THE INTRACELLULAR DOMAIN OF EPHB1 IS REQUIRED FOR AXON PATHFINDING AT THE OPTIC CHIASM AND CORPUS CALLOSUM

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THE INTRACELLULAR DOMAIN OF EPHB1 IS REQUIRED FOR AXON PATHFINDING AT THE OPTIC CHIASM AND CORPUS CALLOSUM

By

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“Education consists mainly in what we have unlearned.”

-Mark Twain

For Mom and Dad

Acknowledgements

First and foremost, I would like to thank my family. Truly, it has been my parents and siblings that have guided me through life. I cannot adequately express the gratitude I feel for the time and energy my parents devoted in my upbringing. I do not think I would change anything about my childhood. I got to grow up with a great tight-knit family and good group of friends in a sun-loving community (sure maybe raising a little bit of trouble every now and again), but there was always a solid base to which I could return.

It was at home that I could always count on my brother Nathan to take me fishing (sometimes against my will because he needed the company), or my sister Yvette to tag along surfing the Texas and Mexican coast (it was great to watch her turn from a grommet into a skilled surfer). They are now pretty much fully grown. Nathan has a career, a loving wife, and beautiful kid, and my sister in now engaged in Minnesota of all places (how a Texas Surfer could end up there, I will never understand). Hopefully, some of that will rub off on me, so I can start my adult life. I have been a student for three quarters of an eternity now, and I am beginning to think that may be enough.

Next, I definitely need to thank Mark. He took me into his laboratory more than a few years ago and has allowed me to take my time in becoming a fully functioning and independent minded scientist. Until the end, he would never push too hard but instead gave me room to experiment the way I desired even allowing me to stay on after I temporarily dropped out of graduate school. I may have stumbled along the way a few

times, but he was always ready to give advice if I came by his office. Because of this, I now feel confident that I can solve most any problem in the laboratory.

I would certainly be vexed if I did not mention my labmates, the Henkemites as we now seem to be called. Kelly, Tracey, Tim, Jan, Sonal, and Chris all have helped me in their own ways. Kelly I think kept the laboratory sane when it needed to be and then injected some crazy when it did not. A tough thing to judge, but she was always up to the task.

Tracey could always be counted on to say something hilarious when things became too quiet. Sure it was often inappropriate, but she would just smile and somehow get away with saying anything.

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I will be eternally grateful to Jan because she unified the laboratory in a way that no one else could. Her instruments were alcohol, happy hours, and dancing, and we were all disciples eager to learn her arts. It is too bad she has since stopped being a mentor (well, bad for us anyway, but probably good for her and the husband).

Sonal, well Sonal is a complex entity that I expected to crack at some point and inflict some serious damage to whomever was within range yet never did. She sat behind

me from day one, and when she first joined the laboratory, a post-doc, Mike Chumley, would shout just to startle her. Day after day, "Hey Sonal!" would be catapulted towards her, and each day she would jump as if struck upon the brow. Nevertheless, every single day, she would simply smile and shrug it off. Chris will vouch that she is indomitable, as I have seen him try to test this over the years.

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Publication No._____

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Supervising Professor: Mark Henkemeyer, Ph.D.

In this dissertation I present evidence of the importance of EphB1 mediated signaling in retinal and callosal axons while attempting to reach their targets. EphB receptor tyrosine kinases direct axonal pathfinding through interactions with ephrin-B proteins following axon-cell contact. Since EphB:ephrin-B binding leads to bidirectional signals, the contributions of signaling into the Eph-expressing cell (forward signaling) or the ephrin-expressing cell (reverse signaling) cannot be assigned using traditional protein-null alleles. To determine if EphB1 is functioning as a receptor during axon pathfinding, I created a new knock-in mutant mouse, *EphB1*$^{\text{T-lacZ}}$, that expresses an intracellular-truncated EphB1-β-gal fusion protein from the endogenous locus. As in the *EphB1*$^{-/-}$ protein-null animals, the *EphB1*$^{\text{T-lacZ/T-lacZ}}$ homozygotes fail to form the ipsilateral projecting subpopulation of retinal ganglion cell (RGC) axons. This indicates

that forward signaling through the intracellular domain of EphB1 is required for proper axon pathfinding of RGC axons at the optic chiasm. Further analysis of other *EphB* and *ephrin-B* mutant mice shows that EphB1 is the preferred receptor of both ephrin-B1 and ephrin-B2 in mediating axon guidance at the optic chiasm despite the coexpression of EphB2 in the same ipsilaterally projecting RGC axons.

In addition to analyzing the axon pathfinding defect at the optic chiasm, the *EphB1*$^{T\text{-}lacZ}$ mice were also used to analyze another phenotype associated with *EphB1*$^{-/-}$ protein-null animals, a failure to properly form a corpus callosum. I will show that the intracellular domains of EphB1 and EphB2 are important for the guidance of callosal axons across the midline during the formation of the corpus callosum. However, opposite to the above mentioned optic chiasm phenotype, these animals have axons that fail to project contralaterally choosing to remain on the ipsilateral hemisphere.

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Prior publications that appear in this work

Chenaux, G., M. Henkemeyer. (Submitted). "Forward signaling mediated by the EphB1 intracellular domain is required to form the ipsilateral projection at the optic chiasm."

Thakar, S., G. Chenaux, M. Henkemeyer. (Submitted). "Critical roles for EphB:ephrin-B bidirectional signaling in retinocollicular mapping."

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AgCC – agenesis of the corpus callosum

B – BamHI (restriction enzyme)

BAC – bacterial artificial chromosome

β-gal – beta-galactosidase

bGH – bovine growth hormone

bp – base pairs

CAM – cell adhesion molecule

CCI – chronic constrictive injury

CV – capture vector

CRD – cysteine rich domain

°C – degrees centigrade

Cdc42 – cell division control protein 42 homolog

CGC – cingulate cortex

CSPG – chondroitin sulfate proteoglycans

DiI – 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate

d - dorsal

DT-A – diphtheria toxin-α

DB-PH – Dbl homology-pleckstrin homology

EBD – ephrin binding domain

ECM – extracellular matrix

E. coli – Escherichia coli

Eph – ephrin receptor (a.k.a. erythropoietin-producing hepatocellular receptor)

ephrin – Eph receptor interacting protein

ES – embryonic stem

Fc – fragment crystallizable

FGF – fibroblast growth factor

FN-III – Fibronectin III

gDNA – genomic deoxyribonucleic acid

GAP – GTPase activating proteins

GDP – guanosine diphosphate

GEF – guanine nucleotide exchange factor

GPI – Glycosylphosphatidylinositol

GTP – guanosine triphosphate

GW – glial wedge

H – HinDIII (restriction enzyme)

HC – hippocampal commissure

HSPG – heparan sulfate proteoglycan

IgG – immunoglobulin G

IGG – indusium griseum

JM - juxtamembrane

LGN – lateral geniculate nucleus

LHA – left homology arm

LTV – large targeting vector

MS – midline sling

mV – millivolts

MZG – midline zipper glia

n – nasal

N – NcoI (restriction enzyme)

NMDA – n-methyl-d-aspartic acid

ON – optic nerve

ORF – open reading frame

OX – optic chiasm

P – PsiI (restriction enzyme)

PDZ – postsynaptic discs large zona occludens

PGK – phospho-glycerate kinase

Ptpro – protein tyrosine phosphatase receptor type O

Rac – Ras-related C3 botulinum toxin substrate

RGC – retinal ganglion cell

RHA – right homology arm

RhoA – Ras homolog gene family member A

SAM – sterile alpha motif

SC – superior colliculus

SH2 – Src Homology 2

Shh – Sonic hedgehog

t – temporal

TK – thymidine kinase

TM – transmembrane

TV – targeting vector

v – ventral

X-gal – 5-bromo-4-chloro-3-indolyl-beta-D-galacto-pyranoside

Chapter 1

Introduction

Axon pathfinding is an extremely elaborate process where mixtures of attractive and repulsive cues culminate to guide the axon of a differentiated neuron to its termination zone. The axon must navigate a precise preordained path to correctly establish synapses necessary for a functional nervous system. My studies have focused primarily on the repulsive guidance cue EphB1 and the role of its intracellular domain in axon pathfinding. Here, I will introduce the Eph family of receptor tyrosine kinases and their activators, the ephrins, will discuss some general features of axon pathfinding, and will then conclude with some of the known functions of Ephs and ephrins.

Ephs and ephrins

The Eph family is the largest group of receptor tyrosine kinases, and they play major roles in development following cell-cell interaction. Initially cloned and named due to their over-expression in an erythropoietin producing human hepatocellular carcinoma cell line (Hirai et al., 1987), this family is separated into two subclasses, A and B, which are distinguished by their membrane-anchored ephrin ligands. A-subclass ephrins interact promiscuously with all EphA receptors and are anchored to the extracellular membrane via glycosyl-phosphatidylinositol (GPI) linkage, while B-

subclass ephrins are transmembrane proteins that tend to prefer binding to EphB receptors (Fig. 1.1). There are, however, a few known examples of cross binding; for example, EphA4 binds B-class ephrins (Kullander and Klein, 2002) and EphB2 binds to ephrinA5[1] (Zimmer et al., 2003; Himanen et al., 2004). Early studies of these proteins were performed in a variety of species resulted in an overlapping list of names until the Eph Nomenclature Committee established a standard naming system that is still in effect (Eph Nomenclature Committee, 1997).

While EphB:ephrin-B interactions are generic, it remains unclear how much cross communication occurs between EphB receptors if coexpressed. When EphB1 and EphB2 were expressed in the same cells and were stimulated by ephrin-B1-Fc, both EphB1 and EphB2 were immunoprecipitated independently. Neither seems to form a cis-heterophilic complex with the other protein (Stein et al., 1998) suggesting that EphB proteins function independently of one another. This excludes EphB6, which has been shown to form complexes with other EphB receptors (Freywald et al., 2002) where this interaction is necessary, as the kinase domain of EphB6 is non-functional. In order to become activated, EphB6 must be transphosphorylated by another EphB receptor. It remains unclear what regions stimulate or inhibit these EphB cis interactions, but I will expand on the known functional domains below.

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[1] Additionally, there is limited evidence of interactions with other proteins such as the ability of ephrin-A5 to become activated by bone derived neurotrophic factor and interact with TrkB to induce retinal axon branching or synapse formation in the hippocampus independent of EphA binding (Marler et. al., 2008).

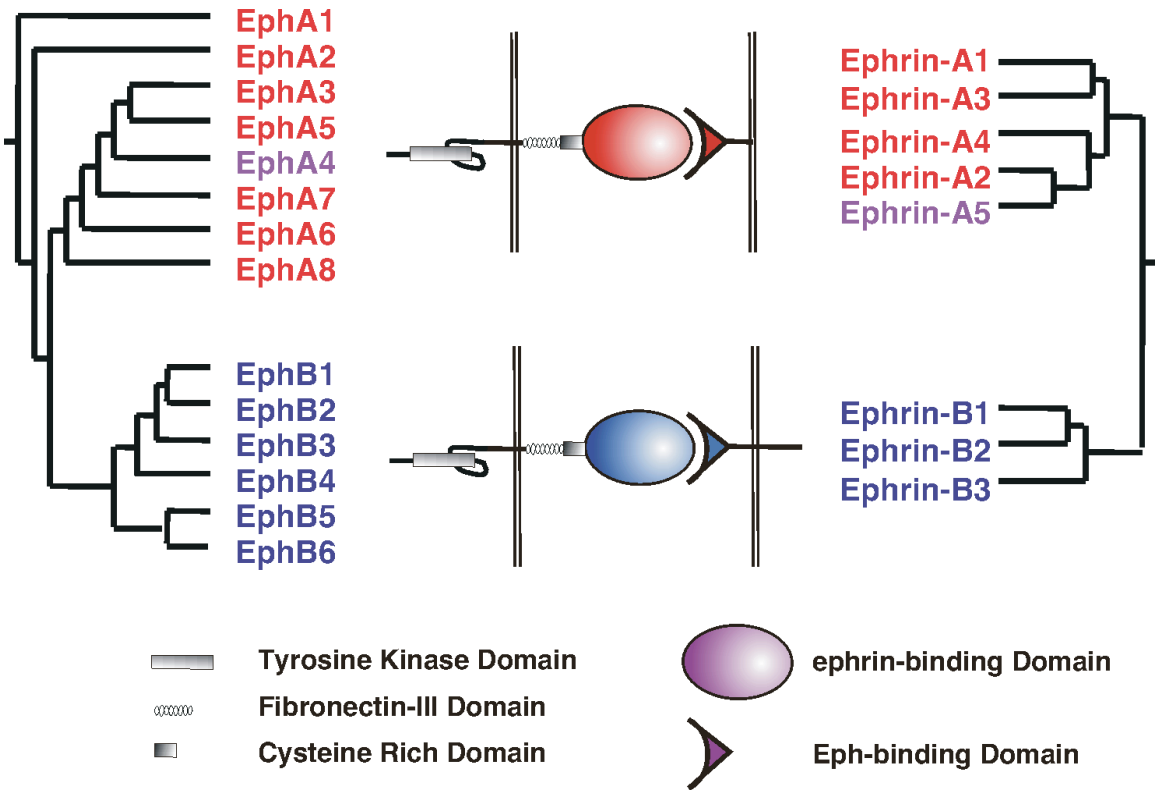


Fig 1.1: The two subclasses of Ephs and ephrins.

Represented above is the phylogenetic tree of Ephs and their ligands, the ephrins, based on sequence conservation. Ephs and ephrins are separated into two unique subclasses: A and B. While ephrin-A molecules are bound to the cell membrane via a GPI anchor, ephrin-B molecules have a transmembrane domain so contain both an extra- and intra-cellular domain. A-subclass Eph receptors tend to interact promiscuously with all ephrin-As while EphB receptors tend to bind exclusively to ephrin-Bs. The Eph proteins are traditionally referred to as the receptors even though ephrins exhibit receptor-like functions by transducing their own "reverse signal."

Structure of Eph Receptors

Both being single pass transmembrane receptors, EphA and EphB proteins share the same core elements: an N-terminal ephrin binding domain, a cysteine rich domain, two fibronectin type III repeats, a transmembrane domain (TM), a juxtamembrane (JM) segment with Src homology 2 (SH2) domain binding motifs, a tyrosine kinase catalytic domain, a sterile alpha motif (SAM) domain, and a postsynaptic density, discs large, zona occludens (PDZ) domain binding site at the extreme C-terminal tail (Himanen and Nikolov, 2003).

As the name suggests, the ephrin binding domain is what allows the Eph to function as a receptor by binding neighboring cells that express ephrins. The binding process is initiated when the ephrin G-H loop contacts two folded β-sheets within the Eph protein. A second weaker interaction occurs between the Eph receptor's H-I loop and the ephrin's C-D loop. The two stage binding process is important because there is evidence that the H-I loop is responsible for the binding specificity of A and B subclass Eph receptors. The H-I loop in EphA molecules is 13 residues long while in EphB proteins it is 17 AA in length (Blits-Huizinga et al., 2004). Additionally, when the H-I loop of EphA3 was transplanted to EphB2, the chimeric protein was able to bind both A and B subclass ephrins (Himanen et al., 1998).

The cysteine rich domain and fibronectin-III domains are thought to be important in aiding in dimerization and higher order clustering after ephrin binding. They may also be crucial in establishing larger lipid raft complexes (Lackmann et al., 1998; Smith et al., 2004; Himanen et al., 2010). Lipid raft complexes function as special small microdomains of the plasma membrane with unique glycosphingolipid and cholesterol

compositions. This can alter the function of proteins contained within them (Scicolone et al., 2009).

Upon ephrin binding, the tyrosine kinase domain autophosphorylates key tyrosines residues within the protein, and the juxtamembrane tyrosines are of specific importance. Before binding, the tyrosines within these motifs remain unphosphorylated, and a hydrophobic pocket covers and inhibits the catalytic activity of the tyrosine kinase domain (Wybenga-Groot et al., 2001). Binding to their ligands brings the kinase domains into close contact with one another allowing phosphorylation. The additional negative charge from the phosphate group releases the auto-inhibitory JM segment (Himanen et al., 2001; Blits-Huizinga et al., 2004), and Ephs then gain the ability to transduce a forward signal through the SH2 binding domain site (Pawson, 2002). SH2 domain containing proteins have a positively charged pocket that interacts with a specific four-peptide sequence (e.g. the motifs in EphB1 are YIDP and YEDP). The addition of a phosphate group to the juxtamembrane tyrosine converts the SH2 domain binding site within Eph receptors into a suitable negatively charged target ready for binding (Schlessinger and Lemmon, 2003).

Upon activation, the kinase domain is required to autophosphorylate neighboring Eph receptors, yet it remains unclear when the kinase domain is required to phosphorylate other proteins or solely its own family. For example, the kinase domain of EphB2 has been shown to directly phosphorylate syndecan-2 and synaptojanin-1 *in vitro* (Ethell et al., 2001; Irie et al., 2005), but other tyrosine kinases such as Focal Adhesion Kinase are recruited to the complex upon ephrin stimulation (Moeller et al., 2006). Thus, it is difficult to precisely determine which is the active kinase under *in vivo* conditions.

While Eph receptors autophosphorylate to become activated, they must also be negatively regulated to maintain balance, and, sure enough, activity is regulated by protein tyrosine phosphatases (PTP) to terminate signaling. For example, protein tyrosine phosphatase receptor type O (Ptpro) has been shown to dephosphorylate JM tyrosines *in vitro*, and the overexpression or blocking of activity of PTPs leads to errors in axon pathfinding in mouse retina and chick motor neurons. PTPs are potent regulators of intracellular Eph signaling, as they are ubiquitously expressed and have been shown to dephosphorylate both A- and B-subclass Eph receptors (Stepanek et al., 2005; Shintani et al., 2006).

By using point mutation analyses, it is known that if the juxtamembrane tyrosines of EphA4 were mutated to glutamic acid, this will mimic the charged state of phosphorylated tyrosines in an active form of the Eph receptor. Thus, clustering will be initiated prior to ephrin stimulation (Egea 2005). Hence, although ephrins are important in initiating the clustering to induce signaling, the subsequent activation of Eph receptors tyrosine kinase domain is also crucial in the maintenance of clustering.

Continuing down the length of the protein, the function of the SAM domain remains somewhat unclear, as its loss does not display an obvious phenotype (Boyd and Lackmann, 2001; Kullander et al., 2001). Nevertheless, the SAM domain does contain another phosphorylation site that may also be crucial in the formation of tetramers and other functions (Himanen and Nikolov, 2003). Finally, the PDZ domain-binding site is located at the extreme C-terminus of the molecule. Forward signaling is sometimes dependent upon the ability of the receptor to bind to PDZ domain containing proteins. This is typically kinase independent and is necessary for the recruitment and clustering of

cytoplasmic proteins necessary for signaling (Hock et al., 1998; Cowan et al., 2000). While there are many aspects to Eph "forward" signaling in the Eph-expressing cell, it becomes more complicated due to signaling occurring simultaneously in the ephrin-expressing cell.

Bidirectional signaling in Ephs and ephrins

Ephrins were initially treated solely as the ligands of Eph receptors until key studies revealed Eph receptors can also function as ligands. Mutant mice lacking EphB2 fail to form the posterior tract of the anterior commissure, and this phenotype is shared with another mutant mouse that retains the extracellular domain by replacing the intracellular domain of EphB2 with β-gal (Henkemeyer et al., 1996). A subsequent study also showed that the ephrin-B intracellular domain became phosphorylated upon binding the EphB extracellular domain (Holland et al., 1996). When B-subclass ephrins bind their Eph receptors, not only will the aforementioned forward signal be initiated through the Eph receptor but a "reverse" signal will also be initiated back through the ephrin expressing cell.

As tetrameric clusters and higher order aggregates form, Src family kinases are recruited, and they will phosphorylate conserved intracellular tyrosines of the B-subclass ephrins. The phosphorylation event disrupts a β-hairpin structure that then allows the binding and recruitment of SH2 domain containing proteins that are able to transduce the reverse signal (Noren and Pasquale, 2004; Klein, 2009). In addition, ephrin-B proteins possess a C-terminal PDZ binding motif, so PDZ domain containing proteins remain constitutively bound in preparation of transducing a reverse signal upon Eph binding

(Kullander and Klein, 2002; Noren and Pasquale, 2004). A-subclass ephrins have also been shown to transduce reverse signals despite the lack of an intracellular domain, but it is less well understood, yet it likely occurs via lipid raft complexes that form upon Eph-ephrin clustering at the membrane (Gauthier and Robbins, 2003).

To further research the role of individual functional domains of Eph receptors, the Henkemeyer laboratory has been focusing on making a wide array of knockout and insertion mutant mice. When an ephrin-B or EphB protein is completely removed, it is difficult to assign a function to either molecule as a receptor or ligand, as both reverse and forward signaling are removed. To remove only forward or reverse signals, the intracellular domain can be replaced with β-gal, while the extracellular and transmembrane domains are unaltered. β-gal naturally forms a tetramer (Appel et al., 1965), which allows the extracellular Eph or ephrin domain to mimic the natural bound and activated state of EphB:ephrin-B interactions. This simultaneously removes the ability to transduce a canonical reverse or forward signal in ephrin-Bs or EphBs, respectively (Fig 1.2). Mice with these mutations have proven quite valuable in separating individual roles of EphB and ephrin-Bs as receptors or ligands (Yokoyama et al., 2001; Cowan et al., 2004; Dravis et al., 2004; Chumley et al., 2007; Xu and Henkemeyer, 2009).

Interestingly, if ligand and receptor are coexpressed in the same cells, this can lead to a canceling out of forward and reverse signaling. There have been several explanations proposing why cis-interactions between Ephs and ephrins coexpression in the same cell leads to the simultaneous inhibition of forward and reverse signaling between two neighboring cells. First, it is possible that the cis-expressed ephrin may be

able to interact with the Eph receptor via its ephrin-binding domain effectively masking its presence from the trans-expressed ephrin. Alternatively, when present on the same membrane, ephrin-A molecules have been shown to preferentially bind EphA receptors at the fibronectin-III region and block activation (Yin et al., 2004; Halloran and Wolman, 2006). Additionally, there may exist either steric hindrance from the cis-expressed ephrin that blocks the ephrin-binding domain from interacting with the trans-expressed ephrin ligand, or the cis-expressed ephrin may simply sequester the Eph receptor away from being functionally relevant. In this scenario, the trans-expressed ephrin may still potentially bind the Eph receptor, so a reverse signal may be initiated (Egea and Klein, 2007). However, there is a limited ability to respond if the cis-expressed ephrin blocks transendocytosis between the contacting cells (the role of endocytosis will be expanded upon below). At this point, I intend to switch gears and briefly discuss some key aspects relating to neurons, which will then lead to my key area of interest, how their axons pathfind to help form neural circuits.

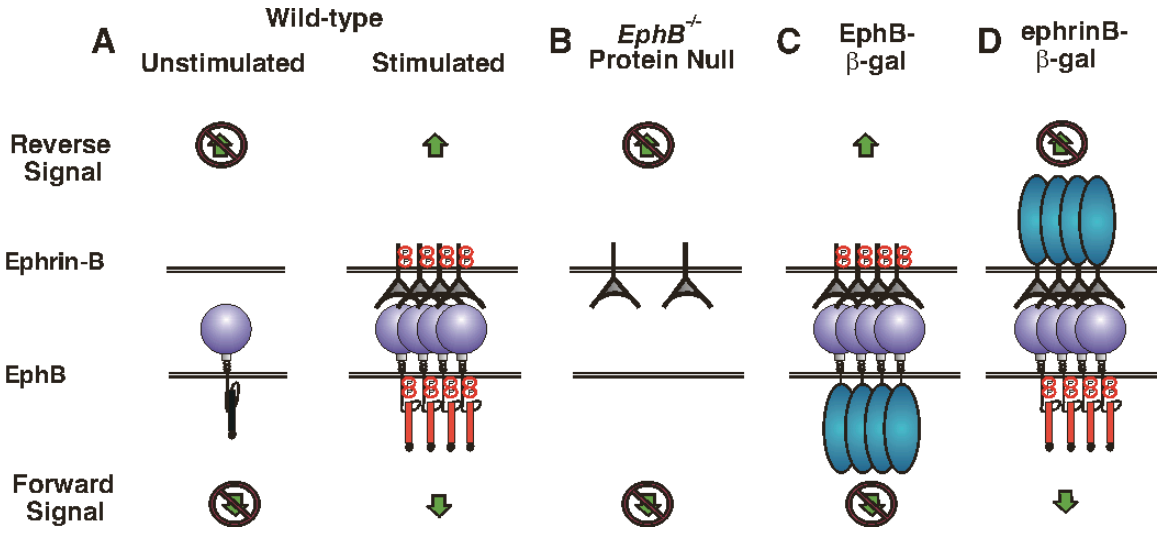


Fig 1.2: Diagram of forward and reverse signaling mutants

A. A representative image of unstimulated WT EphB and stimulated WT EphB receptor. When bound ephrin-B, a forward signal is transduced by EphB while ephrin-Bs simultaneously transduce a reverse signal in the opposing cell. **B.** *EphB*$^{-/-}$ protein null mice lose the ability to transduce a forward signal or stimulate a reverse signal. **C**. Truncated EphB-β-galactosidase fusion proteins cannot transduce a forward signal yet still stimulate ephrin-B mediated reverse signals. **D**. Ephrin-B-β-gal molecules are still able to stimulate a forward signal but lose a reverse signal.

What is a neuron?

In order to create a functional adult human brain, roughly one hundred billion neurons must perform the astonishing feat of forming an intricate circuitous network via interactions with potentially thousands of other cells (Mattson, 1989). While we now know the importance of the neuron and its ability to form synapses to enable the nervous system to function, this was once under heavy contention. In the 19th century, the reticular theory was the accepted explanation. This stated that the brain becomes wired by the fusion of cells into a functional net where there is no individual cell but more of a functioning hive (Toombs, 2003). Santiago Ramón y Cajal (1852–1934), an extraordinary Spanish neurophysiologist I will mention several times in this document, and Wilhelm His developed an alternate theory that the neuron is the critical functional cell in the brain[2], and it functions independently by communicating through vast networks of close contact connections (Hamburger, 1981). This theory could not be confirmed until the direct imaging of synapses with electron microscopes in the 1950's (De Robertis and Bennet, 1954; Palade and Palay, 1954).

There are many methods of classifying types of neurons that can be based on structure, function, connectivity, and direction of signaling, but at their core, all neurons are essentially chemically or electrically excitable cells capable of sending signals over short to very long distances. A typical differentiated and functional neuron has three

[2] Ironically, Camillo Golgi, an Italian anatomist who developed the Golgi staining method that Ramón y Cajal used to great effect, was a strident supporter of the reticular theory and even used his time on the pulpit when receiving the Nobel prize in 1906 with Ramón y Cajal to insist that this theory was the correct one (Hamburger, 1981).

basic parts: a soma, dendrites, and an axon. The soma is the cell body that contains the genetic information and is where transcription and the majority of translation occurs. Dendrites are protrusions that stem and then branch out from the cell to sense the surrounding environment. The axon is a single process that is responsible for transmitting the information to the appropriate target cells (Kandel et al., 2000; Polleux and Snider, 2010). An essential process in the maintenance of adult and growing neurons is their ability to translocate proteins, organelles, mRNA transcripts, cytoskeletal polymers, and many other molecules via axonal transport. The direction of movement is distinguished as either anterograde or retrograde (i.e. away from or to the soma) (Brown, 2003).

In a stereotypical neuron, dendrites extend from the soma to sense the environment via synapses with other neurons and sometimes glial cells. A synapse is a junction between two cells, one being the sender of information (presynaptic) and the other receiving the signal (postsynaptic). When stimulated, a presynaptic cell will release a chemical signaling agent, a neurotransmitter, from the postsynaptic cell into a small gap between the two cells, termed the synaptic cleft. These neurotransmitters will then activate the postsynaptic cell and will typically alter its membrane potential, which may lead to an action potential as discussed in the next segment (Araque et al., 2001; Hille, 2001; Cook, 2008).

Excitable membranes and action potentials

A membrane potential is created when there is a strong difference in the percentage of ions outside of the cell compared to the inside (i.e. low intracellular to high

extracellular concentrations for sodium, calcium, and chloride ions while potassium is at a high intracellular to low extracellular concentrations). In a neuron, the majority of its energy in the form of ATP is used by the sodium-potassium pump, which actively transports three sodium ions out of the cell for every two potassium ions taken into the cell. This in addition to other ion pumps creates the voltage gradient across the plasma membrane. This dissimilarity of ion concentrations creates the resting potential, which is usually held around -70 millivolts (mV) for most neurons. This feature is essential for neurons to perform their main function, transmitting an action potential (Hille, 2001).

As mentioned above, a neurotransmitter will stimulate a postsynaptic cell and can induce either depolarization or hyperpolariziation of the dendrites. If the intended response is to be excitatory, depolarization will occur in the dendrite. In a stereotypical cell (e.g. a pyramidal cell of the hippocampus), this depolarization will continue to the cell body. If a strong enough overall signal is obtained, the soma will become depolarized at the axon hillock, the region where the soma and axon meet. It is here that an action potential will commence and continue in an all or none response (Kandel et al., 2000; Hille, 2001; Cook, 2008).

An action potential begins when outside stimuli open enough select ion channels to cause a local change in membrane potential. For example, NMDA receptors receive excitatory signals from glutamate and allow calcium ions into the cell, which will depolarize it (Hardingham and Bading, 2003; Zhang and Linden, 2003). When a change in polarization reaches the axon hillock and the threshold of excitation (typically around -55mV) is reached, voltage-gated sodium channels open. At this point, the steep sodium concentration gradient induces a rush of sodium into the axon, which depolarizes the

membrane potential rapidly, so that all neighboring voltage-gated sodium channels open. During this rapid change in membrane polarity, the sodium channels close and voltage gated potassium channels open allowing the axon to revert to a hyperpolarized state. This cycle is repeated down the length of the axon until it reaches a pre-synapse. At the pre-synapse, voltage gated calcium channels open allowing for an influx of calcium. This triggers a complex process leading to the release of neurotransmitters. These will then transmit their signal to the proper postsynaptic target cell (Kandel et al., 2000; Hille, 2001; Spruston, 2008). However, the ability of a neuron to fire is moot unless its axon has found the correct target through axon pathfinding.

Axon guidance

Over a century ago, Ramón y Cajal, cementing his legacy as a premiere neuroscientist, was the first to associate the growth cone with axonal pathfinding. He described it as "concentration of protoplasm of conical form" as having "chemical sensitivity, rapid amoeboid movements, and a certain motive force" from viewing only fixed cells and tissue (Ramón y Cajal, 1890; Hamburger, 1981). It has since been confirmed that the growth cone leads the axon by detecting minute changes in concentrations over a gradient (Mattson, 1989; Rosoff et al., 2004). It has three basic components: a central core, lamellipodia, and filopodia. The central core is where the growth cone meets the axon. This is less motile than the other regions partly because it contains larger structures such as organelles for translation and providing energy. Lamellipodia are flat sheet-like structures composed mostly of actin polymers spread out

to resemble a net. From this, filopodia, finger-like projections, further extend the sensory area of the growth cone (Mogilner and Keren, 2009).

Growth cone motility is highly dependent upon the ability to organize actin and tubulin (Kalil and Dent, 2005). When an axon is at a decision point, the growth cone will grow in size and become more complex in arrangement. There will be an increase in the amount of lamellipodia and filopodia in these regions that is specific only to neurons undergoing a decision point. Other axons passing through the areas that are not undergoing redirection will continue on without stalling indicating that not all axons will react to changes in environment, but only specific cells intended will respond. This has been observed in several animal models like chickens (Tosney and Landmesser, 1985), frogs (Holt, 1989), and mice (Bovolenta and Mason, 1987).

Actin and tubulin are both chemicals that are able to form long polymers. G-actin (the G is for globular) is a single monomer present in high concentrations throughout the growth cone. When bound to ATP, it can be added to the head of helical polymer chain of actin referred to as F-actin (filamentous actin). It will rapidly transition to ADP-P_i-actin and then ADP-actin. At this point, the actin is readily dissociated from the filament chain, and it is this inherent instability of the polymer chain that gives it a dynamic quality. Microtubules are inherently more stable polymer chains that form in a similar manner except GTP bound β-tubulin and tyrosinated α-tubulin will first form a dimer before being added to a long linear array. The specific manner in which this dimer is added to form the polymer grants these plate-like chains an inherent polarity and creates microtubule directionality, with the poles referred to as the plus and minus end. An attribute used by transport mechanisms to distinguish anterograde versus retrograde

routes. Microtubules will also age becoming GDP bound and detyrosinated, and this will usually precede the recruitment of stabilizing factors such as minus end capping proteins that can make microtubules extremely stable, especially those polymers located within an established axon (Dent and Gertler, 2003; Kalil and Dent, 2005).

To smaller extent than actin, microtubules explore the growth cone and will interact with actin chains while the growth cone is pathfinding (Dent and Gertler, 2003). The current model proposes that actin is the initial pusher of the growth cone via a treadmill of actin being recycled from the front to the rear, which is constantly recycled to the polymer chains. These unstable areas then recruit microtubules to become stabilized. Evidence for this is based on direct visualization of actin polymerization and microtubule exploration occurring in a growth cone undergoing pathfinding and the presence of microtubule stabilizing proteins within the growth cone (Kalil and Dent, 2005; Mogilner and Keren, 2009).

Another key molecule in axon growth cone dynamics is myosin. A repulsive guidance response will lead to both F-actin destabilization and myosin contraction. This myosin contraction is necessary to constrict the size of growth cone through an increase in membrane tension. The collapse will also push actin debris to the opposing side of the growth cone to continue outgrowth in that direction (Mogilner and Keren, 2009).

Guidance cues

The decision a growth cone makes along its journey is decided by the overall response surrounding attractive and repulsive cues. They can be separated into two basic groups, diffusible and membrane anchored. A diffusible cue is a soluble chemical

released at a distance from the growing axon, and over the course of its diffusion, a high to low chemical gradient is established. A chemoattractive response will result in the axon turning toward the increasing gradient while a chemorepulsive response will cause the axon to be repelled from the source. A membrane anchored guidance cue on the other hand will only be initiated by direct axon-cell contact. This contact can also lead to an attractive or repulsive response that induces adhesion or repulsion only as long as contact is maintained. Similarly, fasciculation or extracellular matrix adhesion may occur only when the axon contacts a previously established axonal tract or glial cell barrier, respectively. These structures produce an environment that is conducive to outgrowth via growth promoting molecules on the cell surface (Dodd and Jessell, 1988; Kandel et al., 2000; Raper and Mason, 2010).

For the sake of simplicity and because a large portion of my project deals with it, I will briefly describe several examples of guidance cues utilized for retinal ganglion cell (RGC) axon pathfinding from the retina to the thalamus and colliculus (or optic tectum in frogs and birds). In fact, RGC axon pathfinding has long been used as a model to understand axon guidance in the rest of the nervous system, in large part because it is an isolated system that has clearly distinguished stereotypical decision points (e.g. exiting the optic disc or the midline decision at the optic chiasm, a major brain commissure) (Erskine and Herrera, 2007; Bao, 2008). Similar to all pathfinding axons, RGC axons must follow long-range guidance cues (e.g. semaphorins, slits, netrins, and neurotrophins) and contact mediated guidance cues (e.g. matrix associated proteins like laminins and proteoglycans or cell associated molecules like cell adhesion molecules (CAMs), cadherins, and Ephs (Scicolone et al., 2009)).

Serving as an attractive cue, Netrin-1 is expressed around the optic disk while its receptor DCC is expressed on RGC axons. Without either of these, RGC axons never exit the retina through the optic disk (Deiner et al., 1997; Lauderdale et al., 1997). The semaphorin Sema5A is a repulsive cue. When blocked by antibodies, RGC axons will stray from the main bundle (Oster et al., 2003). This indicates it is important not to just have attractive cues, but repulsive cues to keep axons in line. In zebrafish, Sema3D, which is expressed and secreted at the optic chiasm, has been shown to be a repulsive cue that keeps RGC heading in a contralateral direction by blocking RGC axons that have reached the midline from crossing back to the ipsilateral side (Sakai and Halloran, 2006). Slit is a secreted protein that inhibits outgrowth of Robo expressing RGC axons, and it has been found to be important in regulating RGC axon exit from the optic fiber layer to the optic disk into the optic nerve (Thompson et al., 2006b)). Slit-Robo signaling is also crucial in maintaining a fasciculated state of the optic nerve and optic tract where loss of one or the other results in a less organized bundle or mistargeting within the brain in mice (Thompson et al., 2006a; Thompson et al., 2006b) and in zebrafish (Fricke et al., 2001).

Besides being dependent on the ability to directly follow cues from glial structures, a major determining factor in the ability of axons to properly find and innervate their targets depends on their ability to follow pioneer axons. The importance of pioneering axons was first demonstrated by observing their growth in grasshopper neural development (Bate, 1976), and pioneering axons have since been shown to be critical in advanced organisms as well (McConnell et al., 1989; Burrill and Easter, 1995). Along the optic tract, L1 is expressed in retinal axons and seems critical for fasciculation,

for inhibiting its function with antibodies results in pathfinding errors through the optic nerve (Brittis et al., 1995).

As another example of contact mediated guidance, proteoglycans such as chondroitin proteoglycans (CSPGs) and heparan sulfate proteoglycans (HSPGs) are compounds with a polypeptide backbone that have complex glycosaminoglycan molecule chains composed mostly of disaccharide units, which are negatively charged from sulfation. These can be enormous in size and may be up to 95% sugar chains in total composition (Bovolenta and Fernaud-Espinosa, 2000). Transported to the ECM, proteoglycans are produced through various enzymatic pathways and can be very structurally diverse as the length, composition, and core proteins can all be altered with different resulting functions (Inatani, 2005). The compounds regulate the interactions between receptors and their ligands and have more recently been shown to initiate cell signaling independently of other receptors (Elfenbein and Simons, 2010), and this assisting role makes it is difficult to assign method of function for these complexes. CSPGs have been shown to be inhibitory factors in retinal axon pathfinding (Snow et al., 1991), and the removal of CSPGs cause improper sorting at the chiasm of RGC axons at E15 and a misdirection of RGC axons into the ventral diencephalon (Chung et al., 2000) and anterior diencephalon (Ichijo and Kawabata, 2001).

Ephs in axon guidance

Returning to the star of this thesis, the Eph receptor, I will now discuss in more detail the known functions of Eph receptors in axon pathfinding. EphB and ephrin-B molecules are traditionally viewed as contact mediated repulsive guidance cues. If

ephrin-B-Fc is added to EphB expressing retinal axons, growth cone collapse will occur (Williams et al., 2003). Likewise, if EphB-Fc is added to ephrin-B expressing retinal axons, there will be a repulsive effect (Birgbauer et al., 2001). However, EphBs have also been demonstrated to have an attractive response in ephrin-B expressing retinal axons (Mann et al., 2002), and the distinction was shown to be dependent on cyclic GMP levels. Specifically, the induction of a forward signal led to an increase in cyclic GMP levels while induction of ephrin-Bs by EphB-Fc caused a decrease (Mann et al., 2003).

The most highly studied pathway for Ephs and ephrins within RGC axons is their task in retinotectal map formation. In a developing chick embryo, EphA3 is known to display a high temporal to low nasal expression pattern while EphA4 and A5 are expressed uniformly throughout. At the retinal axon target the optic tectum, ephrin-A2 and A5 are expressed in a high posterior to low anterior gradient (Flanagan and Vanderhaeghen, 1998). Ephrin-A reverse signaling is dependent upon the recruitment of the src kinase Fyn via the formation of a lipid raft. The response is typically attractive (Scicolone et al., 2009). This process is somewhat conserved in the comparable tissue of rodents, the superior colliculus. *Ephrin-A2/A5*$^{-/-}$ protein null and *EphA5* mutant mice have errors in retinotectal mapping at the superior colliculus of ipsilateral and contralateral projections (Frisen et al., 1998; Feldheim et al., 2004; Haustead et al., 2008). These mice are unable to properly integrate the two groups of axons at the superior colliculus, so ephrin-As seem key to this process. In chicks, it has been shown that a gradient of EphAs and ephrin-As is key to proper retinotectal pathfinding. EphA3 is expressed in a low nasal to high temporal pattern through the retina while EphA4 and EphA5 are expresses uniformly. These RGC axons will then encounter a low anterior to

high posterior expression pattern for ephrin-A2 and ephrin-A5 in the optic tectum (Flanagan and Vanderhaeghen, 1998), so EphAs and ephrin-As are key regulators of retinotectal mapping.

When looking at B-subclass Ephs and ephrins at the colliculus, there is a high dorsal to low ventral expression pattern for ephrin-Bs in the retina and high medial to low lateral expression gradient at the superior colliculus. At the same time, EphB receptors show opposing expression pattern gradients in the same regions (Scicolone et al., 2009). Mutant mice have revealed that both reverse and forward signaling are crucial in the ability of RGC axons to topographically map (Hindges et al., 2002; Thakar et al., 2011, Submitted). It has been shown that this EphB signaling is attractive in nature to ephrin-B expressing retinal axons (Birgbauer et al., 2001; Mann et al., 2002). This is in conjunction with Wnt and Ryk gradients that appear to function in a repulsive manner, for blocking Ryk activity leads to mistargeting of retinal axons within the optic tectum (Schmitt et al., 2006).

When an EphA receptor is unbound to an ephrin, it may still bind to the GEF ephexin and is thought to help induce axon outgrowth. However, upon ephrin binding, the EphA molecule becomes phosphorylated and recruits Src kinase, which then phoshorylates and activates ephexin that stimulates RhoA activity and induces growth cone collapse (Egea and Klein, 2007). Sometimes phosphorylation is required for SH2 interactions to occur (e.g. Kalirin only binds to phosphorylated EphB2 (Penzes et al., 2003)). Alternatively, binding can also occur before ephrin activation (e.g. intersectin, which is constitutively bound to EphB2 but remains inactive until EphB-ephrin-B binding occurs (Noren and Pasquale, 2004; Irie et al., 2005). Other Eph interacting GEFs

like Vav, ephexin, intersectin, kalirin, and Tiam1 have dbl homology-pleckstrin homology (DH-PH) domains that bind protein to Rho family GTPases (Murai and Pasquale, 2005).

Possibly the main downstream factor for axon guidance that Eph:ephrin signaling controls is the regulation of Rho family GTPases (members of this family that have proven important in Eph signaling are RhoA, Rac1, and cdc42). These enzymes fluctuate between an activated form when bound to GTP and an inactive form when bound to GDP. This process is regulated by GEFs and GAPs. An active GEF will transfer a GTP group to an inactive Rho GTPase, which will then be transported from the cytoplasm to the cell membrane where it will induce its signal. One of the key functions of Rho GTPases is to induce actin polymerization when in an active state (Etienne-Manneville and Hall, 2002). To stop the signal, a GAP will then help alter GTP to GDP to return the Rho GTPase to an inactive state (Nikolic, 2002).

It is counterintuitive to think that two bound extracellular molecules with high binding affinity on neighboring cells would result in a repulsive effect. After all, when two disparate strands of Velcro come into contact, the result is an adhesive response, so it seems the likely reaction for Eph-ephrin interactions is to have an attractive role, yet there is often a clearly repulsive response. This problem began to be resolved when it was discovered that upon binding to EphA receptors ephrin-A2 can become cleaved by the metalloprotease Kuzbanian/ADAM10. If the cleavage site on ephrin-A2 is altered, the expressing fibroblasts do not exhibit the normal repulsive response (Hattori et al., 2000), and other proteases were discovered that are able to cleave B-subclass ephrins (Pascall and Brown, 2004; Georgakopoulos et al., 2006). This was a major breakthrough

in the understanding of how Eph-ephrin interactions can result in repulsion despite strong intercellular binding. Once the ligand is cut, separation can commence.

In addition to cleavage, experiments monitoring fibroblasts and endothelial cells *in vitro* showed that EphB4 became internalized when EphB4 was stimulated with ephrin-B2. In addition to the Eph-ephrin complex, an entire portion of the donor cell plasma membrane will be phagocytosed by the accepting EphB expressing cell. This process was shown to be dependent upon on the Arp2/3 complex and Rac activity since transendocytosis of the ephrin ligand would not occur without Rac dependent actin rearrangement (Marston et al., 2003). In the same publication, endocytosis via vesicular trafficking was shown to occur in both directions, as full length EphB will also be transendocytosed into the ephrin-B expressing cell upon mutual stimulation in co-cultured cells. Furthermore, internalization into the EphB expressing cell is dependent on tyrosine kinase catalytic activity, and cells that express a truncated form of ephrin-B do not accept donor EphB2 as well as full length does (Zimmer et al., 2003). Without transendocytosis of the Eph-ephrin complex, there is a failure of cells to undergo contact-contact mediated repulsion. Instead, an attractive response is initiated (Pitulescu and Adams, 2010). It is not well understood how this same machinery can initiate two opposing reactions, but it is known that for a repulsive response to happen, so too must endocytosis of the Eph:ephrin signaling complex from the cell surface occur.

The roles of Ephs in the synapse formation

Eph receptors seem to regulate synaptogenesis, for increasing levels of EphB2 in culture conditions results in GluR2 clustering, which in turn results in increased spine

formation. EphB receptors have also been shown to interact directly with NMDA receptors, which regulate the amount of calcium that enters a cell (Dalva et al., 2000), and if EphB2 is removed, there is a decrease of NMDA at synapses (Lai and Ip, 2009). Additionally, ephrin-A stimulation will lead to spine retraction through RhoA dependent activity (Lai and Ip, 2009). This suggests that Eph receptors have the ability to modulate synapses. This was further confirmed when *EphB1/2/3* triple null mice were shown to have a decrease in the number of spines and synapses in the hippocampus (Henkemeyer et al., 2003).

In addition to participating in synapse formation in the brain, EphB receptors may also regulate synapse formation in the peripheral nervous system. When ephrin-B-Fc is injected intrathecally into an adult rat or mouse, this results in a hyperalgesic state that mimics sciatica in humans. These rodents become temporarily hypersensitive to stimuli in their hindpaws. Moreover, when an alternate neuropathic pain model is used where the sciatic nerve is damaged but still allowed to signal, the intrathecal injection of EphB-Fc can block neuropathic pain (Battaglia et al., 2003; Song et al., 2008a). Additionally, EphB1 and ephrin-Bs are normally expressed in the dorsal horn of the spinal cord and upregulated when neuropathic pain is induced by nerve injury (Battaglia et al., 2003; Kobayashi et al., 2007; Song et al., 2008b; Song et al., 2008a). Finally, the loss or reduction of EphB1 results in loss of this hyperalgesic state in mice (Han et al., 2008). Although the mechanism causing hyperalgesia has not been proven, it is likely dependent on NMDA signaling and may require synaptic remodeling within the dorsal horn.

Goals of thesis

When complete null mouse models are analyzed, phenotypes cannot be used to differentiate between a loss of EphB forward signaling or ephrin-B reverse signaling. Since the loss of expression of EphB1 results in errors in the guidance of axons at the optic chiasm and the corpus callosum, I have chosen to study this by creating a mutant mouse that replaces the genetic sequence encoding the intracellular domain of EphB1 with a *lacZ* cassette. The modified form of EphB1 expressed under its endogenous locus will be truncated and fused to β-gal but will still reach the cell surface. Essentially, half of EphB1's function will remain, so when compared to the preexisting *EphB1*$^{-/-}$ null mutant mouse, any differences or similarities in phenotypes can be ascribed to the required function of only the extracellular domain or both the extra- and intracellular domains, respectively. The following chapters detail the construction of this mutant and the tests performed around this and other related preexisting EphB and ephrin-B mutant mice to determine their roles in axon guidance during the neural development of mice.

Chapter 2

Generation of mutant mice

Summary

As EphB:ephrin-B binding induces signaling in a bidirectional manner, the contributions the Eph-expressing cell (forward signaling) or the ephrin-expressing cell (reverse signaling) are impossible to determine in protein-null mutant mice. To determine when EphB1 and ephrin-B2 are functioning as receptors, I created a new knock-in mutant mouse and point mutant mouse, termed *EphB1*$^{T\text{-}lacZ}$ and *ephrin-B2*$^{6YF\Delta V}$, respectively. In this chapter, I will present the strategies and the targeting and screening methods used in their creation. I will also describe the experiments performed to confirm *EphB1*$^{T\text{-}lacZ}$ is functioning as expected, for the truncated β-gal fusion protein needs to reach the cell surface and be transported down the axon. Additionally, I will present data illustrating that this protein does not phenocopy a neuropathic pain defect visible in complete *EphB1*$^{-/-}$ null animals.

Mice as a model system

The first method used to generate mutations in the genome for phenotypic analysis relied on exposing Drosophila to ultraviolet light (Muller, 1927). Since then, more mutagens such as ethylnitrosourea or ethylmethanesulphonate have been used to

generate mutations in simple animal models from *Caenorhabditis elegans* to more complex animal models such as mice; unfortunately, these methods all depended on random DNA damage events. While screens with these chemicals could produce a great deal of information, the amount of time and energy that needs to be invested to mutate and then cross and screen following generations for phenotypes was often overwhelming. A desire for easier ways to modify the genome lead to the development of transgenic mice (Gordon et al., 1980), which have foreign DNA randomly integrate into the genome of developing mouse blastocysts. These mice can then misexpress any protein of interest to label and/or modify relevant cells. While transgenic mice remain powerful tools, gene targeting became the gold standard by allowing the precise removal and/or replacement of specific regions of the genome. This was accomplished by precisely modifying the mouse genome through homologous recombination and then injecting the altered embryonic stem (ES) cells into blastocysts (Hooper et al., 1987; Kuehn et al., 1987; Aizawa, 2008). With this ability to modify the genome, many genes have already been removed or modified, and there is now a project underway to create a knockout mouse for every protein-coding gene within the mouse known as the Knockout Mouse Project (reviewed in (Guan et al., 2010).

Mice have long been a preferred model organism in scientific studies because of their rapid cycles of replication and the pre-existence of inbred strains from fancy mouse breeders across the world (Steingrimsson et al., 2006). Inbred strains were required because they display a consistency in development and behavior that is useful for consistency in complex biochemical and behavioral analyses. Interestingly, it was pure chance that first allowed the creation of gene targeting methods, for this method was

largely dependent on the ability of 129 mouse strain ES cells to retain pluripotency after extensive time grown *in vitro*. In fact, it is only recently that another model organism, the rat, had its genome modified through ES cell based recombination (Tong et al., 2010), so it is staggering to ponder where our understanding of genes and proteins would currently be without the use of gene-targeted mice and the existence of 129 strain mice[3].

Description of mutant mice generated

To determine whether EphB1 functions as a receptor or ligand, two new mutant lines were created. The first, a knock-in mutant has the entire region encoding the intracellular domain of EphB1 replaced with in-frame *lacZ* sequences encoding beta-galactosidase (β-gal), and the second is an ephrin-B2 point mutant that has all intracellular tyrosine codons replaced with those corresponding to phenylalanine while simultaneously removing the C-terminal valine residue codon. Previous studies from our laboratory have shown that similar *lacZ* mutant mice that express C-terminal truncated EphB2-β-gal, ephrin-B2-β-gal, and ephrin-B3-β-gal fusion proteins lose their ability to transduce cell autonomous signals that require the intracellular domain of the targeted protein (Yokoyama et al., 2001; Cowan et al., 2004; Dravis et al., 2004; Chumley et al., 2007; Xu and Henkemeyer, 2009). However, unlike protein null mutants, these truncated

[3] 129 strain mice were first generated by Leslie Dunn at Columbia in 1928 and were then acquired in 1945 by the Jackson laboratory, the powerhouse in mouse studies with over 5000 mouse strains and the source of the mouse genome database. It was again simple luck that this strain was not lost when a forest fire destroyed most of the laboratory in 1947 (Simpson et. al., 1997).

EphB-β-gal and ephrin-B-β-gal fusion proteins retain their respective extracellular and transmembrane domains allowing them traffic to the plasma membrane and act as ligands to stimulate reverse or forward signaling, respectively. This is dissimilar to a solely truncated from, which will not be properly targeted to the plasma membrane, so does not transduce its own ligand-like signal (Adams et al., 2001; Cowan et al., 2004). Furthermore, since these truncated fusion proteins are expressed from the endogenous genes, they are present at physiological spatial and temporal patterns. This eliminates possible artificial results obtained by other methodologies (e.g. viral injection) where proteins are over-expressed, miss-expressed, or knocked down.

Referring to the second mutant mouse, canonical ephrin-B2 reverse signaling is disrupted, as both interactions with SH2 domain containing proteins and PDZ domain proteins fail to be initiated. This *ephrin-B2*$^{6YF\Delta V}$ allele has point mutations in the exon that encodes the intracellular cytoplasmic tail that changes the cytoplasmic tyrosine residues into phenylalanines and also deletes the C-terminal valine residue. As a result, this mutant allele expresses a protein that is unable to become tyrosine phosphorylated or interact with either SH2 or PDZ domain-containing downstream signaling proteins. Previous studies have shown that similar mutations in *ephrin-B1* and *ephrin-B3* express proteins that lose their ability to transduce canonical ephrin-B reverse signals but maintain their ability to act as ligands to transduce forward signals in adjacent EphB expressing cells (Makinen et al., 2005; Bush and Soriano, 2009; Xu and Henkemeyer, 2009; Bush and Soriano, 2010).

Recombineering strategy

Traditionally, cloning a targeting vector (TV) relied on finding unique restriction enzyme sites naturally occurring in the mouse genome. If a unique site could not be found, a researcher would be limited in how they might be able to modify the genetic sequence. To solve this problem, the Copeland laboratory created a special strain of *E. coli* with a defective prophage expression system (Yu et al., 2000) while expressing recombination proteins exo, bet, and gam, from the λ phage (Copeland et al., 2001; Liu et al., 2003). All of which were integrated into the bacteria's chromosomal DNA. The recombination proteins were placed under the control of λP_L promoter, which is repressed by the temperature-sensitive repressor λ*cl857*. When cultured at 32°C, this promoter is repressed, but when exposed to temperatures of 42°C, the recombination proteins are expressed. Any linear double stranded piece of DNA present within the bacteria at this time will have the potential of undergoing recombination. This allows for a high degree of specificity in choosing how to create plasmids, for a small portion of homologous DNA (~50 – 500 bp) can be synthesized by PCR and placed 5' and 3' of any sequence.

By choosing the portions of DNA carefully, these sequences can in theory be placed anywhere in the genome where recombination can occur. This method is not viable in high-copy vectors, but instead is only useful in single-copy vectors like BACs. Considering that the entire mouse genome of several strains of mice has been inserted into BAC libraries, it is possible to use recombineering to create almost any modification within the genome imaginable, so our laboratory chose to use this method in the creation of the TVs for two mutant mice. The first described is for a truncated EphB1-β-gal

fusion protein termed *EphB1*$^{T\text{-}lacZ}$ that was completely created by me. The second TV for a modified form of ephrin-B2 will not be described in detail here, as that was created by Rebekkah Warren, but I will discuss the integration into the genome and screening of that mouse termed *ephrin-B2*$^{6YF\Delta V}$.

Creating the targeting vectors

First, traditional cloning was used to create a minitargeting vector (MTV) based on the plasmid pL452, which contains a lox-P flanked neo positive selection cassette under a bacterial and mammalian PGK promoter with a bGH pA cassette. Transformed bacteria exhibit kanamycin resistance, which greatly improves the ability to find positive clones. The LHA of the MTV was designed to incorporate the reading frame of *EphB1* exon 9 immediately following the codons encoding the transmembrane domain but before the JM segment while the RHA was designed to target a region 3' of exon 9. An insert containing the ORF of *lacZ* was cloned in frame with the ORF of exon 9. The proper construction of the MTV was verified by digestion analysis and DNA sequencing (Fig. 2.1).

Previously, Dr. Henkemeyer had experienced great difficulty in the creation of both the *EphB1*$^{-/-}$ and *EphB1*^{lacZ} mutant mice, so based on this and sequencing data, which shows that the intronic gDNA of 129 strain mice is surprisingly not homologous to that of Black 6[4], I decided to use a 129 strain based BAC to create the TV for *EphB1*$^{T\text{-}lacZ}$. To do so, a 129 mouse strain based BAC library from Invitrogen was screened by PCR

[4] I have noticed as much as a 10% difference over 100bp stretches when I compared sequencing results between 129 and Black 6 strain mice (data not shown).

and two positive cones found. BAC 209F22 showed the largest coverage of gDNA surrounding *EphB1* and was chosen. This BAC was purified and transformed into the *E. coli* recombination strain EL250, the strain where all of the following recombineering mentioned occurred.

Recombineering was used to recombine the MTV into the BAC 209F22 creating the large targeting vector (LTV). A capture vector (CV) was then created via traditional cloning into the plasmid pL254. Created by Michael Halford, this plasmid is pL253 based, so it still contains the pBluescript backbone and the thymidine kinase (TK) cassette under the P_{MC-1} promoter. It also includes an additional negative selection cassette for diphtheria toxin α (DT-A) under the P_{eIF4A1} promoter that is constitutively expressed in mammalian cells. The CV was designed to remove a LHA that was 5.9 kb upstream of the MTV insertion site and a RHA that was 8.6 kb downstream of the MTV insertion site.

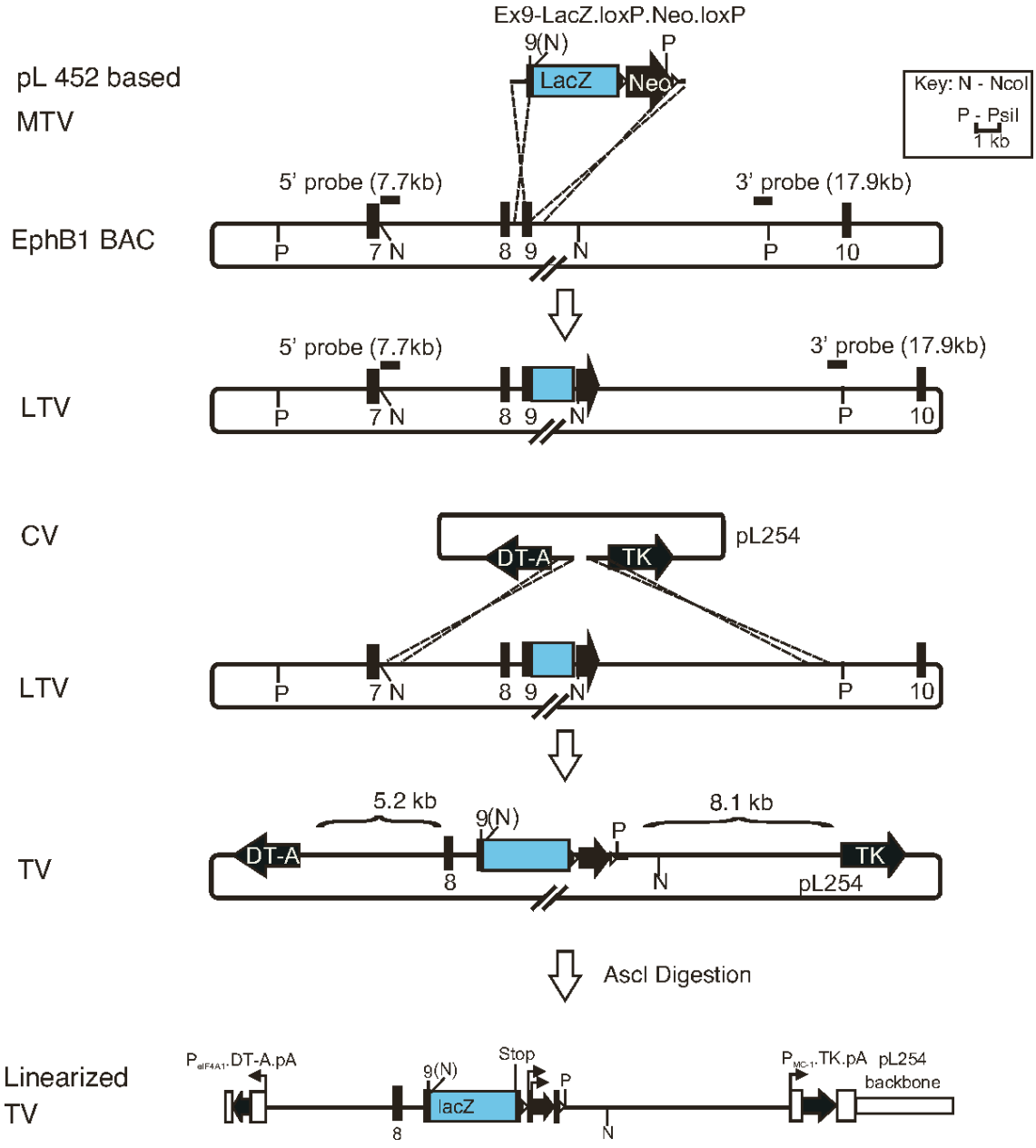

pL 452 based
MTV
Ex9-LacZ.loxP.Neo.loxP
9(N)
P
LacZ
Neo
Key: N - NcoI
P - PsiI
1 kb
5' probe (7.7kb)
3' probe (17.9kb)
EphB1 BAC
P
7 N
8 9
N
P
10
LTV
5' probe (7.7kb)
3' probe (17.9kb)
P
7 N
8 9
N
P
10
CV
pL254
DT-A
TK
LTV
P
7 N
8 9
N
P
10
5.2 kb
8.1 kb
9(N)
P
TV
DT-A
8
N
TK
pL254
AscI Digestion
Linearized
TV
Stop
9(N)
lacZ
P
8
N
pL254
backbone

Fig 2.1: Strategy for the creation of TV for *EphB1*$^{T\text{-}lacZ}$

To create the MTV, small regions of homology around exon 9 of *EphB1*'s ORF and the *lacZ* ORF were cloned into the plasmid pL452 so that, once inserted within the mouse genome, β-gal would be translated in lieu of the intracellular domain of EphB1. Using recombineering, the MTV was recombined into a BAC encompassing the entire 3' half of *EphB1* creating the LTV. Also with recombineering, a pL254 based CV that was designed to create a LHA of 5.2 kb and a RHA of 8.1 kb gDNA, which contained the negative selection cassettes for DT-A and TK, was used to create the TV. The TV was then linearized via a unique AscI site present in the plasmid pL254.

Integration into the genome and verification of proper insertion and expression

Using this CV, a TV was retrieved, amplified, purified, linearized (Fig 2.2 A), and electroporated into murine embryonic stem (ES) cells. The ES cells were screened by southern blot and two lines that exhibited proper homologous recombination were identified. Targeted ES cells were injected into blastocysts, and the resulting chimeric mice were bred to obtain germ-line transmission. The proper targeting of this new mutation, termed *EphB1*$^{T\text{-}lacZ}$, was verified using additional southern blots (Fig 2.2 B), PCR analysis, and sequencing (data not shown). The loxP-flanked positive selection neomycin cassette was then removed by crossing the mice to a germline cre-expressing mouse.

To validate expression of the resulting EphB1-β-gal fusion protein, whole protein lysates from wildtype (WT) and *EphB1*$^{T\text{-}lacZ/+}$ heterozygote littermates collected at embryonic day 11.5 (E11.5) were probed in immunoblot with anti-β-gal antibodies, and expression was confirmed at the expected protein size of 180 kilodaltons (Fig. 2.3 A). To confirm that expression of the fusion protein matches expected expression patterns of endogenous EphB1, whole mount embryos were collected at three time-points and stained with X-gal, and overall expression levels increase throughout the mouse from E10.5 to E12.5. Additionally, β-gal enzymatic activity is especially visible in the developing nervous system where changes in expression are evident in the developing mesencephalon and spinal cord with an altered low to high expression from E10.5 to E11.5 and E12.5 (Fig. 2.3 B). This suggests that the EphB1-β-gal fusion protein is expressed and regulated in the proper spatial and temporal pattern (Mori et al., 1995).

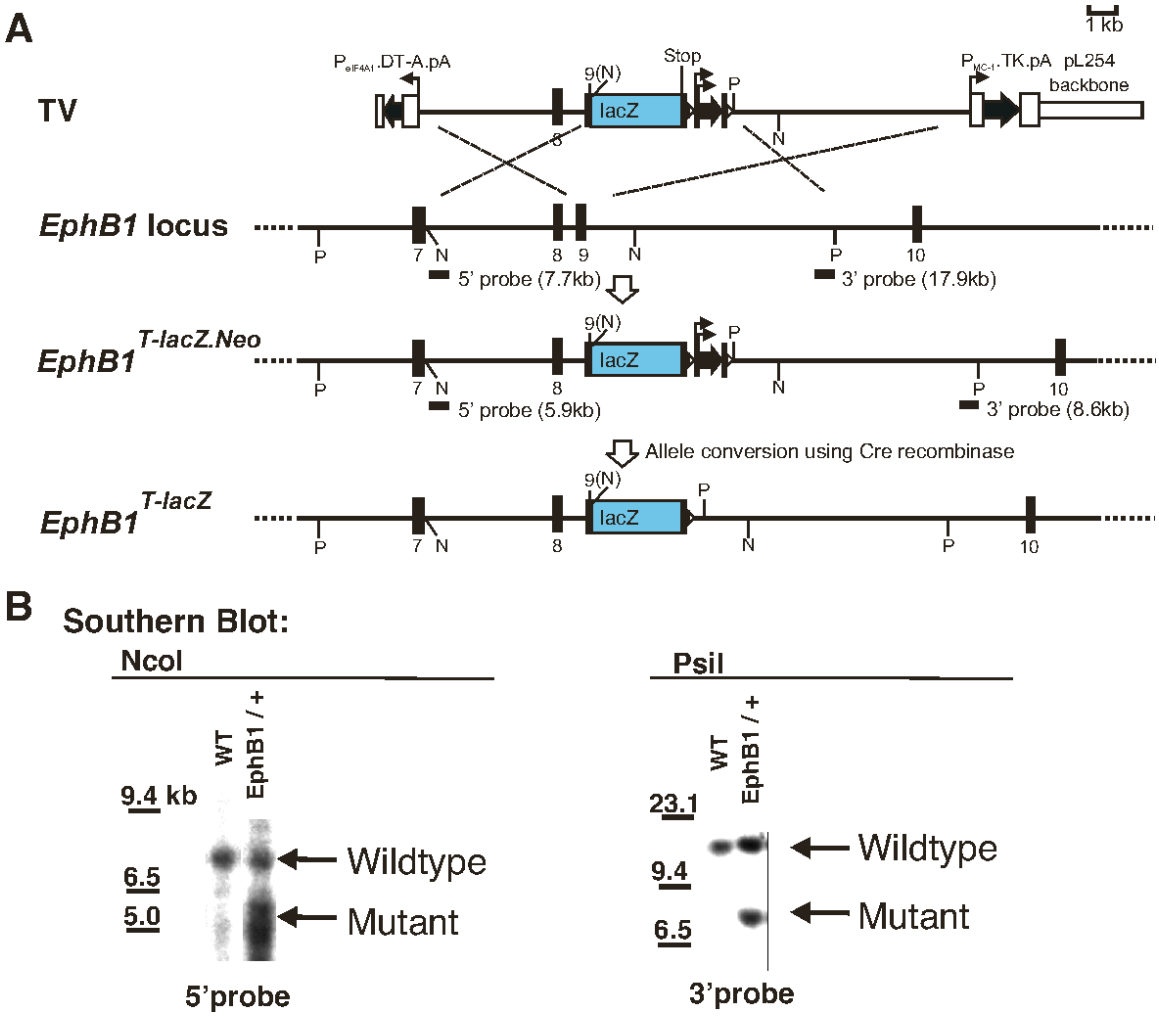


Fig 2.2: Insertion of *EphB1*$^{T\text{-}lacZ}$ TV into the mouse genome

A. Southern blot strategy that utilized homologous recombination in ES cells to insert lacZ open reading frame into *EphB1* reading frame directly following transmembrane region resulting in the replacement of the entire intracellular domain with β-gal. **B**. Confirmation of proper 5' and 3' end TV integration in the mouse genome by southern blot analysis.

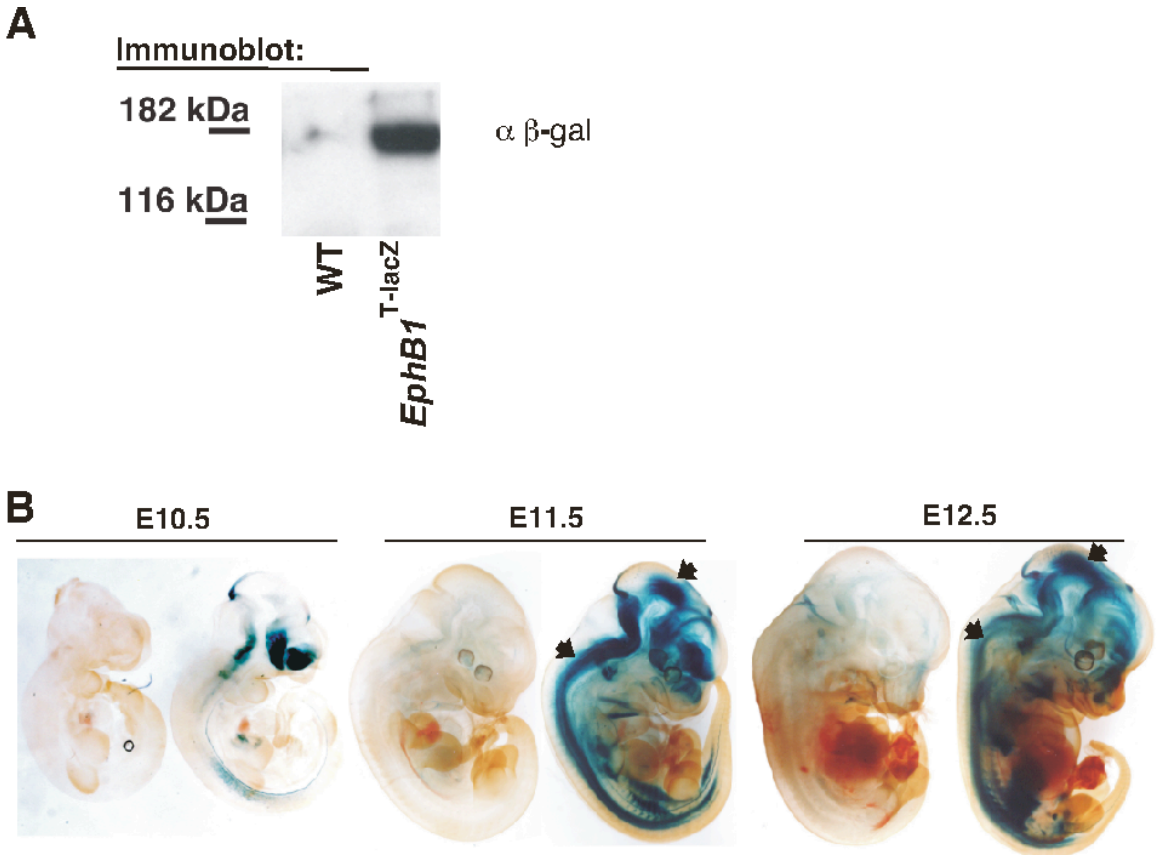


Fig 2.3: Verification of β-gal expression of *EphB1*$^{T\text{-}lacZ}$ mutant mice

Generation of truncated EphB1 mutant mice. **A**. Immunoblot against β-gal shows new fusion protein is being produced in *EphB1*$^{T\text{-}lacZ}$ mice at the expected mass of 180 kiloDaltons. **B**. WT (left) and *EphB1*$^{+/lacZ}$ (right) embryos were stained with X-gal at three stages of development: E10.5, E11.5, and E12.5. Expression of the EphB1-β-gal fusion protein increases in the developing nervous system from E10.5 to E12.5 (arrows).

EphB1-β-gal traffics to the cell surface

To verify that the C-terminal truncated EphB1-β-gal fusion protein is properly localized to the plasma membrane, cells from whole brains of E13.5 *EphB1*$^{+/T\text{-}lacZ}$ heterozygote mice and WT littermates were dissociated and cultured for 24 hours and then exposed to NHS-biotin to biotinylate all cell surface proteins. Total protein lysates were prepared and biotinylated proteins purified with strepavidin-coated beads. The biotinylated fraction and whole cell lysates were probed in immunoblots with antibodies against β-actin, EphB2, and β-gal. Functioning as a negative control, β-actin was only detected in the whole cell lysates indicating that the biotinylation specifically labeled only proteins exposed to the cell surface. Serving as a positive control because it is trafficked to the cell surface, EphB2 was detected in both the whole-cell and biotinylated lanes. The truncated EphB1-β-gal fusion protein at the expected size of 180 kilodaltons was also present in both the whole-cell and biotinylated lanes from *EphB1*$^{+/T\text{-}lacZ}$ mice but not in the WT lanes (Fig. 2.4 A). This demonstrates that the truncated EphB1-β-gal fusion protein trafficks to the plasma membrane as expected.

To investigate if the EphB1-β-gal fusion protein is transported throughout cellular processes, its expression pattern and subcellular localization was compared to the previously generated *EphB1*^{lacZ} protein-null mutation, which expresses an unconjugated β-gal protein confined to the cell body. In X-gal stained sections of the adult hippocampus both mutations reported expression of EphB1 within CA3 pyramidal neurons and neural progenitors in the subgranular zone (SGZ) of the dentate gyrus (Fig. 2.4 B). Importantly, the β-gal staining observed in *EphB1*$^{T\text{-}lacZ}$ mice was visible in both the cell bodies and cellular processes including the axonal projections of the CA3

neurons that target the CA1 region and the cell body extensions of the SGZ progenitor cells that reach into the dentate molecular layer. This data shows the EphB1-β-gal fusion protein is transported into axons and other cellular processes.

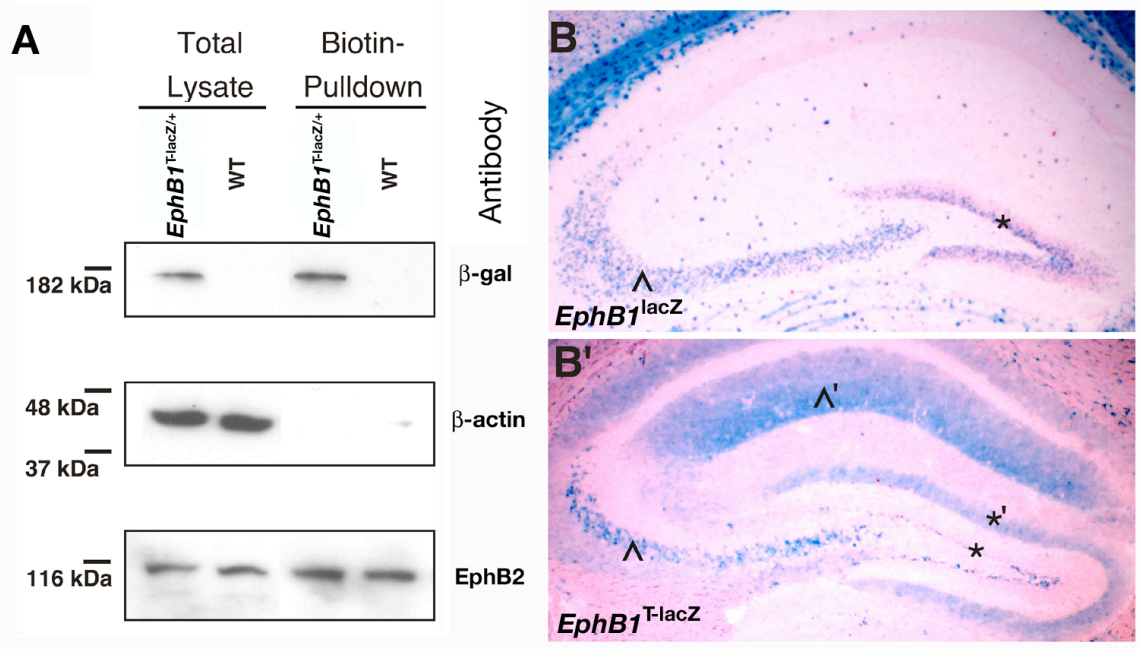


Fig 2.4: The EphB1-β-gal fusion protein is expressed on the cell surface in axons and other cellular processes

A. Immunoblot of proteins isolated from primary cells whose extracellular proteins were labeled with biotin and purified with streptavidin-agarose beads. Total protein lysates (left) of cells from WT and *EphB1*$^{+/lacZ}$ heterozygote littermate embryos collected at E13.5 were compared to the purified biotin-labeled fraction (right) using antibodies raised against β-gal for EphB1-β-gal fusion protein, β-actin as intracellular control, and EphB2 as extracellular control. **B.** X-gal stains of hippocampal sections of adult *EphB1*^{lacZ} mice (**B**) where β-gal activity is localized only in the cell bodies of CA3 neurons (^) and neural progenitors of the dentate gyrus (*) and *EphB1*$^{T\text{-}lacZ}$ mice (**B'**) where staining is visible in both the cell bodies and their processes, including CA3 axons that target into the CA1 region (^') and in the extensions of SGZ progenitor cells that reach into the molecular layer of the dentate gyrus (*').

EphB1$^{T\text{-}lacZ/T\text{-}lacZ}$ mice do not phenocopy hyperalgesia seen in *EphB1*$^{-/-}$ null mice

Neuropathic hyperalgesia is a physical condition where sensory input becomes adversely heightened resulting in an inappropriately sensitive pain response. A mouse model has been generated for sciatica, a specific type of this disorder where the sciatic nerve becomes over activated by normal stimulation. This model used was chronic constrictive injury (CCI) to simulate neuropathic hyperalgesia. In this model, several ligatures were tied around a mouse's left sciatic nerve. In a normal hyperalgesic state, the left foot will become hypersensitive to an applied heat source in the while the right has a normal response. This is measured by the time it takes the mouse to retract a hindpaw from the heat source. The retraction latency between the two feet can be directly compared. For reasons yet to be determined, mice with a loss or reduction of EphB1 (i.e. *EphB1*$^{-/-}$ protein null and *EphB1*$^{+/-}$, respectively) do not display this hyperalgesic response (Han et al., 2008).

In addition to the phenotype seen in *EphB1*$^{-/-}$ protein null and *EphB1*$^{+/-}$ heterozygous mice, the intrathecal administration of ephrin-B1-Fc, a conjugate of ephrin-B1 ECD to human immunoglobulin G-crystallizable fragment (IgG-Fc), results in the temporary induction of neuropathic pain (Battaglia et al., 2003; Song et al., 2008a). This suggests that the activation of EphB leads to increased pain response, so it appeared that EphB1 forward signaling is crucial in the generation of the hyperalgesic state. However, the *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ mice show no difference in retraction latency between the two feet when compared to WT (Fig. 2.5).

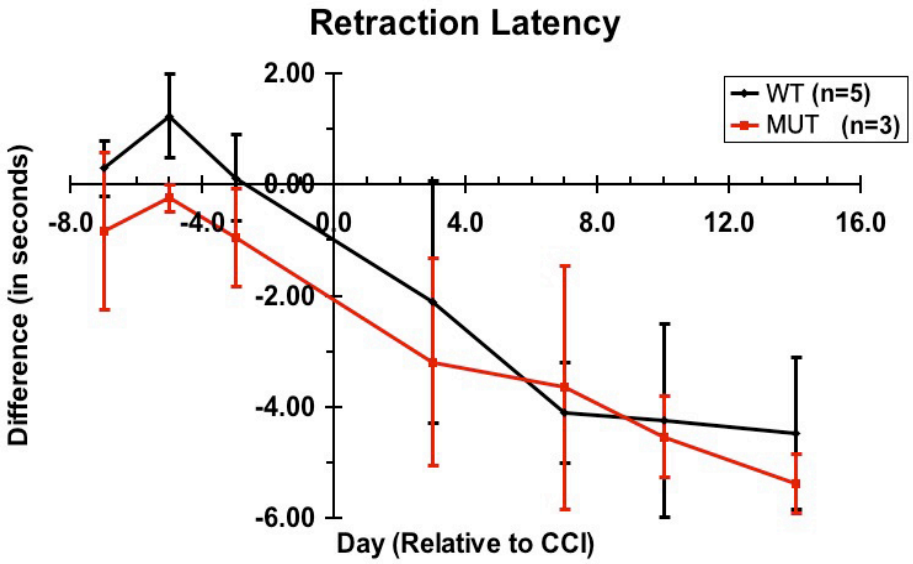


Fig 2.5: *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ mutant mice exhibit normal neuropathic pain response

WT (black line) and *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ (red line) mice had their sensitivity to heat stimulus of their right and left hindpaw measured before and after CCI procedure was performed on left sciatic nerve, and the resulting difference (in seconds) of left to right is shown. Both the WT and mutant mice showed a marked increase in sensitivity to pain in their left hindpaw following the procedure.

Generation of ephrin-B2$^{6YF\Delta V}$ mutant mouse

Rebekkah Warren was responsible for the creation of the TV for this mutant, and I targeted and screened the ES cells. The process relied on recombineering similar to the methods described above (Fig. 2.6 A). Of 400 clones screened, 2 positives were retrieved and injected into blastocysts of which one was successfully integrated. Proper integration was confirmed by southern analysis (Fig. 2.6 B&C), PCR, and sequencing (data not shown). Additionally, mice that are homozygous for this mutation are semi-viable. While all *ephrin-B2*$^{-/-}$ protein null and *ephrin-B2*$^{lacZ/lacZ}$ mice fail to live past birth, some *ephrin-B2*$^{6YF\Delta V/6YF\Delta V}$ homozygotes have survived to adulthood. Additionally, mice that are heterozygous for the truncated-β-gal mutation and the 6YFΔV mutations do not exactly phenocopy mice that are truncated-β-gal homozygotes. These data suggest that this new *ephrin-B2*$^{6YF\Delta V}$ mutant protein is functional, so the modified form of ephrin-B2 is making it to the cell surface and is operating as hoped.

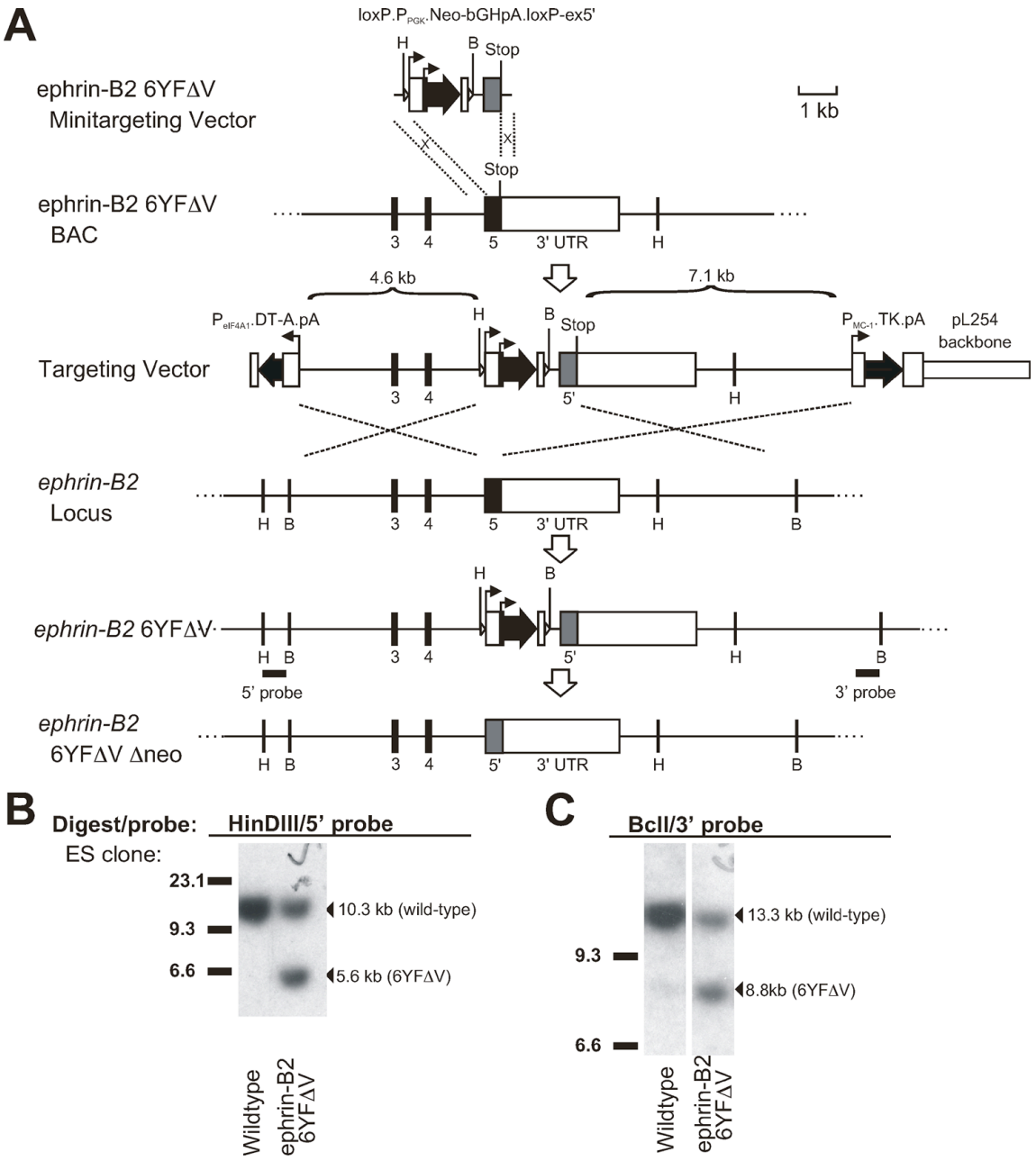

A
loxP.P$_{PGK}$.Neo-bGHpA.loxP-ex5'
H
B
Stop
ephrin-B2 6YFΔV
Minitargeting Vector
1 kb
Stop
ephrin-B2 6YFΔV
BAC
3
4
5
3' UTR
H
4.6 kb
7.1 kb
P$_{eIF4A1}$.DT-A.pA
P$_{MC-1}$.TK.pA
pL254
backbone
Targeting Vector
5'
ephrin-B2
Locus
ephrin-B2 6YFΔV
5' probe
3' probe
ephrin-B2
6YFΔV Δneo
B
Digest/probe:
HinDIII/5' probe
ES clone:
23.1
9.3
6.6
10.3 kb (wild-type)
5.6 kb (6YFΔV)
Wildtype
ephrin-B2
6YFΔV
C
BclI/3' probe
13.3 kb (wild-type)
8.8kb (6YFΔV)

Fig 2.6: Generation of *ephrin-B2*$^{6YF\square V}$ mutant mice

A. The cloning and southern blot strategy that was used in the creation of the *ephrin-B2*$^{6YF\Delta V}$ mutation. Recombineering was used to convert the codons for all intracellular tyrosines of ephrin-B2 to phenylalanine and to remove the C-terminal valine residue to disrupt interactions with SH2 domain and PDZ domain containing proteins, respectively.

B and **C.** Southern blot results for a WT and *ephrin-*$^{B26YF\Delta V/+}$ heterozygote mouse when screening the 5' end (B) and the 3' end (C).

Discussion

Mutant mice are correctly targeted and retain partial function

I verified the insertion of the *EphB1*$^{T\text{-}lacZ}$ mutation into its respective loci through southern blot, PCR, sequence, and β-gal expression analysis. While I expected this mutant to display some overlap in the phenotype with the *EphB1*$^{-/-}$ protein null, there still remains an intact extracellular domain. As will be discussed in detail later, the lack of the intracellular domain is sufficient to phenocopy the null mutant in other aspects. However, it does not seem necessary for the role EphB1 plays in generating neuropathic pain in mice, for previous studies have shown that the complete removal of EphB1 results in the inability of mice to develop hyperalgesia (Han et al., 2008).

Not only does this experiment show that canonical EphB1 forward signaling is not necessary for the generation of neuropathic pain in mice, it also shows that the new truncated EphB1-β-gal fusion protein is still functionally relevant. Because *EphB1*$^{+/-}$ heterozygous mice show a phenotype (Han et al., 2008), even slight alterations in the amount of functional EphB1 at the cell surface can result in a phenotype. However, the *EphB1*$^{T\text{-}lacZ/lacZ}$ mice do not show any noticeable phenotype. This greatly enhances the argument that these newly created mice are not only making functionally relevant EphB1 ECD, but also that it is being trafficked to the membrane and transported down axons to the appropriate targets at biologically relevant levels.

The *ephrin-B2*$^{6YF\Delta V}$ mutation was also verified by southern blot, PCR, and sequencing analysis. Furthermore, preliminary data shows that this point mutant produces a protein that reaches the cell surface (data not shown). Moreover, as in the

EphB1$^{T\text{-}lacZ}$ mutant mice, these mutants do not phenocopy *ephrin-B2*$^{-/-}$ protein null mice. While *ephrin-B2*$^{-/-}$ protein null mice die in early embryonic development (Adams et al., 2001; Cowan et al., 2004), *ephrin-B2*$^{6YF\Delta V/6YF\Delta V}$ can reach adulthood and even produce progeny. Nevertheless, there remains a partial lethality in the homozygous state, as these mice do not reproduce at Mendelian ratios. This is similar to the previously established *ephrin-B2*$^{lacZ/lacZ}$ homozygous mutant, which will outlive the protein null but do not live past birth (Adams et al., 2001; Cowan et al., 2004). This clearly suggests that *ephrin-B2*$^{6YF\Delta V}$ mutant mice are producing a protein that is able to reach the cell surface and retain partial function.

Chapter 3

The intracellular domain of EphB1 is essential for proper axon pathfinding at the optic chiasm

Summary

EphB receptor tyrosine kinases direct axonal pathfinding through interactions with ephrin-B proteins following axon-cell contact. As in the *EphB1*$^{-/-}$ protein-null animals, the *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ homozygotes fail to form the ipsilateral projecting subpopulation of retinal ganglion cell (RGC) axons. This indicates that forward signaling through the intracellular domain of EphB1 is required for proper axon pathfinding of RGC axons at the optic chiasm. Further analysis of other *EphB2*^{lacZ} forward signaling mutant mice shows that EphB1 is the preferred receptor in mediating axon guidance at the optic chiasm despite the coexpression of EphB2 in the same ipsilaterally projecting RGC axons.

Ipsilateral RGC projections and depth perception

Within the retina, sensory rods and cones are stimulated by light and activate bipolar, horizontal, and amacrine interneurons that will then activate retinal ganglion

cells (RGCs). These retinal ganglion cells will then take this input and send the information to the thalamus in the midbrain, where another group of neurons that will receive the information from synapses with RGC axons and will then carry the information to the primary visual cortex. It is in the cortex that all input is assimilated and visual perception achieved (Kandel et al., 2000). For this circuit to be established, RGC axons must find their way from the retina to their targets in the optic tectum in animals like fish, frogs, and birds or the lateral geniculate nucleus (LGN) and superior colliculus in mammals.

In animals with binocular vision, a subset of RGC axons will project to the ipsilateral hemisphere, and animals that have a higher degree of overlapping vision will in turn have a larger percentage of retinal axons project ipsilaterally at the optic chiasm (Jeffery and Erskine, 2005). The information from this ipsilateral originating group will be incorporated with the corresponding contralateral hemisphere projection, and the coordination of the images will impart the ability for depth perception (Kandel et al., 2000; Blake and Wilson, 2010). This process is highly dependent upon the sorting of optic nerve axons to sort to either the ipsilateral or contralateral hemisphere.

The optic chiasm is the midline choice point where retinal axons choose whether or not to cross. Being a white matter tract, the optic nerve and chiasm are obvious structures to the naked eye and thus caught the attention of anatomists through history. The optic chiasm earned its name in ancient Greece due to its resemblance to the greek letter chi (χ) and was even referred to as such by the philosopher Aristotle (Yanoff and

Duker, 2004). Remarkably, it was correctly theorized by the 13th century C.E[5]. that the optic nerve partially decussates at the optic chiasm. Furthermore, another of the greatest minds in science, Isaac Newton, studied and expanded on the importance of the partial decussation of the optic nerve in his book "Opticks: Or a Treatise of the Reflections, Refractions, Inflections & Colours of Light" (Jeffery, 2001). Not be left behind, Ramón y Cajal also examined the optic nerve and created a beautiful diagram of its partial decussation at the optic chiasm while attempting to understand how neuronal axons navigate through the brain (Ramón y Cajal, 1898).

Development of visual system

In the mouse RGCs begin to form at E11 (Drager and Olsen, 1980) and by E12 start sending early pioneer axons that all cross to the contralateral hemisphere (Silver, 1984; Godement et al., 1990; Marcus and Mason, 1995), but around E14.5, a change occurs and a subset of VT-RGC axons make the decision to project ipsilaterally (first shown in Drager and Olsen, 1980). By E15.5, there is an ipsilateral projection established, and more RGC axons continue to pathfind at the optic chiasm choosing which hemisphere to target until around E17.5 when most ipsilateral RGC axons have

[5] Albert the Great, a Germanic nobleman and scholar, observed a soldier with a head injury on his left temple that resulted in the loss of vision in his right eye. From this, he postulated that the optic nerve did not entirely project to the ipsilateral side as was the commonly held supposition of that era (the importance of contralateral projections to the brain not having been established), but that some of it may project contralaterally (Theiss et. al., 1994).

finished pathfinding (This process was nicely diagrammed in developing mice in (Sretavan, 1990). After the ipsilateral tract is established, RGCs continue to send axons through the optic chiasm until birth (Colello and Guillery, 1990). Interestingly, RGC axons tend to sort relative to when they began pathfinding, and age-related ordering of axon bundles is lost when axons enter the decision point at the chiasm. This is not regained until after axons exit the chiasm (Colello and Guillery, 1998).

So began the quest to determine why some retinal axons choose to cross while another subset growing at the same time would be repelled at the chiasm. One early hypothesis for controlling retinal axon laterality was that this subset was dependent upon the tracts stemming from the opposite, which is logical considering that where these two nerves meet is the choice point. However, this was disproved when the removal of one eye was shown not to alter the formation of ipsilateral projections at the chiasm. Furthermore, this study showed that when an RGC axon reaches the optic chiasm, it takes about 10-20 minutes for it to be deflected to the same hemisphere. Additionally, contralateral projections will sometimes stall while passing through the optic chiasm while ipsilateral projections do not (Sretavan and Reichardt, 1993). This stalling suggested a signaling mechanism present within the chiasm itself where growth cones facing guidance cues had to make a choice.

This was followed by further proof of the importance of glial cells within the chiasm, for at E15, a wall of SSEA-1 positive radial glia cells forms which deflects VT-RGC axons rendering them unable to cross (Marcus et al., 1995). This glial structure corresponds to the previously discovered line ~100-200 μm where RGC axons normally diverge when projecting ipsilaterally (Godement et al., 1990). Furthermore, RGC axons

that normally cross were unfazed when cultured with optic chiasm cells while VT retinal explants projected axons that avoided optic chiasm cells (Wang et al., 1995). Although this repulsive effect from these glial cells caused the midline decision candidates to be narrowed, the potential list of molecules operating at the chiasm was still large. For example, the regulation of sonic hedgehog signaling was also demonstrated to effect the ability of the RGC axons to cross at the chiasm. In mice without Pax2, levels of Shh remain elevated at the chiasm when they should be downregulated and as a result all RGC axons project ipsilaterally (Torres et al., 1996). As increased expression of Shh also inhibits RGC axon outgrowth in chicks (Trousse et al., 2001), this was a candidate of interest.

It was, however, an experiment in frogs that first linked EphB and ephrin-B molecules to the guidance of retinal axons at the chiasm, for ephrin-B expression at the chiasm proved essential in determining retinal axon laterality (Nakagawa et al., 2000). (These experiments are discussed in more detail in the following chapter.) EphB function showed to be conserved in mammals, for *EphB1*$^{-/-}$ protein null mutant mice display a severe failure in the ability of retinal axons to project ipsilaterally (Williams et al., 2003). Additionally, mice that lack both Vav2 and Vav3 show a decrease in the percentage of ipsilaterally projecting RGC axons. Furthermore, Vav interacts directly with EphB proteins and regulates their ability to be endocytosed and thus for repulsion to occur (Cowan et al., 2005), so these Vav mutant mice confirmed the role of EphB:ephrin-B mediated axon repulsion at the chiasm and elucidated on the mechanism of function. The importance of EphB1 signaling was further established when dorsal retinal axons that

otherwise are directed contralaterally were redirected to the ipsilateral hemisphere when EphB1 was misexpressed (Petros et al., 2009).

Although critical, EphB:ephrin-B interactions are not the exclusive regulators to determine RGC axon laterality. For example, Nr-CAM is expressed in contralaterally projecting RGC axons. When Nr-CAM is blocked, there is an increase in the percentage of ipsilaterally projecting cells, which was shown to operate in a unique pathway from Eph-ephrin interactions (Williams et al., 2006). Similarly, Semaphorin 3D is secreted in the chiasm and aids contralaterally crossing fibers in zebrafish (Sakai and Halloran, 2006), but this is likely linked to Nr-CAM function. Furthermore, the addition of α-CD44 to E13 and E14 embryonic optic chiasm to block normal CD44 functioning, which normally acts as a negative regulator of axon growth (Sretavan et al., 1994), resulted in a large decrease in the percentage of contralaterally projecting RGC axons (Lin and Chan, 2003).

In the following sections of this chapter, I will focus on the role EphBs perform at the chiasm. I will show that EphB1 forward signaling is necessary to direct ventrotemporal (VT) RGC axons ipsilaterally at the optic chiasm, and I will show that EphB2 forward signaling is also involved in forming the ipsilateral projection although its role is subservient to that of EphB1.

Results

Measuring minor changes in percentage of ipsilateral projections

In order to carefully examine the role of EphB1 and possibly of EphB2 in the formation of the ipsilateral RGC axon projection, a sensitive *in vivo* assay was created to measure a relative percentage of ipsilaterally projecting axons that reach the LGN. Using adult mice ~2 months of age, RGCs in the right eye were labeled with cholera-toxin subunit-B bound to AlexaFluor 555 (CTB-555, red), while RGCs in the left eye were simultaneously labeled with CTB-488 (green). After allowing the tracers to undergo anterograde transport for two days, the entire LGN of WT and mutant mice was visualized in serial coronal vibratome sections. Both the ipsilateral and contralateral projections are visible in one image where green in the left hemisphere LGN of each section represents the ipsilateral projections while red represents the contralateral projections, and vice versa for the corresponding right hemispheres.

To verify that this method can distinguish subtle differences in the percentage of ipsilateral to contralateral projections reaching the LGN, this technique was performed on WT mice with different pigments, for albino mice have a diminished percentage of RGC axons that project to the ipsilateral hemisphere (Drager, 1985; Jeffery, 2001). For our studies, I used combinations of two strains of mice, albino CD1 outbred mice and pigmented 129 inbred mice, which have four known alleles that effect coat color: white bellied agouti (A^{w}), pink eyed dilution (p), chinchilla (c^{ch}), and albino (c). When isolated, the coat colors for each of these mice are brown with dark eyes, light yellow with pink eyes, grey with dark eyes, and non-pigmented with pink eyes, respectively

(Porter et al., 1991; Bultman et al., 1994; Simpson et al., 1997; Brilliant, 2001; Steingrimsson et al., 2006). Mice with brown coat color display a larger percentage of ipsilaterally projecting RGC axons reaching the LGN than mice with other pigmentation (Fig. 3.1 A,B). Further, this data is quantifiable and the difference is statistically significant for the majority of the LGN, specifically for the more caudal portion of the LGN (Fig. 3.1 C). These data show that this method can be applied to quantify minor changes in the ability of RGC axons to target the ipsilateral hemisphere and suggests that the A^w allele partially regulates the ability of RGC axons to properly target the LGN.

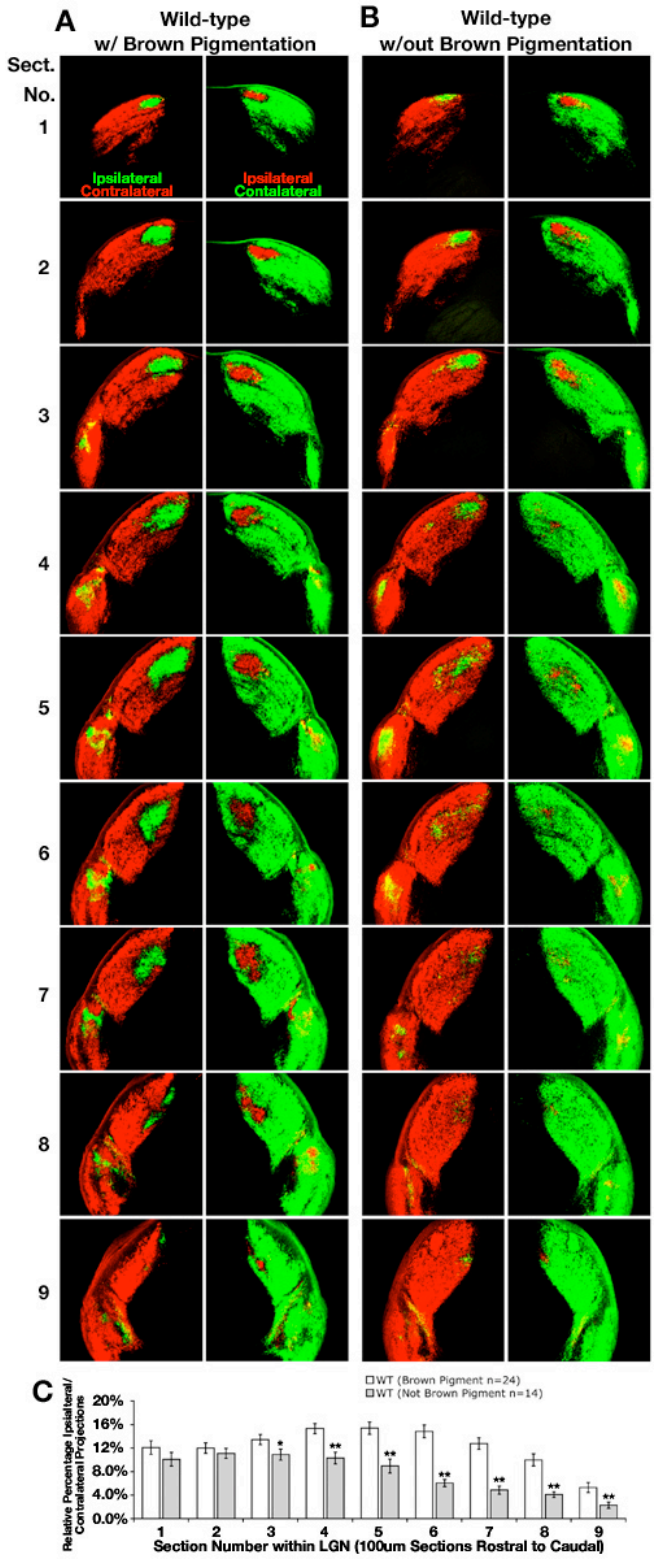

A
Wild-type
w/ Brown Pigmentation
B
Wild-type
w/out Brown Pigmentation
Sect.
No.
1
2
3
4
5
6
7
8
9
Ipsilateral
Contralateral
Ipsilateral
Contalateral
C
WT (Brown Pigment n=24)
WT (Not Brown Pigment n=14)
Relative Percentage Ipsilateral/
Contralateral Projections
20%
16%
12%
8.0%
4.0%
0.0%
1
2
3
4
5
6
7
8
9
Section Number within LGN (100um Sections Rostral to Caudal)

Fig 3.1: Brown pigmented mice possess a larger percentage of ipsilateral projections

A-B. Serial sections through the LGN of a WT mouse with brown pigmentation (**A**) and a WT mouse without brown pigmentation (**B**). The RGCs were labeled with CTB-555 (red) anterograde dye in right eye and CTB-488 (green) in the left eye to compare changes in the amount of ipsilaterally projecting RGC axons. **C**. Quantitative analysis showing the differences in relative percentage of ipsilaterally projecting RGC axons throughout the entire LGN of each genotype (* indicates $p<0.05$, ** indicates $p<0.01$).

EphB1$^{T\text{-}lacZ}$ mutants have a strongly reduced ipsilateral projection

When compared with WT, *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ mutant mice displayed a strongly reduced ipsilateral projection that was equivalent to the *EphB1*$^{-/-}$ protein-nulls (Fig. 3.2 A-C; Fig. 3.3). Both mutants showed a statistically significant decrease from WT throughout the entirety of the LGN (Fig. 3.2 E), and there was no statistically significant difference evident between these two groups (Fig. 3.2 F). Thus, mice lacking the intracellular domain of EphB1 phenocopy *EphB1*$^{-/-}$ null mutant mice. This data strongly indicates that the intracellular domain of EphB1 is required to transduce forward signals necessary to deflect ventrotemporal RGC axons away from the optic chiasm such that they project ipsilaterally.

The ipsilateral projection is more reduced in *EphB1*$^{T\text{-}lacZ}$: *EphB2*^{lacZ} compound mutants

Because EphB2 is also expressed in ventral RGCs at the time their axons are being directed to the ipsilateral hemisphere (Holash and Pasquale, 1995; Henkemeyer et al., 1996; Birgbauer et al., 2000), the potential role of this receptor was examined. Compared with *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ single mutants, *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$:*EphB2*$^{lacZ/lacZ}$ compound mutants expressing two intracellular truncated EphB-β-gal fusion proteins showed a more extreme reduction in the percentage of ipsilaterally projecting RGC axons where some sections of the LGN showed no visible ipsilateral projections remaining (Fig. 3.2 D). This decrease in the ipsilateral projection of compound mutants was statistically significant when compared with *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ single mutants in 4 of the 9 sections through the LGN (Fig. 3.2 F). This data suggests EphB2 may assist EphB1 in controlling

RGC axon pathfinding decisions at the optic chiasm. In another group of mice analyzed separately based on effect from coat color (see methods), no observable or significant difference between WT and *EphB2*$^{lacZ/lacZ}$ single mutant mice was found (Fig. 3.4). Thus, unlike *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ single mutants, loss of the intracellular domain of EphB2 alone does not appear to affect the ipsilateral projection.

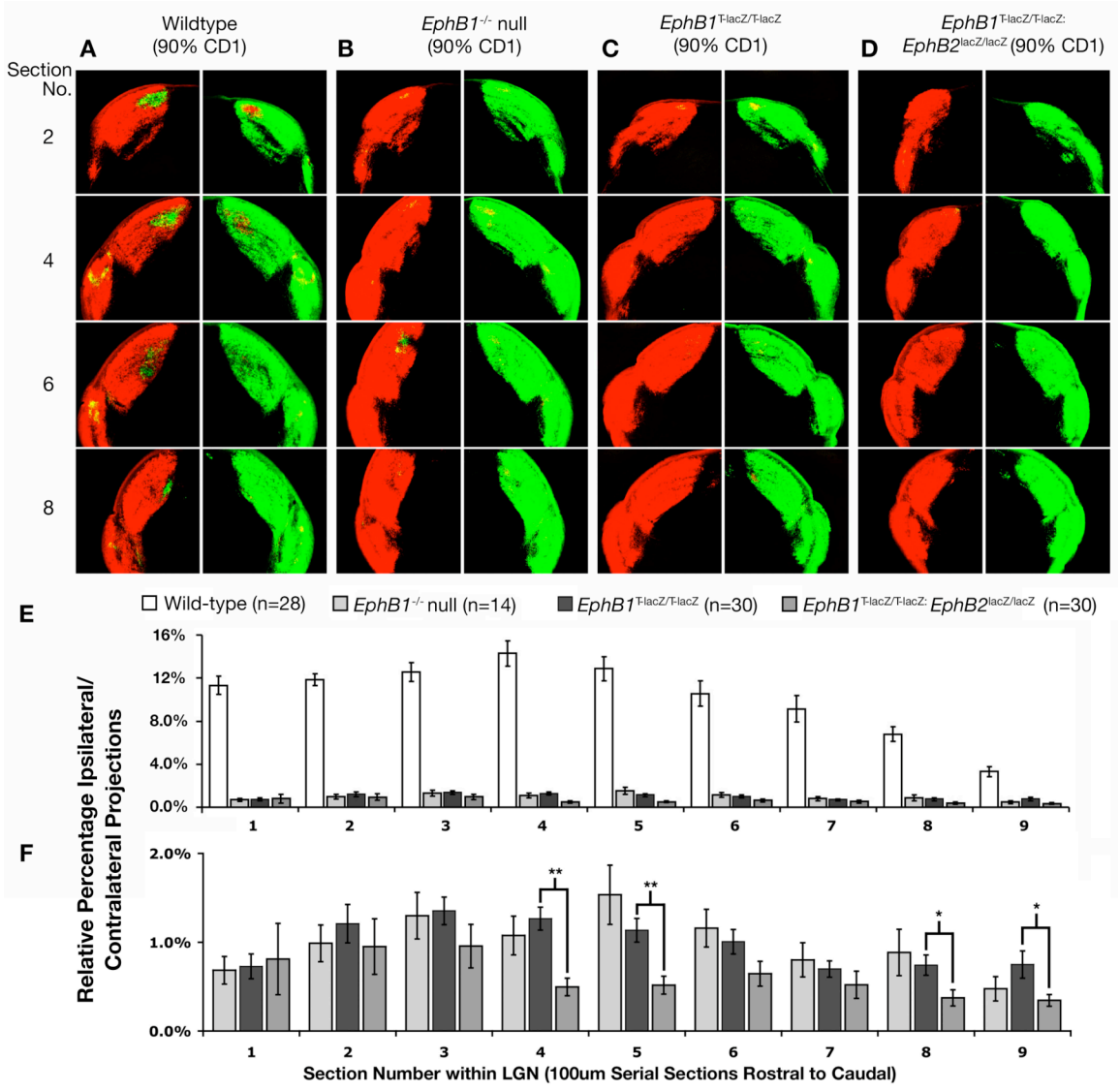

A
Wildtype
(90% CD1)
B
EphB1-/- null
(90% CD1)
C
EphB1TlacZ/TlacZ
(90% CD1)
D
EphB1TlacZ/TlacZ:
EphB2lacZ/lacZ (90% CD1)
Section No.
2
4
6
8
E
Wild-type (n=28)
EphB1-/- null (n=14)
EphB1TlacZ/TlacZ (n=30)
EphB1TlacZ/TlacZ: EphB2lacZ/lacZ (n=30)
16%
12%
8.0%
4.0%
0.0%
1
2
3
4
5
6
7
8
9
F
Relative Percentage Ipsilateral/ Contralateral Projections
2.0%
1.0%
0.0%
**
**
*
*
1
2
3
4
5
6
7
8
9
Section Number within LGN (100um Serial Sections Rostral to Caudal)

Fig 3.2: *EphB1*$^{\text{T-lacZ/T-lacZ}}$: *EphB2*$^{\text{lacZ/lacZ}}$ compound mutant mice show a more drastic decrease in ipsilateral projections than EphB1 single mutant mice

EphB1$^{\text{T-lacZ/T-lacZ}}$ mutant mice show a drastic decrease in the ipsilateral projection. **A-D**. Representative regions of serial coronal sections through the LGN of WT (***A***), *EphB1*$^{-/-}$ null homozygote (**B**), truncated *EphB1*$^{\text{T-lacZ/T-lacZ}}$ homozygote (**C**), and *EphB1*$^{\text{T-lacZ/T-lacZ}}$: *EphB2*$^{\text{lacZ/lacZ}}$ compound homozygote (**D**) mice. All RGCs were labeled with CTB-555 (red) in right eye and CTB-488 (green) in the left eye and allowed to anterogradely label their terminations in the LGN to compare changes in the amount of ipsilaterally projecting RGC axons. **E**. Quantitative analysis showing the differences in relative percentage of ipsilaterally projecting RGC axons throughout the entire LGN of each genotype (All mice are 90%CD1 strain; * indicates $p<0.05$, ** indicates $p<0.01$).

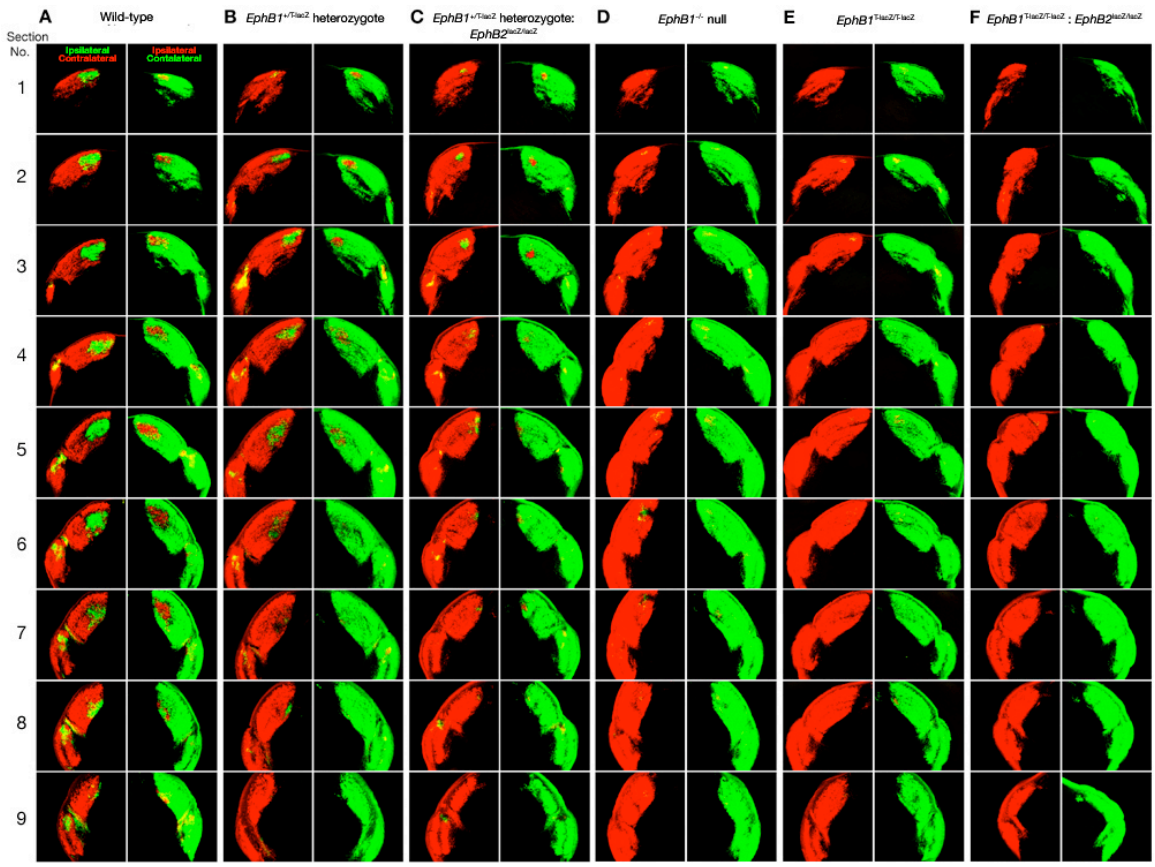


Fig 3.3: Entire LGN of EphB mutant mice

A-F. Serial sections through the entire LGN of WT (**A**), *EphB1*$^{+/T\text{-}lacZ}$ heterozygote (**B**), *EphB1*$^{+/T\text{-}lacZ}$ heterozygote: *EphB2*$^{lacZ/lacZ}$ homozygote (**C**), an *EphB1*$^{-/-}$ homozygote (**D**), *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ homozygote (**E**), and *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$: *EphB2*$^{lacZ/lacZ}$ compound mutant (**F**) mice. The RGCs were labeled with CTB-555 (red) anterograde dye in right eye and CTB-488 (green) in the left eye to compare changes in the amount of ipsilaterally projecting RGC axons (All mice are 90% CD1.)

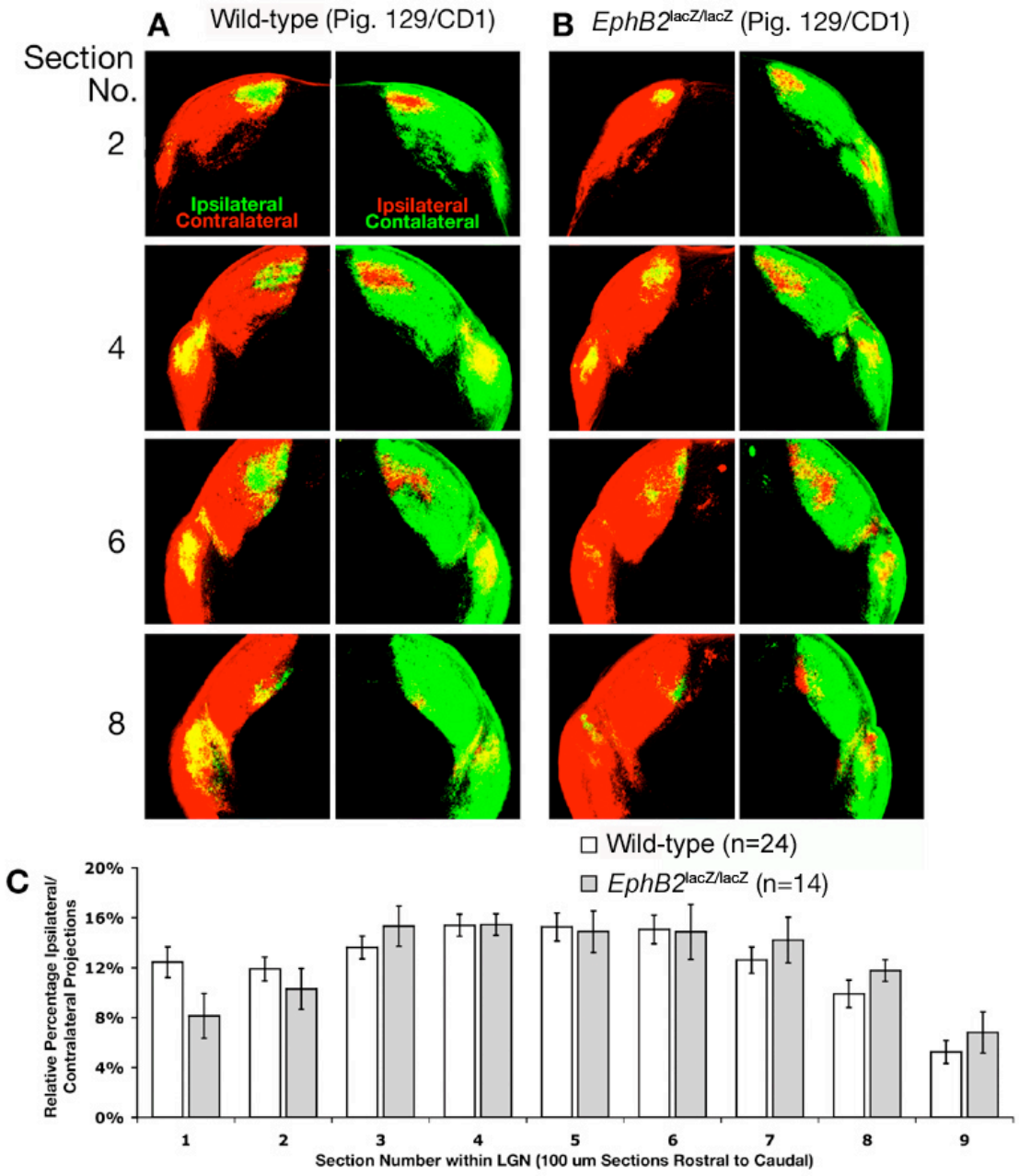

A
Wild-type (Pig. 129/CD1)
B
EphB2lacZ/lacZ (Pig. 129/CD1)
Section No.
2
4
6
8
Ipsilateral
Contralateral
Ipsilateral
Contalateral
C
Wild-type (n=24)
EphB2lacZ/lacZ (n=14)
Relative Percentage Ipsilateral/ Contralateral Projections
20%
16%
12%
8%
4%
0%
1
2
3
4
5
6
7
8
9
Section Number within LGN (100 um Sections Rostral to Caudal)

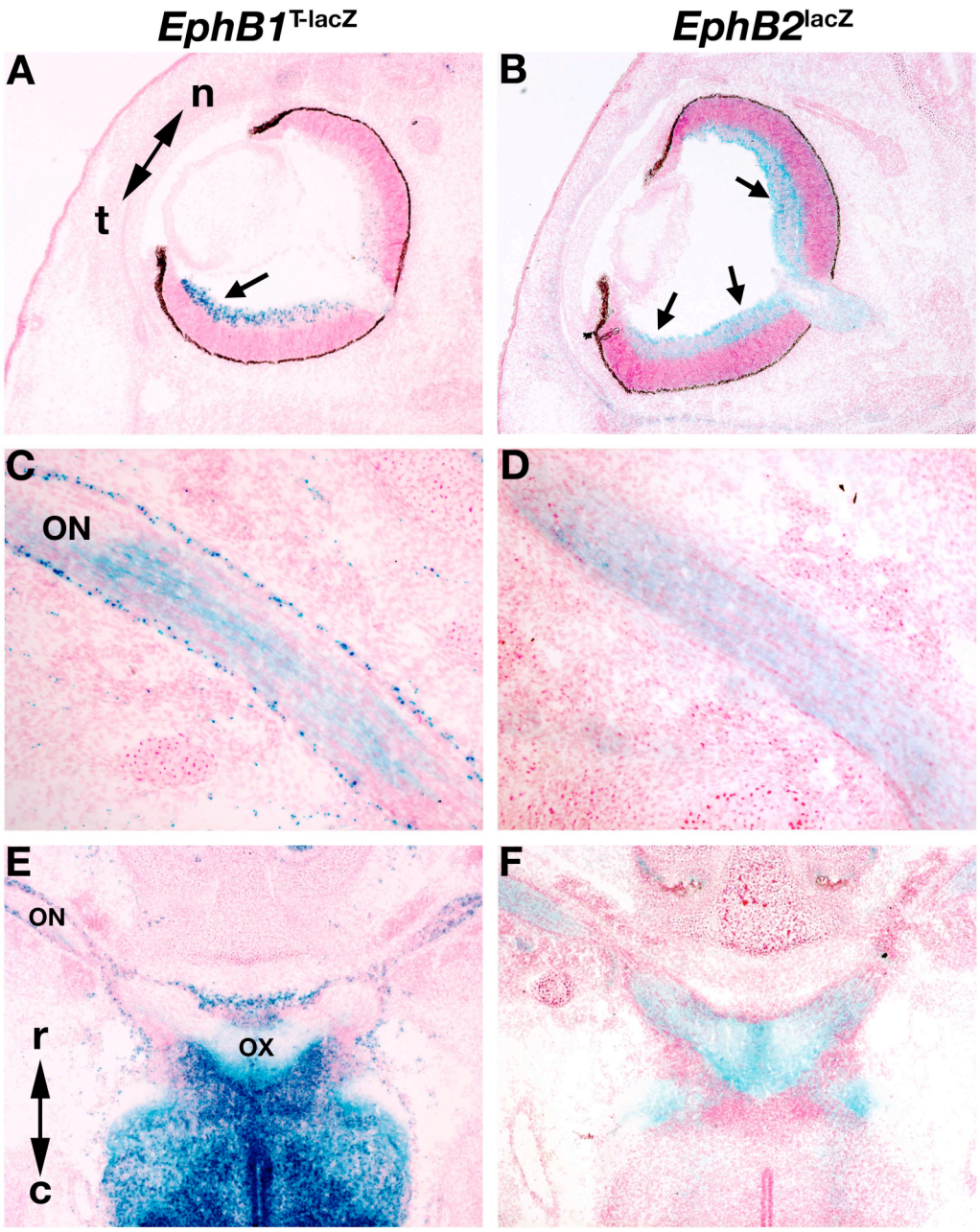
Fig 3.4: No difference visible in the percentage of ipsilaterally projecting RGC axons of WT and EphB2$^{lacZ/lacZ}$ mutant mice

Serial sections through the LGN of WT (**A**) and truncated *EphB2*$^{lacZ/lacZ}$ homozygote (**B**) mutant mice of 129/CD1 brown pigmented strain. The RGCs were labeled with CTB-555 (red) anterograde dye in right eye and CTB-488 (green) in the left eye to compare changes in the amount of ipsilaterally projecting RGC axons. **C**. Quantitative analysis showing the differences in relative percentage of ipsilaterally projecting RGC axons throughout the entire LGN of each genotype (No statistically significant difference was found for any region).

EphB1 and EphB2 expression patterns in the retina, optic nerve, and optic chiasm

In early stages of retinal axon pathfinding (up to E14.5), all RGCs axons cross to the contralateral hemisphere, and then, around E15.5/E16.5 axons originating from the VT portion of the retina are deflected from the main bundle at the optic chiasm to instead project to the ipsilateral side of the brain (Godement et al., 1990; Sretavan, 1990). To better characterize when and where EphB1 and EphB2 are functioning while directing RGC axons at the optic chiasm, the expression patterns of both receptors were examined at the retina, optic nerve, and optic chiasm by staining for the highly sensitive EphB1-β-gal and EphB2-β-gal fusion proteins.

X-gal stains of horizontal cryosections of E16.5 mutant mouse embryos revealed highly localized expression in the temporal region of the ventral retina for EphB1-β-gal (Fig. 3.5 A) while EphB2 was expressed throughout the ventral retina (Fig. 3.5 B). EphB1-β-gal and EphB2-β-gal were also both visible throughout the length of the optic nerve (Fig. 3.4 C&D) and reached the optic chiasm (Fig. 3.5 E&F). This data suggests that both EphB1 and EphB2 are expressed in RGCs and are transported down their axons through the optic nerve to reach the optic chiasm. Therefore, they are both present when RGC axons are deciding which hemisphere to target.



E16.5 Horizontal Sections

Fig 3.5: EphB1 and EphB2 are present in the eye, optic nerve, and optic chiasm at E16.5

A-B. X-gal stains of horizontal sections at E16.5 at the ventral region of the eye. *EphB1*$^{T\text{-}lacZ}$ mice (**A**) show higher β-gal activity confined temporally (t) while *EphB2*^{lacZ} mice (**B**) have present β-gal activity from temporal to nasal (n) portions of the retina. **C-D**. Horizontal sections show that both EphB1 (**C**) and EphB2 (**D**) β-gal fusion proteins are transported down the optic nerve (ON). **E-F**. Horizontal sections through the optic chiasm (OX) show that both EphB1 (**E**) and EphB2 (**F**) β-gal fusion proteins are present at the optic chiasm originating from the ON, while EphB1 is uniquely present in the area surrounding the OX.

EphB1 and EphB2 are coexpressed on the same RGC axons

To determine whether EphB1 and EphB2 are coexpressed on the same RGC axons projecting from the VT region of the retina, retinal explants from the dorsal and VT regions of the retina were dissected at E15.5, cultured to promote axon outgrowth for 16-20 hours, and labeled for EphB1 and EphB2 expression. Due to the lack of a specific antibody for EphB1, this was not previously possible; however, with the truncated EphB1-β-gal fusion, the inherent β-gal activity was used to specifically label EphB1 expressing axons by exposing cultured retinal explants to the substrate 5-6 X-gal (Mohler and Blau, 1996), which is converted in the presence of β-gal into an insoluble fluorescent byproduct. Cells were simultaneously labeled with an α–EphB2 antibody to identify EphB2 protein and Cy5-conjugated phalloidin to label all axonal projections.

While low expression of both EphB1 and EphB2 was detected in all axonal projections from explants taken from the dorsal region of the retina (Fig. 3.6 A), higher levels of both EphB1 and EphB2 were present in axons from explants of the VT region of the retina (Fig. 3.6 B). EphB2 expression was consistently elevated in all axons in the VT explants, and there were some axons with high relative levels of EphB1 and others with lower levels (Fig. 3.6 B). As negative controls, explants from embryos that were *EphB2*$^{-/-}$ null did not show any axonal staining with the α–EphB2 antibody (Fig. 3.6 C), and explants from animals WT for EphB1 did not show any β-gal related fluorescence on axonal projections or cell bodies (Fig. 3.6 D). As the subset of EphB1 expressing RGC axons from the VT explants also express EphB2, the potential exists for EphB2 to also function in guidance of axons at the optic chiasm.

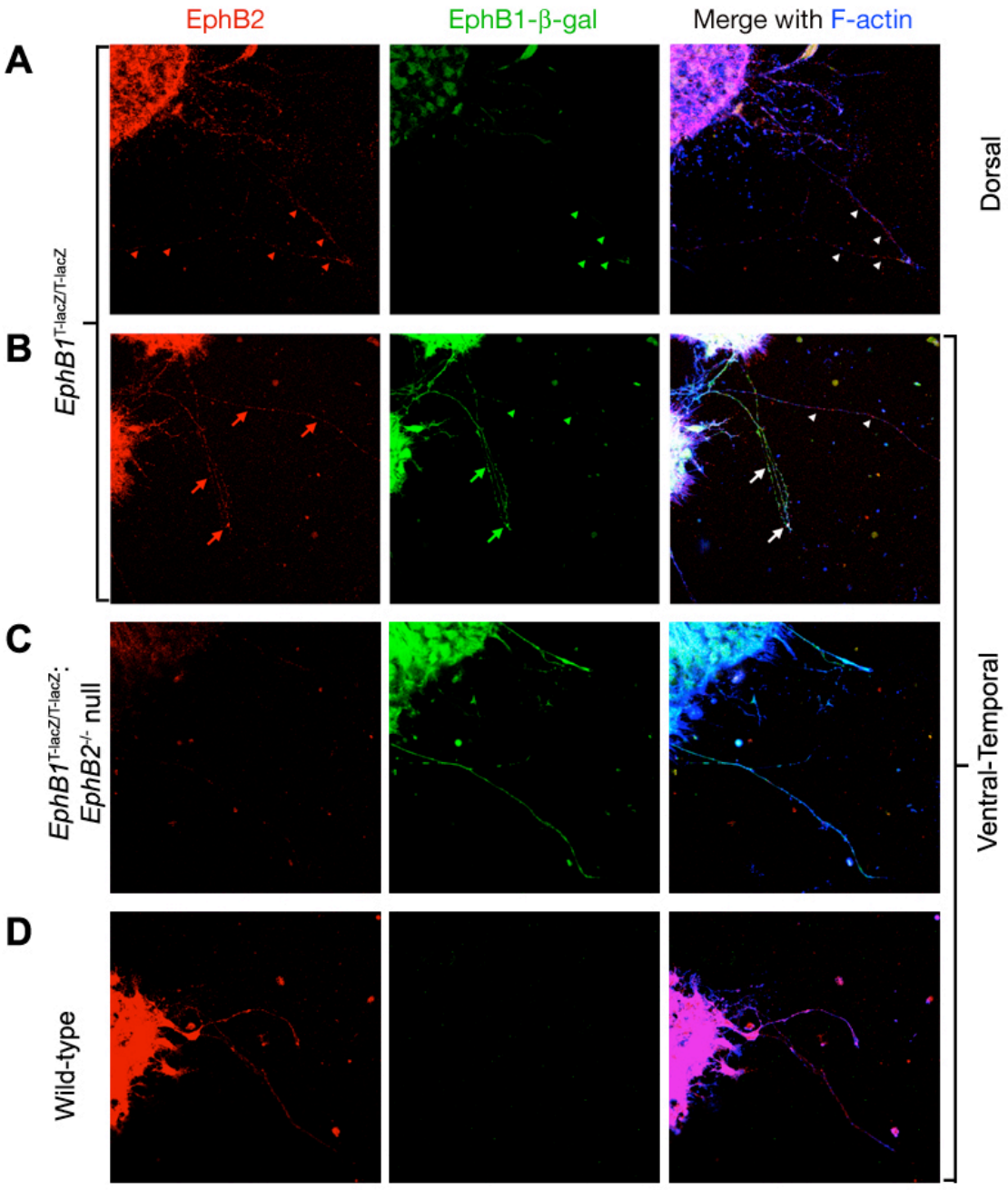

EphB2
EphB1-β-gal
Merge with F-actin
A
B
C
D
EphB1T-lacZ/T-lacZ
EphB1T-lacZ/T-lacZ; EphB2-/- null
Wild-type
Dorsal
Ventral-Temporal

Fig 3.6: EphB1 and EphB2 are coexpressed in VT RGC axons

Retinal explants from *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ (**A**&**B**), *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$: *EphB2*$^{lacZ/lacZ}$ (**C**), and wild-type (**D**) E15.5 stage embryos were removed, cultured, exposed to a fluorescent substrate recognized by β-gal (green), immunostained with α-EphB2 antibodies (red), and exposed to phalloidin conjugated to cy-5 (blue). **A**. RGCs from the dorsal portion of the retina extended axons that co-express (white arrowheads) low levels of EphB2 (red arrowheads) and EphB1-β-gal (green arrowheads). **B**. RGCs from the VT portion of the retina extended axons with higher levels of EphB2 (red arrows), but only a subset of these coexpress high levels of EphB1-β-gal (green/white arrows). **C**. *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$: *EphB2*$^{-/-}$ mutant RGC axons from the VT portion of the retina project axons with little to no visible EphB2 staining but high levels of β-gal activity. **D**. WT RGC axons from the VT portion of the retina project axons with high levels of EphB2 and no visible β -gal activity.

Reduced levels of EphB1 results in a diminished ipsilateral projection

To further explore this apparent interdependency of EphB1 and EphB2 for axon pathfinding at the optic chiasm, two combinations of mutant mice that were heterozygous for the *EphB1*$^{T\text{-}lacZ}$ mutation were analyzed. When compared to WT mice (Fig. 3.7 A), *EphB1*$^{+/T\text{-}lacZ}$ heterozygote mutant mice displayed a consistent decrease in the ratio of ipsilaterally projecting RGCs visible throughout the LGN (Fig. 3.7 B) although the decrease was largely not statistically significant (Fig. 3.7 E). However, if the *EphB1*$^{+/T\text{-}lacZ}$ heterozygote mutants was compounded with the *EphB2*$^{lacZ/lacZ}$ forward signaling mutant mice, the relative percentage of ipsilateral to contralateral RGC axons was strongly reduced in comparison to both WT and *EphB1*$^{+/T\text{-}lacZ}$ heterozygote mutants (Fig. 3.7 C), and this difference was statistically significant through the majority of the LGN when compared to the *EphB1*$^{+/T\text{-}lacZ}$ heterozygote (Fig. 3.7 E). Importantly, this reduction in ipsilateral axons reaching the LGN was still not as drastic as the complete loss of the EphB1 intracellular domain alone (Fig. 3.7 D), which showed a near complete ablation of the ipsilateral projection throughout the entirety of the LGN (Fig. 3.7 E).

By examining mice that are heterozygous mutants for the EphB1-β-gal fusion protein, the amount of functional EphB1 forward signaling was reduced but not abolished. A trend for the reduction of ipsilaterally projecting axons to the LGN was then detectable, yet it was only when reduced levels of EphB1 were compounded in mutant mice also lacking the EphB2 intracellular domain that there was a statistically significant reduction in the percentage of ipsilateral axons projecting to the LGN. Still, mice with normal expression for EphB1 but lacking the intracellular domain of EphB2 displayed no difference in the ratio of ipsilateral to contralateral projections at the LGN

compared to WT mice (Fig. 3.4). Together, this genetic data indicates that EphB1 is the key regulator while EphB2 plays a supporting role in their function as receptors to deflect VT RGC axons at the optic chiasm.

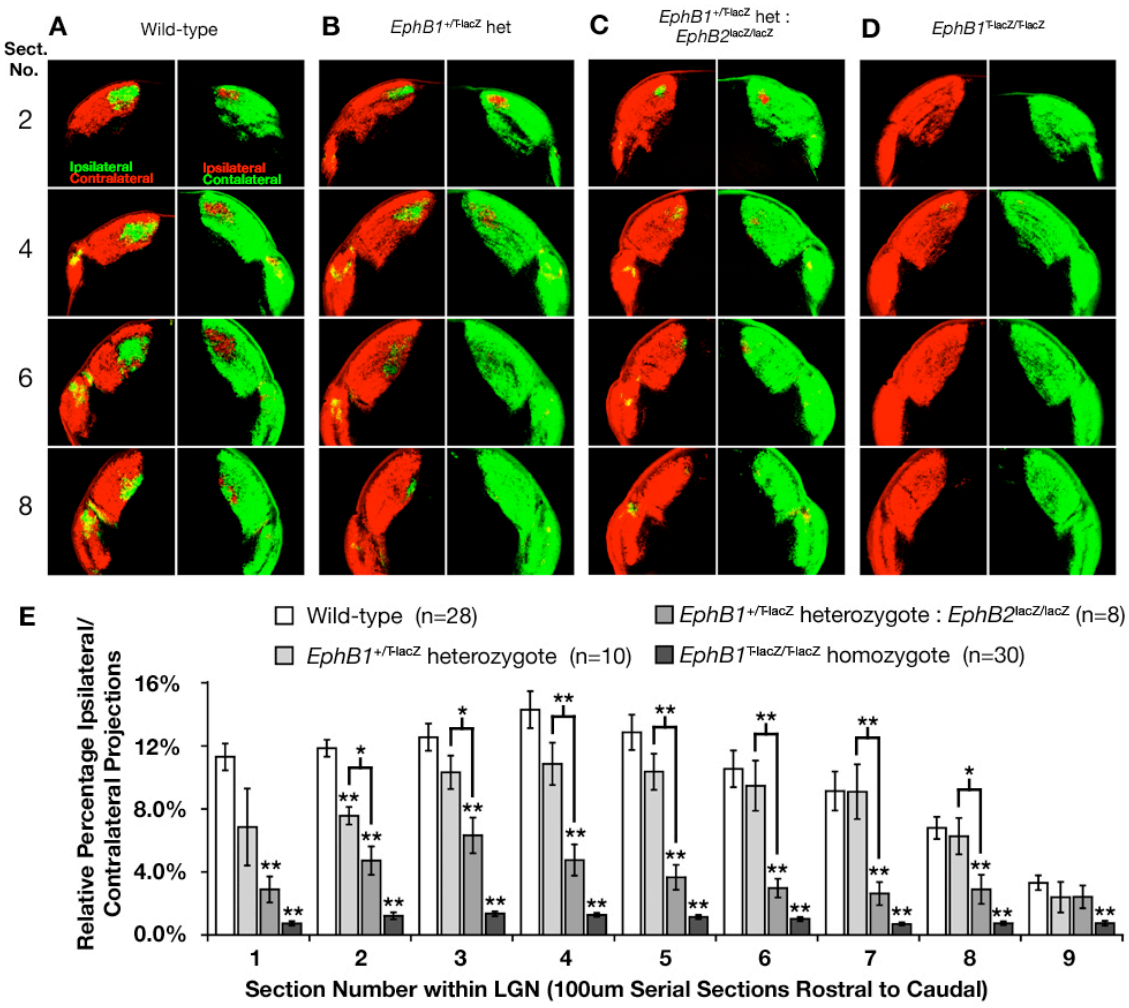

A
Wild-type
B
EphB1+/TlacZ het
C
EphB1+/TlacZ het : EphB2lacZ/lacZ
D
EphB1TlacZ/TlacZ
Sect. No.
2
4
6
8
Ipsilateral
Contralateral
Ipsilateral
Contalateral
E
Wild-type (n=28)
EphB1+/TlacZ heterozygote (n=10)
EphB1+/TlacZ heterozygote : EphB2lacZ/lacZ (n=8)
EphB1TlacZ/TlacZ homozygote (n=30)
Relative Percentage Ipsilateral/ Contralateral Projections
16%
12%
8.0%
4.0%
0.0%
1
2
3
4
5
6
7
8
9
Section Number within LGN (100um Serial Sections Rostral to Caudal)

Fig 3.7: Reduced ipsilateral projections in mice with diminished EphB1 and EphB2 forward signaling

A-D. Serial coronal sections through representative regions the LGN of WT (**A**), *EphB1*$^{+/T\text{-}lacZ}$ heterozygous (**B**), *EphB1*$^{+/T\text{-}lacZ}$ heterozygote: *EphB2*$^{lacZ/lacZ}$ (**C**), and EphB1$^{T\text{-}lacZ/T\text{-}lacZ}$ homozygous (**D**) mice. All RGCs were labeled with CTB-555 (red) in the right eye and CTB-488 (green) in the left eye to compare changes in the amount of ipsilaterally projecting RGC axons. **E**. Quantitative analysis showing the differences in relative percentage of ipsilaterally projecting RGC axons throughout the entire LGN of each genotype (All mice are 90%CD1 strain; * indicates $p<0.05$, ** indicates $p<0.01$).

Discussion

The purpose of this study was to determine under *in vivo* conditions and expression levels if EphB1 functions as a receptor or ligand in its role in directing RGC axons to the ipsilateral hemisphere at the optic chiasm. To directly address this, a germline mutant mouse was created that removes the intracellular domain of EphB1 while leaving the extracellular and transmembrane domains unchanged. These *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ forward signaling mutant mice recapitulated the phenotype seen in *EphB1*$^{-/-}$ protein null mice clearly illustrating that EphB1 dependent forward signaling is necessary to determine the laterality of RGC axons at the optic chiasm. Surprisingly, EphB2, which is also expressed on VT RGC axons, is not required to deflect these axons unless the levels of EphB1 are also functionally reduced.

EphB1 forward signaling directs RGC axons to the ipsilateral hemisphere

Using a protein null mutant mouse, it was previously established that EphB1 directs VT RGC axons at the optic chiasm to the ipsilateral hemisphere (Williams et al., 2003). Given the ability of EphB:ephrin-B interactions to activate both forward and reverse intracellular signals upon axon-cell contact, it was crucial to determine whether EphB1 was functioning as a receptor or ligand in guidance at the optic chiasm. To accomplish this, a mutant mouse was created that expresses an intracellular truncated fusion protein under the endogenous locus of EphB1. While the EphB1-β-gal fusion protein can still act as a ligand to stimulate ephrin-B reverse signaling, it cannot transduce a canonical forward signal that requires its intracellular domain. However, the

truncated fusion protein does possess the potential to transduce a non-canonical forward signal, for EphB receptors interact in cis with the NR1 subunit of the NMDA receptor via the extracellular regions of both proteins and do not require the intracellular domain of EphB molecules (Dalva et al., 2000; Grunwald et al., 2001). Nevertheless, as the *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ mice recapitulate the phenotype seen in *EphB1*$^{-/-}$ protein null mice, our data demonstrate that canonical EphB1 forward signaling is required to deflect VT RGC axons at the optic chiasm to the ipsilateral hemisphere. Interestingly, when this mutation was combined with an EphB2 forward signaling mutant mouse line, which displayed retinal axon pathfinding defects at the superior colliculus (Hindges et al., 2002; Thakar et al., Submitted), there was a sharper decrease in the percentage of ipsilateral to contralateral RGC axons reaching the LGN (Representative cartoon in Fig. 3.8).

EphB1 and EphB2 are expressed on the same subset of VT RGC axons

The increased defect seen in *EphB1/B2* double mutant mice suggests EphB1 and EphB2 have an overlapping function, yet *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ mice display a phenotype while *EphB2*$^{lacZ/lacZ}$ mice do not. I therefore sought to determine if EphB1 is distinctly expressed from EphB2 at the chiasm, as previous genetic studies have established some functional redundancy between EphB family receptors (Orioli et al., 1996; Cowan et al., 2000; Henkemeyer et al., 2003; Chen et al., 2004; Dravis et al., 2004; Chumley et al., 2007). By performing X-gal stains of developing tissue on the *EphB1*$^{T\text{-}lacZ}$ and *EphB2*^{lacZ} mutant mouse reporters, axonal expression patterns of both EphB1 and EphB2 can be seen within the optic nerve and at the optic chiasm when RGC axon laterality is being determined. However, to share redundant functions, both EphB1 and EphB2 must be

expressed in the same cell, yet this was not previously determined due to a lack of a specific antibody against EphB1. Utilizing *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ mice, EphB1 and EphB2 are visibly co-expressed within the same VT RGC axons based on the retinal explant cultures. Therefore, EphB2 has the potential to operate in tandem with EphB1 at the chiasm.

EphB1 receptor is preferred over EphB2 at the optic chiasm

Even though EphB1 and EphB2 are co-expressed in these same VT RGC axons, the inability of EphB2 to rescue function in *EphB1*$^{-/-}$ null and *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ mice implies that EphB2 is not required at the optic chiasm. However, mice that have reduced levels of functional EphB1 (i.e. *EphB1*$^{+/T\text{-}lacZ}$ heterozygotes) while expressing unaltered levels of EphB2 display a close to normal ipsilateral population. Furthermore, if the *EphB1*$^{+/T\text{-}lacZ}$ heterozygote is compounded with *EphB2*$^{lacZ/lacZ}$, there is a statistically significant decrease in the percentage of ipsilaterally projecting RGCs. This shows that EphB2 does function in pathfinding at the chiasm, but only if the dosage of EphB1 is reduced. Moreover, *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$:*EphB2*$^{lacZ/lacZ}$ compound mutants display an even stronger reduction in the percentage of ipsilaterally projecting axons than in *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ single mutants. In aggregate, this genetic evidence suggests that EphB2 does possess a partial ability to guide axons at the chiasm.

Alternatively, there exists the potential that truncated EphB2 is acting in a dominant negative manner in the heterozygous *EphB1*$^{+/T\text{-}lacZ}$ mice, but this is unlikely because truncated *EphB2*$^{lacZ/lacZ}$ single mutant mice have no discernible difference from WT mice. If the truncated form of EphB2 was functioning in a dominant negative

fashion, a mild phenotype should be evident in these mice due to the impairment of EphB1 function. As EphB2 function in the chiasm is revealed in *EphB1*$^{+/T\text{-}lacZ}$ heterozygous state, the data indicate a role for EphB2 that is subservient to EphB1.

Since the intracellular truncated *EphB1* and *EphB2* mutants both show a phenotype, it is doubtful that the unique factor of EphB1 stems from the intracellular domain. Previous studies have shown that EphB-mediated axon retraction is heavily dependent upon the downstream regulation of Rho GTPases (Etienne-Manneville and Hall, 2002; Noren and Pasquale, 2004). Furthermore, blocking Rho-kinase signaling, which can be activated by EphB2 (Shi et al., 2009), stops the retraction response of VT RGC axons stimulated with ephrin-B2 (Petros et al., 2010). Moreover, a likely direct downstream target of EphB1 at the chiasm is the Rho family GEF Vav2, which directly binds EphB2 (Cowan et al., 2005). These data further indicate that the intracellular domain of both EphB1 and EphB2 are equally able to induce axon repulsion at the optic chiasm through Rho-GTPase dependent signaling. In addition, Petros et al. reported that non-VT RGCs ectopically transfected with WT EphB1 cDNA results in a percentage of these axons being redirected ipsilaterally. When a chimeric protein composed of the extracellular domain of EphB2 and intracellular domain of EphB1 is expressed, the RGC axons are not redirected as well as WT EphB1. Instead, the chimera redirects at a reduced level similar to full length EphB2 (Petros et al., 2009). This and our data suggest a unique factor in the extracellular domain of EphB1 regulates the specificity of this receptor in directing RGC axons at the optic chiasm.

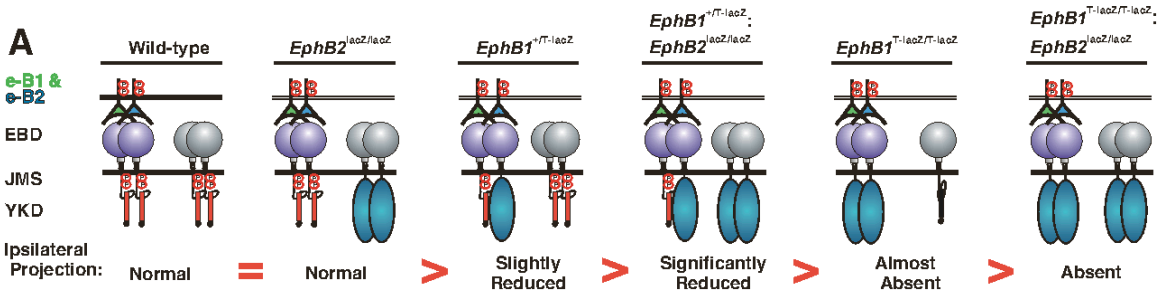


Fig 3.8: Diagram of phenotypes observed

This is a representative diagram of the EphB1 and EphB2 forward signaling genotypes assayed for reduction of ipsilateral projections at the optic chiasm. When EphB1 is not bound to an ephrin-B, it is in an auto-inhibited state where the kinase domain is prevented from being activated. Upon binding ephrin-B1 and B2 at the optic chiasm EphB1 becomes activated and induces a forward signal that results in redirection of the VT-RGCs. The loss of a functional intracellular domain of EphB2 (*EphB2*$^{lacZ/lacZ}$) shows no difference compared in determining the laterality of RGC axons. A partial loss of EphB1 forward signaling does reduce the overall percentage of ipsilaterally projecting RGC axons, but this loss is slight unless this mutation is compounded with the loss of EphB2 forward signaling. However, the complete loss of the EphB1 intracellular domain results in a severe reduction in the percentage of ipsilaterally projecting cells, and this was further reduced with the additional loss of EphB2 forward signaling in the *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ:}$*EphB2*$^{lacZ/lacZ}$ compound mutant.

Chapter 4

Ephrin-B1 and ephrin-B2 act as ligands in determining RGC axon laterality

Summary

In order to determine the potential regulators of EphB1 and EphB2 at the optic chiasm, I examined various ephrin-B mutant mice. Both *ephrin-B1*$^{-/-}$ homozygotes and *ephrin-B2*$^{+/-}$ heterozygotes displayed a reduction in the percentage of RGC axons projecting to the ipsilateral hemisphere, while *ephrin-B3*$^{-/-}$ mutants displayed no difference from WT mice. Furthermore, two *ephrin-B2* reverse signaling mutants analyzed were observed to retain their ipsilateral projecting population of RGC axons. Taken together, this shows that ephrin-B1 and ephrin-B2 mediated forward signaling but not ephrin-B2 reverse signaling is crucial in creating a partial decussation of the optic nerve at the optic chiasm.

Ephrins in the optic nerve and at the optic chiasm

Before an elegant study performed on Xenopus laevis, the proteins responsible for deciding RGC axon laterality remained largely unknown. In the early life phase of a

frog, tadpoles have lateralized eyes that view a wide visual field well designed for the evasion of predators, but adult frogs have binocular vision that is suited for their life as a predator. During the metamorphosis between these two developmental stages, the eyes will shift forward, and a subset of temporal RGC axons will project to the ipsilateral optic tectum (Hoskins and Grobstein, 1985). When metamorphosis stage RGC axons that normally project to the ipsilateral optic tectum were transplanted to earlier stage developing tadpoles, all transplanted RGC axons projected to the contralateral hemisphere along with the endogenous population. This revealed that that the unique factor guiding ipsilateral projections did not alone depend on the RGC axons. Further studies revealed that the optic chiasm expresses ephrin-B molecules during metamorphosis, and if ephrin-B is misexpressed in premetamorphosized tadpoles, RGC axons will be redirected from a contralateral path to an ipsilateral path (Nakagawa et al., 2000). This study clearly established the link to EphB and ephrin-B molecules in determining RGC laterality.

After these studies in frogs, experiments relying on *in situ* expression data and *in vitro* growth cone collapse assays suggested that ephrin-B2 is functioning as the sole repulsive ligand at the optic chiasm to interact with VT RGC axons (Williams et al., 2003; Petros et al., 2010). However, there was no concrete link marking ephrin-B2 as the ligand at the chiasm. Additionally, although EphB forward signaling is essential for RGC axons to be directed ipsilaterally (see above), this does not preclude a potential role for ephrin-B reverse signaling in this process. Here I will show that both ephrin-B1 and ephrin-B2 act as ligands at the chiasm, and that ephrin-B2 reverse signaling is not required to determine RGC axon laterality.

Results

Ephrin-B1 and ephrin-B2 mutant mice display a reduced ipsilateral projection

In addition to examining the role of EphB1 and EphB2 receptors, the ephrin-B molecules that are involved in directing the laterality of RGC axons was determined *in vivo* by using the same sensitive LGN measuring method described above directly testing *ephrin-B1*, *ephrin-B2*, and *ephrin-B3* mutant mice. *Ephrin-B3*$^{-/-}$ null mice did not display a noticeable reduction in the ratio of ipsilaterally projecting RGCs compared to WT littermates (Fig. 4.1). However, I found that *ephrin-B1*$^{-/Y}$ hemizygous and *ephrin-B1*$^{-/-}$ null mutant mice displayed a statistically significant decrease in the ratio of ipsilaterally projecting RGCs compared to WT (Fig. 4.2 A,B,E). Although *ephrin-B2*$^{-/-}$ null mice could not be analyzed as they exhibit early embryonic lethality, *ephrin-B2*$^{+/-}$ heterozygotes are viable and were found to exhibit a slight decrease in the percentage of ipsilaterally projecting RGCs compared to WT (Fig. 4.2 C), yet it was not statistically significant through most of the LGN (Fig. 4.2 E). These data suggest that ephrin-B1 and ephrin-B2 function in the proper pathfinding of VT RGC axons while ephrin-B3 is uninvolved.

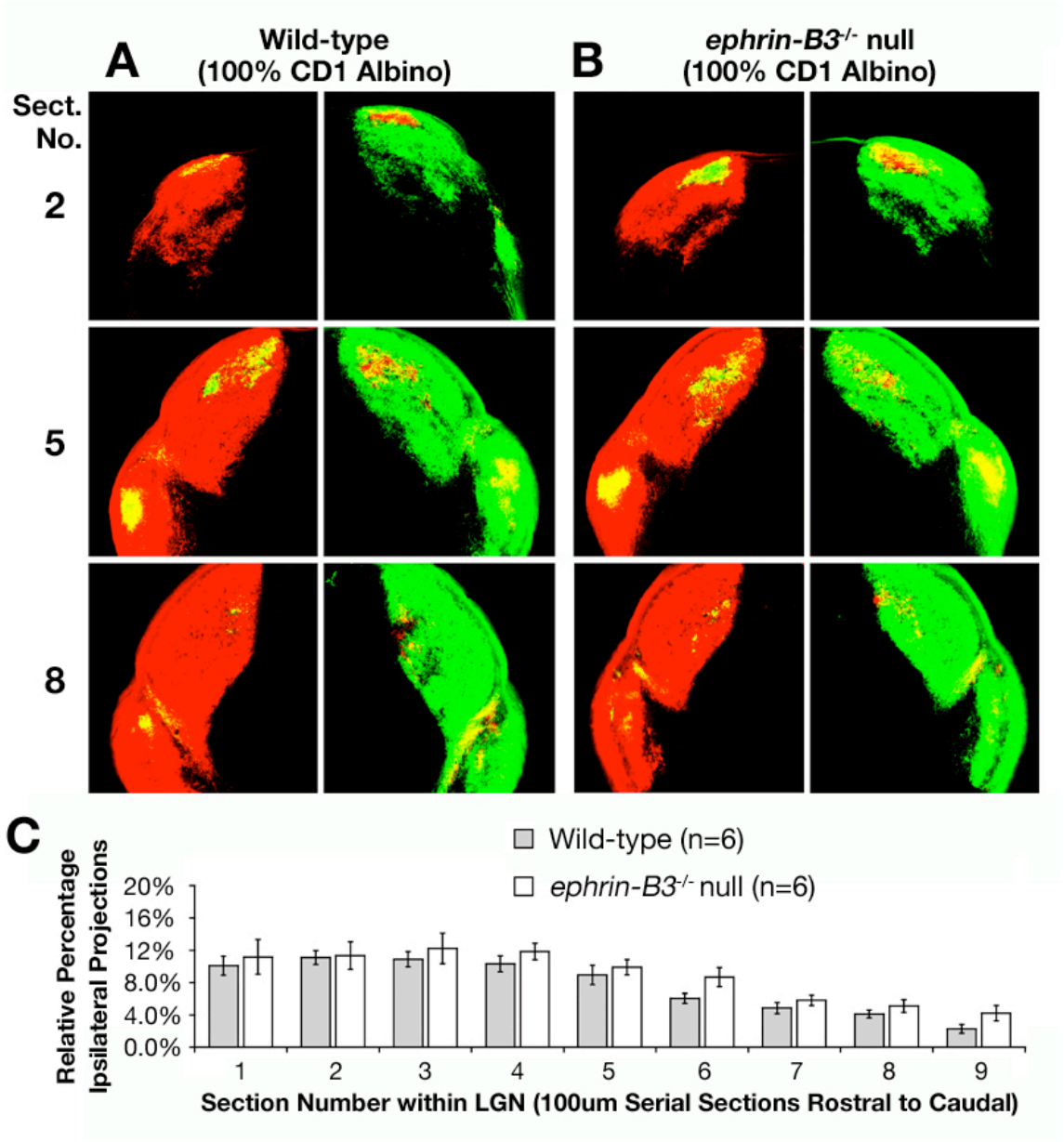

A
Wild-type
(100% CD1 Albino)
B
ephrin-B3-/- null
(100% CD1 Albino)
Sect.
No.
2
5
8
C
Wild-type (n=6)
ephrin-B3-/- null (n=6)
Relative Percentage
Ipsilateral Projections
20%
16%
12%
8.0%
4.0%
0.0%
1
2
3
4
5
6
7
8
9
Section Number within LGN (100um Serial Sections Rostral to Caudal)

Fig 4.1: *Ephrin-B3*$^{-/-}$ mutant mice display no difference in percentage of ipsilaterally projecting RGCs when compared to WT mice

A-B. Serial sections through the LGN of WT (**A**) and *ephrin-B3*$^{-/-}$ null (**B**) albino CD1 strain mutant mice whose RGCs were labeled with CTB-555 (red) anterograde dye in right eye and CTB-488 (green) in the left eye to compare changes in the amount of ipsilaterally projecting RGC axons. **C**. Quantitative analysis showing the differences in relative percentage of ipsilaterally projecting RGC axons throughout the entire LGN of each genotype (All mice are 100% CD1 albino strain; no statistically significant differences found for any region).

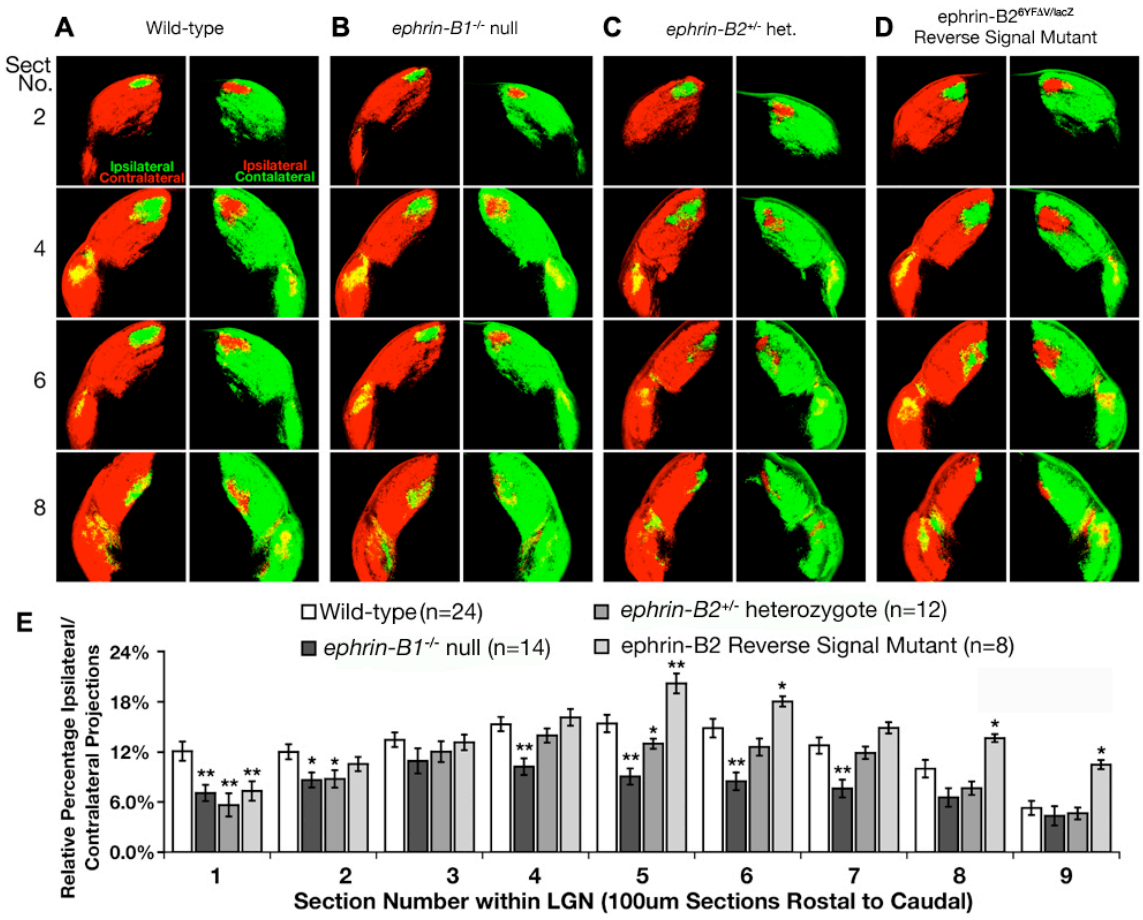


Fig 4.2: *Ephrin-B1* and *ephrin-B2* mutant mice display reduced ipsilateral projections

A-D. Serial sections through the LGN of WT (**A**), *ephrin-B1*$^{-/-}$ null (**B**), *ephrin-B2*$^{+/-}$ heterozygote (**C**), and ephrin-B2$^{6YF\Delta V/lacZ}$ reverse signaling (**D**) mice whose RGC axons were labeled with CTB-555 (red) in the right eye and CTB-488 (green) in the left eye to compare changes in the amount of ipsilaterally projecting RGCs. **E**. Quantitative analysis showing the differences in relative percentage of ipsilaterally projecting RGC axons throughout the entire LGN of each genotype (All mice are 90%CD1 strain; * indicates $p<0.05$, ** indicates $p<0.01$).

Ephrin-B2 reverse signaling mutants retain an ipsilateral projection

In addition to expression at the optic chiasm, ephrin-B2 is also expressed to high levels in the dorsal retina (Birgbauer et al., 2000; Thakar et al., Submitted). Moreover, EphB1 is also expressed at the chiasm (Fig 3.4 E). This allows for the possibility that EphB1 mediated reverse signaling may direct retinal axon laterality. To determine whether ephrin-B2 is functioning in guidance at the optic chiasm as a receptor to transduce reverse signals, mutant adult mice lacking the ability to transduce intracellular signals were also analyzed.

Ephrin-B2^{lacZ} mutant mice express an intracellular truncated β-gal fusion protein that is unable to transduce a canonical reverse signal, but may still stimulate forward signaling (see cartoon in Fig 1.2 D). Unfortunately, mice homozygous for this mutation do not survive past birth, so these mice could not be analyzed through the sensitive LGN measuring assay. To determine if the complete loss of ephrin-B2 reverse signaling results in the loss of the ipsilateral projection at the optic chiasm, E18.5 WT and mutant embryos had their entire right optic nerve labeled with DiI at the optic disk. This was allowed to diffuse past the optic chiasm, which was subsequently visualized directly. Both WT and *ephrin-B2*$^{lacZ/lacZ}$ embryos retained an ipsilateral projection (Fig 4.3).

Unlike *ephrin-B2*$^{lacZ/lacZ}$ mutants, combinatorial *ephrin-B2*$^{lacZ/6Yf_{\Delta}V}$ reverse signaling mutant mice analyzed were viable adults. These mice carried one copy of the *lacZ* allele and one copy of the 6YFΔV mutant that has key point mutations within the intracellular domain of ephrin-B2 implicated in reverse signaling. In these mice, all six intracellular tyrosines are replaced with phenylalanine and the C-terminal valine is deleted. This mutant mouse produces a protein that can neither be tyrosine

phosphorylated and bind SH2 domain containing proteins nor recruit PDZ domain containing proteins to its C-terminal tail (Thakar et al., Submitted). Interestingly, the *ephrin-B2*$^{lacZ/6YF\Delta V}$ mice exhibited, if anything, a slight increase in the percentage of ipsilaterally projecting RGC axons (Fig. 4.2 D,E).

These data indicate that reverse signaling is not necessary for guidance of VT axons at the optic chiasm, and that perhaps either the truncated ephrin-B2-β-gal fusion protein or the ephrin-B2-6YFΔV mutant protein may have a slightly greater ability to stimulate forward signaling (see below). Nevertheless, ephrin-B2 does not require a functional intracellular domain to direct VT RGC axons at the optic chiasm.

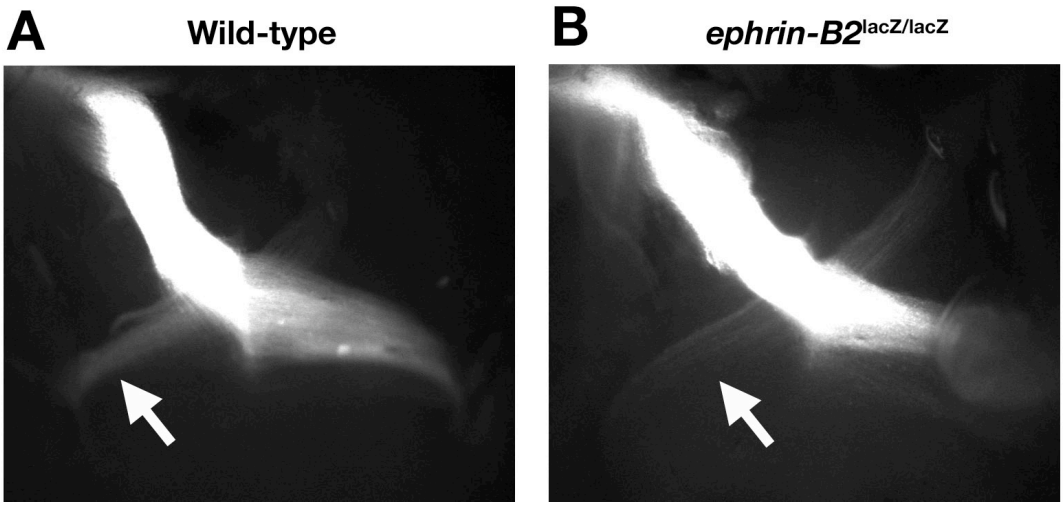


Fig 4.3: Direct visualization of optic chiasm of *ephrin-B2*$^{lacZ/lacZ}$ mutant mice

WT and *ephrin-B2*$^{lacZ/lacZ}$ reverse signaling mutant mice had one of their optic nerves completely labeled with DiI and optic chiasm exposed. An ipsilateral projection (arrows) is clearly visible in WT (**A**) and truncated ephrin-B2-β-gal (**B**).

Discussion

Both ephrin-B1 and ephrin-B2 function as ligands at the chiasm

Along with EphB1 and EphB2, the potential role of three ephrin-Bs ligands at the optic chiasm was also examined. Our study reveals that both ephrin-B1 and ephrin-B2 function as ligands at the optic chiasm while ephrin-B3 does not (Phenotypes outlined in cartoon in Fig. 4.4). A key element of the EphB extracellular domain is the ephrin-binding domain. As the dissociation constants for specific EphB:ephrin-B interactions tend to vary depending on the proteins involved (Blits-Huizinga et al., 2004), not all EphB:ephrin-B binding is of equal strength. Therefore, if a solitary ephrin-B protein were the sole ligand involved, it could be reasonably surmised that EphB1 is its preferred receptor at the optic chiasm. However, I have shown *in vivo* that both ephrin-B1 and ephrin-B2 act as ligands for EphB1 in directing RGC axons ipsilaterally. Thus, it is unlikely that the specification of EphB1 activation over EphB2 is dependent on a particular EphB:ephrin-B interaction.

The fact that ephrin-B1 is also involved in determining RGC axon laterality at the optic chiasm assists in explaining why the overall reduction of ipsilaterally projecting RGC axons seen in the *ephrin-B2*$^{+/-}$ heterozygous mice is largely non-significant, for ephrin-B1 can rescue normal function by stimulating EphB1 forward signaling even with reduced levels of ephrin-B2. The decrease in ipsilateral projections reaching the LGN is similar to that seen in the EphB1$^{+/T\text{-}lacZ}$ heterozygote mice, which EphB2 has the ability to rescue. However, unlike the dependency of EphB2 on EphB1, ephrin-B1 and ephrin-B2 both seem to be capable of stimulating a forward signal response independently.

Ephrin-B reverse signaling is not required to determine RGC axon laterality

In further evidence of the importance of forward signaling, the statistically significant increase in ipsilaterally projecting RGC axons reaching the LGN in the ephrin-B2 reverse signaling mutant mice may be due to a gain of function from the ephrin-B2 truncated β-gal mutant mice. β-gal naturally forms a tetramer (Appel et al., 1965; Juers et al., 2000), so the ephrin-B2 extracellular domain would be in a state that mimicked activated ephrin-B2 before EphB1 binds. This pre-clustered ephrin-B2 extracellular domain would then have the potential to act as an improved ligand since clustering is a key factor in transducing a forward signal (Himanen et al., 2007). Thus, RGC axons that would normally take a contralateral pathway are redirected to the ipsilateral hemisphere from an enhanced forward signal.

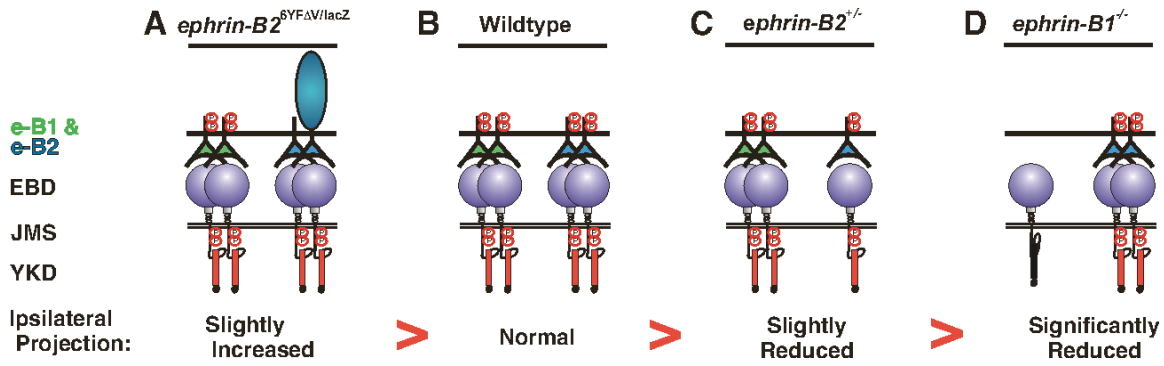


Fig 4.4: Diagram of phenotypes observed

When the intracellular domain of ephrin-B2 is replaced with β-gal, there seems to be a slight increase in the percentage of ipsilaterally projecting RGC axons compared to WT, which may be due to an increased ability to act as a ligand. The partial loss of ephrin-B2 results in a largely non-significant but still detectable reduction. The complete loss of ephrin-B1 results in a more pronounced statistically significant reduction in the percentage of ipsilaterally projecting RGC axons.

Chapter 5

EphB and ephrin-B mutant mice develop agenesis of the corpus callosum

Summary

The corpus callosum is a major brain commissure that connects and allows communication between the two cerebral hemispheres. Previous studies showed that EphB and ephrin-B mutant mice developed agenesis of the corpus callosum (AgCC), but the extent of these roles and the expression patterns during development remained vague. Unlike other Eph and ephrin related agenesis studies, I have distinguished a region specific AgCC phenotype that is unique to each EphB and ephrin-B mutant mouse group analyzed. Here, I will present preliminary finding suggesting that EphB forward signaling and ephrin-B reverse signaling are required for callosal axons to cross the midline, as mutant mice lacking these signaling abilities develop AgCC.

Evolution of the corpus callosum

The corpus callosum is the largest white matter tract in the brain with ~200 million axons connecting the two cortical hemispheres in humans. As it is only present in placental mammals (eutheria), but not marsupials (metatheria) or monotremes (prototheria), it is a relatively novel adaptation. Eutheria split ~125 million years ago

(Johnson et al., 1982; Aboitiz and Montiel, 2003), and the brain seems to have radically changed because of the addition of the corpus callosum. For example, reptilian brains are much more dependent on tectal commissures while the eutherian anterior and hippocampal commissures remain smaller relative to overall brain size (Aboitiz and Montiel, 2003). The corpus callosum granted increased connectivity between hemispheres allowing them to develop topographically structured sensory areas of increased complexity (Houzel and Milleret, 1999; Mihrshahi, 2006). Indeed, there is a school of thought that it may even be the corpus callosum that has permitted humans to reach our current level of intellectual advancement (Gazzaniga, 2000).

Defects from agenesis of the corpus callosum

There are at least 50 known congenital syndromes that lead to agenesis of the corpus callosum (AgCC) (Richards et al., 2004), but it is difficult to glean the function of the corpus callosum from case studies on these patients, as there are often defects present in other areas of the brain. In fact, there are patients whose MRI scan shows complete AgCC, but they exhibit no quantifiable defects in intelligence or behavior (Paul et al., 2007). However, other studies have revealed losses in normal neural function in patients without a functional corpus callosum. Many of these patients were studied after undergoing a surgical treatment for severe seizures that was to cut the corpus callosum. This led to callosal disconnection syndrome, which could then be compared to people born with AgCC (Gazzaniga, 2005). These patients had some interesting defects that could be surprisingly subtle including an inability to understand jokes, specifically word play such as puns (Brown et al., 2005). More concretely measurable, the corpus callosum

is known to be important for the following functions: dual hand usage coordination, sound localization, precise rapid hand movements, correct word usage in writing and speaking, and integration of three-dimensional vision[6] (Gazzaniga, 2000; Richards et al., 2004; Mihrshahi, 2006; Paul et al., 2007). Interestingly, the corpus callosum also seems to control right- or left-handedness based on the asymmetrical dominance from the motor cortex (Derakhshan, 2003). There is currently some debate as to whether the majority of the synaptic connections between hemispheres are excitatory or inhibitory, but the current consensus leans to excitatory. Inhibitory connections are thought to be crucial in maintaining the independent function of each hemisphere while excitatory connections are thought to integrate information from the two hemispheres (Bloom and Hynd, 2005).

Development of corpus callosum

In order to properly form an interhemispheric connection, there are several fundamental stages that must occur that include basic formation and separation of the brain into two halves, the adhesion of the midline at key regions, the birth, migration, and

[6] This role in three-dimensional vision integration is intriguing to me because I have shown above that the intracellular domain of EphB1 is critical pathfinding of RGC axons involved in depth perception, and I will discuss below how the intracellular domain of EphB1 is important in proper formation of the corpus callosum. This may make EphB1 the most important protein in depth perception. However, there need to be studies to confirm patients with depth perception problems have an intact ipsilateral projection at the optic chiasm, for the role of EphB1 in temporal RGC axon pathfinding is likely conserved in humans (Lambot et. al., 2005) as this corpus callosum phenotype may be.

specification of neurons, and then the proper pathfinding and crossing of axons (Paul et al., 2007). For my discussion on the formation of the corpus callosum, I will focus mainly on the axon guidance, for, as mentioned in detail above, Ephs and ephrins have proven critical in axon guidance.

The decision points can be broken down for proper axon contralateral targeting into eight steps. The cortex is composed of 6 layers and the majority of callosal fibers project from layers 2/3 and 5. The differentiated neuron must first send its axonal projection in a ventral direction away from the cortex, and this process is mainly regulated via repulsion from secreted Sema3A onto neuropilin-1 expressing callosal fibers (Polleux et al., 1998). At this point, either the axon can project to the ipsilateral hemisphere to innervate other areas or it can take a contralateral path to the cingulate cortex. Interestingly, axons will bifurcate at this decision point sending out two growth cones, one of which will be pruned when the choice is later determined (Garcez et al., 2007). The cingulate cortex is where pioneer axons are born in the earliest stage of pathfinding. While the majority of axons composing the corpus callosum will cross the midline in stages beginning at the most rostral portion followed closely by the caudal region (Kier and Truwit, 1996, 1997), the pioneering axons seem to become established across the length of the structure all at once (Richards et al., 2004). Axons from the cortical region will fasciculate via L1 and other CAMs with this pre-established tract to head across the midline to the contralateral cingulate cortex (Kamiguchi et al., 1998), and, to a lesser extent, attractive cues like netrin-1 will guide DCC expressing callosal axons (Serafini et al., 1996). Once across, axons follow growth factors like FGF8 to reach their target, which is usually in a region homotipic to the site of origin (Hutsler and

Galuske, 2003; Innocenti and Price, 2005). Finally, activity dependent pruning will fine tune targeting and finalize synapses (Koralek and Killackey, 1990).

In animals that develop AgCC, callosal axons will attempt to cross but become lost and can wander looking for the proper path. In this time, they will wrap around themselves and seem to eventually give up. Instead of collapsing and degenerating, these axons will remain and even become myelinated, which makes them stand out as a prominent defect named Probst bundles, named after the Austrian anatomist Moriz Probst (Schmahmann and Pandya, 2007).

It is around E15 that the first pioneer axons begin to cross midline. First, the midline zipper glia (MZG) must induce adhesion and fusion between the two hemispheres, for mice lacking this ability develop AgCC (Silver and Ogawa, 1983). The only known attractive cue for these early axons is neuropilin-1, which is attracted by a semaphorin gradient (Hatanaka et al., 2009; Piper et al., 2009). For repulsive cues, the glial wedge (GW) and indusium griseum (IGG) are structures that block axons from projecting into the ventral hemisphere and dorsal hemisphere, respectively. This is accomplished in part via repulsion through Slit-Robo and Wnt-Ryk repulsive signaling. Callosal fibers express Robo and RYK receptors while surrounding glial cells send a Slit and Wnt gradient that directs axons through repulsion (Shu et al., 2003; Keeble et al., 2006).

There are also physical structures that guide the pioneering axons across. The hippocampal commissure is an interhemispheric connection that forms just before the corpus callosum. If this structure fails to form, there is AgCC (Ozaki and Wahlsten, 1992; Livy and Wahlsten, 1997). The hippocampal commissure can even rescue the

ability of callosal fibers to cross in mice who were exposed to high doses of gamma irradiation at E16, which ablates the ability of callosal fibers to cross except those directly over the hippocampal commissure (Abreu-Villaca and Schmidt, 1999). The midline sling is a more controversial guidance structure found to be important in directing growing callosal axons, as its absence in mice leads to AgCC. It was initially believed to be glial in nature (Silver and Ogawa, 1983), but it is now believed to be composed of neuronal cells (Shu et al., 2003).

The role of Ephs in corpus callosum development

Through analysis of mutant mice, EphB2 and EphB3 were the first Eph receptors identified to play a role in formation of the corpus callosum (Orioli et al., 1996). Later studies showed that *EphB1* and *ephrin-B3* mutant mice also develop AgCC, and that these receptors and ligands were often co-expressed in the same glial cells and callosal axons (Mendes et al., 2006). Furthermore, mutant mice with an altered ephrin-B1 PDZ domain binding site also develop AgCC in a strain dependent manner[7]. While ephrin-B1 is expressed on callosal axons, these *ephrin-B1* reverse signaling mutant mice have defects in the formation of the indusium griseum, but, confusingly, ephrin-B1 is apparently not expressed there (Bush and Soriano, 2009). Additionally, EphA5 mutant mice show a reduction in the percentage of callosal fibers that project contralaterally (Livy and Wahlsten, 1991; Hu et al., 2003), yet the phenotype was not as severe as the

[7] The importance of the glial structures mentioned above mostly stem from studies conducted on mouse strains that developed AgCC over the course of inbreeding. This includes certain 129 sub-strains of mice (Livy and Wahlsten, 1991).

AgCC seen in EphB mutant mice. It remains unclear whether Ephs and ephrins are acting as repellants, attractants, or are necessary for migration and formation of the glial barriers. However, EphB2 is also known to act as a repellent against ephrin-B2 expressing axons from the posterior tract of the anterior commissure stopping them from projecting into the ventral forebrain (Henkemeyer et al., 1996; Ho et al., 2009).

Below, I show that EphB1 and EphB2 forward signaling and ephrin-B2 and ephrin-B3 reverse signaling are necessary in the ability of the corpus callosum to form. Without functional intracellular domains, mutant mice callosal fibers fail to project contralaterally and AgCC develops.

Results

EphB mutant mice show a range of AgCC phenotypes

In mice, the properly formed corpus callosum is a connection between the two cortical hemispheres that spans a large portion of the brain in both a medial-lateral and rostral-caudal direction (Fig 5.1 A). In order to properly form, callosal axons must project contralaterally and will then typically innervate homotipic regions in the opposite hemisphere. Previously, it was revealed that *EphB* protein null mutant mice can develop different degrees of AgCC. Namely, mutants can show no defect, a partial defect where only a portion of the corpus callosum fails to properly form, or a complete defect with no callosal fibers crossing the midline (Mendes et al., 2006).

In addition to these criteria, there are more subtle defects visible when EphB forward signaling mutants are analyzed more closely. Similar to the *EphB1*$^{-/-}$ protein null

mutant mice, complete and partial AgCC was also observed in *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ homozygous mutant mice, and this was typically accompanied by Probst bundles (Fig 5.1 B,C). The *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ mutant mice that displayed partial AgCC could display two unique defects. Either AgCC only occurred in the rostral portion while the caudal portion was normal or AgCC in rostral and caudal extremes while the middle region of corpus callosum formed correctly (Table 5.1). The hippocampal commissure was the structure located in the rostral-caudal midline used to distinguish these regions. Agenesis occurring before the hippocampal commissure was termed rostral was agenesis after it was termed caudal agenesis. Interestingly, *EphB2*$^{lacZ/lacZ}$ homozygous mutant mice displayed only partial AgCC confined to the caudal region (Fig 5.1 D).

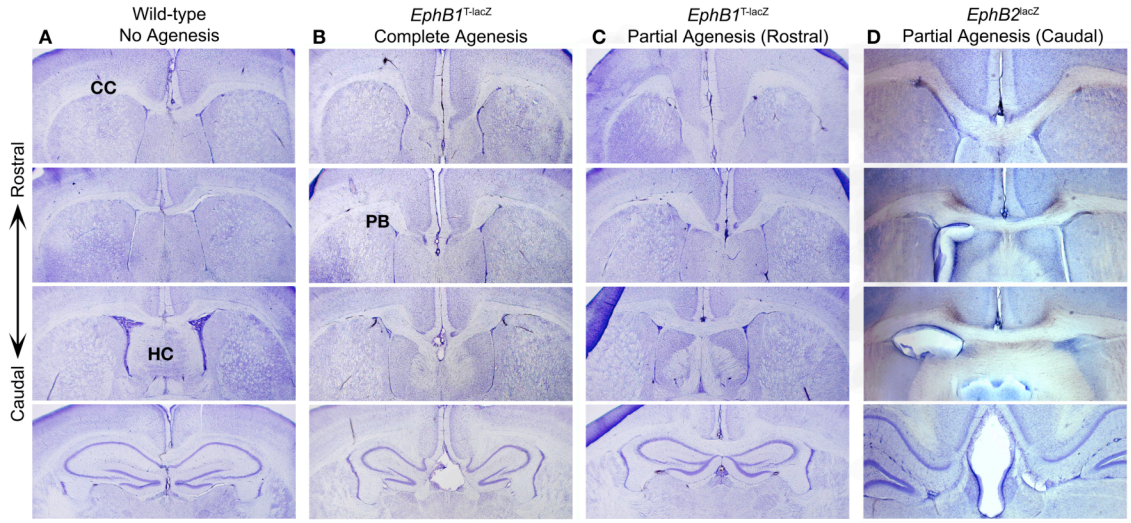


Fig 5-1: Types of AgCC defects in Eph and ephrin mutant mice

A-D. Nissl stains of serial coronal sections through a WT mouse with a normal corpus callosum (**A**), *EphB1*$^{\text{T-lacZ/T-lacZ}}$ homozygous mutant mice with complete agenesis (**B**) or partial agenesis within the rostal portion (**C**), and an *EphB2*$^{\text{lacZ/lacZ}}$ homozygous mutant mouse with partial agenesis where the caudal portion failed to form (**D**) (CC, corpus callosum; HC, hippocampal commissure; PB, probst bundle).

Genotype	Total No. of Mice	Agenesis			
		No AgCC	Rostral	Caudal	Complete
WT	27	26	0	1	0
EphB1$^{-/-}$ null	12	5	3	3	3
EphB1$^{T\text{-}lacZ}$	33	24	4	3	5
EphB2^{lacZ}	7	4	0	3	0
EphB1$^{T\text{-}lacZ}$:*EphB2*^{lacZ}	16	2	0	11	5
EphB1$^{-/-}$:*EphB2*$^{K\text{-}VEV}$	11	0	1	3	8
EphB1$^{-/-}$:*EphB2*^{K661R}	3	0	0	0	3
ephrin-B1 null	7	7	0	0	0
ephrin-B2 $^{6YF\Delta V/LZ}$	4	3	1	0	0
ephrin-B3 null	7	7	0	0	0
EphB1$^{T\text{-}lacZ}$:*eB3*$^{3YF\Delta V}$	7	3	2	0	2

Table 5.1: Numbers of mice and different types of defects seen in corpus callosum

A table detailing the numbers of WT and various EphB and ephrin-B signaling mutants that displayed either a normally developed corpus callosum (No AgCC), displayed partial agenesis in either the rostral or caudal portion of the corpus callosum, or completely failed to form the corpus callosum.

Forward signaling mutants display AgCC

To expand on the function of EphB1 and EphB2 forward signaling, a variety of mutants ranging from protein nulls to point mutants had phenotype penetrance compared (Table 5.1, Fig 5.2). Some 129 sub-strains mice display spontaneous AgCC even when WT, so, considering that the mice used in these studies are mixtures of 129 and CD1 strain, WT mice were taken from each strain analyzed over the course of the analyses mentioned below. These WT mice display very little spontaneous AgCC where only 1 in 27 observed developed AgCC, and this specific mouse had a severe defect in the development of the hippocampus not shared by any other mouse from this study. Interestingly, only approximately one in four *EphB1*$^{\text{T-lacZ/T-lacZ}}$ mutant mice develop AgCC. This is a lower penetrance when compared with the complete protein null mutant (Fig 5.2). In between *EphB1*$^{-/-}$ null and *EphB1*$^{\text{T-lacZ/T-lacZ}}$ mutants, *EphB2*$^{\text{lacZ/lacZ}}$ mutant mice show partial AgCC in forty percent of the animals observed, but there is no complete agenesis and the partial AgCC that does occur is confined to the caudal region of the corpus callosum. However, the overall percentage is increased to ninety percent in *EphB1*$^{\text{T-lacZ/T-lacZ}}$:*EphB2*$^{\text{lacZ/lacZ}}$ compound mutants.

To address if the catalytic activity of the kinase domain and the PDZ binding domain site were involved in the formation of the corpus callosum, *EphB2*$^{\text{K-VEV/K-VEV}}$ and *EphB2*$^{\text{K661R/K661R}}$ mutant mice were also analyzed. The *EphB2*$^{\text{K661R}}$ mutant mouse expresses a form of EphB2 with a point mutation converting a lysine within the kinase domain to arginine, which disrupts its ability to transfer a phosphate group from ATP to the target protein. This effectively renders the kinase domain inactive. In addition to the kinase inactive mutation, the *EphB2*$^{\text{K-VEV/K-VEV}}$ mutant mouse expresses another point

mutated form of EphB2 that has the extreme C-terminal amino acids valine, glutamic acid, and valine truncated, which disrupts the ability of this protein to bind PDZ domains, (Genander et al., 2009). 100% of *EphB1*$^{\text{T-lacZ/T-lacZ}}$:*EphB2*$^{\text{K-VEV/K-VEV}}$ and *EphB1*$^{\text{T-lacZ/T-lacZ}}$:*EphB2*$^{\text{K661R/K661R}}$ compound mutant mice analyzed displayed partial or complete AgCC (Fig 5.2; Table 5.1).

These data show that the loss of forward signaling in either or both EphB1 and EphB2 results in AgCC. Furthermore, this process is likely to be dependent upon the catalytic activity of the kinase domain of EphB2. However, as there is a difference in the penetrance of *EphB1*$^{-/-}$ null and *EphB1*$^{\text{T-lacZ}}$ mutant mice, the role of reverse signaling cannot be disregarded.

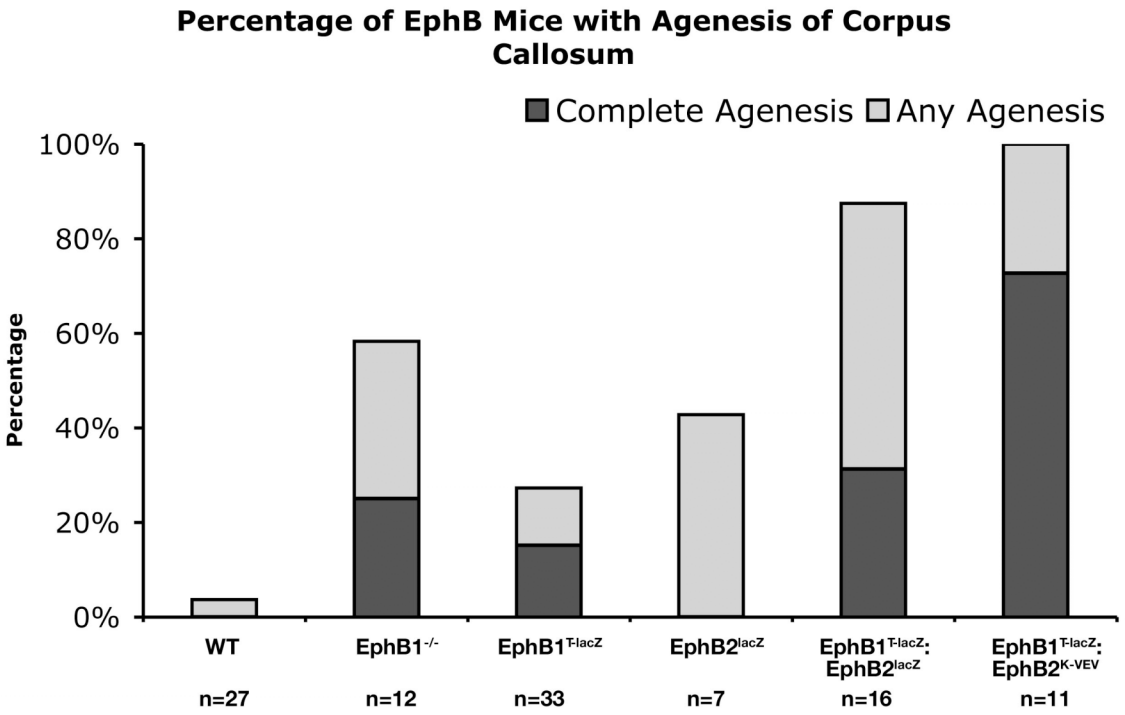


Fig 5.2: Percentage of EphB mutant mice with AgCC

A chart based on table 5.1 showing the percentages of *EphB* mutant mice that displayed either partial or complete AgCC.

Reverse signaling *ephrin-B* mutants display AgCC

Along with EphB forward signaling mutants, several ephrin-B mutant mice were also analyzed for AgCC. While *ephrin-B1*$^{-/-}$ protein null mutant mice did not show any signs of AgCC, the *ephrin-B2*$^{6YFDV/lacZ}$ reverse signaling mutant (described above) did show partial AgCC in the rostral portion of the corpus callosum (Table 5.1; Fig 5.3). Interestingly, in contradiction to previously reported data (Mendes 2003), *ephrin-B3*$^{-/-}$ mutant mice did not develop AgCC. However, when compared to *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ single mutant mice, there is an increase in the penetrance of mice with AgCC in *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$:*ephrin-B3*$^{3YF\Delta V/3YF\Delta V}$ compound mutants. *Ephrin-B3*$^{3YF\Delta V}$ mutant mice are similar to *ephrin-B2*^{6YFDV} mutant mice since they are unable to bind SH2 domain and PDZ containing proteins, but only three of the conserved tyrosines are mutated to phenylalanine. These data suggest that in addition to EphB mediated forward signaling ephrin-B reverse signaling is also critical in the ability of the corpus callosum to cross to the contralateral hemisphere.

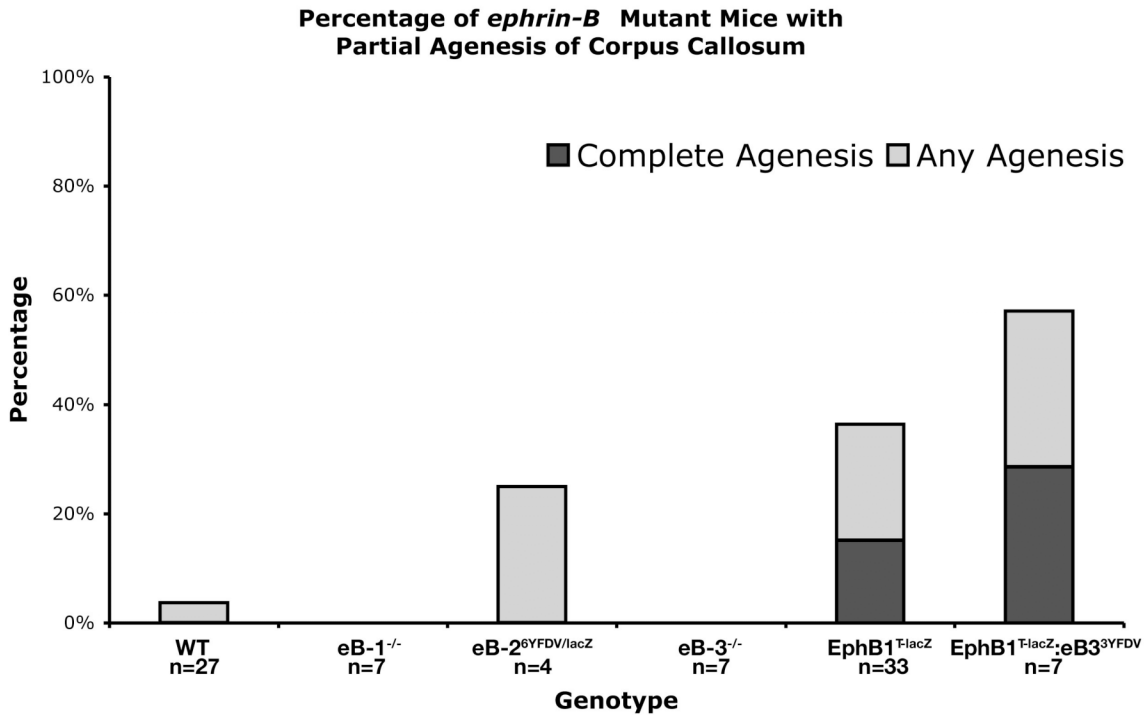


Fig 5.3: Percentage of ephrin-B mutant mice with AgCC

A chart based on table 5.1 showing the percentages of *ephrin-B* mutant mice that displayed either partial or complete AgCC. This includes a direct comparison of EphB1 forward signaling mutant mice next to *EphB1*$^{\text{T-lacZ}}$:*ephrin-B3*$^{3\text{YF}\Delta\text{V}}$ forward and reverse signaling compound mutant mice.

Expression of EphB1 and ephrin-B3 at the corpus callosum

To better understand how EphB1 forward and ephrin-B3 reverse signals are involved in the development of the corpus callosum, their patterns of expression were monitored during callosal axonal pathfinding. E16.5 stage brains from *EphB1*$^{T\text{-}lacZ}$ and *ephrin-B3*^{lacZ}, both of which express an intracellular truncated β-gal fusion protein in place of EphB1 and ephrin-B3, respectively (cartoons in Fig 1.2 D), mutant embryos were coronally cryosectioned and X-gal stained. Ephrin-B3-β-gal was visibly expressed in the ventral portion of the glial wedge and in the midline sling, two glial structures important for development of the corpus callosum. Additionally, there is expression in the ventral midline zipper glial cells and lower expression levels in the dorsal midline zipper glia. Although it appeared faint comparatively, there is also ephrin-B3-β-gal expression within callosal axons throughout the more mature callosal fibers dorsal and lateral to the cingulate cortex (Fig. 5.4A).

Although not in the same areas, the EphB1-β-gal fusion protein was present in the important glial structures, the indusium griseum and the dorsal portion of the glial wedge (Fig 5.4 B). It was also expressed within the ventral midline zipper glia like ephrin-B3 yet, uniquely, was solely expressed at the midline. Additionally, EphB1-β-gal was found in the cingulate cortex and extended to an area matching the path of axons stemming from this region during callosal axon pathfinding (Fig. 5.4 C).

On a large scale, ephrin-B3-β-gal was expressed by the glial structures to form a ventral barrier while EphB1-β-gal presented a dorsal barricade. Simultaneously, they are also both likely to express on the callosal axons themselves, yet at different regions within the brain. Interestingly, both molecules were expressed within the midline zipper

glia. This structure is required for adhesion to occur to allow fusion of the two hemispheres of the brain, so EphB1 and ephrin-B3 may participate in the joining of the hemispheres.

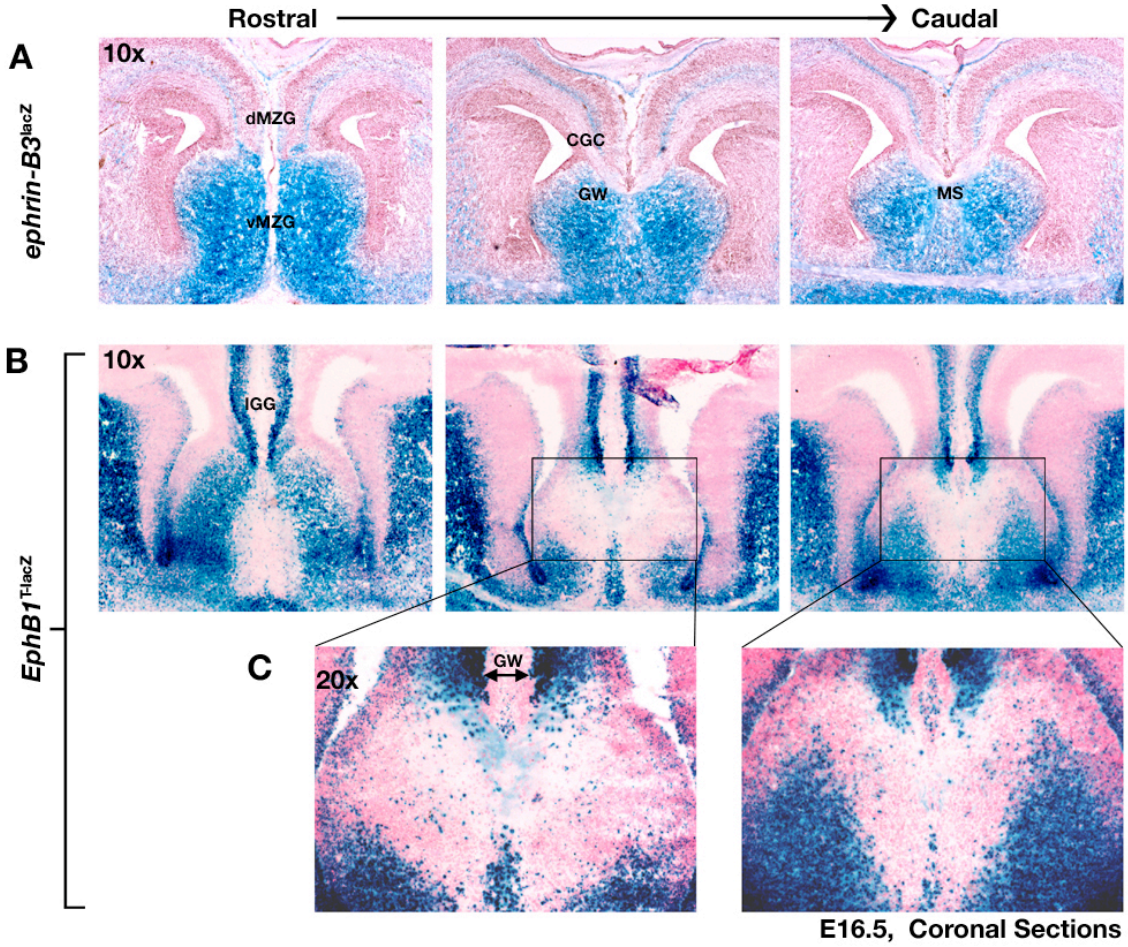


Fig 5.4: Expression of ephrin-B3 and EphB1 in the developing corpus callosum

E16.5 mouse brains were sectioned coronally through the developing corpus callosum and X-gal stained. Representative images from rostral to more caudal portions of the developing corpus callosum are presented. **A.** *Ephrin-B3*^{lacZ} mice show heavy expression around the ventral midline zipper glia (vMZG), glial wedge (GW), and the midline sling (MS) and lighter expression at the dorsal midline zipper glia (dMZG). **B.** *EphB1*$^{T\text{-}lacZ}$ mutant mice display expression at the indusium griseum (IGG), GW, vMZG, and within the cingulate cortex (CGC). **C.** Higher magnification of the x-gal staining ventral to the cingulate cortex and dorsal to the ventral portion of the glial wedge.

Discussion

The data presented here is the first to illustrate the importance of EphB forward signaling in the development of the corpus callosum. My data also adds to a previous study that showed ephrin-B1 reverse signaling through PDZ domain interactions is required for callosal fiber crossing (Bush and Soriano, 2009), for our ephrin-B2 signaling mutant mice develop AgCC. Additionally, the ephrin-B3 PDZ mutation leads to an increase in the percentage of mice if compounded with the EphB1 forward signaling mutation. Furthermore, this is the first data to show a clear role for EphB2, as previous data suggested that *EphB2*$^{lacZ/lacZ}$ mutant mice did not display a phenotype (Mendes 2006). Moreover, it is now clear that the kinase domain of EphB receptors is necessary to direct callosal axons as they pathfind to the contralateral hemisphere.

It remains unclear how and when forward or reverse signaling are initiated. The expression pattern of ephrin-B3 and EphB1 encompass the entirety of the developing corpus callosum. AgCC may be the result of defect in adhesion at the midline, but this is not likely. Although *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ mutant mice show split brains (data not shown), this phenotype did not occur consistently in mice with AgCC. More likely is that EphB and ephrin-B molecules are coexpressed on growing axons. While axons are pathfinding across the midline, an EphB1 wall is presented dorsally while an ephrin-B3 marker is located ventrally. Whether these interaction lead to attractive or repulsive mechanisms remains to be determined.

Chapter 6

Conclusion and future directions

On determining how EphB1 and EphB2 are uniquely activated at the chiasm

By analyzing various mutant mice, I have determined that the intracellular domain of EphB1 is required for VT RGC axons to be repelled at the optic chiasm, so these axons can be directed to the ipsilateral hemisphere. Additionally, I have shown that EphB2 is involved in this process yet does not participate nearly as robustly as EphB1.

Considering that the extracellular domain of EphB1 is important in its specificity of function at the optic chiasm (Petros et al., 2009), this should be an area to focus our attention. My data showed that ephrin-B1 and eprhin-B2 are functioning as ligands at the optic chiasm. Thus, it is unlikely that a specific ephrin-B2:EphB interaction justifies the preference of EphB1 activation at the chiasm. Since ephrin-B2-Fc can stimulate EphB2 expressing ventronasal and EphB1 and EphB2 expressing VT RGC axons to undergo growth cone collapse (Williams et al., 2003; Petros et al., 2010), why will it not do so at the chiasm *in vivo*? It may depend on the modification of EphB1 or EphB2 by an additional unidentified factor.

When grown on laminin alone, retinal axons will be repelled by Eph-Fc (Birgbauer et al., 2001) but if they are exposed to L1 this response is lost. If L1 and laminin are both added together, EphB2-Fc will cause the axon to pause but not undergo

growth cone collapse (Suh et al., 2004). This may help in explaining how EphB2 expressing cells are not repelled by the ligands ephrin-B1 and ephrin-B2 at the optic chiasm, for similar regulation may be occurring there. It does not necessarily have to be L1 that it responsible, but could be any regulatory molecule. Additionally, Ephs and ephrin extracellular domains are glycosylated. Without carbohydrates, there is a decrease in the affinity between Eph-ephrin binding domains (Blits-Huizinga et al., 2004). This could also explain why there is a difference in the binding of EphB1 and EphB2. EphB2 may not become bind sufficiently to ephrin-B1 and ephrin-B2 to become activated because it lacks a necessary recognition site for glycosylation or another modification.

As mentioned before, proteoglycans such as NrCAM have been shown to be crucial in the ability of RGC axons to project contralaterally. Additionally, the removal of the heparan sulfate transferase, Hs6st1, results in mice that have an increased percentage of contralaterally projecting RGC axons (Pratt et al., 2006). Likewise, when treated with a chondrotinaseABC, which removes all CSPGs, at E15.5 VT-RGC axons were not repelled at the chiasm as in untreated mice (Ichijo and Kawabata, 2001). The potential role proteoglycans play in Eph-ephrin signaling needs to be further examined.

If a proteoglycan similar to Nr-CAM is regulating EphB2 by inhibiting its activation by ephrin-Bs, that would clearly explain how EphB1 is the preferred receptor. Moreover, this would explain why *EphB1*$^{+/\text{T-lacZ}}$ heterozygotes have a statistically significant higher percentage of ipsilateral RGC axons reaching the LGN than the *EphB1*$^{+/\text{T-lacZ}}$:*EphB2*$^{\text{lacZ/lacZ}}$ compound mutants. In the single heterozygote mutant, EphB1 is able to become activated and transphosphorylated EphB2, but in the compound mutant mice, EphB2 remains inactive and repulsion at the chiasm is not initiated. When EphB2

forward signaling is lost, there is no reduction in the ability of retinal axons to be repelled at the chiasm. Furthermore, the combined loss of forward signaling in *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$:*EphB2*$^{lacZ/lacZ}$ double homozygote mutant results in a stronger reduction than *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ single mutant mice despite a clear presence of EphB2 in all ventral retinal axons.

Why is EphB2 showing a partial defect in these mice? It is likely due to a low ability of activation from ephrin-B ligands. This suggests that EphB2 is somewhat functional, but nowhere near as much as EphB1. To this point, if clustered ephrin-B-Fc is added to ventral retinal explant cultures, there is growth cone collapse initiated in both the VT and ventronasal quadrants. Although there is an increased responsiveness in VT RGC axons, ventronasal axons still respond to ephrin-B-Fc at higher concetrations. Thus, EphB2 is there and able to be activated. The intracellular machinery is equipped in these neurons, but they do not experience collapse and redirection to the ipsilateral hemisphere at the chiasm. There is a unique region most likely in the extracellular domain of EphB2 or EphB1. Afterall, EphB1 and EphB2 can both interact with the known downstream target Vav (Cowan et al., 2005). If the unique factor is in EphB2, it inhibits the stimulation through ephrin-B binding, but this effect must be temporary, as EphB2 is required for retinotectal mapping later in development and must then be able to respond to ephrin-B molecules (Hindges et al., 2002; Thakar et al., Submitted). Additionally, there may be a unique site on the extracellular domain of EphB1 that stimulates it as a ligand for ephrin-Bs. Indeed, glycosylation has been shown to be important for activity (Blits-Huizinga et al., 2004), so the modifcation may be unique to either EphB1 or EphB2.

Future studies involving EphBs at the optic chiasm

In order to determine which region is important, more work needs to be performed to identify this unique domain. Unfortunately, this may be difficult to perform *in vitro*, for the regulation of expression levels from expression vectors is difficult to regulate in order to mimic *in vivo* levels. Additionally, all ventral retinal explants respond to higher doses of ephrin-B-Fc (Williams et al., 2003). Still electroporation experiments similar to those performed by Petros et. al. are likely the best way to begin (Petros et al., 2009).

Precise point mutations will need to be made to accurately determine the exact point of uniqueness in EphB1 and EphB2. Preliminary experiments can be performed electroporating the expression vectors, but a point mutant will need to be made in mice to verify the function. The addition of a myc, flag, or HA tag to the modified chimera protein would then allow pull-down experiments to be performed *in vivo*. This will be necessary as this is likely a unique interaction that only occurs at the chiasm and may not be expressed in other areas, as EphB2 is functional in retinal axons at the superior colliculus (Birgbauer et al., 2000; Hindges et al., 2002; Thakar et al., Submitted). Binding partners of tagged WT EphB1 and chimeric EphB1/B2 can then be compared and contrasted to find this interacting protein. If this interaction is extremely subtle and time-sensitive, this may be the one of few limited alternatives to discovering what makes EphB1 and EphB2 uniquely stimulated at the optic chiasm.

On roles of EphB forward and ephrin-B reverse signals in callosal axon pathfinding

The experiments performed analyzing defects in the corpus callosum provided

interesting preliminary results. They demonstrated the importance of the intracellular domains of both EphB and ephrin-B molecules. As such, they provide direct evidence that both forward and reverse signaling are important in the ability of callosal axons to project to the contralateral hemisphere. Indeed, the compound *EphB1*$^{\text{T-lacZ}}$:*ephrin-B3*$^{\text{3YF}\Delta\text{V}}$ mutant mice seem to display an additive defect over the *EphB1*$^{\text{T-lacZ}}$ single mutants. Considering this mutant more closely matched the penetrance of *EphB1*$^{-/-}$ protein null mutant mice, this shows that EphB1 dependent reverse signaling is also required for proper corpus callosum formation. Indeed, EphBs and ephrin-Bs may have distinct functions each requiring their respective intracellular domains.

Furthermore, it is clear now that all future studies on the role of Ephs and ephrins in corpus callosum development need strenuous control over the strains of mice utilized. Some 129 sub-strains will autonomously develop AgCC even when WT (Livy and Wahlsten, 1997; Clapcote and Roder, 2006). Additionally, ephrin-B1$^{\text{5YF}\Delta\text{V}}$ reverse signaling mutant mice only develop AgCC in certain mouse strains while others show no phenotype (Bush and Soriano, 2009), and I failed to see AgCC in mice that completely lacked ephrin-B1. Strain disparity also likely explains why the *ephrin-B3*$^{-/-}$ mice analyzed here did not show any AgCC while Mendes et. al. reported that these mice do have this defect (Mendes et al., 2006).

Future studies of EphB and ephrin-B related AgCC

In addition to controlling strain variance, future studies need to focus on ephrin-B3 mutant mice. Our laboratory already has developed *ephrin-B3*$^{\Delta\text{V}}$ PDZ binding null and *ephrin-B3*$^{\text{3YF}}$ SH2 interacting null mutant mice. These should be crossed to the

EphB1$^{T\text{-}lacZ}$ mouse strain to determine which region of ephrin-B3 is responsible for the increase in penetrance of AgCC. Most likely, it will prove to be the PDZ binding domain site, which would agree with the previous report on *ephrin-B1* PDZ binding null mutant mice (Bush and Soriano, 2009). However, given the complex array of phenotypes and penetrance seen in EphB and ephrin-B mutants when examining AgCC, nothing should be assumed.

Furthermore, it will be necessary to find exact expression patterns of EphB and ephrin-B expression during corpus callosum development. There seems to be EphB1 expression on pioneering axons that bridge the midline, but this should be tested by culturing these axons *in vitro*. Since all callosal pioneer axons express neuropilin-1 (Richards 2004), they may be easily distinguished in culture. The fluorescent β-gal reporter 5-6 X-gal in retinal explant cultures can then be used in callosal neuron cultures to mark expression patterns for EphB1, EphB2, EphB4, ephrin-B2, and ephrin-B3 from the β-gal reporter mutant mice. Finally, by performing immunohistochemical stains with the available antibody to neuropilin-1 (Pratt et al., 2006; Piper et al., 2009), it will be possible to determine if there are any errors in the ability of pioneer axons to properly navigate. If this is the explanation, there would be errors evident throughout the *EphB1*$^{T\text{-}lacZ}$ mutants, while *EphB2*^{lacZ} mutants would only display errors in the more caudal portion of the developing callosum.

It is rather interesting to me that the loss of the intracellular domain of EphB1 can result in the failure of retinal axons not to cross chiasm and, sometimes in the same mutant animal, result in the failure of callosal axons to cross. This succinctly provides an example of how one protein can be utilized to function with altering consequences. Put

bluntly, in physics there may be an equal and opposite reaction for every action, but in the world of biology, every action seems capable of producing a multitude of reactions.

The role of EphB1 in neuropathic pain

Based on the preliminary behavioral studies conducted here, the loss of canonical EphB1 forward signaling is not required for mice to develop neuropathic pain. Further analysis will be required to determine if EphB1 mediated reverse signaling is required to develop neuropathic pain. However, when ephrin-B-Fc (which is composed of the extracellular domain of ephrin-B joined to the constant fragment of human IgG) is injected intrathecally, neuropathic pain develops. Moreover, the addition of Eph-Fc to rodents experiencing hyperalgesia temporarily relieves the heightened pain response (Battaglia et al., 2003; Song et al., 2008a). Both of these experiments implicate EphB1 forward signaling as the cause of hyperalgesia.

It is possible that EphB:ephrin-B interactions stimulate NMDA receptors in these nociceptive neurons. This would then lead to an influx of calcium ions, which will depolarize the cell and increase the likelihood of action potentials firing, and this increased sensitivity of nociceptive neurons to stimulus is transferred into hyperalgesia. Importantly, EphB:NMDA receptor interactions can occur independently of the intracellular domain of EphB receptors (Dalva et al., 2000). Thus, ephrin-B dependent activation of NDMA receptors would occur equally well in either WT or *EphB1*$^{\text{T-lacZ/T-lacZ}}$ mice while *EphB1*$^{-/-}$ protein null mice would not be able to be stimulated by ephrin-Bs, which matches the results obtained.

To better understand this phenotype, future experiments need to focus on the potential ligands for EphB1 activation. A likely candidate is ephrin-B2, which was shown to be expressed presynaptically in the dorsal horn while EphB1 is expressed postsynaptically (Battaglia et al., 2003). Interestingly, *ephrin-B2*$^{+/lacZ}$ heterozygote mutant mice have anti-social tendencies that were initially thought to be related to aggression, but preliminary studies did not reveal aggressive behavior (data not shown conducted by Sonal Thakar). These mice, however, do seem to behave in a sluggish almost dour manner that resembles mice that had surgery performed to induce neuropathic pain. As I have demonstrated the capability for *ephrin-B*$^{6YFDV/lacZ}$ mice to stimulate increased EphB1 forward signaling at the optic chiasm, it is also possible that these mice develop neuropathic pain due to overactivation of EphB1 within the dorsal horn by an overactive ephrin-B2-β-gal ligand. This can be tested directly by analyzing *ephrin-B2*$^{+/-}$ heterozygotes, for even though these animals will not be complete nulls, *EphB1*$^{+/-}$ heterozygotes display a phenotype (Han et al., 2008). Thus, if ephrin-B2 is the ligand, *ephrin-B2*$^{+/-}$ heterozygotes should also fail to develop neuropathic pain.

Concluding remarks

There is a sort of irony intrinsically built into neuroscience in that that we are using our brains while attempting to understand how our brain works. This is a sometimes-startling paradox to contemplate, for the complexity with which our mind functions seems to be more than we are currently capable of comprehending (Cook, 2008). Thus far, neuroscientists have laid out the map of how regions in the brain are connected with a high level of specificity, shown which types of cells are essential in

connecting these circuits, and have even discovered many of the molecules that allow these cells to function. Nevertheless, a true understanding of how our brain as a whole is able to function and allow us to form such complex thoughts still eludes us.

The standard operating procedure adopted thus far to tackle this daunting undertaking has been to focus on small individual pieces rather than immediately try to solve the larger puzzle of how the brain works. My small piece to aid in the understanding of the nervous system has been to focus on the protein EphB1 and its role in regulating axon pathfinding. The main long-term goal is that the information presented here will be applied to benefit medical science. While the experiments performed on axon pathfinding at the optic chiasm bore the most fruit, I hope that experiments into the role of EphB:ephrin-B interactions in the development of neuropathic pain and AgCC are continued, for the potential uses of the mutant mice described here to aid in understanding the workings of neuropathic pain and the development of the corpus callosum are quite promising.

Materials and methods:

Generation of EphB1$^{T\text{-}lacZ}$ mice. 129 mouse strain genomic DNA was obtained from Invitrogen Mouse BAC DNA Library Pools (Invitrogen cat# 96021RG) by screening bacterial artificial chromosome (BAC) superpools and pools via PCR to find a BAC clone 209575, which included exon 6 to 16 of *EphB1*. A pL452 based minitargeting vector (MTV) plasmid with a loxP flanked neo cassette (Liu et al., 2003) was constructed. A bacterial *lacZ* sequence was inserted in frame within exon 9 immediately following the codon corresponding to murine EphB1 amino acid #578 (EphB1: …YSDKL/ MARDD…: β-gal). Using recombineering the MTV was then inserted into the BAC, and a targeting vector (TV) was retrieved into the vector pL254 (based on pL253 including a diphtheria toxin-alpha negative selection expression cassette 5' of the left homology arm in addition to a tk negative selection cassette 3' of the right homology arm). The TV was electroporated into R1 strain mouse embryonic stem (ES) cells (Nagy et al., 1993), and 960 ES colonies were screened by southern blot analysis at the 5' and 3' ends using PCR synthesized probes. One clone (2C1) exhibiting homologous recombination was identified and injected into blastocysts. Resulting chimeric mice were mated and germline transmission of the initial *EphB1*$^{T\text{-}lacZ.Neo}$ allele was verified by southern analysis, PCR (Fwd: tgaatcctctgcccaagggaatgt, Rev mut: gaaacgccgagttaacgccatcaa (660 bp), Rev WT: tgcagaaggtaatcttccaccagg (450bp)), and sequencing. The loxP flanked neo cassette was removed by crossing *EphB1*$^{T\text{-}lacZ.Neo}$ mice to a transgenic mouse that expresses Cre-recombinase in the germline, which generated the *EphB1*$^{T\text{-}lacZ}$ allele.

Mice and breeding. Other mice used in this study have been previously described: *EphB1*^{lacZ} and *EphB1*$^{-}$ (Williams et al., 2003), *EphB2*$^{-}$ and *EphB2*^{lacZ} (Henkemeyer et al., 1996), *ephrin-B1*^{loxP} (Davy et al., 2004), and *ephrin-B2*^{T} and *ephrin-B2*^{lacZ} (Dravis et al., 2004), *ephrin-B2*$^{6YF\Delta V}$ (Thakar et al., 2011) *ephrin-B3*^{lacZ} and *ephrin-B3*$^{-/-}$ (Yokoyama et al., 2001), *ephrin-B3*$^{3YF\Delta V}$ (Xu and Henkemeyer, 2009), and *EphB2*$^{K\text{-}VEV}$ and *EphB2* ^{K661R} (Genander et al., 2009). *Ephrin-B2*^{T} mice are effectively protein null mutants since the truncated form of ephrin-B2 does not reach the cell surface so are referred to as *ephrin-B2*$^{-}$. All mice were housed in the Animal Resource Center at the University of Texas Southwestern Medical Center at Dallas and the experiments on them followed protocols approved by the Institutional Animal Care and Use Committee.

X-gal staining. To stain embryos as whole mounts, pregnant females containing fetuses at the desired time-points where the day of plug day was considered embryonic day 0.5 (E0.5) were euthanized with CO2 and specimens were removed, washed in wash buffer (2mM $MgCl_2$, 0.02% nonidet-P40, 0.1M PO_4 pH 7.3) fixed for 20 minutes at RT in fixative buffer (2% gluteraldehyde, 2mM $MgCl_2$, 5mM EGTA, 0.1M PO_4 buffer pH 7.3), and then placed in X-gal solution (wash buffer w/ 1mg/mL X-gal (Roche), 2.12 mg/mL K4[Fe(CN)6]•3H2O, 1.64 mg/mL K3[Fe(CN)6] (Sigma)) o/n at 37 °C. Specimens were washed, postfixed in 4% paraformaldehye (PFA) in PO_4 buffer o/n at RT, and were then dehydrated and clarified using methyl salicylate (Polysciences Inc.) prior to imaging.

To stain cryosections, unfixed brain tissue was quickly harvested and frozen under OCT medium or else cryopreserved in 30% sucrose in 0.1M PO_4 buffer pH 7.3 prior to freezing. Tissue was then sectioned on a cryostat (10 to 25 μm), immediately

mounted on charged slides, and allowed to air dry. Sections were then washed and placed in X-gal solution o/n at 37 °C. Slides were washed, post-fixed in 4% PFA, and counterstained with nuclear fast red.

Biotinylation assay. Primary cells were harvested from E13.5 fetuses by dissociating with trypsin and culturing *in vitro* for 24 hours. Cell surface proteins were then exposed and covalently linked to biotin by the addition of sulfo-NHS-SS-biotin (Pierce prod. #89881), and total cell protein lysates were then prepared in lysis buffer (1% Triton, 100mM NaCl, 50mM NaF, 50mM Tris-HCl pH 7.5) containing Complete (Roche) protease inhibitor cocktail. Streptavidin coated beads (Pierce) were used to pull down all biotinylated cell surface proteins, which were resolved on 6% Tris-glycine gels, transferred to PVDF (Millipore) membranes, and then immunoblotted with rabbit α-β-gal (Chemicon), goat α-EphB2 (R&D Systems), and mouse α-β-actin (Sigma) antibodies followed by secondary antibodies conjugated to horseradish peroxidase (Jackson).

Anterograde labeling the LGN. For cholera-toxin subunit B (CTB) labeling, three unique strain combinations groups were used, 50% 129/ 50% CD1 (all brown pigmented mice), 90% CD1/ 10%129 strain (25% brown pigmented, 50% gray pigmented, and 25% albino mice), and 100% CD1 (all albino mice) (More information about coat color alleles available at http://jaxmice.jax.org/jaxnotes/index.html). Each group was analyzed independently. Adult mice 8-12 weeks of age were anaesthetized with ketamine/xylazine and injected with 2-4 μL of 0.5% CTB-AlexaFluor 555 (red) in PBS in the right eye and CTB-488 (green) in the left eye (Sigma C-22843 and C-22841, respectively). Tracer was

allowed to undergo transport for two days and mice were cardiac perfused with 4% PFA. Brains were removed, post-fixed o/n, serially sectioned at 100 μm throughout entire LGN spanning ~1100 μm, and mounted in aquapolymount. The LGN was visualized under confocal microscopy using a Zeiss 510 LSM at 10x magnification. Images were quantified using ImageJ by taking pixel intensity of entire LGN's ipsilateral projection in one section and dividing the number by the corresponding contralateral measurements for corresponding tracer. P-values were determined by student's t-test with 0.05 being the minimum criteria for statistical significance.

Retinal explant cultures. E15.5 fetuses that were WT, *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$, or *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$: *EphB2*$^{-/-}$ had their retinas removed and explants isolated from either the most dorsal or ventral-temporal regions. Explants were grown for 16-24 hours on laminin and poly-O-ornithine coated glass coverslips in DMEM/F12 media supplemented with 1% BSA, insulin-transferrin-sodium selenite (Sigma cat# I1884), and 0.4% methylcellulose. Explants were then exposed to 250 μg/mL 5-bromo-6-chloro-3-indolyl-β-D-galactopyranoside (5-6 X-gal) (Sigma cat# B8931) to detect expression of EphB1-β-gal fusion protein for 1 hour at 37°C in media, washed 2x in PBS, fixed for 5 min in 4% PFA, immunostained with α-EphB2, and phalloidin conjugated to cy5 (Invitrogen #A22287), and then imaged by confocal microscopy at 63x magnification.

Generation of ephrin-B2 mutant mice. To construct a targeting vector, a BAC contig encompassing genomic DNA surrounding *ephrin-B2* exon 5, which encodes the entire intracellular domain of ephrin-B2, was first assembled in pBeloBAC11 from gDNA by

combining lambda phage clones EB2.9 and EB2.22 by using recombineering in *E. coli* strain EL 250 (Liu et al., 2003). Using a pL452-based MTV that contained a positive selection Neo cassette, a wild-type 300 bp 5'-arm of exon 5, and a 1200 bp 3'-arm containing base pair modifications corresponding to the desired 6YFΔV mutations (Y255F, Y307F, Y314F, Y319F, Y333F, Y334F, and Δ336V was recombined with endogenous ephrin-B2 sequence. A targeting vector was retrieved into pL254 (a modified form of pL253 with a 3' TK and additional
DT-A negative selection cassette and an AscI restriction enzyme site for linearization). R1 ES cells (Nagy et al., 1993) were electroporated and colonies screened by southern blotting. Germline transmission was obtained from chimeric mice generated by blastocyst injection. The loxP floxed neo cassette was deleted in the mouse by crossing to a germline expressing cre recombinase mouse. Genotypes were confirmed by sequencing and then PCR using the following primers: Forward 5'- GGC GTT TAA AGA CGG ACA TAT AAC A -3' Reverse 5'- CCT CAA GGT CCA ATG CTC ATA C-3' (data not shown).

*Immunoblotting***.** Whole protein samples were isolated from E11.5 WT and *EphB1*$^{T\text{-}lacZ}$ heterozygote embryos homogenized in an extracellular lysis buffer with a protease inhibitor cocktail. These were run on a 6% gel, transferred to PVDF membrane, and immunostained using rabbit α-β-gal (Chemicon) at 1:1000 in 1% milk then 1:2000 donkey α-rabbit peroxidase (Jackson) in 1% milk.

DiI labeling the optic chiasm. P0 or E18.5 pups were fixed o/n in 4% PFA. The solidified eye humors were removed and a small crystal of DiI was placed on the optic disk. DiI was allowed to trace for 7-10 days and the optic chiasm was exposed and visualized.

Measuring thermal sensitivity of mice. 2 month old mice were pre-tested twice in testing chambers to precondition the mice to their surroundings. Testing chambers were transparent plexiglass boxes ~12 x 6 x 6 cm in dimension over a temperature controlled glass plate. Mice then had their right and left hindpaw exposed to a focused radiant heat source generated by an IITC Model 336 Analgesia Meter (Life Science, Series 8, Woodland Hill, CA) when their paw was flush with the floor and the animal was neither moving nor sleeping, and the heat stimulus was automatically shut off after 20 seconds to avoid tissue damage. The latency of retraction was measured in seconds 7, 5, and 3 days prior to surgery. Neuropathic pain was produced using a rat-based CCI model where mice were anaesthetized with 50 mg/kg sodium pentabaritol had their left sciatic nerve exposed at mid-thigh level and three ligatures of 6-0 chromic gut suture was loosely tied around it in 0.5mm increments. Wounds were sutured after surgery. 3, 7, 10, and 14 days after surgery, all mice had their hindpaw retraction times measured at least 3 times for both the left and right paws, and the average differences and standard errors for WT and *EphB1*$^{\text{T-lacZ/T-lacZ}}$ mice were calculated.

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