenotypes are observed in the *single-minded* (*sim*) and *slit* mutants [7, 15, 16]. In *sim* mutants, the midline cells fail to differentiate and commissural tracts cannot form. This results in a collapse or fusion of the longitudinal tracts at the CNS midline [15]. In *slit* mutants, the midline cells initially differentiate but become displaced ventrally. The commissures form at first but as the midline cells are displaced, the longitudinal tracts begin to fuse and the CNS collapses onto the midline [8, 17]. Mutations in *orthodenticle* (*otd*) affect a subset of the midline cells. In *otd*, the VUM cells degenerate and the axons that pioneer the posterior tracts are no longer able to extend across the midline, either remaining on their own side of the CNS or joining the anterior commissure [8] (fig. 2A).

In the vertebrate, association and commissural neurons extend towards the floor plate at the ventral midline of the CNS. Commissural neurons cross the floor plate

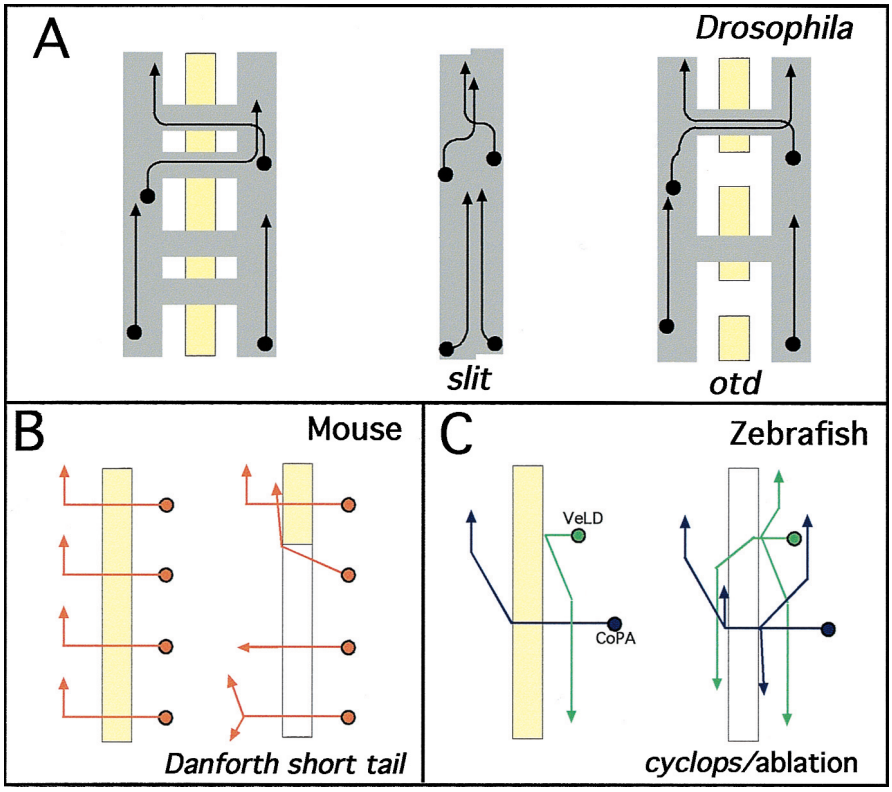

Figure 2. Schematic diagrams illustrating axon behaviour caused by loss of midline cells. (*A*) In the *Drosophila* CNS, the majority of axons are commissural and extend across the midline (yellow shading) while a minority extend ipsilaterally and do not cross. In *slit* mutants, the midline cells are displaced ventrally and the CNS collapses onto the midline. Both commissural and ipsilateral axons grow towards and along the midline (54a; D. Hartley and G. Tear, unpublished observations). In *otd* mutants, a subset of midline cells, the VUM cells, degenerate, so that the posterior commissure fails to form. (*B*) In the mouse, commissural axons cross the midline and extend rostrally. In the *Danforth short tail* mutant, much of the floor plate and notochord fail to develop. Many commissural axons can reach the midline, often taking an aberrant pathway along the circumference of the neural tube, but then fail to make the correct turning decisions. Where floor plate tissue remains, commissural axons will turn to project directly to these cells. (*C*) In the zebrafish, both commissural neurons (CoPA) and ipsilaterally projecting neurons (VeLD) are affected by the removal of the midline by laser ablation or in *cyclops* mutants. CoPA neurons reach the midline but can then make incorrect turning decisions, extending either longitudinally on their own side or close to the midline on the contralateral side. The VeLD neurons can cross the midline when midline cells are missing and usually make their correct turn on the contralateral side. The VeLD neurons also make incorrect turning decisions on their own side.

before turning into a longitudinal pathway whereas axons from the association neurons extend ventrally but turn to join the ipsilateral longitudinal pathway prior to crossing the midline. Both types of neuron are affected by manipulations that remove midline structures (fig. 2B). In the mouse mutant *Danforth short tail*, both the floor plate and the underlying notochord are missing. In these animals, the commissural axons can reach the midline but they make incorrect pathway choices, often failing to turn longitudinally, and may project out of the spinal cord [18] (fig. 2B). Similarly, if floor plate development is prevented in the chick by removal of the notochord, the axons that reach the midline make turning errors [19]. In both mouse and chick embryos that lack a floor plate, the commissural axons that reach the midline do so by extending along the circumference of the neural tube. This pathway is the same as that normally taken by the earliest-born commissural neurons and differs from the trajectory taken by later-born commissural neurons. These neurons initially extend ventrally close to the lateral edge of the neural tube in dorsal regions of the spinal cord and then turn to extend ventromedially directly towards the floor plate (fig. 1). In the absence of the floor plate, the later-born commissural axons cannot identify this direct route.

In the zebrafish embryo, removal of floor plate cells in the *cyclops* mutant or by laser ablation also induces errors in the migration of the commissural primary ascending (CoPA) neurons and the association-like ventral longitudinal descending (VeLD) neurons [20, 21].

The CoPA neurons normally cross the midline floor plate cells and turn anteriorly, while VeLD neurons extend to the midline and turn posteriorly without crossing the midline (fig. 2C). In the midline-deficient embryos, approximately 25% of the CoPA axons fail to cross the midline and 15% of the VeLD axons now aberrantly cross the midline. Furthermore, many of the axons make errors in their choice of turn into a longtiudinal pathway: CoPA axons turn to extend posteriorly and 22% of the VeLD axons extend anteriorwards [20, 21] (fig. 2C). Again, the axons do appear to be able to reach the midline in the absence of the midline cells but once there, make incorrect pathway choices.

The midline provides positive and negative cues to guide axons

The cells that lie at the CNS midline are required for the correct routing of axons in the CNS. The ablation experiments reveal that the midline cells are required in the embryo to both attract commissural axons from a distance and provide cues to direct the axons once they reach the midline. This suggests that the midline supplies long- or short-range signals to direct axonal guidance. Recently, a number of different experimental approaches including genetic screens, in vitro investigations and in vivo observations have begun to reveal the nature of some of these signals. The data confirm that the cells at the midline guide axons by producing signals that can either attract or repel certain growth cones.

Positive and negative guidance cues at the invertebrate midline

A large-scale genetic screen was conducted in *Drosophila* to identify genes that when mutated give rise to defects in the formation of CNS axon pathways [14]. In this screen, several mutations were identified that affect the development of the commissural and longitudinal axons that extend near to or across the midline. Attention has focused on mutations in two genes, *commissureless* (*comm*) and *roundabout* (*robo*), because they have dramatic and complementary phenotypes in which guidance of axons at the midline is specifically affected. In *comm* mutant embryos, commissural axon tracts are completely absent while other aspects of CNS development are unaffected, including midline and other CNS cell fates and formation of the longitudinal axon tracts. Some commissural axons initially extend a short distance toward the midline; however, these axons appear to be unable to extend across the midline and they turn to join the longitudinal tracts on their own side [14, 22]. The ipsilateral projections taken by these misdirected neurons are not as precise as they would normally be. They are unable to fasciculate correctly within the tracts as they would had they crossed the midline into the contralateral longitudinal pathway [14]. The failure of axons to cross the midline in *comm* mutants suggests that the *comm* gene product is required as part of a positive attractive signal produced by the midline that normally guides commissural axons across *comm* might also be required in a mechanism whereby midline cells impart some information to commissural axons allowing them to change their behaviour to recognise cues on the contralateral side of the embryo that they do not perceive on the ipsilateral side.

comm has been cloned and encodes a transmembrane protein (fig. 3) that is expressed in the midline glia cells when the commissural axons are extending towards these cells [22]. The *comm* phenotype and expression reveal that one role of the midline cells is provision of attractive signals that allow axons to cross the midline. Intriguingly, the Comm protein also appears to accumulate on the cell surface of the commissural axons. This suggests the protein is transferred to these axons as they cross the midline cells.

Mutations in the *robo* gene cause a dramatic misrouting of axon pathways at the midline so that axons that normally project solely on their own side now cross the midline [14, 23]. Moreover, the longitudinal pathways appear to contain fewer axons than normal. Not only do those axons that normally project ipsilaterally extend across the midline in *robo* mutant embryos but axons such as SP1 that normally cross the midline once can recross the midline and may cross multiple times to circle it [24]. However, all other aspects of development including neuronal and midline cell fate differentiation in these animals appear normal. In the *robo* mutant, medially located ipsilaterally projecting neurons, e.g. pCC, initially extend as normal but then begin to cross the midline approximately where the anterior commissure crosses the midline. Axons that project in lateral fascicles do not appear affected in *robo* mutants. This phenotype is quite unlike other mutations that affect attractive longitudinal axon guidance cues, in which there is no inappropriate midline crossing [14, 25]. The dramatic turn by medial axons towards the midline suggests Robo may be required as part of a repulsive signal that normally keeps ipsilateral axons away from the midline and prevents contralateral axons from recrossing the midline.

The presence of a repulsive activity at the midline is also suggested by the observation in *comm* embryos that some commissural growth cones extend a short distance towards the midline but fail to completely cross. The attractive role of Comm could be an ability to overcome this repellent activity and allow commissural axons across the midline. Further evidence of the need for

commissural growth cones to overcome a midline repellent is suggested by observation of the behaviour of the Q1 commissural axon growth cone at the midline in the grasshopper [26]. The Q1 axon turns from an initial anterior projection and heads directly to the midline. When the Q1 growth cone contacts the midline it stops or retracts in a response similar to contact inhibition [27]. The growth cone remains at the midline until the inhibitory reaction is overcome, in this case by contact with its contralateral homologue.

Robo encodes a transmembrane protein with five immunoglobulin (Ig) domains and three fibronectin domains extracellularly and a large intracellular domain [23] (fig. 3). Unlike other members of the Ig superfamily, Robo does not appear to act as a cell adhesion molecule (CAM). The possession of a large intracellular region places Robo in a group of Ig superfamily members that act as receptors for extracellular signals [24]. The Robo protein is expressed at high levels on the surface of growth cones and axons within the longitudinal tracts but at low levels on the commissural regions of the axons. The *robo* phenotype and its structure and expression suggests Robo acts as a receptor for a midline-derived repellent signal.

Embryos that are doubly mutant for both *comm* and *robo* display a phenotype that is identical to that of embryos mutant for *robo* alone [14]. This suggests there is a balance of attractive and repellent activities at the midline that ensures the appropriate axons cross or avoid it. As Robo appears to act downstream of Comm, the repellent activity of the midline may be the predominant force that has to be overcome to allow axons to reach the midline. A similar balance of positive and negative guidance cues also appears to exist at the midline of the vertebrate embryo.

Positive and negative guidance cues at the vertebrate midline

In vitro and in vivo experiments have demonstrated the ability of the floor plate to guide axons via the production of both positive and negative cues [5]. These cues

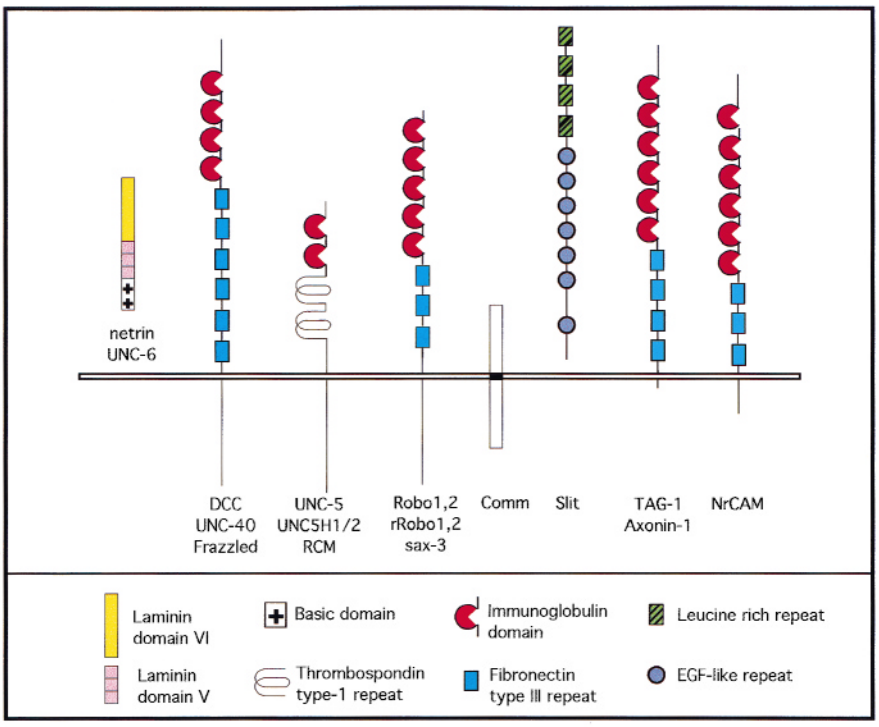

Figure 3. Molecules with a role in axon guidance at the midline. The netrin chemoattractant is conserved across species and is related to the amino-terminal domains of laminin. The DCC and UNC-5 families of netrin receptors are also highly conserved. Both groups of receptors include immunoglobulin (Ig) domains combined with either fibronectin type III (FN) or thrombospondin domains. A similar tandem arrangement of Ig or FN domains is also present in the cell adhesion molecules (CAMs) Axonin-1 (TAG-1) and NrCAM. These CAMs have shorter intracellular domains than the receptor molecules. The repellent receptor Robo also includes Ig and FN domains extracellularly. Slit, the putative ligand for Robo is a large protein including four leucine-rich repeats and epidermal growth factor (EGF)-like motifs known to be important in protein/protein interactions. Comm is a transmembrane protein with no readily identifiable motifs.

can be secreted and act over a distance to guide axons through a chemotropic or chemorepellent action or be produced at the floor plate to provide short-range local guidance cues. The different populations of axons that extend at the midline make differential use of these cues to navigate their appropriate pathways.

Culture of floor plate cells in a three-dimensional collagen-based in vitro culture system, which allows gradients of diffusible substances to be established, has revealed that the floor plate can secrete activities that attract or repel specific axons towards or away from the midline. Initial use of this culture system revealed that explants of rat floor plate encouraged extension of commissural axons from rat dorsal spinal cord [28]. Further experiments revealed that this activity not only promotes outgrowth but can reorient commissural axon growth within the explant [29]. This chemotropic activity is specific to the commissural axons, as the floor plate has no turning effect on the association neurons that do not project to it. Consistent with these results is the observation that spinal commissural axons reorient to grow toward ectopically transplanted floor plate cells in the chick embryo [30, 31]. This chemotropic activity is not restricted to the floor plate cells within the spinal cord but also appears to be a property of floor plate cells in the brain. Explants of floor plate cells from rat rhomboencephalon can promote axonal outgrowth, and reorientation, from corresponding explants from dorsal rhomboencephalic regions when they are cocultured [32].

Not only does the floor plate produce a chemotropic activity but it also secretes an activity that is able to repel axons. In the neural tube, motor neurons differentiate on either side of the floor plate cells. All of the motor neurons project their axons ipsilaterally and are initially directed away from the midline before exiting the neural tube to innervate their target muscles. Motor axons also divert around floor plate transplanted between their cell bodies and their normal exit from the neural tube [33]. To demonstrate whether this repulsion of motor axons required contact with the floor plate, explants of rat ventral hindbrain or spinal cord were cocultured with rat floor plate tissue in a collagen gel. Motor axons turned within the explanted tissue to exit from it on the side that faced away from the floor plate cells. Similar coculture experiments demonstrate that mesencephalic floor plate can act at a distance to repel mesencephalic alar and basal plate axons [32].

In addition to its ability to produce long-range signals, the floor plate also expresses local cues that provide positive and negative signals that regulate axonal growth at the midline. These signals have been revealed at the chick floor plate by experiments that disrupt an interaction between two CAMs from the Ig superfamily. The floor plate cells express NrCAM, while Axonin-1 (the chick homologue of rat TAG-1), a heterophilic binding partner for NrCAM, is expressed on commissural growth cones (fig. 4). Injection of function-blocking antibodies against these CAMs into the spinal cord of chick embryos during commissural growth in ovo results in the failure of commissural axons to cross the midline [34]. In the absence of the normal interaction between NrCAM and Axonin-1, commissural axons now turn along the ipsilateral border of the floor plate, suggesting this interaction normally provides a positive signal to allow commissural axons to extend across the floor plate; without it, the floor plate appears inhibitory to the growth cone.

Thus, the floor plate cells at the midline of the vertebrate CNS, in common with invertebrate midline cells, are able to produce both positively and negatively acting guidance cues that bring certain axons to the midline and direct others away. Recent experiments in a number of species have begun to reveal the molecular nature of the guidance cues produced by midline cells and indicate how these signals are recognised by growth cones.

Molecules that guide axons towards or away from the midline

Purification of the chemoattractant, netrin, produced by the floor plate cells has revealed that there is extensive molecular conservation of the guidance cues that guide axons to and away from the midline. The netrins were isolated from chick brain as an activity that can promote commissural axon outgrowth from rat dorsal spinal cord [35]. Two related proteins, Netrin-1 and Netrin-2, each possess the same activity observed for the floor plate in vitro. *netrin-1* is expressed in the floor plate while *netrin-2* is expressed in the ventral two-thirds of the spinal cord but excluded from the floor plate. Netrin-1 is secreted into the medium from COS cells transfected with recombinant *netrin-1* yet a substantial fraction remains associated with the cell surface [36]. This suggests that association with cell surface or extracellular matrix proteins may regulate the diffusion of the netrins. This may aid the production of a gradient of Netrin-1 expression in vivo with a peak at the midline. Netrin-1 probably contributes the chemoattractant activity of the floor plate, since when *netrin-1* function is removed in mice, many commissural axons fail to reach the midline [37].

The netrins are predicted to include an N-terminal region with high homology to domain V and VI of the laminin B2 chain and a positively charged C-terminal region (fig. 3). These molecules are highly homologous to a nematode protein UNC-6 that is also required for circumferential guidance of axons [35, 38]. Netrin ho-

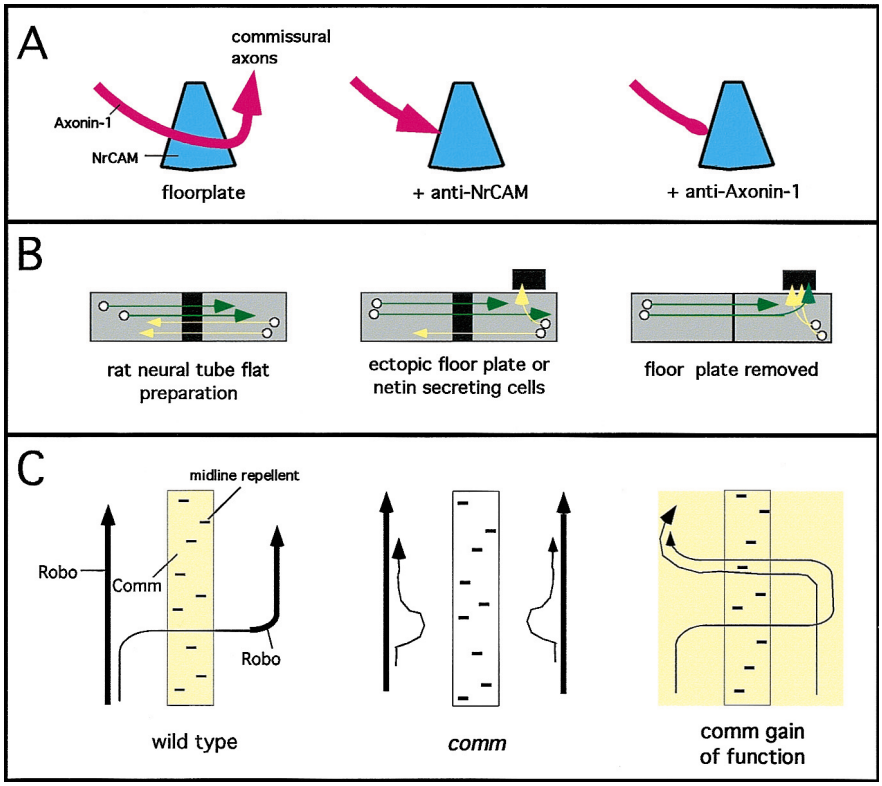

Figure 4. Diagrams illustrating interactions that regulate axon behaviour at the midline. (*A*) In the chick spinal cord, commissural axons cross the floor plate and turn longitudinally. The floor plate cells express NrCAM on their surface and the commissural axons express Axonin-1. These molecules bind heterophilically. Following interference of this interaction by administration of antibodies against NrCAM or Axonin-1, commissural axons fail to cross the midline—the growth cones either stall (anti-NrCAM) or collapse (anti-Axonin-1) [55]. (*B*) Commissural axons will extend in an open-book preparation of rat neural tube that has been cut dorsally. Axons that extend to the floor plate on each side can be differently labelled with DiI (green) or DiO (yellow). Addition of floor plate tissue or netrin-secreting cells next to one side of the preparation causes axons on the ipsilateral side to turn to the cells. However, the axons that have crossed the floor plate are not responsive to the ectopic source of chemoattractant. If the floor plate is removed from the neural tube, axons from the contralateral and ipsilateral side turn towards the ectopic source [56]. (*C*) In the wild-type *Drosophila* embryo, Comm and a midline repellent (Slit) are expressed at the midline. The repellent receptor Robo is expressed on ipsilateral axons from the outset of their growth. Commissural axons only express high levels of Robo once they have crossed the midline. In *comm* mutants, commissural axons initially orient towards the midline but are unable to cross. Comm normally provides a signal that overrides the repellent signalling to allow axons to cross. Overexpression of Comm reveals Comm can downregulate Robo protein levels allowing crossing and recrossing of the midline. This behaviour is identical to that observed in *robo* mutant embryos.

mologues have been identified in *Drosophila* where they are also expressed and required at the ventral midline [39, 40]. *Drosophila* has two netrin genes, *netrin-A* and *netrin-B*, located close to one another on the X chromosome. Embryos deficient for this region have defective commissures with many axons failing to cross the midline. Re-expression of either *netrin-A* or *netrin-B* in the midline cells can rescue this phenotype, whereas misexpression of the genes throughout the nervous system causes disruption of commissural and longitudinal axon tracts [39]. Thus a localized source of netrin is required at the midline to direct commissural axons; widespread expression results in a failure of axons to correctly find their normal pathway.

Further analysis of netrin function has revealed that these molecules are bifunctional and able to act both as chemoattractants and chemorepellents for different populations of axons at the midline. The initial evidence for this bifunctionality came from genetic analysis in *Caenorhabditis elegans* [41, 42]. In the nematode, mutations in *unc-6* and two other genes, *unc-5* and *unc-40*, affect guidance of axons that extend ventrally towards or dorsally away from the ventral midline. Mutations in *unc-5* affect dorsal migration while *unc-40* mutants primarily display defects in ventral guidance. However, different *unc-6* mutations can affect guidance of axons that extend either towards or away from the midline while null mutations disrupt both migrations [42]. This analysis

suggested UNC-6 is a common component required at the midline to attract or repel axons dependent on the type of receptor they express. Similarly, in vertebrates, Netrin-1 can repel the growth cones of trochlear motor axons that originate near the floor plate but extend dorsally [43].

Genetic studies in the worm suggest that *unc-5* and *unc-40* encode candidate receptors for UNC-6/netrin [41, 42]. Both *unc-5* and *unc-40* encode proteins that are members of the Ig superfamily [44, 45] (fig. 3). UNC-5 has two Ig domains and two thrombospondin domains with a short intracellular region. UNC-5 is required in neurons and its misexpression in lateral neurons causes them to be repelled by UNC-6 to which they are normally insensitive [46]. Vertebrate homologues of *unc-5* have been identified—*unc-5H1*, *unc-5H2* and *rcm*—all of which can bind netrin [47, 48]. Mutations in *rcm* result in abnormal neuronal migration suggesting these molecules also act in the vertebrate to guide neurons [48].

UNC-40 has four Ig domains and six fibronectin type III domains and a large ($\sim$ 300 amino acids) intracellular region [45]. Two molecules with a homologous structure to UNC-40 exist in vertebrates, DCC (deleted in colorectal cancer) and neogenin, while a single homologous molecule *frazzled* has been identified in *Drosophila* [45, 49]. DCC is the best candidate vertebrate receptor to receive the netrin signal secreted from the midline. DCC binds netrin and is expressed in commissural neurons [50]. If DCC function is removed, the majority of commissural axons fail to reach the midline [51]. Furthermore, antibodies against DCC inhibit the turning of axons towards a source of netrin in vitro [52]. The *Drosophila* homologue *frazzled* is also expressed in neurons and mutations in *frazzled* cause an embryonic phenotype very similar to that observed in embryos deficient for the *netrin* genes [49].

Despite the identification of DCC as the neuronal receptor that guides axons to the netrin source it is not yet clear how activation of the receptor guides axonal growth. Examination of the pharmacological sensitivity of DCC-positive *Xenopus* cortical axons extending towards a netrin source suggests that transduction of the netrin signal may be modulated by regulation of cyclic nucleotide levels. Inhibition of protein kinase A can convert axons from responding to netrin as an attractant to responding as if it were a repellent [53]. This also leads to the speculation that different receptors may cause axons to respond differentially to a netrin signal by differential regulation of the same downstream pathway. Indeed, it is unclear whether UNC-5 can itself act as a receptor to transduce the netrin signal. Genetic evidence in the nematode suggests that UNC-5 might function alongside UNC-40 to produce a complex response that interprets the UNC-6/netrin signal as repellent [54].

The expression and structure of Robo suggests it acts as a receptor that detects a repulsive signal secreted from the midline. However, it appears unlikely that netrin is a ligand for Robo since in *Drosophila* there is no evidence of any genetic interaction between *robo* and *netrin* [23]. Recently, however, a genetic interaction has been identified between *robo* and *slit* [54a]. Slit is a large extracellular matrix protein that is secreted from the midline cells and can be found associated with commissural axons [16] (fig. 3). In the absence of *slit*, many axons extend to the midline but fail to leave, leading to a collapse of the CNS (fig. 2A). Slit will bind to Robo [54b] suggesting that it is the repulsive ligand for Robo. The stronger phenotype of *slit* loss-of-function mutants when compared with similar *robo* mutants suggests that Slit may also be necessary for axons to leave the midline. One candidate for a second Slit receptor is Robo2, another receptor closely related to Robo that is also expressed in the CNS [23]. Vertebrate homologues of Slit have also been identified and these are able to bind the vertebrate Robo molecules. It remains to be seen if Slit and Robo function in vertebrates as they do in *Drosophila* to guide axons away from the midline.

Regulation of axon behaviour at the midline

The midline is not the final target for commissural axons but acts as a choice point or intermediate target (for a further discussion of intermediate targets see the review by O'Connor in this issue). Commissural axons follow attractive guidance cues to the midline but do not remain there, crossing it to leave to follow a longitudinal path on the contralateral side (fig. 1). These axons rarely return to recross the midline. A number of growth cones are also attracted to the midline but never cross, turning instead to join an ipsilateral pathway close to the midline. This change in the pattern of growth cone extension at the midline reveals that axons alter their behaviour after crossing the midline. What molecules mediate such a change? What are the cues that allow axons to cross over midline cells that produce potent attractants and repellents?

In the chick, the floor plate cells express NrCAM which is required for axons to cross the midline. When function-blocking reagents disturb a heterophilic interaction between NrCAM at the midline and Axonin-1 on commissural axons, the floor plate becomes inhibitory to the commissural growth cones [34, 55] (fig. 4A). This suggests that an association of Axonin-1 and NrCAM allows crossing by overcoming a repellent activity at the midline. Yet, the qualitative nature of the axonal failure to cross the midline varies depending on whether Axonin-1 or NrCAM activity is blocked [55]. Anti-Axonin-

1 causes commissural growth cones to collapse on contact with the floor plate while addition of anti-NrCAM causes the growth cones to stall (fig. 4A). Axonin-1 might have more partners than NrCAM, since interference with Axonin-1 function appears to remove all positive interactions, leading to collapse, while blocking only NrCAM interactions leaves some Axonin-1 interactions possible and the floor plate now appears non-permissive. Thus, growth cones may react to the midline in various ways dependent on the types of receptors they express. To cross they must express receptors that identify positive cues or lack receptors that recognise negative cues. Furthermore, it appears it would be possible to adapt axon behaviour at the midline by regulating their repertoire of receptor types. At the chick floor plate, interaction with the midline cells may induce alterations in the population of surface IgCAMs to disrupt the Axonin-1/NrCAM interaction and unmask the inhibitory action of the floor plate. Changes in surface IgCAM expression have been identified when axons grow on different substrates [34]. This dynamic regulation of surface proteins may alter the behaviour of the growth cone to allow turning or extension across or away from the midline.

Rat commissural axons actually switch expression from the surface antigen TAG-1 (homologous to Axonin-1 in the chick) to another IgCAM, L1, as they cross the floor plate [2]. This switch may allow the axons to choose their appropriate pathway on the contralateral side or ensure that once across the midline they are unable to recross. Commissural axons rarely recross the midline; this might be due in part to the downregulation of their ability to respond to floor plate-derived netrin. Commissural axons that have crossed the floor plate are no longer attracted to either an explant of floor plate or cells expressing *netrin* placed on the contralateral side [56] (fig. 4B). However, there is no downregulation of DCC as the commissural axons cross the floor plate [50] suggesting that a second receptor might be upregulated. One candidate could be a member of the Robo family of repulsive receptor molecules [23].

Robo is another example of a receptor whose level of expression is regulated along the length of axons [23]. In *Drosophila*, high levels of Robo are present on ipsilaterally projecting axons from the outset of their growth where it acts to ensure these axons do not cross the midline. However, commissural axons upregulate Robo expression to high levels once they have crossed the midline thus ensuring they only cross the midline once. Such upregulation renders the commissural axons sensitive to Slit, the putative midline-derived repellent molecule [54a]. How do the commissural axons ensure that they are able to cross the midline through a repellent signal? In *Drosophila*, Comm suppresses the inhibitory signal at the midline—if Comm is absent no axons can cross. Double-mutant studies reveal that Comm activity is only required if Robo is present; if Robo function is lacking, Comm is not needed for axons to cross the midline [14]. Thus, Comm normally ensures commissural axons can cross the midline by antagonising Robo function. Examination of Comm and Robo expression reveals that Comm is expressed at the midline where Robo levels are low [22, 23]. This complementary pattern of expression is created by the ability of Comm to downregulate Robo protein levels. If Comm is overexpressed throughout the CNS, Robo protein levels are reduced and axons can freely cross the midline as they do in *robo* mutants [24] (fig. 4C). In the normal embryo, Comm is observed to transfer from the midline cells to the commissural axons as they cross the midline. This may be a result of Comm binding to Robo or other receptor molecules on the commissural surface and subsequently brought into the neuron. Thus Comm appears to act locally to ensure Robo levels remain low on the commissural axons by targeting Robo for degradation, or inhibiting *robo* translation. In this way, commissural axons can cross the midline through a source of repellent activity. It is also possible that Comm, or another signal transmitted by the midline cells, may modify the commissural axons to allow them to turn and make their correct pathway choice within the longitudinal tracts once across the midline.

Transfer of macromolecules between floor plate cells and commissural axons has also been observed in vertebrates [57, 58], suggesting complex communication between midline cells and commissural axons during midline crossing. A possible secretory activity for floor plate cells has been proposed based on the numerous vesicles they contain. Moreover, processes from the floor plate tightly enwrap the axons during midline crossing suggesting that signals could be transmitted via macromolecular transfer [5]. However, the molecules identified as being transferred do not yet have known roles in axon guidance. To date, no homologues of Comm have been identified in vertebrates, so it is not known whether the floor plate cells use the same molecules as the midline cells in *Drosophila* to communicate information to the axons. The mutant and ablation studies suggest there is communication between the floor plate cells and the commissural axons but how this signalling takes place remains to be discovered.

Robo molecules are present in vertebrates and the nematode where they may also provide a negative signal to prevent axons from crossing the midline or to drive axons across the midline. Mutations in *sax-3*, the *C. elegans robo* homologue, cause axons that normally express *sax-3* to misroute across the ventral midline of the worm suggesting that Robo function to prevent midline crossing is highly conserved [59]. However, the

nematode ventral nerve cord lacks commissures and so may have not evolved a mechanism to overcome Robo activity to allow crossing—no readily identifiable Comm homologue exists in the now complete *C. elegans* genomic sequence.

A Robo orthologue *r-robo1* is expressed in commissural axons during development of commissures in the rat [23]. However, until an antibody becomes available, we will not know whether the protein is regulated in a similar manner as in the fly. It is possible that Robo levels are reduced in those axons that cross the midline and upregulated by a Comm-like mechanism as they cross. Alternatively, interaction with the floor plate cells may disrupt a heterophillic interaction between Robo and another IgCAM (e.g. TAG-1) freeing Robo to act as a repulsive receptor. Homologues of Slit, the postulated Robo ligand, have been identified in vertebrates and these also bind vertebrate Robo molecules [54b] suggesting that Robo-mediated mechanisms are highly conserved.

Summary

The cells at the midline of the CNS provide an essential role in guiding axons that extend towards, across, away or near to the midline. These specialised cells produce both short- and long-range cues that can attract or repel growth cones. Many elements of these signals are conserved at a molecular level although their regulation may differ among species. Repellents such as netrin and the Robo ligand ensure non-crossing axons stay away from the midline or turn ipsilaterally alongside it. Netrin can also act as a long-range attractant to bring commissural axons to the midline, while short-range molecules such as NrCAM or Comm provide positive signals to allow extension across the midline. Correct guidance of axons at the midline requires a differential interpretation of the many positive and negative signals that the midline cells present (fig. 5). For different classes of axons, the balance of positive or negative signals can be tipped one way or the other. Ipsilaterally projecting axons are more sensitive to repellent signals, while commissural axons might express the receptors that allow them to experience positive cues. Alternatively, ipsilateral and commissural axons may experience the same signals but react differently. Some cell surface molecules that regulate growth decisions at the midline do appear to be dynamically expressed. Receptors can be differentially expressed along the axon and regulated in response to midline signals. The signals communicated by the midline may also effect the changes in responsiveness that allow commissural axons to adjust their behaviour to follow cues on the contralateral side that they had ignored on their own side of the CNS. Comm is an example of such a signal in *Drosophila*; however, the nature of similar floor plate-derived signals is as yet unknown.

Studies of axon guidance at the midline have revealed much about how axons might be guided to or away from particular targets. The midline is also an example of a choice point at which axons have to make guidance decisions. Similar decisions are made throughout the nervous system as axons seek out their appropriate targets. It is likely that many of the mechanisms used at the midline are also used in other areas of the nervous system that are perhaps less amenable to experimental analysis. Continued work to characterise how guidance cues are recognised to produce the appropriate guidance response at the midline will contribute to further-

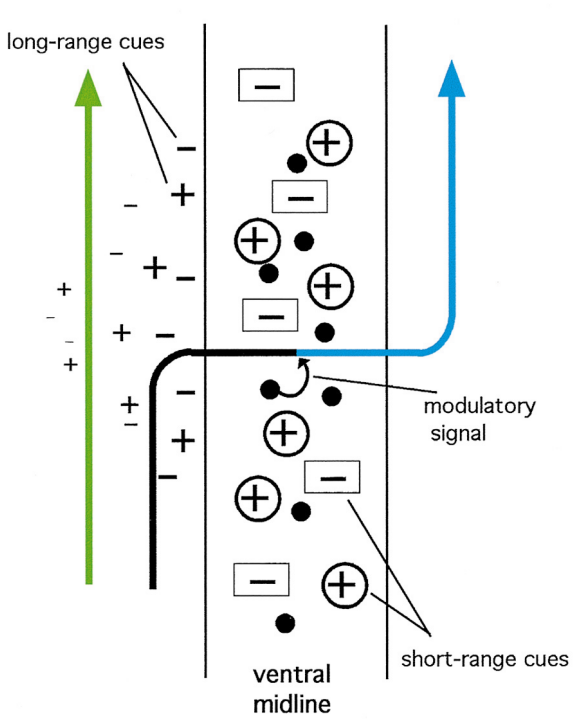

Figure 5. Mechanisms that guide axons at the midline. Axons experience a number of signals at the midline. These signals can be long- or short-range attractants (+) or repellents (−). Ipsilateral (green) or commissural (black/blue) axons dynamically express specific receptors on their surfaces allowing them to react differentially to the same signals presented by the midline (illustrated by the switch from a black to a blue surface). Axons that do cross the midline appear to receive midline signals (black circle) that regulate axonal responsiveness. This regulation may be important to allow these axons to cross and leave the midline. Furthermore, the midline signals might play a role in ensuring the contralaterally projecting axons are able to correctly respond to the longitudinal signals that they had ignored on their own side of the CNS.

ing our understanding of how similar guidance decisions are made at other choice points within the nervous system.

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