ROLE OF EPHB FAMILY RECEPTORS IN REGULATING AXON GUIDANCE IN THE MAMMALIAN CENTRAL NERVOUS SYSTEM

by

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A thesis submitted in conformity with the requirements for the degree of Doctor of Philosophy
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ABSTRACT

Neural function depends on precise wiring of axon during development. Previous studies have demonstrated that the erythropoietin producing hepatocellular carcinoma (Eph) family of tyrosine receptor kinases is crucial for the proper development of a number of neural circuits in the mammalian central nervous system (CNS).

Mice lacking Eph receptors have been shown to exhibit deficits in pathways which include the thalamocortical, callosal, retinal and corticospinal tract. Due to the large number of Eph family members, the relative contribution of each receptor to axon pathfinding and neural function remains elusive. In this thesis, I have addressed the function of EphA4, EphB2 and EphB3 in the regulating the formation of interhemispheric projections within the forebrain and motor axon connections within the spinal cord using EphA4, EphB2 and EphB3 and combinatorial null mice. To perform a detailed examination of the process of axon guidance regulated by these receptors within the forebrain, high resolution magnetic resonance imaging (MRI), immunofluorescence and in vivo stereotactic fluorescent labeling were performed. This work resulted in the development and validation of MRI-based analytic tools performed using EphB2 mutants which we have previously shown to exhibit specific morphologic defects in the anterior commissure (AC). Analysis of EphA4 null mice using high resolution MRI revealed for
the first time that in addition to errors of midline crossing, loss of EphA4 activity results in a positional reorganization of the rostral AC. Results demonstrate that while EphB2 and A4 each regulate distinct aspects of guidance within ACpp, these receptors also operate cooperatively to control the guidance of axons in the pars anterior of the AC, a pathway not been previously implicated in Eph-mediated guidance. With respect to the spinal cord, mice deficient in EphB2 and EphA4 display prominent axon guidance errors in the medial subsets of the lateral motor column (LMCm); neurons which normally innervate ventral limb musculature. Finally, I have addressed the functional effect which Eph mutants exhibit with respect to motor behavior by examining a detailed set of motor coordination parameters.
ACKNOWLEDGEMENTS

I would like to acknowledge collaborations outside the lab that were part of this journey. Natasa, Leila (MICe), Monica (Nagy lab), Andrew Elia (Mak lab), Andras Nagy (Faculty of Pharmacy), Carrie Causing (Wranna Lab).

I would like to thank my supervisor, Dr. J.T. Henderson, for his guidance, mentorship and patience. It is a privilege to have been trained in his laboratory.

I would also like to thank my advisory committee members, Dr. David Hampson, Dr. John Roder, Dr. Zhengping Jia and Dr. Joseph Culotti for their valuable suggestions and constructive comments throughout the program.

Many thanks to my labmates for enduring all my moments and for being my good friends. Thanks to Patrick Ng, Anish Kanungo, Kelvin Hui, Ashlin Kanawaty, Lily Huang, Gennie Wang, Dan Cojocari, Erin Chan, Jacqueline Wong, Ciric To, Melissa Shih, Jessica Chung, Melissa Chan, Maria Michalowsky and Stephano Chang.

Finally, I am indebted to my loving parents, Amy and Cecil, and dearest husband, Marvin, for their continual support and endless sacrifices.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>AC</td>
<td>Anterior commissure</td>
</tr>
<tr>
<td>ACpa</td>
<td>Pars anterior branch of the AC</td>
</tr>
<tr>
<td>ACpp</td>
<td>Pars posterior branch of the AC</td>
</tr>
<tr>
<td>ADAM10</td>
<td>A-disintegrin and metalloprotease 10</td>
</tr>
<tr>
<td>A-P</td>
<td>Anterior-posterior axis</td>
</tr>
<tr>
<td>AOB</td>
<td>Accessory olfactory bulb</td>
</tr>
<tr>
<td>C.elegans</td>
<td>Caenorhabditis elegans</td>
</tr>
<tr>
<td>Cdk5</td>
<td>Cyclin-dependent kinase 5</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CPG</td>
<td>Central pattern generator</td>
</tr>
<tr>
<td>CST</td>
<td>Corticospinal tract</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CXCR4</td>
<td>Chemokine receptor 4</td>
</tr>
<tr>
<td>DAB</td>
<td>3,3'-diaminobenzidine</td>
</tr>
<tr>
<td>DAPI</td>
<td>4'-6-Diamidino-2-phenylindole</td>
</tr>
<tr>
<td>DCC</td>
<td>Deleted in colorectal cancer</td>
</tr>
<tr>
<td>DH</td>
<td>Dbl homology</td>
</tr>
<tr>
<td>DiI</td>
<td>1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine</td>
</tr>
<tr>
<td>DiD</td>
<td>1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate salt</td>
</tr>
<tr>
<td>dLGN</td>
<td>Dorsal lateral geniculate nucleus</td>
</tr>
<tr>
<td>Dsh</td>
<td>Dishevelled</td>
</tr>
<tr>
<td>D-V</td>
<td>Dorsal-ventral axis</td>
</tr>
<tr>
<td>ECL</td>
<td>Enhanced luminol-based chemiluminescent</td>
</tr>
<tr>
<td>EGF</td>
<td>Epidermal growth factor</td>
</tr>
<tr>
<td>EGFP</td>
<td>Enhanced green fluorescent protein</td>
</tr>
<tr>
<td>EMPRESS</td>
<td>European Mouse Phenotyping Resource for Standard Screens</td>
</tr>
<tr>
<td>Eph</td>
<td>Erythropoietin producing hepatocellular carcinoma</td>
</tr>
<tr>
<td>Ephexin</td>
<td>Eph-interacting exchange proteins</td>
</tr>
<tr>
<td>Ephrin</td>
<td>Eph family receptor interacting protein</td>
</tr>
<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
</tr>
<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase-activating proteins</td>
</tr>
<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GDNF</td>
<td>Glial cell derived neurotrophic factor</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GIT</td>
<td>G protein-coupled receptor kinase interacting protein</td>
</tr>
<tr>
<td>Grb2</td>
<td>Growth factor receptor-bound protein 2</td>
</tr>
<tr>
<td>Grb4</td>
<td>Growth factor receptor-bound protein 4</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine triphosphate</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>JM</td>
<td>Juxtamembrane domain</td>
</tr>
<tr>
<td>KD</td>
<td>Kinase domain</td>
</tr>
</tbody>
</table>
Kuzbanian domain metalloproteinase
LMCm Lateral motor column (lateral)
LMCl Lateral motor column (medial)
LMW-PTP Low molecular weight protein tyrosine phosphatase
LTP Long-term potentiation
MAPK Mitogen activated kinase
MICe Mouse Imaging Centre
MMC Medial motor column
MNI Montreal Neurological Institute
MRI Magnetic resonance imaging
MSP Major sperm protein
N-T Nasal-temporal axis
N-WASP Neural Wiskott-Aldrich syndrome protein
NCAM Neural cell adhesion molecule
NPN Neuropilin
PBS Phosphate buffered saline
PDZ Postsynaptic density protein/disc large/zona occludens
PDZ-RGS3 PDZ-regulator of G-protein signaling 3
PFA 4% paraformaldehyde in 0.1M PBS
PH Pleckstrin homology
PI3K Phosphatidylinositol 3-kinase
PICK1 Protein interacting with C kinase 1
PN Peroneal nerve
PNA Peanut agglutinin
PSA Polysialic acid
PTK Protein tyrosine kinase
PTP Phosphotyrosine phosphatase
Ptpro Protein-tyrosine phosphatase receptor type O
RasGAP p120-Ras GTPase-activating protein
RBD Receptor binding domain
RGC Retinal ganglion cell
RTK Receptor tyrosine kinase
S1 Somatosensory 1 area
SAM Sterile alpha motif
SC Superior colliculus
SDF-1 Stromal cell derived factor 1
SFK Src-family of kinases
SH2 Src-homology 2 domain
SHEP1 SH2-domain-containing Eph receptor-binding protein 1
SHIRPA Smithkline Beecham, MRC Harwell, Imperial College, the Royal London hospital Phenotype Assessment)
SHP1 SH2-domain containing PTP2
SN Sural nerve
TN Tibial nerve
VEGF Vascular endothelial growth factor
VNO Vomeronasal organ
CHAPTER 1: INTRODUCTION

1.1 Axon guidance in mammalian central nervous system

Of all the body’s components, it is the central nervous system (CNS) that imparts identity to the individual. The collective inter-connection of neural cells endues individuals with their unique social characters. A critical step in the proper assembly of this nervous system is the formation of neural networks with distinct patterns of connectivity (Dodd and Jessell, 1988). This process relies on the ability of the growing axons and dendrites to locate and recognize appropriate synaptic partners. This process is also critical to the appropriate re-innervation of neurons following injury, as inappropriate innervation has been associated with pathological conditions such as epilepsy, phantom limb pain, hearing disorders and changes in emotive behavior.

During development, neural loci initially send pioneer axons into permissive territories which subsequently serve as guideposts for the growth cones of later differentiating neurons. Correct guidance of these initial axons to their targets critically depends upon their responsiveness to appropriate environmental cues (Tessier-Lavigne and Goodman, 1996). These guidance cues can act to either attract or repel growing axons, operating at either proximally or in a diffusible manner over greater distances. Over the years, biochemical and genetic studies have led to the identification of four major families of guidance molecules: (receptor/ligand) deleted in colorectal cancer (DCC)/netrins, Robo/Slits, Plexin/semaphorins and Eph/ephrins.
The netrins are a family of laminin-related molecules. The identification of netrins initially arose from studies of circumferential axon guidance in *Caenorhabditis elegans* (*C. elegans*) (Hedgecock et al., 1990; Ishii et al., 1992). Subsequent studies have demonstrated that netrins can mediate both attraction (netrin1-DCC) and repulsion (Unc5-Unc6) (Kennedy, 2000). Homologs of these proteins have also been identified in mammals, suggesting that they have been conserved for over 600 million years. In mammals, genetic ablation studies have shown that guidance cue signals provided by netrins can be attractive or repulsive in nature (Serafini et al., 1996). Mice carrying a hypomorphic allele of *netrin-1* result in severe axon guidance defects in several CNS structures (Serafini et al., 1996). By contrast, *netrin* null mutants completely lack the corpus callosum, hippocampal commissure, anterior commissure and some pontine nuclei. Mice lacking DCC exhibit a similar phenotype as *netrin-1* deficient mice, suggesting that DCC mediates the majority of netrin-mediated effects in these neurons (Fazeli et al., 1997).

Slits represent a family of secreted proteins which signal through the Roundabout (Robo) receptors. Members of the Robo family are a group of highly conserved transmembrane glycoproteins that make up a small subgroup of the immunoglobulin (Ig) superfamily. Roundabout was first identified in genetic screens for midline guidance defects in *Drosophila* (Kidd et al., 1998; Seeger et al., 1993). Unlike netrins, the interaction of Robo and Slit triggers axon repulsion (Kidd et al., 1998). The best understood functions of Slit proteins are in regulating midline guidance in *Drosophila* and formation of the optic chiasm in vertebrates. In *Drosophila*, Slit is dynamically expressed at the ventral midline. It is expressed following initial migration of midline axons. This expression prevents ipsilateral axons expressing Robo from recrossing the body midline (Battye et al., 1999; Kidd et al., 1999). Mice deficient in *Slit1* and *Slit2* lack of any obvious morphologic defects in midline guidance, however, exhibit striking
defects in the formation of the optic chiasm (Plump et al., 2002). Because of this, it has been suggested that expression of Slit1 and Slit2 in cells surrounding the chiasm act to repel ipsilateral and contralateral retinal axons (Erskine et al., 2000; Plump et al., 2002; Ringstedt et al., 2000).

Semaphorins represent a large family of cell surface and secreted guidance molecules consisting of at least 30 members which interact principally with neuropilin or plexin receptors to mediate axon repulsion, fasciculation, branching and synapse formation (Chen et al., 1997; Cheng et al., 2001; Murakami et al., 2001). Genetic analyses of semaphorin function in flies and mice suggest that they primarily act as short-range inhibitory cues which prevent inappropriate axonal migration (Cheng et al., 2001; Raper, 2000). Mice deficient in SemaIII have been shown to exhibit aberrant termination of sensory axons within the spinal cord (Behar et al., 1996). Additional axonal defects have been noted within the trigeminal, facial, vagus, and accessory nerves (Taniguchi et al., 1997). Although semaphorins are highly expressed in motor neuron within the spinal cord, mice lacking these proteins appear to exhibit normal motor axon innervation (Catalano et al., 1998).

Together with their respective receptors, the above families of guidance molecules have been shown to guide axons either through the promotion of growth cone elongation, or retraction by regulating cytoskeletal dynamics. The temporal pattern of expression for many of these genes is tightly regulated in order to create the appropriate environment cues for axon guidance. In many instances, the exact signal transduction mechanisms which these guidance molecules regulate have not yet been wholly determined. In order to gain insight into the mechanism by which the CNS connections are constructed, I have focused my studies upon a group of the largest family of axon guidance receptors; the erythropoietin producing hepatocellular carcinoma derived tyrosine kinases known as the Eph receptor group.
1.2 Structure of Eph receptors and ephrins

Receptor tyrosine kinase (RTKs) and their ligands govern an array of properties with respect to cellular communication both during and subsequent to development. The largest known family of these receptor tyrosine kinases is the Eph subgroup. Together with their ligands (ephrins-Eph family receptor interacting proteins), a total of sixteen Eph receptors and nine ephrins have been identified in vertebrates (Pasquale, 2004). Eph receptors and their ligands are highly conserved and homologs have been found in species as diverse as C.elegans (George et al., 1998) and Drosophila (Scully et al., 1999). In contrast to mammals, these organisms express only a single Eph receptor gene, suggesting extensive diversification of this group occurred during the course of mammalian evolution (Drescher, 2002).

A unique feature that distinguishes the Eph-ephrin system from other RTKs is that ephrins are membrane bound typically, limiting Eph-mediated signaling to cell-to-cell communication. Eph receptors can be further subdivided based upon the nature of their ephrin ligands. EphrinA ligands are tethered to the outer cell surface by a glycosyl-phosphatidyl-inositol (GPI) linkage, whereas ephrinB ligands contain a transmembrane motif. EphA receptors typically bind to ephrinA ligands, while EphB receptors interact with ephrinB ligands to initiate signaling as shown in Figure 1.1. Exceptions to this rule do exist, notably that EphA4 also binds ephrinB ligands (Pasquale, 2005). Cross-class interactions have also been reported for the EphB2 with ephrinA5 (Himanen et al., 2004). Interestingly, the affinity of EphB2 for ephrinA5 is 2 fold higher than that for ephrinB3, the expected B-class ligand (see Table 1.1). Within a given subgroup, binding interactions are relatively promiscuous, as a single EphA receptor can
Figure 1.1 Binding interactions between Eph receptors and ephrins.

Known binding profiles for each Eph receptor and ephrin ligand are shown. Receptor-ligand interactions can be promiscuous within Eph A and B classes. Exceptions in the binding discrimination between classes are the bindings between EphA4 and ephrinB2 and B3 and between EphB2 and ephrin A5 (shown in red).
Table 1.1 Eph-ephrin binding affinities

**EphA-ephrinA**

<table>
<thead>
<tr>
<th>EphA</th>
<th>EphrinA1</th>
<th>EphrinA2</th>
<th>EphrinA3</th>
<th>EphrinA4</th>
<th>EphrinA5</th>
</tr>
</thead>
<tbody>
<tr>
<td>EphA1</td>
<td>2.67X10E-9M (Gale et al 1996)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>EphA2</td>
<td>1.33X10E-9M (Gale et al 1996)</td>
<td>2.0X10E-8M (Gale 1996)</td>
<td>9.5X10E-10M (Gale et al 1996)</td>
<td>n/a</td>
<td>n/a</td>
</tr>
<tr>
<td>EphA3</td>
<td>n/a</td>
<td>n/a</td>
<td>5.0X10E-9M (Kozlosky et al 1994)</td>
<td>5.0X10E-12M (Kozlosky et al 1994)</td>
<td>6.9X10E-12M (Himanen et al 2004)</td>
</tr>
<tr>
<td>EphA4</td>
<td>3.9X10E-10M (Gale et al 1996)</td>
<td>3.96X10E-9M (Gale et al 1996)</td>
<td>3.0X10E-9M (Gale et al 1996)</td>
<td>n/a</td>
<td>5.9X10E-7M (Himanen et al 2004)</td>
</tr>
<tr>
<td>EphA5</td>
<td>1.19X10E-9M (Gale et al 1996)</td>
<td>1.31X10E-9M (Gale et al 1996)</td>
<td>1.78X10E-9M (Gale et al 1996)</td>
<td>n/a</td>
<td>1.5X10E-9M (Himanen et al 2004)</td>
</tr>
<tr>
<td>EphA7</td>
<td>2.4X10E-9M (Gale et al 1996)</td>
<td>1.0X10E-9M (Gale et al 1996)</td>
<td>1.47X10E-9M (Gale et al 1996)</td>
<td>n/a</td>
<td>3.1X10E-11M (Himanen et al 2004)</td>
</tr>
</tbody>
</table>

**EphB-ephrinB**

<table>
<thead>
<tr>
<th>EphB</th>
<th>EphrinB1</th>
<th>EphrinB2</th>
<th>EphrinB3</th>
</tr>
</thead>
<tbody>
<tr>
<td>EphB1</td>
<td>1.2X10E-9M (Gale et al 1996)</td>
<td>1.12X10E-9M (Gale et al 1996)</td>
<td>n/a</td>
</tr>
<tr>
<td>EphB3</td>
<td>n/a</td>
<td>n/a</td>
<td>1.5X10E-9M (Bergemann et al 1998)</td>
</tr>
<tr>
<td>EphA4</td>
<td>n/a</td>
<td>8.6X10E-9M (Gale et al 1996)</td>
<td>6.0X10E-9M (Bergemann et al 1998)</td>
</tr>
</tbody>
</table>
bind to several ephrinA ligands. This broad overlap of binding specificity within and between subclasses demonstrates the potential redundancy of the system.

EphA and EphB family receptors exhibit similar overall structure and characteristics, however differ in their precise recognition motifs. Like other RTK families, the Eph family exhibits several conserved binding motifs within the ectodomain (see Figure 1.2), a glycosylated N-terminal ligand binding motif (Himanen et al., 2001; Lackmann et al., 1998), forming a globular β-jellyroll structure (Himanen et al., 1998) principally regulates a cysteine-rich linker, and is followed by an epidermal growth factor (EGF)-like region and two fibronectin repeats. Just beyond the transmembrane region, the cytoplasmic domain contains two juxtamembrane (JM) tyrosines residues (Ellis et al., 1996), the phosphorylation of which is critical for receptor activation (Lackmann et al., 1998). When phosphorylated, the juxtamembrane region serves as docking sites for Src homology 2 (SH2)-domain containing proteins (Holland et al., 1997). In its inactive, unphosphorylated conformation, the juxtamembrane region represses catalytic function of the kinase domain (KD) through the formation of an amino acid loop which blocks ATP access. Based upon the crystal structure of the EphB2 receptor, it is proposed that activation of the EphB2 kinase domain occurs following dissociation of the amino acid loop following phosphorylation of the JM region (Wybenga-Groot et al., 2001). Consistent with this, substitution studies in which the JM tyrosine residues were mutated to phenylalanines significantly decreases ephrin-induced Eph kinase activity (Binns et al., 2000).
Figure 1.2 Structural architecture of Eph receptor and ephrin ligands.

General structural features of Eph receptors and ephrin ligands are illustrated. Both Eph receptors and ephrin ligands are membrane bound. The extracellular region of Eph receptors includes: ephrin-binding globular domain at the N-terminus, cysteine-rich region containing an epidermal growth factor-like motif and fibronectin type-III repeats. The cytoplasmic region is composed of a highly conserved (among Eph family members) juxtamembrane sequence, followed by the kinase domain, sterile alpha-motif, and postsynaptic density protein/disc/large zona occludens domain at the C-terminus. EphrinB ligands are bound to the membrane by a transmembrane segment, followed by a short cytoplasmic domain with conserved tyrosine phosphorylation sites. By contrast, while ephrinA ligands have an extracellular organization similar to ephrinBs, they are attached to the cell surface through a GPI-anchor.
Eph Receptor

Globular domain
Cys-rich region
Fibronectin Type-III repeats

Juxtamembrane region
Kinase domain
SAM domain
PDZ-binding site

PDZ-binding site

IC

EC

A A

B B
C-terminal to the kinase domain, Eph receptors contain a 70 amino acids sterile alpha-motif (SAM) domain (Hubbard and Till, 2000). The functional relevance of this low affinity domain has been suggested to be an assistance in receptor homo-oligomerization (Stapleton et al., 1999). At high local concentrations, homodimerization of Eph receptors has been suggested to be enhanced by the SAM domain (Stapleton et al., 1999). In addition, the SAM domain also acts as binding site for low molecular weight protein tyrosine phosphatase (LMW-PTP) (Stein et al., 1998) and Grb10 (Stein et al., 1996).

At the Eph receptor C-terminus, lies the postsynaptic density protein/disc large/zona occludens (PDZ)-binding domain. It is 90 amino acids in length and serves as target site for many scaffolding proteins and cytoplasmic proteins that are linked to cytoskeleton, for example, protein interacting with C kinase (PICK1) and syntenin. These functional protein interactions occur independently of receptor phosphorylation. At present, the functional relevance of this domain is unclear, as truncation of the PDZ-binding motif has not shown disruption of receptor function in vitro or in vivo (Park et al., 2004).

With respect to the ephrin ligands, the N-terminal receptor binding domain (RBD) (Figure 1.2) are highly conserved, even among the A and B class ligands (Himanen and Nikolov, 2003). Crystallographic studies of the EphB2-ephrinB2 complex show that the RBD is composed of an eight stranded β-barrel (Himanen et al., 2001). Mutational studies have subsequently confirmed the subdomains responsible for receptor binding (Himanen et al., 2001; Smith et al., 2004). In ephrinA ligands, a stretch of ~40 amino acids following the receptor binding domain, harbors the recognition site for Eph-triggered cleavage by a-disintegrin and metalloprotease (ADAM10) (see below) which allows for termination of signal (Hattori et al., 2000). In contrast, ephrinBs possess a transmembrane region followed by a highly conserved cytoplasmic domain.
composed of five conserved tyrosine residues and a C-terminal PDZ-binding motif, important for ephrin signaling as described below (Figure 1.2) (Lin et al., 1999; Torres et al., 1998).

1.3 Eph receptor activation

The current model of Eph-ephrin interaction involves the formation of a tetrameric Eph-ephrin complex where each ephrin ligand forms contacts with two Eph receptors and each receptor with two ephrin ligands (Himanen and Nikolov, 2003). Eph is the only family of RTK in which tetramer formation is required for biological activity (Davis et al., 1994; Stein et al., 1998). Consistent with this, x-ray crystallographic studies showed two distinct ephrin-binding sites located on opposite sides of the EphB2 ectodomain. Biophysical solution studies suggest that the EphB-ephrinB binding site with the larger interface mediates the initial, high affinity (nanomolar) association of ephrinB2 with EphB2, while the second site mediates further assembly of two EphB2-ephrinB2 heterodimers into an activated circular tetramer (Himanen and Nikolov, 2003; Himanen et al., 2001) (Figure 1.3). In contrast to the tetrameric EphB2-ephrinB2 complex, the EphB2-ephrinA5 complex only form dimers as a consequence of structural differences in the binding domain of ephrinA5 and ephrinB2 (Himanen et al., 2004; Lackmann et al., 1998). Thus, the low-affinity interface contains important determinants of subclass specificity. Additional residues have been suggested to play a role in stabilizing the final receptor-ligand complex, including the Cys-rich domain (Lackmann et al., 1998; Smith et al., 2004), SAM (containing low-affinity dimerization interface) (Thanos et al., 1999) and PDZ-binding domain (may bind to scaffolding proteins) (Bruckner et al., 1999; Lin et al., 1999).
Figure 1.3 Model of Eph/ephrin binding interfaces.

Two Eph-ephrin dimers join to form a tetramer, in which each ligand interacts with two receptors and each receptor interacts with two ligands. The Eph and ephrin molecules are precisely positioned and orientated in these complexes, promoting higher-order clustering and initiation of bidirectional signaling.
These regions provide weak interactions which could promote the association of the receptor-ligand complexes into an interconnected network (Himanen and Nikolov, 2003). Despite these data, the actual stoichiometry of Eph-ephrin receptor complexes in vivo remains controversial, though these complexes do exhibit a slow activation phase consistent with a nucleation event.

Prior to cell-cell contact, there is evidence to suggest that Eph receptors are loosely pre-clustered on lipid rafts within the cell membrane and unbound ephrins form low-affinity homodimers (Toth et al., 2001) (Figure 1.4A). Following initial contact, both EphB2 and ephrinB2 undergo conformational changes to expose the receptor-binding interfaces. Eph-ephrin heterodimerization then creates complementary interaction surfaces that results in the joining of dimer pairs into tetrmeric complexes. Together, these actions are thought to form the active tetramer. They are oriented in such way to allow trans-phosphorylation of the receptors. First phosphorylation events occur at the tyrosine residues of the juxtamembrane region (Figure 1.4B). Phosphorylation of these residues causes distortion of a helix which disrupts the inhibitory interactions of the juxtamembrane segment with the kinase domain. In brief, the phosphorylation causes steric and electrostatic forces that push the juxtamembrane segment away from the kinase, relieving the structural constraints that distort the active site (Figure 1.4C). In addition to enhancing kinase activity of the receptor, the exposed juxtamembrane region becomes available for interactions with downstream signaling proteins (Noren and Pasquale, 2004) (Figure 1.4C).

1.4 Eph receptor-mediated forward signaling

A major developmental role of Eph receptors and ephrin signaling is to mediate cell-contact-dependent repulsion to prevent migrating cells or neuronal growth cones from
Figure 1.4 Autoinhibition by the juxtamembrane region and receptor activation.

In the absence of ephrin binding, the kinase domain of the Eph receptor is maintained in an inhibited state through interaction of the juxtamembrane region (and the carboxy tail of the kinase regions) (A). Following ligand-mediated dimerization of the Eph receptors, EphB2 cytoplasmic domains become juxtaposed potentially with higher order Eph aggregates containing active kinase domains. This allows trans-phosphorylation of tyrosine residues in the juxtamembrane region to occur (B). Following phosphorylation, conformational changes in the inhibitory segment from the juxtamembrane region removes the autoinhibition, and allows potential recruitment of SH2 domain proteins (C). In the proximity of correctly orientated kinase domains enables trans-phosphorylation of the tyrosine residues on the juxtamembrane regions.
entering ephrin-expressing territories; thus confining them to an appropriate pathway. However, studies have shown that adhesive responses can also occur as a result of Eph-ephrin interaction. For example, ephrinB1 has been shown to both repel and attract different populations of EphB-expressing neural crest cells (Santiago and Erickson, 2002). Similarly, ephrinA5 has been shown to mediate the repulsive guidance of motor axons to distal limb musculature (Eberhart et al., 2002), but provides positive guidance for motor axons which project to axial muscles (Eberhart et al., 2004). Finally, ephrinA2 has been shown to both promote and inhibit Eph-expressing retinal axons (Hansen et al., 2004). The biochemistry regulating these alternative responses is only now being investigated.

Binding of a transmembrane receptor to a ligand tethered to the surface of an opposing cell might be expected to promote adhesion. However, most of the mechanisms through which these Eph receptors are known to be involved are repulsive in nature. Two mechanisms have been proposed to address the problem of receptor disengagement following receptor activation (Figure 1.5). In one suggested mechanism, interaction of EphA3 and ephrinA2 leads to proteolytic cleavage in the juxtamembrane domain of ephrinA2 by ADAM-10, a kuzbanian (KUZ) domain metalloproteinase (Hattori et al., 2000). KUZ has been shown to associate with ephrinA2 (not Eph receptors) prior to cell-cell contact. Release of the ephrin ectodomain by ADAM-10 has been shown to require the presence of complex formation between ephrinA2 and EphA3 receptor on the opposing cell (Hattori et al., 2000) (Figure 1.5 C). This breaks the adhesive bond between the opposing cells enabling detachment. Consequently, inhibition of ADAM-10 would result in the maintenance of adhesion between by Eph/ephrin, which could promote neurite growth. At present, such mechanisms have been suggested to play little role in
Figure 1.5 Mechanism of Eph/ephrin-mediated repulsion versus adhesion.

(A) Cells expressing Eph receptors and ephrin ligands prior to cell-cell interaction. (B) Upon contact, Eph receptors and ephrins bind on opposing cells resulting in phosphorylation events that lead to activation of signaling downstream. (C) One mechanism allowing disengagement of cells joined by Eph-ephrin complexes is proteolysis of the ephrinAs by ADAM10. After interaction EphA and its ligand, ADAM-10 comes and cuts ephrinA2, permitting retraction/detachment. (D) Termination of EphB-ephrinB complexes is facilitated by transcytosis of both the receptor and ligand. (E) Direct adhesion promotes attraction upon contact with a truncated Eph receptor.
A Prior to cell contact

EphrinA
EphrinB
IC
EC
EphA
EphB
IC

B Signaling

Signal

C Repulsion

1. Proteolysis

D Repulsion

2. Endocytosis

Vesicle

E Adhesion
regulating ephrinBs’ termination of adhesion, instead they are known to be regulated through a different mechanism.

In this second mechanism, EphB-ephrinB interactions at the cell surface can be terminated through endocytosis of the receptor-ligand complex into either the EphB or ephrinB expressing cells (Marston et al., 2003; Zimmer et al., 2003). This bidirectional transcytosis involves engulfment of membrane vesicles from one cell to its neighbor (Figure 1.5D). *In vitro* studies showed that when both EphB2 and ephrinB2 are truncated, complexes were not endocytosed, resulting in strong adhesion (Zimmer et al., 2003). Mutants lacking the ephrinB cytoplasmic domain result in a complex that was internalized only into EphB2-expressing cell. Whether ephrinB activation is required for endocytosis into the ephrinB-expressing cell is still unclear. This form of signal ablation, which removes adhesive complexes from the cell surface, also occurs in EphA-ephrinA signaling and has been shown to require the Cbl ubiquitin ligase as an adaptor (Sharfe et al., 2003; Wang et al., 2002). However, unlike the EphB-ephrinB transcytosis, this phenomenon occurs only in the direction of EphA-expressing cells. The mechanism proposed is likely to involve Rac1-mediated retraction as disruption of Rac1 pathway interfered with ligand-induced endocytosis and growth cone collapse in retinal neurons (Jurney et al., 2002).

In contrast to the roles of Eph-ephrin interaction in mediating cell repulsion, in other situations they can act as positive guidance cues for migrating cells and neuronal growth cones. The physiological importance of Eph adhesive effects is highlighted by the defects that are observed in midline fusion in the neural tubes of *ephrinA5* null mutants (Holmberg et al., 2000). Adhesion can result from the activation of effectors downstream of Eph receptors that lead to cell adhesion to the ECM or axon extension (Figure 1.5E). Attraction can also arise from poor
activation of the signaling pathways that promote cell detachment. Consistent with this, splice forms of EphA7 that lack kinase activity shifts cellular response from repulsion to adhesion (Holmberg et al., 2000). Interestingly, different level of Eph forward signaling can lead to shifts between cell repulsion and adhesion (McLaughlin et al., 2003).

1.4.1 Eph signaling through Rac/Rho family GTPases

The principal model whereby Eph receptors regulate growth cone dynamics is through their regulation of the activity of GTPases of the Rho family. Rho GTPases cycle between an active GTP-bound conformation, and an inactive GDP-bound conformation. These GTPases control cell shape and movement by altering the formation of stress fibers (Rho), lamellipodia (Rac) and filopodia (Cdc42) (Nobes and Hall, 1999). In neurons, Rho activation induces growth cone collapse by altering actin polymerization (Luo, 2000; Yuan et al., 2003) while Rac and Cdc42 promotes the formation of F-actin network in growth cone lamellipodia and filopodia extension (Yuan et al., 2003). Substantial evidence has linked Eph receptors and their ligands to several members of Rho family (Irie and Yamaguchi, 2002; Penzes et al., 2001a; Shamah et al., 2001; Wahl et al., 2000; Zhang et al., 2005), and differential regulation of Rho, Rac and Cdc42 has been observed in Eph receptors upon ligand stimulation.

EphA receptors

EphA receptors can also directly activate Rho GTPases through the Rho GTPase exchange factor (Rho-GEF) ephexin (Shamah et al., 2001). Rho-GEFs function to activate GTPases by catalyzing the replacement of bound guanosine diphosphate (GDP) with guanosine
triphosphate (GTP). In the EphA signaling, ephexin binds constitutively to the kinase domain of EphA4 through its Dbl homology (DH) and pleckstrin homology (PH) domains (Shamah et al., 2001) (Figure 1.6). Upon EphA receptor activation by ephrinA ligands, ephexin has been shown to mediate the activation of RhoA in cultured cortical neurons resulting in growth cone collapse (Wahl et al., 2000). Consistent with this, EphA can lead to downregulation of Rac and Cdc42 activation (Shamah et al., 2001; Wahl et al., 2000) and to inactivation of downstream effectors of Rac, Pak (Jurney et al., 2002) (Figure 1.6). Decreasing Rac activity results in retraction of lamellipodia and filopodia, thereby further potentiating the repulsive signaling. Ephexin is highly expressed in retinal ganglion cells at the time when these neurons establish their topographic connections to the visual system, consistent with a possible role in guiding these axons to their targets (Shamah et al., 2001).

In the Vav family of exchange factors, Vav2 also plays a role in regulating growth cone collapse in retinal ganglion cells (Cowan et al., 2005). Vav2 binds directly to phosphorylated tyrosine residues in the EphA4 receptor juxtamembrane region and is transiently activated. Binding of Vav2 to EphA4 initially results in an adhesive interaction which is then converted into a repulsive response (Cowan et al., 2005). This is accomplished through the Vav2-mediated activation of Eph-ephrin complex endocytosis, ultimately resulting in growth cone collapse. In Vav2 null mice, endocytosis is inhibited and significant defects in the ipsilateral retinogeniculate projections were observed (Cowan et al., 2005). Functional redundancy has been demonstrated between Vav2 and Vav3 and axonal projections from the retina to the dorsal lateral geniculate nucleus (dLGN) were observed to be altered in Vav2-Vav3 double knockouts (Cowan et al., 2005). These findings suggest an important role for Vav family GEFs as regulators of ligand-receptor endocytosis and determinants of repulsive signaling during axon guidance.
Figure 1.6 EphA receptors differentially regulate Rho, Rac and Cdc42.

Domain and structure of EphA receptors involved in activating GEFs in downstream signaling pathway. Ephexin binds constitutively to the kinase domain of EphA4 receptor and is activated following ephrinA stimulation of the receptor. In turn, it promotes RhoA activation and simultaneously decreases both Rac and Cdc42 activities, together resulting in growth cone collapse. The recruitment of Vav2 to activated Ephs inhibits Rac-dependent actin polymerization and promotes endocytosis.
EphrinA

EphA receptors

Vav2

Ephexin

RhoA-GDP

RhoA-GTP

GAPs

Cdc42-GTP

Rac-GTP

Cdc42-GDP

Rac-GDP

Growth Cone Collapse

EC

IC

Extracellular domains
Kinase domains
SAM domains
Phosphorylation
Recent findings have also identified a Rac-specific GTPase activating protein (GAP), $\alpha_2$-chimaerin, as a mediator of EphA4-mediated axonal growth cone collapse (Beg et al., 2007; Iwasato et al., 2007; Wegmeyer et al., 2007). In these studies, animals lacking the $\alpha_2$-chimaerin gene display a phenotype similar to that of EphA4 mutant mice, including aberrant midline axon guidance and defective spinal cord central pattern generator activity. Unlike ephxin, $\alpha_2$-chimaerin binds to phosphorylated Eph receptors. Upon binding to EphA4, $\alpha_2$-chimaerin inactivates Rac1 and inhibits downstream signaling resulting in axonal retraction. Recent reports provide evidence of a cyclin-dependent kinase 5 (Cdk5), known to interact with $\alpha_2$-chimaerin (Brown et al., 2004), in regulating of EphA4-mediated dendritic spine retraction. This raises the possibility that a combination of proteins may be recruited to the Eph-$\alpha_2$-chimaerin complex, and together function to regulate specific neuronal behavior. The ultimate control of actin dynamics by the Eph/ephrin signaling appears to depend upon a balance between activation of Rho-GEF (leading to growth cone collapse) and inactivation of Rac-GAP (resulting in neurite extension). Interestingly, ablation of ephxin1 or Vav-2/Vav-3, which couple to activated Epha4 receptors, does not phenocopy the locomotor defects in EphA4 and $\alpha_2$-chimaerin mice. Rather, these mutants exhibit defects in retinal ganglion cell axon guidance (Cowan et al., 2005). This may be explained by cell-type specificity of EphA4 signaling.
EphB receptors

There are several RhoGEFs with which EphB receptors can interact. In hippocampal neurons, EphB2 has been shown to associate with the exchange factors intersectin (Irie and Yamaguchi, 2002) and kalirin (Penzes et al., 2003) (Figure 1.7). Intersectin activates Cdc42 and kalirin activates Rac, both leading to the regulation of EphB-mediated morphogenesis and dendritic spine maturation. Intersectin associates with the kinase domain of EphB2 independently of receptor activation (Irie and Yamaguchi, 2002). Studies have shown that intersectin is activated by the synergistic actions of EphB2 with neural Wiskott-Aldrich syndrome protein (N-WASP) (Cory et al., 2002). Kalirin is predominantly expressed in the nervous system and plays a role in dendritic morphogenesis through binding to EphB2 receptors (Penzes et al., 2003; Penzes et al., 2001b). Unlike ephexin and intersectin, kalirin binds only to activate EphB2 receptors. However, the region mediating the EphB2 and kalirin interaction has yet to be determined. Upon ephrinB1 stimulation of hippocampal neurons, phosphorylation of kalirin has been observed, which is followed by activation of Rac1, to activate PAK downstream. This suggests that kalirin regulates Rac1 activation downstream of EphB2 receptors. Consistent with this, hippocampal neurons expressing dominant negative forms of either Rac1 or PAK showed inhibition of spine morphogenesis in response to ephrinB stimulation (Penzes et al., 2003). Thus, both kalirin and intersectin are important mediators of EphB-ephrinB induced actin rearrangement in dendritic spines.
Figure 1.7 EphB receptors and Rho family GTPases.

EphB receptors mediate growth cone turning through activation of Rac1 and Cdc42 via GEFs, intersectin and kalirin. While intersectin binds constitutively to the cytoplasmic domain of the EphB receptor, kalirin is recruited following ephrinB stimulation. These Rho GTPases activate Cdc42 and Rac1 respectively. This scenario favors axon outgrowth.
1.4.2 Eph signaling through Ras

Eph receptor can also signal through the Ras family of GTPases. Ras GTPases have previously been shown to be important players in regulating cell migration, neurite outgrowth and axon guidance through activation of the mitogen-activated protein (MAP) kinase cascade (Chang and Karin, 2001; Forcet et al., 2002). Eph receptors have been shown to engage both positive and negative regulators of the MAPK pathway. Depending on the cell type, Eph-mediated MAPK activation can lead to a decrease of ECM adhesion (Pratt and Kinch, 2002) or a stimulation of cell proliferation (Yu et al., 2003). Eph receptors can also negatively regulate Ras/MAPK activity, suppressing cell proliferation (Miao et al., 2001) or neurite retraction (Elowe et al., 2001). Eph receptors are among the few receptor tyrosine kinases known to negatively regulate H-Ras and the downstream MAP kinase pathway. In neuronal cells, EphB2 mediates suppression of H-Ras activation, which is required for RhoA-mediated neurite retraction (Elowe et al., 2001; Tong et al., 2003). Another mechanism by which EphB receptors negatively regulate the Ras/MAP kinase pathway is by favoring negative signaling of p120RasGAP (Figure 1.8). p120RasGAP is recruited to the activated EphB receptors and suppresses H-Ras activity within minutes of ephrin treatment (Elowe et al., 2001; Holland et al., 1997; Tong et al., 2003). Furthermore, cells lacking p120RasGAP or expressing dominant negative p120RasGAP failed to inhibit MAP kinase activity in response to ephrinB1 stimulation, suggesting that p120RasGAP is a critical mediator of H-Ras inactivation downstream of EphB2 (Elowe et al., 2001). Eph receptors not only regulate H-Ras but they also impact the activity of other Ras family proteins. In hippocampal neurons, EphB2 activation inhibits integrin-mediated adhesion through phosphorylation of a tyrosine in the effector domain of R-Ras, which can then no longer bind and activate the MAPK activator Raf1 (Grunwald et al., 2004; Zou et al., 1999).
Figure 1.8 Positive and negative regulation of the MAPK pathway by Ephs.

(A) EphB1 receptor has been shown to activate the MAPK cascade through recruitment of the Grb2/Sos complex. Recruitment of Grb2 to the activated receptor results in engagement of Grb2/Sos and promotes activation of MAPK. (B) Recruitment of p120RasGAP to EphB2 receptor inhibits Ras activation and terminates downstream signaling of MAPK.
Positive regulation of MAPK

Negative regulation of MAPK

Extracellular domains
Kinase domains
SAM domains
Phosphorylation
One candidate molecule that may link EphB2 to R-Ras is the signaling protein SHEP1 (Dodelet et al., 1999). SHEP1 binds directly to EphB2 and, in addition, binds to R-Ras and Rap1A, suggesting that SHEP1 localizes these GTPases at the sites of EphB2 activation. Under some circumstances, however, the Eph receptors activate rather than inhibit the MAP kinases. Overexpression of EphB receptors in neuronal cells has been reported to activate Erk1/Erk2 MAP kinase (Grunwald et al., 2001; Miao et al., 2001). In most cases, the activating effect of Eph receptors is mediated by the recruitment of the Grb2-Sos1 complex, which acts as a Ras-specific GEF (Pratt and Kinch, 2002) (Figure 1.8). Overexpression of EphB2 in neuronal cell-lines has reported to activate Erk1/Erk2 MAP kinases through its strong association with growth factor receptor-bound protein2 (Grb2) and inability to bind RasGAP (Miao et al., 2001). In sum, the Eph-mediated regulation of the MAP kinase pathway depends on the particular combination of signals to which the cell is subjected and on the distinct cell types.

1.4.3 *Cis* versus *trans* interaction

Interactions between Eph receptors and ephrins expressed on opposing cells are known as *trans* interactions, whereas ligand-receptor interaction on the same cell are termed *cis* interactions. Initial *in vitro* studies on retinal ganglion cells (RGC) have shown a loss of sensitivity to stimulation when EphA receptors and ephrinAs are expressed in *cis*. When coexpressed with EphAs in culture, ephrinAs interacted directly in *cis* via their binding domain with adjacent EphA receptors rendering them unavailable for *trans* interactions (Carvalho et al., 2006) (Figure 1.9B). However, the mechanism underlying the Eph-ephrin mediated loss of sensitivity in these RGC is still unknown. In the motor system, spinal motor axons that innervate limb muscles have been shown to coexpress EphA4 receptor and ephrinA ligands, raising the
Figure 1.9 Cis versus trans interaction between ephrins and Eph receptors.

(A) The interaction *in trans* of ephrin and Eph receptors expressed in opposite cells resulting in bidirectional signaling. (B) Coexpression of Eph receptors and ephrins in the lower cell leads to *cis* interaction which suppresses forward signal transduction and blocks interaction with ephrin *in trans*. (C) Coexpressed Eph and ephrin proteins with ability to signal opposing effects on the growth cone.
A. Reverse signaling

B. Forward signaling

C. GC spreading

GC collapse
question of the role of cis interaction (Marquardt et al., 2005). In vitro studies have demonstrated that while motor neuron explants when challenged with preclustered ephrinAs exhibited growth cone collapse, the addition of preclustered EphAs to these explants resulted in an opposite effect that induced significant neurite extension (Marquardt et al., 2005). This suggests that subcellular arrangements of Eph-ephrin proteins enable axons to discriminate between cis- versus trans-configurations of ligand/receptor proteins, thereby allowing opposite actions (Figure 1.9C).

1.4.4 Negative regulation of Eph forward signaling

The regulation of Eph signaling depends on the balance of protein tyrosine kinase (PTK) and phosphatase (PTP) activities within the cell at a given time. Following endocytosis of Eph/ephrin complex, signaling is terminated by the dephosphorylation of tyrosine residues at the juxtamembrane domain, converting it to the autoinhibited state. Several PTPs are known to regulate Eph/ephrin signaling pathways, in particular the Low Molecular Weight (LMW)-PTP. Through dephosphorylation of EphA2, LMW-PTP has been shown to negatively regulate ephrinA1-mediated signaling by inhibiting cell proliferation, cell adhesion and spreading, as well as the formation of retraction fibers (Kikawa et al., 2002; Miao et al., 2001). In contrast, recruitment of LMW-PTP to oligomeric Eph/ephrin clusters is required for EphB1-mediated integrin adhesion (Becker et al., 2000) and EphB2-directed vascular network assembly (Stein et al., 1998). LMW-PTP has also been shown to interfere with ephrin-mediated MAP kinase signaling through its inhibition of p120RasGAP binding to activated EphA2 (Parri et al., 2005).

Other PTPs known to associate with Eph/ephrins include the SH2-domain containing PTP2 (SHP1) (Luo et al., 2001). SHP1 binds constitutively to EphB6, one of two Eph receptors
lacking kinase activity. Even in the absence of kinase activity, EphB6 clustering has been shown to lead to Cbl dephosphorylation through association with SHP1 (Freywald et al., 2002).

A recently identified phosphatase, protein tyrosine phosphatase receptor type O (Ptpro) is also involved in Eph/ephrin downstream signaling. Overexpression of Ptpro in the chick retina decreased sensitivity of EphA expressing temporal axons towards ephrinA2 (Shintani et al., 2006). Moreover, overexpression of the inactive form of Ptpro increased signaling response to ephrinA2. However, the importance of this negative regulation of Eph receptors has yet to be determined in vivo. Taken together, emerging evidence for roles of PTPs in modulating Eph/ephrin signaling suggests the importance of equilibrium in the net level of tyrosine phosphorylation in regulating Eph/ephrin activity.

1.4.5 Cross-talk in Eph/ephrin signaling

Cross-talk between Eph/ephrins has been demonstrated with several RTK signaling pathways. The first demonstration of communication was described for cross-talk between the Eph and fibroblast growth factor (FGF) family in regulating cell adhesion. In embryo culture studies, FGF has been shown to inhibit ephrin-induced dissociation of blastomeres (Jones et al., 1998). Activated FGF receptor directly binds to ephrinB inducing its phosphorylation on tyrosine residues, which in turn inhibits the ability of ephrinB to induce cell dissociation (Chong et al., 2000). In vivo relevance of this interaction was illustrated in the formation of the eye field in Xenopus. Moody et al. (2004) showed that the lack of ephrinB1 phenocopied the repression associated with activated FGF receptor to restrict retinal progenitors from migrating into the eye field (Moody, 2004). These studies point to an antagonistic interaction between FGF and ephrin pathways (Figure 1.10). More recently, the interplay between FGF and Eph/ephrin
Figure 1.10 Antagonistic and synergistic cross-talk with Eph/ephrin signaling.

Schematic diagram depicts interactions of Eph receptors with other cell surface receptors and the regulation of downstream effectors and biological processes. Plus signs indicate synergistic regulation while minus signs indicate antagonistic regulation of downstream effectors.
Ryk, Eph, and FGFR are involved in various cellular processes such as axon guidance, cleft palate formation, asymmetric division, oocyte maturation, and cell migration. The diagram illustrates the interactions between these signaling pathways and their respective effects on cell behavior.
signaling has been demonstrated in asymmetric cell division in *Ciona* embryos (Picco et al., 2007). The work of Picco et al. (2007) demonstrated that the antagonistic relationship may also be mediated further downstream through differential regulation of ERK activation in promoting notochord fate. In this system, Eph/ephrin signaling acts to attenuate FGF-Ras-ERK pathway to initiate asymmetric cell division leading to acquisition of distinct cell fates (Picco et al., 2007).

Another potential signaling interaction for Eph/ephrin activity is Ryk. Ryk null mice exhibit craniofacial defects similar to those observed for *EphB2B3* double knockouts (Halford et al., 2000; Orioli et al., 1996). The authors proposed that Ryk interacts directly with EphBs to facilitate recruitment of AF-6 to the receptor, thereby facilitating activation downstream (Halford and Stacker, 2001). However, the exact mechanism by which Ryk influences EphB/ephrinB signaling is still unclear (Figure 1.10).

Given that the ERK/MAPK pathway represents a point of convergence for many different signaling pathways, it is not surprising that the ability of Eph/ephrin to modulate the activity of ERK/MAPK could impinge on multiple signaling systems. An example of this is the ability of EphB2 and EphB4 to antagonize the migration and proliferation of endothelial cells by vascular endothelial growth factor (VEGF) and angiopoietin (Kim et al., 2002) (Figure 1.10). Another example is the antagonism of major sperm protein (MSP)-mediated oocyte maturation by VAB-1 (the *C. elegans* Eph receptor) (Miller et al., 2003) (Figure 1.10).

EphA4/ephrinA has also been shown to interact cooperatively with glial cell-derived neurotrophic factor (GDNF)/Ret signaling to mediate the repulsive guidance of motor axons (Kramer et al., 2006). Genetic ablation studies demonstrated that absence of either Ret or EphA4 produces phenotypes which vary in severity, while the absence of both receptors resulted in a more severe phenotype of axon misrouting during limb innervations (Kramer et al., 2006).
authors postulate that the Ret and EphA4 converge to modulate a common downstream effector, ephexin1 (Figure 1.10). These experiments, however, do not rule out the possibility of other points of convergence downstream.

1.5 Reverse signaling of Eph receptors

Forward signaling is clearly a major mechanism in Eph signaling. However, several in vivo studies have demonstrated that Eph signal can occur in the reverse direction, through ephrin-mediated signaling (Henkemeyer et al., 1996; Kullander et al., 2001b). Evidence for this has been observed for several neural systems, including neural crest cell migration (Davy et al., 2004), cerebellar progenitor migration (Lu et al., 2001), synaptic plasticity (Armstrong et al., 2006; Grunwald et al., 2004), and axon guidance (Cowan et al., 2004). In this mechanism, upon binding to the EphB receptors, ephrinB cytoplasmic domain becomes phosphorylated on conserved tyrosine residues through the activity of associated Src family kinases (SFKs) (Holland et al., 1996; Kalo et al., 2001) (Figure 1.11). Eph receptor binding is thought to structurally alter the β-hairpin conformation of the ephrin cytoplasmic domain, leading to the recruitment of Src-family kinases and phosphorylation of tyrosine residues on the ephrin cytoplasmic domain (Kalo et al., 2001; Palmer et al., 2002). These phosphorylated residues on ephrinB ligands serve as a docking sites for Src-homology 2 domain-containing proteins, such as growth factor receptor bound protein 4 (Grb4), initiating a cytoskeletal reorganization (Cowan and Henkemeyer, 2001). EphrinB reverse signaling has been shown to promote spine maturation through a Grb4 activated pathway which includes the G protein-coupled receptor kinase interacting protein (GIT) 1 and the exchange factor for Rac GTPase, β-PIX.
Figure 1.11 Phosphorylation-dependent reverse signaling by ephrinB.

(1) Activation of ephrinB proteins leads to recruitment of Src-family kinases (SFKs) which phosphorylate the tyrosine residues on the cytoplasmic tail of ephrinB ligands. Subsequently, Grb4 is recruited to phosphorylated ephrinBs and initiates a cascade of signaling events that regulate cytoskeleton dynamics. (2) Much after activation, phosphatase PTP-BL dephosphorylates ephrinB and inactivates Src, which terminates downstream signaling.
Presynaptic ephrinBs have also been shown to be necessary for the induction of mossy fiber long term-potentiation (LTP) (Contractor et al., 2002). In this respect, it is interesting to note that ephrinB reverse signaling has been shown to increase glutamate release resulting in subsequent increase in calcium influx into the postsynaptic neuron (Palmer et al., 2002). Other known signaling pathways downstream of ephrinB1 involves Dishevelled (Dsh), acting through Grb4 to regulate the migration of retinal progenitor cells during eye field formation (Lee et al., 2006; Tanaka et al., 2003).

Like Eph receptors, ephrin signaling is terminated by phosphotyrosine phosphatase, such as PTB-BL. This molecule acts to dephosphorylate ephrinB and inactivate Src kinases (Palmer et al., 2002) (Figure 1.12). First functional evidence for this mechanism was provided by the regulation of cerebellar granule cell migration (Lu et al., 2001). During the development of the cerebellum, ephrinB activation renders postmitotic granule cells insensitive to stromal cell derived factor 1 (SDF-1) activation of chemokine receptor 4 (CXCR4) by its association with a GTPase-activating protein for heterotrimeric G protein, PDZ-regulator of G protein signaling 3 (PDZ-RGS3) (Lu et al., 2001) (Figure 1.12). Through its PDZ domain, PDZ-RGS3 binds constitutively to the PDZ-binding motif of ephrinB1. Binding to active ephrinB1 enhances PDZ-RGS3 catalysis of GTP to GDP in the alpha subunit of heterotrimeric G proteins. This results in an inhibition of CXCR4 activation of SDF-1 at the pial surface of granule cells.

Controversy exists as to whether ephrinA ligands can convey its own signal to modify cellular behavior since these ligands do not possess an intracellular domain. Studies have suggested that ephrinAs are targeted to lipid rafts, where they presumably assemble into protein complexes that transduce intracellular signal (Marquardt et al., 2005). It has been speculated that the clustering of ephrinA to lipid rafts recruits Src family kinases, Fyn and is accompanied by
Figure 1.12 Phosphorylation-independent reverse signaling by ephrinB.

PDZ-RGS binds constitutively to ephrinBs and links regulation of G-protein-coupled signaling to ephrins. The chemoattractant SDF-1 interacts with CXCR4 receptor to induce Gα subunit exchange GDP to GTP, which promotes migration of cerebellar neurons. Activation of ephrin and PDZ-RGS, together reverse G-protein induced signaling and return it to its inactive form, inhibiting cell migration.
redistribution of vinculin, activation of MAP kinase and increase cell substrate adhesion (Davy et al., 1999; Knoll et al., 2001). However, genetic evidence is conflicting and the mechanism underlying signal transduction via ephrinAs remains unproven in vivo.

1.6 Functional diversity of Eph-ephrin interactions

Eph receptors and ephrins have been shown to regulate a wide array of cell-cell contacts which extend beyond axon guidance. Eph receptors are involved in an array of developmental cell sorting processes in the skeletal, cardiovascular and vascular systems as well as synaptic plasticity (Henderson et al., 2001; Palmer and Klein, 2003). In addition, Eph receptors have been implicated in learning and memory (Gerlai, 2002), bone homeostasis (Zhao et al., 2006), and insulin secretion (Konstantinova et al., 2007).

Work from our laboratory has shown that EphB receptors can direct the sorting of intestinal epithelial cells originating in the villus crypts traveling towards the lumen in the mammalian intestine (Batlle et al., 2002; Holmberg et al., 2006). Opposing gradients of EphB receptor and ephrinB expression along the intestinal crypt serves to restrict intermingling between proliferative and differentiated cells (Figure 1.13) (Batlle et al., 2002). EphB3 is specifically localized in Paneth cells, which migrate to the bottom of the crypts. Mice lacking the EphB3 gene have Paneth cells randomly distributed along crypts instead of being normally restricted to the base of the crypt. Combinatorial null mutants of EphB2 and EphB3 exhibit aberrant intermingling of the proliferative and differentiated population of epithelial cells, suggesting a critical role for these receptors in guiding the proper location of different cell population within the small intestine.
Figure 1.13 Regulation of intestinal epithelial cell migration by EphB/ephrinB signaling.

Shown is the schematic representing the adult small intestinal crypt (figure adopted from Palmer and Klein 2003). In wild-type, stem cells in the crypt give rise to proliferative cells co-expressing EphB2 and ephrinB1, B2 in an inverse, position-dependent pattern. These cells migrate toward the villus, where they initiate differentiation. Paneth cells expressing EphB3 receptors migrate toward the base of the crypt, below the putative stem cell zone. In mice lacking EphB2 and EphB3 receptors, proliferative cells expressing high expression of ephrinBs invade the bottom of the crypt, while Paneth cells are found throughout the crypt.
Paneth cells

Proliferative cells

Stem cells

*Wild-type*

Cript-villus junction

EphB3

Base

*Protein expression*

EphB2

ephrinB

Wnt

*EphB2B3-/-,-/-*

49
In the developing vasculature, Eph/ephrin signaling are important for angiogenic remodeling, and represent the earliest known markers of venous versus arterial cell identity. *In vivo* studies showed that mice homozygous for null alleles of *ephrinB2* and *EphB4* closely phenocopy one another, exhibiting embryonic lethality at E10.5 due to severe defects in angiogenic remodeling (Gerety et al., 1999). Interestingly, the reciprocal pattern of expression by EphB4 (in veins) and ephrinB2 (in arteries), suggests a repulsive mechanism of action for the segregation of the distinct vessel types. However, this interpretation may be overly simplified given the expression of EphB3 in the developing veins and ephrinB1 in both arterial and venous endothelium (Adams et al., 1999). Indeed, a portion of *EphB2<sup>-/-</sup>*<sup>B3<sup>-/-</sup></sup> mice exhibit vascular defects (Adams et al., 1999). Nevertheless, the mechanism by which these Eph receptors and ligands act together in controlling angiogenic remodeling remains unclear.

### 1.6.1 Role of Eph receptors in axon guidance within the CNS

Eph receptors and ephrins are primarily expressed in the developing and adult nervous system in mammals, reflecting their prominent role in neural development and function (Becker et al., 1994; Ciossek et al., 1995; Ganju et al., 1994; Henkemeyer et al., 1994; Lai and Lemke, 1991; Maisonpierre et al., 1993; Nieto et al., 1992; Pasquale et al., 1992). Due to their ability to trigger cytoskeletal remodeling, they have been shown to play a crucial role in axon guidance in a number of systems. The most studied activity of Eph receptors and ephrins is in the establishment of topographic connections within the CNS, at sites such as the retina, vomeronasal organ, thalamocortical neurons and muscle innervations. In this context, topographic organization describes specific spatial arrangement resulting in organized innervation of one structure to another. Eph receptors and ephrins are essential for this process.
which allows interhemispheric communication at sites such as the corpus callosum, corticospinal tract (CST) and anterior commissure (AC).

**Retina**

One of the most well studied Eph-mediated topographic system is the projection of retinal ganglion cells (RGC) to the superior colliculus (SC) along the anterior-posterior (A-P) and dorsoventral (D-V) axes. Anterior-posterior (A-P) topographic patterning of retinal axons is controlled in part by a graded distribution of EphA and ephrinA members (Pasquale, 2005). EphrinAs are expressed in a low-high A-P gradient in the SC, whereas a complementary low-high gradient of EphA receptors (EphA3, A5 and A6) is expressed along the nasal-temporal (N-T) axis of the retina (Cheng et al., 1995; Feldheim et al., 1998). Axons expressing high levels of EphA receptors project to the anterior midbrain of tectum (in chick) or superior colliculus (SC: in mammals); regions with low levels of ephrinA2 and A5 expression. Axons expressing low levels of EphA receptors project to the posterior regions of the SC region which exhibit high expression of ephrinA (Drescher et al., 1995; Feldheim et al., 2000) (Figure 1.14). *In vitro*, EphA signaling has been shown to induce collapse of retinal growth cones (Drescher et al., 1995; Flanagan and Vanderhaeghen, 1998). Consistent with this model, loss of ephrinA2 and ephrinA5 cause retinal axons to overshoot their appropriate target area projecting to posterior regions of the SC in *ephrinA2/A5* double knockouts. In this model, the shift of axons to ectopic sites within the SC increases the local competition with other axons at that site; causing further misprojection (Feldheim et al., 2000). With respect to Eph receptors, genetic studies have yet to reveal defects in retinotopic mapping comparable to those seen in *ephrinA2/A5* double mutants. However, targeted knock-in of EphA3 into a subset of RGCs, disturbing the EphA gradient in the retina,
Figure 1.14 Eph receptors and ephrins in retinotopic mapping.

In the map connecting the retina to the optic tectum (chicken)/superior colliculus (mammals), EphA receptors and ephrinAs are expressed in opposing gradient. (A) Along the anterior-posterior axis, retinal ganglion cells in the nasal retina expressing high levels of EphAs project their axons to more posterior part of the superior colliculus with lower level of ephrinAs. (B) In contrast, high levels of EphB in the ventral retinal axons project to medial regions of the superior colliculus which express abundant ephrinBs, suggesting an attractive signal by EphB/ephrinB interaction.
concomitantly caused projection errors of both EphA3-overexpressing and wild-type RGCs (Brown et al., 2000). Together, these findings suggest that positional information is defined by the relative levels of EphA on neighboring axons, rather than the absolute Eph-level in individual cells.

Whereas the EphAs and ephrinAs are involved in establishing the A-P axis during retinocollicular mapping, analysis of EphB family of receptors have revealed a role for these members in specifying proper D-V innervation. Studies in chick and mouse have revealed low-to-high gradients of EphBs (B2, B3 and B4) expression along the D-V axis of the retina (Birgbauer et al., 2000; Connor et al., 1998; Henkemeyer et al., 1996). EphrinB1 is expressed in a low-to-high gradient along the lateral-to-medial (L-M) axis in the SC (Hindges et al., 2002) (Figure 1.14). In contrast to the repulsive guidance mediated by EphA/ephrinA, ventral axons expressing high level of EphB project to target with high ephrinB level, suggesting an attractive response. Consistent with this, EphB2B3 double knockouts show aberrant RGC axon extension resulting in a lateral shift of the axon termination zones within the SC (Hindges et al., 2002) (Figure 1.14). Mice with kinase-inactive EphB2 have similar D-V mapping defects; indicating that forward rather than reverse signaling is required for this guidance as part of the retinal axons.
Vomeronasal organ

Another well-characterized topographic map consists of the vomeronasal organ (VNO) innervation to the accessory olfactory bulb (AOB). Vomeronasal organ is a distinct and bilaterally symmetric structure of the ventral nasal septum in terrestrial vertebrates that is mainly responsible for detection of smell (Knoll et al., 2001). VNO axons originate in the vomeronasal epithelium, travel along the septum, cross the cribiform and project to the AOB. EphrinA5 is expressed at a higher level in the apical versus basal aspect of the VNO, while EphA6 is expressed at a higher level in the anterior than in the posterior part of the AOB. \textit{In vitro} guidance studies have demonstrated that VNO axons expressing high levels of ephrinA5 project to regions of the AOB with high level of EphA6; suggesting that the Eph/ephrin interaction at this site is attractive rather than repulsive (Knoll et al., 2001). In support of this, genetic ablation of ephrinA5 resulted in the inability of VNO axons to reach the appropriate domain within the AOB. Taken together these \textit{in vitro} and \textit{in vivo} experiments demonstrate an important role for EphA/ephrinA complexes in the topographic establishment of the vomeronasal system.

Thalamocortical connections

Similar to the retina, the development of connections between thalamic afferents and the cortex occurs in a highly organized manner. Thalamocortical projections arise from the ventrobasal nucleus within the thalamus and terminate in cortical layer 4, hence the name “thalamocortical” (Mann et al., 2002). Studies using ephrinA5 knockouts demonstrated a contribution of EphA/ephrinA signaling to the thalamocortical projections (Uziel et al., 2002). Normally, limbic thalamic neurons in the lateral-dorsal nuclei avoid the sensorimotor cortex and project to the cingulated cortex. In absence of ephrinA5, these thalamic neurons formed aberrant
projections to the sensorimotor cortex. This suggests a guidance role for ephrinA5 in restricting thalamic axons from connecting with inappropriate neocortical areas (Uziel et al., 2002). In addition, the connections between the thalamus and the neocortex were seen to be even more perturbed in mice lacking both EphA4 and ephrinA5 genes. In these animals, axons from medial ventrobasal nucleus project to more medial domains in the somatosensory S1 area of the cortex (Dufour et al., 2003). Together, these findings suggest that expression of ephrinA5 within the cortex is acting as a repulsive cue for thalamocortical axons expressing EphA4 (and other EphAs) to generate a precise point-to-point somatosensory map.

**Spinal cord**

As we have seen, neurons in many part of the nervous system form topographically ordered maps of connections onto their targets. This is also true for somatic motor neurons within the spinal cord, which project to specific muscle targets in the hindlimb (Lance-Jones and Landmesser, 1981). Motor neurons are organized in longitudinal columns within the ventral spinal cord and are topographically positioned both rostrocaudally and mediolaterally in relation to the location of the specific muscles that they innervate. Motor neurons in the medial motor column (MMC) extends their axons to the body wall and back muscles along the entire length of the spinal cord, whereas, the motor neurons in the lateral medial motor column (LMC) innervate the limb muscles at brachial and lumbar level (Tsuchida et al., 1994). The LMCs are further divided into motor pools, which innervates individual muscles (Landmesser, 1978; McHanwell and Biscoe, 1981; McKenna et al., 2000; Romanes, 1951). Motor pools belonging to the medial LMC (LMCm) innervate muscles derived from the ventral limb, while those in the lateral LMC (LMCl) innervate muscles that are dorsally derived. Initially, axons from both LMC pools of
motor neurons extend along a common trajectory to the base of the limb. Upon reaching the limbic plexus, these axons undergo intricate sorting and adapt two distinct pathways to innervate the developing hindlimb. LMCl axons enter the dorsal pathway while LMCm axons enter the ventral pathway. The faithfulness of these axons is thought to be tightly controlled by expression of guidance cues in the limb mesenchymal cells and motor axons (Lance-Jones and Landmesser, 1981).

A number of factors have been implicated in the guidance of growing motor axons including peanut agglutinin (PNA)-binding glycoproteins and chondroitin sulfate proteoglycans (Davies et al., 1990; Oakley and Tosney, 1991), T-cadherin (Fredette et al., 1996), polysialic acid (PSA) (Tang et al., 1994), neural cell adhesion molecule (NCAM) (Chao et al., 2003), semaphorins (Kitsukawa et al., 1997), GDNF (Kramer et al., 2006) and ephrins (Wang and Anderson, 1997). The Eph family of receptors and their ligands, the ephrins, have emerged as important players in topographic patterning of the motor system. Both EphA receptors and ephrin ligands have been shown to be expressed by subsets of motor neurons (Kilpatrick et al., 1996; Ohta et al., 1996; Olivieri and Miescher, 1999) and developing limbs (Eberhart et al., 2000). For instance, EphA4 is expressed by lateral LMC neurons, which innervate a target that express low levels of ephrinA5 (Helmbacher et al., 2000). Ectopic expression of EphA4 in medial LMC neurons led to aberrant dorsal projection of ventrally fated motor axons while inactivation of the EphA4 protein disrupts the normal dorsal trajectory by LMCl axons (Eberhart et al., 2002). Loss of EphA4 in mice resulted in loss of innervation of muscles in the dorsal hindlimb since axons were misguided to the ventral part of the limb (Helmbacher et al., 2000). Gathering all evidence, the current model suggests that EphA4 is required by LMCl axons to be repelled by ephrinA-expressing ventral limb. The precise mechanism of EphA-mediated
dorsoventral guidance is, however, not yet resolved. EphrinAs are expressed not only by ventral limb mesoderm but also on motor axons and both EphA4 and EphA7 are expressed in the dorsal part of the limb (Araujo et al., 1998; Helmbacher et al., 2000). Therefore, ephrinA/EphA-mediated signaling may involve both axon-axon and axon-mesoderm interactions.

In addition to playing a major role in topographic map formation, Eph/ephrin signaling also mediates axon guidance of various tracts, such as the corticospinal tract (CST). CST is a collection of axonal projections that connects the brain with the spinal cord and provides central control over body movement. CST neurons arise from layer V (of sensorimotor cortex) in the neocortex and extend their axons through the forebrain, midbrain, and hindbrain and terminate at various levels of the spinal cord. It originates in one lobe of neocortex, crosses the midline at the pyramidal decussation and terminates in dorsal horn (gray matter) of the contralateral spinal cord. EphA4 is expressed by CST axons and ephrinB3 is expressed by spinal cord midline (Kullander et al., 2001a; Yokoyama et al., 2001). Inactivation of EphA4, its kinase domain or ephrinB3 results in inappropriate crossing of midline by CST axons (Dottori et al., 1998; Kullander et al., 2001a; Yokoyama et al., 2001). These findings suggest the requirement of EphA4 kinase domain for normal CST formation, as well as indicate a role for ephrinB3 in repelling EphA4-expressing CST axons at the level of the SC to restrict their connections to the ipsilateral side of the SC. Interestingly, this morphological defect in mutant mice correlates with a striking lost of reciprocal hindlimb movement (Dottori et al., 1998), which prompted subsequent studies on their local neuronal circuitry in the spinal cord. These studies revealed the presence of EphA4 in the excitatory component of a local neuronal circuit that controls rhythmic walking termed CPG. The CPG can regulate rhythmic activity independent of the brain input (Kullander et al., 2003). In mice lacking EphA4 or ephrinB3, abnormal synchronous rhythm
rather than left-right alternation was observed (Kullander et al., 2003). In addition, the synchronous rhythm in the mutants was reversed to left-right alternation by chemical strengthening of the inhibitory components of the CPG. It was suggested that midline ephrinB3 acts to repel EphA4-positive neurons from connecting to the ipsilateral side of the spinal cord (Kullander et al., 2003).

The exchange of information between the cerebral hemispheres in mammals relies upon the commissural fibers. These long-distance connections in the mammalian forebrain are made by axons that traverse the telencephalic midline, principally in three commissural tracts, the corpus callosum, the hippocampal commissure and the anterior commissure. The formation of these tracts, like that of all major connections in the brain, are highly organized and regulated by a complex interplay of long-range and short-range guidance cues. Eph/ephrin interactions have been shown to participate in the formation of commissural projections, such as the corpus callosum and the anterior commissure (Palmer and Klein, 2003).

Corpus callosum neurons reside in the layers II/III and V of the cortex. Glial Wedge and the indusium griseum, situated ventral and dorsal to corpus callosum, have been shown to guide callosal axons by preventing the tract from entering adjacent structures. These structures provide guidance by secreting chemorepellents such as Slit2 (Shu and Richards, 2001; Shu et al., 2003). The midline zipper glia located at the midline regulates fusion of the brain hemispheres and facilitates the traversing commissural axons (Shu and Richards, 2001). Recently, EphA4 has been found to be expressed in the glial wedge (Shen et al., 2006). The spatial and temporal expression pattern suggest EphA4 involvement in formation of the corpus callosum, however, further analysis is required to identify the specific function in callosal development. In contrast, evidence for EphB1, B2, B3, A5 and ephrinB3 in formation of the corpus callosum has been
provided in genetic ablation studies (Hu et al., 2003; Mendes et al., 2006). Mice lacking either EphB1 or ephrinB3 displayed aberrant callosal projections and the formation of Probst bundles (Mendes et al., 2006). Moreover, in absence of ephrinB3, radial glial cells were found erroneously in the pathway of the growing callosal fibers, suggesting a role for radial glia in inhibiting the path of these axons.

Eph receptor signaling has also been implicated in the establishment of the anterior commissure (AC). Contralateral cerebral cortical projections through the anterior commissure integrates sensory and many neuronal inputs of the two brain hemispheres. The AC is composed of two tracts, namely the pars anterior (ACpa), which connects the olfactory lobes and pars posterior (ACpp) connecting the two temporal lobes. Fibers of the anterior commissure have been shown to express both Eph receptors and ephrin ligands (Cowan et al., 2004). Formation of the anterior commissure has been shown to require the presence of EphB2 in territories through which AC axons migrate (Henkemeyer et al., 1996). Mice lacking either EphB2 or ephrinB2 exhibited a significant reduction in the pars posterior AC fibers (Cowan et al., 2004; Henkemeyer et al., 1996). EphA4 has also been shown to be expressed adjacent to the AC tract (Greferath et al., 2002), and it has been proposed that mice lacking EphA4 exhibit reductions in AC axons (Dottori et al., 1998); however the precise nature of these defects is unknown. Interestingly, defects in the anterior commissure were rescued in mice carrying truncated intracellular domains of either EphA4 or EphB2 receptors, suggesting that the Eph receptors are acting as ligands to ephrin-mediated reverse signaling (Cowan et al., 2004; Kullander et al., 2001b).
1.7 Role of magnetic resonance imaging in murine studies

Magnetic resonance imaging has become an invaluable tool in the routine diagnosis of many disease processes due to its non-invasive nature and high soft-tissue contrast and discrimination. The physical principle that forms the basis of MRI is the interaction of nuclei which have a magnetic moment and angular momentum with a magnetic field. The nucleus of an atom such as hydrogen atom of water provides the highest sensitivity in MRI due to high natural abundance and high gyromagnetic ratio. In the past few years, the field strengths for small bore MRI instruments have increased to such a degree that imaging of murine brains can now routinely be performed at a voxel resolution of \((30-60\mu m^3)\). Such instruments have become an invaluable three-dimensional (3D) tool in anatomical imaging studies of murine neural mutants due to its remarkable sensitivity for identifying alterations in cellular pathology. Its principal advantage in these studies is that it provides anatomical information of high spatial resolution with strong delineation of brain parenchymal versus axonal structures. Such data is also free of distortion and sectioning artifacts, which plague classic stereotactic sectioning procedures. Thus, these data can be used to determine neural structure and to analyze complex volume changes in spatial patterns of axon fiber bundles in 3D throughout the entire brain. In addition, due to its non-destructive nature, MRI is an ideal complement to standard histologic methods.

Beyond traditional, anatomical atlases based on post-mortem tissue, modern brain atlases are being developed that incorporate flexible, computable system, which accommodate the variation in a population. To establish the degree of significance between wildtype versus mutant data sets, the limits of natural variability must first be determined for each experimental genetic background. Comparisons of such limits obtained in wildtype data sets can then be compared to
the relevant mutant data and be used to determine the degree of any significant difference between the two data sets in three dimensions. Such comparisons can be utilized to perform a quantitative analysis of the morphology, site and distribution of any definable neural loci.

Consistent with this, variational MRI atlases have been generated for different strains of mice, including inbred 129S1/SvImJ, inbred C57Bl/6J and outbred CD1 by several investigators (Benveniste et al., 2000; Kovacevic et al., 2005; MacKenzie-Graham et al., 2003). These atlases provide a well-defined basis against which mutant outliers can be compared.
1.8 Thesis rationale and research objectives

Growing number of roles have been identified for Eph/eprhin signaling within the central nervous system. Despite a number of advances, the integrated role that these receptors play in controlling axon guidance is still not fully understood. Thus, understanding how these molecules act to regulate neural connectivity during development will be important not only for understanding the basic mechanism of axon guidance but also how these processes may be modified following neural injury. The aim of my thesis is to study the mechanism by which Eph-family members EphB2, B3, and A4 regulate the development and maintenance of topographic motor projections in the CNS and peripheral musculature. The motor system has been selected as it represents a set of relatively well-defined neural circuits of significant clinical interest in which the above Eph receptors are known to be expressed. I have focused my investigations to the EphB subclass of Eph family due to their similar functional characteristics and pattern of expression. Because of this, in addition to studying their individual roles in topographic patterning, I have also examined the combinatorial contributions of these receptors with respect to the above process.

My primary hypotheses are as follows:

1. EphB receptors control the primary developmental pattern of inter-hemispheric tract formation within the mammalian forebrain.

2. EphB-family receptors regulate the pattern of somatotopic innervation of ventral hindlimb musculature.
CHAPTER 2

MATERIALS AND METHODS
2.1 Murine strains and backgrounds

Eight-weeks old inbred 129S1/SvImJ (Jackson Laboratories, Bar Harbor, ME, USA), C57Bl/6J (Charles River Laboratories, Inc., Wilmington, MA, USA), outbred CD1 (Charles River Laboratories, Inc.) and \textit{Eph} mutant lines were housed in the animal colony at Mount Sinai Hospital and Spadina Crescent in a controlled environment with 12 hours light-dark cycle. All studies were performed in accordance with the Canadian and Ontario Animal Care guidelines. Animals lacking EphB2, EphB3, EphA4 or a combination of these were generated from the appropriate heterozygous and homozygous intercrosses. Mice homozygous for the null allele of \textit{EphB2} gene are designated as \textit{EphB2}^{-/-}. Mice carrying a targeted mutation for \textit{EphB2} lacking the kinase, SAM and PDZ-binding motifs are designated \textit{EphB2}^{N2}, as previously described (Henkemeyer et al., 1996).

2.2 Murine brain imaging

2.2.1 Sample preparation

Animals for perfusion were first anesthetized with an overdose of 2.5\% tri-bromoethanol (Avertin \textregistered{}) injected intraperitoneally causing a lack of deep tendon responsiveness. Animals were then pinned down and the thoracic cavity quickly opened to expose the heart. Animals were then perfused through the left ventricle (exit opening in the right atria) with 10 cc of 0.1 M phosphate buffer (pH 7.4), 0.9\% NaCl (PBS) to flush vascular fluids, followed by 30 cc 4\% paraformaldehyde in 0.1 M PBS (PFA) at room temperature. Following perfusion, whole heads or brains were removed and postfixed at room temperature for an additional 60 minutes. For low resolution MRI and CT scans, dissections were performed in such a manner as to preclude the...
introduction of magnetic or paramagnetic material into the sample. For high resolution MRI scans, brains were excised and fixed for in 4% PFA.

2.2.2 MR imaging

Heads were scanned in a varian (Varian Instruments, Palo Alto, CA) MRI scanner at a resolution of 121 µm using a magnet of field strength 7.0-T (Tesla) (Magnex Scientific, Oxford, UK). Collection coil diameter was 3 cm. All scans were collected at the Mouse Imaging Centre (MICe), Hospital for Sick Children. The parameters used for $T_2$-weighted 3D spin-echo scans were as follows: TR/TE = 1660/30 ms, single average, field of view = 32 x 16 x 16 mm, matrix size = 264 x 132 x 132; resulting in a final isotropic resolution of 121 µm.

Following low resolution MRI and high-resolution micro-computed tomography (CT) image acquisition, isolated fixed brains were placed into low-melting PFA-agarose filled with a proton-free susceptibility-matching fluid (Fluorinert FC-77, 3M Corp., St Paul, MN) and imaged using the 7.0-T (Tesla), 30 cm bore magnet (Magnex Scientific, Oxford, UK), with a 12 mm custom solenoid coil (Idzaiak and Haeberlen, 1982) of appropriate inner bore diameter gradient set (Tesla Engineering Ltd., Storrington, Sussex, UK). Parameters used for brain scans were optimized for maximal contrast between grey matter and white matter in the mouse brain at 7.0-T (Guilfoyle et al., 2003). $T_2$-weighted, three-dimensional spin-echo sequence was set for: with TR/TE = 1600/35 ms, single average, field-of-view = 12 x 12 x 24 mm and matrix size = 200 x 200 x 400 giving an isotropic resolution of 60 µm$^3$. Average imaging time was 18.5 hours per brain.
2.2.3 CT imaging

CT images of the murine heads were obtained at Sunnybrook and Women’s College Health Sciences Centre. Prior to imaging, heads were embedded in paraformaldehyde agarose and were scanned by micro-computed tomography using a MS-8 system, at 80 kVp and 80 uA. For each sample, 905 views were obtained, averaging 3 frames per view, using 1x1 binning. Additional parameters were as follows: angle of increment 0.4, exposure time 2 sec., source to detector distance: 236 mm, source to object distance: 118 mm, CCD detector spacing: 35 µm. The total scanning time was 1.5 hours.

2.2.4 Image registration and analysis

Image sets were registered as described previously (Kovacevic et al., 2005). Briefly, individual brain images comprising a given group were first normalized and registered to the global average using the 9-parameter affine registration method of Woods et. al (1998); using the software package AIR5.2.2. (University of California). Subsequent, non-linear alignments were performed using the multi-resolution, multi-scale ANIMAL methodology, initially developed at the Montreal Neurological Institute (MNI) (Kovacevic et al., 2005). Using this procedure, individual image matrices were compared to (deformed into) the appropriate control average, with tracking of the resulting difference. Results of this process were recorded in terms of deformation value, a vector sum which describes the magnitude of the adjustment required for any one point of the experimental matrix in matching its complementary control matrix; as previously described (Kovacevic et al., 2005). In accordance with standard practices, the magnitude of relative spatial transformation is displayed as a function of spectral color, with cool colors (e.g. purple) indicating low levels of spatial variability, and warm colors (e.g. red).
indicating higher levels of spatial variability between comparator sets. Jacobian analyses were performed as described previously (Chen et al., 2006). An absence of change in the comparator sets is indicated in green, while expansion of the experimental versus control matrix is indicated by progressively warmer colors. Similarly, contraction of the experimental matrix is shown by cooler colors.

2.2.5 3D image reconstruction

Following full registration of image sets, MRI-definable anatomic structures were delineated using the automated expert system of Kovacevic and Henderson 2005 as previously described (Kovacevic et al., 2005). Structures comprising the forebrain were additionally cross-checked through manual delineation in each of the three orthogonal planes; using the software package Display (Montreal Neurological Institute, Montreal, Canada). Nomenclature of the structures was based upon that described by Franklin and Paxinos (Franklin and Paxinos, 1997), and as previously described (Henkemeyer et al., 1996). Two-dimensional visualizations of the results obtained were made using Display / Register (Montreal Neurological Institute, Montreal, Canada). Three dimensional surface renderings of data were constructed using AMIRA (TGS, San Diego, CA).

2.3 Stereotactic micro-injection of fluorescent neural tracers

Adult animals were anesthetized with 2.5% Avertin (180 µL/10g body weight) via intraperitoneal injection. Once anesthetized, the scalp hair was removed and an incision made along the dorsal midline. Murine heads were secured for stereotactic procedures using a standard Cunningham mouse stereotactic unit (Cunningham and McKay, 1993) as shown in figure 2.1A.
Figure 2.1 Stereotactic micro-injection of fluorescent neural tracers.

(A) Image depicting surgical set up used for stereotactic micro-injection of tracer into the brain under anesthesia. (B) A mineral oil sealed neural tracer delivery system. On one end of this system, a 25μL gas tight Hamilton syringe is connected to the Teflon tubing through a microelectrode holder. At the other end of the system, a pulled borosilicate glass microcapillary with outer diameter of 1.5 mm is attached the Teflon tubing via another microelectrode holder.
Following alignment of the head to $x = 0$, $y = 0$, $z = 0$, referred standards (Chen et al., 2006), placements were performed in accordance with the stereotactic coordinates determined from our developed atlases for the appropriate genetic backgrounds (Chan et al., 2007). At the appropriate location, a 500 $\mu$m diameter opening in the skull was made using a carbide tipped dental drill.

Based upon our cranial alignment scheme, the following coordinates were employed for injection of tracers into the temporal cortex: $X: 0.40$ mm rostral of bregma, $Y: +3.75$ mm lateral to midline, $Z: 3.77$ mm ventral from dura. For the olfactory bulb, the coordinates employed were: $X: 4.15$ mm rostral of bregma, $Y: 0.84$ mm lateral to midline, $Z: 1.25$ mm ventral from dura. Following interruption of the underlying dura with a sterile 30 gauge needle, 100-150 nl of fluorescent tracers (DiI or Emerald Green – Molecular Probes) were injected into target sites.

Dyes were delivered using a sealed oil microinjection system (Figure 2.1B) connected using a microelectrode housing to a borosilicate glass microcapillary with outer diameter of 1.5 mm. Following delivery of the neural tracers, capillaries were held in place for 3 minutes, then slowly retracted under positive pressure to avoid dye displacement. The incision was sutured closed, and animals allowed to recover for 72 hours; at which time they were sacrificed and the brains were removed for analysis.

2.4 Retrograde tracing

Spinal motor neurons were retrogradely labeled through direct application of fluorescent lipophilic carbocyanine crystals or fluorescent dextrans on Gelfoam (Upjohn, Kalamazoo, MI) to peroneal nerve (PN), tibial nerve (TN), or sural nerve (SN) at the level of the knee (Figure 2.2). Prior to all operative procedures, adult mice were anesthetized by intra-peritoneal injection of 180 uL/10g body weight of 2.5% Avertin (11 mg of 2,2,2 tribromomethanol and 22.5 ml of
Figure 2.2 Anatomical location of the sciatic nerve in the hindlimb of an adult mouse.

(A) Dorsal view of the musculature of hindlimb in an adult mouse following fixation and removal of the skin and the outer muscular layer, showing details of the sciatic nerve sheath. (B) Photomicrograph of the sciatic nerve sheath following surgical opening under anesthesia, opened to reveal the three branches of the sciatic nerve: peroneal, tibial and sural.
A) Sciatic nerve

B) Sciatic nerve

Peroneal nerve

Tibial nerve

Sural

3mm
tert-amyl alcohol per 180 μL of 2.5% Avertin). The thigh of the experimental animal was shaved. A small incision was made through the skin and the right sciatic nerve and its braches were exposed by opening the fascial plane between the gluteal musculature. Each component of the sciatic nerve, tibial, peroneal and sural (in the order of decreasing in diameter) was carefully isolated and either pressure injected or transected and applied to the distal end of the nerve stump. For experiments requiring retrograde labeling of three different motor groups, the peroneal nerve was labeled using 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine perchlorate (DiI crystals), the tibial nerve labeled with 2% (w/v) dextran Oregon Green, and the sural nerve labeled with 2% (w/v) 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarbocyanine, 4-chlorobenzenesulfonate salt (DiD) (Molecular probes, Eugene, OR).

Retrograde labeling studies in embryos were performed in conjunction with immunohistochemistry of antisera directed against LIM homeodomain transcription factors. For retrograde labeling, 2 μm DiI crystals were injected into the tibialis anterior muscles of embryonic day (E)13.5 embryos and preparations were incubated for 14 days in 4% PFA in PBS (pH7.4) at 37°C. All injections and analyses were done blinded with respect to embryo genotypes.

2.5 Immunohistochemistry

For adult samples, animals were sacrificed with an overdose of 2.5% Avertin. Upon the loss of deep tendon responses, mice were transcardially perfused with 0.1 M PBS to flush vascular fluids, followed immediately by 4% PFA in PBS at room temperature (25°C). Following perfusion, brains were excised and post-fixed in 4% PFA in PBS overnight at 4°C. For embryonic samples, tissues were dissected and fixed overnight at 4°C in 4% PFA in PBS.
Both adult and embryonic samples for frozen section were equilibrated in 30% sucrose and embedded in OCT (Tissue Tek). Floating sections were typically collected at 30 µm while thaw-mount sections were obtained at thickness of 12-16 µm on a Leica CM3050S cryostat. Sections were washed with PBS and blocked in 5% serum and 0.2% tween-20 for 30 minutes prior to overnight incubation with the designated primary antisera at 4°C. Antisera utilized for these studies were as follows: anti-ephrinA1, A2 (1:25; Santa Cruz Biotechnology, Santa Cruz, CA) anti-EphA4 (1:20; R & D Systems, Minneapolis, MN), anti-beta-galactosidase (1:200; Promega: Madison, WI) and anti-phospho-EphB (1:200; (Henderson et al., 2001). Following washes, sections were incubated with fluorescent secondary antisera at a dilution of 1:200 for 2 hours prior to visualization. Alternatively, some sections were visualized using 3,3′-diaminobenzidine (DAB) histochemistry. Prior to horse radish peroxidase (HRP)-DAB reaction, sections were incubated in 3% H2O2 in PBS for 30 minutes to remove endogenous peroxidase. Vectastain ABC ELITE avidin-biotin-HRP in 0.1 M PBS was prepared 30 minutes prior to use (Vector Laboratories; Burlingame, CA). Tissues were incubated for 45 minutes in the avidin-biotin-peroxidase complex at room temperature and HRP detection was performed by adding 0.5 mg/ml DAB solution with 0.3% H2O2 and 0.5% nickel leading to a black reaction product. Sections were analyzed using a Nikon Eclipse E1000 motorized microscope equipped with a 270° rotating stage, a Hamamatsu C4742-95 camera, Nemarski contrast optics, and fluorescent excitation and emission filters appropriate for the detection of chromophores in the ranges of: 4′-6-Diamidino-2-phenyindole (DAPI), EGFP, FITC/Cy2, TRITC/Cy3, and Cy5 (excitation / emission filters used for data shown were: EGFP: EX-470, EM-525 and TRITC/Cy3: EX-528, EM-600). Dual color fluorescent images shown represent direct composites from the single channel images combined using Photoshop 7.0 software.
2.6 LacZ staining

For lacZ staining, tissues were fixed for 20 minutes in 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl\textsubscript{2} in 0.1M phosphate buffer, rinsed 3 times in wash buffer (2 mM MgCl\textsubscript{2}, 0.02% NP-40 in PB) and incubated at 37\textdegree C in lacZ staining buffer (wash buffer containing 1 mg/ml X-gal (MBI Fermentas), 2.12 mg/ml potassium ferrocyanide, and 1.64 mg/ml potassium ferricyanide) until developed. Tissues were rinsed with 0.1 M NaCO\textsubscript{3} to stop lacZ development for 15 minutes. Tissues were postfixied in 4% paraformaldehyde in PBS.

2.7 Western analysis

Total lysate were prepared from homogenizing EphB\textsubscript{2}\textsuperscript{N2/N2} and wild-type cortical and hippocampal tissues at P7 in lysis buffer (50mM Tris-HCl, 150mM NaCl, 1% Nonidet P40, 1mM EDTA, 1mM Na orthovanadate with cocktail of protease inhibitors including 5ug/ml Aprotinin, 10ug/ml Leupeptin, 1mM PMSF) and incubated for 40 minutes at 4\textdegree C. Lysate protein concentration was determined using BCA assay (Pierce, Rockford, IL). Total protein concentration among various samples was equalized using SDS sample buffer. Proteins were separated by SDS-PAGE on an 8% polyacrylamide gel and electrotransferred (constant current (35 mA, 300 V) to a nitrocellulose filter. Filters were blocked using 5% casein in Tris-buffered saline with 1% Tween 20 (TBS-T) and incubated overnight at 4C with the appropriate primary antibody. Following washing and incubation with the appropriate HRP-labeled secondary antibodies, filters were developed using enhanced luminol-based (ECL) chemiluminescence reagents (Pierce, Rockford, IL) as per manufacturer’s instructions.
2.8 Gait analysis

Gait dynamics were recorded using a customized ventral plane videography, illustrated in figure 2.3. Briefly, in conjunction with Mr. Andras Nagy (Faculty of Pharmacy), I developed a motor-driven treadmill with a transparent tread way (156 cm (L) x 5 cm (w)). A digital video camera was mounted below the transparent tread way to film gait dynamics. A plexiglas chamber (20 cm (L) x 9 cm (H) x 5 cm (W)) was mounted over the tread way to allow animals to move freely within view of the camera. A scale was mounted to the bottom of the display box. Illumination was provided by a 20 Watt halogen lamp. Digital video images of the tread were collected at 60 frames per second.

Each animal was weighed (mean weight 30 ± 5g) and allowed to acclimatize for 3 minutes. Treadmill speed was then set to 5.3 cm/s, 7.7 cm/s or 10.9 cm/s depending upon the experimental series and images collected. Greater than one minute of images were collected for each mouse. Videos were then converted to avi files using Bink and Smaker software and analyzed using Aviedit software. Spatiotemporal analysis was performed on a frame-by-frame basis (66 msec per frame) using Aviedit and Adobe photoshop. Only those frames where mouse is in persistent motion were taken into consideration for analysis.

Toe spread, stride base width, stride length, and interstep distance (the distance from one paw until the paw on the opposite side) were measured between two sequential foot strikes (Figure 2.4). Gait data were collected and pooled from both the left and right forelimbs and left and right hindlimbs.
Figure 2.3 Dynamic gait apparatus.

Shown is the sideview of gait system. Speed is controlled by a movable belt track to follow performance at controlled speeds. The digital camera attached below records gait properties.
Figure 2.4 Gait dynamics recorded using ventral plane videography.

(A) A still frame capturing the ventral view of adult 129S1/SvImj mouse on a tread way, walking at a speed of 10.9 cm/s. Red circle indicating the right paw and blue circle highlighting the left paw of the subject. (B) Diagram depicting features measured.
2.9 Tests of motor performance

2.9.1 Rotarod performance (static)

The rotarod test is used to assess motor coordination and balance using the latency of the mouse to fall from the rotating rod at accelerating speed as an indicator. Following a 3 minute habituation period, mice were subjected to different static speeds (0 rpm, 8 rpm, or 15 rpm) for a period of 2 minutes each trial. Latency to fall was recorded for each speed and the ability of the animal to passively rotate (complete revolution) without falling was denoted.

2.9.2 Hindlimb extension reflex

Mice were suspended by their tails at a distance of 10 cm from a supportive surface and the extent of hindlimb extension observed on 5 successive lifts. A score of 0 corresponds to the absence of hindlimb extension or the mouse clutches limbs close to body. A score of 1 corresponds to a hindlimb extension reflex but no extension upon nose/whisker contact. A score of 2 corresponds to a normal extension reflex in both hindlimbs including splaying of toes upon whisker contact. A score of 3 corresponds to hindlimb extension immediately prior to whisker contact. A score of 4 corresponds to early vigorous hindlimb extension by the animal.

2.9.3 Edge performance test

Motor coordination and balance were evaluated by the ability of mice to traverse a graded series of narrow beams to reach an enclosed safety platform (Carter et al., 1999). In this study, mice were placed on a 3 mm wide beam 30 cm long at the height of 28 cm. Mice were examined for maneuverability on a stable platform located at either end of the runway. A score of 1 was
given to animal which crossed the edge surface easily and could reverse on the 3 mm edge, reaching the platform from a distance of 15 cm easily. A score of 2 was given to animal that reached the stable platform with significant difficulty and can only reach the platform from distances of less than 7 cm. A score of 3 was given to animals who exhibited substantial difficulty (tremor) in edge navigation and progressed toward edge but were unable to maintain edge grip for more than 8-10 seconds. A score of 4 was given to animals which were unable to maintain balance on the grip.

2.9.4 Hindlimb grip response

Forelimbs of experimental animals were placed on a platform 6.8 cm in height and hindlimb grip response was measured by their ability to grip onto a 3mm lowered metal rod 3.5 cm in height. A score of 0 corresponds to full grip onto bar. A score of 1 corresponds to partial grip onto bar and that grip does not immediately occur as a function of dynamic load. A score of 2 corresponds to inability of digit to grip onto bar. Any abnormal flexure of the foot as a function of dynamic load was noted.

2.9.5 Grip strength

A commercially available grip strength meter (dynamometer) was used to measure the grip strength of mice. Slow and steady traction was applied until animal’s grip was released. The maximum grip strength was recorded in grams. Two types of measurements were performed in the grip strength test: (1) forelimb measurement and (2) forelimb and hindlimb measurement. Scores were determined and averaged for the highest 4 out of 7 measures.
2.9.6 Wire platform performance

Mice were examined for their ability to move across a wire mesh composed of 2 cm square grids (mesh 2 mm thick). The number of foot falls through the mesh by both fore and hindlimbs were recorded as the animal traversed the platform over a 60 second period. Animals were induced to actively traverse the platform by gentle stimulation of the body.

2.9.7 Platform performance at 90 degrees

Animals were introduced to the bottom of a 90° inclined wire mesh platform (2 cm square grids) and scored for the time (in seconds) required to traverse to the top.

2.9.8 Statistical Analysis

Data were processed and analyzed in a database (Graphpad software Prism 3.02, SanDiego, USA) for the parameters described. Data are presented as means ± SEM. Statistical comparisons were made with a Student’s two tailed t-test for unpaired values and a paired t-test for paired values (Gaussian distribution verified by normality test). Differences were considered significant at $P<0.05$. 
EphB receptors regulate formation of the principal inter-hemispheric tracts of the mammalian forebrain


3.1 Introduction

Proper assembly of the mammalian central nervous system is critically dependent upon the ability of projecting axons to appropriately innervate their targets (Dodd and Jessell, 1988). Over the past decade, several conserved families of molecules have been identified as important regulators of developmental CNS axon guidance and among the largest of these are Eph receptors. Previously, our laboratory demonstrated that EphB2 activity is required for proper development of the posterior branch of the anterior commissure and that axon guidance was mediated by ephrinB-induced reverse signaling (Henkemeyer et al., 1996). EphA4 has also been shown to be expressed adjacent to the AC tract, and it has been suggested that mice lacking EphA4 exhibit reductions in AC axons (Dottori et al., 1998; Greferath et al., 2002); however the precise attribute of these defects is unknown. To uncover the nature by which Eph receptors regulate axon guidance of the murine anterior commissure, detailed three-dimensional structures of all axon tracts and the anatomic origin of these tracts were examined in vivo.

The anterior commissure, a forebrain tract connecting olfactory and basal telencephalic regions, decussates at the anterior end of the third ventricle and consists of ACpa and ACpp branch (Jouandet and Hartenstein, 1983). Fibers coursing through the anterior limb of the anterior commissure stem predominantly from cells of the anterior olfactory nucleus and the piriform cortex (Alheid et al., 1984). Most brain areas known to project through the AC have been implicated in reproductive and behavioral functions. Thus, attention has been paid to examine structural changes within the AC. For instance, structural differences in the AC have been found in relation to gender (Noonan et al., 1998), gonads (Larriva-Sahd et al., 1998) and development (Guadano Ferraz et al., 1994). During development, pioneer AC axons must make several pathfinding decisions to reach their contralateral targets. In the mouse, commissural
axons originating from differentiated neurons in the cortical plate first extend forward into the white matter around embryonic day 15. These axons then change their direction and travel toward the midline. Upon reaching the midline, the axons cross over, reenter the grey matter of the contralateral side at specific positions and form synapses with appropriate targets. Disruptions at any of these critical choice points will likely lead to pathfinding and targeting errors.

To understand the mechanism regulating axon guidance, analysis of detailed axonal navigation is required. Traditional comparative analyses in the mammalian CNS have consisted of flat plane histologic analyses of these sections. For such analyses, detailed comparison of neural structure is difficult due to section to section variability, labor intensive serial histologic sectioning, data capturing and analysis. These factors have severely limited the ability to analyze axonal tracts. To efficiently detect and quantify changes in axonal organization within the murine CNS that are due to genetic manipulation in mice, high-resolution magnetic resonance imaging (MRI) was performed on Eph mutants and controls. MRI examines the relative differences in local water and lipid content compared to surrounding cell soma (Neema et al., 2007). It is ideally suited for this study because it allows for non-destructive visualization of axon tracts in situ and detection of structural deviations observed within a given sample to be later examined histologically. While this method is useful, prior to initiation of my dissertation, there were no standard MR atlases available for the strains of mice of my interest. To determine the level of variation between MRI data from animal to animal of the same genetic background, as part of a joint effort between our laboratory and Dr. Henkelman’s laboratory at Toronto Centre for Phenogenomics, I participated in the development of the first variational atlases for several important strains of inbred and outbred mice (CD1, 129S1/SvImJ, C57Bl/6) and data analysis
protocols which would allow us to determine, in a relatively high throughput manner, changes in neural tracts within the murine CNS (Chen et al., 2006; Kovacevic et al., 2005). Next, to determine the practical limits of morphologic resolution using these MRI scanning and analysis protocols, I have examined an axon guidance mutant previously characterized in our laboratory, the EphB2 nulls. In addition, I played a significant role in the development of the first interactive MRI/CT (computed tomography) based atlases of the mouse brain/skull and dynamic reference planes for use in stereotactic surgical planning (Chan et al., 2007). Historically, stereotactic placements within murine CNS have been performed based upon coordinates derived from two major skull landmarks; the lambda and bregma sutures, obtained from a single genetic background (C57Bl/6J) (Franklin and Paxinos, 1997). Only minor scalar corrections were performed in the event of significant deviation in the lambda and bregma distance between experimental subjects of difference genetic backgrounds. In contrast, our analysis of inbred and outbred murine strains had indicated that there were substantial non-linear and non-isotropic differences in the morphology of the brain and skull among these groups (Chen et al., 2006). These differences extended to the relative intracranial position of CNS structures and neural loci with respect to external landmarks (Chan et al., 2007). For these reasons, we sought to develop strain-specific interactive three-dimensional stereotactic atlases for the important genetic strains composing our Eph mutant mice, 129S1/SvImJ and C57Bl/6J. Not only does 129Sv represent the genetic background from which the embryonic stem cells for the generation of Eph mutants were obtained, but it also represents the principal strain from which the large majority of embryonic stem cells are derived. Both 129Sv and C57Bl/6J are the principal backcross strains in our Eph mutations whose genomes have been completely sequenced at present. In addition, C57Bl/6J also represents the most thoroughly studied strain of mice with respect to neurologic mutations.
Hence, the development of 3D stereotactic atlases for these strains would not only be useful for our lab but beneficial to a variety of neurologic investigators.

To uncover the mechanisms by which Eph receptors regulate axon guidance within the murine CNS, for the first time, high-resolution MRI on $Eph$ mutant brains were performed. Both single and combinatorial $EphB2$ and $EphA4$ null mice were examined to unmask the possibility of redundancy among EphB family members. In this study, I have faithfully mapped the three-dimensional anatomy of AC in $EphB$ mutants and have accurately quantified changes in AC morphology between controls and $EphB$ knockouts. To identify the anatomical origin leading to aberrant AC projections, I have stereotactically labeled the relevant neural fields in vivo, via microinjection of fluorescent neural tracers. Using these methods, in combination with immunohistochemistry, I have developed a detailed three-dimensional picture of how Eph receptors regulate inter-commissural forebrain axon guidance. These results demonstrate that while EphB2 and A4 each regulate distinct aspects of guidance within ACpp, these receptors operate in a redundant manner to regulate guidance of axons in the pars anterior of the AC, a pathway not previously implicated in Eph receptor function. Analysis of $EphA4$ null mice using high resolution MRI reveals for the first time that, in addition to errors of midline crossing, loss of EphA4 activity results in a positional reorganization of the rostral AC. Taken together, the results demonstrate that each of the principal guidance decisions within both anterior and posterior tracts of the AC can be accounted for by the individual and combinatorial actions of EphB2/A4 receptors. These data also demonstrate the power of MRI to analyze complex anatomical phenotypes.
3.2 Results

3.2.1 Development of the MRI atlases

As a first step to examine integral changes in neural structures in 3D within the mouse brain, work conducted by our laboratory in conjunction with the laboratory of Dr. Henkelman was aimed at quantifying intra- and inter-strain anatomical variation using MRI for these studies were focused on the mouse genetic backgrounds; 129/SvImJ and C57Bl/6 (n ≥ 9 per each strain), which represent strains commonly utilized in knockout and random mutagenesis studies. In addition, we examined CD1, one of the most widely utilized outbred murine strains (Chen et al., 2006; Kovacevic et al., 2005). These studies were a necessary first step to understanding and quantifying the levels of natural variability present within the CNS of parental strains used for our genetic studies.

To determine the limits of natural variation, initially, raw MRI data sets of eight-week-old male outbred CD1, inbred C57Bl/6J, and inbred 129S1/SvImJ mice were collected at a resolution of 60μm³ (voxel size). Image registration for deriving unbiased group averages was performed to create the average image and to determine the standard deviation of the wildtype specimens. This average image is composed of individual data sets compiled into a single file, which is then used to extract commonalities among individual brain specimens and filters out idiosyncrasies. The final registered data set providing a representation of average anatomy as well as the range of anatomical variation present within a particular sample population. Using such average representations, I have manually delineated a number of CNS structures that were distinguishable on MRI according the accepted nomenclature from the literature (Franklin and Paxinos, 1997). A total of 40 neural structures were segmented (Table 1), such that each
Table 3.1 List of murine CNS structures identified by MRI

<table>
<thead>
<tr>
<th>Segmentation labels</th>
<th>CNS structures</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3rd and 4th ventricles, cerebral aqueduct</td>
</tr>
<tr>
<td>2</td>
<td>Amygdaloid region-dorsal</td>
</tr>
<tr>
<td>3</td>
<td>Amygdaloid region-ventral</td>
</tr>
<tr>
<td>4</td>
<td>Anterior Commissure (pars anterior)</td>
</tr>
<tr>
<td>5</td>
<td>Anterior commissure (pars posterior)</td>
</tr>
<tr>
<td>6</td>
<td>Arbor vitae of cerebellum</td>
</tr>
<tr>
<td>7</td>
<td>Brain Stem</td>
</tr>
<tr>
<td>8</td>
<td>Cerebellum (gray matter)</td>
</tr>
<tr>
<td>9</td>
<td>Cerebral peduncle</td>
</tr>
<tr>
<td>10</td>
<td>Corpus callosum</td>
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<tr>
<td>11</td>
<td>Cortex</td>
</tr>
<tr>
<td>12</td>
<td>Dentate gyrus</td>
</tr>
<tr>
<td>13</td>
<td>Diencephalon</td>
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<tr>
<td>14</td>
<td>Facial nerve and nucleus</td>
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<tr>
<td>15</td>
<td>Fimbria</td>
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<tr>
<td>16</td>
<td>Fornix</td>
</tr>
<tr>
<td>17</td>
<td>Globus pallidus</td>
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<tr>
<td>18</td>
<td>Habenulo-peduncular</td>
</tr>
<tr>
<td>19</td>
<td>Hippocampus</td>
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<tr>
<td>20</td>
<td>Inferior colliculus</td>
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<tr>
<td>21</td>
<td>Internal capsule</td>
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<tr>
<td>22</td>
<td>Lateral septum</td>
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<tr>
<td>23</td>
<td>Lateral ventricles</td>
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<tr>
<td>24</td>
<td>Longitudinal Fasciculus of pons</td>
</tr>
<tr>
<td>25</td>
<td>Mammalothalamic tract</td>
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<tr>
<td>26</td>
<td>Medial lemniscus</td>
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<tr>
<td>27</td>
<td>Medial septum</td>
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<tr>
<td>28</td>
<td>Nucleus accumbens</td>
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<td>29</td>
<td>Olfactory Bulb</td>
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<td>Olfactory tract</td>
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<tr>
<td>31</td>
<td>Optic tract</td>
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<tr>
<td>32</td>
<td>Periaqueductal gray</td>
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<td>Pre/para subiculum</td>
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<td>34</td>
<td>Posterior comissure</td>
</tr>
<tr>
<td>35</td>
<td>Region determined as floor of forebrain</td>
</tr>
<tr>
<td>36</td>
<td>Stria medullaris thalami</td>
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<tr>
<td>37</td>
<td>Stria terminalis</td>
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<tr>
<td>38</td>
<td>Striatum</td>
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<tr>
<td>39</td>
<td>Superior colliculus</td>
</tr>
<tr>
<td>40</td>
<td>Ventral pallidum</td>
</tr>
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</table>
structure corresponded in all three orthogonal planes (in coronal, sagittal and horizontal) (Figure 3.1 A-C). More advanced visualization in three-dimensions was obtained from surface renderings of individual structures, as shown in (Figure 3.1D-E). These annotations, in addition to allowing comparisons between individual brains, also enable accurate volumetric measurement of each CNS structure. This consisted of automatically counting the number of voxels with a given segmentation label for each member of a given data set and comparing these to the average to obtain a measure of variability. The results demonstrate that volumes of a number of CNS structures, in particular axonal tracts, were found to be quite similar (<5% difference) across the three reference strains (shown in Table 3.2 for axonal structures). These included defined axon tracts of the anterior commissure, posterior commissure, fimbria, stria terminalis and optic tract. In addition to determining the absolute volume of MR-definable neural structures, the relative position of these structures within the brain were determined for wildtype strains. For this analysis, the degree of positional shift for a given structure with respect to its representation in the appropriate average was reported as a function of deformation magnitude (DM). The DM represents a vector field which denotes the relative magnitude and direction of the displacement incurred. For each sample, a three-dimensional map of the relative deformation magnitude (positional displacement of centre of mass) with respect to a defined coordinate system is generated. By generating maps of DM, I have identified and provided a quantitative measure of natural variability among the three reference strains. The relative magnitudes of the spatial transformation are displayed referenced to a spectral color scale. Cooler colors (towards purple) are indicative of low levels of spatial variability whereas, warmer colors (towards red) denotes high level of spatial variability.
Figure 3.1 MRI annotations of murine CNS structures.

(A-C) Two-dimensional slices through a 129S1/SvImJ murine brain showing examples of anatomical labels in the coronal (A), horizontal (B) and sagittal (C) views. Each label color corresponds to a specific CNS segmentation structure. (D) Surface rendering of the anatomical labels of cerebellum (orange), olfactory bulb (light green) and cortex (red) and their spatial relationships.
Table 3.2 Table of average volumes (% of total volume ± % standard deviation) for selected axon tracts for the three reference strains

<table>
<thead>
<tr>
<th>CNS tracts</th>
<th>129S1/SvImJ (mean ± σ %)</th>
<th>C57Bl/6J (mean ± σ %)</th>
<th>CD1 (mean ± σ %)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anterior commissure (pars anterior)</td>
<td>0.13 ± 0.008</td>
<td>0.13 ± 0.008</td>
<td>0.13 ± 0.005</td>
</tr>
<tr>
<td>Anterior commissure (pars posterior)</td>
<td>0.056 ± 0.006</td>
<td>0.053 ± 0.003</td>
<td>0.060 ± 0.003</td>
</tr>
<tr>
<td>Fimbria</td>
<td>0.63 ± 0.02</td>
<td>0.63 ± 0.02</td>
<td>0.67 ± 0.04</td>
</tr>
<tr>
<td>Optic tract</td>
<td>0.22 ± 0.01</td>
<td>0.19 ± 0.01</td>
<td>0.21 ± 0.01</td>
</tr>
<tr>
<td>Posterior commissure</td>
<td>0.020 ± 0.001</td>
<td>0.021 ± 0.003</td>
<td>0.020 ± 0.003</td>
</tr>
<tr>
<td>Stria terminalis</td>
<td>0.048 ± 0.003</td>
<td>0.05 ± 0.005</td>
<td>0.05 ± 0.003</td>
</tr>
</tbody>
</table>
In addition to measuring absolute volume and centre of mass position for the MR-definable neural loci, the relative morphologic distribution of each MR-definable structure was also determined as a Jacobian function. This Jacobian analysis (JA) defines from a field of Jacobian determinant values the relative expansion or contraction at each voxel derived as a product of the deformation field. As such, this analysis was used to localize shifts in morphologic distribution within a given MR definable structure (without a requisite change in absolute volume or overall position within the CNS). Cool colors represent contraction of local volume and warm colors represent expansion of local volume. Together these scalar measures provide summary voxel-wise measures of the combination of shape and size and location differences across wildtype strains.

3.2.2 Validation of MRI-based tools for morphologic analyses

As a check of the fidelity and resolution of the MRI-based tools of analysis, I examined the previously characterized EphB2\textsuperscript{−/−} mutants (Henkemeyer et al., 1996), in order to compare the MR results obtained with those determined histologically. Raw MRI data sets were collected at a resolution (voxel size) of 60 \(\mu\text{m}^3\) for both the EphB2 mutants \((n = 7)\) and the control littermates \((n = 6)\). To visualize and to compare data sets between the mutant and control brains, average images were generated for both groups using the registration procedures described above (Kovacevic et al., 2005). The average images generated are shown in figure 3.2. In this method, the average image was formed through a series of registration steps involving initial global normalization of brains with respect to their orientation, size and MR intensity, and subsequent multiple non-linear registrations of individual brains to the average (Figure 3.2). The average image in EphB2 controls (heterozygous littermates) showed an apparent improvement in image
Figure 3.2 Generation of averaged image and individual associated deformation fields.

Illustration of basic process of data set comparisons. Lines between each of the images indicate deformation fields determined by non-linear registration. The deformation fields to the control average were used for image analysis and generation of statistical maps (deformation magnitude (DM) analysis and Jacobian analysis (JA)).
Individual mutant MR images

Wildtype MR images

Wildtype Average

Mutant Average

Wildtype deformation fields (DM. JA) denoting natural variability

Mutant deformation fields (DM. JA) denoting mutation associated variability

Mutant deformation fields (DM. JA) denoting variability among mutants
quality compared to the individual image (Figure 3.2). Consistent with our previous findings, both MR plane sections and volume renderings of EphB2\(^{-/-}\) mutants demonstrated a substantial reduction in the extent of the ACpp (>85%, \(P > 0.001\)) (Figure 3.3 B, D, G-H: 3D representation) compared to EphB2\(^{+/+}\) mutants or wildtype mice (Figure 3.3 A, C, E-F: 3D representation). These data demonstrate that the MRI field strength and the parameters utilized are sufficient to effectively detect variation in axon tracts of 150-200 \(\mu\)m in any plane. Utilizing the MRI-based multivariate analysis tools that I have helped develop, structural differences in the AC of EphB2\(^{+/+}\) and EphB2\(^{-/-}\) mice were examined. As mentioned above, data sets were created from the resulting deformation fields including comparisons of positional shifts in neural structures and changes in local volume between individual and averaged MR samples. These changes in local size, shape and position were presented in terms of colors. The deformation magnitude (DM) captures the magnitude and direction of anatomical differences among individual CNS loci and translates the displacement in micrometers into a relative colorimetric scale (Figure 3.4 A-B). Areas with warm colors represent regions with large displacements compared to the average, while cooler colors denote regions with lower relative levels of displacements. Volumetric comparisons were assessed using Jacobian analysis (JA), which provides a measure of local structural expansion or contraction (Chung et al., 2001). Expansion is indicated by warmer colors, whereas contraction is indicated by cool colors.

Results of deformation field analysis for EphB2\(^{+/+}\) versus EphB2\(^{-/-}\) MR data are shown in figure 3.4. The left and right columns show the horizontal view of the average MR images of EphB2\(^{+/+}\) and EphB2\(^{-/-}\) mutants at the level of the anterior commissure respectively. Deformation magnitude of EphB2\(^{+/+}\) brains, shown in figure 3.4, demonstrates the limits of normal variation seen in the
Figure 3.3 Comparisons between MR data and histology in the AC of EphB2 mutants.

(A-B) Horizontal view of histologic sections through the level of the AC showing significant reduction in the ACpp in EphB2\textsuperscript{−/−} mutants (B; black arrows) compared to EphB2\textsuperscript{+/−} controls (A). (C-D) Average MR images of EphB2\textsuperscript{−/−} (C) and EphB2\textsuperscript{+/−} (D) mutant depicting the AC shown for comparison. White arrows indicate significant loss of the ACpp, consistent with previous characterization of these mutants using histology (black arrow). (E-F) 3D reconstruction of the anterior commissure for average EphB2\textsuperscript{+/−} (E) and average EphB2\textsuperscript{−/−} (F) MR images demonstrated similar defects in the ACpp as in histologic analyses, confirming the fidelity and resolution of the MRI system. ACpa (red) and ACpp (green). Asterisk represents $P < 0.001$ by $t$-test.
Figure 3.4 Deformation magnitude and Jacobian analysis in *EphB2* mutants at the level of the anterior commissure. Left column illustrates the unbiased *EphB2*+/− average MR image shown at the level of the AC in horizontal, sagittal and coronal views. Columns A and B depict the mean deformation magnitude (DM) from individual control images to control average (*EphB2*+/− average) and mutant images to control average (*EphB2*−/− average) of the boxed region represented in the MR images. The magnitude of deformation (in μm) is depicted as a function of colors, where warmer colors correspond to larger structural displacements. A displacement of ~250 μm is observed in all three planes of the ACpp in the *EphB2*−/− mutants. Local positional differences between the individual images and the averaged image are depicted by Jacobian data (JA). The amount of spatial adjustment required for a structure of the average image to match with that of individual images is represented by spectral colors, where regions of contractions are indicated by cool colors and areas of expansion are denoted by hot colors. Respective color scales are depicted below.
<table>
<thead>
<tr>
<th></th>
<th>DM of B2 (+/-) Average</th>
<th>DM of B2 (-/-) Average</th>
<th>JA of B2 (+/-) Average</th>
<th>JA of B2 (-/-) Average</th>
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<td><img src="image15.png" alt="Image" /></td>
<td><img src="image16.png" alt="Image" /></td>
</tr>
</tbody>
</table>

**Legend:***
- DM: 100 200 300 400 500 600 700µm
- JA: -1 0 +1

**Images:**
- MR image of B2 (+/-) Average
- Horizontal
- Sagittal
- Coronal
heterozygotes. Areas of greater relative variability (green) appear along the outer surface of the brains and at the point of flexure such as between the cortex and cerebellum. This is expected for excised brain samples. By contrast, in interior regions, displacements of <50 μm were observed in the region such as the anterior commissure. Figure 3.4 shows the DM of the mutant average compared against the heterozygous average. Among these groups, a displacement of approximately 280 μm was observed within the ACpp. By Jacobian Analysis, the displacement appears to have been a local contraction in the ACpp, as evidenced by the blue-purple color surrounding the ACpp in EphB2<sup>+/−</sup> mutants (Figure 3.4). Together, all three measures (volume, DM and JA) demonstrated a reduction of the ACpp in EphB2<sup>+/−</sup> brains, consistent with histological studies at a similar resolution. With the exception of the reductions seen in the ACpp, no additional structural anomalies were observed by MRI within the forebrain. In conjunction with our MR atlases of the natural variation in murine strains (Chen et al., 2006), these findings support the utility of the MR parameters and procedures employed to examine the whole brain in detail in three dimensions and to provide an internal test of the true dimensional limits to which changes in tract fine structure could be determined. In addition, these analyses helped define the nature, extent, and significance of any structural deviation observed within mutant versus control comparisons.

### 3.2.3 MRI analyses of EphA4 mutants

Utilizing this MRI tool of analysis, forebrains of EphA4 mutant mice were examined for the first time in three-dimensions. Analysis of MRI data from EphA4<sup>+/−</sup> mice also demonstrated no significant differences compared to wild-type littermates or 129S1/SvImJ atlas controls (n ≥ 8 mice per genotype, data not shown). As shown in figure 3.5A, EphA4<sup>+/−</sup> mice exhibit a morphologically normal AC, with little variation (< 150 μm) among individuals with respect to
Figure 3.5 MRI analysis of *EphA4* mutants.

(A-F) Horizontal views of the forebrain at the level of the anterior commissure in *EphA4*+/− (A-C) and *EphA4*−/− (D-F) mice. MR images of *EphA4*+/− (A) and *EphA4*−/− (D) mice showing the AC structure. The deformation magnitude (DM) is a measure of the magnitude of positional changes within a CNS structure of mutant compared to controls MR images and represents the displacement (in μm) into a colorimetric scale. Areas with warm colors represent regions with large displacements compared to the natural variational average, while cooler colors denote regions with lower relative levels of displacements. The Jacobian analysis (JA) represents the distribution of local changes in volume within a given structure in comparison to controls. DM and JA from individual *EphA4*+/− MR images (*n* = 8) against the control average image (B-C) and mutant MR images (*n* = 8) against the control average image (E-F) are illustrated as a color coded map. In these panels, the relative positions of the AC tract from the corresponding MR view are indicated by a dotted line. (E) Displacements of ~200-400 μm are evident in the ACpa *EphA4*−/− compared to controls (grey arrows representing vectors of deformation). Note the apparent loss of ACpp density in the *EphA4*−/− mice (arrow in D) compared to *EphA4*+/− (A). Changes in local volume are observed in structures surrounding the ACpa in *EphA4*−/− (F) compared to *EphA4*+/− (E). (G) Spectral color scale for deformation magnitude analyses with units in microns (B, E). (H) Spectral color scale for Jacobian volume analyses (C, F). Scale bars denote a distance of 1 mm.
MR image Deformation Magnitude Jacobian Analysis

A A4(+/-) Avg

D A4(-/-) Avg

G DM

H JA

100 200 300 400 500 600 700µm

106
either the AC or surrounding structures in comparison to the composite average (Figure 3.5B) or Jacobian analysis (Figure 3.5C) \((n = 8)\). By contrast, all \(EphA4^{+/−}\) mice \((n = 8)\) exhibited substantial variation in several regions of the AC (Figure 3.5D) with respect to the relative position of the AC (indicated by deformation magnitude up to 400 µm in Figure 3.5.E), and the distribution of axons within the ACpa tract (Jacobian analysis, Figure 3.5F). The total volume of the ACpa was not significantly different \((P = 0.13)\) in \(EphA4^{−/−}\) mice versus \(EphA4^{+/−}\) controls \((0.42 ± 0.10 \text{ mm}^3 \text{ versus } 0.5 ± 0.05 \text{ mm}^3 \text{ respectively})\). Within the ACpp however, \(EphA4^{−/−}\) mice exhibited a 43% reduction in tract volume compared to heterozygous controls by MRI \((0.15 ± 0.05 \text{ mm}^3 \text{ versus } 0.27 ± 0.04 \text{ mm}^3 \text{ respectively, } P < 0.01)\).

To better understand the nature of the morphologic defects observed in \(EphA4\) null mice, three dimensional representations of the entire anterior commissure were generated for all \(EphA4^{−/−}\) mutants and controls. In contrast to wild-type and \(EphA4^{+/−}\) littermates (Figure 3.6A, B), \(EphA4^{−/−}\) mice (Figure 3.6C, D) showed perturbations in both the distribution and axonal organization of the ACpa tract (arrowheads). Tract perturbations reminiscent of de-fasciculation are a common feature in \(EphA4^{−/−}\) mice along the caudal aspect of the ACpa tract (arrowhead). The full magnitude of these perturbations of the ACpa tract can be seen in the \(EphA4^{−/−}/EphA4^{+/−}\) overlays (Figure 3.6E, F). To determine the relative morphologic significance of the increase in ACpa tract displacement from the midline seen in \(EphA4^{−/−}\) mice, this distance was determined for \(EphB2\) and \(A4\) mutants and controls, together with CD1 \((n = 10)\), C57Bl/6J \((n = 10)\) and 129S1/SvImJ \((n = 10)\) wildtype (WT) mice. The ACpa in \(EphA4^{−/−}\) mice exhibited a lateral displacement of greater than 400 µm from the midline compared to \(EphA4^{+/−}\) mice (Figure 3.7). This lateral displacement of the ACpa from the CNS midline is fairly consistent across a number of genetically different comparator groups. This displacement is significantly increased
Figure 3.6 Three dimensional representations of the anterior commissure in \textit{EphA4} mice.

(A-D) Three dimensional renderings of the anterior commissure from MRI data were performed for \textit{EphA4}^{+/−} (A, B) and \textit{EphA4}^{−/−} (C, D) mice. The lateral and horizontal planes are shown respectively. (E, F) Heterozygotes /homozygote overlays of \textit{EphA4} null mice are shown in comparison to the WT in lateral view (E) and horizontal view (F). Note \textit{EphA4}^{−/−} mice exhibit tract deviations within the ACpa (arrowheads in C, D), a ventral shift in ACpa (black arrow in E), and enhanced lateral displacement in ACpa (black arrow in F) compared to \textit{EphA4}^{+/−} littermate controls. Scale bars indicate 1 mm (A-F).
Figure 3.7 Analysis of ACpa tract displacement from the midline.

For each of the genotypes (129S1/SvImJ (n = 10), C57Bl/6J (n = 10), CD1 (n = 10), EphB2\textsuperscript{+/−} (n = 7), EphB2\textsuperscript{−/−} (n = 8), EphA4\textsuperscript{+/−} (n = 8) and EphA4\textsuperscript{−/−} (n = 8)) average displacement from the midline is indicated. The width was measured at midway between its caudal limit and the cortical boundary (white arrow in Figure 3.6F). The bar on the left end represents the mean (±s.e.m.) distance to the inside boundary of the ACpa, while the bar on the right end is the mean (±s.e.m.) distance to the outside boundary of the ACpa (** \( P < 0.01 \); \( t \)-test).
Tract displacement from sagittal midline (mm)

Genotype

CD1
EphB2+/-
EphB2-/

C57Bl/6J
EphA4+/
129Sv/JmJ
EphA4-/

111
in \textit{EphA4}^{+/−} mice compared to each of the comparison groups, consistent with the deformation magnitude results for these mutants (Figure 3.5D).

\textbf{3.2.4 Disruption of axon pathfinding in \textit{EphB} family mutants}

Knockout mice have been widely used to assess the role of a particular gene product in a given biochemical or cellular process. To examine the effects of neuropharmacologic substances and to examine complex behaviors, targeted knockout technology is, thus, a powerful method for studying the physiologic properties of a given protein. Hence, we have used \textit{Eph} mutant mice as tools to define or clarify the \textit{in vivo} function of these receptors. As indicated above, the MRI parameters used provided a means to quantitatively analyze global positional and/or structural changes in MR-definable objects of sufficient magnitude. However, to understand the mechanisms by which these deviations arise, it was necessary to clearly determine the path of axons innervating each AC tract. I therefore performed stereotactic microinjections of fluorescent neural tracers into anesthetized wild-type and \textit{EphB} family mutants as described, into sites within the temporal cortex and olfactory bulb. Tracing was performed for a period of 4 days, at which time animals were sacrificed and serially sectioned in the horizontal (transverse) plane. Temporal and olfactory microinjections of \textit{EphA4}^{+/−} (Figure 3.8A) and wildtype (data not shown) mice show robust labeling of the ACpp and ACpa tracts respectively. Similar tracing studies performed in \textit{EphB2}^{−/−} mice revealed a substantial reduction in ACpp axons (Figure 3.8B), consistent with previous observations (Henkemeyer et al., 1996). Those axons which do arrive at the midline in \textit{EphB2}^{−/−} mice, however, persist in forming a discreet ACpp tract which crosses the midline properly (Figure 3.8B). In contrast, ACpp axons in \textit{EphA4}^{−/−} mice exhibit aberrant axon guidance upon contact with ACpa axons (Figure 3.8C-E) where the ACpp axons in
these mice now freely intermingle with those of the ACpa tract. As a result, in EphA4<sup>−/−</sup> mice, approximately one-half of all ACpp axons become mis-directed along the ACpa tract (Figure 3.8D); while the remainder exit the midline and resume projection to the contralateral hemisphere. Interestingly, as shown in the progressively more ventral levels of the AC among different EphA4<sup>−/−</sup> mice (Figure 3.8C-E), the character of ACpp and ACpa intermingling changes, becoming more stratified within the ventral AC. The aberrant behavior of ACpp axons in EphA4<sup>−/−</sup> mice as seen by in vivo neural tracing is consistent with both the MRI-based volumetric reduction of the ACpp tract, and the disappearance of MR contrast intensity along the AC midline seen in EphA4<sup>−/−</sup> mice (Figure 3.5D, arrow). Despite this deviation, ACpp axons of EphA4<sup>−/−</sup> mice are still observed to project only along the well defined tracts of the AC (ACpp, ACpa) rather than projecting randomly within a given zone, as observed for ACpp axons of EphB2<sup>−/−</sup> mice (Henkemeyer et al., 1996). In vivo tracing of axons also demonstrates that the apparent tract fasciculation events seen along the caudal aspect of the ACpa tract in EphA4<sup>−/−</sup> mice using MRI, actually represents bundles of mis-projecting ACpp axons which project through the ACpa tract (Figure 3.8E). As shown in figure 3, aberrant axonal projection is not observed for ACpa axons in EphA4<sup>−/−</sup> mice, similar to EphB2<sup>−/−</sup> mice. With respect to other EphB family members known to be expressed within the forebrain, neural tracing experiments performed using EphB3<sup>−/−</sup> mice (Figure 3.8F), and mice homozygous for a targeted deletion of the EphB2 intracellular domain (EphB2<sup>N2/N2</sup>, (Henkemeyer et al., 1996)) (data not shown), show axonal tracing patterns identical to that seen in wildtype and heterozygous controls.
Figure 3.8 Axonal pathfinding defects in Eph family mutants.

(A-F) Fluorescent tracers were employed to map the *in vivo* axonal distribution within the component tracts of the AC, following stereotactic injection in the olfactory (green) or temporal (red) compartments. (A) Fluorescent labeling of the anterior commissure in EphA4+/− mice shown in the horizontal plane. (B) Labeling in EphB2−/− mice, demonstrating the reduction in ACpp labeling. (C-E) Labeling pattern seen in three independent EphA4−/− mice, showing progressively more ventral planes within the AC to demonstrate intermingling of ACpp axons with those of the ACpa tract. (F) Tract labeling observed in EphB3−/− mice. Scale bar denotes a distance of 400 µm (A-F).
Recently, several groups have demonstrated that α-2 chimerin is a downstream target of EphA4, raising the possibility that α-chimerin mediated signaling may also act to regulate AC formation (Beg et al., 2007). Histologic observation by the authors indicated no gross morphologic perturbations of the AC of α-2 chimerin null mutants. However, due to the nature of the targeting defects we observed in the EphA4 null mice (which would not be observed histologically), we performed in vivo neuro-anatomic tracing in mice homozygous for an inactivating point mutation in the GAP domain of the α-chimerin gene (α-1 and 2 chimerin null mutant), to determine whether an α-chimerin deletion results in similar axon guidance defects observed for EphA4 null mice (Iwasato et al., 2007). Analysis of serial sections from the AC from these animals demonstrated that both the anterior and posterior tracts of the AC were normal in α-1 and 2 chimerin null mice (Figure 3.9).

3.2.5 Regulation of axon guidance by Eph family members in a redundant manner

As indicated above, genetic deletion of EphB2 and A4 (but not B3), exhibits unique effects on axon guidance within the anterior commissure. To examine potential interactions between Eph family members in this system, a series of combinatorial EphB-family mutants were generated (Figure 3.10A). As indicated in the figure, with the exception of EphB2/A4 double knockouts (DKO), each of the combinatorial lines examined exhibited either a wildtype pattern of innervation, or recapitulated the phenotype of one of the single knockouts. EphB2/A4 double heterozygotes exhibited wildtype patterns of axon guidance in both the ACpa and ACpp (Figure 3.10B). While the ACpp axons in EphB2/A4 DKO exhibited an additive pattern of the axon guidance defects seen in EphB2−/− and EphA4−/− mice, the ACpa axons in DKO animals exhibited a new pattern of aberrant axon guidance not seen in either of the single null mutants. A
Figure 3.9 Normal formation of the anterior commissure in \textit{\(\alpha\)-chimerin} mutant mice.

Shown are neural tracings of both ACpa (in green) and ACpp (in red) and sectioning at the level of the anterior commissure of adult \(\alpha\)-chimerin wildtype (A) and transgenic (B, (Iwasato et al., 2007)). Results demonstrate that formation of the AC is independent of \(\alpha\)-chimerin activity.
Figure 3.10 Synergistic effects of EphB-family receptors.

(A) List of combinatorial EphB-family mutants and controls examined by in vivo stereotactic neural tracing (column headings), together with the ACpp and ACpa phenotypes observed (W, wildtype/control pattern; A1, axon pathfinding defect typified by EphB2−/− mutants; A2, axon guidance defect typified by EphA4−/− mutants; A3, synergistic/non-additive axon guidance defect). (B-E) Fluorescent photomicrographs of the AC in the horizontal plane, demonstrating the pattern of labeling observed for EphB2+/−A4+/− (B), and EphB2−/−A4−/− mice (C-E). ACpp axon labeling has been removed for clarity. Note that ACpa axons in EphB2−/−A4−/− mice become redirected along the intertwined ACpp pathway (arrowheads C and D). (E) Higher magnification example of the ACpa mis-directed axons shown in (C). Scale bar represents 400μm (B-E).
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</table>

**B** EphB2+/-A4+/-

**C** EphB2-/-A4-/-

**D** EphB2-/-A4-/-

**E** EphB2-/-A4-/-
subgroup of ACpa axons in EphB2/A4 DKO was redirected into the ACpp pathway (Figure 3.10C-E). Similar to the behavior seen in EphA4−/− mice, misdirected ACpa axons in EphB2/A4 DKO only project along the pathway defined by the ACpp. Thus, while EphB2 and EphA4 regulate distinct aspects of axon pathfinding in ACpp axons, both receptors participate in regulating the guidance of ACpa axons.

### 3.2.6 Expression of Eph receptors and ephrin ligands in the developing forebrain

Results obtained from MRI and stereotactic tracing analyses of Eph family mutants suggested a mechanism by which these Eph receptors interact to control axon guidance. To further define the nature of these interactions, we examined in three dimensions, the pattern of EphB2 and EphA4 and ephrin ligands staining within the developing forebrain at embryonic day (E) 15, time at which ACpa and ACpp tracts are still forming. The pattern of EphB2 expression was determined using histochemistry in EphB2N2/+ knock-in mice, as previously described (Henkemeyer et al., 1996). In these animals, the β-galactosidase gene is fused with the EphB2 coding sequence downstream of the juxtamembrane tyrosine residues. The results show that while low levels of EphB2 expression persist dorsal to the principal region of the AC, the domain of highest local EphB2 expression (Figure 3.11A) exists just ventral and lateral to the commissural midline under the primary projection path of AC (Figure 3.11A). Within the forebrain, the region of high EphB2 expression extends caudal to the AC, but tapers rostrally beyond the immediate vicinity of ACpp axons (Figure 3.11B). With respect to EphA4, immediately surrounding the midline, high EphA4 expression is observed adjacent to the AC tract consistent with previous reports (Kullander et al., 2001b); while more laterally (Figure 3.11E, arrow), a roughly triangular domain of elevated EphA4 expression is observed in the
Figure 3.11 Eph and ephrin ligands expression in developing forebrain from E13-E16.

(A) Pattern of EphB2 expression at E15 in the sagittal plane at the level of the midline at the AC in whole mount specimen. High level of EphB2 expression was seen lateral and immediately ventral to the AC tract, as shown by beta-galactosidase histochemistry. (B) Brightfield photomicrograph in the horizontal plane showing the normal projection of the ACpa tract in control forebrain at E15. At this stage of development, the ACpa tract lies just ventral to base of the lateral ventricles. (C) Pattern of anti-beta galactosidase staining in EphB2^{N2/N2} embryos at E15 shown in the horizontal plane, illustrating EphB2 expression in the midline and lateral regions below the AC tract. This pattern is consistent with pattern seen in the wholemount lacZ staining (A). (D) Pattern of ephrinB1/B2 staining at E15 shown in the horizontal plane. Note that ephrinB expression is observed in both ACpa and ACpp tracts consistent with previous reports (Kullander et al., 2001b). (E) Pattern of EphA4 expression at E15 shown in the horizontal plane at the level ventral to the AC. Within the path of the ACpa, a region of elevated EphA4 expression which extends lateral to the ACpa tract is denoted (white arrow). (F) Overlay of EphA4 and EphB2 expression. Low levels of EphA4 expression in ACpp but not ACpa axons at E15; as shown in the horizontal plane. (G) Development of ACpa and ACpp axon tracts within the forebrain. Injection of lipophilic tracers into fixed tissues from E13.5-E16.5, demonstrates that axons of the ACpp and ACpa tracts project toward and reach the sagittal midline in a synchronous manner. Section is shown in the horizontal plane at E14.5. (H) Schematic model of the pattern of EphB2 and EphA4 expression observed within the developing forebrain, together with ephrin ligands. With respect to the ACpa tract, the domain of EphA4 expression lies just rostral to the domain of elevated EphB2 expression. Scale bar indicates a distance of 200 μm (A-G).
region ventrolateral to the AC tract. Analysis of ephrinB expression using antisera which recognizes ephrinB1/B2, demonstrates that axons within both the pars anterior and pars posterior express ephrinBs (Figure 3.11D). Thus, both ACpp and ACpa axons possess (at a minimum) the ability to respond to the repulsive effects of EphB2. Within the components of the temporal lobe which gives rise to axons of the ACpp tract, we also observed ephrinBs expression (Figure 3.11D, arrowhead). Despite previous suggestions that EphB/ephrin signaling may assist in the guidance of cortical axons, none of the single or double mutant combinations examined (Figure 3.10A), exhibited significant perturbations in the intitial coalescence of the ACpp (or ACpa) tract.

Free intermingling between ACpp and ACpa axons in the absence of EphA4, prompt us to examine the pattern of EphA4 in ACpp and ACpa axons. In wild-type mice, ACpp axons (but not ACpa) expressed EphA4 (Figure 3.11F), suggesting a possible mechanism for EphA4-mediated axon sorting between ACpp and ACpa axons during development. The stringent segregation between ACpp and ACpa axons could reflect response to either the coincident contact of growing ACpp/ACpa axons along the CNS midline, or represent a subsequent segregation for late arriving axons. The intermingling of ACpp and ACpa axons seen in EphA4−/− mice suggested to us that this deviation might arise as a result of the coincident arrival of ACpp and ACpa axons at the AC junction. We therefore examined the pattern of ACpp and ACpa axon innervation toward the AC junction using lipophilic neural tracers in fixed whole brains from E13.5-E16.5. For the genetic backgrounds examined, no significant innervation at the AC junction was observed prior to E13.5-13.75. Neural tracings performed at E13-E14.5 suggested coincident or near coincident arrival of ACpp and ACpa projections (Figure 3.11G). The data obtained suggests the pattern of Eph/ephrin expression (Figure 3.11H), in which a domain of
high EphA4 expression lies immediately rostral to that of EphB2. Both axons of the ACpa and ACpp express ephrinB ligand, with axons of the ACpp expressing low levels of EphA4.

3.3 Discussion

3.3.1 Generation of MRI-based tool of analyses

The digital average atlas and analysis tools presented here represent a foundation for detecting and quantifying subtle yet significant anatomical differences. The system of analysis allowed the volumes of annotated regions to be reliability measured and compared. The resulting deformation magnitudes calculated was used to identify the position and the degree of any perturbations present. In addition to serving as a hypothesis generation method for focused histological and molecular examinations, we have shown via analysis of the EphB2 and EphA4 null mice that MRI can be used to analyze subtle shift in axonal tract in 3D within the murine CNS as this has never been able to do before in 2D. Little was previously known about how these axons were arranged in relation to other structures in 3D, thus, understanding the arrangement may provide structural and molecular insights into how axons behave when they are misguided in vivo. In addition, high-resolution MRI has enabled the development of the digital surgical atlases (129S1/SvImJ and C57Bl6). The 3D surgical atlas was a significant step forward in the production of accurate stereotactic surgical models in mice. The new stereotaxic coordinate system based on the lambda-rcs sutures was less prone to individual morphological errors created by the natural variation within the examined mouse populations. This system increased our success in positioning the microinjection system into the murine CNS for anterograde labeling of the AC tract.
3.3.2 Regulation of AC formation by EphA4 and EphB2 receptors

Previous *in vivo* analyses of Eph/ephrin-mediated axon guidance within the anterior commissure by laboratories including our own have largely been confined to gross histologic examination. While such analyses provide a valuable overview of the structural morphology of the anterior commissure in genetic mutants, they do not allow the specific origin, innervation or path taken by individual axons to be determined. To more clearly determine the mechanism by which EphB-type receptors act to control AC axon guidance, we have for the first time performed quantitative 3D magnetic resonance imaging of EphB2, B3, A4 and combinatorial null mutants in concert with *in vivo* stereotactic tracing.

These results demonstrate that the loss of EphA4 activity results in the development of several previously unrecognized axon guidance defects within the anterior commissure; including ACpa, ACpp axon segregation. Specifically, high-resolution MR imaging demonstrates for the first time that loss of EphA4 results in a significant ventral and lateral displacement of the ACpa tract in relation to surrounding forebrain structures compared to both heterozygous and wild-type controls, demonstrating that EphA4 plays an important role in positioning the ACpa tract within the dorsoventral and mediolateral axes. In the absence of a means to globally position fine structures of the AC with a high degree of confidence within the CNS, and without corresponding measures of natural variability, such determinations are difficult to assess with confidence. High-resolution MRI analyses therefore enable a new level of quantitative structural analyses to be performed in the indicated axon guidance mutants.

With respect to guidance cues regulating the interaction of ACpa and ACpp axons at the sagittal midline, analysis of *EphA4* null mutants demonstrates that loss of EphA4 normally expressed in ACpp axons, results in a stochastic intermingling of ACpp axons with those of the
ACpa tract. Thus, EphA4 normally regulates the midline segregation of ACpp from ACpa axons during their coincident developmental arrival through a repulsive mechanism. We observe that this aberrant projection persists, continuing into the adult period. It is interesting to note that the degree of tract intermingling appears higher in the central and the dorsal aspect of the AC midline compared to ventral regions, suggesting the potential involvement of additional axon guidance molecules. Beyond the sagittal midline, in EphA4 null mice, mis-projecting ACpp axons can continue to project along the ACpa tract. However the converse projection (ACpa axon projection along the ACpp tract beyond the midline) is prohibited. Our expression studies suggest that this difference in axon guidance may relate to the greater sensitivity of ACpa versus ACpp axons to EphB2-mediated repulsion. The repulsion of ACpp axons appears to involve the gradient of EphB2 expression seen lateral to the midline within the ACpp tract territory.

In the absence of EphA4 expression beyond the midline segregation point for ACpp and ACpa axons, mis-projecting ACpp axons either travel along the main body of the ACpa tract, or continue to proceed laterally until they encounter a region of elevated EphB2 expression (summarized in Figure 3.12C). Upon reaching this point, these axons cannot project laterally or caudally due to the high local levels of EphB2. They, therefore, proceed anteriorly, frequently rejoining the ACpa tract at its rostral aspect. As such, the ACpa tract in EphA4 null mice appears to exhibit de-fasciculated bundles of the ACpa tract. Analysis of stereotactic tracing in such animals demonstrates however that these “de-fasciculated bundles” are in fact composed of mis-projecting ACpp axons. This also explains the true origin of the so-called “U-turn” projections seen in ACpa axons in EphA4 null mice, as described previously by Kullander (2001). The present study demonstrates that such projections are not ACpa tract element U-turns, but rather separate ACpp tract elements coalescing just lateral to the sagittal midline.
**Figure 3.12 Eph-mediated interactions within the developing forebrain.** Schematic representations of the behavior of axons within the AC based upon the individual and cumulative affects of EphA4 and EphB2 during development. EphA4 (A4) and EphB2 (B2) domains are indicated (A). As indicated previously (Henkemeyer et al., 1996) the absence of EphB2, axons from temporal neurons comprising ACpp tract fail to encounter EphB2-mediated repulsion as they approach the sagittal midline; resulting in aberrant projection to the floor of the forebrain (1) (B). In the absence of EphA4, several axonal deviations occur. Normally, the coincident developmental projection of the ACpp and ACpa tracks results in segregation of ACpp and ACpa axons. In the absence of EphA4, axons of the ACpp exhibit a loss repulsive contact between ACpa axons, resulting in free intermingling of ACpp and ACpa axons (2). As a result of this intermingling, axons of the ACpp stochastically mix with ACpa axons, ultimately resolving into either continued projections towards the contralateral temporal cortex, or aberrant projection along the ACpa tract (3 and 4). Aberrant ACpp axons can either project within the displaced (see below) ACpa toward the rostral forebrain (3), or clusters of ACpp axons can continue along a more lateral course from the midline, due to the absence of EphA4 expression in the region immediately rostral to the EphB2. In such instances, because of the more ventral position of the ACpa tract compared to the ACpp, these axons continue until they encounter the rostral perimeter of the high EphB2 expressing domain. As these axons cannot project caudally due to high levels of EphB2, they turn in an anterior direction, typically rejoining the ACpa tract (4) (C). With respect to ACpa axons, the absence of EphA4 results in ventrolateral deviation of the ACpa tract (5) due to loss of the forebrain EphA4 domain indicated. In addition, while ACpp and ACpa axons can freely intermingle at the midline, only ACpp axons can project into ACpa territory in the absence of EphA4. ACpa axons are blocked from the converse behavior (ACpp
tract entry), due to their apparently greater sensitivity to EphB2-mediated repulsion. (E) In the absence of both EphB2 and EphA4, each of the individual guidance errors described above occurs; however in addition, axons of the ACpa can now enter ACpp territory (6) due to removal of the remaining EphB2 barrier. However, in contrast to the stochastic intermingling observed in ACpp axons, only a minority of ACpa axons (~20%) ultimately enter the ACpp tract in the absence of EphB2 and A4.
Taken together, both the genetic ablation and immunohistochemical expression studies support a model where ephrinB expressing ACpa axons are repelled through ventrolateral interaction with EphA4 expressing regions adjacent to ACpa tract (summarized in Figure 3.12D). This model suggests that in the absence of rostral EphA4 expression, ACpa axons originate from the piriform cortex of olfactory bulb project ventral and lateral to their normal path, projecting caudally until they reach a zone of high EphB2 expression (Figure 3.12D). At this point they are redirected toward the midline (Figure 3.12D-4). The loss of this rostral EphA4 domain (Figure 3.12C) also allows the ventrolateral deviation of mis-projecting ACpp axons from the midline (see below).

Interestingly, we observed concomitant expression of both EphA4 and ephrinB expression within axons of the ACpp. Consistent with this, previous reports have demonstrated ephrinB2 expression within the ACpp (Cowan et al., 2004). This poses an intriguing problem with regard to how ACpp axons respond to the dual expression of a given Eph receptors and its ligand within the same tract; as these axons do not appear to exhibit significant self-repulsion. Previous in vitro studies have provided evidence of down-regulation of Eph/ephrin signaling in cis (Carvalho et al., 2006). Another potential explanation involves mutual exclusivity of EphA4 and ephrinBs. An example of this is seen in the work of Marquardt et al. (2005) who examined EphA and ephrinAs co-expresssion in motor axons. It was proposed that EphA and ephrinAs were segregated into separate membrane domains and were capable of simultaneously binding their respective binding partners presented in trans (Marquardt et al., 2005). Whether internal repulsion arising from the relatively low levels of EphA4 in ACpp axons (relative to adjacent domains such as the ACpa tract) is overridden by surrounding Eph-ephrin interactions or through the influence of additional signaling molecules remains to be determined. However recently,
several laboratories have identified Rac-GTPase α2-chimerin as a key mediator of ephrinB3/EphA4 forward signaling in the development of motor circuits (Beg et al., 2007; Iwasato et al., 2007; Wegmeyer et al., 2007). Our tracing analysis of these mutants demonstrates that unlike the corticospinal tract, α-1 and 2 chimerin do not mediate the actions of EphA4 with respect to the anterior commissure. The fact that our axonal tracings in α-Chn null mice did not result in similar phenotype to that seen in EphA4 knockouts, supports evidence for a reverse signaling mechanism. Consistent with this, reverse signaling through ephrinB2 has previously been shown to mediate the repulsion of ACpp axons from the ventral regions (Cowan et al., 2004).

Analysis of ACpp axons in both EphB2 and EphA4 null mice, demonstrates that these similar signaling complexes impart unique guidance activities to this axonal population. From a developmental perspective, it is remarkable that two receptors of such similar structural character with largely overlapping ligand binding and expression profiles should exert such selective control over a given group of axons. Such findings highlight the complex and variegated nature of the Eph/ephrin receptor system. That these signaling interactions are strictly separable with respect to the guidance of ACpp axons is illustrated by the fact that ACpp axons of EphB2/A4 double knockout mice exhibit no additional perturbation with respect to axon guidance beyond the addition of each of the single knockout phenotypes.

While we have demonstrated that EphA4 and EphB2 play distinct roles in the guidance of ACpp axons, our analysis of the EphB2/A4 double knockout demonstrates for the first time, a redundant effect for these receptors with respect to the guidance of a subpopulation of ACpa axons. In addition to exhibiting the guidance errors seen in each of the corresponding single knockouts, ~20 ± 7% of the ACpa fibers in EphB2/A4 double knockout now leave the ACpa tract
and enter ACpp territory (Figure 3.12D). In contrast to the stochastic behavior (50% of the axons) seen for ACpp axons in *EphA4*−/− mice, the mis-projection seen in the double knockout represents approximately half this number, suggests that functional heterogeneity may exist within the ACpa population. The mis-projection of less than 50% of ACpa axons in *EphB2/A4* double knockouts suggests that additional guidance molecules may regulate this process. In this respect, it is interesting to note that like the *EphA4* null mutant, mice lacking *Sema3B* also exhibit a ventral and lateral shift in the ACpa tract (Falk et al., 2005). In the absence of a comparable quantitative determination of ACpa tract morphology in *Sema3B* null mutants, it is difficult to determine with certainty whether the extent these deviations are similar to those seen in *EphA4* null mice. However, the ability of *Sema3B* ablation to phenocopy at least some of the features seen in *EphA4* null mutants suggests that these disparate receptors ultimately act to affect common downstream mechanisms regulating axon guidance. With respect to Eph-mediated axon guidance, the altered projection of ACpa axons seen in the *EphB2/A4* double knockouts strongly implies that these receptors have compensatory roles in regulating the anterior-posterior guidance for at least some ACpa axons. However, it does not necessarily indicate that they share an identical mode of action, as suggested by their distinct pattern of expression and the differential effects on ACpa tract morphology. As shown in the model, we postulate that in the absence of EphB2, ACpa axons are restricted from entry into the ACpp through interaction with EphA4-expressing ACpp axons at the midline due to their expression of ephrinA3 (Kudo et al., 2005). By contrast, in the absence of EphA4 at the midline, ACpa axons are not formally repelled from entry into the ACpp tract. We demonstrate that at least a subgroup of these axons do not continue along the ACpp tract due to elevated levels of EphB2 expressed lateral and caudal to the midline.
While our analysis of Eph mutants identifies their role both alone, or in combination, in regulating the key steps involved in properly guiding forebrain axons to the contralateral hemisphere, these data also highlight the importance of additional regulators in controlling tract formation. In none of the single or combinatorial Eph receptor mutants examined was a complete loss of the ACpa or ACpp tract observed, suggesting the influence of axon guidance molecules in regulating initial projection of AC axons. Consistent with this, mice lacking netrin1 fail to extend axons to either the ACpa or ACpp (Serafini et al., 1996). In addition, the absence of the semaphorin ligand Sema3F (Sahay et al., 2003) or its principal receptor Npn2 (Chen et al., 2000; Giger et al., 2000), results in a substantial reduction in numbers of both ACpa and ACpp axons projecting to the anterior commissure. In contrast to the indicated EphB receptors, these additional guidance cues appear to exert a more general influence on axonal behavior within the CNS. Thus, while EphB-class receptors regulate discreet aspects of the projection of both ACpa and ACpp axons, as well as tract segregation, the process of commissure formation is influenced particularly with respect to initial projection by an array of additional guidance cues.

In addition to the Eph null mutants indicated above, EphB3 and a series of additional combinatorial null mutants (Figure 3.8F) were examined in several different murine backgrounds (C57Bl/6J, 129Sv/ImJ, mixed). Within the forebrain EphB3 null mutants did not exhibit statistically significant defects in axon guidance with respect to the analyses performed. Similarly, none of the combinatorial mutants listed demonstrated additional axon guidance defects beyond that which we have described for the indicated single mutants. Taken together, these data provide a basis for understanding the mechanism by which EphB-class receptors regulate not only ACpp axon guidance, but the position and trajectory of the ACpa tract, as well as anterior-posterior tract segregation within the anterior commissure. Specifically, EphB2 and
A4 exert both unique and synergistic activities with respect to inter-hemispheric connection of the forebrain.
CHAPTER 4

Role of EphA4 and B2 in regulating somatotopic organization of motor axons within the lumbar spinal cord
4.1 Introduction

Controlled voluntary movement depends upon many levels of neural control, from proper connections within the motor and association cortices to appropriate motor connections and innervations to peripheral musculature. Connections of lower spinal motor nerves are organized in a topographic manner in that the position of a given motor neuron along the rostrocaudal/medial-lateral axis of the spinal cord correlates with the relative position of its muscular target within the limb (Lance-Jones and Landmesser, 1981). These connections are highly regulated and initiated shortly after the genesis of motor neurons in the ventral spinal cord. Groups of these motor neurons become organized into discrete groups along the anteroposterior axis. The median motor column (MMC) innervates axial muscles of the body wall, whereas the lateral motor column (LMC) innervates muscles of the limbs. The LMC can be further divided into lateral (LMCl) and medial (LMCm) components, which send axons to the dorsal and ventral of the limb muscles respectively (Tosney and Landmesser, 1985). These groups can be distinguished by their combinatorial expression of LIM homeodomain proteins (Kania et al., 2000; Sockanathan and Jessell, 1998). The LMC projections are first established as axons exit the spinal cord via the ventral root, extend along a common sciatic sheath, ultimately converging on the sciatic plexus at the base of the limb. At this point, dorsal and ventral LMC axons diverge and innervate specific muscular targets (Tosney and Landmesser, 1985). During this process, LMC axons diverge following contact with limb mesenchyme. The precision and specificity of these interactions implies an intricate coordination of guidance and expression cues, both spatially and temporally. To date, the mechanism by which guidance cues direct motor axons to appropriate dorsal or ventral muscular targets in the limb remains elusive. An
example has been shown for EphA-ephrinA interaction mediating dorsal pathfinding of LMCl axons to dorsal musculature (Eberhart et al., 2002; Feng et al., 2000; Kania and Jessell, 2003).

EphA receptors, such as EphA4, are known to be expressed in subsets of developing motor neurons (Ohta 1996, Kilpatrick 196, Olivieri and Miescher 1999) and the developing limbs (Eberhart 2000). The EphA-ephrinA interactions have been shown to influence neuromuscular synapse formation and maintenance (Lai 2001), as well as affect the migration of neural crest cells (Smith et al., 1997). Targeted deletion of the EphA4 gene has been shown to result in axon guidance defects in the corticospinal tract (CST- the principal descending motor pathway), resulting in altered motor behavior (hopping gait) (Dottori et al., 1998). A similar phenotype seen in ephrinB3 null mutants is thought to reflect the key role which EphA4/ephrinB3 interaction play in preventing premature defasciculation of motor axons from the CST tract (Yokoyama et al., 2001).

With respect to the lateral motor column, ectopic expression of EphA4 in chick LMCm neurons leads to aberrant dorsal projection of ventrally-fated motor axons, while inactivation of EphA4 has been shown to disrupt the normal LMCl axon projection (Eberhart et al., 2002). The current model suggests that EphA4 is required for LMCl axons to be repelled by ephrinA-expressing domains within the ventral limb. Nevertheless, these gain and loss-of-function experiments suggest that EphA4/ephrinB3 signaling does not account for all aspects of motor axon pathfinding in the rodent hindlimb. In particular, the mechanisms that instruct axons of LMCm motor neurons to project to the ventral mesenchyme remain obscure. To determine whether EphA4 or EphB-family members play a role in regulating this aspect of motor axon guidance, single and combinatorial Eph mutants were examined for their pattern of dorsoventral hindlimb innervations.
In the present study, I show that EphB2-ephrinB signaling is required for the correct dorsoventral decision made by a subpopulation of LMCm axons. Furthermore, I demonstrate that the loss of EphB2 alone or EphB2/EphA4 in combination result in specific mistargeting of unique population of LMCm motor axons within the lumbar spinal cord.
4.2 Results

4.2.1 Expression of Eph receptors and cognate ephrin ligands in lumbar motor neurons of the spinal cord and limb musculature

To explore whether the identified groups of Eph receptors play a role in regulating the dorsoventral decision making of motor axons, the expression of EphB receptor and ephrinB ligands was first examined in limb mesenchyme and LMC neurons. Analyses performed using phosphospecific (denoting activated Eph receptor) antibodies to the juxtamembrane region of the EphB receptor (Henderson et al., 2001; Holland et al., 1996) demonstrated strong immunoreactivity in both LMCm and LMCl neurons at embryonic (E) day 11.0-13.5 (Figure 4.1B). In addition, robust phosphospecific EphB expression was seen in motor axons exiting the sciatic plexus and into the limb mesenchyme (Figure 4.1C). The fact that activated EphB receptor expression continues to be observed in motor axons following the period of initial innervation, raises the possibility that, in addition to potentially regulating dorsoventral axon guidance, EphB may act to maintain some ongoing aspects of neuromuscular innervation. I then sought to determine whether cognate ligands (ephrinBs) were expressed in either spinal cord motor neurons or the limbs. Cross-sections of wildtype embryos at E13.5 demonstrated that ephrinB ligands were detectable in dorsal mesenchyme at E13.5 (Figure 4.1D). By contrast, only low levels of ephrinB expression were detected in ventral mesenchyme (Figure 4.1D). Together, these data indicate that EphB receptors and several of their cognate ligands are expressed in the developing limb.
**Figure 4.1 Expression of EphB receptors and ephrinB ligands in the developing limb from E10-E13.5.** (A) Schematic representation of the spinal cord cross-section during development (B-D) Immunohistochemical analyses of transverse sections of wildtype mouse embryo at the level of the limbic plexus with antisera directed against phosphorylated pan-EphB receptors (B-C) and pan-ephrinB (D). At the limbic plexus, EphB receptors are detected on both LMCm and LMCl motor axons while ephrinBs span the region of the dorsal mesenchyme. (E) Representative whole-mount lacZ stain of EphB2<sup>N2/+</sup> embryo, illustrating the expression of EphB2 receptors is first seen within specific motor neurons only after E10 and extending throughout the entire length of the spinal cord by E10. (F-Q) Co-immunofluorescence of EphB2 and motor neuron population markers, Lim3 (F-H), Islet1,2 (I-K), HB9 (L-N) and Raldh2 (O-Q) at lumbar level of spinal cord of an E11.5 EphB2<sup>N2/N2</sup> embryo. The observed expression patterns of EphB2/Islet1,2, EphB2/HB9 and EphB2/Raldh2 and indicate that EphB2 is present in both LMCm and LMCl neurons. Scale bars in (A-Q) denote 100μm.
Because no reliable antibody exists which specifically recognizes EphB2, I definitively determined the pattern of EphB2 expression by taking advantage of the EphB2^{N2/+} animals which express the β-galactosidase (LacZ) gene fused in-frame to the juxtamembrane region of the endogenous EphB2 gene (Henkemeyer et al., 1996). In whole-mounts, EphB2 expression was first detected within mouse spinal cord at E9.5 (Figure 4.1E), the period during which motor neurons first exit the cell cycle and begin organizing themselves into topographic groups. By E10, robust EphB2 expression was observed throughout the entire length of the spinal cord (Figure 4.1E). At this stage, motor neurons begin to project their axons to the limbic plexus but typically have not yet reached their final targets in the periphery. To determine whether EphB2 receptors are restricted to a columnar class of motor neurons, I compared the pattern of β-gal in EphB2^{N2/+} animals with expression of differential motor neuron antigens Lim3, Islet1,2, HB9 and Raldh2 (Kania et al., 2000; Tsuchida et al., 1994). EphB2 and HB9 expression was found to overlap throughout the ventral spinal cord, indicating that EphB2 is expressed in most LMC motor neurons (Figure 4.1O-Q). To differentiate MMC from LMC neurons, I compared EphB2-lacZ staining with the MMC marker Lim3, and pan-LMC marker Raldh2. While a low level of EphB2 expression was observed in Lim3 positive neurons (Figure 4.1F-H), the majority of EphB2 expression coincided with Raldh2 (Figure 4.1L-N), suggesting a preferential expression of EphB2 by LMC neurons. Because LMCm neurons express both Isl1 and Isl2, and LMCl neurons express only Isl1, these two LMC populations can easily be distinguished by their pattern of Islet1,2 using the well-characterized antisera (39.4D5: DSHB). As shown by combinatorial staining in figure 4.1I-K, EphB2 is expressed in both LMCm and LMCl motor neurons, as distinguished by its co-expression with both strongly and weakly labeled Islet-1,2.
neurons. Thus, both LMC1 and LMCm classes of spinal motor neurons express EphB2 at similar levels.

4.2.2 The rostrocaudal distribution of motor neurons in EphB-deficient mice is normal

The reciprocal pattern of activated EphB receptors and ephrinB ligands within motor neurons and the developing limb prompted the examination of the possibility that these interactions might regulate the dorsoventral fate of LMC motor axons. To test this hypothesis, the distribution of labeled motor neurons along the lumbar spinal cord was determined following retrograde tracing of the peroneal nerve innervating a subset of dorsal muscles in EphB family mutants. In wildtype and EphB+/- controls, distinct columnar distribution of spinal motor neurons was observed as described previously (McHanwell and Biscoe, 1981). To assess the normal pattern of motor neuron innervation and ensure that labeling procedures were being performed reproducibly, I analyzed the rostrocaudal arrangement of motor neurons in adult wildtype (n = 6) lumbar spinal cord. Unique neural tracers were deposited into three different branches of the sciatic nerve (Figure 4.2A) resulting in labeling of three distinct population of motor neuron. Each of these exhibited their own discrete rostrocaudal distribution within the lumbar spinal cord. Serial cross-sections through the entire extent of these labeled spinal cords demonstrated that the largest population of labeled motor neurons arose from the tibial nerve (3.7 mm ± 0.3, spanning vertebrae levels T13-L2), followed by those of the peroneal nerve (1.4 mm ± 0.1, spanning vertebrae levels T13-L1) and finally, the sural nerve (0.5 mm ± 0.1, spanning vertebrae level L1) (Figure 4.2A). The results observed are in agreement with the known distances of these motor neuron pools where the tibial nerve innervating the gastrocnemius, tibialis posterior, plantaris and soleus muscles contains a greater axonal population than that of either the
Neither single nor combinatorial deletion of the EphA4, B2, or B3 receptors altered the rostrocaudal distribution of the peroneal labeled motor pools within the lumbar spinal cord. Motor pools were mapped with respect to the vertebral segment by retrograde transport following deposition of fluorescent dye. (A) Diagram depicting the normal rostrocaudal position of retrogradely labeled motor neurons traced from the tibial nerve (green), peroneal nerve (red) and sural nerve (blue) with respect to the vertebrae level. (B) Comparison of rostrocaudal distribution of retrogradely labeled motor neurons in different Eph mutants. Graph illustrates the average vertebrae level of observed retrograde labeling following peroneal nerve labeling in adult Eph mutants and wildtype (error bar ± s.e.m., * $P > 0.05$ t-test). No significant difference (confidence level and statistical test used) were observed in the extent of the rostrocaudal distribution of labeled peroneal motor somas between wildtype, heterozygous and Eph mutant mice.
Genotypes

Wildtype ($n = 6$)
EphA4$^{+/-}$ ($n = 12$)
EphB2$^{-/-}$ ($n = 13$)
EphB2$^{N2/N2}$ ($n = 7$)
EphB3$^{-/-}$ ($n = 10$)
EphB2$^{-/-}$B3$^{-/-}$ ($n = 8$)
EphB2$^{-/-}$A4$^{-/-}$ ($n = 4$)
EphB2$^{N2/N2}$A4$^{-/-}$ ($n = 4$)
peroneal innervating the tibialis anterior muscles or the sural nerve innervating the lower lateral plantaris muscles.

Subsequently, axon tracing studies were performed in each of the single and combinatorial EphB-family mutants to determine whether the rostrocaudal topographic distribution of motor neurons was altered in absence of each of these components. Deposition of fluorescent tracers onto either tibial or peroneal nerves, followed by serial sectioning of the entire extent of the labeled region is summarized in figure 4.2B. No significant shift (a shift of more than one vertebral level) in the rostrocaudal distributions of labeled motor neurons were observed for labeling of the tibial and peroneal nerves between EphB mutants and wildtype mice (Figure 4.2B). Similarly, analysis of EphA4 null mutants on several genetic backgrounds in our lab demonstrate no perturbation in the rostrocaudal pool of labeled peroneal motor neurons and the presence of normal well-developed tibial nerve, in contrast to previous reports (Eberhart et al., 2002; Helmbacher et al., 2000).

4.2.3 EphB2 regulates proper innervation of a subgroup of LMCm axons to ventral muscles in the hindlimb

As indicated above, the LMCI and LMCm populations represent a level of positional identity within the ventral spinal cord with respect to the selection of ventral and dorsal axonal trajectories during target innervation (Kania and Jessell, 2003). Based upon these analyses, I have constructed a map of the relative location of the motor columns according to their pattern of target innervation (Figure 4.3A). Represented in a hemi-spinal cord cross-section are regions delineating the mediolateral relation of motor groups that send projections to the tibial and peroneal nerves (Figure 4.3A). These zones were determined by pooling all labeled motor
neurons contributing to the tibial (zone T), peroneal (zone P) and sural (zone S) nerves respectively at a confidence level of 95% for each motor population. This map was then used to analyze the retrograde labeling of EphB mutants.

To test the requirement of Eph receptor activity in assigning the dorsoventral fate to LMC axons, motor neuron connectivity in Eph mutants was analyzed by performing in vivo retrograde labeling of the tibial (ventral nerve) and peroneal (dorsal nerve) using fluorescent tracers. Tibial labeling studies showed that in addition to labeled motor neurons in the LMCm (zone T), a significant percentage of labeled motor neurons were found in the LMCl (zone P) in EphA4−/− (27.0 ± 6.7%, $P < 0.01$) compared to wildtype (3.3 ± 2.4%) (Table 4.1). Consistent with previous reports, these findings confirmed the role for EphA4 in regulating the dorsal guidance of LMCl axons (Kania and Jessell, 2003). Interestingly, a significant increase of labeled motor neurons was observed in the LMCl (zone P) in EphB2 null mice (40.0 ± 9.5%, $P < 0.01$) compared to controls (3.3 ± 2.4%) (Table 4.1), demonstrating, for the first time, a role for EphB2 in regulating LMCl axons patterning. In contrast, no significant difference in the pattern of labeled motor neurons was observed between those of EphB3−/− (6.0 ± 2.7%) and controls (3.3 ± 2.4%) indicating the dispensable role of EphB3 within these neurons (Table 4.1).

In absence of EphA4, retrograde tracings of the peroneal nerve demonstrated a significant increase in the labeling of LMC motor neurons in the zone T (13.0 ± 2.2% versus 7.0 ± 1.9%, Table 4.1), which motor neurons found in this zone would normally project to the tibial nerve. EphA4+/− mice also demonstrated a trend towards higher levels of peroneal/tibial mis-guidance compared to controls (7.0 ± 1.9% versus 4.0 ± 2.0%, Table 4.1). However, this did not have a statistical significance for the number of animals studied. This finding correlates with the low
Motor pools were identified following retrograde tracing of fluorescent dyes into the peroneal (P), tibial (T) and sural (S) nerves. (A) Example of probability constructs at one level of the spinal cord for labeled neural populations in wildtype animals. The interface boundary for T and P populations is determined by defining the probability of finding a labeled motor neuron from retrograde tracing of that specific nerve within the indicated zone. Probability zones were established from the analyses of 38 wildtype animals. (B-D) Representative cross section of an adult wildtype spinal cord showing the approximate area of labeling following injection of each nerve; peroneal (red, B and C), tibial (green, C) and sural (blue, D). (E-H) Schematic representations of actual labeled motor neuron population (represented by the red circles) following peroneal nerve injections in EphB2<sup>+/−</sup> (E), EphB2<sup>−/−</sup> (F), EphB2<sup>−/−</sup> A4<sup>+/−</sup> (G), and EphB2<sup>N2/N2 A4<sup>−/−</sup> (H) at the same spinal level. Red lines denote the peroneal zone, green lines denote tibial zone. Scale bars represent 200µm.

**Figure 4.3 Medial-lateral mapping of motor pools in wildtype and Eph mutants.**
PT O EF G H


Wildtype P P and T P and S

A B C D

E F G H

151
### Table 4.1. Quantification of labeled motor neurons following retrograde tracing of the tibial and peroneal nerve in wildtype and Eph mutant mice

<table>
<thead>
<tr>
<th>Genotypes</th>
<th>n</th>
<th>% labeled MN found in zone P</th>
<th>% labeled MN found in zone T</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
<th>p value&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% labeled MN found in zone O</th>
<th>p value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Retrograde labeling of Tibial Nerve</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype (+/+)</td>
<td>6</td>
<td>3.0 ± 2.4</td>
<td>97.0 ± 2.4</td>
<td>------</td>
<td>------</td>
<td>0</td>
<td>------</td>
</tr>
<tr>
<td>EphA4&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>3</td>
<td>27.0 ± 6.7*</td>
<td>73.0 ± 6.7</td>
<td>3.5 x 10&lt;sup&gt;−1&lt;/sup&gt;</td>
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<td>60.0 ± 9.5</td>
<td>9.7 x 10&lt;sup&gt;−4&lt;/sup&gt;</td>
<td>------</td>
<td>0</td>
<td>------</td>
</tr>
<tr>
<td>EphB3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>4</td>
<td>5.0 ± 2.7</td>
<td>95.0 ± 2.7</td>
<td>0.54</td>
<td>------</td>
<td>0</td>
<td>------</td>
</tr>
<tr>
<td><strong>Retrograde labeling of Peroneal Nerve</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wildtype (+/+)</td>
<td>6</td>
<td>96.0 ± 2.0</td>
<td>4.0 ± 2.0</td>
<td>------</td>
<td>------</td>
<td>0</td>
<td>------</td>
</tr>
<tr>
<td>EphA4&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>12</td>
<td>93.0 ± 1.9</td>
<td>7.0 ± 1.9</td>
<td>0.41</td>
<td>0.05</td>
<td>1.0 ± 0.5</td>
<td>0.46</td>
</tr>
<tr>
<td>EphA4&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>12</td>
<td>87.0 ± 2.2</td>
<td>13.0 ± 2.2*</td>
<td>0.74</td>
<td>&lt;0.01</td>
<td>2.0 ± 1.6</td>
<td>0.22</td>
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<tr>
<td>EphB2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>10</td>
<td>95.0 ± 1.6</td>
<td>5.0 ± 1.6</td>
<td>0.01</td>
<td>0.66</td>
<td>0</td>
<td>------</td>
</tr>
<tr>
<td>EphB2&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>13</td>
<td>82.0 ± 2.7</td>
<td>16.0 ± 2.7*</td>
<td>0.66</td>
<td>&lt;0.01</td>
<td>0</td>
<td>------</td>
</tr>
<tr>
<td>EphB2&lt;sup&gt;−/−&lt;/sup&gt;/N2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>5</td>
<td>97.0 ± 2.5</td>
<td>3.0 ± 2.5</td>
<td>0.09</td>
<td>1.0 ± 0.7</td>
<td>0</td>
<td>------</td>
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<tr>
<td>EphB3&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>3</td>
<td>94.0 ± 3.2</td>
<td>6.0 ± 3.2</td>
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<td>0.35</td>
<td>2.0 ± 0.9</td>
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<td>10.0 ± 2.6</td>
<td>0.01</td>
<td>0.09</td>
<td>0</td>
<td>------</td>
</tr>
<tr>
<td>EphB2&lt;sup&gt;−/−&lt;/sup&gt;/A4&lt;sup&gt;−/−&lt;/sup&gt;</td>
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<td>82.0 ± 3.2</td>
<td>16.0 ± 3.2*</td>
<td>0.44</td>
<td>0.29</td>
<td>0</td>
<td>------</td>
</tr>
<tr>
<td>EphB2&lt;sup&gt;−/−&lt;/sup&gt;/A4&lt;sup&gt;+/−&lt;/sup&gt;</td>
<td>2</td>
<td>91.0 ± 2.0</td>
<td>9.0 ± 2.0</td>
<td>0.1</td>
<td>0.02</td>
<td>2.0 ± 1.0</td>
<td>0.12</td>
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<tr>
<td>EphB2&lt;sup&gt;−/−&lt;/sup&gt;/A4&lt;sup&gt;−/−&lt;/sup&gt;/N2&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>4</td>
<td>64.0 ± 8.0*</td>
<td>34.0 ± 8.0*</td>
<td>2.0 x 10&lt;sup&gt;−3&lt;/sup&gt;</td>
<td>0.1</td>
<td>19.0 ± 1.5*</td>
<td>2.4 x 10&lt;sup&gt;−7&lt;/sup&gt;</td>
</tr>
<tr>
<td>EphB2&lt;sup&gt;−/−&lt;/sup&gt;/A4&lt;sup&gt;−/−&lt;/sup&gt;/A4&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>4</td>
<td>42.0 ± 2.2*</td>
<td>39.0 ± 2.2*</td>
<td>3.4 x 10&lt;sup&gt;−6&lt;/sup&gt;</td>
<td>0.01</td>
<td>14.0 ± 1.6*</td>
<td>3.6 x 10&lt;sup&gt;−6&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>p values: values determined against WT animals, Student t-test, two-tailed
<sup>b</sup>p values: values determined against heterzygotes of respective genotypes, Student t-test, two-tailed
level of EphA4 previously observed within the LMCm motor neurons (Eberhart et al., 2002; Helmbacher et al., 2000). A similar situation was observed in mice lacking EphB2. In these mutants, a significantly higher number of labeled motor neurons were found in the zone T following peroneal tracing (16.0 ± 2.7%) compared to controls (5.0 ± 1.6%, Table 4.1). Though smaller than the effects seen for EphA4 and EphB2, loss of EphB3 also resulted in some misguidance of the tibial motor axons into the peroneal nerve (10.0 ± 2.6%, Table 4.1). Thus, loss of each of the above receptors results in some mis-projection of a sub-population of tibial motor axons into the peroneal pathway. To determine whether forward or reverse signaling is disrupted in the EphB2 null mutants, I assessed retrograde labeling in mice that selectively lack the cytoplasmic domain of the EphB2 receptor (EphB2<sup>N2/N2</sup>) (Henkemeyer et al., 1996). In EphB2<sup>N2/N2</sup> mutants, a pattern of axonal mis-direction similar to that seen for EphB2 null mice was observed. Specifically, 15.0 ± 3.4% of tibial motor axons were found to project aberrant to the peroneal nerve, indicating that the extracellular and juxtamembrane regions of EphB2 receptor alone is insufficient to rescue the aberrant phenotype seen in EphB2 knockouts (Table 4.1).

To assess the potential redundancies among the Eph family receptors with respect to dorsoventral guidance, fluorescent tracing of the peroneal nerve was performed on combinatorial Eph mutants. As shown in Table 4.1, while EphB2<sup>+/−</sup>B3<sup>+/−</sup> mice demonstrated comparable numbers of labeled LMC1 motor neurons (2.0 ± 2.5%) (data not shown) to controls (4.0 ± 2.0%), EphB2<sup>−/−</sup>B3<sup>−/−</sup> mutants showed a pattern of motor neuron labeling (16.0 ± 3.2%) that was similar to that seen in EphB2 null mice (16.0 ± 2.7% in zone T, Table 4.1). These findings suggest that EphB3 does not function to exacerbate the axon guidance seen in the absence of EphB2. In contrast, a marked increase in aberrant motor neurons labeling was observed in
EphB2\textsuperscript{−/−}A4\textsuperscript{−/−} and EphB2\textsuperscript{N2/N2}A4\textsuperscript{−/−} mice (39.0 ± 2.2% and 36.0 ± 4.9% respectively) compared to respective controls (Table 4.1). Interestingly, in addition, a dramatic increase in the number of labeled motor neurons outside of the LMCs was observed in these mutants (19.0 ± 1.5% and 14.0 ± 1.6% respectively) defined as zone “O” in figure 4.3A. These findings demonstrate that \textit{in vivo}, combinatorial loss of EphA4 and EphB2 results in a synergistic enhancement of motor axon guidance defects seen in each single mutant and the development of new defects in axon pathfinding not seen in either mutant alone.

\textbf{4.2.4 EphA4 and EphB2 do not mediate their effects by selectively altering motor neuron survival}

Next, I sought to determine whether changes observed in the population of labeled motor neurons following loss of EphB2 and EphA4 might reflect an effect of these receptors on the survival of specific motor subpopulations. Stereologic counts of the number of labeled peroneal motor neurons were performed on \textit{Eph} mutants and controls. The average number of labeled motor neurons in each peroneal pool did not significantly differ between mutants (\textit{EphA4}\textsuperscript{−/−} (n = 12, 19 ± 1.8), \textit{EphB2}\textsuperscript{−/−} (n = 13, 25 ± 1.5) and \textit{EphB3}\textsuperscript{−/−} (n = 10, 25 ± 1.9)) and controls (\textit{EphA4}\textsuperscript{+/−} (n = 12, 19 ± 1.0), \textit{EphB2}\textsuperscript{+/−} (n = 10, 23 ± 2.3) and \textit{EphB3}\textsuperscript{+/−} (n = 3, 20 ± 2.7) and wildtype (n = 6, 14 ± 1.5) (Figure 4.4). In contrast, a 2-fold increase in labeled peroneal motor neurons was observed in \textit{EphA4} and \textit{EphB2} combinatorial knockouts (\textit{EphB2}\textsuperscript{−/−}A4\textsuperscript{−/−} (n = 4, 53 ± 7.4) and \textit{EphB2}\textsuperscript{N2/N2}A4\textsuperscript{−/−} (n = 4, 49 ± 5.1)) (Figure 4.4). The fact that an increase in motor neuron number was observed suggests that while the correct number of motor neurons are projecting to the dorsal muscles, motor neurons from other area of the spinal cord are also inappropriately projecting dorsally.
Figure 4.4 Stereologic counts of the number of labeled peroneal in *Eph* mutants.

Following injection of neural tracer to the peroneal nerve, stereologic counts of labeled motor neurons within the lumbar spinal cord was performed. No significant difference was observed between single *Eph* mutants and controls. In contrast, a significant increase in the number of labeled motor neurons was seen in combinatorial mutants suggesting an increase in the population of motor neurons projecting to the peroneal nerve.
Average numbers of fluorescently labeled motor neurons in T13-L1 vertebral level


* *
4.2.5 EphB2 regulates dorsoventral decisions of LMCm axons during development

Based on the above results, these aberrant mediolateral distributions of labeled motor neurons seen in EphB2 null mice could potentially have arisen from either the defective navigation of LMC axons at the limbic plexus, or through a change in motor neuron identity during developing. To rule out this latter possibility, the pattern of motor neuron markers, Islet1 and 2 was examined in Eph mutants following retrograde labeling of the dorsal hindlimb muscles at E13.5. Islet1,2 antisera were utilized to distinguish between LMCl from LMCm neural populations as it strongly labels LMCm motor neurons (Islet-1/Islet-2 positive) while weakly labeling LMCl motor neurons (Islet-2 positive). Antisera against Lim3 were also employed to identify MMC motor neurons within the developing spinal cord. Whereas fluorescent dye injection into the dorsal muscles of control embryos labeled specifically the LMCl neurons, as identified by overlapping expression with the low level of the antibody Islet1/2, a similar procedure performed on EphB2 null embryos labeled both the LMCl and LMCm neurons (Figure 4.5). In addition, no labeled motor neurons were seen in MMC (identified by Lim3 expression) (data not shown). Taken together, these results indicate that a substantial fraction of LMCm neurons undergo inappropriate projection into the dorsal hindlimb in the described EphB mutants. This also definitively demonstrates that EphB2 is required for the establishment of correct dorsoventral projections for at least a subgroup of LMCm motor axons.
Figure 4.5 Aberrant projections of the LMCm axons into dorsal muscles in E13.5 EphB2−/− embryos. (A) Schematic illustrating motor column represented by labeled motor neurons within the spinal cord. (B and E) Retrograde labeling using DiI into the hindlimb dorsal muscles in EphB2+/− and EphB2−/− embryos at E13.5 respectively. Notice the marked difference in pattern of labeled motor neurons. (C and F) Double immunostaining with Islet1, 2 antibody to define the two populations of LMC neurons (LMCl and LMCm) in EphB2+/− and EphB2−/− embryos respectively.
E13.5 Wildtype

E13.5 EphB2⁻/⁻
4.3 Discussion

*In vivo*, targeted innervation of limb muscles by motor axons occurs in a temporal and topographic manner through the regulated expression of axon guidance molecules. One key point in this process occurs at the level of the limbic plexus where the axon innervations of dorsal and ventral muscles diverge to specific sub-tracts. Developmental failures in this process can result in motor neuron death leading to limb atrophy and/or impaired motor control.

In the present study, I have identified for the first time, EphB2 as guidance signal regulating topographic projection of LMCm axons. The results of these experiments demonstrate that LMCm neurons express elevated levels of EphB2 and normally project to ventral limb territories expressing low level of ephrinB ligands. I demonstrate that mice lacking *EphB2* or expressing the kinase dead form of the receptor exhibit aberrant projection of LMCm axons into dorsal projection zones, while removing both EphB2 and EphB3 receptors *in vivo* did not enhance the dorsoventral axon defect seen in *EphB2* single mutants. By contrast, ablation of both EphB2 and EphA4 receptors enhanced the misprojection of the LMCm axons. Taken together, the results demonstrate that EphB2 and EphA4 both play complementary and redundant roles in regulating the guidance of LMC axons in dorsal versus ventral limb trajectories.

4.3.1 EphB signaling is required for LMCm axon dorsoventral guidance

While previous studies have demonstrated that in developing limbs, EphA-ephrinA signals are required for the proper guidance of a sub-population of LMC1 axons innervating dorsal limb targets (Kania and Jessell, 2003), the guidance decisions regulating the remainder of this motor population and the LMCm neurons remains largely unknown. It has previously been shown by ourselves and others that EphB2 is highly expressed within the motor neurons of the
ventral spinal cord during the period of developmental target innervation. However, a functional role for EphB2 in these motor neurons has not been demonstrated. Analysis of $EphB2^{N2/+}$ mice demonstrates that strong EphB2 expression is observed in both the LMCl and LMCm motor neurons beginning at E9.5, the period of terminal differentiation. Analysis of motor neuron identity in $EphB2$ null mice versus controls demonstrates that the motor aberrations seen in $EphB$ mutants do not occur as a result of changes in motor neuron class identity, but rather through changes in axon guidance. Analysis of EphB2 expression during development demonstrates that innervating axons express this receptor during the period of projection through the limbic plexus, a critical site of dorsoventral guidance cues. In the absence of EphB2, LMCm axons which are normally prevented from innervating dorsal muscles do so due to the reduction in EphB2 (Figure 4.6). This aberrant dorsal projection in $EphB2$ null mice is consistent with the inability of these axons to respond to ephrinB signals present within dorsal limb mesenchyme. A similar mechanism affecting a different sub-population of motor neurons appears to account for the increase in LMCm axon projection to the dorsal limb in $EphA4$ null mice (Luria et al., 2008).

### 4.3.2 EphA4 and EphB2 cooperate to regulate dorsoventral projection of a population of LMC axons

While loss of either EphA4 or EphB2 produces defects in axon guidance of LMCm axons with severity of 13% and 16% respectively, loss of both receptors results in an enhancement in the number of LMCm axons misprojecting to the dorsal targets (39%) that could be explained by the synergistic interactions of these receptors (Figure 4.6). This suggests that while there is a distinct population of LMCm neurons which respond to EphB2 or EphA4 alone, there also exist
Figure 4.6 Schematic of axon guidance defects seen in *EphB*-family mutants.

(A) Summary diagram illustrating aspects of dorsoventral pattern of innervation by motor neurons of the LMCl (red) and LMCm (green) axons following tracer injection retrograde labeling of the peroneal nerve (orange arrow). (B) Diagram depicting changes in motor innervation seen in *EphA4*<sup>−/−</sup>, *EphB2*<sup>−/−</sup> and *EphB2<sup>N2/N2</sup> mutants. Lack of either receptor results in the misprojection of LMCm axons dorsally. (C) Summary of LMC axon guidance defects seen in the absence of both EphA4 and EphB2 receptors. Combinatorial null mutants exhibit higher levels of LMCm axons misprojecting dorsally (green solid line), together with increased aberrant innervations by LMC neurons not seen in individual mutants. Together, these data suggest both complimentary and synergistic effects between the EphA4 and EphB2 receptors with resepect to motor axon guidance *in vivo*. 
A) Wild-type

**Limbic plexus**

Tibialis anterior muscles

Gastrocnemius muscles

B) EphB2 (-/-), EphA4 (-/-), EphB2 (N2/N2)

13-16%

C) EphB2A4 (-/-, -/-), EphB2A4 (N2/N2, -/-)

36-39%

14-19%
an additional population in which these receptors serve a redundant function with respect to axon guidance. This is supported by the overlapping expression of EphB2 and EphA4 receptors seen within the LMCm region and the observed expression of ligands common to both receptors present within critical guidance regions such as the dorsal limb mesenchyme. The fact that a kinase dead version of the EphB2 receptor is able to mimic the effects seen in the EphB2 null mice suggests that in LMCm motor neurons guidance results as a response to events triggered by receptor autophosphorylation. This is in sharp contrast to the effects seen in the previous investigation of EphB2 guidance within the mammalian forebrain. In fact, these results demonstrate the first axon guidance effects mediated by EphB2 which is strictly dependent upon receptor kinase activity.

### 4.3.3 Influence of EphA4 and EphB2 receptor interaction beyond the LMC

Current literature suggests that, during development, EphA4-expressing dorsal LMCl axons are normally repelled from ventral ephrinA-expressing mesenchyme (Kania and Jessell, 2003). Consistent with this, my analysis supports this finding in that a significant number of LMCl axons in EphA4 null mice are misrouted ventrally (Table 4.1). As indicated above, combinatorial loss of EphB2 and EphA4 results in an enhancement of dorsal mis-projection of LMCm axons to approximately 39%. Given that removal of all axon guidance cues would reduce dorsoventral tract entry to a stochastic process (50:50), it can be seen from these studies that in vivo EphA2 and EphA4 regulate the majority (though not exclusively) of axon decision making with respect to dorsoventral pathfinding at the level of the limbic plexus. However, the combinatorial loss of EphB2 and EphA4 has an additional and unique effect on axon guidance pathfinding beyond the lateral motor groups. Double knockouts exhibit a 10 to 20 fold
enhancement in the number of motor neurons labeled outside of the LMC (zone T and zone P) which now apparently also project to the peroneal nerve. Such effects indicate that the combinatorial loss of these receptors produces more widespread and profound disruption of motor axon guidance than either receptor alone. In the case of these motor neurons, whose axons do not normally project to either the peroneal or tibial nerve, misprojection is seen only in the case of EphB2/A4 double mutants and not in either EphB2 or EphA4 single receptor knockouts. The axon guidance seen in these motor neurons appears to be controlled through the activity of these EphB-type receptors in a redundant manner. Similar to the effects seen in the lateral motor column, guidance of these additional motor groups is mediated by forward signaling as demonstrated by the similar levels of axon mis-routing seen in EphB2^{N2/N2}A4^{-/-} and EphB2^{-/-}A4^{-/-} knockouts.

The mechanism underlying the observed redundancy between EphB2 and EphA4 may rely on a converging signaling pathway of downstream effectors. An example of this is the guanine nucleotide exchange factors that have been demonstrated to be required for both EphA4- and EphB2-mediated forward signaling for growth cone collapses (Penzes et al., 2001a; Sahin et al., 2005). Another potential common downstream effector is Src kinase (Knoll and Drescher, 2004). Recent studies have also pointed to alpha-chimaerin 2 as the downstream effectors of EphA4 signaling while Dishevelled and p120RasGAP as effectors for EphB signaling (Kim et al., 2002; Tanaka et al., 2003).

This analysis has focused on Eph signaling events controlling projections within the hindlimb. However, it is likely that a similar set of signals coordinate dorsoventral axonal trajectories within regions such as the forelimbs. The fact that LMCm axons do not completely reach a stochastic distribution even in EphB2A4 double knockouts suggests the presence of
additional signaling moieties directing dorsoventral patterning in these axons. Semaphorins (Sema) ligands and their neuropilins (Npn) receptors represent one such possibility. Neuropilin2 and Sema3F have previously been shown to regulate the ventral patterning of LMCm axons within the forelimb (Kim et al., 2002). These findings demonstrate that expression of Sema3F in dorsal mesenchyme was required to repel neuropilin expressing LMCm axons to the ventral territories. Hence, both EphB2-ephrinB and neuropilin-Sema3F are important in coordinating the dorsoventral guidance of the LMCm axons, however, the respective contribution of either one signaling system or the possibility of cross-talk between them remain to be determined and will require further examination.
CHAPTER 5

Motor behavior of *Eph* mutants
5.1 Introduction

Proper motor control is a function of organized neuronal signaling correctly translated into specific muscular targets. Many motor disorders, including Kallmann syndrome (Quinton et al., 1996), Cerebral palsy (Carr, 1996) and neurologic diseases, such as Parkinson’s and Huntington’s diseases, have been shown to be associated with loss of the appropriate pattern of neural innervation. Behavioral studies in both human and animal have aided our understanding of how neuronal networks are regulated in response to specific stimuli. In conjunction with murine gene-targeting methods, behavioral assessments of specific mutant strains have proven invaluable in elucidating roles which specific gene products play in controlling physiologic functions.

To examine and quantify specific motor behaviors, tests have been introduced to characterize activity and screen for motor impairments in rodents. Of these, the most comprehensive and systematically validated is the SHIRPA (SmithKline Beecham, Harwell, Imperial College and Royal London Hospital phenotype assessment) battery which performs a wide range of standardized assessments and a broad screen for mouse phenotypes. It is an excellent tool for identifying phenotypic abnormalities following ENU mutagenesis (Rogers et al., 1999; Rogers et al., 2001). The protocol consists of a three-stage screening procedure that involves quantitative analyses of neurologic and motor function. The primary SHIRPA screen covers simple measurements of physical and neurological health, weight and sensory and motor function such as global disturbances in gait, posture and muscle tone deficits. The secondary screen incorporates several of the motor tests including performance on rotarod, spontaneous locomotor activity test and general reflexes. The tertiary assessment focuses on psychiatric and cognitive aspects of behavior which includes pain tolerance, Morris water maze task and
prepulse inhibition. SHIRPA test is an excellent initial screen for mice with unknown phenotype, and in conjunction with additional behavioral assessments, these battery of tests have been widely applied to examine changes in motor performance resulting from genetic modification. An example of this is shown by EphA4, which is known to regulate axon guidance within the motor system. Analysis of animals lacking either EphA4 or ephrinB3 (a cognate ligand), has demonstrated abnormal synchronized gait behavior in the hindlimbs due to preconscious ipsilateral branching of descending corticospinal axons within the dorsal spinal cord (Dottori et al., 1998; Kullander et al., 2001a). The prominent expression of several Eph receptors and ephrins in the developing motor cortex, cerebellum and spinal cord further suggests a possible role for Eph in the formation and maintenance of motor circuitry (Coonan et al., 2001; Eberhart et al., 2000; Eberhart et al., 2002; Moreno-Flores et al., 2002). While Eph mutants have received significant characterization at the histologic level, very few motor performance measures have been studied. Together with my earlier studies demonstrating the perturbed dorsoventral targeting of motor axons in Eph combinatorial null mice, I elected to investigate the motor properties of Eph mutant mice. To understand the influence of Eph on motor function, I have systematically evaluated EphB mutants on a range of motor tests obtained from SHIRPA, with differing degrees of coverage and complexity, which include examination of their dynamic gait, coordination of movement (beam balance, rotarod), neuromuscular function (inclined plane, fore and whole body grip strength test), fine motor control (wired platform performance), and neurologic effects (hindlimb extension reflex test). However, prior to characterization of these Eph mutants, there is a crucial need for identifying the contribution of different genetic backgrounds of inbred and outbred strains to the phenotypes of these mutant mice. While there are many strain comparison studies that have focused on task performance on motor parameters.
(Brooks et al., 2004) (McFadyen et al., 2003) (Dierssen et al., 2002), there is presently no comprehensive database for motor behavior characteristics of mice composed of several background strains, more specifically those contributing to our $Eph$ mutants. To isolate motor performance resulting from genetic manipulations apart from those due to differences in genetic backgrounds, I have also conducted motor tests of all parental strains composing the $Eph$ mutants (C57Bl/6, 129S1/SvImJ and CD1) to initially assess the naturally occurring allelic variants.

Together, I have provided, for the first time, a detailed analysis of motor behavior for $EphA4$, $EphB2$ and $EphB3$ mutants and their parental background strains.
5.2 Results

5.2.1 Dynamic gait in Eph mutants

Dynamic gait is a detailed analysis of motor coordination and synchrony. Instead of utilizing the traditional “foot printing” approach, where the fore and hind paws are painted with dyes of different colors and the mouse is encouraged to walk in a straight line, I have opted a more reliable approach which is to use an under mount video camera to measure foot pattern while mouse is walking on a treadmill. Gait dynamic was initially analyzed in wildtype animals at several different speeds (at 5.3, 7.5 and 10.9 cm/sec) in order to determine the speed that provides the most sensitivity in detecting real differences between experimental groups. No significant differences (with increasing speed) were detected in the gait parameters measured; toe spread, stride width, stride length and reciprocal stride (Figure 5.1A-B). Therefore, a speed of 10.9 cm/sec was selected and utilized in my analyses as it provides the greatest sensitivity with respect to determining differences in gait. To minimize confounding variables beyond strain differences on gait dynamics (Leblond et al., 2003), age-matched wildtype mice of different genetic backgrounds (C57BL/6J, 129S1/SvImJ and CD1) were examined. No significant differences in left vs. right side measurements were observed in either the forelimbs or hindlimbs for any group. Mean toe spread, stride width, stride length and reciprocal stride distance for both fore- and hindlimbs were consistent across the above mentioned genetic backgrounds (data not shown). In addition, no significant differences in measured gait parameters were observed in EphB2\(^{-/-}\) or EphB3\(^{-/-}\) mice compared to controls. In contrast, marked reduction in the reciprocal stride length in both fore- and hindlimbs and the stride length of forelimbs was evident in EphA4\(^{-/-}\) mice compared to heterozygous and wildtype controls (Figure 5.2).
Figure 5.1 Gait analysis of wildtype mice at different treadmill speeds.

(A) A still frame capturing the ventral view of adult 129Sv/Imj mouse on a treadmill, walking at a speed of 10.9 cm/s. Red circle indicating the right paw and blue circle highlighting the left paw of the subject. (B) Diagram depicting features measured. (C) Measurements of toe spread, stride width, stride length and reciprocal stride distance for both fore- and hindlimbs for wildtype mice ($n = 10$) at different treadmill speeds (5.3, 7.5 and 10.9 cm/sec).
A

B

Cross-reciprocal

Step 1

Stride Length

Step 2

Stride Width

Toe Spread

Step 1

Step 2
C

**Forelimb**

- **Toe spread**
  - 5.3 cm/sec
  - 7.5 cm/sec
  - 10.9 cm/sec

- **Stride width**
  - 5.3 cm/sec
  - 7.5 cm/sec
  - 10.9 cm/sec

- **Stride length**
  - 5.3 cm/sec
  - 7.5 cm/sec
  - 10.9 cm/sec

- **Reciprocal stride**
  - 5.3 cm/sec
  - 7.5 cm/sec
  - 10.9 cm/sec

**Hindlimb**

- **Toe spread**
  - 5.3 cm/sec
  - 7.5 cm/sec
  - 10.9 cm/sec

- **Stride width**
  - 5.3 cm/sec
  - 7.5 cm/sec
  - 10.9 cm/sec

- **Stride length**
  - 5.3 cm/sec
  - 7.5 cm/sec
  - 10.9 cm/sec

- **Reciprocal stride**
  - 5.3 cm/sec
  - 7.5 cm/sec
  - 10.9 cm/sec
Figure 5.2 Examination of dynamic gait in wildtype and *Eph* mutants.

*EphA4*−/− mice (*n* = 5) exhibit a significant reduction in stride length (A) and reciprocal stride (B) of the forelimbs compared to wildtype mice (CD1 (*n* = 5), 129Sv (*n* = 5), and C57Bl/6J (*n* = 5)), heterozygotes of *EphA4* (*n* = 5) and *EphB2* (*n* = 5) and *EphB2* null mutants (*n* = 5). Asterisk denotes *P* < 0.0001 by *t*-test.
**A** Stride Length in forelimbs

![Bar chart showing stride length in forelimbs for different strains and genotypes.](image)

**B** Reciprocal stride in forelimbs

![Bar chart showing reciprocal stride in forelimbs for different strains and genotypes.](image)
5.2.2 Rotarod performance (constant-speed) in Eph mutants

To further assess motor performance, Eph mutants and controls were examined using a fixed-speed rotarod (8, 15, 25 and 30 rpm). Rotarod performance specifically measures motor coordination and balance in rodents and is one of the most commonly used tests of motor function in mice. CD1 wildtype mice exhibited the strongest rotarod performance at all speeds (Figure 5.3). Similarly, no significant differences in the latency to fall between EphB3\textsuperscript{+/−} and EphB3\textsuperscript{−/−} mice and wildtype were observed in the rotatrod test (data not shown). By contrast, C57Bl/6J mice (8 weeks, averaged weight of 23 grams) showed significant decrease in motor performance at the highest speeds compared to CD1 (at 25 and 30 rpm) (Figure 5.3). As shown in figure 5.3 and D, EphB2\textsuperscript{+/−} and EphA4\textsuperscript{+/−} mice exhibited similar motor performance profiles at all speeds tested, suggesting that these values reflect the native ability of the genetic background of these strains. In addition, these mice quickly learned to keep themselves on the rotating rod and were able to maintain balance on all the speeds tested. While EphB2\textsuperscript{−/−} mice were able to perform at wildtype levels at speed of 8 rpm, they showed significant decreased in performance at higher speeds (at 15, 25 and 30rmps) (Figure 5.3). In contrast, EphA4\textsuperscript{−/−} mice exhibit significant difficulties at even the lowest speed tested (Figure 5.3, \(P < 0.01\)). Similar performance was observed in Eph combinatorial null mice suggesting a recapitulation of the EphA4 null phenotype (Figure 5.3, \(P < 0.01\)). Because the rotarod is a complex motor task that involves coordination, balance and motor learning, additional tests were performed to assess individual aspects of motor function.
Figure 5.3 Eph mutant mice exhibit altered rotarod performance.

(A) C57Bl/6J (n = 8), CD1 (n = 9), (B) EphB3+/− (n = 5), EphB3−/−(n = 10), (C) EphB2+/−(n = 10), EphB2−/−(n = 10), (D) EphA4+/−(n = 10), EphA4−/−(n = 10), EphB2A4+/−/−/−(n = 2) mice were tested at constant speeds of 8, 15, 25 and 30 rpm with respect of rotarod performance. EphB2+/−, EphA4+/− and EphB2A4−/−/− mice showed decreasing motor performance at all speeds tested. Asterisks denote *P* < 0.01 by *t*-test.
**Performance on rotarod**

**Speed 8 rpm**

- C57Bl/6J: 60 ± 10 sec
- CD1: 60 ± 10 sec
- B2+/−: 50 ± 10 sec
- B2−/−: 50 ± 10 sec
- A4+/−: 40 ± 10 sec
- A4−/−: 10 ± 10 sec
- B2A4 DKO: 10 ± 10 sec

**Speed 15 rpm**

- C57Bl/6J: 60 ± 10 sec
- CD1: 50 ± 10 sec
- B2+/−: 50 ± 10 sec
- B2−/−: 20 ± 10 sec
- A4+/−: 40 ± 10 sec
- A4−/−: 10 ± 10 sec
- B2A4 DKO: 10 ± 10 sec

**Speed 25 rpm**

- C57Bl/6J: 40 ± 10 sec
- CD1: 40 ± 10 sec
- B2+/−: 40 ± 10 sec
- B2−/−: 10 ± 10 sec
- A4+/−: 40 ± 10 sec
- A4−/−: 10 ± 10 sec
- B2A4 DKO: 10 ± 10 sec

**Speed 30 rpm**

- C57Bl/6J: 20 ± 10 sec
- CD1: 20 ± 10 sec
- B2+/−: 20 ± 10 sec
- B2−/−: 10 ± 10 sec
- A4+/−: 20 ± 10 sec
- A4−/−: 10 ± 10 sec
- B2A4 DKO: 10 ± 10 sec
5.2.3 Motor performance tasks are altered in EphA4 and B2-deficient mice

Prior to performing the motor tests on Eph null mutants, responses to each test were performed on groups of wildtype representing the background strains comprising lines of or Eph mutants (C57Bl/6J and CD1). No prior data exist regarding the comparisons of motor assessment for these strains for the tests indicated. In these studies, weights of C57Bl/6J mice were observed to be consistently lower than that of age matched, sex matched CD1 mice (19.9 ± 0.69 grams vs 31.3 ± 0.64 grams). This difference prompted the assessment of whether differences in weight would significantly affect motor test results. As such, C57Bl/6J animals were re-examined at 4 months of age, where their weights had become comparable to that of the CD1 controls (27.2 ± 0.64 grams). No significant difference in the performance scores for hindlimb extension was observed (Figure 5.4A), edge performance (Figure 5.4B), hindlimb grip response (Figure 5.4C), climbing of a 90° angled platform (Figure 5.4D) and crossing wired platform (Figure 5.4E) between C57Bl/6J (1), C57Bl/6J (2) and CD1 mice, suggesting little variation among these strains within the performance limits tested. These data also suggest that it is maturation level and not absolute weight which determines performance in these tests. In contrast, significant differences in grip strengths were observed which correlated with differences in animal weight. Figure 5.5 shows marked differences in the grip strength (forelimb and combined limbs) between C57Bl/6J (1) and those measured for adult mice with greater weight average (C57Bl/6J (2) and CD1).
Figure 5.4 Motor coordination in wildtype C57Bl/6J and CD1.

No significant difference was observed in the hindlimb extension (A), edge performance (B), hindlimb grip response (C), 90° platform performance (D) and wire platform performance (E) between C57Bl/6J (1) (20 grams, \( n = 8 \)), C57Bl/6J (2) (30 grams, \( n = 8 \)) and CD1 (30 grams, \( n = 9 \)).
A. Hindlimb extension

B. Edge performance

C. Grip response

D. 90° angled platform

E. Wire platform
Figure 5.5 Differences in grip strength correlated with weight differences.

(A and B) Bar graphs illustrating measured grip strength for forelimbs (A) and combined limbs (B) for C57Bl/6J (1) (20 grams, \( n = 8 \)), C57Bl/6J (2) (30 grams, \( n = 8 \)) and CD1 (30 grams, \( n = 9 \)) animals. Asterisks denote \( P < 0.01 \) by \( t \)-test.
Grip strength (forelimb)

Grip strength (combined)
Hindlimb extension performance has previously been shown to be altered in a number of transgenic and knockout mice which exhibit alteration in somatic motor neuron structure. To determine whether Eph receptors play a role in regulating motor neurons morphology, Eph null mutants and controls were examined. Both male and female animals were tested. Since no gender differences were seen among the resulting data, the results are presented together. Eph mutants tested exhibited a normal hindlimb extension reflex (Figure 5.6A). With respect to edge performance, both EphA4\(+/-\) and EphA4\(-/-\) mice were capable of crossing the 3mm beam and received similar scores, as the test is defined (Figure 5.6B). However, while scores may be similar, the total time and behavior on the beam was strikingly different. Control mice walked the beam with ease, while EphA4 null mice exhibit much higher latency to cross. In addition, although adult EphA4\(-/-\) could maintain their balance on the beam, substantial tremor was observed. In contrast, both EphB2\(+/-\) and EphB3\(+/-\) performed normally with respect to edge performance. All Eph mutants exhibited similar performance on the 90\(^\circ\) incline platform (Figure 5.6C).

With respect to grip strength, as shown in figure 5.7, significant differences were observed in the forelimb grip strength between EphA4\(-/-\), EphB2A4, EphB2B3 double knockouts compared to controls (Figure 5.7). In addition, combined grip strength was significantly reduced in EphA4\(-/-\) and EphB2A4 double knockouts compared to heterozygous mice (Figure 5.7A and B). To verify that the difference in grip strength observed was not due to a change in fatigue, data from individual trials were analyzed. Repeated trials did not show a decrease in observed grip strength suggest that it is not likely due to overt fatigue.
Figure 5.6 Motor performance tasks in Eph mutants.

Adult Eph mutants (EphA4$^{+/−}$ (n = 10), EphA4$^{-/−}$ (n = 10), EphB2$^{+/−}$ (n = 10), EphB2$^{-/−}$ (n = 10), EphB3$^{+/-}$ (n = 5), EphB3$^{-/-}$ (n = 10), EphB2$^{-/-}$B3$^{-/-}$ (n = 10), EphB2$^{-/-}$A4$^{-/-}$ (n = 6)) were assayed for hindlimb extension (A), performance on a 3mm edge (B) and performance on a 90° inclined platform (C). No significant differences were observed among Eph mutants for these tasks.
**Hindlimb extension**

- **Genotype**
  - A4+/-
  - A4-/
  - B2+/-
  - B2-/
  - B3+/-
  - B3-/
  - B2B3
  - B2A4

- **Time (sec)**
  - 0.0
  - 2.5
  - 5.0
  - 7.5

**Edge performance**

- **Genotype**
  - A4+/-
  - A4-/
  - B2+/-
  - B2-/
  - B3+/-
  - B3-/
  - B2B3
  - B2A4

**90° Incline**

- **Genotype**
  - A4+/-
  - A4-/
  - B2+/-
  - B2-/
  - B3+/-
  - B3-/
  - B2B3
  - B2A4

Averaged Scores

- Hindlimb extension:
  - A4+/-
  - A4-/
  - B2+/-
  - B2-/
  - B3+/-
  - B3-/
  - B2B3
  - B2A4

- Edge performance:
  - A4+/-
  - A4-/
  - B2+/-
  - B2-/
  - B3+/-
  - B3-/
  - B2B3
  - B2A4

- 90° Incline:
  - A4+/-
  - A4-/
  - B2+/-
  - B2-/
  - B3+/-
  - B3-/
  - B2B3
  - B2A4

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Figure 5.7 Grip strength test.

(A and B) Bar graphs showing grip strength (forelimb and both limbs) for Eph mutants ($EphA4^{+/-}$ ($n = 10$), $EphA4^{-/-}$ ($n = 10$), $EphB2^{+/-}$ ($n = 10$), $EphB2^{-/-}$ ($n = 10$), $EphB3^{+/-}$ ($n = 5$), $EphB3^{-/-}$ ($n = 10$), $EphB2^{-/-}$ $B3^{-/-}$ ($n = 10$), $EphB2^{-/-}$ $A4^{-/-}$ ($n = 6$)). Significant differences were observed for $EphA4^{-/-}$, $B2B3^{-/-}$ and $B2A4^{-/-}$ mice compared to heterozygous controls. Asterisks denote $P < 0.01$ by $t$-test.
A  Grip strength (forelimb)

B  Grip strength (combined)

Genotype

Grip strength (grams)
Foot fall placement is a measure of sensorimotor function. Analysis of the number of foot fall while crossing an open wire platform for 60 seconds demonstrated significant increases in errors for $EphA4^{-/-}$, $EphB2^{-/-}$, $EphB2B3^{-/-}$ and $EphB2A4^{-/-}$ mice (Figure 5.8). The number of foot falls seen in $EphB2A4$ double knockouts was similar to that seen in $EphA4$ null mutants alone (Figure 5.8). By contrast, the increased number of fall seen in $EphB2B3$ double knockouts was greater than that seen in $EphB2^{-/-}$ or $EphB3^{-/-}$ mice alone, suggesting that loss of these genes leads to synergistic effect.
Figure 5.8 Open wire platform performance of *Eph* mutants.

Performance on open wire platform was measured for *Eph* mutants (*EphA4*+/− (n = 10), *EphA4*−/− (n = 10), *EphB2*+/− (n = 10), *EphB2*−/− (n = 10), *EphB3*+/− (n = 5), *EphB3*−/− (n = 10), *EphB2*+/−*B3*+/− (n = 10), *EphB2*+/−*A4*+/− (n = 6)). Each bar represents the number of foot fall while animal is crossing on a wired mesh for 60 seconds (± s.e.m.). Asterisks denote *P* < 0.01 by *t*-test.
Wire platform performance

Number of foot fall

Genotype

5.3 Discussion

In the present study, I have extended previous observations on the behavior of adult EphA4 null mice and have presented for the first time, a detailed analysis of the motor performance of Eph mutant mice and requisite controls. These data lay the foundation for a series of quantifiable measurements for murine knockouts against defined genetic strains. The data indicate that 129S1/SvImJ C57Bl/6J and CD1 have consistent and quantifiable motor performances properties which can be assessed with respect to potential motor mutants. In this respect, I have measured the performance of our EphB-class mutants as a function of their strain controls. These studies demonstrated that the motor tests used vary with respect to their ability to distinguish changes in motor function among Eph mutants tested.

The focus of previous studies has been to describe the synchronous movement of the hindlimbs in the EphA4 null mice (Akay et al., 2006; Dottori et al., 1998; Kullander et al., 2001a; Kullander et al., 2001b). Aberrant recrossing of the corticospinal tract in these animals renders them unable to have specific unilateral control movement in their hindlimbs. Instead, signals from one side of the motor cortex provide bilateral input to both sides of the body. This phenotype accounts for the significant reduction in the reciprocal stride distance observed in our dynamic gait measurements. Apart from observing the well-known synchronous stepping effect previously discussed in the EphA4 null mice, I have also observed a significant reduction in the reciprocal stride length in the forelimbs of EphA4 nulls compared to controls. This is consistent with the recent study using high-speed video recordings that reported large variability in coordination of the forelimbs in EphA4 null mutants, ranging from synchrony to alternation (Akay et al., 2006). This may be explained by a perturbation in the central pattern generator in producing rhythmic forelimb movements. While normal alternative rhythm may be intact in the
CPG of the forelimb, input from abnormal patterning in the hindlimb and from abnormal sensory feedback arising form the modified movement in the body resulting from the hopping gait may have contributed to changes in the animal’s forepaw reciprocal stride. Alternatively, this may reflect the abnormal recrossing of the CST in more rostral level of the spinal cord in EphA4−/− mutants, which can result in a defect in reciprocal forelimb movement that has not been previously detected.

Furthermore, I observed a marked reduction in the forelimb stride length in EphA4−/− mice. Reduction in stride length has previously been shown to be a characteristic of defects affecting the basal ganglia in conditions such as Parkinson’s disease (Lewis et al., 2000; Morris et al., 1996) and degenerative striatal processes as observed in Huntington’s disease (Koller and Trimble, 1985; Tison et al., 1995). In mice, there has previously been shown a significant correlation between reduction in stride length and substantial nigra par compacta cell loss (Fernagut et al., 2002). Whether the reduction in stride length seen in EphA4−/− mutants is a result of loss of nigrostriatal pathway guidance remains to be elucidated.

Alteration in hindlimb extension has previously been observed in mice with significant altered somatic motor neuron number. Mice with such defects are frequently observed to retract their hindlimbs when suspended. All Eph knockouts examined exhibited normal hindlimb extension, suggesting no such motor neuron deficit in the spinal cord. This is consistent with all my histologic studies in the lumbar spinal cord.

EphA4, EphB2B3 and EphB2A4 null mice exhibited significantly reduced grip strength and performed more poorly than controls on the open wired platform performance compared to littermates. Poor performance in these tests may be linked to perturbed neuromuscular function
(directly) or altered spinocerebellar or sensory function (indirectly) (Lalonde et al., 2005; Levedakou et al., 2005). These results thus suggest other aspects of EphB control in vivo.

With respect to rotarod performance, I demonstrated that EphA4−/− and EphB2−/− mice exhibit significantly reduced motor performance with respect to controls at 15, 25 and 30 rpms. These motor deficits could involve either central or peripheral perturbations of motor integration. With respect to EphA4 null mutants, disturbance in the central connections may be correlated with defasciculation in the corticospinal tract (Dottori et al., 1998) while peripheral deficits may be correlated with abnormal dorsoventral connections to the hindlimb (Kania and Jessell, 2003). Similarly, we have shown deficit in the dorsoventral guidance of LMCm axons in EphB2 null mice, which may partly explain the motor deficits observed on the rotarod performance (Chapter 4). Furthermore, decrease in latency on the rotarod may be related to the alterations in cerebellar cytoarchitecture shown in our MRI analysis of EphB2−/− mice (Chapter 3). In fact, cerebellar defects have been shown to be associated with performance deficits on the rotarod test (Lalonde et al., 1995; Lalonde et al., 2003; Lalonde and Strazielle, 2003). In mutants with defects in the neuroanatomy of the cerebellum, such as staggerer, hot-foot and lurcher, all perform poorly on the rotarod task with latencies to fall off in 0-100 sec, as compared to latencies of 300-400 sec for wild-type controls (Lalonde et al., 1996). Thus, disturbance in the foliation observed in the EphB2 null mutants strongly correlates with decrease in latency to fall when tested on the rotarod. Here, I have shown motor deficits in EphB2 null mice that may be a result of defective cerebellar connectivity.
CHAPTER 6: CONCLUSION AND FUTURE PERSPECTIVES

Magnetic resonance imaging provides a substantial amount of non-invasive structural information in clinical applications. Recent improvements in the strength and resolution of the MRI have revolutionized our ability to investigate brainstructures in small specimen such as the rodent brains. This technique is not only non-invasive but also allows for longitudinal studies of a given animal. It has become an important research tool in neuroscience to study the CNS of strains with targeted genetic alterations. In my studies, neuroanatomic and morphometric anlaysis of the murine brain was greatly assisted by the development of a robust three-dimensional atlas in which analyses performed in different genetic backgrounds could be directly compared. Application of this three-dimensional CNS atlas has allowed us to investigate complex structures, such as the anterior commissure within the brains of Eph mutants.

Collectively, the data from this thesis have established the importance of EphA4 and EphB2 in the formation of the anterior commissure and topographic innervation by spinal motor neurons and have elucidated underlying molecular mechanisms. The redundant actions of these receptors observed in both the forebrain and lumbar spinal cord shed light on the detailed interactions that exist among Eph receptors and their ligands in vivo. While the contribution of individual Ephs and their ephrin ligands in development has been studied, a true appreciation of the role that these receptors play in the development and maintenance of the mammalian CNS can only emerge from such combinatorial studies in vivo. The fact that my data reveal evidence for differential sensitivity of LMCl neurons to the loss of EphA4 and EphB2 signaling, suggests that they may regulate different downstream signaling pathways. Future studies will be to
examine the differential requirement of downstream effectors by EphA4 and EphB2 receptors in mediating the fidelity of axon extension. Questions also remain regarding the molecular events that link the Eph forward signaling, or ephrinB reverse signaling, to axon guidance. Here, I have demonstrated that proper formation of the anterior commissure is kinase-independent, whereas EphB2 and EphA4 forward signaling is involved in the dorsoventral guidance decision of LMC motor neurons. The ability to trigger a bi-directional signaling cascade raises even more plausible mechanism of actin cytoskeletal regulation. It is therefore important to further investigate the role of ephrins in guiding the commissural fibers and the mechanism of signaling. This may be accomplished by examining interhemispheric connections in the brains of animals with targeted deletion of specific ephrin ligands. Finally, other families of guidance molecules are also expressed within the developing forebrain and spinal motor neurons, where they have been shown to play a role in the formation of these connections. Semaphorins and neuropilins represent such candidates. Neuropilin2 and Sema3F have previously been shown to regulate the ventral patterning of LMCm axons within the forelimb (Kim et al., 2002), suggesting a possibility of crosstalk between the semaphorins and Ephs. Further examination of the ventral patterning of LMC axons using mutants lacking both these signaling systems is required to decipher the relative contribution of these receptors in axon guidance.
References


APPENDICES
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<tr>
<th>Gene</th>
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<th>Method of creation/description of genetic modification</th>
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<tr>
<td>EphA2</td>
<td>EphA2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Gene-trap strategy U3B-geo A provirus, containing Betageo, inserted into an intron 1.8 kb downstream of the alternatively spliced exon 5.2.</td>
<td>No obvious phenotype</td>
<td>Chen 1996</td>
</tr>
<tr>
<td>EphA2</td>
<td>EphA2&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Gene-trap –ROSAN B-geo inserted into exon 1</td>
<td>Kinky tails. Defective notochord formation. Notochord is abnormally bifurcated at the caudal end at E12.5</td>
<td>Naruse-Nakajima 2001</td>
</tr>
<tr>
<td>EphA3</td>
<td>EphA3&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>The coding sequence for EphA3 and a floxed neo cassette was inserted into the endogenous locus and placed under the regulatory elements of Isl2.</td>
<td>Mice surviving perinatal period develop normally with no cardiac or other abnormalities and normal grip strength, motor column organization and spinal nerve projections.</td>
<td>Brown 2000</td>
</tr>
<tr>
<td>EphA3</td>
<td>EphA3&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Refer to Vaidya et al. 2003</td>
<td>EphA3 knockouts have significant defects in the development of their atrial septa and atrioventricular endocardial cushions, and that these cardiac abnormalities lead to the death of approximately 75% of homozygous EphA3&lt;sup&gt;-/-&lt;/sup&gt; mutants.</td>
<td>Vaidya 2003</td>
</tr>
<tr>
<td>EphA4</td>
<td>EphA4&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>A neomycin selection cassette replaced exon 3, corresponding to nucleotides 217-880 of the encoded transcript.</td>
<td>Adult null mutants exhibit abnormal hopping gait CST axons in forelimb exit normally, whereas the hindlimb populations branch and exit too early.Evidence of recrossing of contralateral CST axons.</td>
<td>Dottori 1998</td>
</tr>
<tr>
<td>EphA4</td>
<td>EphA4\textsuperscript{\text{lacZ}}, EphA4\textsuperscript{KD}</td>
<td>Exon 1 was replaced by a cassette containing the lacZ coding seq followed by neomycin gene (lacz/pgk/neo). In addition, a frameshift mutation in exon 3 was introduced. (C57BL/6J blastocysts and crossed chimeras to DBA2 or 129 Sv mice)</td>
<td>EphA4 mutant animals show peroneal muscular atrophy correlating with the absence of the peroneal nerve, the main dorsal nerve of the hindlimb.</td>
<td>Helmbacher 2000</td>
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<tr>
<td>EphA4</td>
<td>EphA4\textsuperscript{\text{SAM}}, EphA4\textsuperscript{EE}</td>
<td>A cDNA encoding a kinase dead mutant protein with a substitution of lysine 653 for methionine was fused in frame into exon 3 of the gene followed by a poly (A) site and a loxP flanked neomycin selection cassette.</td>
<td>EphA4 is required for CST formation as a receptor for which it requires an active kinase domain. In contrast, the formation of the AC is rescued by kinase-dead EphA4.</td>
<td>Kullander 2001</td>
</tr>
<tr>
<td>EphA4</td>
<td>EphA4\textsuperscript{EE}</td>
<td>A cDNA encoding a kinase dead mutant protein with a substitution of phenylalanines for tyrosines 596 and 602 was fused in frame into exon 3 of the gene followed by a poly (A) site and a loxP flanked neomycin selection cassette.</td>
<td>SAM domain is not required for EphA4 functions.</td>
<td>Kullander 2001</td>
</tr>
<tr>
<td>EphA5</td>
<td>EphA5 (K-)</td>
<td>Truncated EphA5 receptor fused to eGFP under transcriptional control of a neuron-specific a-tubulin promoter. The transgene retains the extracellular and ---</td>
<td>Knockin mice expressing a mutant form of EphA4 (EphA4(EE)), whose kinase is constitutively activated in the absence of ephrinB ligands, are deficient in the development of thalamocortical projections and some aspects of central pattern generator rhythmicity.</td>
<td>Egea 2005</td>
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<tr>
<td>EphA5</td>
<td>---</td>
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<td>In EphA5 (K-) transgenic mice, medial hippocampal axons terminate more ventrally compared with the wildtype.</td>
<td>Yue 2002</td>
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<tr>
<td>Gene</td>
<td>Mutant Description</td>
<td>Manipulation Details</td>
<td>Phenotypic Effect</td>
<td>Reference</td>
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<tr>
<td>EphA5</td>
<td>EphA5&lt;sup&gt;lacZ/lacZ&lt;/sup&gt;</td>
<td>The endogenous gene was disrupted by the insertion of a cassette containing lacZ and PGK-neo. The protein putatively lacks the entire tyrosine kinase and C-terminal domain, but the kinase-independent functions are expected to remain intact.</td>
<td>Retinal axon mapping abnormalities with temporal axons shifted posteriorly and nasal axons anteriorly, the entire target, the superior colliculus, remained filled with retinal axons</td>
<td>Feldheim 2004</td>
</tr>
<tr>
<td>EphA6</td>
<td>EphA6&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Standard homologous recombination strategy was used to delete exon 1 of EphA6.</td>
<td>Using a fear conditioning training paradigm, mice deficient in EphA6 did not acquire the task as strongly as did wild type (WT) mice.</td>
<td>Savelieva 2008</td>
</tr>
<tr>
<td>EphA7</td>
<td>EphA7&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Exon 1 of EphA7 was replaced with a loxP-flanked tk/neo selection cassette.</td>
<td>EphA7 mutant mice the retinocollicular map is disrupted, with nasal and temporal axons forming additional or extended TZs, respectively</td>
<td>Rashid 2005</td>
</tr>
<tr>
<td>EphA8</td>
<td>EphA8&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>The exon encoding the extracellular domain was replaced with sequence encoding the transmembrane domain of the human NTRK1 receptor followed by lacZ and neo genes.</td>
<td>Abnormal ipsilateral projection of some superior colliculus axons into cervical spinal cord. Superior colliculus axons fail to project to target in the contralateral inferior colliculus.</td>
<td>Park 1996</td>
</tr>
<tr>
<td>EphB1</td>
<td>EphB1&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Exon 3 has been replaced by a neomycin resistance gene which causes a frame shift in addition to the exon loss.</td>
<td>EphB1 null mice exhibit a dramatically reduced ipsilateral projection by 43%, both at E17.5 and at birth</td>
<td>Williams 2003</td>
</tr>
<tr>
<td>Gene</td>
<td>Phenotype</td>
<td>Description</td>
<td>Outcome</td>
<td>Reference</td>
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<tr>
<td>EphB3</td>
<td>EphB3&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Replacement of four exons and part of a fifth exon containing sequences encoding part of the kinase domain with a PGK-neomycin resistance cassette.</td>
<td>Failure of axons of CC to cross midline.</td>
<td>Orioli 1996</td>
</tr>
<tr>
<td>EphB2/B3</td>
<td>EphB2&lt;sup&gt;-/-&lt;/sup&gt;, EphB3&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Crossed</td>
<td>Abnormal pathfinding of retinal ganglion cell RGC within the retina, with dorsal RGC axons being more affected then ventral axons.</td>
<td>Birgbauer 2001</td>
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<tr>
<td>EphB2/B3</td>
<td>EphB2&lt;sup&gt;-/-&lt;/sup&gt;, EphB3&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>Crossed</td>
<td>The boundary between the proliferative and differentiated cells was largely absent.</td>
<td>Battie 2002</td>
</tr>
<tr>
<td>EphB4</td>
<td>EphB4&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>A tau-lacZ-PGK-neo cassette replaced exon 1 and was fused in frame to the translation start codon via homologous recombination.</td>
<td>A targeted mutation in EphB4 essentially phenocopies the mutation in ephrin-B2.</td>
<td>Gerety et al. 1999</td>
</tr>
<tr>
<td>EphB6</td>
<td>EphB6&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>A portion of exon 1 and all of exons 2 and 3 were replaced by a floxed neo cassette inserted by homologous recombination. The deleted region included the translational start codon.</td>
<td>Developed normally, revealed no abnormality in general appearance, and were fertile.</td>
<td>Shimoyama 2002</td>
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<td>EphrinA2</td>
<td>ephrin-A2&lt;sup&gt;-/-&lt;/sup&gt;; ephrin-A5&lt;sup&gt;-/-&lt;/sup&gt; double mutants</td>
<td>Focal retinal labeling reveals moderate map abnormalities when either gene is disrupted.</td>
<td></td>
<td>Feldheim 2000</td>
</tr>
<tr>
<td>EphrinA2, A5</td>
<td>ephrin-A2&lt;sup&gt;-/-&lt;/sup&gt;; ephrin-A5&lt;sup&gt;-/-&lt;/sup&gt; double mutants</td>
<td>Frisen 1998; Feldheim 2000</td>
<td>In double homozygotes, anteroposterior order is almost though not completely lost. Dorsoventral topography is also impaired.</td>
<td>Feldheim 2000</td>
</tr>
<tr>
<td>EphrinA2/EphA7</td>
<td><em>ephrin-A2</em>&lt;sup&gt;−/−&lt;/sup&gt; and <em>EphA7</em>&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>Feldheim et al. 2000; Holmberg et al. 2000</td>
<td>Cells in the neural stem cell niche in the adult brain proliferate more and have a shorter cell cycle in mice lacking ephrin-A2 and EphA7.</td>
<td>Holmberg 2005</td>
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<tr>
<td>EphrinA5</td>
<td><em>ephrin-A5</em>&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Insertion of a neomycin selection cassette deleted sequence encoding amino acids 42 through 129 as well as a 5' splice acceptor.</td>
<td>The majority of ephrin-A5&lt;sup&gt;−/−&lt;/sup&gt; mice develop to adulthood, are morphologically intact, and have normal anterior-posterior patterning of the midbrain. A subpopulation of ephrin-A5 null mice display neural tube defects resembling anencephaly (failure of the neural folds to fuse in the dorsal midline).</td>
<td>Frisen 1998</td>
</tr>
<tr>
<td>EphrinA5</td>
<td><em>ephrin-A5</em>&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Frisen 1998</td>
<td></td>
<td>Frisen 1998</td>
</tr>
<tr>
<td>EphrinA5/EphA4</td>
<td><em>ephrin-A5</em>&lt;sup&gt;−/−&lt;/sup&gt; and <em>EphA4</em>&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
<td>Frisen 1998 and Kullander 2001</td>
<td>Disruption of Intra-Areal topographic map. Projections of rostral TC axons in ephrin-A5/EphA4 DKOs was caudalized in the ventral telencephalon.</td>
<td>Dufour 2003</td>
</tr>
<tr>
<td>EphrinB1</td>
<td>Conditional <em>ephrinB1</em>&lt;sup&gt;lox/lox&lt;/sup&gt; x PGK-Cre</td>
<td>Exon 2 was flanked by loxP sites (black triangles) and fused to a human ephrinB1 cDNA with polyadenylation signal (pA). The neomycin resistance cassette (Neo) in conditional (Lox) mice was removed by Flp-mediated recombination on Heterozygous (ephrinB1&lt;sup&gt;lox+/−&lt;/sup&gt;), homozygous (ephrinB1&lt;sup&gt;lox/lox&lt;/sup&gt;), and hemizygous (ephrinB1&lt;sup&gt;lox&lt;/sup&gt;) offspring proved to be viable and fertile. However, a small fraction of homozygous or hemizygous mice (10%) died within 24 hr after birth, apparently because of cleft palate</td>
<td>Compagni 2003</td>
<td></td>
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</table>
| EphrinB1 | Efnb1  
Conditional ephrinB1 KO x Cre-Meox2  
Conditional ephrinB1 KO x Cre-Wnt1 | Cre-mediated recombination removes the floxed exons 2-5 in Ephb1<sup>tm1Sor</sup>, leaving a PGK-neo-bpA cassette flanked by FRT sites. To generate ephrinB1-deficient mice, conditional (Lox/+ or Lox/Lox) mutants were bred to Meox2-Cre and Wnt-Cre transgenic animals | Perinatal lethality phenotype with cleft palate and body wall closure defect, abnormal sternum and rib development. Defect in NCC-derived tissues. They also exhibited polydactyly. | Davy 2004 |
|---|---|---|---|---|
| EphrinB2 | ephrinB2<sup>−/−</sup>  
EphrinB2/lac Z removal of exon 1 cause complete deletion of the gene |  | Mice lacking ephrinB2 have enlarged heart and die in utero. Mutant capillaries failed to delaminate from basal endoderm where capillaries in head are dilated and arrested at primary plexus stage. | Wang et al 1998 |
| EphrinB2 | ephrinB2<sup>ΔC</sup>  
Replaced the endogenous gene by cDNAs encoding either carboxyterminally truncated (ephrinB2(DeltaC)) |  | Loss of the ephrinB2 C-term of gene resulted in midgestation lethality similar to ephrinB2 null mutants | Adams 2001 |
| EphrinB2 | Tie2-Cre<sup>+</sup> x ephrinB2<sup>loxP/loxP</sup>  
A loxP site was inserted 45 bp upstream in the 5' UTR and a floxed PGKneo cassette was inserted downstream of the first exon. | Endothelial-specific ephrinB2 knockout embryos (ephrinB2<sup>lacZ/loxP</sup>;Tie2-Cre<sup>+</sup>) show an arrest in intersomitic vessel angiogenesis at the primary plexus stage |  | Gerety 2002 |
<p>| EphrinB2 | ephrinB2&lt;sup&gt;ΔV/ΔV&lt;/sup&gt; mice ephrinB2&lt;sup&gt;5F/5F&lt;/sup&gt; mice | Knock-in mice: ephrinB2DeltaV mice | homozygous mutant mice survived the requirement of ephrinB2 in | Makinen 2005 |</p>
<table>
<thead>
<tr>
<th>EphrinB2</th>
<th>ephrin-B2lacZ</th>
<th>A lacZ cassette was flanked with loxP sequences and then inserted in-frame at the introduced SalI site in the modified ephrin-B2 exon to delete codons 264–336 and create an ephrin-B2-βgal fusion.</th>
<th>Mice lacking ephrinB2 exhibit severe anorectal malformations characterized by an absence of the terminal-most hindgut (rectum) and formation of a fistula that aberrantly connects the intestines to the urethra at the base of the bladder. Ephrin-B2 reverse signaling is required for the pathfinding of axons that form the posterior tract of the anterior commissure.</th>
<th>Dravis 2004 Cowan 2004</th>
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<tbody>
<tr>
<td>EphrinB3</td>
<td>efnb3−/−</td>
<td>The coding region of exon 1 was deleted and replaced with a lacZ-PGK-neo cassette.</td>
<td>EphrinB3 null mice exhibited abnormal locomotion by P8-10: the gait had significantly decreased in stride length. EphrinB3 deficient mice had the same phenotypes as EphA4 −/− mutants.</td>
<td>Kullander 2001</td>
</tr>
<tr>
<td>EphrinB3</td>
<td>efnb3lacZ</td>
<td>The lacZ cassette disrupted codons 264 through 340 which make up the majority of the cytoplasmic domain.</td>
<td>Truncated ephrin-B3 protein lacking its cytoplasmic domain did not lead to hopping, indicating that reverse signaling is not required for corticospinal innervation.</td>
<td>Yokoyama 2001</td>
</tr>
<tr>
<td>EphrinB3</td>
<td>efnb3lacZ</td>
<td>EphrinB3-β-galactosidase fusion protein that retains the extracellular and transmembrane domains but lacks the conserve cytoplasmic segment and cannot transducer While in WT, the infra-pyramidal bundles initially grow through the entire length of the CA3 region, but then retract and prune to join the supra-pyramidal bundles, EphrinB3lacZ mutant mice lack the</td>
<td>Xu 2009</td>
<td></td>
</tr>
<tr>
<td>EphrinB3</td>
<td>efnb3&lt;sup&gt;3F&lt;/sup&gt; and efnb3&lt;sup&gt;5F&lt;/sup&gt;</td>
<td>Modify the last (5&lt;sup&gt;th&lt;/sup&gt;) exon by replacing the tyrosines in the cytoplasmic domain with phenylalanine.</td>
<td>Efnb3&lt;sup&gt;3F&lt;/sup&gt; and Efnb3&lt;sup&gt;5F&lt;/sup&gt; mice had long IPB axons indicating that tyrosine phosphorylation is required for pruning for mossy fibers.</td>
<td>Xu 2009</td>
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<tr>
<td>reverse signals requiring tyrosine phosphorylation/SH2 and c-terminal/PDZ protein-protein interactions. (Yokoyama 2001)</td>
<td>ability to cause pruning.</td>
<td></td>
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