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# Commissure formation in the mammalian forebrain

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Commissural formation in the mammalian brain is highly organised and regulated both by the cell-autonomous expression of transcription factors, and by non-cell-autonomous mechanisms including the formation of midline glial structures and their expression of specific axon guidance molecules. These mechanisms channel axons into the correct path and enable the subsequent connection of specific brain areas to their appropriate targets. Several key findings have been made over the past two years, including the discovery of novel mechanisms of action that 'classical' guidance factors such as the Slits, Netrins, and their receptors have in axon guidance. Moreover, novel guidance factors such as members of the Wnt family, and extracellular matrix components such as heparan sulphate proteoglycans, have been shown to be important for mammalian brain commissure formation. Additionally, there have been significant discoveries regarding the role of FGF signalling in the formation of midline glial structures. In this review, we discuss the most recent advances in the field that have contributed to our current understanding of commissural development in the telencephalon.

## Addresses

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## Introduction

The mammalian brain requires neuronal inter-hemispheric connections to coordinate activity between contralateral areas. These long-distance connections in the mammalian forebrain are made by axons that traverse the telencephalic midline, principally in three commissural tracts, the corpus callosum, the hippocampal commissure and the anterior commissure (Figure 1). Formation of these tracts, like that of all major connections in the brain, is tightly regulated by a complex interplay of long-range and short-range guidance cues. Disturbances in guidance can cause brain abnormalities

including defective development (dysgenesis) or complete absence (agenesis) of one or more commissures. Such malformations are likely to result from intrinsic defects in receptor expression and signalling at the growth cone, or from failure to produce an extracellular environment that is suitable for navigation (i.e. gradients of molecular guidance cues and scaffolds of glia and pioneering axons). Recent progress in gene targeting methods, the development of axonal tracers and even more recent advances in high-resolution brain imaging techniques have together revealed the role of certain genes in regulating axon guidance and the formation of commissures. Here, we discuss advances in the field with a focus on findings from the past two years that are helping to elucidate the molecular mechanisms underlying development of the telencephalic commissures.

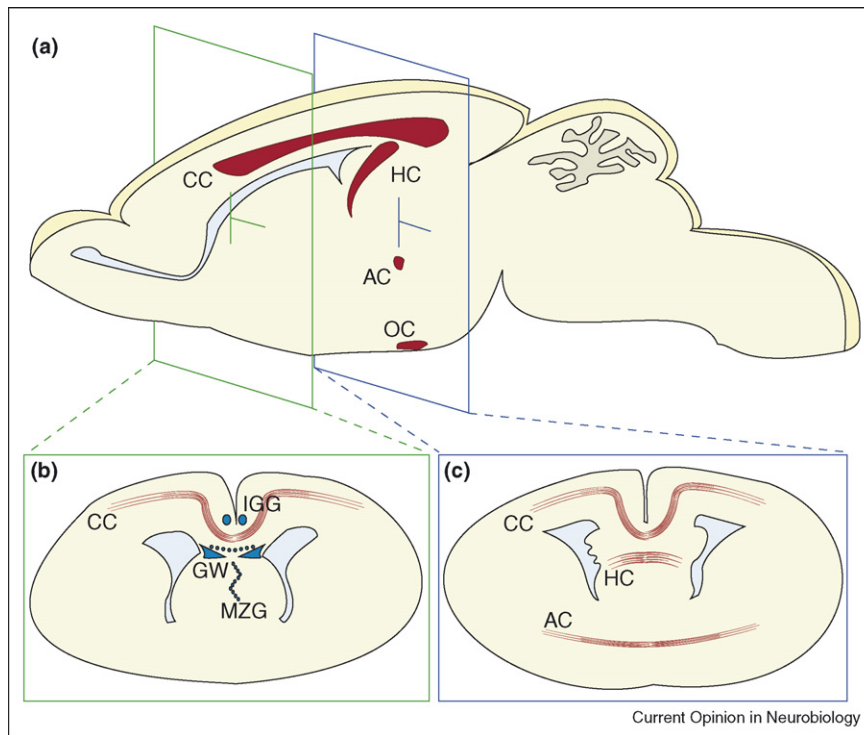
## Midline glial development and commissure formation

### Glial structures in developing nervous systems

During development, strategically located glial structures mature to form cellular boundaries that surround forebrain commissure tracts (Figure 1). These glial structures have key roles in commissural formation, by acting as sources of guidance cues that prevent axons from leaving the tract and entering adjacent structures. Such midline glia have been demonstrated in mice [1–3], in human embryos [4,5], in the *Drosophila* CNS [6,7], and in the zebrafish forebrain [8<sup>••</sup>]. During mammalian development, midline zipper glia located at the telencephalic midline are hypothesised to regulate fusion of the brain hemispheres. Midline zipper glia could therefore facilitate the passage of traversing commissural axons. Other midline structures such as the glial wedge and indusium griseum glia, situated ventral and dorsal to the corpus callosum, respectively (Figure 2), guide callosal fibres by secreting guidance molecules such as the chemorepellant Slit2 [1,9]. Recently, novel genes have been found to be expressed in the glial wedge, along with others already known for their importance in brain development such as Notch, FGFR1, NFIB and EphA4 [10]. The spatial and temporal expression of these genes suggests that they are involved in formation of the corpus callosum; however, further analysis is required to identify their specific functions in callosal development.

During zebrafish development, cells that express glial fibrillary acidic protein (GFAP) span the forebrain midline in positions where axons of the post-optic commissure and the anterior commissure will later cross [8<sup>••</sup>]. These glia provide a substrate for the guidance of

Figure 1



Schematic sagittal (a) and coronal (b,c) depiction of a mouse brain, showing the location of the three principal mammalian forebrain commissures: the corpus callosum (CC), the hippocampal commissure (HC) and the anterior commissure (AC). The optic chiasm (OC) is also illustrated. (b) Also shows the position of midline glial structures (blue) involved in callosal formation: the indusium griseum glia (IGG), the subcallosal sling (represented by the dots below the corpus callosum), the glial wedge (GW) and midline zipper glia (MZG).

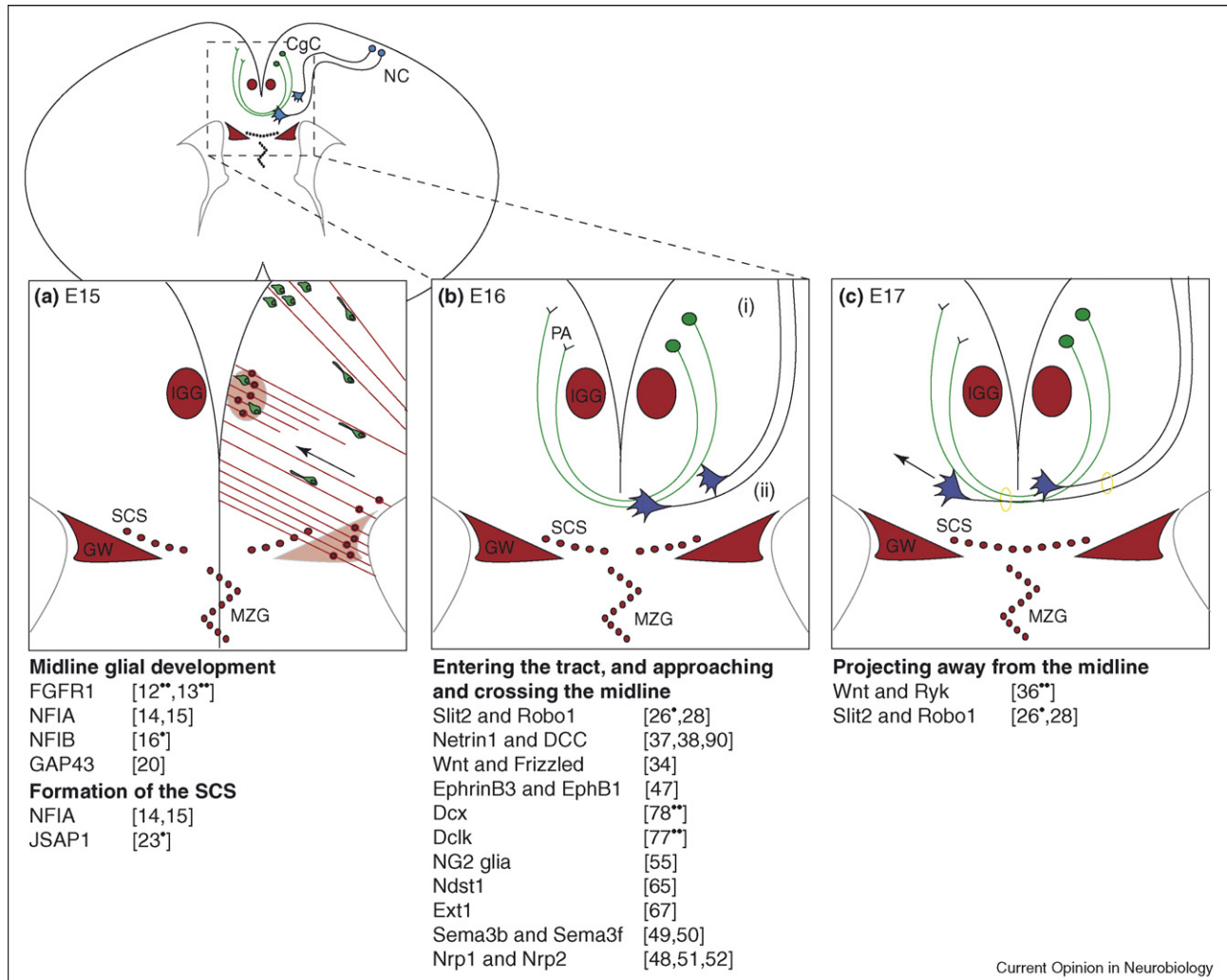
commissural axon growth and are specifically positioned by the expression of Sonic hedgehog (Shh) and Slit proteins [8<sup>••</sup>]. Disrupting Slit signalling causes disorganisation of these midline glia and severe commissural axon guidance defects. In *belladonna* (*bel*) mutant zebrafish, which lack expression of the transcription factor Lhx2, midline glia are highly disorganised [11]. In these mutants, forebrain axons of the post-optic and anterior commissures fail to cross the midline and instead associate with misplaced glia. These mutants also display aberrant expression of the axon guidance molecules Slit2, Netrin1a and Sema3d, but it is not clear whether this is caused by the incorrect positioning of midline glia.

#### FGF signalling in the formation of midline glial structures

Other studies have demonstrated that the disruption of midline glial structures also leads to defects in the development of mammalian forebrain commissures (Table 1). Signalling by fibroblast growth factors (FGFs) is crucial during midline glial development and commissure formation. In two independent studies, brain-specific *FGF receptor 1* (*Fgfr1*) null and glial-specific *Fgfr1* deficient mice lacked major commissural tracts, including the corpus callosum [12<sup>••</sup>,13<sup>••</sup>]. The failure of callosal axons to

turn towards and cross the midline in both these studies was correlated with striking defects in midline glial development. GFAP staining revealed that the *Fgfr1* deletion resulted in a loss of midline structures including the glial wedge, indusium griseum glia and the midline zipper glia. The absence of the indusium griseum glia was due to a disruption in the somal translocation of radial glia from the ventricular zone (VZ) to the subpial region at the midline, where these glia differentiate (Figure 2) [12<sup>••</sup>]. This aberrant formation would be expected to result in a loss or reduction of key guidance molecules, which could account for the failure of commissural axons to cross the midline. However, despite the lack of *Fgfr1*, no deficiencies in expression of *Slit2*, *Slit3*, *Robo1*, *Bone morphogenetic protein 4* (*Bmp4*) or *Growth-associated protein 43* (*GAP43*) were found at the telencephalic midline, and only a slight decrease in *Netrin1* expression was observed [12<sup>••</sup>,13<sup>••</sup>]. Another possible cause of the defect in commissural midline crossing is the displacement of midline glial structures and consequently a displacement of short-range guidance cues. Nevertheless, heterozygous *Fgfr1* mutants have normal midline glial structures but the corpus callosum and the hippocampal commissure fail to cross the midline [13<sup>••</sup>]. This indicates that an FGF dependent mechanism, separate from the gener-

Figure 2



Coronal sections of mouse brain, with the boxed region illustrated at three time points of corpus callosum formation (E15–E17). The molecules described in this review are aligned with the developmental time point at which they are most significant. **(a)** Development of midline glial structures (red) at E15 involves formation of the glial wedge (GW), somal translocation and detachment of radial glial ventricular endfeet to form the indusium griseum (IGG), and development of the subcallosal sling (SCS). Newborn cells (green) migrate along radial glia from the ventricular and subventricular zones to populate the cortex (arrow). **(b)** At E16, pioneering axons extending from neurons in the cingulate cortex (CgC) have crossed the midline and projected into the contralateral hemisphere (i); axons extending from neurons of the neocortex (NC) are guided into the callosal tract and across the telencephalic midline (ii). The growing fibres are prevented from entering surrounding structures by repulsive cues secreted from or expressed by surrounding structures (i.e. the GW, IGG and SCS). **(c)** The cortical axons form tight fascicles (illustrated by yellow rings) with pioneering axons (PA) and with each other. After crossing the midline, the cortical axons project into the contralateral hemisphere.

ation of midline glia, might be required for callosal and hippocampal axons to cross the midline. *Fgfr1* is also expressed by cingulate cortical neurons, implying that FGFR1 is required for the differentiation of callosal pioneering axons [13\*\*], but neuron-specific *Fgfr1* deletion had no effect on the formation of either the corpus callosum or midline glial structures [12\*\*]. Taken together, these results indicate that FGFs are important for the formation of midline glial structures, particularly the indusium griseum glia, although the exact role of FGF signalling in commissure formation remains to be elucidated.

#### Disruption of midline glial structures coincides with commissural defects in the brain

The nuclear factor I (NFI) family of transcription factors also regulates midline glial development. Both *Nfia* and *Nfib* knockout mice display an acallosal phenotype in addition to midline glial defects [14,15,16\*]. Transcription factors of the NFI family bind to the *GFAP* promoter and can thereby regulate *GFAP* expression [17,18]. Moreover, in rat cortical precursor cells, NFI genes regulate *GFAP*-mediated astrocyte differentiation [18]. A reduction in midline *GFAP* expression was observed in

Table 1

Genes involved in mammalian telencephalic commissure formation<sup>a</sup>

Genes knocked out or mutated	Midline glial structure affected			Subcallosal sling affected	Commissure affected			Comment	References
	GW	IGG	MZG		CC	HC	AC		
Diffusible molecules and their receptors									
<i>Slit2</i>	N	N	N	-	Y	N	N		[28]
<i>Robo1</i>	N	N	N	-	Y	Y	N		[26*]
<i>Netrin1</i>	-	-	-	-	Y	Y	Y		[37,38]
<i>DCC</i>	-	-	-	-	Y	Y	Y		[90]
<i>Fzd3</i>	-	-	-	-	Y	Y	Y		[34]
<i>Ryk</i>	N	N	-	-	Y	-	-		[36**]
<i>Wnt</i>	-	-	-	-	-	-	-	Wnt5a is required for Ryk-mediated repulsion <i>in vitro</i>	[36**]
<i>Fgfr1</i>	Y	Y	Y	-	Y	Y	Y	Glial-specific <i>Fgfr1</i> deficiency does not affect the GW or MZG	[12**,13**]
Short-range guidance molecules									
<i>Sema3b and Sema3f</i>	-	-	-	-	-	-	Y		[49,50]
<i>Nrp1</i>	-	-	-	-	Y	Y	-	Defective Semaphorin binding in <i>Nrp1</i> knockin mice	[48]
<i>Nrp2</i>	-	-	-	-	N	N	Y		[51,52]
<i>Celsr3</i>	-	-	-	-	N	N	Y		[53]
<i>Efnb3 (EphrinB3)</i>	?	?	-	-	Y	-	-	Abnormal positioning of glial cells in <i>Efnb3</i> knockout mice	[47]
<i>Ephb1</i>	?	?	-	-	Y	-	-		[47]
Transcription factors									
<i>Nfia</i>	Y	Y	N	Y	Y	Y	Y		[14,15]
<i>Nfib</i>	Y	Y	Y	-	Y	-	-		[16*]
Heparan sulfation enzymes									
<i>Ndst1</i>	-	-	-	-	-	Y	Y		[65]
<i>Ext1</i>	-	-	-	-	Y	Y	Y		[67]
Cytoskeletal elements and intracellular signalling molecules									
<i>GAP43</i>	Y	-	-	-	Y	Y	Y	GW cells failed to express GFAP	[21]
<i>JSAP1 (Mapk8ip3)</i>	N	N	-	Y	Y	N	Y		[23*]
<i>Mtap1b (MAP1B)</i>	-	-	-	-	Y	Y	Y		[45]
<i>Mena</i>	-	-	-	-	Y	Y	N	<i>Mena</i> ;VASP double knockouts have AC defects	[74]
<i>Dcx</i>	-	-	-	-	-	-	-	<i>Dcx</i> <sup>+/-</sup> ; <i>Dclk</i> <sup>+/-</sup> and <i>Dcx</i> <sup>-/-</sup> ; <i>Dclk</i> <sup>-/-</sup> mice have abnormal CC, AC and HC	[78**]
<i>Dclk</i>	-	-	-	-	Y	Y	N		[77**]

<sup>a</sup> This table reviews recent findings and is provided as an update to Table 2 in [83]. Abbreviations: AC, anterior commissure; CC, corpus callosum; GW, glial wedge; HC, hippocampal commissure; IGG, indusium griseum glia; N, no; Y, yes; -, not investigated; ?, midline glial defects noted but not specified.

both *Nfia* and *Nfib* mutants, implying that these genes could be involved in the regulation of both midline glial development and GFAP expression [14,16\*]. Evidence against a role for GFAP in directly regulating glial formation is that *GFAP* knockout mice do not display malformations in glial development or commissure formation [19]. Another molecule that regulates cell cycle exit and the subsequent maturation of radial glia is *GAP43* [20]; *GAP43* is also required for GFAP expression in these cells. Absence of *GAP43* during development results in agenesis of the corpus callosum [21] but *GAP43* null axons respond normally to *Slit2*, indicating that the midline crossing defect is a result of impaired glial development.

An important structure associated with callosal formation is the subcallosal sling (also known as the glial sling). It is positioned just ventral to the corpus callosum and might provide a guidance substratum for extending callosal fibres [2,22]. Sling cells were initially described as glial precursors [2], but many of the cells within the sling are neurons [22]. Initial formation of the sling occurs between embryonic day (E) 15 and E17 in mice, generating a continuous band of cells at the telencephalic midline [2]. In *Nfia* knockout mice however, sling cells fail to form this structure and instead migrate aberrantly into the septum [14]. Another molecule known to be required for proper formation of the sling is the JNK-interacting protein

JSAP1 (also known as Mapk8ip3 or JIP3) [23<sup>\*</sup>]. Importantly, *JSAP1* deficiency leads to a failure of callosal axons to cross the telencephalic midline. The altered configuration of the sling might account for this failure, but how NFIA and JSAP1 function to regulate sling formation remains to be investigated.

### Commissural axon guidance

Long-range diffusible factors have been known for over a decade to have a significant role in guiding nascent axons towards and across the CNS midline. Knockout mice that lack guidance ligands or their receptors, such as Netrins, deleted in colorectal cancer (DCC), Slits and Roundabout (Robo) proteins, have demonstrated the fundamental role that these molecules play during commissural development in both vertebrates and invertebrates. In addition to these ‘classic’ guidance molecules, morphogens such as Shh, FGFs and Wnts also provide cues for axon guidance and regionalisation during neural development (see [24] for a recent review). Further complexity is likely to occur through the interaction of diffusible guidance factors and morphogens with heparan sulfates, chondroitin sulfates and their core glycoproteins. Heparan and chondroitin sulfates could provide differential signalling or localisation of these ligands to the appropriate regions. Moreover, mice that lack enzymes involved in the production of heparan sulfates and chondroitin sulfates display axon guidance errors and commissural defects in several fibre tracts. Short-range axon guidance cues that require cell–cell contact, including members of the Eph and Ephrin families and the Neuropilin, Plexin and Semaphorin families, also have key roles in axon guidance. The functions of these molecules have been well described in other systems, but only recently have they been shown to play a role in forebrain commissure formation.

#### Slits

Slits are chemorepellent ligands expressed at the midline in the invertebrate nerve cord and in the floor plate of the mammalian spinal cord, where they prevent commissural axons from re-crossing the midline after they have already crossed. Robo proteins are upregulated on axons after they have crossed the midline, enabling these axons to respond to Slits and causing repulsion of their growth cones [25]. Recent characterisation of the dynamics of Robo expression in the developing mouse CNS has revealed diverse expression patterns in axons navigating across the midline in the corpus callosum, anterior commissure and hippocampal commissure [26<sup>\*</sup>,27]. In a similar phenotype to *Slit2* knockout mice [28], *Robo1* knockouts display aberrant projections of callosal axons. However, instead of forming typical Probst bundles (longitudinal axon bundles of callosal axons that fail to cross the midline) [29], both callosal and hippocampal axons are misdirected at the midline, overlapping and

forming large intermingled fascicles. *Slit2* knockout mice were not reported to show a similar disruption of the hippocampal commissure [28], but further investigation will be required to establish whether this commissure is completely unaffected. If the *Slit2* knockout mice have a normal hippocampal commissure, it might be that formation of this commissure involves Slit proteins other than Slit2, or that in the absence of Slit2, other molecules at the midline can act in concert with Robo signalling to direct hippocampal axons contralaterally.

A major issue in axon guidance is how axons are guided first to the midline, and then away from it once they have crossed. In the developing mammalian spinal cord, Robo3 (also known as Rig1) modulates responsiveness to Slits at the midline [30,31]. Upregulation of *Robo3* protein in axons before they cross reduces Slit-induced repulsion, and removal of *Robo3* prevents axons from crossing the midline. Indeed, Slit signalling through a Robo–DCC complex reduces the responsiveness of axons to Netrin1 [25]. Furthermore, in *Drosophila*, longitudinal axons (which extend parallel to the nerve cord) are attracted towards the next segment by Netrin that is localised at the midline. As the axons grow past the segmental boundary, Robo allows them to respond to Slit expressed adjacent to the commissure and suppress the attractive effects of Netrin, preventing the axons from crossing the midline [32<sup>\*</sup>]. Such signalling mechanisms might also have a role in the development of brain commissures, although it is important to note the differences in commissure formation between the *Drosophila* nerve cord and the mammalian brain. In contrast to the mammalian spinal cord, in the brain there is evidence that Robo proteins are expressed on commissural axons before, during and after crossing to the contralateral side [9,26<sup>\*</sup>]. Moreover, callosal axons do not grow through the region of Slit expression as in the spinal cord, but rather, Slit proteins are expressed by glial structures adjacent to where callosal axons cross the midline [33]. Callosal axons are therefore likely to be channelled into the correct path using a mechanism distinct from Slit–Robo guidance in the spinal cord.

#### Wnts

In murine morphogenesis, canonical Wnt signalling occurs through the binding of Wnts to the Frizzled receptor. Using diffusion tensor imaging (DTI) in combination with genetically directed neuronal labelling, Frizzled3 (Fz3) was recently found to be important for commissural formation [34]. In the brains of *Fz3* knockout mice, major axon tracts — including the corpus callosum, hippocampal commissure and anterior commissure — were absent or greatly reduced, indicating a requirement for Wnt–Fz3 signalling. The Derailed (Drl) guidance receptor also regulates midline crossing of the *Drosophila* anterior commissure projection through repulsion upon binding to its ligand Wnt5 [35].



There is also a non-canonical Wnt signalling pathway, acting through the vertebrate Wnt receptor Ryk, a Derailed (Drl) orthologue. Ryk has been found to transduce a repulsive axon guidance signal during formation of the corpus callosum [36<sup>••</sup>]. Wnt5a is expressed in a similar pattern to Slits, in the glial wedge and indusium griseum at the callosal midline, and Ryk is present on callosal axons. In *Ryk* knockout mice, callosal axons become defasciculated and are unable to project away from the midline after crossing, suggesting that Ryk is required for repulsion. Furthermore, axons growing from cortical explants are repelled by Wnt5a at E18, but not at E16 or E17. Because many callosal axons have crossed the midline by E18, this result, together with *in vivo* findings, suggest that *Ryk* expression is upregulated after crossing to guide axons away from the midline.

### Netrins

Netrins were first identified as cues that guide commissural axons in the developing mammalian spinal cord, by attracting them at long-range from the dorsal spinal cord towards the ventral the floor plate [37,38]. Callosal axons express the Netrin receptor DCC throughout development [39] but at present there is no direct evidence to suggest that they are guided towards the midline by Netrins, although *Netrin1* knockout mice display agenesis of all forebrain commissural projections [38]. Recent evidence has also demonstrated the presence of a gradient of Netrin protein extending from the spinal cord floor plate [40<sup>•</sup>]. Additional attractive cues can guide commissural axons towards the floor plate along with Netrin. The morphogen Shh, which is expressed in a gradient from the floor plate, regulates commissural axon guidance in the mammalian spinal cord by acting as a chemoattractive agent in collaboration with Netrin1 [41]. Shh also has roles in the guidance of longitudinal axons in the spinal cord and the guidance of retinal ganglion cell axons [42,43].

Netrins have additionally been shown to provide a short-range attractive cue to guide commissural axons across the midline in *Drosophila* [44<sup>••</sup>]. In this study, Netrin was tethered to cell membranes at the midline, and commissure formation proceeded normally, indicating that in this system Netrin does not necessarily act as a long-range guidance cue. In *Drosophila*, Netrin is also relocated to regions just lateral to the CNS midline, where it is bound by axons in the longitudinal tracts that express the Netrin receptor Frazzled [32<sup>•</sup>]. However, whether Netrins can act as short-range cues in the mammalian brain is unknown. Netrin-mediated guidance can be further modulated by the downstream phosphorylation of microtubule-associated protein 1B (MAP1B) [45]. Microtubule-associated proteins are characteristically involved in stabilising microtubules, and *Map1b* knockout mice are deficient in forebrain commissures that include the corpus callosum, anterior commissure and hippocampal commissure. This finding provides additional evidence

that a Netrin signalling pathway is important for axon guidance at the midline.

### Short-range molecules

Ephrins and Eph receptors represent a family of short-range guidance molecules expressed at the midline in the developing mouse forebrain. These molecules provide repulsive cell–cell contact functions and have the ability to initiate bidirectional signalling [46]. Evidence for the requirement of EphrinB3 and EphB1 in formation of the corpus callosum has come from an extensive study of mice deficient in genes that encode multiple Eph receptors and B-class Ephrins [47]. Knockout mice lacking either *Ephb1* or *Efnb3* (the gene encoding EphrinB3) display aberrant callosal projections and the formation of Probst bundles. Furthermore, in *Efnb3* knockout mice, glia that display the radial glial markers RC1 and RC2 are found in the pathway of growing callosal axons [47]. This implies that radial glia affect growing callosal fibres in *Efnb3* mutants by blocking their path, or by failing to guide the axons properly.

Guidance by Semaphorins is crucial for axonal pathfinding during cortical development and is regulated by interactions with their transmembrane receptors, the Neuropilins [48,49]. Although some Semaphorins have the ability to act as soluble ligands, the majority act as short-range chemorepulsive or chemoattractive signals that rely on cell–cell contact. Specifically, interactions of Sema3b and Sema3f with Neuropilin2 (Nrp2; also known as Npn2) are required for proper positioning of the anterior commissure [49,50]. In mice, Nrp2 deficiency leads to severe malformation of the anterior commissure without affecting the corpus callosum or the hippocampal commissure [51,52]. Thus, Nrp2-mediated guidance in response to class 3 Semaphorins can affect specific neuronal populations that contribute axons to the anterior commissure. Additionally, *Nrp1* knockin mutant mice, whose axons are unable to bind Semaphorins, display malformations of the corpus callosum and hippocampal commissure [48]. Sema5A is another Semaphorin that is essential for axon guidance, and will be discussed further in the following section.

In *Drosophila*, the *Celsr* gene is important for the coordinated organisation of cells within an epithelial plane [53]. The mouse orthologues *Celsr1–Celsr3* encode seven-pass transmembrane protocadherins and are expressed in the developing brain [54]. *Celsr3* mutant mice have severe malformations of axon bundles, including lack of the anterior commissure and internal capsule, implicating protocadherins in axon tract formation [53]. However, despite being expressed in the developing cortex and hippocampus [54], *Celsr3* deficiency does not affect formation of the corpus callosum or the hippocampal commissure, suggesting that its role in axon formation is specific to certain tracts.

### Extracellular-matrix molecules and proteoglycans

Commissural fibres are guided not only by chemoattractive and chemorepulsive ligand expression from surrounding structures but also by the growth substrate within the tract. NG2-positive glia, which express the cell surface proteoglycan NG2 and the extracellular matrix (ECM) molecules laminin and fibronectin, are present in the corpus callosum of the developing rats, where they interact with callosal growth cones [55]. NG2-positive glia also promote axonal outgrowth *in vitro* [55]. Axon guidance in *Drosophila* and *Caenorhabditis elegans* is further regulated by heparan sulfate proteoglycans (HSPGs) [56,57]. Mutations of the HSPG Syndecan 1 in these animals led to axon guidance errors that resembled those in mutants of Slit or Robo genes in *Drosophila* and their homologues in *C. elegans*. Moreover, Syndecan 1 affected the distribution of Slit proteins in a way that suggests a role in stabilising diffusible gradients [57]. Sema5A has also been found to interact physically with both HSPGs and chondroitin sulfate proteoglycans (CSPGs) [58]; HSPGs mediated the attractive effects of Sema5A, whereas CSPGs converted this cue to an inhibitory one in regulating the guidance of axons in the fasciculus retroflexus. Indeed, recent evidence has shown that chondroitin sulfate is expressed spatially and temporally in hamsters around the developing hippocampal commissure [59]. Furthermore, these and other ECM molecules that influence rodent axonal guidance are localised to commissural tracts in human foetal brains [4]. These molecules could act by binding diffusible proteins such as Slits and Netrins to provide so-called 'molecular tunnels' for commissural axons to grow through [4]. Alternatively, they could locally modulate responsiveness to these diffusible cues as the growth cone navigates through a specific region.

Heparan sulfates bind multiple growth factors, including Slits [60], and are considered to be co-receptors for numerous ligands. Morphogens such as Shh, FGFs and Wnts interact with heparan sulfate and are important during cortical development [61–64]. Grobe *et al.* [65] suggest that interactions between these molecules and heparan sulfate give rise to the diverse and severe phenotypes observed when heparan sulfate is removed completely. Using mice that lack the sulfotransferase *Ndst1*, which is important for the generation of sulfated ligand-binding sites on heparan sulfate, Grobe *et al.* presented evidence for the involvement of heparan sulfate in mammalian brain commissure formation. *Ndst1* knockouts have significant brain commissural defects including an absent or hypoplastic anterior and hippocampal commissures, with axons dispersing on the ipsilateral side instead of crossing the midline [65]. A less severe phenotype was observed in mice that lack the heparan sulfotransferase enzyme *Hs6st1*, which controls the responsiveness of retinal ganglion cell growth cones to Slits *in vitro* [66]. *Hs6st1* knockout mice display axon guidance errors at the

optic chiasm but have normal corpus callosum and anterior commissure projections. Complete removal of heparan sulfate by knockout of *Ext1* results in the absence of the corpus callosum, anterior commissure and hippocampal commissure [67]. *Ext1* knockouts are similar in this respect to the *Ndst1* mutants: both phenotypes are severe and the mice possess significant craniofacial and cerebral defects, including a lack of olfactory bulbs. *Ext1*<sup>+/-</sup> and *Slit2*<sup>-/-</sup> single mutant mice exhibit normal optic chiasm projections, but when they are crossed together the resulting *Slit2*<sup>-/-</sup>; *Ext1*<sup>+/-</sup> double mutants possess significant guidance errors at the optic chiasm, indicating a molecular or genetic interaction between these two genes. Given its ubiquitous expression and seemingly fundamental role in many facets of development, it is unlikely that broad knockout studies will determine the exact role of heparan sulfate in axon guidance. However, different modifications of sugar residues on heparan sulfate might provide specificity for certain ligands during axon guidance, using a heparan sulfate 'sugar code' [68], and directed mutation of the genes responsible for these modifications could provide useful insight. Indeed, different heparan sulfate sulfotransferase genes are differentially expressed in regions of the mouse brain [69]. Thus, identifying the modifying enzyme or the particular heparan sulfate chain responsible might help to characterise further the role of heparan sulfate in axonal guidance.

### Cytoskeletal elements

Modulation of the cytoskeleton is important for both neuronal migration and axon extension. Microtubules form long rigid structures that extend along the axon shaft to stabilise the axon and enable intracellular transport from the soma to the growth cone. Correct extension of microtubules is therefore important for axon growth. Actin filaments are also abundant in the navigating growth cone and are essential for the correct formation of both filopodia and lamellipodia. Signalling responses to extracellular cues enable the reorganisation of actin filaments within the growth cone and thereby control navigation through the substrate. Enabled (*Ena*) and Vasodilator-stimulated protein (*VASP*) bind to F-actin, preventing capping and promoting the formation of long filaments at filopodial tips [70]. *Ena* and *VASP* proteins and their homologues also influence *Drosophila* and *C. elegans* axon guidance downstream of guidance receptors, including Robo receptors [71] and DCC [72]. Additionally, *Mouse enabled* (*Mena*; also known as *Enah*) knockout mice display Probst bundles of the corpus callosum and aberrant formation of the hippocampal commissure [73]. *VASP* single knockout mice do not show any commissural malformations [74], but *Mena*;*VASP* double knockouts have a more severe neurological phenotype than single *Mena* mutants, with complete absence of the corpus callosum, hippocampal commissure and anterior commissure, the severity of the phenotype depending on gene dosage

[74]. These data suggest a cooperative role for Mena and VASP in commissure formation.

Doublecortin (Dcx) and the highly homologous doublecortin- and calcium/calmodulin-dependent protein kinase-like 1 (Dclk; also known as Dcamk1) are microtubule-associated proteins. Their roles in development have been debated because of phenotypic differences between mice and humans: *DCX* mutations in humans result in inadequate neocortical migration with severe disruption to cortical cytoarchitecture [75], whereas no such defect has been observed in *Dcx* knockout mice [76]. *Dclk* single mutants mice display agenesis of the corpus callosum and the formation of Probst bundles [77<sup>••</sup>]. However, cortical neuron migration and the structure of the radial glial scaffold, indusium griseum glia and the glial wedge seem to be normal in the *Dclk* mutants. Therefore, *Dclk* alone might be required for axonal initiation or elongation rather than for regulation of extrinsic axonal guidance signals. Moreover, *Dcx;Dclk* double knockout mice have severe malformations of all three major commissures, suggesting a functional redundancy between Dcx and Dclk [78<sup>••</sup>].

### Targeting of callosal axons in the contralateral cortex

After commissural axons cross the midline and project into the contralateral cortex, they must identify specific target areas in which to terminate and form synapses with neurons in the appropriate layer. The mechanisms underlying this formation have been studied best in the thalamocortical system. Afferent thalamocortical projections grow into the cortical plate through the intermediate zone, where they then penetrate the deep layers and terminate in layer 4 (reviewed in [79]). Inter-hemispheric neural projections are generally organised in a homotopic fashion, and thus commissural axons terminate in the contralateral hemisphere according to the cortical region from which they originate. An electron microscopic study [80] of callosal fibres in the neonatal hamster has demonstrated that radial glia might serve as a guidance substrate for such projections; however, the molecular mechanisms that underlie targeting of callosal axons to a specific layer are unknown. Similarly, no specific genes have been identified as defining neocortical regionalisation for callosal projections. However, several studies have revealed gradients of molecules, such as Pax6, Emx2, FGF8, Eph receptors and Ephrins, that might provide regional cues for afferent connections (reviewed in [81,82]). These molecules are thought to regulate the guidance of axons to their appropriate cortical area. It is also possible that molecules known to be involved in guiding callosal axons across the midline also provide guidance for commissural axon targeting in the contralateral hemisphere. However, this is difficult to investigate using mutant mice because in many cases callosal axons fail to cross the midline, and thus the role of these molecules in axon targeting in the opposite

hemisphere cannot be investigated [83]. Further research is needed using area-specific or temporally regulated knockdown of genes to define these mechanisms.

In addition to the molecular guidance mechanisms that specify neural circuits, electrical activity is important during the development of synaptic connectivity (reviewed by [84]). Thus, guidance cues might provide an initial crude targeting mechanism (i.e. in finding the proper cortical area), followed by activity-dependent mechanisms to refine these connections. In the cat visual cortex, callosal connections initially form an exuberant projection that is later pruned back to produce the adult pattern of connections [85]. The primary visual areas within the cortex, Brodmann areas 17 and 18, are subject to prominent developmental exuberance of callosal axons. Later in development, only those axons targeting the border region of areas 17 and 18 are retained into adulthood. Studies using eyeless or neonatally enucleated mice, where electrical input to the visual cortex is absent, show that although many aspects of callosal connectivity are maintained, the refinement of connections is lost [86]. These results suggest that activity from the retina is required to sculpt the callosal projections to the contralateral visual cortex. Such refinement of connections has been suggested to occur in callosal projections to other areas of cortex, where transient projections are formed and reach their target region but never enter the cortex [87].

Neuronal activity has also been found to modulate guidance of axons in response to 'classical' diffusible molecular cues [88]. Electrical stimulation of *Xenopus* spinal neurons enhances their attraction to Netrin1, and converts myelin-associated glycoprotein (MAG)-mediated repulsion into an attractive response. More recently, spontaneous electrical activity has been found to have a role in the dorsoventral pathfinding of chick spinal motoneurons during development [89]. In this study, blocking the spontaneous rhythmic bursts of electrical activity in these neurons by *in ovo* drug application resulted in fasciculation defects and axon pathfinding errors. Furthermore, expression of EphA4 and polysialylated neural cell adhesion molecule (NCAM) was altered on these axons as a result of blocking electrical activity, further supporting its role in axon guidance. It is not yet known whether these mechanisms have a role in commissural formation, but it remains an important area in cortical development that deserves further attention.

### Conclusions

A growing list of molecules has now been implicated in commissural development. These molecules regulate all aspects of axonal growth and guidance, including the formation of permissive and repulsive substrates, growth cone dynamics, and structures required by commissural fibres to choose the correct path of growth. The precise mechanism by which midline glial structures form and to what extent



commissural axons directly interact with these structures is an important aspect of brain development that requires further exploration. In addition, growth cone responses to guidance molecules are likely to be far more complex than is currently understood. A clear example of this can be seen from recent discoveries of the involvement of spontaneous electrical activity during development [88,89]. Our challenge now is to incorporate the new wealth of data that relate to molecular pathways and guidance mechanisms to formulate new models of how all commissures develop in the brain. It is important to identify and understand similarities and differences between the molecular regulation of commissure formation in the mammalian brain and that in other systems. There are both similarities and differences among the genes that regulate the formation of the corpus callosum, hippocampal commissure and anterior commissure, and the common mechanisms must be characterised. Experimental insight in the field is progressing rapidly, making this an exciting time for the exploration of commissure formation in the mammalian brain.

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