Multiple B-Class Ephrins and EPH Receptors Regulate Midline Axon Guidance in the Developing Mouse Forebrain

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MULTIPLE B-CLASS EPHRINS AND EPH RECEPTORS REGULATE MIDLINE AXON GUIDANCE IN THE DEVELOPING MOUSE FOREBRAIN

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Ephrins and Eph receptors have been implicated in a number of developmental processes including axon growth and guidance. One important guidepost is the central nervous system midline, where ephrins and Eph receptors have been implicated. At the embryonic midline, axons either cross into the contralateral central nervous system (CNS) targeting appropriate partners on the opposite side or remain ipsilateral extending either rostrally or caudally. In these studies, we examine a major forebrain commissure called the corpus callosum (CC). Agenesis of the CC is a rare birth defect that occurs in isolated conditions and in combination with other developmental cerebral abnormalities. Recent identification of families of growth and guidance molecules has generated interest in the mechanisms that regulate callosal growth. One family, ephrins and Eph receptors, has been implicated in mediating midline pathfinding decisions; however, the complexity of these interactions has yet to be unraveled. This dissertation sheds light on which B-class ephrins and Eph receptors function to regulate CC midline growth, and how these molecules interact with important guideposts during development. We also show that multiple Eph receptors (B1, B2, B3, and A4) and B-class ephrins (B1, B2, and B3) are present and function in developing forebrain callosal fibers based on both spatial and temporal expression patterns and analysis of gene-targeted knockout mice. Defects are most pronounced in the combination double knockout mice, suggesting that
compensatory mechanisms exist for several of these family members. Furthermore, these CC defects range from mild hypoplasia to complete agenesis and Probst’s bundle formation. Further analysis of the ephrinB3 gene revealed that Probst’s bundle formation may reflect aberrant glial formations which alter the normal architecture of midline glia resulting in one potential mechanism of this abnormal phenotype. Another potential mechanism we discovered is a role for EphB1 receptor in the altered sensitivity of CC axons to midline guidance cues. Removal of this receptor resulted in cortical axons responding to GW guidepost cells with increased sensitivity. Our results support a significant role for ephrins and Eph receptors in CC development and may provide insight to possible mechanisms involved in axon midline crossing as well how failed molecular and genetic mechanisms may contribute to human CC disorders. Lastly, we show that one fiber tract that remains ipsilateral in the forebrain may use distinct midline guideposts to regulate proper growth and guidance. These findings implicate additional ephrins and Eph receptors in CC midline guidance than previously known and reveal novel mechanisms in mice, which may be pertinent to human disease states that result in agenesis of the CC.
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Abbreviations

CNS: central nervous system
CC: Corpus callosum
e: embryonic
PD: postnatal day
pAC: Posterior limb of anterior commissure
aAC: Anterior limb of anterior commissure
Pfp: Perforating pathway
DCC: Deleted in colorectal cancer
Robo: Roundabout
GS: ‘glial’ sling
GW: Glial wedge
Ig: Indusium griseum
Mzg: Midline zipper glia
GS: glial sling
ACC: Agenesis of the corpus callosum
β-gal: Beta-galactosidase
lacZ: β-galactosidase
CST: Corticospinal tract
Ctx: cortex
KO: knockout
WT: wild-type
GFAP: glial fibrillary acidic protein
Publication Note

The work reported in parts of Chapter II, III, and IV were done in collaboration with Daniel J. Liebl and Mark Henkemeyer, and published as:

Chapter I
Introduction

Early embryonic development

The central nervous system (CNS), which consists of structures called the brain and spinal cord, is a complex cellular organization. The CNS is involved in motor functions as well as complex sensory functions such as gustation, vision, audition, and olfaction (Purves et al., 2000). These CNS functions are primarily mediated by cell types called neurons that communicate motor and sensory information between each other through long processes called axons. Neurons and their axons began to be well-described in the late 19th century by pioneering scientists such as Santiago Ramon y Cajal. Through innovation and optimization of existing histochemical techniques, Ramon y Cajal and other scientists meticulously examined and documented CNS components, which included neurons and their connective axonal structures (Ramon y Cajal, 1888).

Since the first observations of neurons and the intricate axonal networks that connect these cells, a fundamental question that has captured the attention of scientists for decades is how neurons extend their axons during development to produce a consistent and reproducible neural network?

To begin investing neurons and the establishment of their connections, it is important to understand the stages of brain and spinal cord development. Early in vertebrate embryonic development, the CNS develops from the cells within the outer ectoderm layer called the neural plate, which later begins to fold inward until the edges fuse. The internal cavity of this tube becomes the ventricular region with the surrounding cellular region, called the neuroepithelium, providing progenitor cells. At similar
developmental time points a midline begins to form that separates the left and right CNS. The neural tube differentiates and future CNS regional specializations begin to develop, which include the prosencephalon (forebrain), mesencephalon (midbrain), and rhombencephalon (hindbrain). The rhombencephalon continues to differentiate eventually resulting in the formation of the metencephalon (pons and cerebellum) and myelencephalon (medulla) with the remaining portion of the neural tube forming the spinal cord (Hatten et al., 1999; Richards et al., 2004). Following regionalization of the neural tube, cells within the neuroepithelium continue to proliferate on either side of the prosencephalon forming bulges that will become the two telencephalic hemispheres or forebrain. In the developing telencephalic hemispheres, some cells within the ventricular region become postmitotic, develop into neuroblasts, and begin a radial migration pathway forming cortical layers (I-VI). Upon reaching the final destination within the cortex, the neurons will then communicate with cellular partners in various CNS regions via axon connections (Hatten et al., 1999).

**Overview of axon growth and guidance families**

Developing neurons can project their axons over a significant distance with remarkable accuracy (Tessier-Lavigne and Goodman, 1996). This is accomplished by the response of a specialized structure within the axon called a growth cone that responds to a variety of coordinated repulsive and attractive cues at specific guideposts throughout the growth pathway (Huber et al., 2003; Dickson et al., 2002). These growth and guidance cues may be either membrane bound initiating signals upon direct contact (e.g. ephrins and Eph receptors) or diffusible (e.g. netrins and slits) capable of initiating signals through established protein gradients in the extracellular matrix (ECM) (Dickson,
One important guidepost for commissural axons is the CNS midline, where several families of guidance molecules function to attract or restrict midline crossing (Bagri et al., 2002; Kaprielian et al., 2001; Serafini et al., 1996; Henkemeyer et al., 1996). Midline crossing occurs in both rostral (e.g. forebrain) and caudal (e.g. spinal cord) CNS regions where sensory information is transferred from one side of the body (ipsilateral) to the other (contralateral) through axon connections (Imondi et al., 2001; Richards et al., 2002). Together these guidance factors expressed in the ECM instruct the growth cone at various points (i.e. choice points) with each successive step leading to appropriate target selection.

Axon growth and guidance families include, in part, molecules such as netrins, slits, ephrins and Eph receptors, receptor-like protein tyrosine phosphatases (RPTPs), and semaphorins. These molecules have been found to direct axon growth and guidance through either repulsive or attractive mechanisms. The netrins function as diffusible chemoattractants to axons (Serafini et al., 1994; Kennedy et al., 1994). These molecules interact with both deleted in colorectal cancer (DCC) and Neogenin receptors initiating intracellular signals that involve the small GTPases Rac1 and Cdc42, which aid in reorganization of the actin cytoskeleton thereby promoting neurite outgrowth (Shekarabi et al., 2005). Ephrins and erythropoietin producing hepatocellular (Eph) receptors, the largest class of receptor tyrosine kinases, function in contact-mediated repulsion and adhesion. These molecules have been found to play roles in axon growth and guidance in regions such as the hippocampus, thalamocortical system, central pattern generator, and retinotectal system (Murai et al., 2003; Grunwald et al., 2004; Bolz et al., 2004; Kullander et al., 2003; McLaughlin et al., 2005). Tyrosine phosphorylation used by
many guidance receptors must be tightly regulated during development (Johnson et al., 2003). Receptor-like protein tyrosine phosphatases (RPTPs) are molecules that regulate tyrosine phosphorylation and genetic screens in Drosophila have implicated the Type II-V RPTPs in axon guidance (Johnson et al., 2003). In the chick embryo, ribonucleotide (RNA) interference experiments focusing on members of the typeII and typeIII subfamilies have shown that downregulation of RPTP translation results in fasciculation errors of the dorsal motor nerve in the limb bud (Stepanek et al., 2005). Semaphorins, divided into eight subclasses based on sequence structure, function as both secreted and membrane bound guidance factors (Pasterkamp et al., 2003). The semaphorin subclasses 3-7 are found in vertebrates and function in both axon attraction and repulsion (Dickson, 2002). The subclass 3A semaphorins are mostly known for their role in axon repulsion and experiments focusing on this subclass have elucidated the critical receptor complexes involved in signaling, which include plexins and neuropilins (Tamagnone et al., 1999). Neuropilin receptors directly bind with semaphorins and are incapable of transmitting signals, while plexins act as obligate co-receptors in a complex with neuropilins. This receptor complex initiates signals involving molecules such the GTPases Rac and Rho, which regulate the actin cytoskeleton, either inducing actin polymerization or depolymerization, respectively (Liu et al., 2001). Deletion of the receptors for semaphorins 3A and 3F results in defects in the targeting of dentate granule cell axons to the cornu ammonis (CA3) region of the mouse hippocampus (Cheng et al., 2001).

Cell adhesion molecules (CAMs) are a large class of proteins and these include subfamilies such as cadherins, Ig superfamily CAMs (L1 and neural cell adhesion molecule (NCAM)), and integrins. The cadherin subfamily consists of more than 80
cadherins and cadherin-like molecules, but the most well-characterized are the classical cadherins, which include members such as endothelial (E)- and neuronal (N)-cadherin (Ranscht et al., 2000). These transmembrane molecules, which consist of a highly conserved intracellular domain and five extracellular cadherin domains, interact through \( \text{Ca}^{+2} \) dependent homophilic binding to initiates intracellular signal cascades involving \( \alpha \) and \( \beta \)-catenin (Hirano et al., 1992; Goda, 2002). In vitro experiments using cerebellar neurons have revealed that this molecule stimulates neurite outgrowth when presented as a substrate (Williams et al., 1994). L1 and NCAM are CAMs that have been studied for their developmental role during patterning of the CNS (Crossin et al., 2002). Other Ig superfamily members have also been examined for their role in axon growth and guidance and include contactin, DCC receptors, and Tag-1 (Cunningham, 1995). Contactin and Tag-1 are glycosylphosphatidylinositol (GPI)-linked to the membrane, while DCC receptors are linked via a transmembrane domain. Binding of these molecules with their respective ligands regulates events such as axon outgrowth, adhesion and fasciculation. The ligands for contactin and Tag-1 include extracellular matrix proteins such as tenascin-R and tenascin-C, and phosphacan, while those for DCC receptors include chemoattractant netrin-1 (Povlsen et al., 2003). The structure of L1 consists of six-Ig like domains, five fibronectin repeats, and a highly conserved intracellular domain with the extracellular domain of the molecule carrying out homophilic binding interactions as well as heterophilic interactions with molecules such as neurocan and integrin (Crossin et al, 2002; Oleszewski et al., 2000; Silletti et al., 2000). L1 has also been shown to be a positive regulator of neurite outgrowth in vitro (Lagenaur and Lemmon et al., 1987). Furthermore, L1 knockout mice exhibit abnormal
targeting of retinal axons in the superior colliculus (SC) supporting a role for this molecule in vivo (Demyanenko et al., 2003). NCAM, which consists of 5 Ig-like domains and 2 fibronectin repeats, carries out similar homophilic binding interactions and contributes to axon adhesion (Johnson et al., 2004). NCAM, which includes three isoforms that result from alternative splicing, is capable of engaging in both homophilic and heterophilic interactions. Homophilic interactions involve binding between NCAM molecules on two adjacent cells, while heterophilic ligands of NCAM include L1 and the fibroblast growth factor (FGF) receptor (Ranheim et al., 1996). Recent investigations have also shown direct interactions between NCAM and FGF receptors expressed in the same cell, and suggest that FGF signaling may be involved in NCAM mediated neurite outgrowth (Williams et al., 1994). In vitro experiments using cerebellar neurons have revealed that NCAM promotes neurite outgrowth when presented as a growth substrate (Williams et al., 1994). Other heterophilic ligands for this molecule include the ECM chondroitin sulfate proteoglycans (CSPG) and heparan sulfate proteoglycans (HSPG) and interactions between NCAM and these molecules result in cell to cell adhesion (Kiryushko et al., 2004). CSPG and HSPG are membrane bound or soluble heteropolysaccharides that are often localized to regions which axons avoid suggesting that these molecules play a role in axon repulsion (Anderson et al, 1998; Yamada et al., 1997). Lastly, integrins bind ECM molecules such as laminin and fibronectin leading to effects such as cell adhesion and axon extension, two critical events in the targeting of axons to appropriate regions in the developing CNS (Cheresh et al., 1994). There are over 20 different integrin receptors with each receptor consisting of different combinations of a α and β-subunit (Miao et al., 2000).
These growth and guidance cues are a sampling of molecules that play a role in neuron development. To complicate the scenario, these guidance receptors also interact with each other either directly (i.e. receptor-receptor) or indirectly (i.e. signaling crosstalk) allowing for integration of signals at the growth cone (Guan et al., 2003). For example, neuropilin-1 receptors interact with L1 and this is critical for the growth cone collapsing effect of semaphorin3A (Castellani et al., 2004; Castellani et al., 2002). L1 mutations inhibit this interaction which results in the blocking of semaphorin3A repulsion. In addition, genetic evidence from studies in *Drosophila* suggests that integrins may regulate the responsiveness of axons to repellant slits (Stevens et al., 2002). Specifically, this study showed that when integrin gene levels are reduced in mutants there is an increase in the probability of midline guidance errors when slit gene levels are simultaneously reduced. Individual axons are subjected to multiple guidance cues at similar developmental time points. These examples begin to reveal that despite numerous guidance families and complex receptor interactions, growth cones are able to integrate guidance signals and continue to orchestrate proper axon connections.

**Netrin family**

The netrins represent a major family of diffusible midline attractant cues for developing axons (Livesey et al., 1997; De la Torre et al., 1997). In vertebrates, netrin-1 and netrin-2 are expressed by specific guideposts (regions which express guidance molecules and instruct axon targeting) and interact with developing axons that express the receptors Deleted in Colorectal Cancer (DCC), Neogenin, and Unc-5 (Gad et al., 2000; Gitai et al., 2003; Dickson et al., 2002; Livesey et al., 1997; Keino-Masu et al., 1996). The embryonic spinal cord midline of both chicken and mouse embryos have
served as widely used experimental systems for studying midline axon guidance (Kaprielian, 2000). In fact, one of the first developmental events examined that involve binding of netrin-1 with DCC was the attraction of commissural axons to the floorplate at the midline of the embryonic spinal cord (Kennedy et al., 1994). In addition to axon guidance at the spinal cord midline, netrins have also been shown to attract axons in forebrain regions such as the internal capsule and ganglionic eminence (Metin et al., 1997; Richards et al., 1997). Interestingly, the netrins and their receptors have also been implicated in the development of major commissures such as the corpus callosum (CC), which connects neuronal populations in the two cerebral hemispheres, and anterior commissure (AC), a tract that allows communication between the left and right temporal cortices.

Netrin-1 mRNA transcripts are present at appropriate time points and in specific midline guidepost regions to potentially play a role in the formation of the CC and AC (Richards et al., 2002; Serafini et al., 1996). DCC receptors are expressed in the axons that make up the CC, AC, and hippocampal commissure (Shu et al., 2000). The importance of netrin-1 and DCC expression is supported by functional data from mutant animals. In netrin-1 or DCC deficient mice, the CC fails to develop and instead axons stall at the midline forming whorls or Probst’s bundles, which are characteristic abnormal CC phenotypes in certain mutant mice (Richards et al., 2000). These data support a role for netrin-DCC interactions in the growth and guidance of callosal axons, though whether netrin-1 molecules serve as attractive cues for DCC-expressing callosal axons remains to be investigated (Serafini, 1996; Fazeli, 1997).
**Slit family**

The slits are the antagonists to netrins and these molecules function instead to repel axons at the midline of both the spinal cord and forebrain (Kidd et al., 1999; Kennedy et al., 1994; Serafini et al., 1996; Shu et al., 2003b). In vertebrates, three distinct slit genes (slit-1, slit-2, and slit-3) and three roundabout (Robo) receptor genes (Robo-1, Robo-2 and Robo-3/Rig-1) are expressed in the CNS. Robo-1 and Robo-2 receptors bind all three slit ligands while it remains unknown whether Robo-3/Rig-1 binds these ligands (Sabatier et al, 2004). Robo receptors mediate repulsion through interactions with intracellular signaling proteins such as the Rho family of GTPases. These Rho GTPase family members include RhoA, Rac1 and Cdc42, which regulate the actin cytoskeleton that lies beneath the growth cone plasma membrane (Nguyen Ba-Charvet et al., 1999; Guthrie et al., 2004; Patel et al., 2002). *In vitro* and *in vivo*, the full length 200kD slit-2 ECM proteins are spliced into 140kD N-terminal and 55-60kD C-terminal cleavage fragments that bind Robo receptors (Brose et al., 1999). Experiments focusing on slit-2 have shown that these cleavage fragments have different cell association characteristics with the 140kD N-terminal fragment remaining membrane bound compared to the diffusible 55-60kD C-terminal fragment. *In vitro* studies using olfactory and retinal axons have shown that full length slit-2 and the 140kD cleavage fragment both mediate axon repulsion, while the 55-60kD cleavage fragment did not result in repulsion (Nguyen Ba-Charvet et al., 2001; Erskine et al., 2000). It remains unknown whether other slit proteins (i.e. slit-1, slit-3) are cleaved in a similar manner, or whether full length and cleaved slit proteins need to be present at the same time to mediate growth cone collapse. Investigations using *Drosophila* initially characterized the...
Robo receptors as ligands for the slit repellent guidance cues (Kidd et al., 1998; Kidd et al., 1999). These studies revealed that Robo-1 and Robo-2 receptors binding with their slit ligands regulated the crossing of commissural axons because they prevented the re-entering of the ipsilateral CNS after crossing the midline. Deletion of any of these genes resulted in aberrant re-crossing of the midline. Recent evidence suggests that the function of this guidance family may be regulated through interactions with intracellular sorting proteins. For example, in *Drosophila*, the repulsive activity of Robo receptors is regulated by interactions with the sorting receptor commissureless (Comm), which functions to regulate both Robo-1 and Robo-2 receptor membrane expression prior to crossing the midline. Comm recognizes specific lumenal and/or transmembrane domains in receptors and directs delivery of Robo receptors from the trans-Golgi network to late endosomes for recycling (Keleman et al., 2005; Keleman, et al., 2002; Myat et al., 2002). After crossing the midline, Comm protein expression is downregulated while Robo-1 and Robo-2 expression is upregulated and repulsive activity is re-established.

Using mouse models, recent studies have determined Robo-3/Rig-1 receptors may have functions during axon guidance that are separate from axon repulsion. This is based on the expression of Robo-3/Rig-1 receptors on axons before crossing the midline and downregulation of the receptor after crossing the midline, which is completely opposite from the expression patterns of Robo-1 and Robo-2 (Sabatier et al, 2004). In addition, functional data from Robo-3/Rig-1 knockout mice indicate that pre-crossing commissural axons prematurely respond to slit-2 ligands. These investigators showed that Robo-3/Rig-1 receptors function to inhibit the ability of pre-crossing commissural axons to sense slit repellents allowing axons to successfully cross the midline. Analysis of Robo-
3/Rig-1 knockout mice further supported a role for this receptor in regulating the repulsive mediators Robo-1 and Robo-2 because commissural axons failed to cross the floorplate. The exact mechanism of how Robo-3/Rig-1 regulates the ability of Robo-1 and Robo-2 receptors to sense slit guidance cues remains unknown, but it is clear that there are differences in the function of each Robo receptor. Robo-1 and Robo-2 are necessary to prevent commissural axons from re-crossing once they have entered the contralateral CNS, while Robo-3/Rig-1 receptors regulate the repellent activity of these receptors prior to midline axon crossing. Separate experiments have revealed that slit-1/slit-2 double knockout mice show no abnormalities in spinal cord midline crossing. However, triple mutant mice for slit-1, slit-2, and slit-3 did have defects in midline crossing suggesting that all three ligands compensate for one another during midline crossing (Long et al, 2004). In addition, this study also revealed a distinct function for Robo-1 receptors in regulating midline crossing, while Robo-2 receptors regulated extension and positioning of axons away from the midline once they have crossed and continue their extension towards rostral targets. In *Xenopus*, *in vitro* experiments have revealed that another mechanism used by commissural axons involves a direct interaction between Robo-1 and DCC receptors (Stein and Tessier-Lavigne, 2001). Through analysis of spinal cord midline axon guidance, it is apparent that as axons cross the midline there is a switching of sensitivity from netrin to slit responsiveness. Using an *in vitro* assay in which the turning angle of growth cones in response to gradients of guidance cues can be quantitated, investigators showed that slit-2 inhibits or silences netrin-1 mediated axon attraction suggesting that there is a hierarchical arrangement of these two guidance families with Robo regulating DCC receptor signaling. Further
examination revealed that the silencing activity of slit-2 was due to a direct interaction between the cytoplasmic domains of the Robo-1 and DCC receptor. The model established from these experiments suggests that netrin mediated attraction is silenced once axons reach the floorplate due to activation of Robo receptors by slit. This dynamic interplay between attractive and repulsive factors may permit commissural axons to traverse the CNS midline in a stereotypical manner. However, similar mechanisms involving a direct interaction between DCC and Robo receptors and a switching of sensitivity between guidance cues in vertebrates have yet to be determined.

In the developing CC, the expression of slits and their receptors at critical developmental time points and gene targeted knockout mice which exhibit midline axon crossing defects suggests an important function for this growth and guidance family. First, slit-2 mRNA has been detected in critical CC midline guideposts (Shu et al., 2001a; Shu et al., 2003b; Bagri et al., 2002). Second, expression of Robo receptor mRNA has been detected in the neocortex during callosal neuron developmental time points, but more importantly Robo-1 and Robo-2 receptor proteins are expressed in callosal axons (Sundaresan et al., 2004; Shu et al., 2003b). Functional data from mutant animals indicate that the expression of these molecules is important for CC development. In slit-2 deficient mice, callosal fibers fail to extend appropriately across the midline with axons aberrantly projecting through known repulsive guideposts into ventral regions of the forebrain (Bagri et al., 2002). In a set of elegant experiments, Richards and colleagues (2003) demonstrated that unlike the situation in the spinal cord, in the forebrain slit-2 repels axons before and after crossing the midline (Shu et al., 2003b). Using injection of anti-sense oligonucleotides in utero to downregulate slit-2 expression, the investigators
revealed that downregulation of slit-2 resulted in defasciculation of axons with both pre-
crossing and post-crossing axons aberrantly entering the septum. Together, these data
show that slit mediated repulsion is important in both rostral and caudal midline regions,
while the function of slits before and after midline axon crossing differs depending on the
population of axons examined.

**Ephrins and Eph receptors**

Recently, membrane bound guidance cues such as ephrins and Eph receptors have
also been implicated in axon growth and guidance (Nakagawa et al., 2000; Kullander et
al., 2001; Yokoyama et al., 2001; Kaprielian et al., 2001). Eph receptors are receptor
tyrosine kinases that function together with their ephrin ligands in a number of vertebrate
developmental processes; including axon growth and guidance, neuronal migration, and
synaptogenesis (Wilkinson, 2001; Kullander, 2002). These functions are achieved
through cell-cell contact since both receptor and ligand are membrane-bound molecules
(O'Leary et al., 1999; Davis et al., 1994; Zimmer et al., 2003). Eph receptors are the
largest group of receptor tyrosine kinases (RTK). They contain an extracellular N-
terminal domain, cysteine-rich EGF-like domain, and two fibronectin type III motifs
(Poliakov et al., 2004). Ephrins are divided into two subtypes, the A-class and B-class,
which differ in the way they are anchored to the plasma membrane (Polizakov et al.,
2004). In vertebrates, there are 9 EphA (A1-A9) receptors while in the B-class there are
6 EphB (B1-B6) receptors, which interact with 6 ephrinA (A1-A6) and 3 ephrinB ligands
(B1-B3), respectively (Holder et al., 1999; Klein et al., 2004) (Fig. 1).
Structural classes of Eph receptors and ephrins and their binding specificities.

The B-class ephrins are ligands that are linked to the membrane via a transmembrane domain and have cytoplasmic region capable of recruiting intracellular signaling molecules (Martinez et al., 2005). By contrast, the A-class ephrins are linked to the membrane via a GPI linkage (Zimmer et al., 2003). Within each subtype, it is also well known that the interactions between receptors and ligands are promiscuous with EphA receptors interacting with a number of ephrinA ligands and EphB receptors capable of interacting with various ephrinB ligands (Fig. 1) (Blitz-Huizinga et al., 2004; Martinez et al., 2005). Exceptions to this rule include EphA4 receptors that are known to interact with both A- and some B-class ephrins in vitro and in vivo, and EphB2 that binds and is activated by ephrinA5 in vitro (Fig. 1) (Gale, 1996; Gale, 1996; Kullander, 2003; Himanen, 2004). Ephrins interact with other ephrin molecules forming a complex of two ligands called a dimer. Eph receptors also interact with each other in a similar fashion also forming a complex of two receptors. During binding, it is believed that these resulting dimers of ligands and receptors interact at an interface forming a tetrameric complex of ligands and receptors. Whether all the binding interactions that occur between ephrins and Eph receptors result in both activation of the molecules and a physiological function remains to be investigated (Blitz-Huizinga et al., 2004; Himanen et al., 2003). Studies have demonstrated that soluble ephrins must be pre-clustered to activate Eph receptors in vitro suggesting that dimerization is critical for function (Birgbauer et al., 2001). Recent x-ray crystallography data suggests that higher order clustering of ephrins and Eph receptors beyond heterotetramers (i.e two ligands binding
two receptors) may occur because additional receptor-receptor and ligand-ligand interaction domains in these molecules have been discovered (Himanen et al., 2001).

_Eph receptor forward and ephrin reverse signaling_

Interactions between B-class ephrins and Eph receptors can generate bidirectional intracellular signals that lead to cytoskeletal changes within the growth cone which alter the growth and guidance of axons (Lu et al., 2001; Lu et al., 2004; Cowan et al., 2002; Klein et al., 1999) (Fig. 2). To examine the importance of either the extracellular or intracellular region of the proteins, ephrins and Eph receptors have been examined using various truncation mutants (Davy et al., 2005). Truncation mutants are often created by replacing the intracellular coding region of the ephrin or Eph receptor with the β-galactosidase (β-gal) gene. Binding of B-class Eph receptors with their ligands during cell-cell contact normally results in the initiation of ‘bidirectional’ signaling (Murai et al., 2004). Intracellular signaling downstream of Eph receptors is known as forward signaling, while signaling downstream of B-class ephrins is known as reverse signaling (Davy et al., 2005).

Genetic manipulation of the extracellular, intracellular, or the entire coding region of these receptors and ligands has been critical in our understanding of how these molecules function during development. For example, deletions of the entire Eph receptor gene eliminates both forward and reverse signaling since the receptor neither signals itself nor is it capable of mediating ephrin reverse signaling. By contrast, deletion of the intracellular coding region of the receptor gene eliminates only forward signaling, since the extracellular domain of the Eph receptor is still capable of binding ephrins and
mediating reverse signaling. Similarly, deletions of the entire ephrin gene eliminates both reverse and forward signaling since the ligand neither signals itself nor is it capable of mediating Eph forward signaling. Ephrin truncation mutants, where the intracellular region of the B-class ligand is lost, fail to initiate signals in the reverse direction. These truncation mutants retain the ability to mediate Eph receptor forward signaling since the extracellular binding domain of the ligand is still present (Murai et al., 2004). Studies using these truncation mutants have helped our understanding of whether the extracellular or intracellular domain of either the ephrin or Eph receptor are important during specific developmental events (Murai et al., 2003). Experiments taking advantage of these mutants have uncovered forward and reverse signaling functions for both receptors and ligands in this gene family.

*Eph receptor and ephrin functional studies*

Using complete null mice as well as truncation mutants, investigators have uncovered important functions for both the extracellular and intracellular regions of ephrins and Eph receptors in CNS development. For example, studies focusing on EphB2 receptors showed that during development of the posterior limb of the anterior commissure (pAC- the part of the tract that connects the temporal cortices), EphB2 can activate reverse signaling in pAC axons. This was best demonstrated *in vivo* using EphB2<sup>lacZ</sup> mice, where the intracellular domain of the EphB2 receptor was replaced with β-gal creating a truncation mutant. This mutant retains the capability of binding and initiating ephrin reverse signaling because the extracellular domain is still present. Evaluation of these truncation mutants revealed that the pAC did not have abnormalities with axons continuing to extend appropriately into regions of the temporal cortex.
Henkemeyer et al., 1996). These studies showed that the catalytic region of the receptor was not necessary for proper development of the pAC, suggesting that reverse signaling by ephrinB1 ligands, which are expressed by pAC axons, is an important function of EphB2 during pAC pathfinding (Davy et al., 2005). EphA4 receptor domains have also been studied using similar techniques. Studies have demonstrated EphA4 acts as a ligand for ephrin-expressing AC axons and forward signaling from this receptor is not necessary for proper development of either the pAC or anterior limb of the AC (aAC- the part of the tract that connects the olfactory bulbs) since axons in both regions of the AC project appropriately to either the olfactory bulb or temporal cortex, respectively (Dottori et al., 1999; Kullander et al., 2001). By contrast, the development of the corticospinal tract (CST) appears to depend on EphA4 forward signaling since both kinase defective and homozygous mouse mutants exhibit similar defects (Kullander et al., 2001).

Ligands such as ephrinB3 have also been studied using similar kinase defective mutants. EphrinB3 is expressed at the midline during CST development (Yokoyama et al., 2001). In ephrinB3 homozygous mutant mice, CST axons aberrantly re-crossed the midline after decussating, a defect that was restored by replacing the extracellular domain of ephrinB3, confirming that only the extracellular domain of this ligand was required to bind Ephs and mediate forward signaling allowing proper CST development (Kullander et al., 2001; Yokoyama et al., 2001). These studies revealed important functions for the extracellular domain of ephrinB3 during development, and began to reveal that the extracellular and intracellular domain of ephrins and Eph receptors are important during axon guidance.
Eph receptor signaling mechanisms

Following activation, ephrins and Eph receptors are capable of regulating axon growth and guidance and other cellular events by initiating specific intracellular signaling cascades (Cowan et al., 2001; Cowan et al., 2002; Murai et al., 2003). Specifically, the small GTPase RhoA and its effector Rho kinase (ROCK) have been shown to be critical factors in the ability of ephrinAs to induce growth cone collapse in chick retinal ganglion axons that express EphA5 receptors (Wahl et al., 2000). EphA5 receptors inactivate Rac1 while at the same time activating RhoA and its downstream effector ROCK, which ultimately results in actin depolymerization and growth cone retraction. Inhibitors of RhoA or ROCK eliminate the ability of ephrinA ligands to stimulate growth cone collapse. Other investigations of EphA receptors have shown that other signaling proteins that are phosphorylated and activated downstream of these receptors include focal adhesion kinase (FAK) and p130<sup>cas</sup> proteins (Carter et al., 2002). FAK is a protein which binds, phosphorylates, and activates intracellular signaling proteins, and in vitro experiments have revealed that this molecule is a critical component of the integrin signaling cascade which regulates cell adhesion and spreading (Parsons et al., 2000). The molecule P130<sup>cas</sup> is a multiadaptor protein that assembles a second large complex of proteins and allows amplification of integrin signaling (Harte et al., 1996). This indicates that Eph receptors not only function to direct cellular events via their own pathways, these molecules also can modulate signaling complexes activated downstream of other guidance receptors. Using Chinese hamster ovary (CHO) cells, investigators showed that EphB1 receptors recruit the focal adhesion proteins FAK and paxillin (Vindis et al., 2004; Huynh-do et al., 1999). This recruitment occurs through a complex signaling
cascade involving the Nck-interacting kinase (NIK), which phosphorylates paxillin and leads to FAK activation. Similarly, EphB1 receptors may also phosphorylate c-Src kinase, which is a second pathway leading to activation of paxillin. These signaling cascades result in cytoskeletal changes within the cell that promote cell migration \textit{in vitro}. EphB2 receptors have been implicated in actin depolymerization by activating intracellular signaling cascades that downregulate the mitogen activated protein kinase (MAPK) pathway (Tong et al., 2003; Elow et al., 2001). Using NG-108 cells and ephrinB1 ectodomains, investigators showed that autophosphorylated EphB2 receptors are capable of downregulating the Ras-MAPK signaling cascade through activation of the SH2-adaptor containing protein p120-Ras-GTPase activating protein (GAP). These activated proteins reduce the amount of GTP-bound Ras, downregulate the MAPK pathway, which results in neurite retraction. Adding to these signaling mechanisms another investigation showed that EphA signaling cascades involves molecules such as the guanine nucleotide exchange factor (GEF) ephexin, which regulates small GTPases of the Rho family (e.g. RhoA, Rac1, Cdc42) (Shamah et al., 2001). Activation of Eph receptor forward signaling recruits ephexin, which through an undescribed mechanism downregulates the activity of Cdc42 and Rac1 and upregulates the activity of RhoA. This signaling cascade promotes actin disassembly and this is one mechanism that may contribute to growth cone retraction (Sahin et al., 2005; Huot et al., 2004). EphB2 receptors have been shown to interact with a GEF specific for Rac1 called kalirin (Penzes et al., 2003). Activation of EphB2 receptors with ephrinB ligands causes phosphorylation of kalirin and subsequent activation of Rac1. Activated Rac1 is then able to activate the p21-activated kinase (PAK), which then initiates actin polymerization events that promote dendritic spine
morphogenesis. Other intracellular molecules downstream of activated EphB2 receptors and also involved in spine morphogenesis include a GEF called intersectin as well as the neural-Wiscott-Aldrich protein (N-WASP). Cdc42 is activated downstream of intersectin, which results in actin polymerization (Irie et al., 2002). N-WASP has been shown to promote the interaction of Cdc42 with the actin nucleation Arp2/3 complex resulting in actin polymerization (Rohatgi et al., 1999). Dominant-negative forms of any of these key players inhibit the actin polymerization and spine morphogenesis.

**Ephrin signaling mechanisms**

*In vitro* experiments have revealed that ephrin reverse signaling activates a host of intracellular molecules including PDZ-RGS3, which is capable of binding B-class ephrins and contains a regulator of heterotrimeric G-protein signaling (RGS) domain (Lu et al., 2001). PDZ-RGS3 inhibits the G-protein signaling that occurs downstream of the activated CXCR4 chemokine receptor by preventing the interaction of Gα with the Gβγ subunits, the three key heterotrimeric G-protein components. Functional studies showed that using this mechanism, PDZ-RGS3 inhibited the G-protein coupled chemoattraction of cerebellar granule neurons that normally occurs after SDF-1 ligands interact with CXCR4 receptors. Another intracellular signaling molecule activated during ephrin reverse signaling is a GEF specific for Rac1 called Tiam (Tanaka et al., 2004; Habets et al., 1994). This molecule interacts with the intracellular domain of ephrinB1, and when dominant-negative forms of Tiam are expressed in e14 cortical neurons neurite outgrowth promoted mediated by ephrinB1 is abolished (Tanaka et al., 2004). Other ephrin reverse signaling proteins have been uncovered by taking advantage of yeast-two hybrid screens. Specifically, these screens revealed that the SH2/SH3 adaptor protein Grb4 is a key
player in the reverse signaling that occurs after ephrin activation. This molecule interacts with a number of molecules that regulate the actin cytoskeleton including PAK1, axin, and heterogeneous ribonuclear protein K (hnRNPK) (Cowan et al., 2001). These intracellular signaling molecules have been implicated in actin remodeling and cell adhesion (Hobert et al., 1994; Ikeda et al., 1998; Hamada et al., 1999). Stimulation of NG108 cells with ephrinB1 leads to a loss of focal adhesions, rounding up, and a disassembly of F-actin filaments. Expression of a dominant-negative form of Grb4 blocks these cellular responses suggesting that this molecule plays a central role in ephrin reverse signaling. In sum, these data show that both Eph receptors and ephrins activate a multitude of intracellular signaling components that function to induce either actin assembly or disassembly.

**Ephrin and Eph receptor signaling in axon adhesion and repulsion**

Ephrins and Eph receptors function in numerous CNS events including patterning of rhombomeres, synaptogenesis, and axon fasciculation (Wilkinson et al., 2001). Based on the various axon growth and guidance roles of these molecules, it is clear that ephrin and Eph receptor interactions may result in either repulsion or adhesion (Holberg et al., 2002). During axon extension for example, ephrins and Eph receptors interact, which initially leads to adhesion. EphB2 receptor mediated adhesion was demonstrated in the cell-cell adhesion that occurs during tubularization of the urethra and partitioning of the urinary and alimentary tracts (Dravis et al, 2004). Deletion of the EphB2 receptor results in incomplete midline fusion in the urethra and severe abnormalities in the reproductive system. However, for repulsion to occur the adhesive ephrin-Eph receptor complex must be cleaved or endocytosed (Wilkinson et al., 2003). Using *in vitro* methods, investigators
have revealed that the termination of adhesion and the promotion of repulsion involve a
Rac-dependent internalization of the entire receptor-ligand complex (Zimmer et al., 2003;
Marston et al., 2003). More recently, a molecule called Vav2 has been implicated in the
signaling of Eph receptor-ephrin internalization during axon guidance and growth
(Cowan et al., 2005). This molecule is a GEF, and Vav2 is recruited to the Eph receptor
intracellular domain following ephrin binding. Deletion of Vav2 in mouse retinal
ganglion neurons eliminates the ability of ephrin-Eph receptor complexes to be
internalized suggesting that this molecule is an important link between Eph receptor
activation and repulsion.

Using *Xenopus* dorsal retinal neurons that express ephrinBs, investigators also
have shown that application of EphB2 ectodomains to *Xenopus* neurites growing *in vitro*
induces fast collapse of growth cones involving intracellular cyclic guanine
monophosphate (GMP) intracellular signaling molecules (Mann et al., 2003). Application of cGMP analogues such as 8-bromo-cGMP, which increases the amount of
cGMP, eliminated the collapsing ability of EphB2. These data suggested that the ability
of activated ephrinB1 to induce growth cone collapse depended on low levels of
intracellular cGMP. By contrast, using the same experimental system except with
*Xenopus* ventral retinal neurons that express EphB receptors, the investigators showed
that ephrinB1 ectodomains induced slow growth cone collapse. This effect of ephrinB1
ectodomains on *Xenopus* growth cones depended on high levels of intracellular cyclic
GMP because the cGMP inhibitor Rp-8-pCPT-cGMP blocked the collapsing effect of
activated EphB2 receptors. Separate studies have suggested that guidance cues such as a
netrin function by activating protein degradation (Campbell et al., 2001). Similarly, these
investigators also showed that the growth cone collapsing ability of EphB2 in *Xenopus* neuron cultures required proteosome function because application of proteasome inhibitors such as lactacystin prior to application of EphB2 ectodomains blocked growth cone collapse (Mann et al., 2003). Studies such as these have lent support to the notion that activation of ephrins and Eph receptors leads mainly to growth cone collapse and axon repulsion (Klein et al., 2001).

However, more recently some studies have revealed that ephrins and Eph receptors may have dual effects on axon guidance depending on the concentration and/or spatial expression of the ephrins or Eph receptors. Recently, investigators showed that ephrins and Eph receptors may be co-expressed within the same cell and these molecules are separated into distinct membrane pools with ephrins segregated from Eph receptors (Marquardt et al., 2005). This study further showed that activation of either ephrinA ligands or EphA receptors in chick ventral spinal cord explants with appropriate Eph- or ephrin-Fc (fusion protein consisting of extracellular domain of Eph or ephrin and human immunoglobulin G) had opposite effects on growth cone morphology. Activation of endogenous ephrinAs increased the size of growth cones compared to untreated cultures. By contrast, activation of EphA receptors led to collapse of growth cones compared to untreated neurons. Increasing or decreasing the levels of ephrinA ligands expressed in the same cell did not alter the collapsing ability of EphA receptors. These studies suggest that ephrinA and EphA receptor effects may be functionally uncoupled with ephrins and Eph receptors capable of initiating either neurite outgrowth or growth cone collapse, respectively (Klein et al., 2005). Similarly, Flanagan and colleagues (2004) showed that low levels of ephrinA ligands actually induce retinal neurite outgrowth whereas high
concentrations of ephrinA ligands inhibit axon outgrowth (Hansen et al., 2004). This study revealed that there is a concentration dependent mechanism that regulates growth cone initiation versus collapse (Hansen et al., 2004).

Other studies demonstrate that ephrin and Eph receptor interactions may also promote neurite outgrowth depending on the embryonic stage or neuronal cell type. Using substrates coated with ephrinA5 or ephrinB1-Fc molecules, investigators showed that these molecules increased neurite outgrowth compared to uncoated substrates (Zhou et al., 2001). In addition, using cell lines stably expressing recombinant ephrinA2, A3, and A5, investigators showed that these molecules inhibited the outgrowth of embryonic (e) day 18 olfactory and striatal neurites, while ephrinA2 and A5 promoted the outgrowth of neurites in e18 sympathetic neurons (Zhou et al., 2001). Lastly, using 3T3 fibroblasts stably expressing recombinant ephrinA5, investigators showed that this molecule was capable of stimulating neurite outgrowth from retrogradely labeled neurons (Hu et al., 2003). In vivo data from this same study revealed that in EphA5 kinase deletion mutants, CC fibers failed to enter the commissural tract (Hu et al., 2003). Together, these in vitro and in vivo data suggest that ephrinA5 ligands promote callosal axon outgrowth and that the disruption of ephrinA5 and EphA5 interaction may eliminate the ability of CC axons to initiate axon outgrowth and extend across the midline (Hu et al., 2003). These studies begin to reveal that ephrins and Eph receptors have a broader range of effects than simply growth cone collapse and repulsion, and may promote axon extension in specific CNS regions during development.
Eph receptors and ephrins in midline axon crossing

Various studies have implicated ephrins and Eph receptors in midline axon guidance (Williams et al., 2003; Kaprielian, 2000). Unlike netrins and slits, the ephrins and their receptors regulate midline axon growth and guidance through direct cell-cell interactions (Gale et al., 1997; Gerlai et al., 2001). These interactions function to regulate axons such as the crossing of commissural interneuron (CIN) fibers that make up part of the central pattern generator (CPG) in mice. Tracing experiments have revealed that EphA4, which is expressed by CINs, is a key player in the routing of CIN axons because deletion of this gene results in CIN axons that aberrantly cross the spinal cord midline (Kullander et al., 2003). Separate experiments have implicated B-class ephrins and Eph receptors in regulating the dorsoventral position of longitudinally projecting spinal cord sensory afferents (Imondi et al., 2001).

In CNS regions rostral to the spinal cord, studies investigating mouse development have implicated ephrinB3 and EphA4 receptors in the midline decussation of corticospinal tract (CST) fibers. EphrinB3 is expressed at developmental time points when CST fibers are crossing the midline in the spinal cord, while one of its binding partners, EphA4, is expressed in CST axons (Yokoyama et al., 2001; Dottori et al., 1998). Deletion mutants for ephrinB3 or EphA4 exhibit CST axons that aberrantly recross the spinal cord. These data suggest that ephrinB3 acts as a repulsive guidance factor preventing recrossing (i.e. re-entering the ipsilateral spinal cord) of the midline once CST axons have entered the contralateral side of the spinal cord (Yokoyama et al., 2001; Kullander et al., 2001). Other studies using the optic chiasm to examine midline axon guidance have shown that EphB1 and ephrinB2 direct the ipsilateral routing of retinal
axons through repulsive interactions preventing aberrant midline crossing (Williams et al., 2003). Specifically, these investigators showed that only ipsilateral axons expressed EphB1 while ephrinB2 was confined to midline regions. EphrinB2 prevents the crossing of EphB1-expressing retinal axons allowing proper establishment of ipsilateral retinal projections. Finally, in the developing forebrain, knockout mice reveal important roles for EphB2 and EphB3 in the formation of the CC and AC in the mouse (Williams et al., 2003; Orioli et al., 1996; Henkemeyer et al., 1996). In sum, these studies show that multiple axons are regulated by ephrins and Eph receptors during development and further research will likely uncover additional roles for this family in the embryonic CNS.

**Midline glia important for callosal neuron axon growth and guidance**

In the mouse, callosal axons are mainly derived from neurons in cortical layers 2, 3, and 5 which project axons that reach the midline at around e15 (Richards et al., 2002). A variety of growth and guidance cues have been implicated in this process, including but not limited to the slits, netrins, semaphorins, ephrins and Eph receptors (Richards et al., 2004) (Fig. 3). At e15, pioneer callosal fibers interact with glial guideposts as they project to the forebrain midline. By late e17, most callosal fibers have crossed the midline and begin defasciculating from the main CC bundle as they continue their trajectory toward appropriate contralateral targets (Richards et al., 2004). During development of the CC, a few midline glial guideposts function to direct callosal axons. One important midline guidepost for CC development is the glial wedge (GW), which consists of radial glia derived cells (Fig. 3). This guidepost expresses the axonal repellent slit-2 (Richards et al., 2000). The GW begins expressing glial fibrillary acidic protein (GFAP) at e13 in the mouse and GW cells stop being generated by postnatal day 2. By
e17, the GW may be co-labeled with the radial glial markers RC-2, brain lipid-binding protein (BLBP), astrocyte specific glutamate transporter (GLAST), and nestin (Shu et al., 2003a). Recent investigations have determined that slit-2 restricts the growth and guidance of callosal fibers before and after crossing the midline (Shu et al., 2003b).

The ‘glial’ sling (formed by migrating neuronal cells) also functions as a guidepost structure instructing callosal fibers at all stages of embryonic development of the CC. Investigators showed that at early CC developmental stages (i.e. e15) lesioning of the ‘glial’ sling resulted in callosal axons that failed to cross the midline instead forming whorled neuromas called Probst’s bundles (Silver et al., 1983). After surgical disruption, replacement of the ‘glial’ sling by aligning a cellulose bridge between the two hemispheres, where ‘glial’ sling cells normally reside, resulted in a redirecting of axons within the Probst’s bundles appropriately across the midline. These data implicated the ‘glial’ sling in the proper extension of callosal fibers across the midline. Recent data indicate that development of this structure actually involves the migration of neuronal cells to the midline beginning at e14, which remain present throughout CC formation (Shu et al., 2003b,c; Rash et al., 2001; Shu et al., 2001a,b; Silver et al., 1983). These cells form a bridge-like structure that is located ventral to the developing CC, extends medially and spans the distance between the lateral ventricles (Fig. 3). This guidepost has been reported to function as a repellent structure preventing aberrant migration of CC axons into ventral forebrain regions, yet cells within the sling may also have growth promoting functions (Richards et al., 2004; Silver et al., 1983). The exact role of the ‘glial’ sling during development remains to be investigated.
The next major guidepost, which also expresses slit-2, necessary for CC development is the indusium griseum (Ig) (Fig. 3). The Ig begins expressing the marker GFAP at e14 and by e17 BLBP and GLAST are also expressed by this guidepost. Together the GW and Ig guideposts direct callosal fiber growth across the corticoseptal boundary using ‘surround repulsion’ mechanisms that are thought to be mediated mainly by direct interactions between slit-2 expressed in GW and Ig with Robo receptors in callosal axons (Shu et al., 2003b; Shu et al., 2001a; Richards et al., 2002; Bagri et al., 2002).

Lastly, midline zipper glial (Mzg) cells are generated in small numbers between e14 and relatively few are generated at e17 (Shu et al., 2003a). Mzg cells have been proposed to secrete molecules that regulate midline fusion, and this event is critical in providing a substrate for crossing of midline guiding axons (Richards et al., 2004). Whether or not this structure plays a direct role in callosal axon growth or guidance remains to be investigated. While the function of Robo receptors and slits in the CC and midline glial guideposts has been well studied, little is known about the role ephrins and Eph receptors play in the GW, Ig, ‘glial’ sling, and Mzg.

**Eph receptors and ephrins in CC development**

What is known of ephrins and Eph receptors in midline axon pathfinding mainly is derived from studies of commissural pathways such as the corticospinal tract, spinal cord sensory pathway, inner ear efferents, and optic chiasm (Cowan et al., 2000; Yokoyama et al., 2001; Imondi et al., 2000; Williams et al., 2003). Few studies have examined ephrins and Eph receptors in the more rostral regions of the CNS and those few
have mainly focused on the postnatal morphology of the AC and CC (Henkemeyer, 1996; Orioli, 1996). Using knockin mice that have the intracellular domain of Eph receptors or ephrins replaced by the \( \beta \)-galactosidase (\( \beta \)-gal), investigators are able to localize the expression patterns of this fusion protein by reacting tissue with the substrate for the \( \beta \)-gal enzyme and detecting the blue color product (Henkemeyer et al., 1996). Studies have discovered EphB2 fusion protein and EphB3 mRNA expression in the CC during the development of this tract (Beckmann et al., 1994; Orioli et al., 1996; Hankin et al., 1988; Rash et al., 2001; Shu et al., 2001; Koester et al., 1994; Henkemeyer et al., 1996). In both mouse and rat, EphA4 receptor expression has been detected in callosal fibers during development, yet the function of this receptor is not clear (Martone et al., 1997; Greferath et al., 2002). EphA3, EphA5, and EphA7 receptors have also been detected in the cell bodies of callosal neurons suggesting a role for these EphA receptors in the development of the CC (Hu et al., 2003; Kudo et al., 2005).

Previous studies have examined a functional role for ephrins and Eph receptors in CC development. In particular, EphB2 knockout (EphB2\(^{\text{KO}}\)) and EphB3\(^{\text{KO}}\) mice exhibit defects in the development of the CC and AC, while mice deficient in both receptors exhibit severe defects. In these double knockout mice, callosal axons fail to cross the midline and instead stall forming large swirls of axons or Probst’s bundles in a region near the GW and Ig (Orioli et al., 1996). This was the first study demonstrating compensatory mechanisms or overlapping functions in this gene family. Other studies have shown that the deletion of the intracellular domain of the EphA5 receptor results in some callosal fibers that fail to enter the commissure, a phenotype known as CC hypoplasia (Hu et al., 2003). While there are clear roles for EphB2, EphB3, and EphA5
receptors in CC development; the developmental expression, ligand-receptor interactions and functions of EphB1, EphA4 and B-class ephrins in the embryonic CC are relatively unknown.

Another fiber tract that develops in the forebrain is called the perforating pathway (Pfp), and this tract has an interesting projection pattern compared to the CC. The Pfp projects ipsilaterally through the corticoseptal boundary at the same developmental time period as callosal fibers, but differs in axon trajectory and target innervations (Shu et al., 2001b). The Pfp, which is a bidirectional pathway, consists of neurons in both the medial septum/diagonal band of Broca and cingulate cortex that extend axons beginning at e15 to dorsal and ventral targets, respectively. Dye tracing experiments have revealed that the majority of fibers that make up this tract are derived from neurons in the medial septum that project axons to the cingulate cortex (Shu et al., 2001b). It remains unknown what mechanisms differ between Pfp fibers and callosal fibers to allow proper establishment of two tracts that develop in a similar time period yet have completely different trajectories (Shu et al., 2001b). Understanding the roles of ephrins and Eph receptors may shed light on these differences between CC and Pfp axons.

**CC defects in mice**

In mice, telencephalic hemispheres are fused at ventral regions and this fusion is believed to be directed by molecules released by the Mzg (Richards et al., 2004). This fusion event is critical in creating a substrate over which CC fibers migrate similar to the events previously described. Malfunctions in any of the critical steps of telencephalic formation, neuronal migration, or axon extension can lead to defects such as agenesis (failure of axons to cross the midline) of the CC. A number of genes are associated with
defects in the development of the CC and these may be categorized as guidance molecules and their receptors, transcription factors, intracellular molecules, and growth factors (Richards et al., 2004). L1, a member of the CAM superfamily, is one axon growth and guidance molecule that has been studied for its role in CC development, with deletion of this gene resulting in decreased dorso-ventral thickness of the CC (i.e. hypoplasia) (Demyanenko et al., 1999). Nuclear factor I-A (Nfia) and Vax1 are examples of transcription factors that have been implicated in CC development because deletion of these genes results in agenesis of this commissure (das Neves et al., 1999; Bertuzzi et al., 1999). Microtubule associated protein 1B (Map1B), an intracellular signaling protein, has been also been implicated in the development of the CC because these mice also exhibit Probst’s bundle formations (Meixner et al., 2000). This molecule interacts with both microtubule and actin filaments, yet a clear mechanism explaining the role of this molecule in callosal axon growth and guidance is unknown (Pedrotti et al., 1996; Togel et al., 1998).

Three intracellular signaling molecules that include p35 (an activator of cdk5), Mena, and GAP-43 (both Mena and GAP-43 remodel the actin cytoskeleton) have been also been implicated in CC development because knockout mice for each of these molecules develop Probst’s bundles (Lanier et al., 1999; Shen et al., 2002; Kwon et al., 1999). However, the mechanisms by which these molecules are involved during CC development are unknown. After inspection of the genes that regulate CC formation, one apparent characteristic of this system is that there are a plethora of genes that play a role in CC development. This may be due in part to the extensive developmental events that must occur for proper commissure development. Multiple molecules involved in CC
development may also contribute to a dependable mechanism that will ensure proper
development of such a major CNS commissure. Loss of simply one gene may not be as
deleterious to the system as compared to loss of two genes. However, it is also possible
that there may be master regulatory genes that have significant functions in CC
development, and loss of these individual genes may result in major defects.

**CC defects in humans**

In humans, ACC is thought to have autosomal dominant, autosomal recessive, and
X-linked forms with more than 20 autosomal malformation syndromes, including FG
syndrome, Miller-Dieker syndrome, Aicardi syndrome, Donnai-Barrow syndrome,
Joubert syndrome, Kallmann syndrome, Andermann syndrome, and agenesis of the
corpus callosum with peripheral neuropathy. While many of these disorders are mapped
to specific chromosomal regions, studies are only beginning to identify the genetic basis
for these diseases. One example is FG syndrome, which has a gene map locus of Xq12-q21.31. Patients with FG syndrome are affected with mental retardation,
disproportionately large head, hypospadia, cleft lip/palate, congenital hypotonia, and
ACC (Dessay et al., 2002; Opitz et al., 1988). Interestingly, *ephrinB1* is localized to this
same locus (Xq12-q13.1), although it is unclear whether ephrinB1 is responsible for the
ACC phenotype.

One neurological syndrome known to have CC defects and result from *ephrinB1*
missense mutations is craniofrontonasal syndrome (CFNS) (Dessay et al., 2002; Wieland
et al., 2004). Like FG Syndrome, CFNS is also characterized by skeletal abnormalities
and midline abnormalities such as cleft lip/palate and CC hypoplasia. Another molecule
that has been implicated in human CC agenesis is the L1 CAM (Sztriha et al., 2002).
Missense mutations of the L1 gene have been detected in human patients with X-linked hydrocephalus, a genetic disorder which is associated with abnormal CNS phenotypes that include agenesis of the CC (Sztriha et al., 2002). We are just beginning to unravel the role of ephrins and Eph receptors in human diseases.

In this study, we take a comprehensive approach to examine the protein expression of B-class ephrins and Eph receptors in the developing CC and surrounding tissues, and take a genetic approach to examine their roles in midline axon guidance. We demonstrate that ephrins and Eph receptors are expressed in callosal fibers and strategic regions important for regulating midline pathfinding decisions in the developing forebrain. This study also reveals multiple functional redundancies in the interactions between Eph receptors and ephrinB3 that may influence callosal development. The trajectory of CC fibers in the ephrin and Eph receptor knockout mice reveals an important relationship between the residential glial cells and Probst’s bundle formation, and suggests that ephrins and Eph receptors not only regulate growth promoting functions and/or guidance signals but also the cellular organization of local guideposts. Lastly, this study reveals that one important function of Eph receptors may be in regulating the sensitivity of cortical neurons to midline growth and guidance cues.

**Research Objectives**

Although investigations have determined that there are roles for various Eph receptors and ephrins in CC development, the complete list of gene family members and mechanisms that regulate crossing of CC fibers remain unknown. Specifically, this dissertation examined a family of molecules, ephrins and their receptors, for their ability to regulate midline guidance decisions in callosal (corpus callosum) and non-callosal
(perforating pathway) axons. Our central hypotheses are: 1) both ephrins and Eph receptors are required for callosal axon midline pathfinding; 2) ephrins and Eph receptors function to direct the migration and/or signaling of glial midline guideposts; and 3) guideposts distinct from those used by callosal fibers regulate the development of non-callosal perforating pathway axons.

The hypotheses tested in each chapter include:

Chapter II.

a) Multiple B-class ephrins and Eph receptors are expressed in developing CC fibers and/or midline guideposts. This hypothesis will examine the spatiotemporal expression patterns of ephrins and Eph receptors specifically relating their expression with the development of CC fibers and its critical midline guideposts.

Chapter III.

2a) Gene targeted knockout mice will exhibit CC defects with combination-mutant mice exhibiting a higher frequency of CC defects. This hypothesis will investigate compensatory mechanisms that exist within this gene family.

2b) Distinct extracellular and intracellular ephrin and Eph receptor domains are necessary for CC development. This hypothesis will evaluate whether the ephrins and Eph receptors of interest are necessary for forward or reverse signaling.

Chapter IV.

3a) EphrinB3, which is expressed in midline glial guideposts, regulates the CC development by directing glial development.

3b) EphB1 receptors, which are expressed mainly in developing callosal fibers, regulate the sensitivity of callosal axons to midline guidepost cues.
Together, these hypotheses will examine the mechanisms by which ephrins and Eph receptors regulate CC development.

**Chapter V.**

* a) Pfp fibers use similar guideposts (i.e. GW and Ig) necessary for CC development to maintain their ipsilateral orientation in the forebrain. This hypothesis will examine whether ephrin-expressing Pfp fibers interact with Eph receptors at known CC midline guideposts thereby regulating proper ipsilateral routing of this fiber tract.

These studies represent an analysis of how ephrins and Eph receptors may regulate the midline guidance decisions of callosal neurons, as well a potential for these molecules in the development the Pfp within the same developmental region and period. We have discovered members of this gene family expressed in the CC, Pfp and important midline glia (necessary for CC development). Furthermore, we have determined that some of these members are critical for CC development, both forward and reverse signaling are used and novel gene interactions occur during callosal fiber midline guidance. Novel mechanisms involving ephrinB3 and EphB1 have been discovered indicating that this ligand and receptor play roles in radial glial migration and regulating cortical neuron sensitivity to guidance cues, respectively. Deletion of these ligand and receptor genes potentially removes this appropriate function thereby resulting in the CC abnormalities (i.e. CC hypoplasia and/or Probst’s bundle formation).
Figures for Chapter I

Figure 1. Binding interactions between A and B-class ephrins and Eph receptors as well as the critical structural protein components. a) Binding is mainly promiscuous within each of the ephrin and Eph receptor classes (black arrows), with EphA receptors mainly interacting within ephrinA ligands while EphB receptors mainly interact with ephrinB ligands. There are two exceptions that show interactions between members of A and B-class where EphB2 interacts with ephrinA5 (red arrow) while EphA4 interacts with ephrins (B2-B3) (red arrow). b) EphrinA ligands are anchored to the plasma membrane (grey) via a glycosphatidylinositol (GPI) linkage, and the extracellular globular binding domain interacts with the extracellular domain of Eph receptors. EphrinB ligands consist of an extracellular binding domain that interacts with the extracellular domain of Eph receptors. EphrinB ligands are anchored to the plasma membrane via a transmembrane domain while the intracellular portion of the protein contains a kinase recruitment domain that aids in intracellular signaling. B-class ligands also contain a PDZ domain, which carries out interactions with PDZ domain containing proteins. Eph receptors consist of an extracellular domain that consists of a binding domain that interacts with ephrins, cysteine rich region, and 2 fibronectin type III repeats. Eph receptors are tethered to the membrane via a transmembrane domain and the intracellular portion of the protein consists of a kinase domain, which aids in transphosphorylation after receptor dimerization, and a sterile alpha motif (SAM) domain, which is involved in protein-protein interactions. Eph receptors also contain an intracellular C-terminal PDZ-binding sequence that binds to PDZ domain containing proteins.
Figure 2. Diagram of the signaling mechanisms elicited by interactions with complete null and truncated ephrins and Eph receptors. Schematic representation of transmembrane ephrins and Eph receptors, and how truncation of these proteins affects forward or reverse signaling, respectively. The N-terminal extracellular binding domain of the B-class ephrin (orange) is linked to the membrane (grey) via a transmembrane and intracellular domain (black). The C-terminal YYKV (purple) is a motif that is found in the intracellular portion of the ligand recognized by PDZ-containing proteins. Such a motif is also present at the C-terminal tail of Eph receptors. The N-terminal extracellular binding domain of Eph receptors (blue) is linked to the membrane (grey) via a transmembrane and intracellular domain (black). The intracellular domains of Eph receptors contain a kinase region (green) which catalyzes tyrosine phosphorylation (P) of the Eph receptors themselves and a sterile α motif (SAM) domain that aids in receptor dimerization. Removal of the entire or intracellular portion of the gene abolishes signals in that direction (red). (Davy et al., 2005)
Figure 3. Schematic coronal section through the forebrain showing the important midline guideposts and fiber tract trajectories. Axonal and glial midline guidepost populations at the midline. Shown is a schematic view of a coronal section through the developing cortex at e17. A callosal neuron is shown in purple and neurons of the perforating pathway (PFP) are shown in yellow. Cingulate pioneering neurons projecting laterally are shown in green and cingulate pioneering neurons of the CC projecting medially are shown in red. GW= glial wedge; GS= glial sling; Ig= indusium griseum glia. (Richards, 2002)
Chapter II

*B-class ephrins and Eph receptors are expressed in the forebrain during the developmental time points of corpus callosum axon growth*

**Introduction**

During late mouse embryogenesis, callosal neurons project their axons to the contralateral cortex to form cortico-cortical connections. At e15, callosal fibers begin projecting to the midline and extend through the midline by e16. Axon extension and targeting of CC fibers continues through e17 (Rash et al., 2001; Shu and Richards et al., 2001a). Two midline structures, the GW and Ig, are known guideposts that interact with projecting CC fibers that migrate during the same developmental time points (Shu et al., 2003a). A second forebrain tract, the Pfp, also projects through the corticoseptal boundary at around the same developmental time period as CC fibers (Shu et al., 2001b).

To begin investigating the role of B-class ephrins and Eph receptors in the developmental of forebrain commissures, such as the CC and Pfp, we explored the spatial and temporal expression patterns of Eph receptors (B1-B3) and EphA4 as well as ephrins (B1-B3). This chapter will examine the timing and location of ephrin and Eph receptor expression with the developmental patterning of the CC, Pfp and midline guideposts (e.g. GW and Ig).
Materials and Methods

**Transgenic mice, Breeding, and Genotyping**

\[ \text{EphB1}^{\text{KO}}, \text{EphB2}^{\text{KO}}, \text{EphB3}^{\text{KO}}, \text{EphB2}^{\text{LacZ}}, \text{EphA4}^{\text{KO}}, \text{ephrinB3}^{\text{KO}}, \text{ephrin2}^{\text{LacZ}}, \text{and ephrin3}^{\text{LacZ}} \]

CD1 mutant mice and genotyping methods have been previously described (Henkemeyer et al., 1996; Orioli, 1996; Williams et al., 2003; Yokoyama et al., 2001; Dottori et al., 1998; Gale, 1996; Helmbacher et al., 2000; Dravis et al., 2004). At 2 weeks postnatal, animals are anesthetized using halothane and a 2mm piece of tail tissue is obtained. Tails are then placed in 450μL of lysis buffer (50 mM KCl, 10 mM Tris pH 8.3, 2 mM MgCl₂, 0.1 mg/ml gelatin, 0.45% NP-40, 0.45% Tween-20, 100 µg/ml proteinase K) (Sigma, St. Louis, MO) and incubated at 50°C overnight. The following day the lysed tissue was spun at 15,000 revolutions per minute (rpm) at RT for 15 minutes and supernatant placed in 250μL of 100% isopropanol. The solution was then agitated and the visible deoxyribonucleic acid (DNA) placed in 200μL sterile water.

Polymerase chain reaction (PCR) was performed using gene specific primers for each mutant DNA sequence and Taq DNA polymerase (Promega, Madison, WI) to allow genotyping of tissue and determine whether animals were WT, heterozygous, or homozygous. Using available single mutant mice, receptor-receptor double knockout mice were generated, including \( \text{EphB1}^{\text{KO}}/\text{EphB2}^{\text{KO}}, \text{EphB1}^{\text{KO}}/\text{EphB3}^{\text{KO}}, \)

\( \text{EphB1}^{\text{KO}}/\text{EphA4}^{\text{KO}}, \text{EphB2}^{\text{KO}}/\text{EphB3}^{\text{KO}}, \text{EphB2}^{\text{LacZ}}/\text{EphB3}^{\text{KO}}, \text{and EphB3}^{\text{KO}}/\text{EphA4}^{\text{KO}}. \)

Additionally, receptor-ligand double mutants (ephrinB3^{KO}/EphB1^{KO}, ephrinB3^{KO}/EphB2^{KO} and ephrinB3^{KO}/EphA4^{KO}) were generated. Procedures related to
animal use and care were approved by the University of Miami Animal Care and Use Committee.

**Immunohistochemistry and imaging**

Embryos of appropriate time points (embryonic day (e) 15, 16, 17, 18) were obtained from timed pregnant dams. The plug date was defined as e0 and the day of birth as postnatal day (P) 1. On the appropriate embryonic time point pregnant female mice were anesthetized and embryos were removed by caesarian section. Following removal of embryos, the female mice were sacrificed with a lethal injection of ketamine cocktail. Brains were dissected, immersed in 4% paraformaldehyde (PFA) (Fisher Biotech, Hampton, NH) made up in 1X phosphate buffered saline (PBS) for 1 hour. Brains were then cryoprotected in 25% sucrose overnight at 4°C and finally embedded in O.C.T (Tissue-Tech, Hatfield, PA). Cryosections were cut at 10μm and mounted on microslides, permeabized in 0.05% Triton X-100 (Sigma) for 20 minutes, and immersed in 5% bovine serum albumin (BSA) (Fisher Biotech) blocking solution for 30 minutes at room temperature (RT). Antibodies specific for EphB1, 1:25 (Santa Cruz); EphB3, 1:25 (Research & Design (R&D), Minneapolis, MN); EphA4 (R&D) and ephrinB1, 1:100 (Santa Cruz, Santa Cruz, CA) were diluted in 5% BSA/0.1% TritonX-100 and allowed to incubate with sections for 1 hour at RT followed by overnight at 4°C. For single or co-labeling, antibodies against Tag-1, 1:100 (Developmental Studies Hybridoma Bank, Iowa City, IA); GFAP, 1:1000 (DAKO, Glostrup, Denmark); β-galactosidase (β-gal), 1:200, RC-1 or RC-2, 1:200 (Developmental Studies Hybridoma Bank), GAP-43, 1:500, Neurofilament (145kD), 1:500, β-tubulinIII, 1:1000 (Chemicon, Temecula, CA) were used. Sections were then washed 3 times for 5 minutes each in 1X phosphate buffered saline.
saline (PBS) and then incubated with FITC and/or Texas-Red conjugated secondary antibodies (Molecular Probes, Carlsbad, CA) for 1 hour at RT. Sections were washed again in 1X PBS 3 times for 5 minutes each and coverslipped using an aqueous gel-mount (Biomedia, Foster City, CA). Sections for GFAP immunohistochemistry were embedded in 3% agarose (Gibco-BRL, Carlsbad, CA), sectioned at 75μm, immersed in 0.1% Triton X-100 in 1X PBS and transferred to 5% BSA for blocking. Sections were incubated free-floating in GFAP antibody 1:3000 (DAKO) diluted in 5% BSA/0.1% TritonX-100 for 48 hours at 4°C. Sections were then incubated with anti-rabbit biotinylated secondary antibody 1:500 (Vector Laboratories, Burlingame, CA) for 1 hour. Vectastain ABC reagent was then added for 1 hour at RT followed by washes 3 times for 5 minutes each. Sections were then incubated in peroxidase substrate until the desired staining was achieved. Sections were rinsed in distilled water, mounted on microslides, dehydrated in alcohol washes (70%, 80%, 95%, 100% 2 times for 5 minutes each) and finally coverslipped in Sub-X mounting media (Surgipath). 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI) (D-282, Molecular Probes, Carlsbad, CA) was incubated with nitrocellulose membrane (Bio-Rad, Hercules, CA) until all solution was dried on the membrane. DiI soaked membranes were then embedded in the 4% PFA fixed brains for 3-4 weeks at 37°C. Brains were then embedded in 3% agar (Gibco-BRL), vibratome sectioned, mounted on microslides and coverslipped. Sections for fluorescent or non-fluorescent immunostaining were imaged using an inverted microscope (Zeiss Axioscope).
**β-galactosidase histochemistry**

Tissue was prepared and cryosectioned at 10μm as previously described. EphB2, ephrinB2, and ephrinB3 were detected using dated embryos homozygous for the β-gal gene. Sections were incubated with X-gal (Vectra, Prince Edward Island, Canada) staining solution (5mM Kferri/Kferro cyanide, 2mM MgCl₂, 1mg/mL X-gal, 1X PBS) (Sigma) 1-2 hours at RT, then washed in 1X PBS 3 times for 5 minutes and finally dehydrated in an ascending alcohol series (70%, 80%, 95%, and 100%). Sections were coverslipped with Sub-X mounting media (Fisher Biotech) and imaged in bright-field.

**Results**

**Forebrain Development in CD1 Mice**

To begin examining the role of ephrins and Eph receptor in the developing forebrain, we first evaluated the developmental timing of CC growth and appearance of forebrain guideposts in CD1 wild-type (WT) mice. Previous investigations of CC, Pfp, glial sling and midline glial development have focused on the C57BL/6J mouse strain (Rash et al., 2001; Shu et al., 2003a; Silver et al., 1993; Shu et al., 2001b).

Immunolabeling with anti-β-tubulinIII, a general cytoskeletal marker for all axons, showed that at e15 few axons had crossed the midline while by e17 a large population of axons had extended into the opposite hemisphere (Fig. 4a-4c, 4g). This timing was confirmed by tracing the CC axons with the lipophilic tracer, DiI, where fibers projected to the midline at e15 and by e16 a small population of callosal fibers have crossed the midline (Rash et al., 2001) (Fig. 4a’, 4b’).

Another prominent axonal pathway present in the developing forebrain is the perforating pathway (Pfp), which is mainly formed by neurons in the Diagonal band of
Broca/medial septum (DBMS) and cingulate cortex that extend axons dorsally and
ventrally, respectively. At the embryonic midline, axons of the Pfp project ipsilaterally
around the same developmental time point as CC fibers, and predominantly immunoreact
with anti-neurofilament (NF) (145kD) antibodies (Shu et al., 2001b) (Fig. 4d-4f, 4h).
Fibers in this tract remain ipsilateral, bisect the CC and migrate through regions where
the glial sling normally forms at the midline (Fig. 4d-f).

Three glial populations that have been described to function in midline guidance
of CC fibers are born around e14 and have matured by e18, a time point when most or all
CC fibers have crossed the midline (Rash et al., 2001; Shu et al., 2003a,b) (Fig. 4i). As
previously reported, immunolabeling of e18 forebrain tissue with anti-GFAP revealed
that these three glial populations, which include the Ig, GW and midline zipper glia
(mzg), are present during development (Shu et al., 2003b). These studies demonstrate
and confirm that the developing CC and midline structures in CD1 mice are similar to
those previously described for c57BL/6 mice (Rash et al., 2001; Shu et al, 2003a,b; Silver
et al., 1993).

Expression of Ephrins in the Developing Forebrain

To determine whether ephrins are important for formation of the CC, we first
examined whether B-class ephrins are expressed in the commissure or surrounding areas
during development. We found that all three B-class ephrins are expressed in the
forebrain at e15, a time point at which callosal fibers are approaching the midline and
pioneer fibers begin crossing into the contralateral hemisphere. At e15, ephrinB1 was
expressed in the differentiating fields of the frontal neocortex, cingulate cortex,
corticoseptal boundary, and Ig, as well as regions that contain cortical, callosal, and Pfp
fiber projections (Fig. 5a, 5b). At this stage the GW continues to mature and callosal fibers have not yet projected to the region of midline crossing. At e16, ephrinB1 expression was more restricted in the frontal cortex to structures such as the CC, Pfp, Ig, septum, as well as a discrete region of the cortex and superior portion of the lateral ventricle (Fig. 5c). This discrete region of the subventricular zone and cortex is thought to represent projecting radial glia (Shu et al., 2003b; Williams et al., 2003), which was confirmed by immunohistochemistry with the radial glia marker anti-RC1 (Fig. 5d). This RC-1 expression pattern mimics ephrinB1 immunostaining in the region of the dorsolateral ventricle (dLv), but disappears by e17 in development (not shown).

Colabeling ephrinB1 with β-tubulinIII (present in Pfp and CC axons) and Tag-1 (present mainly in CC fibers) supported the expression of ephrinB1 in both Pfp and CC fibers (merged yellow) (Fig. 5e, 5f). This expression is maintained through e17, the period at which many CC fibers have crossed the midline and extended into the contralateral hemisphere (Rash et al., 2001).

EphrinB2 expression was detected using a β-galactosidase (β-gal) enzymatic reaction on ephrinB2\textsuperscript{lacZ} knockin mouse tissue (Dravis et al., 2004), where the intracellular domain of ephrinB2 was replaced with a β-gal molecule. At e15, the ephrinB2 fusion protein was expressed in tissues that correspond to various cortical layers, subventricular zone, Ig, GW, and the region of CC and Pfp fiber growth (Fig. 6a, 6b). At e17, ephrinB2 fusion protein was highly expressed in CC fibers and regions just ventral to the anterior horn of lateral ventricles (Fig. 6c). Unlike other B-class ephrins, ephrinB2 was not found in the septal nuclei, but was maintained in lateral forebrain
regions throughout development. Controls show no β-gal reactivity in e18 wild type CD1 mice (Fig. 6c’).

EphrinB3 expression was detected using a β-gal enzymatic reaction on ephrinB3lacZ knockin mouse tissue (Yokoyama et al., 2001), and showed a similar profile to ephrinB1 in that it was also found in the septum, lateral migratory pathway, Ig, GW, and Pfp between time points e15-e18 (Fig. 7a-7e). Co-labeling using anti-β-gal against the ephrinB3 fusion protein and anti-GFAP confirmed expression in the Ig at e15 (Fig. 7c). EphrinB3 expression was mainly detected in the Pfp during all developmental time points, but little to no expression was observed in the region of CC fiber growth. At the midline, ephrinB3 is expressed both dorsal and ventral to the CC, forming a ‘channel’ through which callosal fibers migrate (Fig. 7d, 7e).

Expression of Eph Receptors in the Developing Forebrain

The expression of Eph receptors was localized to callosal fibers during the period of midline pathfinding in the developing WT forebrain. At e15, EphB1, EphB2, EphB3, and EphA4 receptors are all expressed in the CC fibers prior to midline crossing (Figs. 8 and 9). Both EphB1 and EphB3 receptor expression as determined by antibody labeling were primarily present in callosal fibers beginning at e15 (Fig. 8a, 8b) and continuing through e17 (not shown). EphB2 expression was evaluated using EphB2lacZ mice (Henkemeyer et al., 1996) and this fusion protein was present in superficial cortical layers, callosal fibers at e15-e17, as well as previously unreported areas corresponding to the GW and processes extending from subventricular zone (Fig. 8c-8f). Like EphB2, EphA4 receptor was detected in the GW, Ig, and callosal fibers at e15 (Fig. 9a, 9b), and co-labeled with the axonal marker Gap-43 in callosal fibers at e16 (Fig. 9c) and the glial
marker GFAP at e16 and e17 (Fig. 9d-9f). In addition, EphA4 was expressed in the striatum and the entire subventricular region (Fig. 9a). Immunoreactivity for each of the antibodies for EphA4, EphB1, or EphB3 was not observed in their respective knockout tissues (Fig. 10a-10c).

**Discussion**

In this study, we have shown that B-class ephrins and their respective Eph receptors are expressed in CC axons and surrounding areas during the critical period of CC development (summarized in Fig. 11). Our expression analyses indicates that ephrinB1 and ephrinB2 are located in the callosal fibers, while one of their binding partners EphB2 is located in the GW and Ig. Expression of ephrinB3 was detected mainly in important forebrain regions that include the GW, Ig and regions that make up the pre- and post-callosal perforating pathway. We also observed that EphB1, EphB2, EphB3 and EphA4 are all present in callosal fibers, while their potential binding partners (i.e. ephrinB1, ephrinB2, and ephrinB3) are all expressed in midline guideposts. The complementary expression of multiple ligands and receptors in callosal fibers or midline regions results in an extremely complex pattern of developmental events critical for proper axon guidance.

Co-expression of ephrins and Eph receptors in CC axons and their binding partners in guideposts suggests that both forward and reverse signaling may be necessary in developing axons. Recent investigations examining the co-expression of ligands and receptors in the same cell suggest that each ligand or receptor pool may be activated and signal independent of each other (Marquardt et al., 2005). Furthermore, this study also showed that activation of either ephrin or Eph receptor pools independently resulted in
either axon growth or growth cone collapse. This study examined EphA receptors and ephrinA ligands, so whether similar mechanisms exist in our system is unknown but partitioned membrane expression would be an excellent mechanism to regulate CC and even Pfp fiber attraction or repulsion. Interestingly, Pfp fibers express only B-class ephrins and partitioning of receptor and ligand pools would not occur in the membrane of these axons since the fibers only contain ephrinBs. According to the model developed by Pfaff and colleagues growth cone attraction may occur upon activation of these ligand pools. This would suggest that repulsion would not occur during binding of ephrins in Pfp axons and Eph receptors at guidepost regions suggesting growth cone promotion may be the dominating effect after receptor-ligand interaction.

We have hypothesized that interactions occur between Eph receptors present in callosal axons and ephrins in midline glia and vice versa. We cannot rule out the possibility that interactions might also occur between callosal fibers thereby influencing the levels of axon fasciculation/defasciculation. These molecules have been shown to play a role in the defasciculation of hippocampal axons at the lateral septum and fasciculation of cortical axons in culture (Chen, 2004; Winslow, 1995). We have not assessed whether or not single CC fibers express only ephrins, Eph receptors or both. Expression of complementary ephrins and Eph receptors on individual axon populations within the CC tract may allow for fasciculation or bundling of fibers. For example, EphB1 and ephrinB1 are both expressed in CC fibers, and if this receptor or ligand pair were expressed on separate axon populations and the two molecules were to interact then one result may be the fasciculation of axons before crossing the midline or defasciculation of fibers after crossing the midline. In the Pfp tract, only ephrins are
expressed so fasciculation of fibers within the tract may not occur, but interaction of ephrin (B1-B3) with specific Eph (B2, A4) receptors may allow sorting of these ipsilateral fibers from intermingling with CC fibers migrating through the corticoseptal boundary at the same developmental time period. It is difficult to evaluate axon fasciculation of CC or Pfp axons when abnormal structures such as Probst’s bundles are present, however, more detailed investigations of mutant mice may uncover an additional role for these molecules in either fasciculation or defasciculation.

Figures for Chapter II

Figure 4. Developmental timing of the corpus callosum (CC), perforating pathway (Pfp), and midline glial guideposts is normal in CD1 mice. Few anti-β-tubulinIII (β-tub) labeled callosal fibers crossed the midline at e15 (a) and e16 (b). By e17 many CC fibers have crossed the midline and project to the contralateral cortex (c). Anti-neurofilament (NF) (145kD) labeled Pfp fibers at e16 (d) and e17 (e). High-magnification image of anti-NF labeled Pfp fibers at the midline (f). High-magnification image of anti-β-tub labeled CC fibers (g) and anti-NF labeled Pfp fibers (h) at e17. Callosal axon growth is regulated by glial guideposts, including indusium griseum (Ig), glial wedge (GW), and midline zipper glial (mzg) as visualized using anti-GFAP at e18 (i). (a’) Dil tracing of cortical fibers at e15 show CC axons projecting to the midline but not crossing the midline (dashed line represents midline). (b’) Dil tracing of cortical fibers at e16 show that CC fibers have crossed the midline. (c’) No primary control. Scale bar represents 100μm.
**Figure 5. EphrinB1 is expressed in the developing forebrain.** At e15, ephrinB1 is expressed in forebrain regions corresponding to the cortex (C), septum (Se), indusium griseum (Ig), and lateral-medial tract (lmt) (a,b), while at e16 ephrinB1 is also expressed in regions that include the corpus callosum (CC), perforating pathway (Pfp), Ig, C, Se, and unique regions of the dorsal-lateral ventricle (dLv) (c). RC-1 is also expressed in the dLv (d) similar to ephrinB1 expression. At e16, ephrinB1 (green) co-labels with β-tubulinIII (red) in both the CC and Pfp fibers (yellow) (e). EphrinB1 (green) co-labels with Tag-1 (red) in CC (yellow) and not Pfp fibers (f). Scale bar represents 100 μm.
Figure 6. EphrinB2 is expressed in the developing forebrain. EphrinB2 expression was visualized by examining β-gal expression in the ephrinB2\textsuperscript{LacZ} mice. At e15, ephrinB2 expression is localized to regions corresponding to the C, CC, GW, Ig, Pfp, Striatum (St), and subventricular zone (SVZ), but absent in the Se (a-b). By e17, ephrinB2 expression localized to the C, CC, GW, and St (c). Scale bar represents 100μm.
Figure 7. EphrinB3 is expressed in the developing forebrain. EphrinB3 expression was visualized using ephrinB3^{lacZ} mice. At e15, ephrinB3 was expressed in the Pfp, Ig, Se, and lmt (a,b). Anti-β-galactosidase antibodies (green) colabeled with GFAP (red) in the Ig (yellow) (c). At e16 and e17, ephrinB3 expression is maintained in the Pfp, Ig, and Se, but little to no expression is observed in the CC (d,e). Scale bar represents 100μm.
Figure 8. Eph (B1-B3) receptors are expressed in the developing forebrain. EphB1 (a) and EphB3 (b) are expressed specifically in CC fibers. EphB2 expression was visualized by examining β-gal expression in the EphB2$^{lacZ}$ mice. At e15 and e16, EphB2$^{lacZ}$ expression was detected in areas corresponding to GW, CC, septum (Se), and regions of the subventricular zone (SVZ) (c, e). High-magnification images of EphB2 expression in the GW, CC, and SVZ regions (d, f). Scale bar represents 100μm.
Figure 9. EphA4 receptors are expressed in the developing forebrain. EphA4 was expressed in the regions of the CC, GW, Ig, striatum (St), and SVZ at e15 (a). High-magnification image of EphA4 expression at the midline in the CC, GW and Ig (b). EphA4 (green) and GAP-43 (red) are co-expressed in the CC but not in the GW or Ig at e16 (c). EphA4 (green) co-labels with GFAP (red) in the GW and Ig but not the CC at e16 and e17 (d-f). High-magnification image of EphA4 (green) and GFAP (red) in the GW (f). Scale bar represents 100μm.
Figure 10. Control immunohistochemistry images show antibodies for EphB1, B3 and A4 are specific. (a-c) represent control anti-EphB1, anti-EphB3, and anti-EphA4 immunoreactivity on EphB1KO, EphB3KO, and EphA4KO. Scale bar represents 100μm.
**Figure 11. Summary of expression of B-class ephrins and Eph receptors.** Ephrin and Eph receptor expression patterns superimposed with a schematic coronal cross section showing corpus callosum (CC), perforating pathway (Pfp) and midline guideposts (glial wedge (GW), indusium griseum (Ig), glial sling (gs), and midline zipper glia (mzg)) during development.
Chapter III

Gene targeted deletion of ephrinB3 and/or Eph receptors results in CC pathfinding abnormalities

Introduction

This chapter will examine a functional role for ephrinB3 and its receptors in the developing CC. EphrinB3 and Eph receptors (B1-B3 and A4) are expressed in both the CC and midline guideposts during critical periods of development and these experiments will investigate a functional role for these molecules. First, these experiments will investigate gene targeted knockout mice to determine whether the ephrinB3 and the Eph receptors (Eph (B1-B3 and A4)) observed in the developing forebrain have a functional role in establishment of this tract. These experiments are supported by investigations of growth and guidance molecule families and their role in CC development. For example, deletion of one of the three slit genes, slit-2, in mouse models resulted in CC fibers that form Probst’s bundles while other CC axon populations aberrantly extended through the GW into ventral forebrain regions due to lack of repulsive slit-2 and Robo receptor mediated mechanisms (Bagri et al., 2002). Attractive molecules such as netrin-1 and DCC have also been examined in mouse mutants and deletion of these genes resulted in Probst’s bundle formations; however, CC axons in these mice did not inappropriately grow through GW guideposts (Serafini et al., 1996; Fazeli et al., 1997). Other growth and guidance molecules that function as intracellular signaling proteins in axons such as GAP-43, Mena and Map1B have been investigated in mouse deletion mutants with all
gene targeted knockouts exhibiting similar CC Probst’s bundle formations (Shen et al., 2002; Lanier et al., 1999; Meixner et al., 2000).

Similar studies investigating EphB2 and EphB3 receptors, which were both shown to be expressed in the CC, have revealed that these receptors are necessary for CC development (Dottori et al., 1996). Deletion of both genes resulted in increased frequency of defects (i.e. Probst’s bundle formations) compared to single knockout mice indicating that EphB2 and EphB3 carried out compensatory functions. EphB2 and EphB3 have also been investigated for their function in regulating hippocampal neuron axon fasciculation or bundling while targeting ventral subcortical septal targets and deletion of these genes results in reduced axon bundling in this system (Chen et al., 2004). Furthermore, EphB2 regulates AC development by mediating reverse signaling in ephrinB1-expressing pAC axons (Henkemeyer et al., 1996).

The data in this chapter will explore both single and combination ephrins and Eph receptor knockout mice to determine if additional receptors (i.e. EphB1 and EphA4) or ligands (i.e. ephrinB3) are involved in compensatory mechanisms during CC development. Lastly, forward and reverse signaling functions will be examined using truncated mutants as previously described for CST and AC tracts (Yokoyama et al., 2001; Henkemeyer et al., 1996). In sum, these data will explore novel and complex genetic interactions that occur ephrins and Eph receptors during CC development.

Material and Methods

Cresyl-Violet staining and histological analysis of the corpus callosum

Using time pregnant females, embryonic brains from appropriate time periods were obtained, sectioned and mounted as previously described under immunohistochemical
techniques. Sections were stained in cresyl violet staining solution (0.1% cresyl violet acetate, 1M sodium acetate, 1M glacial acidic acid, 95% ethanol; Sigma, St. Louis, MO) for 1 minute followed by 1-minute rinses in alcohol solutions (50%, 70%, 90%, 95%, 100%, and xylene), and finally imaged using brightfield microscopy. Sample numbers are indicated for each genotype examined.

**Quantification of corpus callosum thickness**

Sections from postnatal day one mice were obtained as previously described for cryosection immunostaining. Sections (30μm) were stained with anti-β-tubulinIII 1:500 (Covance, Denver, PA) antisera diluted in 5% BSA/0.1% TritonX-100 for 1 hr at RT followed by overnight at 4°C. On the following day sections were rinsed 3 times for 5 minutes each in 1X PBS and the incubated with Texas-Red (594nm) conjugated secondary antibody 1:250 (Jackson ImmunoResearch, West Grove, PA). Sections were rinsed 3 times for 5 minutes in 1X PBS, imaged and photographed using a fluorescent microscope. CC thickness (at the level of the hippocampal commissure) was measured from the dorsal to the ventral aspect of CC by measuring the straight line distance between these two points using Zeiss interactive measurement software. At least five coronal sections for each genotype were quantified using this software. Statistical significance was determined using one-way ANOVA with Bonferroni’s correction in Prism 3.02 statistical software.

**Results**

**Genetic analysis of callosal defects in ephrinB3 and Eph receptor knockout mice.**

The data detailed in Chapter 2 indicate that B-class ephrins and their Eph receptors are expressed in both callosal fibers and guidepost regions at the period of
midline pathfinding. To examine how ephrins and Eph receptors may influence midline guidance in the CC, we examined the postnatal (P1) morphology of the CC in various single and double mutant mice as compared to WT littermates (Fig. 12). CC defects were classified into two groups as defined by gross morphological appearance; (1) any visible defect in development of the CC and fewer β-tubulinIII positive fibers at the midline (or hypoplasia) as compared to WT mice, which included both mild and severe phenotypes (Fig. 12b), and (2) mice that had a severe agenesis of the CC and visible Probst’s bundle formations (Fig. 12c) (representative Nissl-stained sections from various single and double-KO mice are listed in Fig. 13). Probst’s bundle formation and hypoplasia have been described in a number of mutant mouse strains and are reliable indicators of CC defect (Richards et al., 2004). First, we evaluated the genetic propensity for CC deformities strictly associated with the CD1 mouse strain, and found no observable defects. We then examined ephrinB3\textsuperscript{KO} mice, since this ephrin is expressed in forebrain guideposts (i.e. GW and Ig) and it may be a key regulator of midline guidance for Eph receptor-expressing CC axons. We found that 84% of ephrinB3\textsuperscript{KO} mice exhibited some form of CC hypoplasia, while 64% had a severe ACC phenotype with Probst’s bundles. Histological analysis was confirmed using the axonal specific antibody β-tubulinIII, which showed that the thickness of the CC at the midline in ephrinB3\textsuperscript{KO} mice (199μm ± 10.72; n=8) was significantly less compared to WT mice (276μm ± 10; n=15) (Fig. 14). CC defects were not observed in ephrinB3\textsuperscript{lacZ} mice, which retain the ability to activate Eph receptors, suggesting that Eph receptor forward signaling is important for CC development. EphB1\textsuperscript{KO}, EphB2\textsuperscript{KO}, EphB3\textsuperscript{KO}, and EphA4\textsuperscript{KO} mice were then evaluated; only EphB1\textsuperscript{KO} (87%) and EphB2\textsuperscript{KO} (61%) showed CC defects with 43% and 13% having
severe ACC defects, respectively. CC hypoplasia in EphB1\textsuperscript{KO} mice was confirmed by measuring CC thickness (113.4\,μm ± 7.527; n=7) at the midline, which was significantly less compared to WT mice. EphB3\textsuperscript{KO} and EphA4\textsuperscript{KO} mice did not show observable CC defects in Nissl stained sections, and quantitation of the CC thickness at the midline in EphB3\textsuperscript{KO} (238\,μm ± 12; n=17) and EphA4\textsuperscript{KO} (204\,μm ± 12; n=5) mice revealed no significant difference as compared to WT mice (276\,μm ± 10; n=15) (Fig. 14).

Interestingly, EphB2\textsuperscript{lacZ} mice did not have CC defects (ACC or hypoplasia) or differences in CC thickness (251\,μm ± 11; n=9) as compared to WT (Fig. 14), suggesting that EphB2-mediated forward signaling is not critical for CC midline guidance. This may reflect an important requirement for ephrinB reverse signaling in CC axons.

To evaluate genetic interactions, we examined multiple combinations of Eph receptor double mutant mice. When EphB1\textsuperscript{KO} was crossed with either EphB2\textsuperscript{KO} or EphB3\textsuperscript{KO} mice (i.e. EphB1\textsuperscript{KO}/EphB2\textsuperscript{KO}, EphB1\textsuperscript{KO}/EphB3\textsuperscript{KO}) there was no gross observabledifference in phenotypic penetrance as compared to EphB1 deficiencies alone. This suggests that EphB2 or EphB3 may not provide compensatory functions in the absence of EphB1. Examination of EphB2\textsuperscript{KO}/EphB3\textsuperscript{KO} mice showed 100% penetrance, supporting a compensatory role for these two receptors and a function for EphB3 in CC midline guidance. However, evaluation of EphB2\textsuperscript{lacZ}/EphB3\textsuperscript{KO} mice did not reveal a similar phenotype, even though these mice are deficient in both EphB2 and EphB3 signaling. This supports a ‘ligand’ like role for EphB2 in regional guideposts, where the absence of EphB2 would result in CC deficits mediated by a reduction in ephrinB reverse signaling in CC axons. Like EphB2, EphA4 receptors are also expressed in regional guideposts. However, unlike EphB2, EphA4 has not been shown to bind ephrinB1 or
ephrinB2 (Blits-Huizinga, 2004). Examination of EphA4 single and double knockout mice did not reveal a function for this receptor in CC midline crossing, which was confirmed by assessing CC thickness where no differences were observed between WT (276μm ± 10; n=15) and EphA4\textsuperscript{KO} (204.1μm ± 11.85; n=5) (Fig. 14) CC thickness, and no Probst’s formations in EphB3\textsuperscript{KO}/EphA4\textsuperscript{KO} mice. Interestingly, examination of EphB1\textsuperscript{KO}/EphA4\textsuperscript{KO} mice revealed a decrease in penetrance as compared to EphB1\textsuperscript{KO} mice, although it remains to be determined why the absence of EphA4 results in a reduction in hypoplasia.

We observed ephrinB3 expression in regional guideposts and Pfp fibers but little to no expression in CC fibers. Mice deficient in both ephrinB3 and EphB1 revealed 100% penetrance in CC defects. The ephrinB3\textsuperscript{KO}/EphB2\textsuperscript{KO} mice were not significantly different from ephrinB3\textsuperscript{KO} mice alone, while ephrinB3\textsuperscript{KO}/EphA4\textsuperscript{KO} mice showed a reduction in penetrance. This further supports a role for EphA4 in CC development, although it is unclear what the role may reflect. Interestingly, in ephrinB3\textsuperscript{KO}/EphB3\textsuperscript{KO} mice there were no animals that displayed CC hypoplasia or Probst’s bundle formation phenotypes. These data were confirmed by axonal staining which showed that the CC thickness at the midline in WT mice (276.4μm ± 10.17; n=15) was not significantly different than ephrinB3\textsuperscript{KO}/EphB3\textsuperscript{KO} mice (301.2μm ± 22.75; n=17) (Fig. 14).

**Discussion**

In this chapter, using gene-targeted knockout mice we show that ephrinB3 and Eph receptors are critical for midline guidance of CC fibers. EphrinB3\textsuperscript{KO} mice have significant defects in CC midline crossing that are not observed in the ephrinB3\textsuperscript{lacZ} knockin mice, suggesting that activation of forward signaling through Eph receptor(s) is
important. This is supported by the expression of ephrinB3 in midline guideposts and expression of Eph receptors in CC axons. Furthermore, this function is consistent with previous observations of CST decussation at the midbrain where ephrinB3 acted as a ligand initiating forward signaling of Eph receptors expressed on CST fibers (Yokoyama et al., 2001). EphB2, EphB3, and EphA4 receptors are all potential binding partners for ephrinB3 and have the possibility of mediating this forward signaling (Blits-Huizinga et al., 2004). However, only EphB2 can independently mediate CC midline crossing because single knockout mice exhibit CC defects. EphB3 seems to be limited to compensatory functions since CC defects are only exhibited when this gene is deleted in combination with another receptor or ligand gene. Previous investigations have evaluated ephrinB3KO mice and report that approximately 20% of knockout mice have CC agenesis (Yokoyama et al., 2001). Our data support a more critical function for this ligand, while other tracts such as the anterior commissure and hippocampal commissure remained unaffected as previously reported.

Earlier reports also indicate EphB2 and EphB3 are important in CC development and these receptors have overlapping functions during CC development (Orioli et al., 1996). Our results support a much more complex set of genetic interactions during development and modify existing genetic data that explain the interaction between EphB2 and EphB3 receptors. We did not observe CC defects in single EphB3KO mice as previously shown (Orioli et al., 1996). By contrast, EphB2KO mice exhibited a higher frequency of CC defects while EphB3KO mice had no observable defects of the CC compared to WT mice. We did confirm previous results showing that removal of both receptors dramatically increased the frequency of defects. One explanation for these
conflicting results may be that our animals were analyzed in a pure CD1 background while previous analyses were performed in a mixed 129/sv and c57B1/6J background. Neither strain have significant incidence of spontaneous CC agenesis so an explanation for these conflicting results remains to be determined. The absence of midline defects in the EphB2\textsuperscript{lacZ} knockin mice, suggests that EphB2 functions in a kinase-independent manner, which is in agreement with previous findings concerning AC formation (Henkemeyer et al., 1996; Cowan et al., 2004). This would support a hypothesis that the expression of EphB2 in midline guidepost cells may be more important for midline axon guidance than its expression in the CC axons. These findings imply that ephrinB1 and/or ephrinB2 signaling in callosal fibers may also be important for CC midline pathfinding and/or growth promoting decisions (unfortunately ephrinB1\textsuperscript{KO} and ephrinB2\textsuperscript{KO} mutant mice were not available for CC analysis). EphrinB1 and ephrinB2, which are known to have repulsive functions in both vertebrate and \textit{Xenopus} model systems, are two good candidates for binding with EphB2 (Williams et al., 2003; Mann et al., 2003). It is unclear whether ephrinB1 and ephrin2 function to repel developing CC axons at the forebrain midline, and additional studies would reveal whether repellent mechanisms initiated by these interactions function to guide CC fibers. Together, our data indicate that CC axons require both reverse and forward signaling to mediate midline guidance.

Combination mutation of EphB1 and either, EphB2, EphB3, or EphA4 did not result in increased frequencies of CC defects compared to single knockout mice. By contrast, EphB2\textsuperscript{KO}/EphB3\textsuperscript{KO} and ephrinB3\textsuperscript{KO}/EphB1\textsuperscript{KO} mice did exhibit overlapping functions since data show a genetic interaction between these gene pairs. Since ephrinB3 is expressed in guideposts and not CC fibers, we interpret these findings to reflect an
absence in signaling from multiple Eph receptors (including EphB1) in CC fibers. The ephrinB3\textsuperscript{KO}/EphB1\textsuperscript{KO} mutation results in complete penetrance with most animals having severe ACC. There was not a significant increase in CC defects observed in the ephrinB3\textsuperscript{KO}/EphB2\textsuperscript{KO} mice, while ephrinB3\textsuperscript{KO}/EphA4\textsuperscript{KO} mice exhibited a decrease in CC agenesis compared to ephrinB3\textsuperscript{KO} mice alone. In ephrinB3\textsuperscript{KO}/EphB3\textsuperscript{KO} mice, which had no abnormalities of either type, there was a complete rescue of the abnormal phenotypes observed in ephrinB3\textsuperscript{KO} mice.

These data suggest that the role of the EphB3 gene during CC development involves extremely complex interactions with both ephrinB3 and EphB2. Removal of EphB3 with the EphB2 gene resulted in 100% CC defects while removal of EphB3 with ephrinB3 reveals completely opposite results. In Chapter II, we showed that EphB2 is expressed in both the CC and GW guideposts while EphB3 is only expressed in the CC. Deletion of both the EphB2 and EphB3 gene prevents EphB2 functions in both guideposts and the CC while EphB3 no longer functions in simply the CC. Thus, removal of both EphB2 and EphB3 may prevent multiple forward and reverse signaling functions for this pair, hence the high frequency of CC abnormalities. By contrast, ephrinB3 is expressed mainly in the GW and Ig and genetic data from β-gal knockin mice suggest an important function for this ligand in activating forward signaling in the CC. Removal of either the ephrinB3 or EphB3 gene only eliminates functions in guideposts or CC fibers, respectively. We could speculate that since this ligand and receptor pair functions to direct CC development in two distinct developmental locations with EphB2 regulating axonal functions and ephrinB3 regulating glial function, Combined deletion is not as deleterious since either axon or glial functions are
individually inhibited. Other receptor or ligand genes present in these locations may compensate for EphB2 and ephrinB3 functions. However, this does not explain the rescue of the abnormal phenotype. Additionally, deletion of EphB3 together with the ephrinB3 gene may allow for molecular interactions to occur between another ephrin, Eph receptor or other axon growth and guidance family members (i.e. slits-2, Robo-1, Robo-2, DCC, or netrin-1). Recently, investigations in *C. elegans* have uncovered direct interactions with a member of the Vab-1 Eph receptor and Sax-3/Robo receptor during embryogenesis (Ghenea et al., 2005). It is unknown whether similar protein interactions occur in vertebrates or even what functions these interactions may serve. However, interactions between Eph (e.g. EphB3) and Robo receptors would need to be regulated and loss of either receptor may result in phenotypic abnormalities such as the Probst’s bundles observed in our mutant mouse models. A mechanism for the observations we observed in EphB3 single or double mutant mice is unknown, but these findings support a complex genetic interaction between multiple ephrins and Eph receptors in regulating CC midline guidance.
Figures for Chapter III

Figure 12. Frequency of CC Probst’s bundle and hypoplasia formation in single and double-knockout ephrin/Eph receptor mice. Representative Nissl-stained coronal section of a normal CC phenotype (a). Nissl-stained section showing a representative CC hypoplasia phenotype from knockout tissue (b). Representative Nissl-stained section exemplifies complete agenesis of the CC (ACC) with greater than 90% of axons not crossing the midline and the formation of Probst’s bundles (c). Frequencies of CC hypoplasia or ACC in single and combination mutant mice at postnatal day one compared to WT CC phenotypes (d).

<table>
<thead>
<tr>
<th>Genotype</th>
<th>% with CC hypoplasia</th>
<th>% with ACC (Probst’s bundles)</th>
<th>n-value</th>
</tr>
</thead>
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<td>wild type</td>
<td>0%</td>
<td>0%</td>
<td>40</td>
</tr>
<tr>
<td>ephrinB3KO</td>
<td>84%</td>
<td>64%</td>
<td>37</td>
</tr>
<tr>
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<td>0%</td>
<td>12</td>
</tr>
<tr>
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<td>87%</td>
<td>43%</td>
<td>47</td>
</tr>
<tr>
<td>EphB2KO</td>
<td>61%</td>
<td>13%</td>
<td>23</td>
</tr>
<tr>
<td>EphB2lacZ KO</td>
<td>0%</td>
<td>0%</td>
<td>15</td>
</tr>
<tr>
<td>EphB3KO</td>
<td>0%</td>
<td>0%</td>
<td>22</td>
</tr>
<tr>
<td>EphA4</td>
<td>0%</td>
<td>0%</td>
<td>5</td>
</tr>
<tr>
<td>EphB1KO/EphB2KO</td>
<td>88%</td>
<td>60%</td>
<td>22</td>
</tr>
<tr>
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<td>20</td>
</tr>
<tr>
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<td>12%</td>
<td>15</td>
</tr>
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<td>67%</td>
<td>7</td>
</tr>
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<td>0%</td>
<td>5</td>
</tr>
<tr>
<td>EphB3KO/EphA4KO</td>
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<td>0%</td>
<td>18</td>
</tr>
<tr>
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</tr>
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<td>0%</td>
<td>18</td>
</tr>
<tr>
<td>ephrinB3KO/EphA4KO</td>
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<td>29%</td>
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Figure 13. Representative Nissl-stained sections from postnatal day 1 ephrin/Eph receptor knockout mice reveal morphological changes of the corpus callosum (CC) at the midline. Coronal sections show normal CC phenotype in wild-type (WT) (a) and EphB3\textsuperscript{KO} (c), ephrinB3\textsuperscript{KO}/EphB3\textsuperscript{KO} mice (h). CC Probst’s bundle formations are shown in EphB1\textsuperscript{KO}/EphB2\textsuperscript{KO} (b), EphB1\textsuperscript{KO}/EphB3\textsuperscript{KO} (d), ephrinB3\textsuperscript{KO} (e), ephrinB3\textsuperscript{KO}/EphB1\textsuperscript{KO} (f), EphB2\textsuperscript{KO} (g), and EphB1\textsuperscript{KO} mice (i). CC hypoplasia phenotypes are shown in ephrinB3\textsuperscript{KO}/EphA4\textsuperscript{KO} mice (j).
Figure 14. Postnatal comparison of CC thickness at the midline in WT compared to single or double-knockout ephrin/Eph receptor mice using β-tubulinIII axon specific markers. CC thickness at the midline was quantified for each genotype listed. Values listed are the average CC thickness at the level of the hippocampal commissure for a minimum of (n=7) mice.
Chapter VI

General Discussion

**Complementary expression patterns of ephrins and Eph receptors**

In the developing forebrain, we have shown that callosal fibers express ephrins (B1-B2) and Eph (B1-B3, A4) receptors. Midline guideposts and callosal axons express ephrins and Eph receptors in a complementary fashion with Ephs (B1-B3, A4) receptors in axons and ephrins (B1-B3) at the midline. Likewise, callosal axons may express the ephrins (B1-B2), while their appropriate binding partners, which include EphB2 and EphA4, are expressed at midline guidepost regions. Contrary to the expression findings in the developing CC, Pfp fibers express only ephrins (B1-B3) during all points of development and may interact with the EphB2 and EphA4 receptors, which are expressed at strategic guidepost regions at the midline.

**Is there topographic mapping of callosal fibers in the forebrain?**

A potential function for this complementary expression of ephrins and Eph receptors in either axons or midline guideposts may be in developing a sort of topographic map of callosal fibers within the cerebral cortex. In the retinal system of the mice for example, investigators have established that one function of complementary Eph receptor and ephrin expression is direct the establishment of topographic maps along the lateral-medial (LM) axis of the superior colliculus (SC) (Hindges et al., 2002; Birgbauer et al., 2000; Lemke et al., 2005). Genetic deletion of receptors such as EphB2 and EphB3 results in disruption of appropriate retinal axon targeting along the LM axis of the SC (Hindges et al., 2002). Examination of the retinotectal system revealed that Eph
receptors are expressed in a gradient pattern along the dorsal-ventral (DV) retina, while complementary B-class ephrins are expressed in countergradient patterns along the LM axis of the SC. Specifically, EphB receptors are expressed in low to high concentrations along the DV axis of the retina, while ephrins are expressed in low to high concentrations along the LM axis of the SC. These studies also showed that one potential function for gradient expression of ephrins is to attract EphB-expressing retinal axons along the LM axis of the SC (Hindges et al., 2002). More recent data investing the retinal ganglion cell development showed that ephrins can stimulate neurite outgrowth at low concentrations while inhibiting growth at high concentrations (Hansen et al., 2004). In the developing CC, pioneer callosal fibers reach the midline by e15, while axons that extend later bundle or fasciculate with each other as they migrate along this pre-established pioneer tract (Richards et al., 2002). After crossing the midline, which occurs at e16, callosal axons must de-fasciculate so that they may target appropriate partners in the contralateral cortex. Our expression data resembles the complementary expression of ephrins and Eph receptors in the retinotectal system. This observation suggests that a potential function for B-class ephrins and Eph receptors may be in directing the targeting of contralaterally projecting callosal axons to appropriate medial or lateral cortical targets. To support this, some of our in vitro results showed that cortical neurons extended longer neurites when grown on ephrinB-Fc substrates, which is similar to the growth promoting functions described for these molecules in the retinal system (see Chapter IV; Hindges et al., 2002). Despite the similarities in complementary expression of ephrins and Eph receptors in the retina and CC, there are several differences between the two systems. First, we did not observe gradient patterns of ephrins or Eph receptors in the developing forebrain as
observed in the retina (McLaughlin et al., 2005). This does not rule out the possibility that ephrins and Eph receptors may be expressed at non-midline guideposts that have yet to be described. In our in vitro studies using WT and EphB1\(^{KO}\) cortical neurons grown on ephrinB-Fc substrates, we did not observe a difference in growth responses to varying concentrations of ephrinB-Fc (6\(\mu\)g/mL vs. 0.6\(\mu\)g/mL). This suggests that unlike in the DV retinal system, the growth promoting functions of EphBs and ephrinBs observed in the CC may be independent of ligand or receptor concentrations in order to cross the forebrain midline (Lemke et al., 2005). Another difference is that we observed CC axons that completely stalled at the midline, which is different than the altered targeting of appropriate regions along the LM axis of the SC (Hindges et al., 2002). Despite these differences, our expression observations along with prevalent knowledge of retinal axon pathfinding suggest that one function of this guidance family in the forebrain may be to attract EphB-expressing axons across the midline to appropriate LM or DV targets expressing ephrinB ligands.

**Roles for co-expression of Eph receptors and ephrins in callosal neurons**

An important observation from our expression data involves the co-expression of both ligands and receptors in the CC. Expression of ephrins and Eph receptors in the same cell requires that subsequent signaling events that occur downstream be tightly regulated to allow proper axon targeting. Recently, Pfaff and colleagues (2005) described that this is accomplished by ephrins and Eph receptors because they are segregated into distinct membrane domains in motor neuron axons, with each Eph receptor or ephrin domain capable of being activated independent of each other (Marquardt et al., 2005). Activation of ephrins resulted in expansion of the growth cone,
while activation of Eph receptors resulted in retraction of the growth cone. These data suggested that ephrin and Eph receptor signaling events can be functionally uncoupled with ephrins capable of directing a specific cellular event (i.e. growth cone spreading) independent of Eph receptors, which direct inhibitory signaling events (i.e. growth cone retraction) in the same cell. Similarly, in the developing CC, ephrin and Eph receptor signaling must be tightly regulated since single callosal neurons may express both B-class ephrins and Eph receptors. We have not investigated how callosal neurons regulate Eph and ephrinB signaling, but one mechanism may involve the uncoupling of Eph versus ephrin signaling as observed in the developing neuromuscular system (Marquardt et al., 2005). For ephrin and Eph receptor mediated mechanisms to occur in callosal neurons as described for developing motor neurons, these molecules must be segregated into distinct membrane domains. We have not conducted confocal microscopy experiments to examine whether a single callosal neuron expresses both ephrins and Eph receptors. In our system, different populations of callosal fibers may instead express ephrins while other populations express Eph receptors which would allow events such as defasciculation and axonal sorting to occur before and after crossing the midline (Chen et al., 2004). We have also not conducted experiments to investigate whether these molecules are segregated into different callosal fiber membrane domains. If ephrins and Eph receptors do have these expression characteristics in developing callosal neurons, then the model established for motor neuron development could be adaptable to the CC, however, another mechanism that may also be useful is the regulation of ephrin or Eph receptor signaling complexes. EphB receptors are capable of developing alternative responses by discriminating dimeric (i.e. unclustered) from multimeric (i.e. clustered)
forms of ephrinB ligands (Stein et al., 1998). This discrimination of different ligand oligomeric forms leads to the recruitment of molecules such as low-molecular weight protein tyrosine phosphatase (LMW-PTP) when higher oligomeric forms (i.e. hexamers) of ligands are used to stimulate Eph receptors as opposed to lower oligomeric forms (i.e. tetramers). Although is not known how downstream signaling proteins such as LMW-PTP are involved in Eph receptor signaling, it is clear that higher order oligomeric forms of ephrins are capable of initiating distinct signaling events. In the developing forebrain, presentation of different oligomeric forms of Eph receptors or ephrins expressed at strategic CC guidepost regions may influence the signaling proteins recruited downstream during forward or reverse signaling (Fig. 22).

Due to the fact many of the guideposts, molecular players, and axonal targets are known, the CC is an ideal model system to examine gradient or partitioned expression of ephrins and Eph receptors and the subsequent effects on signaling and cellular function. It would be interesting to investigate whether any of the mechanisms described for regulating retinal or motor neuron development are also used during CC neurite outgrowth. At face value, our in vivo data do not support differential functions for ephrinB3 and Eph receptors, because all mutant mice show similar hypoplasia or agenesis defects. However, the complexity of both CC axon and guidepost ephrin or Eph receptor expression and their roles in developmental glial migration make it difficult to evaluate independent functions. To aid in evaluating distinct axonal functions for ephrins or Eph receptors, single cell axon tracing using biotinylated dextran amine (BDA) may be useful in evaluating callosal axon phenotypes in different mouse mutants (Parent et al., 2005). Recently, three dimensional imaging techniques such as magnetic resonance
imagery (MRI) technology have been modified to evaluate axonal populations in vivo. This high resolution technology has allowed investigation of AC phenotypes in living EphB2 and EphA4 null mutants. These investigations showed that the axon disruption seen in each of these null mutants actually differed in the abnormal routing of AC axons from one another in several important morphologic respects (Ho et al., 2004). In EphB2<sup>KO</sup> mice, there were defects in the routing of pAC axons, while in EphA4<sup>KO</sup> mice there were mainly disturbances in the defasciculation of aAC axons. These advances in technology may help differentiate alternative functions for independent ephrins and Eph receptors in the developing CC system.

**Combined analysis of expression, genetic, and functional data reveal important developmental roles in axons and glia**

Our studies have demonstrated that the complementary expression of ephrins and Eph receptors in either CC axons or surrounding guideposts is matched with equal complexity in genetic functions. We have determined that individual genes such as those coding for EphB1 and EphB2 receptors are necessary for CC development, while others such as EphB3 and EphA4 do not play critical roles. Deletion of two Eph receptor genes (i.e. EphB2<sup>KO</sup>/EphB3<sup>KO</sup> mice) resulted in increased frequencies of CC defects suggesting these Eph receptors compensate for one another, while combinatorial deletion of other receptors (EphB1<sup>KO</sup>/EphB3<sup>KO</sup>; EphB1<sup>KO</sup>/EphB2<sup>KO</sup>) resulted in no change in frequencies as compared to single knockout mice.

**Proposed roles for Eph receptors and ephrins in callosal axons**

Our data showed that Ephs (B1-B3, and A4) were all expressed in the developing CC. Combining these observations along with our genetic data from single knockout
mice, it is clear that the EphB1 receptor seems to play a major role in the growth of callosal fibers. Furthermore, our *in vitro* data implicate EphB1 receptors, which are only expressed in callosal fibers, in promoting cortical neurite outgrowth. To explain this ability of EphB1-ephrinB interactions on neurite outgrowth, we may explore *in vitro* data that suggest that EphB receptors are capable of activating intracellular signaling cascades that promote actin polymerization and cell motility (Vindis et al., 2004; Parsons et al., 2002; Harte et al., 2002). For example, intracellular signaling cascades involving molecules such as c-Src, NIK, paxillin, and FAK are linked to activated EphB1 receptors. These signaling cascades have been shown to regulate actin polymerization and promote chemotaxis of human renal microvascular endothelial cells (HRMEC) (Vindis et al., 2003). We have shown that EphB1 receptors promote cortical neurite outgrowth and the signaling cascades described for chemotaxis of various cell lines may be used by EphB1 receptors in the endogenous growth cones of developing cortical neurons. *In vitro* studies investigating hippocampal neuron development have also determined that EphB2 receptors regulate intracellular signaling molecules in dendritic spines such as Rac1 and Cdc42, which have been shown to be critical factors in actin polymerization (Penzes et al., 2003). Similar signaling cascades must be investigated in callosal neurons, where receptors such as EphB1, EphB2, or EphB3 are present and may activate molecules in the Rac1 and Cdc42 pathway and promote neurite outgrowth (Fig 23).

Our EphB1 neurite outgrowth data are consistent with experiments that described EphA5-ephrinA5 mediated CC pathfinding mechanisms (Hu et al., 2003), where ephrinA5 promoted the outgrowth of neurites from purified callosal neurons. In EphA5 mutant animals, CC fibers failed to enter the commissure resulting in a thinning of the
CC or hypoplasia suggesting that loss of the Eph-ephrin interactions reduced the ability of axons to grow through the midline. These in vivo results for EphA5 receptors are similar to what we observe in EphB1\textsuperscript{KO} mice, where a high percentage of these animals show hypoplasia phenotypes. Other investigations have shown that e13.5 cortical neurons have increased neurite outgrowth when grown on ephrinA5, ephrinA2, and ephrinB1 (Zhou et al., 2001; Gao et al., 2000), and retinal neurons require low concentrations of ephrins to promote outgrowth (Hansen, 2004). Together, these data and our results expand the role of ephrins and Eph receptors to function as not only repulsive molecules, but also as potential growth promoting factors in the developing CNS. Although we suggest that EphB1 receptors play a role in neurite outgrowth, we cannot rule out the fact that this receptor as well as other Ephs expressed in callosal fibers may mediate repulsive effects during development of the CC. Historically, these molecules have been thought of as primarily repulsive (Kullander et al., 2001; Yu et al., 2001; Wahl et al., 2000). Ephs (B2-B3 and A4) are also expressed in the developing callosal fibers and these receptors may mediate similar growth promoting functions, but based on current evidence in the field, these molecules may also carry out certain repulsive functions during CC targeting (Tong et al., 2003). To support a repulsive role for ephrins and Eph receptors during callosal fiber pathfinding, it is helpful to examine other guidance families in this developing system. The slit ligands have been shown to be important repulsive guidance cues during this developmental event (Bagri et al., 2002). These molecules, which are expressed in guidepost areas (i.e. Ig and GW) surrounding the developing callosal fibers, prevent improper axon targeting into dorsal or ventral forebrain regions directing extension across only the midline. This mechanism
has been termed ‘surround’ repulsion which aids in channeling axons through this region. We have established that ephrins (B1-B3) are expressed in similar patterns while Eph receptors are present in axons setting up a potential function for these molecules in a surround repulsion mechanism. To accomplish this, Eph receptor activation would need to result in repulsion of axons away from sources or ephrin expression and we have not yet shown this to be a function of these molecules. Likewise, we have not investigated the role of ephrins in callosal axons. Ephrins (B1-B2) are expressed in developing callosal fibers, and what role these molecules play remains elusive. Experiments have shown that exposure of retinal axons to the extracellular domains of EphB receptors results in growth cone collapse (Birgbauer et al., 2001). By contrast, in developing motor neurons, activation of ephrinB-reverse signaling results in growth cone expansion (Marquardt et al., 2005). Additionally, the GEF for Rac1 called Tiam has been shown to interact with activated ephrinB1 ligands and this interaction is critical for the outgrowth promoting functions (Tanaka et al., 2004). These studies indicate that ephrins can elicit both repulsive and attractive responses in axons, so a necessary question remains to be answered in the callosal neurons. Does EphB stimulation result in decreases or increases in the size of ephrin-expressing callosal growth cones? Our experiments have not addressed this point, and it would be interesting to understand the role of ephrins in the developing CC as well as the signaling proteins and pathways activated downstream of these ligands. Examination of ephrinB mutant mice would also be useful in answering some of these questions, but we have been unable to examine ephrinB2 and ephrinB1 complete null or truncation mutants. These future experiments as well as the data we have already described may provide clues to the process by which callosal axons fail to
cross the midline in the mouse. These data may also provide clues to the failed mechanisms that contribute to human CNS disorders which exhibit similar phenotypes.

Separate \textit{in vitro} data from our experiments showed that \textit{EphB1}^{KO} cortical neurons have reduced ability to grow on GW cell substrates. This would imply that either cortical neurons developed increased sensitivity to repulsive cues or decreased sensitivity to growth promoting cues. The \textit{in vitro} EphB1-ephrinB interaction data previously described suggest that the latter is a more suitable explanation for these observations. However, these two sets of data cannot be directly linked and to truly show that cortical neurons in knockout animals have decreased neurite outgrowth capabilities, a more suitable experiment may be to knockdown ephrinB1 or ephrinB2 expression in GW feeder layers, which have cortical neurons growing on them, using techniques such as ribonuclease interference (RNAi). This would allow a downregulation of ephrinB1 or ephrinB2 expression in GW feeder layers growing \textit{in vitro}, and aid in determining whether cortical neurons extend longer neurites in the presence of these ligands. This may be a more direct approach to the answer the question of whether cortical neurons from knockout mice do indeed have reduced ability to extend neurites at critical midline structures. Not mentioned previously, one problem with the \textit{in vitro} system that we have chosen to use is that we are not evaluating purified callosal neurons, which make up only approximately 2-3\% of the total neuronal population in the forebrain. Instead we are examining a heterogeneous population of cells, which include various neuronal and glial cells (Richards et al., 2002). A more appropriate system would be to purify callosal neurons from e17 tissue using reagents such green-fluorescent microsphere retrograde tracers. These fluorescently tagged proteins can then be used to isolate callosal neurons
from non-callosal neurons using fluorescence activated cell sorting (FACS) to separate out all fluorescently tagged cells. After this process surviving purified callosal neurons may be cultured in vitro as has been done in the past (Catapano et al., 2004). Using purified callosal neurons, we would then be able to reliably differentiate callosal vs. non-callosal axonal mechanisms. Additionally, injection of DiI into one hemisphere and using this as a retrograde marker of callosal neurons may be another approach to isolate callosal neurons in a similar fashion (Hu et al., 2005). Either of these methods would allow us to truly elucidate whether the function of ephrin-Eph receptor interactions at the midline are responsible for growth promotion in callosal neurons. Additionally, callosal neurons must initiate unique signaling cascades, which result in their axonal pathfinding decisions, and using purified neuronal populations may also provide another advantage in allowing investigation of these novel signaling pathways.

Although, our in vitro data do not confirm a role for EphB1 in regulating callosal neurite outgrowth at the midline, we have determined that this receptor regulates the sensitivity of cortical neurons to ephrin growth and guidance cues. This switch in axonal sensitivity may be attained through regulating signaling cascades activated downstream of other Eph receptors or it may involve interactions with other guidance cue families such as the slits (Stein et al., 1996; Ghenea et al., 2005). Protein interactions between Eph receptors and Robo receptors have been uncovered in C. elegans. In the developing CC, a failure to regulate slit repulsive activity could result in axons that are unable to cross the midline. This has been observed in Drosophila mutants where Robo receptor expression is not downregulated by molecules such as Comm, which directs the subcellular sorting of Robo receptors. Disruptions of this mechanism result in
commissural axons that fail to cross the midline because Robo receptors are present to sense slit repellent cues prematurely (Keleman et al., 2005). There is one caveat to the idea that the Eph/ephrin family may interact with Robo receptors to regulate slit sensitivity. Unlike embryonic spinal cord axons, callosal fibers respond to slit repellent cues before and after crossing the midline (Shu et al., 2003d). This would suggest that slit repulsive activity is not regulated by Eph receptors or ephrins and the slit guidance family functions throughout all time points of CC development.

Integration of guidance signals within the growth cone

Our expression, genetic and in vitro data show that callosal neuron development is regulated by a number of ephrins and Eph receptors. In addition to this, a number of other guidance molecules have been shown to function in CC development and these include GAP-43, slit, netrin, and L1 CAM (Richards et al., 2004). All of these molecules elicit distinct signaling pathways and axonal responses, so it is critical that each signal be integrated properly within the growth cone so that accurate targeting of axons may occur during development (Song et al., 1999). Many axon guidance cues necessary for callosal fiber development have been discovered, so it would also be interesting to determine how ephrin/Eph receptor signals are integrated along with the other guidance receptors that are present at similar time points. These investigations would require that each of these molecules be expressed at similar CC developmental time points (i.e. e15-e18) and also that receptors be able to ‘talk’ or regulate each other’s signaling pathway. This may occur through direct interactions between different guidance receptors or may involve cross-talk between intracellular signaling proteins (Stein et al., 2001; Gallo et al., 2004). Direct protein interactions are involved in Robo and DCC receptor interactions, while
signaling crosstalk has been shown to involve the Rho-family GTPases (RhoA, Rac1, Cdc42). Many of the guidance receptors responsible for proper CC development also use Rho-family GTPases as intracellular signaling proteins (Yuan et al., 2003; Song et al., 1999). During axon growth and guidance, it has been shown that different guidance receptor families regulate each other by controlling the activity of these Rho-family GTPases (Lu et al., 2001). An intriguing question to ask would be whether the integration of signals detected by different guidance receptors within callosal fibers involves direct protein-protein interactions and/or regulation of Rho-family GTPase signaling proteins such as Cdc42, Rac1, or RhoA.

**Proposed role for ephrins and Eph receptors in midline glia**

Our analyses of ephrinB3KO mice revealed that this molecule plays an important role during CC development. First, our expression data showed that this molecule is mainly expressed in areas dorsal (i.e. Pfp) and ventral (i.e. Ig and GW) to developing callosal fibers. Second, our genetic data for ephrinB3 truncation mutants suggested that the extracellular domain of this ligand was important in mediating forward signaling in Eph-expressing callosal axons. Lastly, our *in vivo* data from complete null mice indicated that ephrinB3 ligands expressed in GW and Ig regions are important for regulating positioning of glial cells at the midline. The lack of glial migration defects that we observed in ephrinB3lacZ mice would support an interaction with Eph receptors or other growth and guidance molecules in midline glial regions. Specifically, our observation of glial positioning in complete null mice and not in truncation mutants suggests that the extracellular domain of ephrinB3 interacts with EphB2 and EphA4 receptors, which are also expressed in the GW and Ig guidepost regions. This interaction
would activate forward signaling cascades, which result in maintaining proper positioning of EphA4 and EphB2-expressing glial cells. Loss of ephrinB3 eliminates this interaction and results in aberrant positioning of glial cells that prevent proper callosal fiber development (Fig. 24). EphA4 and EphB2 receptors have been shown to bind ephrinB3 and initiate functional interactions during various developmental processes (Blitz-Huizinga et al., 2005; Gale et al., 1996). Furthermore, these molecules activate forward signaling cascades that regulate cellular processes such as dendritic spine morphology and neural crest cell chemotaxis (Penzes et al., 2003; Smith et al., 1997). Interestingly, evidence from studies evaluating cell migration in zebrafish embryos supports our model that ephrinB3 interactions with EphA4 and EphB2 promote glial positioning in midline guideposts (Mellitzer et al., 1999). This study showed that ephrin-Eph receptor interactions served to restrict cell intermingling in the developing hindbrain. The investigators examined different populations of cells that express EphA4 or EphB2 receptors in one population while the ligands ephrinB1 or ephrinB2 were expressed in another. Disruption of ephrin-Eph receptor interactions using complete-null or truncation mutants resulted in abnormal cell intermingling suggesting that the both the extracellular and intracellular domains of these molecules were necessary for cell positioning. Similar mechanisms have been examined in Xenopus embryos where EphB1 and EphA4 receptors have been shown to interact with ephrinB2, which functions to restrict intermingling of neural crest cells (Smith et al., 1997). In our system, interactions between ephrinB3 and the Ephs (B2 and A4) may act in a similar fashion restricting glial movement into inappropriate regions during development of the CC. One limitation to our model is that we do not know if single glial cells express all three molecules (i.e.
ephrinB3, EphB2, and EphA4) or whether distinct populations of glial cells express different molecules (i.e. ephrinB3, EphB2, or EphA4). Confocal microscopy using glial specific markers such as anti-GFAP and labeling with the ephrin and Eph receptor specific antibodies may allow us to determine whether glial cells express all three molecules or simply one or two. Understanding the spatial distribution of ephrinB3 and Ephs (B2 and A4) would aid in understanding the mechanism of this developmental event and the important signaling pathways that may be activated downstream of this ligand and its receptors. Another limitation to our model is that ephrins and Eph receptors have been shown to have adhesive functions during several developmental events (Poliakov et al., 2004; Irie et al., 2005). Specifically, in zebrafish embryos is has been shown using morphalino oligonucleotides, which eliminate protein expression by blocking translation, that EphA4 receptors are critical for hindbrain boundary formation because blocking these interactions results in improper intermingling of neuronal nuclei from different rhombomeres (Cooke et al., 2005). The investigators further described an adhesive function for EphA4 during positioning of cells within hindbrain rhombomeres. These data imply that ephrinB3 interactions with any of its receptors may also function to promote cell adhesion of glial cells within any of the guideposts. The observation that we observed in ephrinB3\(^{\text{KO}}\) mice could then be due to the inability of cells within the GW, Ig, or other guideposts to adhere to one another and remain appropriately positioned. Another limitation to our analyses of abnormal positioning of midline glia in ephrinB3\(^{\text{KO}}\) mice is that we do not know if these cells actually originate from the GW, Ig or other glial guidepost regions. Injection of viral vectors encoding fluorescently tagged molecules such as yellow-fluorescent protein (YFP) or green-fluorescent protein (GFP)
into GW or Ig guidepost regions during early development time points for these midline guideposts (e.g. e14) and analysis of these tissues at later developmental time points may allow differentiation of exactly what cellular regions these aberrantly positioned glial cells arise. Similarly, injection of Dil crystals into the ventricular space at early embryonic time points in mouse embryos would also allow labeling of GW cells. This technique has been done successfully to determine that GW cells are part of a radial glial scaffold within the developing mouse forebrain (Shu et al., 2003d). This method would benefit our studies because we could potentially label GW cells with Dil in ephrinB3KO mice, which have abnormal positioning of glial cells, and determine if the aberrantly positioned cells we observed in ephrinB3 mutants actually are derived from the GW. This latter method of labeling GW cells would only aid in determining if glial cells in mutant animals arose from the GW region, but it would not help determine whether aberrantly positioned glial cells in our mutants are derived from other glial regions such as the Ig or Mzg.

This novel function of ephrinB3 in the regulation of glial migration at the corticoseptal boundary is consistent with recently published data showing that slit-2 and slit-3 serve to direct glial cell positioning in the zebrafish forebrain (Barresi et al., 2005). Specifically, Robo-expressing glial cells are repelled from slit2/slit3-expressing regions, which restrict these glial cells to bands that span the midline. Removal of slit-2 or slit-3 genes resulted in abnormal positioning of glial cells along the midline, which led to midline crossing defects of postoptic commissure axons. Interestingly, direct protein interactions between Vab-1 Eph receptor and Sax-3/Robo receptor have been discovered in C. elegans, however, the functions of these interactions remain unknown (Ghenea et
Since we have determined that ephrins play a role in the glial positioning necessary for proper midline axon guidance, it would be interesting to investigate whether these molecules cooperate with slit ligands or Robo receptors during CC development. Although the slit and ephrin data in glial positioning are consistent one important difference in the two mechanisms involves the expression profile of the respective ligands and receptors. In the slit model describing glial positioning in the zebrafish, investigators established that slit (slit 1-3) expression was restricted to domains surrounding the glial cells, while the glial cells themselves expressed the receptors for slit (Robo-1 and Robo-2) (Barresi et al., 2005). This is different than what we observe for the expression of ephrinB3 and its receptors EphB2 and EphA4 in the midline guideposts of our system. We observed that glial cell guideposts expressed both ligand and receptors, while no domains of receptor or ligand expression were present surrounding the glial cells as observed in the slit/Robo zebrafish model. The spatial distribution of slits and Robos in zebrafish forebrain during glial cell positioning seems to be quite different than the distribution of ephrins and Eph receptors in the mouse forebrain, suggesting that glial cell movement is maintained via local interactions that result in maintence of proper glial guideposts. Another difference in the two models is that we do not know if Eph receptors and ephrins in our system function to repel or restrict the movement of glial cells as described for the slits and Robos in the zebrafish. If this is not the case, then another function for ephrins and Eph receptors in glial cell positioning may be in adhesion of these cell types, which has been shown to be one function of this family during rhombomere formation (Cooke et al., 2005).
Distinct midline guideposts and independent B-class ephrin expression in Pfp development

We investigated whether ephrins and/or Eph receptors expressed in critical CC midline guideposts also play a role in Pfp development. We have shown interesting ephrin (B1-B3) expression patterns that are specific to Pfp fibers, while two potential binding ligands EphB2 and EphA4 are present at strategic locations to potentially interact and regulate Pfp fiber growth and guidance (Shu et al., 2001b; Blitz-Huizinga et al., 2004). This chapter investigated a) the importance of CC midline guideposts in Pfp axonal pathfinding and b) whether ephrins can regulate axon pathfinding in the absence of Eph receptors in the same cell. EphrinB3 and EphB1 mutants, which both exhibit abnormal midline glial positioning, do not show defects in Pfp axon targeting as shown in Fig. 25.

This observation does not support a role for CC midline glia in mediating Pfp axons growth responses. These results are consistent with previous experiments by other investigators, which show that reduction or loss of the cells that make up the GW and Ig does not disrupt development of the Pfp (Shu et al., 2003d). Previous results as well as our data suggest that unique guideposts at the corticoseptal boundary regulate the ipsilateral routing of this fiber tract. We have hypothesized that Pfp fibers use expression of Eph receptors at strategic guideposts (also used by CC axons) to maintain their ipsilateral orientation. If common guideposts are not used by Pfp fibers, then another potential mechanism that may be used by these fibers to maintain an ipsilateral orientation is the regulated expression of growth and guidance molecules during axonal routing. Mechanisms similar to this have been described in the visual system where
retinal neurons upregulate the expression of EphB1 in ipsilateral routing axons to restrict their growth across the optic chiasm (Williams et al., 2003). In our system, regulated expression of B-class ephrins at the midline crossing decision point would be a useful mechanism to prevent Pfp axons from crossing the midline. Additional expression analyses of ephrinB1 and ephrinB2 would be necessary to determine the temporal expression profile of these molecules in relation to Pfp axon pathfinding at the midline.

Another interesting observation in the Pfp tract was the expression of only B-class ligands within the tract. To test the hypothesis that ephrin reverse signaling independently (no Eph receptor expression in the same cell) regulates the development of an axon tract, we examined the Pfp tract in ephrinB3KO mice. We observed no abnormalities in this axon tract suggesting that this ligand is not critical for Pfp development. This does not exclude the possibility that ephrinB3 may be important for other processes such as postsynaptic structure formation or synaptic plasticity in the Pfp system as described for the hippocampus (Murai et al., 2003; Grunwald et al., 2004; Klein et al., 2004). One explanation for proper Pfp development in the absence of ephrinB3 is that other ephrins may compensate for loss of ephrinB3 function. EphrinB1 and ephrinB2 are both candidates for compensatory functions and it would be of interest to evaluate EphB2KO mice, since absence of EphB2 induced reverse signaling (by ephrinB1 or ephrinB2) may address overlapping functions for these ligands. EphB2 has been shown to have the greatest binding affinity with ephrinB1 and ephrinB2 and would thus play a major role in the activity of these two ligands (Blitz-Huiizinga et al., 2004).

Lastly, sorting of B-class ephrin-expressing Pfp axons from CC axons could also occur at the corticoseptal boundary during development. In this system, B-class ephrins
can potentially interact with EphB2 and EphA4 at the midline resulting in bundling or fasciculation of Pfp axons preventing aberrant growth into the CC tract. This function of Eph receptors and ephrins has been described in hippocampus. Specifically, neurons within the hippocampus send axons to septal targets and defasciculation of these axons was shown to be regulated by EphB2 and EphB3 receptors in hippocampal neurons interacting with ephrinB3 at septal targets (Chen et al., 2004). This ephrin and Eph receptor mediated sorting at the midline would allow for maintenance of axons within proper fiber tracts preventing inappropriate extension of Pfp axons across CC midline crossing regions.

**Future directions**

*A role for multiple growth and guidance family interactions during CC development.*

We have provided extensive evidence indicating that ephrins and Eph receptors contribute significantly to the development of the CC. We have discussed evidence from literature indicating that multiple axon growth and guidance families play a role in CC development. It is well known that during development growth cones integrate multiple signals (Dent et al., 2003; Cooper et al., 2002). Specifically, the netrins and slits play critical roles in CC development (Shu et al., 2003; Bagri et al., 2002; Fazeli et al., 1997; Serafini et al., 1996). It is also well known that interactions between these two growth and guidance families occur during development which result in regulation of axonal attraction and migration of neurons (Stein et al., 2001; Giger et al., 2001; Causeret et al., 2002). Recent evidence from experiments in *C. elegans* indicates that there are also molecular interactions between VAB-1 Eph receptor and SAX-3/Robo receptors and that this interaction is important during embryonic morphogenesis (Ghenea et al., 2005).
Additionally, *in vitro* studies using chick retinal neurons indicate a convergence of signaling pathways used by both Eph receptors and Robo receptors (Wong et al., 2004). Specifically, ephrinA5 and slit2 initiate signals that lead to repulsion, and this signaling pathway converges on phosphatidylinositol 3-kinase (PI3K) and Src family kinases. Pharmacological inhibition prevented both ephrinA5 and slit-2-mediated growth cone collapse. These two forms of evidence involving both direct (receptor-receptor) or indirect (signaling pathway crosstalk) indicate that there is a potential for interactions between the ephrins and Eph receptors and other axon guidance and growth families. Overlapping gradients of growth and guidance cues force axons to integrate multiple axonal cues during embryogenesis (Chisholm et al., 1999).

Mechanisms used by growth cones to integrate and respond to multiple guidance cues remain poorly understood and the CC system seems to be a perfect model system to investigate growth cone integration (Song et al., 2001; Dent et al., 2003). To begin to examine whether interactions between Eph receptors/ephrins occur, co-immunoprecipitation experiments would be conducted. Specifically, interactions between EphB1 receptors, which we showed to be important for CC development, and Robo-1/2 or DCC may be explored. Precise regulation of growth cone attraction or repulsion is critical for proper callosal fiber routing and as we have described all these growth and guidance families are expressed during development in the CC system (i.e. CC axons and midline guideposts) (Richards, 2002). Direct interactions between these proteins would be an excellent way to regulate growth through the midline, which has been described as a mechanism used by the slit and netrin family (Stein et al., 2001).
**Are ephrin and Eph receptor compensatory mechanisms used during Pfp development?**

Our analyses of ephrins and Eph receptors in development of the Pfp did not resolve whether these molecules play a developmental role in the formation of this tract (see Chapter VI). Specifically, we were unable to show that although critical CC midline glial cells were aberrantly positioned in ephrin3 and EphB1 mutant mice, Pfp innervation of the cingulate cortex was altered. Additionally, these experiments also showed that removal of ephrinB3 from Pfp fibers did not result in inability of axons to extend and reach appropriate cingulate targets.

We propose that combinatorial ephrin and Eph receptor mutant mice must be examined to confirm whether or not this family plays a developmental role in the Pfp. Our investigations as well as other studies have shown that compensatory mechanisms exist within the ephrin and Eph receptor family (Dottori et al., 1996). Specifically, it would be interesting to perform DiI tracing experiments in additional single knockouts such as EphB2\(^{KO}\) and EphA4\(^{KO}\) (which are expressed in the GW and Ig, two strategic locations for Pfp axon growth and guidance). Examination of Pfp development in these knockout mice would be advantageous since both receptors are expressed at potential guideposts and this may eliminate interactions with not only ephrinB3 but also ephrin (B1-B2) that are also expressed in Pfp axons and may function to compensate for loss of ephrinB3 ligands.
Figures for Chapter VI

**Figure 22. Schematic representation of a cortical fiber interacting with the GW guidepost.** Callosal axons contain both reverse and forward signaling mediated by ephrins and Eph receptors following interactions with either EphB2 or ephrinB3 signaling complexes in midline guideposts, respectively.
Figure 23. EphB1-ephrin interactions positively regulate cortical neuron neurite outgrowth. Proposed signaling pathways critical for EphB1 induced neurite outgrowth. 
(a) In wild-type neurons, EphB1-expressing cortical axons interact with ephrinB1 or ephrinB2 ligands expressed in the glial wedge guidepost. This interaction may initiate forward intracellular signals that involve molecules such as intersectin, N-WASP, S-Src, NIK, or Kalirin. These molecules may be linked to the activation of Cdc42, paxillin, or Rac1, which may then activate molecules such as Arp2/3, FAK and PAK. The final result of each of these signaling pathways downstream of EphB1 activation is neurite outgrowth. Functional roles for Eph (B2-B3) and EphA4 expressed in callosal fibers remain elusive. 
(b) In EphB1\textsuperscript{KO} neurons, these signaling pathway(s) are not activated, growth cones collapse, and growth extension is inhibited.
Figure 24. EphrinB3-Eph receptor interactions direct midline glial cell positioning.

a) In wild-type mice, interactions between ephrinB3 (green) with either EphA4 (yellow) or EphB2 (red) receptors expressed within the same cell types regulates the positioning of glial cells, which allow proper midline crossing of callosal fibers. 
b) Deletion of the ephrinB3 gene (i.e. ephrinB3^{KO} mice) eliminates the ability of this ligand to interact with EphB2 and/or EphA4 receptors, and results in aberrant positioning of glial cells at the midline and subsequent improper targeting of callosal axons.
Figure 25. Schematic representation of CC and Pfp growth patterns in the ephrinB3 and EphB1 knockout mice. Corpus callosum (CC) fibers stall at the midline forming Probst’s bundles in mutant mice (e.g. ephrinB3\(^{\text{KO}}\), EphB1\(^{\text{KO}}\) and EphB2\(^{\text{KO}}\)). CC fibers in mutants project to the midline but stall prior to crossing the midline comprising glial wedge (GW) and indusium griseum (Ig) guideposts; ‘glial’ sling cells; midline zipper glia (mzg). Perforating pathway (Pfp) axons however, continue to project dorsally and ventrally targeting appropriate cingulate and medial septum targets.
Chapter V

*Perforating pathway fibers use distinct guideposts for ipsilateral routing of axons.*

**Introduction**

The stereotypical growth and guidance of individual fiber tracts is achieved despite the fact that many axon tracts develop during similar time periods each requiring a unique guidance mechanism (Shu et al., 2001b; Demyanenko et al., 2003; Hankin et al., 1988; Long et al., 2004). Some of these fiber tracts include the optic chiasm, corticospinal tract, AC, and CC, which make individual and exact axon trajectories (Yokoyama et al., 2001; Kullander et al., 2001; Henkemeyer et al., 1996; Colamarino et al., 1995; Plump et al., 2002; Williams et al., 2003). Many of these axon fibers cross the midline and enter the contralateral side of the CNS and never cross back, while others never cross the midline and remain ipsilateral (Tear, 1999; Kullander et al., 2001; Shu et al., 2001b). To complicate the scenario, the embryonic midline is encountered by many of these axon tracts at similar developmental time points; however, each fiber tract makes a distinct and predictable decision at this choice point. How this remarkable accuracy is achieved during development remains poorly understood.

In the developing forebrain, two fiber tracts that may be characterized by this complexity include the CC and Pfp with each extending axons to the corticoseptal boundary at similar developmental time points (e15). These two tracts make distinct decisions to either cross the midline extending into the contralateral hemisphere (CC fibers) or remain ipsilateral bisecting the CC during axon extension (Pfp fibers). Development of the Pfp takes place between e15 and e17 with neurons in both the
cingulate cortex and medial septum/Diagonal band of Broca extending axons in ventral and dorsal directions, respectively (Shu et al., 2001b; Hankin et al., 1988). The majority of axons that make up the Pfp in the developing mouse forebrain are derived from neurons in the medial septum/Diagonal band of Broca (Shu et al., 2001b).

The trajectory of Pfp fibers in vivo suggests that certain guidance factors at the midline direct the growth and/or guidance of these fibers, yet the exact guideposts and guidance factors necessary for proper development of Pfp fibers remains completely unknown (Shu et al., 2001b; Richards et al., 2002). Specifically, Pfp fibers have both dorsal and ventral projections in the forebrain avoiding the GW and the midline fusion region (Shu et al., 2001b). These studies suggest that there are guidepost structures and molecules that regulate the development of the tract at these specific regions. A few studies have investigated the molecules and structures that may be involved in development of this tract; however, none of these investigations have determined a clear role for any growth or guidance families in Pfp development. For example, the slit-2 ligands are expressed in the GW and Ig regions during Pfp developmental time points. However, Robo-1 and Robo-2 receptors are not expressed by Pfp neurons, so it remains unknown whether slit ligands play a role in the growth or guidance of Pfp axons (Shu et al., 2003a; Shu et al., 2003b; Shu et al., 2001b; Bagri et al., 2002; Richards et al., 2002). DCC receptors have also been investigated in Pfp axons and this molecule is not expressed in the tract (Shu et al., 2001). Other netrin receptors have not been examined, so it remains unclear whether this growth and guidance family plays a role in Pfp development.
A role for GW and Ig guideposts in Pfp development has also been investigated by taking advantage of developmental abnormalities in nuclear factor I (NFi) knockout mice. The absence of Nfia, which is a transcription factor, results in GW and Ig glial cells that are greatly reduced in number or absent (Shu et al., 2003d). In these mice, the CC forms Probst’s bundles with axons failing to cross the midline, while the Pfp continues to develop normally. These data imply that the glia within the GW or Ig do not play a significant role in Pfp pathfinding; however, unknown cues released from other cell types in or surrounding these guidepost regions may continue to function during Pfp tract formation. The ‘glial’ sling, which begins developing at e15, consists of migrating neurons and is another midline guidepost used by CC axons during development (Silver et al., 1982; Shu et al., 2003d). This structure does not seem to play a role in Pfp axonal pathfinding because these fibers extend directly through this guidepost ignoring cues that may be expressed by this structure (Shu et al., 2001b). Although the cue(s) and choice points used by Pfp fibers are unknown at this point, it is clear that these growth and guidance cues differ from those used by CC axons during development.

Evidence suggests that one class of growth and guidance molecules that may play a role in development of the Pfp is ephrins and Eph receptors. The roles of these molecules have been examined in development of some retinal axons at the optic chiasm that remain ipsilateral similar to Pfp fibers. During growth and guidance, retinal axons make decisions at the optic chiasm to cross in a contralateral trajectory or remain ipsilateral in the CNS. The decision to remain ipsilateral and not cross the optic chiasm depends on mechanisms that involve ephrins and Eph receptors. A specific study by Henkemeyer and colleagues (2003) showed that ephrins are expressed appropriately at
the midline in specific guidepost cells, where EphB1 receptors are expressed in axons that function to regulate the routing of ipsilateral retinal axons and non-EphB1 receptor expressing axons continued to cross the midline (Williams et al., 2003). It is believed that ephrins and Eph receptors regulate these functions through either contact-mediated inhibitory mechanisms or attractive mechanisms (Munoz et al., 2005; Castellani et al., 1998).

Our studies have revealed that a potential mechanism exists to regulate Pfp axon growth and guidance where ephrins expressed in axons interact with Eph receptors expressed at appropriate midline regions (where Pfp fibers make distinct turns), which functions to regulate the ipsilateral routing of Pfp fibers. GW and Ig guidepost regions are two locations where Pfp fibers may encounter growth and guidance cues since these structures are regions where Pfp fibers make distinct turns and are present during Pfp fiber targeting between e15 and e17 (Shu et al., 2001b). In this chapter, we evaluate Pfp development in both EphB1\textsuperscript{KO} and ephrinB3\textsuperscript{KO} mice. Both mutant mice exhibit severe defects in midline glia (i.e. GW and Ig) with radial glial cells inappropriately positioned at the midline. This chapter will investigate whether abnormal migration of these critical CC guideposts affects Pfp fiber targeting that occurs at similar developmental time points and spatial regions. Our studies will focus on ephrinB3, which is mainly expressed in the Pfp and capable of signaling through both EphA4 and EphB2 receptors that are expressed at strategic midline regions (Gale et al., 1996; Himanen et al., 2004).
Materials and Methods

**Immunohistochemistry**

Immunohistochemistry was performed as described in Chapter II. Briefly, postnatal day 1 pups were anesthetized on ice. Brains were dissected, immersed in 4% paraformaldehyde (PFA) (Fisher Biotech) made up in 1X phosphate buffered saline (PBS) for 1 hour. Sections for GFAP immunohistochemistry were embedded in 3% agarose (Gibco-BRL) and incubated free-floating in anti-GFAP 1:3000 (DAKO) or anti-neurofilament (NF) 1:500 (Chemicon) and then incubated with anti-rabbit or anti-mouse fluorescent secondary antibody 1:500 (Molecular Probes) for 1 hour. Sections were then photographed using a fluorescent inverted microscope (Zeiss Axioscope).

**1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) tract tracing or 3,3′-dioctadecyloxacarbocyanine perchlorate (DiO) tract tracing**

DiI tracing was performed similar to Chapter IV. Briefly, postnatal day 1 mice were anesthetized on ice and perfused with 4% PFA and brains were post-fixed for 24 hours at 4°C. For tracing of the corpus callosum, a 10% DiI (D-282) was used to saturate a nitrocellulose membrane (Bio-Rad, Hercules, CA). After drying of the solution in the material, small pieces of the membrane were placed in the medial septum. Brains were then embedded in 3% agarose, vibratome sectioned at 100μm, immunostained with anti-GFAP antibody, and photographed under FITC (GFAP) or Rhodamine (DiI) illumination.
Results

Normal Pfp projection pattern in both WT and ephrinB3\textsuperscript{KO} CD1 mice.

Previous investigations in C57BL mice have shown that Pfp fibers avoid the GW during guidance and make sharp turns when encountering this corticoseptal region remaining ipsilateral in the forebrain (Shu et al., 2001; Hankin et al., 1988). DiI tracing of the Pfp and double-labeling the tissue with anti-GFAP, which labels critical midline guideposts, confirmed that in CD1 WT mice Pfp axons extending ipsilaterally toward ventral (medial septum) and dorsal (cingulate) regions making sharp turns at the GW and avoid crossing of the CNS midline (Fig 19a) (Shu et al., 2001b). Immunolabeling ephrinB3\textsuperscript{KO} mice forebrain tissue with anti-GFAP and anti-NF, which preferentially labels the Pfp, revealed that Pfp fibers continue to extend towards appropriate targets in the cingulate cortex (Fig. 19b). These data indicate that the location of midline guideposts and turning of Pfp fibers is similar in WT CD1 mice and ephrinB3\textsuperscript{KO} mice, suggesting that ephrinB3 is not essential for Pfp pathfinding.

Pfp fibers extend toward appropriate cingulate cortex targets in EphB1\textsuperscript{KO}

To examine whether Pfp fibers are affected by Probst’s bundle formation and glial positioning defects independent of ephrin and Eph receptor signaling, we examined Pfp projections in EphB1\textsuperscript{KO} mice. EphB1 is expressed in the CC but not Pfp fibers or glial guideposts, and EphB1\textsuperscript{KO} mice have abnormal positioning of midline glial structures. To determine whether Pfp fibers were re-routed in EphB1\textsuperscript{KO} mice, we examined the dorsal projection pattern of the fiber tract using anti-NF markers. Immunostaining of the Pfp tract in WT mice revealed that axons extend toward appropriate cingulate cortex targets.
making sharp turns at the GW and avoid midline crossing (Fig. 20a). Similar results were observed in EphB1\textsuperscript{KO}, where Pfp axons projected through and around Probst’s bundles toward appropriate cingulate cortex targets demonstrating that Pfp axons are also a component of the Probst’s bundles (Fig 20b-c). However, unlike CC axons, Pfp axons seem not to terminate in the Probst’s bundle formation, although it is unclear whether these axons represent DBMS or cortical projecting neurons (Fig. 20b-c). These data suggest that Pfp fibers project to appropriate targets despite the formation of CC Probst’s bundles and abnormal migration of glial cells at the midline.

**DiI tract tracing confirms that Pfp fibers reach appropriate targets in ephrinB3\textsuperscript{KO} mice**

Our studies have shown that the Pfp fibers, and not CC fibers, express ephrinB3 ligands that may potentially interact with Eph receptor expressing guideposts. To confirm whether or not Pfp fibers extend towards appropriate targets in ephrinB3 deficient mice, we investigated dorsally projecting Pfp fibers using the axonal tracer DiI. Analysis of Pfp fibers in either postnatal day 1 WT (n=8) or ephrinB3\textsuperscript{KO} mice (n=6) revealed that Pfp fibers projected to appropriate cingulate cortex targets (Fig. 21). Despite the disruption of midline glia and the formation of Probst’s bundles consisting of CC fibers, Pfp axons continued to make sharp turns at appropriate corticoseptal targets migrating through and around Probst’s bundle formations towards the cingulate cortex (Fig. 21a-d). Furthermore, Pfp fibers continue to reach their targets in the absence of ephrinB3 reverse signaling in Pfp neurons. Pfp fibers are visible extending axons near Probst’s bundles and through GFAP-positive processes (Fig. 21b). Interestingly, DiI positive Pfp fibers mainly avoid the GW guidepost, suggesting that there are still
guidance cues present in ephrinB3KO midline tissue. These mechanisms may be mediated by ephrins/Eph receptors, but also may involve slits, or some other repellent growth and guidance cue expressed in midline glial regions.

**Discussion**

These data indicate that Pfp development is unique and involves mechanisms different from those used by callosal neurons. Previous investigations have suggested that Pfp axons respond to GW guidance cue(s) during pathfinding, while avoiding the midline fusion region (Shu et al., 2001b). To examine the hypothesis that Pfp axon growth and guidance is regulated by cues released at these particular midline glial regions, we have examined Pfp development in EphB1KO and ephrinB3KO mice with known CC and midline glial structural defects. Unlike CC axons, Pfp axons uniquely express ephrinB3 (along with ephrinB1 and ephrinB2), and do not express any of the Eph receptors known to bind B-class ephrins. We have determined that ephrinB3 in the absence of Eph receptors does not regulate Pfp pathfinding. We also found that abnormal midline glial structures found in both ephrinB3KO and EphB1KO mice do not alter the pathfinding abilities of Pfp axons. Together, these studies demonstrate that while non-callosal Pfp axons develop in the same spatiotemporal period as callosal fibers, they exhibit independent guidance mechanisms that do not involve ephrinB3 and CC glial influences.

There is evidence from both *in vitro* and *in vivo* studies that show ephrin-expressing fibers are repelled or inhibited when appropriate Eph receptor gradients are encountered during axon extension (Drescher et al., 1995; Wilkinson et al., 2000; Brors et al., 2003; Orioli et al., 1997; Kullander et al., 2003; Birgbauer et al., 2000). EphrinB3
expression in Pfp fibers and reciprocal expression of its binding partners EphA4 and EphB2 in strategically located GW and/or Ig regions supports a potential role for ephrin and Eph receptor interactions at the midline to restrict the growth and guidance of Pfp fibers. We did not observe aberrant growth of Pfp fibers into the contralateral hemisphere or into abnormal ipsilateral targets in ephrinB3KO mice suggesting that ephrinB3 does not solely regulate pathfinding of these fibers. However, we cannot rule out that ephrinB1 and/or ephrinB2 may compensate for lack of ephrinB3 ligands. These two ligands are expressed in the Pfp during development and have been shown to interact with EphB2 and/or EphA4, suggesting a potential compensatory role for ephrins similar to previously described mechanisms used during CC development (Frisen et al., 1997). Further evaluation of combination ephrinB or ephrinB/EphB2 or EphA4 combination mutants may reveal similar compensatory mechanisms that also have been previously uncovered in the CC (see Chapter III).

It is also interesting that in the presence of abnormal glial structures, Pfp fibers seem to grow through and around the Probst’s bundle formation. These findings are supported by studies that examine NFia knockout mice, where there are reduced or complete lack of glial cell development within the GW and Ig (Shu et al., 2001d). In these mice, the Pfp develops normally while callosal fibers fail to cross the midline forming Probst’s bundles similar to what has been observed in our system. This investigation did not support a role for these two guideposts in Pfp development. Similarly, our work does not establish these midline glial guideposts as major components in the Pfp developmental system because disruption of the normal architecture of the glial structures did not result in Pfp abnormalities. Some caveats to
previous studies using Nfia-mutants as well as ours involves the possibility that partial loss or disruption of the GW or Ig glia may still allow for proper Pfp development. Similarly, our studies do not reveal any additional information about the midline guideposts necessary for Pfp development since ephrinB3 and EphB1 mutant mice have intact GW and Ig structures in our analyses. Clearly some signal(s) either attractive (e.g. netrins) or repulsive (e.g. slits, semaphorins, untested Ephs/ephrins) present in guidepost regions and receptors in Pfp fibers must continue to regulate pathfinding of these axons in the mutant mice we evaluated.

In summary, our studies focusing on Pfp pathfinding may require analysis of other single or double-combination mutants since multiple Eph receptor-ephrin interactions may occur during Pfp targeting allowing for compensatory mechanisms that we have previously observed in the CC. Further studies evaluating the exact role of growth and guidance cues released from the GW and Ig regions are necessary. Although is it unclear what role ephrinB3 plays in Pfp development, one clear point is that the midline glial populations necessary for CC may not be critical for proper Pfp development. Further studies are necessary to determine how these axon tracts (i.e. CC and Pfp) that develop at similar time periods use precise localization and timing of ephrins and Eph receptors to establish unique and stereotypical axon trajectories.
Figures for Chapter V

Figure 19. Pfp fibers migrate toward appropriate targets in WT and ephrinB3\textsuperscript{KO} mice. Immunohistochemistry (IHC) and DiI tract tracing reveal that Pfp fibers migrate around Probst’s bundle formations and extend towards cingulate cortex targets. (a) DiI injected in the medial septum and anti-GFAP (green) double labeling in WT mice reveals that Pfp fibers make distinct turns at both the GW and Ig while extending to rostral and caudal ipsilateral forebrain targets. (b) IHC in ephrinB3\textsuperscript{KO} mice reveals that Pfp fibers (green) continue to avoid GW and Ig GFAP-positive regions (red) and Probst’s bundles (Pb) contain Pfp fibers. Scale bar represents 100\textmu m.
Figure 20. Probst’s bundles include Pfp fibers in EphB1\textsuperscript{KO} mice. Neurofilament-145Kd expressing (green) Pfp fibers co-labeled with GFAP expressing (red) guideposts (including GW, Ig and mzg) in WT mice (a). Neurofilament expressing (green) Pfp fibers co-labeled with GFAP expressing (red) guideposts (including GW, Ig and mzg) in EphB1\textsuperscript{KO} mice (b). High-magnification image showing NF-labeled Pfp axons extending through and around the Probst’s bundle (c). Scale bar represents 100μm.
Figure 21. Dil tracing confirms Pfp fibers reach appropriate targets in ephrinB3KO mice. Dil implanted in the medial septum confirms that rostrally migrating perforating pathway (Pfp) fibers (red) in ephrinB3KO mice extend towards the cingulate cortex. (a-d) Representative images of Dil implanted in the medial septum, which labels the majority of the Pfp tract, reveals that Pfp fibers migrate and continue to make distinct decisions at the indusium griseum (Ig) and glial wedge (gw) (green) extending around Probst’s bundles (Pb) towards the cingulate cortex. This observation of the Pfp using Dil tract tracing was observed in ephrinB3KO (n=6) mice. Scale bar represents 100μm.
Complementary expression patterns of ephrins and Eph receptors

In the developing forebrain, we have shown that callosal fibers express ephrins (B1-B2) and Eph (B1-B3, A4) receptors. Midline guideposts and callosal axons express ephrins and Eph receptors in a complementary fashion with Ephs (B1-B3, A4) receptors in axons and ephrins (B1-B3) at the midline. Likewise, callosal axons may express the ephrins (B1-B2), while their appropriate binding partners, which include EphB2 and EphA4, are expressed at midline guidepost regions. Contrary to the expression findings in the developing CC, Pfp fibers express only ephrins (B1-B3) during all points of development and may interact with the EphB2 and EphA4 receptors, which are expressed at strategic guidepost regions at the midline.

Is there topographic mapping of callosal fibers in the forebrain?

A potential function for this complementary expression of ephrins and Eph receptors in either axons or midline guideposts may be in developing a sort of topographic map of callosal fibers within the cerebral cortex. In the retinal system of the mice for example, investigators have established that one function of complementary Eph receptor and ephrin expression is direct the establishment of topographic maps along the lateral-medial (LM) axis of the superior colliculus (SC) (Hindges et al., 2002; Birgbauer et al., 2000; Lemke et al., 2005). Genetic deletion of receptors such as EphB2 and EphB3 results in disruption of appropriate retinal axon targeting along the LM axis of the SC (Hindges et al., 2002). Examination of the retinotectal system revealed that Eph
receptors are expressed in a gradient pattern along the dorsal-ventral (DV) retina, while complementary B-class ephrins are expressed in countergradient patterns along the LM axis of the SC. Specifically, EphB receptors are expressed in low to high concentrations along the DV axis of the retina, while ephrins are expressed in low to high concentrations along the LM axis of the SC. These studies also showed that one potential function for gradient expression of ephrins is to attract EphB-expressing retinal axons along the LM axis of the SC (Hindges et al., 2002). More recent data investigating the retinal ganglion cell development showed that ephrins can stimulate neurite outgrowth at low concentrations while inhibiting growth at high concentrations (Hansen et al., 2004). In the developing CC, pioneer callosal fibers reach the midline by e15, while axons that extend later bundle or fasciculate with each other as they migrate along this pre-established pioneer tract (Richards et al., 2002). After crossing the midline, which occurs at e16, callosal axons must de-fasciculate so that they may target appropriate partners in the contralateral cortex. Our expression data resembles the complementary expression of ephrins and Eph receptors in the retinotectal system. This observation suggests that a potential function for B-class ephrins and Eph receptors may be in directing the targeting of contralaterally projecting callosal axons to appropriate medial or lateral cortical targets. To support this, some of our in vitro results showed that cortical neurons extended longer neurites when grown on ephrinB-Fc substrates, which is similar to the growth promoting functions described for these molecules in the retinal system (see Chapter IV; Hindges et al., 2002). Despite the similarities in complementary expression of ephrins and Eph receptors in the retina and CC, there are several differences between the two systems. First, we did not observe gradient patterns of ephrins or Eph receptors in the developing forebrain as
observed in the retina (McLaughlin et al., 2005). This does not rule out the possibility that ephrins and Eph receptors may be expressed at non-midline guideposts that have yet to be described. In our \textit{in vitro} studies using WT and EphB1\textsuperscript{KO} cortical neurons grown on ephrinB-Fc substrates, we did not observe a difference in growth responses to varying concentrations of ephrinB-Fc (6\,\mu g/mL vs. 0.6\,\mu g/mL). This suggests that unlike in the DV retinal system, the growth promoting functions of EphBs and ephrinBs observed in the CC may be independent of ligand or receptor concentrations in order to cross the forebrain midline (Lemke et al., 2005). Another difference is that we observed CC axons that completely stalled at the midline, which is different than the altered targeting of appropriate regions along the LM axis of the SC (Hindges et al., 2002). Despite these differences, our expression observations along with prevalent knowledge of retinal axon pathfinding suggest that one function of this guidance family in the forebrain may be to attract EphB-expressing axons across the midline to appropriate LM or DV targets expressing ephrinB ligands.

\textbf{Roles for co-expression of Eph receptors and ephrins in callosal neurons}

An important observation from our expression data involves the co-expression of both ligands and receptors in the CC. Expression of ephrins and Eph receptors in the same cell requires that subsequent signaling events that occur downstream be tightly regulated to allow proper axon targeting. Recently, Pfaff and colleagues (2005) described that this is accomplished by ephrins and Eph receptors because they are segregated into distinct membrane domains in motor neuron axons, with each Eph receptor or ephrin domain capable of being activated independent of each other (Marquardt et al., 2005). Activation of ephrins resulted in expansion of the growth cone,
while activation of Eph receptors resulted in retraction of the growth cone. These data suggested that ephrin and Eph receptor signaling events can be functionally uncoupled with ephrins capable of directing a specific cellular event (i.e. growth cone spreading) independent of Eph receptors, which direct inhibitory signaling events (i.e. growth cone retraction) in the same cell. Similarly, in the developing CC, ephrin and Eph receptor signaling must be tightly regulated since single callosal neurons may express both B-class ephrins and Eph receptors. We have not investigated how callosal neurons regulate Eph and ephrinB signaling, but one mechanism may involve the uncoupling of Eph versus ephrin signaling as observed in the developing neuromuscular system (Marquardt et al., 2005). For ephrin and Eph receptor mediated mechanisms to occur in callosal neurons as described for developing motor neurons, these molecules must be segregated into distinct membrane domains. We have not conducted confocal microscopy experiments to examine whether a single callosal neuron expresses both ephrins and Eph receptors. In our system, different populations of callosal fibers may instead express ephrins while other populations express Eph receptors which would allow events such as defasciculation and axonal sorting to occur before and after crossing the midline (Chen et al., 2004). We have also not conducted experiments to investigate whether these molecules are segregated into different callosal fiber membrane domains. If ephrins and Eph receptors do have these expression characteristics in developing callosal neurons, then the model established for motor neuron development could be adaptable to the CC, however, another mechanism that may also be useful is the regulation of ephrin or Eph receptor signaling complexes. EphB receptors are capable of developing alternative responses by discriminating dimeric (i.e. unclustered) from multimeric (i.e. clustered)
forms of ephrinB ligands (Stein et al., 1998). This discrimination of different ligand oligomeric forms leads to the recruitment of molecules such as low-molecular weight protein tyrosine phosphatase (LMW-PTP) when higher oligomeric forms (i.e. hexamers) of ligands are used to stimulate Eph receptors as opposed to lower oligomeric forms (i.e. tetramers). Although is not known how downstream signaling proteins such as LMW-PTP are involved in Eph receptor signaling, it is clear that higher order oligomeric forms of ephrins are capable of initiating distinct signaling events. In the developing forebrain, presentation of different oligomeric forms of Eph receptors or ephrins expressed at strategic CC guidepost regions may influence the signaling proteins recruited downstream during forward or reverse signaling (Fig. 22).

Due to the fact many of the guideposts, molecular players, and axonal targets are known, the CC is an ideal model system to examine gradient or partitioned expression of ephrins and Eph receptors and the subsequent effects on signaling and cellular function. It would be interesting to investigate whether any of the mechanisms described for regulating retinal or motor neuron development are also used during CC neurite outgrowth. At face value, our in vivo data do not support differential functions for ephrinB3 and Eph receptors, because all mutant mice show similar hypoplasia or agenesis defects. However, the complexity of both CC axon and guidepost ephrin or Eph receptor expression and their roles in developmental glial migration make it difficult to evaluate independent functions. To aid in evaluating distinct axonal functions for ephrins or Eph receptors, single cell axon tracing using biotinylated dextran amine (BDA) may be useful in evaluating callosal axon phenotypes in different mouse mutants (Parent et al., 2005). Recently, three dimensional imaging techniques such as magnetic resonance
imagery (MRI) technology have been modified to evaluate axonal populations in vivo. This high resolution technology has allowed investigation of AC phenotypes in living EphB2 and EphA4 null mutants. These investigations showed that the axon disruption seen in each of these null mutants actually differed in the abnormal routing of AC axons from one another in several important morphologic respects (Ho et al., 2004). In EphB2\(^{KO}\) mice, there were defects in the routing of pAC axons, while in EphA4\(^{KO}\) mice there were mainly disturbances in the defasciculation of aAC axons. These advances in technology may help differentiate alternative functions for independent ephrins and Eph receptors in the developing CC system.

*Combined analysis of expression, genetic, and functional data reveal important developmental roles in axons and glia*

Our studies have demonstrated that the complementary expression of ephrins and Eph receptors in either CC axons or surrounding guideposts is matched with equal complexity in genetic functions. We have determined that individual genes such as those coding for EphB1 and EphB2 receptors are necessary for CC development, while others such as EphB3 and EphA4 do not play critical roles. Deletion of two Eph receptor genes (i.e. EphB2\(^{KO}/\)EphB3\(^{KO}\) mice) resulted in increased frequencies of CC defects suggesting these Eph receptors compensate for one another, while combinatorial deletion of other receptors (EphB1\(^{KO}/\)EphB3\(^{KO}\); EphB1\(^{KO}/\)EphB2\(^{KO}\)) resulted in no change in frequencies as compared to single knockout mice.

*Proposed roles for Eph receptors and ephrins in callosal axons*

Our data showed that Ephs (B1-B3, and A4) were all expressed in the developing CC. Combining these observations along with our genetic data from single knockout
mice, it is clear that the EphB1 receptor seems to play a major role in the growth of callosal fibers. Furthermore, our in vitro data implicate EphB1 receptors, which are only expressed in callosal fibers, in promoting cortical neurite outgrowth. To explain this ability of EphB1-ephrinB interactions on neurite outgrowth, we may explore in vitro data that suggest that EphB receptors are capable of activating intracellular signaling cascades that promote actin polymerization and cell motility (Vindis et al., 2004; Parsons et al., 2002; Harte et al., 2002). For example, intracellular signaling cascades involving molecules such as c-Src, NIK, paxillin, and FAK are linked to activated EphB1 receptors. These signaling cascades have been shown to regulate actin polymerization and promote chemotaxis of human renal microvascular endothelial cells (HRMEC) (Vindis et al., 2003). We have shown that EphB1 receptors promote cortical neurite outgrowth and the signaling cascades described for chemotaxis of various cell lines may be used by EphB1 receptors in the endogenous growth cones of developing cortical neurons. In vitro studies investigating hippocampal neuron development have also determined that EphB2 receptors regulate intracellular signaling molecules in dendritic spines such as Rac1 and Cdc42, which have been shown to be critical factors in actin polymerization (Penzes et al., 2003). Similar signaling cascades must be investigated in callosal neurons, where receptors such as EphB1, EphB2, or EphB3 are present and may activate molecules in the Rac1 and Cdc42 pathway and promote neurite outgrowth (Fig 23).

Our EphB1 neurite outgrowth data are consistent with experiments that described EphA5-ephrinA5 mediated CC pathfinding mechanisms (Hu et al., 2003), where ephrinA5 promoted the outgrowth of neurites from purified callosal neurons. In EphA5 mutant animals, CC fibers failed to enter the commissure resulting in a thinning of the
CC or hypoplasia suggesting that loss of the Eph-ephrin interactions reduced the ability of axons to grow through the midline. These in vivo results for EphA5 receptors are similar to what we observe in EphB1^KO^ mice, where a high percentage of these animals show hypoplasia phenotypes. Other investigations have shown that e13.5 cortical neurons have increased neurite outgrowth when grown on ephrinA5, ephrinA2, and ephrinB1 (Zhou et al., 2001; Gao et al., 2000), and retinal neurons require low concentrations of ephrins to promote outgrowth (Hansen, 2004). Together, these data and our results expand the role of ephrins and Eph receptors to function as not only repulsive molecules, but also as potential growth promoting factors in the developing CNS. Although we suggest that EphB1 receptors play a role in neurite outgrowth, we cannot rule out the fact that this receptor as well as other Ephs expressed in callosal fibers may mediate repulsive effects during development of the CC. Historically, these molecules have been thought of as primarily repulsive (Kullander et al., 2001; Yu et al., 2001; Wahl et al., 2000). Ephs (B2-B3 and A4) are also expressed in the developing callosal fibers and these receptors may mediate similar growth promoting functions, but based on current evidence in the field, these molecules may also carry out certain repulsive functions during CC targeting (Tong et al., 2003). To support a repulsive role for ephrins and Eph receptors during callosal fiber pathfinding, it is helpful to examine other guidance families in this developing system. The slit ligands have been shown to be important repulsive guidance cues during this developmental event (Bagri et al., 2002). These molecules, which are expressed in guidepost areas (i.e. Ig and GW) surrounding the developing callosal fibers, prevent improper axon targeting into dorsal or ventral forebrain regions directing extension across only the midline. This mechanism
has been termed ‘surround’ repulsion which aids in channeling axons through this region. We have established that ephrins (B1-B3) are expressed in similar patterns while Eph receptors are present in axons setting up a potential function for these molecules in a surround repulsion mechanism. To accomplish this, Eph receptor activation would need to result in repulsion of axons away from sources or ephrin expression and we have not yet shown this to be a function of these molecules. Likewise, we have not investigated the role of ephrins in callosal axons. Ephrins (B1-B2) are expressed in developing callosal fibers, and what role these molecules play remains elusive. Experiments have shown that exposure of retinal axons to the extracellular domains of EphB receptors results in growth cone collapse (Birgbauer et al., 2001). By contrast, in developing motor neurons, activation of ephrinB-reverse signaling results in growth cone expansion (Marquardt et al., 2005). Additionally, the GEF for Rac1 called Tiam has been shown to interact with activated ephrinB1 ligands and this interaction is critical for the outgrowth promoting functions (Tanaka et al., 2004). These studies indicate that ephrins can elicit both repulsive and attractive responses in axons, so a necessary question remains to be answered in the callosal neurons. Does EphB stimulation result in decreases or increases in the size of ephrin-expressing callosal growth cones? Our experiments have not addressed this point, and it would be interesting to understand the role of ephrins in the developing CC as well as the signaling proteins and pathways activated downstream of these ligands. Examination of ephrinB mutant mice would also be useful in answering some of these questions, but we have been unable to examine ephrinB2 and ephrinB1 complete null or truncation mutants. These future experiments as well as the data we have already described may provide clues to the process by which callosal axons fail to
cross the midline in the mouse. These data may also provide clues to the failed
mechanisms that contribute to human CNS disorders which exhibit similar phenotypes.

Separate *in vitro* data from our experiments showed that EphB1\(^{KO}\) cortical
neurons have reduced ability to grow on GW cell substrates. This would imply that
either cortical neurons developed increased sensitivity to repulsive cues or decreased
sensitivity to growth promoting cues. The *in vitro* EphB1-ephrinB interaction data
previously described suggest that the latter is a more suitable explanation for these
observations. However, these two sets of data cannot be directly linked and to truly show
that cortical neurons in knockout animals have decreased neurite outgrowth capabilities, a
more suitable experiment may be to knockdown ephrinB1 or ephrinB2 expression in GW
feeder layers, which have cortical neurons growing on them, using techniques such as
ribonuclease interference (RNAi). This would allow a downregulation of ephrinB1 or
ephrinB2 expression in GW feeder layers growing *in vitro*, and aid in determining
whether cortical neurons extend longer neurites in the presence of these ligands. This
may be a more direct approach to the answer the question of whether cortical neurons
from knockout mice do indeed have reduced ability to extend neurites at critical midline
structures. Not mentioned previously, one problem with the *in vitro* system that we have
chosen to use is that we are not evaluating purified callosal neurons, which make up only
approximately 2-3% of the total neuronal population in the forebrain. Instead we are
examining a heterogeneous population of cells, which include various neuronal and glial
cells (Richards et al., 2002). A more appropriate system would be to purify callosal
neurons from e17 tissue using reagents such green-fluorescent microsphere retrograde
tracers. These fluorescently tagged proteins can then be used to isolate callosal neurons
from non-callosal neurons using fluorescence activated cell sorting (FACS) to separate out all fluorescently tagged cells. After this process surviving purified callosal neurons may be cultured in vitro as has been done in the past (Catapano et al., 2004). Using purified callosal neurons, we would then be able to reliably differentiate callosal vs. non-callosal axonal mechanisms. Additionally, injection of DiI into one hemisphere and using this as a retrograde marker of callosal neurons may be another approach to isolate callosal neurons in a similar fashion (Hu et al., 2005). Either of these methods would allow us to truly elucidate whether the function of ephrin-Eph receptor interactions at the midline are responsible for growth promotion in callosal neurons. Additionally, callosal neurons must initiate unique signaling cascades, which result in their axonal pathfinding decisions, and using purified neuronal populations may also provide another advantage in allowing investigation of these novel signaling pathways.

Although, our in vitro data do not confirm a role for EphB1 in regulating callosal neurite outgrowth at the midline, we have determined that this receptor regulates the sensitivity of cortical neurons to ephrin growth and guidance cues. This switch in axonal sensitivity may be attained through regulating signaling cascades activated downstream of other Eph receptors or it may involve interactions with other guidance cue families such as the slits (Stein et al., 1996; Ghenea et al., 2005). Protein interactions between Eph receptors and Robo receptors have been uncovered in C. elegans. In the developing CC, a failure to regulate slit repulsive activity could result in axons that are unable to cross the midline. This has been observed in Drosophila mutants where Robo receptor expression is not downregulated by molecules such as Comm, which directs the subcellular sorting of Robo receptors. Disruptions of this mechanism result in
commissural axons that fail to cross the midline because Robo receptors are present to sense slit repellent cues prematurely (Keleman et al., 2005). There is one caveat to the idea that the Eph/ephrin family may interact with Robo receptors to regulate slit sensitivity. Unlike embryonic spinal cord axons, callosal fibers respond to slit repellent cues before and after crossing the midline (Shu et al., 2003d). This would suggest that slit repulsive activity is not regulated by Eph receptors or ephrins and the slit guidance family functions throughout all time points of CC development.

Integration of guidance signals within the growth cone

Our expression, genetic and in vitro data show that callosal neuron development is regulated by a number of ephrins and Eph receptors. In addition to this, a number of other guidance molecules have been shown to function in CC development and these include GAP-43, slit, netrin, and L1 CAM (Richards et al., 2004). All of these molecules elicit distinct signaling pathways and axonal responses, so it is critical that each signal be integrated properly within the growth cone so that accurate targeting of axons may occur during development (Song et al., 1999). Many axon guidance cues necessary for callosal fiber development have been discovered, so it would also be interesting to determine how ephrin/Eph receptor signals are integrated along with the other guidance receptors that are present at similar time points. These investigations would require that each of these molecules be expressed at similar CC developmental time points (i.e. e15-e18) and also that receptors be able to ‘talk’ or regulate each other’s signaling pathway. This may occur through direct interactions between different guidance receptors or may involve cross-talk between intracellular signaling proteins (Stein et al., 2001; Gallo et al., 2004). Direct protein interactions are involved in Robo and DCC receptor interactions, while
signaling crosstalk has been shown to involve the Rho-family GTPases (RhoA, Rac1, Cdc42). Many of the guidance receptors responsible for proper CC development also use Rho-family GTPases as intracellular signaling proteins (Yuan et al., 2003; Song et al., 1999). During axon growth and guidance, it has been shown that different guidance receptor families regulate each other by controlling the activity of these Rho-family GTPases (Lu et al., 2001). An intriguing question to ask would be whether the integration of signals detected by different guidance receptors within callosal fibers involves direct protein-protein interactions and/or regulation of Rho-family GTPase signaling proteins such as Cdc42, Rac1, or RhoA.

**Proposed role for ephrins and Eph receptors in midline glia**

Our analyses of ephrinB3KO mice revealed that this molecule plays an important role during CC development. First, our expression data showed that this molecule is mainly expressed in areas dorsal (i.e. Pfp) and ventral (i.e. Ig and GW) to developing callosal fibers. Second, our genetic data for ephrinB3 truncation mutants suggested that the extracellular domain of this ligand was important in mediating forward signaling in Eph-expressing callosal axons. Lastly, our in vivo data from complete null mice indicated that ephrinB3 ligands expressed in GW and Ig regions are important for regulating positioning of glial cells at the midline. The lack of glial migration defects that we observed in ephrinB3lacZ mice would support an interaction with Eph receptors or other growth and guidance molecules in midline glial regions. Specifically, our observation of glial positioning in complete null mice and not in truncation mutants suggests that the extracellular domain of ephrinB3 interacts with EphB2 and EphA4 receptors, which are also expressed in the GW and Ig guidepost regions. This interaction
would activate forward signaling cascades, which result in maintaining proper
positioning of EphA4 and EphB2-expressing glial cells. Loss of ephrinB3 eliminates this
interaction and results in aberrant positioning of glial cells that prevent proper callosal
fiber development (Fig. 24). EphA4 and EphB2 receptors have been shown to bind
ephrinB3 and initiate functional interactions during various developmental processes
(Blitz-Huizinga et al., 2005; Gale et al., 1996). Furthermore, these molecules activate
forward signaling cascades that regulate cellular processes such as dendritic spine
morphology and neural crest cell chemotaxis (Penzes et al., 2003; Smith et al., 1997).
Interestingly, evidence from studies evaluating cell migration in zebrafish embryos
supports our model that ephrinB3 interactions with EphA4 and EphB2 promote glial
positioning in midline guideposts (Mellitzer et al., 1999). This study showed that ephrin-
Eph receptor interactions served to restrict cell intermingling in the developing hindbrain.
The investigators examined different populations of cells that express EphA4 or EphB2
receptors in one population while the ligands ephrinB1 or ephrinB2 were expressed in
another. Disruption of ephrin-Eph receptor interactions using complete-null or truncation
mutants resulted in abnormal cell intermingling suggesting that the both the extracellular
and intracellular domains of these molecules were necessary for cell positioning. Similar
mechanisms have been examined in Xenopus embryos where EphB1 and EphA4
receptors have been shown to interact with ephrinB2, which functions to restrict
intermingling of neural crest cells (Smith et al., 1997). In our system, interactions
between ephrinB3 and the Ephs (B2 and A4) may act in a similar fashion restricting glial
movement into inappropriate regions during development of the CC. One limitation to
our model is that we do not know if single glial cells express all three molecules (i.e.
ephrinB3, EphB2, and EphA4) or whether distinct populations of glial cells express different molecules (i.e. ephrinB3, EphB2, or EphA4). Confocal microscopy using glial specific markers such as anti-GFAP and labeling with the ephrin and Eph receptor specific antibodies may allow us to determine whether glial cells express all three molecules or simply one or two. Understanding the spatial distribution of ephrinB3 and Ephs (B2 and A4) would aid in understanding the mechanism of this developmental event and the important signaling pathways that may be activated downstream of this ligand and its receptors. Another limitation to our model is that ephrins and Eph receptors have been shown to have adhesive functions during several developmental events (Poliakov et al., 2004; Irie et al., 2005). Specifically, in zebrafish embryos is has been shown using morphalino oligonucleotides, which eliminate protein expression by blocking translation, that EphA4 receptors are critical for hindbrain boundary formation because blocking these interactions results in improper intermingling of neuronal nuclei from different rhombomeres (Cooke et al., 2005). The investigators further described an adhesive function for EphA4 during positioning of cells within hindbrain rhombomeres. These data imply that ephrinB3 interactions with any of its receptors may also function to promote cell adhesion of glial cells within any of the guideposts. The observation that we observed in ephrinB3$^{KO}$ mice could then be due to the inability of cells within the GW, Ig, or other guideposts to adhere to one another and remain appropriately positioned. Another limitation to our analyses of abnormal positioning of midline glia in ephrinB3$^{KO}$ mice is that we do not know if these cells actually originate from the GW, Ig or other glial guidepost regions. Injection of viral vectors encoding fluorescently tagged molecules such as yellow-fluorescent protein (YFP) or green-fluorescent protein (GFP)
into GW or Ig guidepost regions during early development time points for these midline guideposts (e.g. e14) and analysis of these tissues at later developmental time points may allow differentiation of exactly what cellular regions these aberrantly positioned glial cells arise. Similarly, injection of DiI crystals into the ventricular space at early embryonic time points in mouse embryos would also allow labeling of GW cells. This technique has been done successfully to determine that GW cells are part of a radial glial scaffold within the developing mouse forebrain (Shu et al., 2003d). This method would benefit our studies because we could potentially label GW cells with DiI in ephrinB3KO mice, which have abnormal positioning of glial cells, and determine if the aberrantly positioned cells we observed in ephrinB3 mutants actually are derived from the GW. This latter method of labeling GW cells would only aid in determining if glial cells in mutant animals arose from the GW region, but it would not help determine whether aberrantly positioned glial cells in our mutants are derived from other glial regions such as the Ig or Mzg.

This novel function of ephrinB3 in the regulation glial migration at the corticoseptal boundary is consistent with recently published data showing that slit-2 and slit-3 serve to direct glial cell positioning in the zebrafish forebrain (Barresi et al., 2005). Specifically, Robo-expressing glial cells are repelled from slit2/slit3-expressing regions, which restrict these glial cells to bands that span the midline. Removal of slit-2 or slit-3 genes resulted in abnormal positioning of glial cells along the midline, which led to midline crossing defects of postoptic commissure axons. Interestingly, direct protein interactions between Vab-1 Eph receptor and Sax-3/Robo receptor have been discovered in C. elegans, however, the functions of these interactions remain unknown (Ghenea et
al., 2005). Since we have determined that ephrins play a role in the glial positioning necessary for proper midline axon guidance, it would be interesting to investigate whether these molecules cooperate with slit ligands or Robo receptors during CC development. Although the slit and ephrin data in glial positioning are consistent one important difference in the two mechanisms involves the expression profile of the respective ligands and receptors. In the slit model describing glial positioning in the zebrafish, investigators established that slit (slit 1-3) expression was restricted to domains surrounding the glial cells, while the glial cells themselves expressed the receptors for slit (Robo-1 and Robo-2) (Barresi et al., 2005). This is different than what we observe for the expression of ephrinB3 and its receptors EphB2 and EphA4 in the midline guideposts of our system. We observed that glial cell guideposts expressed both ligand and receptors, while no domains of receptor or ligand expression were present surrounding the glial cells as observed in the slit/Robo zebrafish model. The spatial distribution of slits and Robos in zebrafish forebrain during glial cell positioning seems to be quite different than the distribution of ephrins and Eph receptors in the mouse forebrain, suggesting that glial cell movement is maintained via local interactions that result in maintenance of proper glial guideposts. Another difference in the two models is that we do not know if Eph receptors and ephrins in our system function to repel or restrict the movement of glial cells as described for the slits and Robos in the zebrafish. If this is not the case, then another function for ephrins and Eph receptors in glial cell positioning may be in adhesion of these cell types, which has been shown to be one function of this family during rhombomere formation (Cooke et al., 2005).
Distinct midline guideposts and independent B-class ephrin expression in Pfp development

We investigated whether ephrins and/or Eph receptors expressed in critical CC midline guideposts also play a role in Pfp development. We have shown interesting ephrin (B1-B3) expression patterns that are specific to Pfp fibers, while two potential binding ligands EphB2 and EphA4 are present at strategic locations to potentially interact and regulate Pfp fiber growth and guidance (Shu et al., 2001b; Blitz-Huizinga et al., 2004). This chapter investigated a) the importance of CC midline guideposts in Pfp axonal pathfinding and b) whether ephrins can regulate axon pathfinding in the absence of Eph receptors in the same cell. EphrinB3 and EphB1 mutants, which both exhibit abnormal midline glial positioning, do not show defects in Pfp axon targeting as shown in Fig. 25.

This observation does not support a role for CC midline glia in mediating Pfp axons growth responses. These results are consistent with previous experiments by other investigators, which show that reduction or loss of the cells that make up the GW and Ig does not disrupt development of the Pfp (Shu et al., 2003d). Previous results as well as our data suggest that unique guideposts at the corticoseptal boundary regulate the ipsilateral routing of this fiber tract. We have hypothesized that Pfp fibers use expression of Eph receptors at strategic guideposts (also used by CC axons) to maintain their ipsilateral orientation. If common guideposts are not used by Pfp fibers, then another potential mechanism that may be used by these fibers to maintain an ipsilateral orientation is the regulated expression of growth and guidance molecules during axonal routing. Mechanisms similar to this have been described in the visual system where
retinal neurons upregulate the expression of EphB1 in ipsilateral routing axons to restrict their growth across the optic chiasm (Williams et al., 2003). In our system, regulated expression of B-class ephrins at the midline crossing decision point would be a useful mechanism to prevent Pfp axons from crossing the midline. Additional expression analyses of ephrinB1 and ephrinB2 would be necessary to determine the temporal expression profile of these molecules in relation to Pfp axon pathfinding at the midline.

Another interesting observation in the Pfp tract was the expression of only B-class ligands within the tract. To test the hypothesis that ephrin reverse signaling independently (no Eph receptor expression in the same cell) regulates the development of an axon tract, we examined the Pfp tract in ephrinB3\textsuperscript{KO} mice. We observed no abnormalities in this axon tract suggesting that this ligand is not critical for Pfp development. This does not exclude the possibility that ephrinB3 may be important for other processes such as postsynaptic structure formation or synaptic plasticity in the Pfp system as described for the hippocampus (Murai et al., 2003; Grunwald et al., 2004; Klein et al., 2004). One explanation for proper Pfp development in the absence of ephrinB3 is that other ephrins may compensate for loss of ephrinB3 function. EphrinB1 and ephrinB2 are both candidates for compensatory functions and it would be of interest to evaluate EphB2\textsuperscript{KO} mice, since absence of EphB2 induced reverse signaling (by ephrinB1 or ephrinB2) may address overlapping functions for these ligands. EphB2 has been shown to have the greatest binding affinity with ephrinB1 and ephrinB2 and would thus play a major role in the activity of these two ligands (Blitz-Huizinga et al., 2004).

Lastly, sorting of B-class ephrin-expressing Pfp axons from CC axons could also occur at the corticoseptal boundary during development. In this system, B-class ephrins
can potentially interact with EphB2 and EphA4 at the midline resulting in bundling or fasciculation of Pfp axons preventing aberrant growth into the CC tract. This function of Eph receptors and ephrins has been described in hippocampus. Specifically, neurons within the hippocampus send axons to septal targets and defasciculation of these axons was shown to be regulated by EphB2 and EphB3 receptors in hippocampal neurons interacting with ephrinB3 at septal targets (Chen et al., 2004). This ephrin and Eph receptor mediated sorting at the midline would allow for maintenance of axons within proper fiber tracts preventing inappropriate extension of Pfp axons across CC midline crossing regions.

**Future directions**

*A role for multiple growth and guidance family interactions during CC development.*

We have provided extensive evidence indicating that ephrins and Eph receptors contribute significantly to the development of the CC. We have discussed evidence from literature indicating that multiple axon growth and guidance families play a role in CC development. It is well known that during development growth cones integrate multiple signals (Dent et al., 2003; Cooper et al., 2002). Specifically, the netrins and slits play critical roles in CC development (Shu et al., 2003; Bagri et al., 2002; Fazeli et al., 1997; Serafini et al., 1996). It is also well known that interactions between these two growth and guidance families occur during development which result in regulation of axonal attraction and migration of neurons (Stein et al., 2001; Giger et al., 2001; Causeret et al., 2002). Recent evidence from experiments in *C. elegans* indicates that there are also molecular interactions between VAB-1 Eph receptor and SAX-3/Robo receptors and that this interaction is important during embryonic morphogenesis (Ghenea et al., 2005).
Additionally, *in vitro* studies using chick retinal neurons indicate a convergence of signaling pathways used by both Eph receptors and Robo receptors (Wong et al., 2004). Specifically, ephrinA5 and slit2 initiate signals that lead to repulsion, and this signaling pathway converges on phosphatidylinositol 3-kinase (PI3K) and Src family kinases. Pharmacological inhibition prevented both ephrinA5 and slit-2-mediated growth cone collapse. These two forms of evidence involving both direct (receptor-receptor) or indirect (signaling pathway crosstalk) indicate that there is a potential for interactions between the ephrins and Eph receptors and other axon guidance and growth families. Overlapping gradients of growth and guidance cues force axons to integrate multiple axonal cues during embryogenesis (Chisholm et al., 1999).

Mechanisms used by growth cones to integrate and respond to multiple guidance cues remain poorly understood and the CC system seems to be a perfect model system to investigate growth cone integration (Song et al., 2001; Dent et al., 2003). To begin to examine whether interactions between Eph receptors/ephrins occur, co-immunoprecipitation experiments would be conducted. Specifically, interactions between EphB1 receptors, which we showed to be important for CC development, and Robo-1/2 or DCC may be explored. Precise regulation of growth cone attraction or repulsion is critical for proper callosal fiber routing and as we have described all these growth and guidance families are expressed during development in the CC system (i.e. CC axons and midline guideposts) (Richards, 2002). Direct interactions between these proteins would be an excellent way to regulate growth through the midline, which has been described as a mechanism used by the slit and netrin family (Stein et al., 2001).
Are ephrin and Eph receptor compensatory mechanisms used during Pfp development?

Our analyses of ephrins and Eph receptors in development of the Pfp did not resolve whether these molecules play a developmental role in the formation of this tract (see Chapter VI). Specifically, we were unable to show that although critical CC midline glial cells were aberrantly positioned in ephrin3 and EphB1 mutant mice, Pfp innervation of the cingulate cortex was altered. Additionally, these experiments also showed that removal of ephrinB3 from Pfp fibers did not result in inability of axons to extend and reach appropriate cingulate targets.

We propose that combinatorial ephrin and Eph receptor mutant mice must be examined to confirm whether or not this family plays a developmental role in the Pfp. Our investigations as well as other studies have shown that compensatory mechanisms exist within the ephrin and Eph receptor family (Dottori et al., 1996). Specifically, it would be interesting to perform DiI tracing experiments in additional single knockouts such as EphB2\textsuperscript{KO} and EphA4\textsuperscript{KO} (which are expressed in the GW and Ig, two strategic locations for Pfp axon growth and guidance). Examination of Pfp development in these knockout mice would be advantageous since both receptors are expressed at potential guideposts and this may eliminate interactions with not only ephrinB3 but also ephrin (B1-B2) that are also expressed in Pfp axons and may function to compensate for loss of ephrinB3 ligands.
Figures for Chapter VI

Figure 22. Schematic representation of a cortical fiber interacting with the GW guidepost. Callosal axons contain both reverse and forward signaling mediated by ephrins and Eph receptors following interactions with either EphB2 or ephrinB3 signaling complexes in midline guideposts, respectively.
Figure 23. EphB1-ephrin interactions positively regulate cortical neuron neurite outgrowth. Proposed signaling pathways critical for EphB1 induced neurite outgrowth. (a) In wild-type neurons, EphB1-expressing cortical axons interact with ephrinB1 or ephrinB2 ligands expressed in the glial wedge guidepost. This interaction may initiate forward intracellular signals that involve molecules such as intersectin, N-WASP, S-Src, NIK, or Kalirin. These molecules may be linked to the activation of Cdc42, paxillin, or Rac1, which may then activate molecules such as Arp2/3, FAK and PAK. The final result of each of these signaling pathways downstream of EphB1 activation is neurite outgrowth. Functional roles for Eph (B2-B3) and EphA4 expressed in callosal fibers remain elusive. (b) In EphB1\textsuperscript{KO} neurons, these signaling pathway(s) are not activated, growth cones collapse, and growth extension is inhibited.
Figure 24. EphrinB3-Eph receptor interactions direct midline glial cell positioning. 

a) In wild-type mice, interactions between ephrinB3 (green) with either EphA4 (yellow) or EphB2 (red) receptors expressed within the same cell types regulates the positioning of glial cells, which allow proper midline crossing of callosal fibers. b) Deletion of the ephrinB3 gene (i.e. ephrinB3\textsuperscript{KO} mice) eliminates the ability of this ligand to interact with EphB2 and/or EphA4 receptors, and results in aberrant positioning of glial cells at the midline and subsequent improper targeting of callosal axons.
Figure 25. Schematic representation of CC and Pfp growth patterns in the ephrinB3 and EphB1 knockout mice. Corpus callosum (CC) fibers stall at the midline forming Probst’s bundles in mutant mice (e.g. ephrinB3\textsuperscript{KO}, EphB1\textsuperscript{KO} and EphB2\textsuperscript{KO}). CC fibers in mutants project to the midline but stall prior to crossing the midline comprising glial wedge (GW) and indusium griseum (Ig) guideposts; ‘glial’ sling cells; midline zipper glia (mzg). Perforating pathway (Pfp) axons however, continue to project dorsally and ventrally targeting appropriate cingulate and medial septum targets.
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