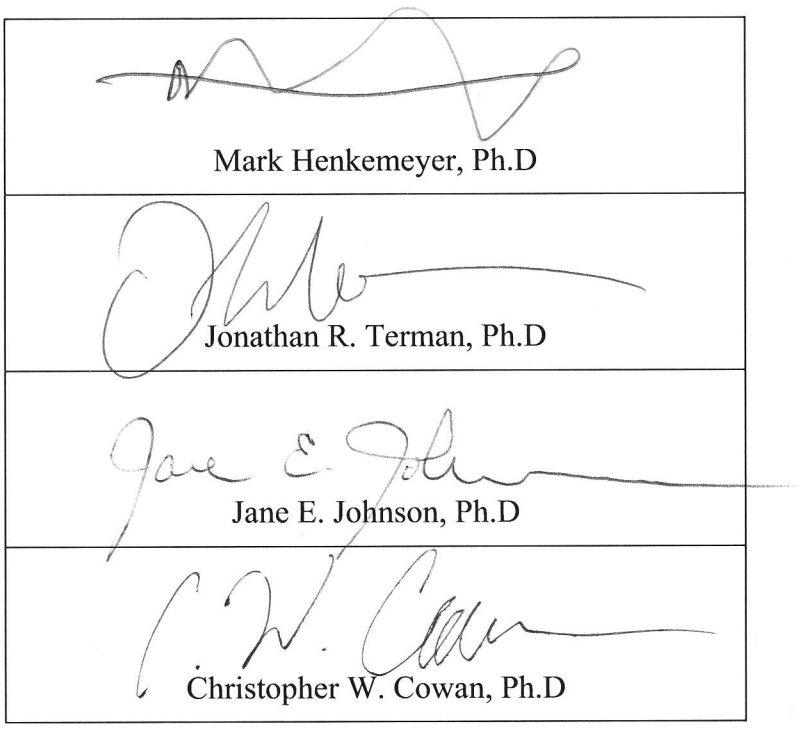
EPHB:EPHRIN-B BIDIRECTIONAL SIGNALING IN RETINOCOLLICULAR MAPPING

Approved by supervisory committee



For Mom, Dad, and Rahulbhai

EPHB:EPHRIN-B BIDIRECTIONAL SIGNALING IN RETINOCOLLICULAR MAPPING

by

SONAL GAUTAM THAKAR

DISSERTATION

Presented to the Faculty of the Graduate School of Biomedical Sciences

The University of Texas Southwestern Medical Center at Dallas

In Partial Fulfillment of Requirements

For the Degree of

DOCTOR OF PHILOSOPHY

The University of Texas Southwestern Medical Center at Dallas

Dallas, Texas

December 1, 2010

ACKNOWLEDGEMENTS

First, I would like to thank Mark for welcoming me into his lab in the spring of 2006 and his continued encouragement for me to be an independent thinker. Mark has always been able to evaluate my data and point out where things could be better or what needs to be done while giving me the freedom to figure out how to make it happen.

I would like to next thank Dr. Victoria Beckner who was responsible for introducing me to the world of scientific research during my sophomore year at The University of Texas at Austin. I joined her clinical psychology research group that studied the affects of stress on undergraduate students' memory so that I could get out of taking Introduction to Biology Lab, which is a crowded course at UT Austin. Her passion for discussion and mentorship was contagious and opened my eyes to a potential career option. The following two years I worked in Dr. Creagh Breuner's behavioral physiology lab. She not only taught me how to go "birding", by the way our model organism were white-throated sparrows migrating through Austin, but also created an environment that encouraged discussion. Thank you Dr. Delia Brownson for seeing leadership potential in a mousy undergraduate and helping me realize what I want to be when I grow up.

To my thesis committee, I would like to thank you for all the fruitful discussion and advice. I respect you all both professionally and personally. Dr. Christopher Cowan, I appreciate all your input and you have always been available to discuss an idea or even a tangent concept of interest that has often become relevant later. Dr. Jonathan Terman, you have not only provided insight into my scientific pursuits, but also allowed me a medium to express my nonscientific interests by letting me illustrate the annual Neuroscience Retreat cartoons the past two years. Dr.

Jane Johnson, you have ensured I stay on track in the neuroscience program and I still remember some of the advice you gave me when I rotated with you my first year. And Dr. Christopher Dravis, it pains me to put a title of respect like "Dr." in front of your name, but you have helped me be a better scientist. Your suggestion to do a complete run through before an oral presentation works wonders as well as your comments on my writing style and content after reading my dissertation helped and perhaps makes me think you deserve the title. Thank you Jan La Westerlund and Franny Prince for your assistance with genotyping.

I don't think I would be who I am today without my family and friends. Mom and Dad, thank you for everything you have taught me. You two have always encouraged me to pursue my interests and be the best I can be, but while doing so to remember to keep healthy and take care of those I care about. Thanks, Mom and Dad, for making sure I become a well-rounded career person. Rahul, Bhai, brotherbear. You were my hero when we were kids. You always saw something in me I could never see. Now, we are older and while you are still five years older, we are friends in addition to being siblings…except when you invoke being the older brother. Thanks for always reminding of my inner *Roy Hobbs* (cue lightning crash). To my sisters from another mother, Rudi Kang and Mitali Kagal Prakash. You two have been in my heart since middle school and still manage to have an anchor caught deep inside. Thanks for being with me when I was a quiet awkward kid and continuing to be there now that I'm a louder awkward adult. "*Ain't no mountain high enough, ain't no valley low enough…to keep me from getting to you babe[s]*".

Finally, to the friends I've made at UTSW who allowed me to maintain sanity after a bad week in the lab. Henkemites from past and present, we helped keep Barley House in business with the amount of patronage we've done. Too bad we stopped going to The Beagle, which

closed down shortly after we stopped going there. Chris, you can *usually* say the right thing to pull me out of a funk and there are few people who know me as well as you do. Jan, Tracey, George, and Tim…you're family to me. Jan and Tracey, we've been through the lows and highs together and let's not let it stop just because of physical distance. Tim and George, my Henkbros, I know you'll miss me even if Tim won't admit it. Nikki, Oliver, Melanie, and Tony…how was I so lucky to meet friends like you? When we first started hanging out I knew this was the beginning a beautiful friendship. Tony's "Lateral who?!" and other verbal outbursts during IM sports are a stark contrast to the even tempered man I know off the field, making those comments all the more funny. Any story of Melanie's is full of detail and interest and I hope I will still have opportunities to hear them. Nikki, sometimes I feel you and I are two peas in a pod sometimes, is it because we share the same birthday month? A loyal and steadfast friend, Oli, you are not that old so stop complaining like an old man. I feel I've made memories for a lifetime with you guys. So, ya'll will Skype, right?

EPHB:EPHRIN-B BIDIRECTIONAL SIGNALING IN RETINOCOLLICULAR MAPPING

Sonal Gautam Thakar

The University of Texas Southwestern Medical Center at Dallas, 2010

Supervising Professor: Mark Henkemeyer, Ph.D

Retinal ganglion cell axon exit into the optic disc and formation of the optic nerve and termination in the superior colliculus is critical for integrating both the visual field and head orientation to mediate appropriate eye movement. Expression of EphB receptor tyrosine kinases and ephrin-B ligands in the visual system signify the potential role for EphB:ephrin-B bidirectional signaling in the development of the visual system. Previous studies of *EphB2*$^{-/-}$*;EphB3*$^{-/-}$ (receptor null mutant) and *EphB2*$^{lacZ/lacZ}$*;EphB3*$^{-/-}$ (EphB2 intracellular signaling deficient mutant) mice established that EphB acting as a ligand mediates RGC axon exit from the optic disc. Use of these compound mutants also showed EphB2 forward signaling mediates ventral RGC axon branching and formation of correct termination zones (TZ) within the medial SC. However, the molecules acting as the receptor to mediate dorsal RGC axon exit into the optic disc are unknown. Also, the EphB2 intracellular component essential for retinocollicular mapping is unknown as are the roles for EphB1, ephrin-B1, and ephrin-B2, which are also expressed in the visual system.

I found that ephrin-B1 and ephrin-B2 reverse signaling has a minor role for directing RGC axon guidance to the optic nerve head. On the other hand, EphB:ephrin-B bidirectional signaling appears to play a major role in dorsoventral RGC axon retinocollicular mapping. DiI labeling of a subset of dorsal or ventral-temporal RGC axons allowed visualization of the termination zone in the superior colliculus in postnatal pups. After a comprehensive analysis of various *EphB* and *ephrin-B* null, intracellular truncation, and point mutants, that affect specific components of forward and reverse signaling, EphB:ephrin-B bidirectional signaling was found to be the key mediator of dorsoventral retinocollicular mapping. Specifically, EphB2 tyrosine kinase catalytic activity alone is critical for ventral RGC axon retinocollicular mapping and EphB1 forward signaling is crucial for ventral RGC axon retinocollicular mapping. Together, EphB1 and EphB2 are the chief mediators of ventral RGC axon retinocollicular mapping. Whereas ephrin-B1 expressed in the SC acts as a ligand to mediate ventral RGC axons, ephrin-B2 expressed in a high dorsal/low ventral gradient in the retina functions as a receptor that is important for both dorsal and ventral RGC axon retinocollicular mapping. Establishing the molecules involved with retinocollicular mapping will focus the analysis of the remaining questions pertaining to the development of the visual system.

Table of Contents

Prior Publications that appear in this work

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

Genander, M., Halford, M.M., Xu, N.J., Eriksson, M., Yu, Z., Qiu, Z., Martling, A., Greicius, G., **Thakar, S.**, Catchpole, T.*, et al.* (2009). Dissociation of EphB2 signaling pathways mediating progenitor cell proliferation and tumor suppression. Cell *139*, 679-692.

List of Figures

List of Tables

List of Abbreviations

ADAM	A-Disintegrin-And-Metalloprotease
ALCAM	activated leukocyte cell adhesion molecule
AP	alkaline phosphatase
β-gal	beta-galactosidase
BMP	Bone Morphogenetic Proteins
C	Caudal
cDNA	complementary DNA
CNS	central nervous system
CST	corticospinal tract
D	Dorsal
DiI	1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate
E	embryonic day
ECD	extracellular domain
Eph	Erythropoietin-producing hepatocellular carcinoma
ephrin	Eph Receptor Interacting Protein
Fc	immunoglobulin Fc region
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factors
GDP	guanosine diphosphate
GPI	glycosylphosphoinositol
Grb4	Growth Factor Receptor Interacting Protein
GTP	guanosine triphosphate

LGN	lateral geniculate nucleus
LMC	lateral motor column
N	Nasal
NMR	nuclear magnetic resonance
OT	optic tectum
P	postnatal day
PCR	polymerase chain reaction
PDZ	type-II Post-synaptic Density-95/Disc large/Zonula Occludens-1
PNS	peripheral nervous system
PTP-BL	Protein Tyrosine Phosphatase BAS-Like
Ptpro	protein tyrosine phosphatase receptor type O
R	Rostral
RGC	retinal ganglion cell
RGS	regulator of G-protein singaling
SAM	sterile alpha motif
SC	superior colliculus
SH2	Src Homology 2
SH3	Src Homology 3
T	Temporal
TZ	termination zone
V	Ventral
VT	ventral-temporal
WT	wild type

Chapter 1

INTRODUCTION

What is Vision?

Vision depends on specifically organized neuronal circuits formed during embryonic and postnatal development that are sustained into adulthood. When visual stimuli reach the eye in the form of light, the retina is the first to receive and process the information through a complex system of photoreceptors, bipolar cells, amacrine cells, and horizontal cells (reviewed in (Sung and Chuang, 2010)). The information is integrated and sent out via retinal ganglion cell (RGC) axons to targets in the brain, such as the visual cortex, lateral geniculate nucleus (LGN), and superior colliculus (SC). RGCs are the sole retinal cell-type carrying visual information into the brain. RGC axons radially project to and funnel out of the optic disc located in the back of the retina to form the optic nerve. Over 50,000 RGC axons in mice and over a million RGC axons in humans must navigate through the brain to find their targets (Sung and Chuang, 2010). The optic nerve reaches the optic chiasm where in mice 3% of the RGC axons project ipsilaterally, while the overwhelming majority crossover to the contralateral side. Once the RGC axons pass this point and reach the brain, the axons navigate to their target regions within the LGN and SC. The LGN then sends axonal projections to the visual cortex and the visual cortex also makes its own synaptic connections in the SC. The visual system is crafted so that the retina projects a two dimensional spatial map of the visual field onto the visual cortex and SC. The LGN processes information on visual perceptions, such as shade, color, relative motion, and depth, that are sent

to the visual cortex, while the SC processes information to control eye movement, pupil size, and circadian photoentrainment (Huberman et al., 2008).

Axon guidance within the retina

The retina is created by an outpouching from the diencephalon during early embryonic development and remains connected by an optic stalk that later develops into the optic nerve. As the development of the retina nears completion, retinal ganglion progenitor cells differentiate into the RGCs and the first RGCs appear at the center of the retina in proximity to what will become the optic nerve head (reviewed in (Oster et al., 2004)). These RGCs radially project their axons into the center of the retina to the optic disc. Although axonogenesis in mammals is a poorly understood process, evidence points towards cadherins, a calcium-dependent family of adhesion molecules, being important for early embryonic retinal development. Cadherins support chick RGC axon growth *in vitro* and dominant-negative expression of cadherin in *Xenopus* RGCs show 70% of the RGC axons fail to project their axon *in vivo* (Hatta and Takeichi, 1986; Matsunaga et al., 1988; Reichardt et al., 1992; Riehl et al., 1996). These data signify the potential for cadherins to support RGC growth as well as ability to initiate and extend their axons and dendrites in higher order vertebrates such as rodents and humans.

The more peripheral RGCs form later so that the last RGC axons to project their axons are at the peripheral edge of the retina. Studies that have labeled RGCs and their axonal projections using DiI (1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate), a lipophilic dye taken up by the cell that can retrograde and anterograde label the full length of the axonal projection, demonstrate how RGC axons all project into the optic disc rather than project

elsewhere within the retina (Birgbauer et al., 2000). As a consequence, the more peripheral RGC axons have a long distance to travel to the optic disc and therefore require guidance cues to help navigate to the optic disc.

Like many axon pathfinding events, RGC axons prefer to form axon bundles and travel as a group, or fasciculate. RGCs closest to the optic nerve head project axons into the optic disc and as a result, more peripheral RGC axons fasciculate with these more proximal axons (reviewed in (Oster et al., 2004)). In rats and goldfish, disruption of fasciculation by blocking L1 or neurolin, two members of the Ig family capable of homophilic binding, causes RGC axons to misproject back into the peripheral retina and/or grow in circles resulting in a subset of RGC axons that do not project into the optic disc (Brittis et al., 1995; Leppert et al., 1999; Ott et al., 1998). These data indicate the importance of fasciculation in RGC axon guidance, as any disruption of fasciculation results in disordered axon pathfinding.

Molecular repellents also serve to facilitate the mass exodus of RGC axons out of the retina by inhibiting migrating axons from meandering into incorrect regions. Chrondroitin sulfate proteogylcan, a negatively-charged extracellular matrix molecule, is located in the periphery of the retina and was one of the first molecules found to inhibit RGC axon extension (Brittis et al., 1992; Snow and Letourneau, 1992). Without it, RGC axons do not project uniformly into the center of the retina and instead project back into the periphery of the retina. Another important RGC axon guidance molecule utilizing cell-axon repulsion is Slit and their receptor Robo, which are both expressed in rat RGCs (Ringstedt et al., 2000). *In vitro* analysis of retinal explants shows Slits repel RGC axons, decrease RGC axon length, and increase RGC axon fasciciulation. Better understanding of how Slit mediates the directionality and fasciculation of RGC axons in the retina *in vivo* is needed.

One family of axon guidance molecules that have been studied both *in vitro* and *in vivo* for their role in RGC axon guidance to the optic disc is the B-subclass of Eph (Erythropoietin-producing hepatocellular carcinoma) receptors and ephrin (Eph Receptor Interacting Protein) ligands. The Eph receptors have a low dorsal/high ventral gradient expression while the ephrin-B ligands have a high dorsal/low ventral gradient expression in the retina. In 20% of the *EphB* receptor null mutants, a subset of dorsal RGC axons defasciculate away from the axon bundle as they approach the optic nerve head. Because EphB2 intracellular truncated mutants exhibited no mistargeting defects, this behavior depends on the EphB receptor extracellular domain acting as a ligand (Birgbauer et al., 2000). *In vitro*, growth cone assays demonstrated EphB extracellular domain (ECD) can induce growth cone collapse (Birgbauer et al., 2001). These data demonstrate the potential role for EphB receptors to act as ligands to mediate repulsion that inhibits aberrant axon extension into the periphery of the retina.

Once axons are able to pathfind to the optic disc, they must exit. Netrin-1 is proposed to attract RGC axons to the optic nerve head so that they will exit the retina and form the optic nerve (Deiner et al., 1997). Netrin-1 is expressed in neuroepithelial cells that are localized at the optic nerve head. The Netrin-1 receptor, DCC, is expressed by RGC axons that course through the optic nerve head interacting with Netrin-1 on the neuroepithelial cells. Mutant mice lacking Netrin-1 and DCC exhibit optic nerve hypoplasia, in which the majority of RGC axons did not exit through the optic disc and instead projected aberrantly in the region surrounding the optic disc resulting in severe optic nerve head developmental malformations. *In vitro*, RGC axons are attracted to transfected cells secreting Netrin-1, further implicating Netrin-1 as the molecular attractant for RGC axons to the optic nerve head.

While the optic nerve must promote RGC axon growth and extension towards its target regions, inhibitory mechanisms must be in place to ensure the RGC axons maintain their path rather than project incorrectly to the surrounding region. Sema5A is a likely candidate for this role because it is expressed in the neuroepithelial cells around the circumference of the optic nerve head and likely inhibits RGC axon growth as *in vitro* growth cone collapse assays have shown (Oster et al., 2003). Silencing Sema5A by *in utero* intraocular injection of anti-Sema5A in embryos, demonstrated the *in vivo* relevance for Sema5A expression around the optic nerve. In mice treated with anti-Sema5A, a subset of RGC axons projected away from the optic nerve bundle, and in some cases whole axon fascicles aberrantly projected from the optic nerve.

The exact mechanism of how RGC axons are attracted to the optic nerve head and then leave to form the optic nerve is not fully understood in mammalian species. Clearly, all the these molecules plus those for which roles have not yet been revealed, must work together to mediate RGC axon radial projection into the center of the retina and exit though the optic disc to form the optic nerve. While some molecules appear to promote RGC axon fasciculation, others function to encourage RGC axons to navigate towards to the optic disc without projecting back into the peripheral retina. Through the balance of these opposing forces in the developing retina, RGC axons are able to exit the eye and carry visual information to the brain.

Guidance of RGC axons into the brain

Historical perspective

Around the turn of the 20th century, the Spanish neuroscientist Santiago Ramón y Cajal first visualized highly ordered neuronal circuits of the retina, optic tectum (OT), hippocampus, and cerebellum using the Golgi method to silver stain, which randomly labels a subset of cells to better visualize individual cells. His artful illustrations prompted questions about how these intricate neuronal connections originated. Scientists, such as Wiesel & Hubel and Roger Sperry, spent the next half-century conducting landmark studies to answer questions about how these neuronal connections arise and relate to visual experiences.

Behavioral tests performed by Sperry in the 1940's, in which he rotated or switched the eyes or uncrossed optic nerve fibers of frogs, demonstrated the positional relation of the eye to the brain. In the course of three papers, Sperry detailed how the frogs with regenerated optic nerves would respond to their target when trying to catch a fly for a meal (Sperry, 1943, 1944, 1945). Frogs with only severed optic nerves would regenerate their nerves and respond appropriately to visual stimuli and be able to capture the fly. Frogs with eyes rotated 180º, switched eyes, or uncrossed optic nerves had an inverted response to the fly. Histological observation of morphological changes after the left and right eyes were switched, lead Sperry to believe the RGC axons pathfind into their original target rather than a new one based on the positional changes. His observations lead to the hypothesis that the optic nerve fibers do not passively following along the path, but rather respond to intrinsic cues expressed in the eye and OT that mediate the ability for axons to navigate and find their precise target region.

To provide his critics with evidence beyond behavioral correlations, Sperry wanted anatomical confirmation for his hypothesis. Using Bodian silver stain, which preferentially labeled regenerating neurons, Sperry and Attardi removed half of the retina or crushed the optic nerve of a goldfish so that they could observe the new axons growing into the brain (Attardi and Sperry, 1963). They observed regenerating axons project back to their appropriate target region even if a more convenient region was accessible. Combining his observations of the retinotectal system, Sperry refined his chemoaffinity hypothesis to state that cells were labeled with at least two markers, one to denote cell type and another to describe its position and that these positional cues between cells projecting axons and the cells of the target regions were expressed in complementary gradients (Sperry, 1963). Many other scientists questioned if his theory based on observations in lower vertebrates would hold true in humans and other higher order mammals.

While the lower vertebrate models able to regenerate neurons provided an ideal system to observe the topographic consequences of optic nerve crush and retina ablation, critics questioned if the observations seen in the frog and fish remain valid in higher order mammals. In a series of three papers in 1963, Wiesel & Hubel performed monocular deprivation in kittens by suturing the eyelids of one side shut. They then followed up with single-cell recordings of the LGN and visual cortex and observed atrophy of cells in the LGN and changes in ocular dominance in the visual cortex compared to normal binocular controls (Hubel and Wiesel, 1963; Wiesel and Hubel, 1963a, b). Their results demonstrated the importance of early visual experiences in the developing brain for anatomical and functional precision.

The introduction of lipophilic axon tracers, such as DiI, in the 1980s as demonstrated by Dennis O'Leary and colleagues revealed when and how the retinotopic map develops. Retinotopic mapping, the specific point-to-point localization of RGC axons to their target region

such as the OT/SC, is critical for interpreting visual stimuli as well as provides a model system to determine function and conditions of the mediators underlying map development. Topographic guidance molecules expressed by the RGC axons and the SC target region are believed to mediate this process, leading to a highly precise two-dimensional representation of the surface of the eye onto the OT/SC that becomes further refined by correlated spontaneous retinal activity and the critical period after eye opening (reviewed in (Huberman et al., 2008)). In lower vertebrates, such as frogs and fish, ventral/dorsal RGC axons target the dorsal (medial)/ventral (lateral) OT and nasal/temporal RGC axons target the posterior/anterior OT. Similarly in rodents and chicks, ventral/dorsal RGC axons target the medial/lateral SC and nasal/temporal RGC axons target the caudal/rostral SC (Figure 1.1).

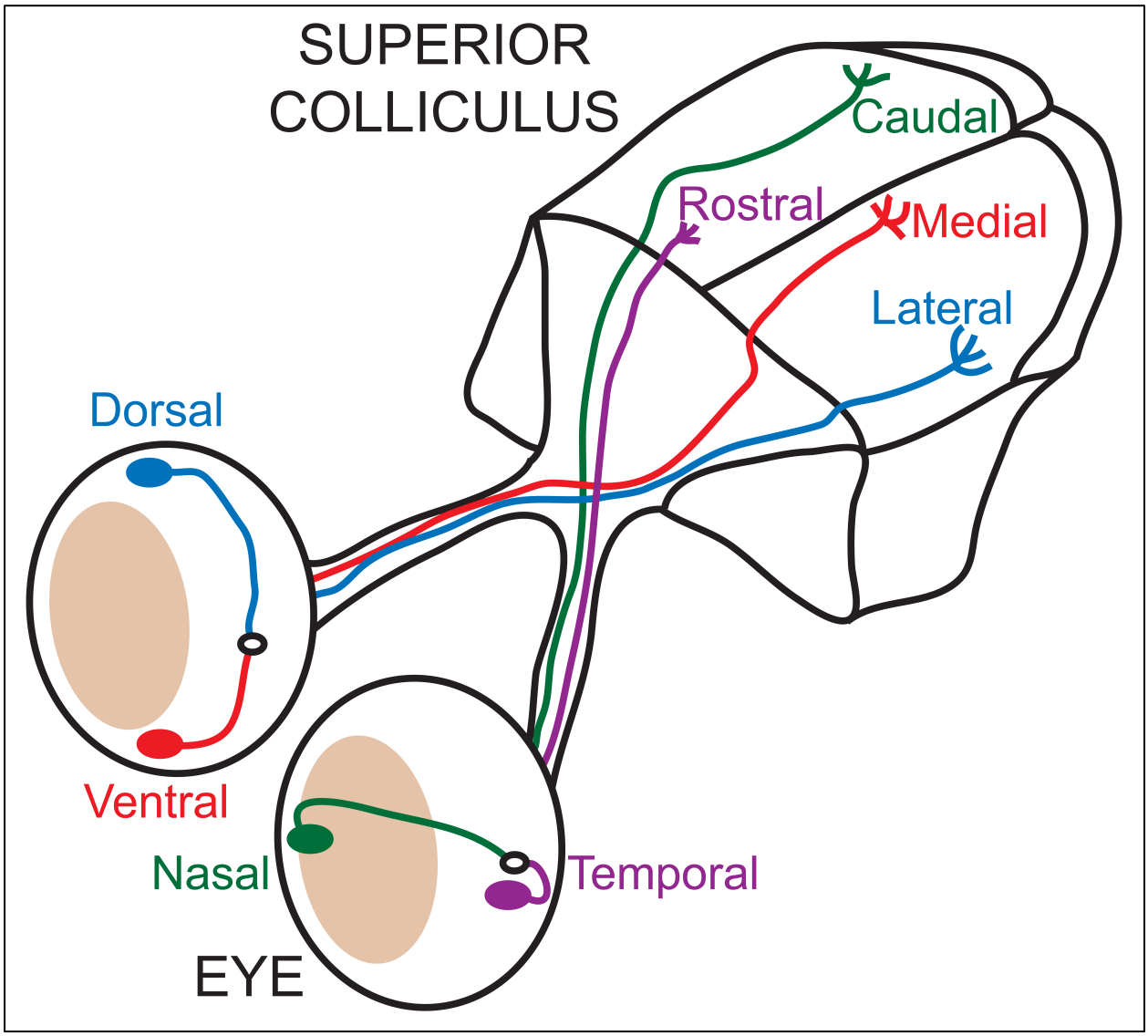


Figure 1.1 Schematic of retinotopic mapping in rodents and chicks

In rodents and chicks, RGC axons along the ventral-dorsal axis of the retina target the medial-lateral axis of the SC and RGC axon along the nasal-temporal axis of the retina target the caudal-rostral axis of SC.

Developmental stages of the topographic mapping

While different species share similar retinotopy, the manner in which they develop are unique. Frog and fish RGCs send axonal projections to the topographically correct position in the OT and then the growth cone arborizes by forming short terminal branches to form the appropriate synaptic connections in the termination zone (TZ) (Fujisawa, 1987; Harris et al., 1987; Kaethner and Stuermer, 1992; O'Rourke and Fraser, 1990). While frog and fish RGC axons target directly to their TZ in the OT, rodent and chick RGC axons initially overshoot and must prune back over time to their TZ in the OT/SC in a multistep process (Hindges et al., 2002; Nakamura and O'Leary, 1989; Simon and O'Leary, 1992a; Yates et al., 2001). From embryonic day 16 (E16) to postnatal day 0 (P0), the RGC axons find the OT/SC and target to the anterior/caudal region. Around P2, interstitial branching emerges along the length of the RGC axons with the majority occurring in the expected region of the TZ. The interstitial branching extends along the medial (dorsal)-lateral (ventral) axis towards their future TZ. Around P4, the segments of the RGC primary axon that are rostral (anterior) or caudal (posterior) to the TZ as well as branches and arbors outside the TZ boundaries begin to be eliminated. The RGC axons and branches are eliminated by Wallerian degeneration through a caspase-6 dependent mechanism (Nikolaev et al., 2009). By P8, a visibly compact TZ is formed resembling the mature TZ. Spontaneous retinal activity and the critical period after eye opening further refine topographic maps (reviewed in (Huberman et al., 2008)).

As the developmental stages of retinotopic mapping were being elucidated in the 1980's and early 90's, a new family of guidance cues was also being discovered. Before discussing how these guidance molecules mediate the stages of retinocollicular development, let me first describe this unique class of Eph receptor tyrosine kinases and their ephrin ligands.

Eph receptor tyrosine kinase and ephrin ligands

Historical perspective

Cell signaling factors have classical been discovered using a bioassay approach that first identifies a cellular behavior and then traces the molecule during a series of cloning or purification steps requiring that the protein to remain in a soluble form. However, Eph receptors were identified during the late 1980's and 90's using molecular techniques. The cleavage of Ephs and ephrins from the membrane results in the loss of their activity likely contributed to them not being discovered earlier. EphA1 was first identified in a cDNA library screen using a kinase domain probe (Hirai et al., 1987) and its kinase domain sequence was used to identify subsequent Eph receptors using cross-hybridization or PCR (polymerase chain reaction) based on sequence homology (Chan and Watt, 1991; Gilardi-Hebenstreit et al., 1992; Lai and Lemke, 1991; Lindberg and Hunter, 1990). Other Eph receptors have been identified using a phosphotyrosine antibody to screen bacterial cDNA expression libraries (Henkemeyer et al., 1994; Letwin et al., 1988; Pasquale, 1991; Pasquale and Singer, 1989; Sajjadi et al., 1991; Zhou et al., 1994).

Following the identification of Eph receptors, focus turned towards identifying their ligands. The ephrin ligands were revealed using the receptor extracellular domain to identify their binding partner. The Eph receptor ECD was fused to a tag that was either the immunoglobulin Fc region (-Fc) or alkaline phosphatase (-AP). The Eph ECD-Fc reagents served as affinity probes to detect several ephrin ligands (Bartley et al., 1994; Beckmann et al., 1994; Bennett et al., 1995; Cheng and Flanagan, 1994; Davis et al., 1994; Kozlosky et al., 1995; Shao et al., 1994; Winslow et al., 1995). The remaining ephrin ligands were discovered based on

sequence homology (Bergemann et al., 1995; Cerretti et al., 1995; Gale et al., 1996; Nicola et al., 1996).

As the Ephs and ephrins were being discovered and classified (refer to figure 1.2 for dendrogram of the mammalian Eph and ephrin molecules), researchers began to realize these molecules formed the largest family of receptor tyrosines and were expressed throughout embryonic and postnatal development. The expression of ephrin-A5 in chick OT drew attention to how these molecules could influence retinotopic mapping (Drescher et al., 1995). In addition, their structure enables these molecules to have unique bidirectional signaling properties that will be detailed in the sections below.

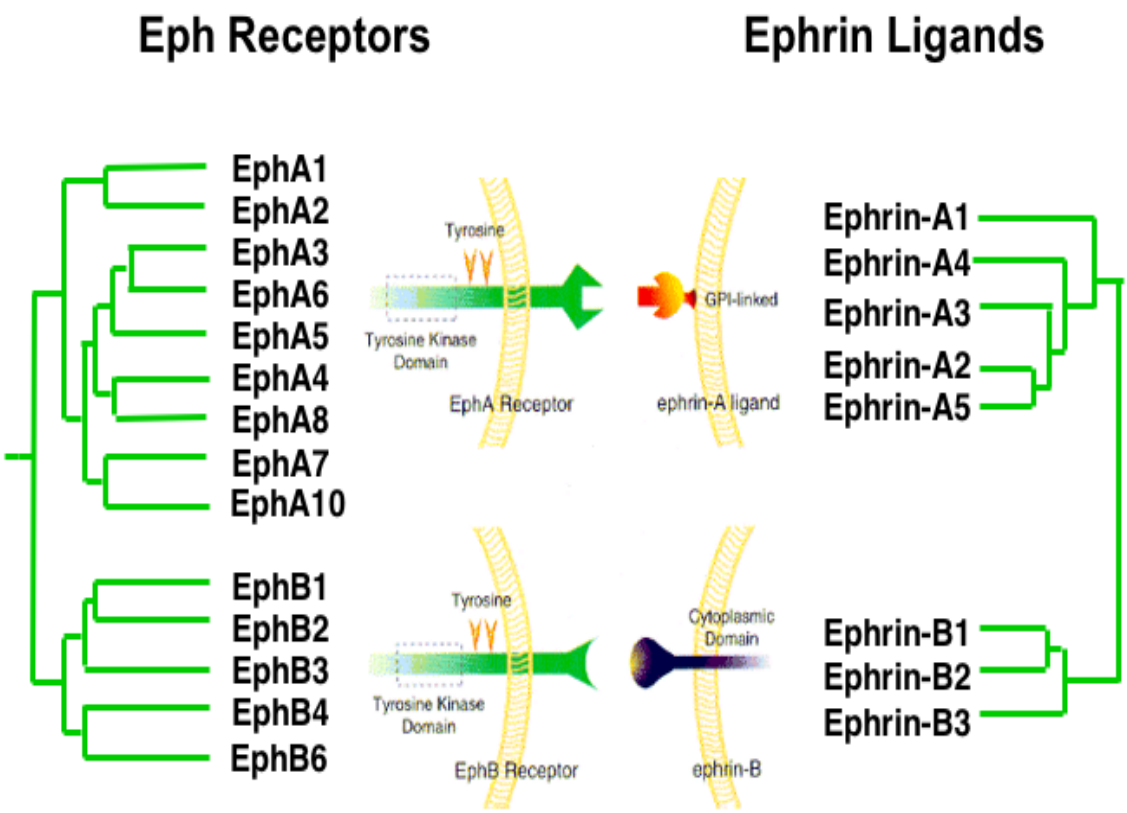


Figure 1.2 Dendrogram of mammalian Eph and ephrin molecules

The Eph receptors are divided into 2 subclasses based on the ligands, ephrin-A and ephrin-B, they bind. Ephrin-A molecules are GPI-linked to the cell membrane surface while ephrin-B molecules have a transmembrane domain and cytoplasmic tail.

Eph receptor and ephrin ligand structure

The Eph receptors constitute the largest family of receptor tyrosine kinases and all share a similar structure consisting of the extracellular domain containing an N-terminal ligand binding domain, a cysteine-rich region, and two fibronectin type III repeats. The intracellular domain includes the juxtamembrane region, tyrosine kinase domain, sterile alpha motif (SAM), conserved tyrosines, and a type-II PSD-95/Disc large/ZO-1 (PDZ) binding motif at the C-terminus (Figure 1.3).

The Eph receptors are divided into the A and B subclasses, and are distinguished by the extracellular region amino acid sequence and therefore binding affinity to structurally distinct ephrin ligands. Ephrin-A ligands attach to the outer membrane by means of a GPI (glycosylphosphoinositol) anchor. On the other hand, ephrin-B ligands contain a transmembrane domain that connects the extracellular ligand binding domain to the intracellular cytoplasmic tail containing conserved tyrosines, and a PDZ binding motif (Figure 1.3).

EphA (for mammals A1-A8 and A10) receptors bind to ephrin-A (A1-A5) while EphB (for mammals B1-B4 and B6) receptors bind ephrin-B (B1-B3). The molecules generally interact promiscuously only within their own subclass, but some exceptions do exist. Both EphA4 and EphB2 are capable of interacting outside their subclass. Whereas Eph:ephrin intraclass high affinity binding is achieved by a hydrophobic Eph channel:ephrin G-H loop interaction, EphA4 binds the ephrin-B molecules with a lower affinity via the ephrin binding channel by an additional stabilizing interaction through a separate polar contact region as illustrated by the crystal structure of EphA4:ephrin-B2 dimer (Chrencik et al., 2006; Qin et al., 2010). EphB2 binds ephrin-A5 through the channel region and has no other known stabilizing interaction resulting in an even lower affinity interaction than EphA4:ephrin-B2 (Himanen et al., 2004; Qin

et al., 2010). While the variety of Ephs able to interact with multiple ephrins may seem like simple biological redundancy, these molecules accomplish signaling specificity by their localized regional expression further described in “Physiological roles of Eph:ephrin bidirectional signaling” section.

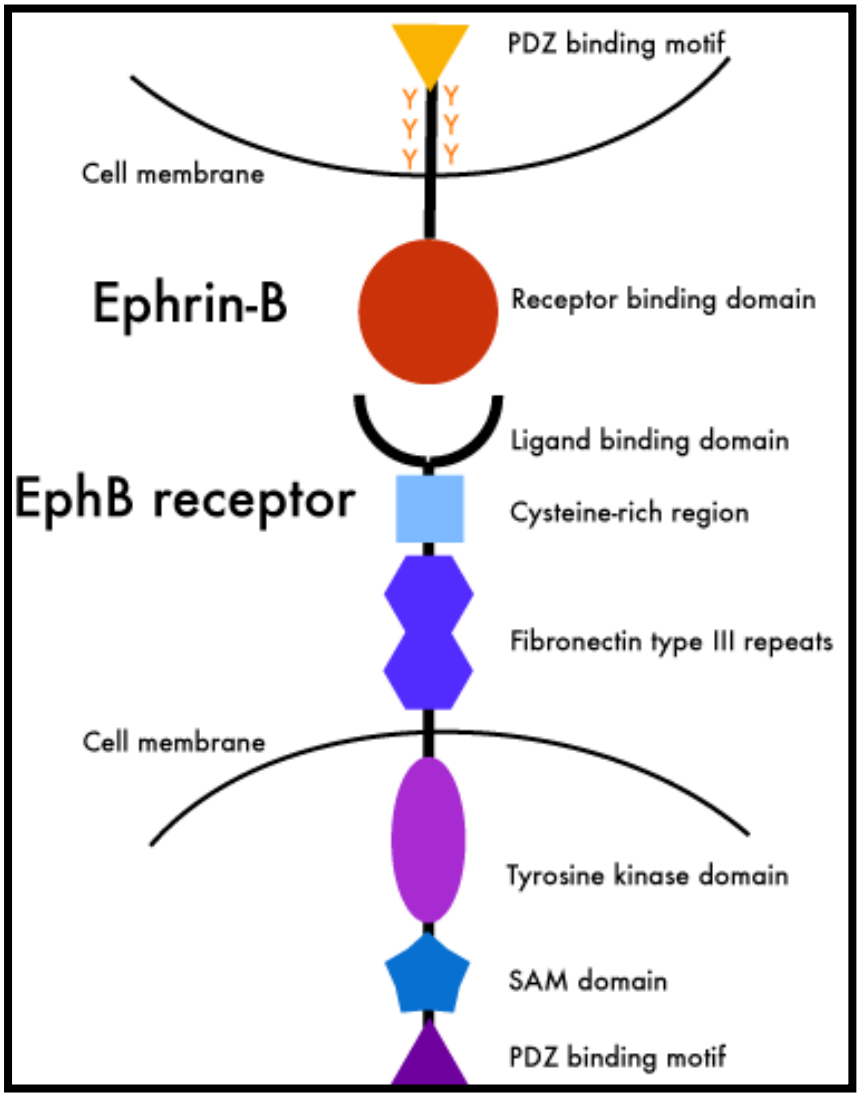


Figure 1.3 Structure of the EphB receptor tyrosine kinase and ephrin-B ligand.

Activation of Eph:ephrin bidirectional signaling

The Eph and ephrin molecules form a unique family of signaling molecules able to transduce signals in not only the receptor-expressing cell, forward signaling, but also the ligand-expressing cell, reverse signaling (Bruckner et al., 1997; Henkemeyer et al., 1996; Holland et al., 1996). These molecules are cell-membrane bound and are capable of interacting on opposing cell-cell or cell-axon membrane surfaces, *in trans*, and within the same membrane, *in cis*. Before binding, Eph and ephrin molecules remain in lipid microdomains/rafts enriched in signaling molecules such as kinases, GTPases, and G proteins (Bruckner et al., 1999; Simons and Ikonen, 1997). After Eph:ephrin binding, the molecules cluster into larger patches of lipid raft domains.

X-ray crystallography has illustrated the conformational changes that lead to activation after Eph:ephrin dimerization. High affinity receptor-ligand dimers cluster together forming lower affinity tetramers that can further oligomerize (Davis et al., 1994; Himanen et al., 2001; Himanen et al., 2007). These interactions might be stabilized by the cysteine-rich region of the extracellular domain and SAM intracellular domain (Himanen and Nikolov, 2003).

Eph receptors and ephrin-B ligands contain conserved tyrosines in their intracellular region that become phosphorylated after binding *in trans* leading to recruitment of SH2 domain containing proteins. In addition, Eph receptors and ephrin-B ligands contain a PDZ binding motif that recruits PDZ domain containing proteins. Ephrin-A ligands lack an intracellular region though they are able to interact with co-receptors to recruit downstream signaling targets. Activation of Eph receptors is much like other receptor tyrosine kinases. Binding of their ligand, ephrin, results in Eph kinase activation and phosphorylation on conserved tyrosines to allow recruitment of SH2 domain containing proteins. Clustering of Eph receptors initiated by ephrin binding induces phosphorylation *in trans* at a conserved tyrosine on the Eph receptor tyrosine

kinase domain (activation loop) and two conserved tyrosines on the juxatamembrane region thus relieving the block on the activation site and thereby allowing for the phosphorylation of conserved tyrosines involved with the recruitment of SH2 domain containing proteins (Binns et al., 2000; Huse and Kuriyan, 2002; Wybenga-Groot et al., 2001). Further emphasized in a more recent X-ray crystallography and NMR (nuclear magnetic resonance) spectrometry mutagenesis study, phosporylation does not completely transition the Eph receptor into an active state, but rather alters the steric hinderance at the kinase domain impeding recruitment of downstream signaling molecules (Wiesner et al., 2006). The conformation changes that occur after ligand binding likely relieve steric hindrance of the C-terminal PDZ binding motif, which allows recruitment of PDZ domain containing proteins (Himanen et al., 2007; Torres et al., 1998).

The ephrin molecules are not limited to only ligand like roles, but are capable of receptor like signal transduction. Formation of the tetramers likely alters the conformation of the ephrin making it permissive to downstream signaling proteins. Ephrin molecules lack a catalytic domain and therefore must recruit activated tyrosine kinases, generally found in the proximity of the lipid raft. While ephrin-A ligands can only signal through co-receptors and other signaling molecules found within the lipid raft because they lack a cytoplasmic tail, Eph bound ephrin-B ligands are phosphorylated by Src family kinases on conserved tyrosines located in the cytoplasmic tail permitting recruitment of SH2 domain containing proteins (Bruckner et al., 1997; Holland et al., 1996; Kalo et al., 2001; Palmer et al., 2002). The phosphorylation is transient due to ephrin-B2 recruitment of PDZ domain containing protein tyrosine phosphatase, PTP-BL (Palmer et al., 2002).

Eph:ephrin bidirectional signaling targets

While receptor tyrosine kinases often target the nucleus to regulate transcription, Eph:ephrin bidirectional signaling primarily targets mediators of cytoskeletal dynamics. GTPases were the first established targets for Eph:ephrin bidirectional signaling and remain to be the major downstream signaling target. Activation of GTPases requires guanine nucleotide exchange factors (GEFs) that exchanges the bound GDP (guanosine diphosphate) for GTP (guanosine triphosphate) (Noren and Pasquale, 2004). GTPase-activating proteins (GAPs) inhibit GTPases by promoting hydrolysis of GTP to GDP. Eph:Ephrin binding typically initiates tyrosine phosphorylation allowing recruitment and/or activation of GEFs and GAPs to regulate GTPase activity.

Eph forward signaling was first found to regulate Ras family GTPases as are most receptor tyrosine kinases. Eph:ephrin signaling generally inhibits Ras activity to promote cell repulsion. For example, ephrin-B1 stimulation of EphB2 in cell cultures initiated the phosphorylation of $p62^{Dok}$ and the formation of a complex between $p62^{Dok}$, RasGAP, and Nck (an SH2/SH3 adaptor protein) (Holland et al., 1997). RasGAP and Nck interacted with activated EphB2 through their SH2 domain and this interaction is dependent on RasGAP binding to the juxtamembrane region of EphB2. Mice lacking *RasGAP* have defects in cellular organization (Henkemeyer et al., 1995). In addition, EphB2 activation by ephrin-B1 binding results in growth cone collapse and neurite retraction in cell cultures by reducing GTP-bound Ras (Dail et al., 2006).

It was later discovered that Eph:ephrin bidirectional signaling primarily employs Rho small GTPases as its downstream signaling target. Rho, Rac, and Cdc42 of the Rho family of GTPases are downstream Eph and ephrin signaling targets that regulate actin cytoskeleton

dynamics involved with cell structure and movement. Rac1 and Cdc42 are generally found to regulate lamellipodia and filopodia extension and are often downstream targets of EphB:ephrin-B forward and reverse signaling (Nobes and Hall, 1995; Noren and Pasquale, 2004). GEFs often facilitate the association between the EphB receptor and the GTPase. For example in hippocampal neurons, ephrin activation of the Eph receptor induces tyrosine phosphorylation and recruitment of GEFs such as intersectin, karlin, and Tiam1 resulting in activation of Cdc42 (intersectin) and Rac1 (karlirin, Tiam1) to modulate cytoskeleton remodeling required for dendritic spine morphogenesis (Irie and Yamaguchi, 2002; Penzes et al., 2003; Tolias et al., 2007).

RhoA generally regulates stress fiber and focal adhesion formation as well as cell contractility. RhoA is a common downstream target of EphA forward signaling via GEFs such as Ephexin (Puschmann and Turnley, 2010; Sahin et al., 2005) and Vms-RhoGEF (Ogita et al., 2003). However, RhoA has also been linked to EphB forward signaling (Nobes and Hall, 1995; Noren and Pasquale, 2004). For example, EphB2 association with SH2 domain containing proteins, Grb4/Nck, leads to recruitment of Dishevelled and activation of Rho. In addition, Vav2, a Rho family GEF shown to interact with both EphA and EphB molecules, promotes growth cone collapse in RGCs (Cowan et al., 2005).

As described earlier, ephrin-B ligands have the unique ability to recruit downstream signaling targets through its cytoplasmic tail. Like the Eph receptors, the ligand contains conserved tyrosines that when phosphorylated can recruit SH2 proteins. For example in cell culture assays, clustered EphB stimulation leads to tyrosine phosphorylation of ephrin-B and recruitment of Grb4/Nckβ/Nck2 causing growth cone collapse (Cowan and Henkemeyer, 2001; Xu and Henkemeyer, 2009). Grb4 contains an SH3 domain and is know to recruit numerous SH3

domain-containing proteins involved with cytoskeleton dynamics, for example PAK1 which interacts with Cdc42 and Rac1 (Cowan and Henkemeyer, 2001; Xu and Henkemeyer, 2009). The ephrin-B signaling cascade plays an important role in biological functions such as ephrin-B3 reverse signaling mediated axon pruning of hippocampal mossy fibers (Xu and Henkemeyer, 2009).

Rho family GTPases have an integral role in the development of the visual system as demonstrated by how the misexpression of Cdc42, Rac1, and RhoA in *Xenopus* RGCs disrupt RGC development and targeting to the OT (Ruchhoeft et al., 1999). Axonogenesis requires Rac1 and is disrupted by the misexpression of all the Rho family GTPases while dendritogenesis depends primarily on both Rac1 and Cdc42. These data emphasize that *Xenopus* RGC axons pathfinding to their target region and maintenance of proper growth cone morphology require a balance between Rac1, Cdc42, and RhoA. Though they are not restricted to these roles, the general model is that Cdc42 and Rac1 promote neurite outgrowth whereas Rho initiates growth cone collapse. The Rho family GTPases works together to create a balance of inhibitory and attractive signals for proper development.

Another mode for Eph receptors and ephrin-B ligands to mediate their bidirectional signaling is through interactions with PDZ domain containing proteins, such as Grip, Pick, mPAR, PDZ-RGS3, Syntenin, PTP-BL/FAP-1, and AF-6/afadin (Cowan and Henkemeyer, 2002; Hock et al., 1998; Torres et al., 1998). PDZ domain containing proteins may aid Eph and ephrin molecules in clustering and localization to the synapse in both the inactive and active states as well as target downstream targets. An established pathway for ephrin-B reverse signaling is EphB2 binding initiated tyrosine phosphorylation of ephrin-B1 and recruitment of PDZ-RGS3 to the ephrin-B1 PDZ binding motif in cerebellar granule cells (Lu et al., 2001). The

C-terminus RGS domain acts like a GAP by hydrolyzing the GTP bound to CXCR4, a G-protein, therefore halting its ability to promote cell migration (Lu et al., 2001).

Silencing Eph:ephrin bidirectional signaling

Eph and ephrin molecules generally interact on opposing cell membrane surfaces creating physical adhesion between the two cell membranes, although many reported functions of Eph:ephrin signaling are inhibitory. Silencing Eph:ephrin bidirectional signaling through cleavage of the ephrin, endocytosis of the Eph:ephrin complex, and dephosphorylation are methods shown to result in the switch from attractive/adhesive effects to growth cone collapse and retraction.

The mode for cleavage was first elucidated in EphAs and ephrin-As. The A-Disintegrin-And-Metalloprotease (ADAM)10 has been shown to co-express with EphA3 and cleaves the ephrin-A ectodomain *in trans*, although the *Drosophila* homolog cleaves ephrin-A *in cis* (Hattori et al., 2000; Janes et al., 2005). While apparent species difference exist, ADAM10 appears to be a mechanism for cleavage in the EphAs and ephrin-As. Ephrin-B intramembrane cleavage is regulated by a different mechanism via aspartic protease γ-secreatase resulting in the translocation of the cleaved intracellular domain to the nucleus (Tomita et al., 2006). While different mechanisms regulate cleavage for the A and B subclasses, bidirectional signaling is interrupted in both cases.

Endocytosis of the Eph:ephrin complex is another mechanism observed to silence Eph:ephrin bidirectional signaling. Two studies showed EphB and ephrin-B molecules interacting on opposing cell surfaces form a complex that was internalized in cell culture (Marston et al., 2003; Zimmer et al., 2003). Endocytosis of the EphB:ephrin-B complex resulted

in the termination of cell-cell adhesion causing the cells to round up and the axons to retract. Endocytosis observed in these studies required the full length EphB and ephrin-B indicating it is independent from cleavage. After cell-cell contact, an increase in tyrosine phosphorylation of Eph receptors along the plasma membrane was detected indicating the activation of EphB:ephrin-B signaling (Marston et al., 2003). Internalization was dependent on actin polymerization and Rac activity, both of which are known to be activated downstream of EphB:ephrin-B signaling.

Just as the Ephs and ephrins are activated by phosphorylation, they can be deactivated by dephosphorylation. Eph receptors have been shown to be dephosphorylated on their juxatamembrane tyrosine by protein tyrosine phosphatase receptor type O (Ptpro) (Shintani et al., 2006). Ephrin-B activation results in the recruitment of PTP-BL via the PDZ binding motif and are subsequently dephosophorylated by PTP-BL (Palmer et al., 2002). While the mechanisms underlying the different cytoskeletal affects observed downstream of Eph:ephrin signaling are still being understood, cleavage, endocytosis, and dephosphorylation may provide the necessary switch in signaling to promote attraction/adhesion or retraction/repulsion.

Physiological roles of Eph:ephrin bidirectional signaling

As the Ephs and ephrins were being discovered during the 1990's their role in neuronal development became apparent based on their expression in neuronal tissue. Invertebrate or vertebrate, rodent or human, all organisms require two key developmental steps: arranging cells in the correct location and establishing neuronal circuitry. These two stages partially depend on Eph and ephrin signaling for cell-cell and cell-axon communication during these processes.

Depending on the biological context, Eph:ephrin signaling can have either an inhibitory or attractive affect on cells and/or axons.

Eph:ephrin bidirectional signaling during embryonic development

Eph receptors and ephrin ligands are expressed throughout the body and have been found to mediate important developmental processes. For example, EphA receptors and ephrin-A ligands are expressed in the closing mouse spinal tube during formation of the neural tube, neurulation, and disruption of their EphA:ephrinA dimerization by cleaving ephrin-A ligands or injecting EphA-Fc/-AP fusion proteins inhibited neural tube closure (Abdul-Aziz et al., 2009). Another example is the role of Eph and ephrin signaling mediating repellent activity to maintain rhombomere segment separation (Xu et al., 1995; Xu et al., 1996).

EphB4 and ephrin-B2 are expressed in the endothelium of veins and arteries, respectively, and play a major role in angiogenesis (Adams et al., 1999; Gerety et al., 1999; Wang et al., 1998). *EphB4*$^{-/-}$ and *ephrin-B2*$^{-/-}$ mutants have severe defects in angiogenesis and die by E10. *Ephrin-B2*$^{lacZ/lacZ}$ mutants deficient in reverse signaling although still able to activate forward signaling survive until birth and embryos do not exhibit defects in blood vessel development (Cowan et al., 2004). The embryonic zebrafish is a simple model system and has the advantage of having a clear observable vasculature system to study how a single vessel splits into the dorsal aorta and cardinal vein. Endothelium cells that express ephrin-B2 sort and segregate into the dorsal aorta because they have limited ability to migrate while the EphB4 expressing endothelium cells migrate into the cardinal vein (Herbert et al., 2009). Observations in the zebrafish indicate the likely function EphB4 and ephrin-B2 have in rodents and humans.

While characterizing the C-terminal truncated ephrin-B2-β-galactosidase (ephrin-B2-β-gal) fusion proteins able to traffic to the cell membrane, researchers observed thick cardiac valves (Cowan et al., 2004). There were twice as many mesenchymal cells in the aortic valve of mutants compared to *wild types*. The mitral valve leaflets also had an increase of mesenchymal cells. The data demonstrated the role of ephrin-B2 reverse signaling in directing the development and migration of the cells forming the valve.

Ephrin-B2 reverse signaling is also critical for lymphatic vasculature development. Mutant mice expressing an ephrin-B2 protein unable to bind PDZ domain containing proteins displayed lymphatic vasculature defects (Makinen et al., 2005). However, mutant mice expressing ephrin-B2 that was unable to interact with SH2 domain containing proteins exhibited no vasculature defects. These data implicating ephrin-B2 mediated reverse signaling via the PDZ binding motif, not SH2 interacting sites, is critical for lymphatic vasculature development.

Eph:ephrin bidirectional plays a major role in midline adhesion. For example, EphB2 and ephrin-B2 are expressed in the midline of the mesoderm cells, in and underneath the endoderm cells of the urorectal region. Ephrin-B2 is highly expressed in the area of the mesoderm that pinches together at the midline. The *ephrin-B2*$^{lacZ/lacZ}$ mutants described above exhibited anorectal defects, hypospadias (incomplete urethral tubulization), and failure in cloacal septation (Dravis et al., 2004). In addition, ephrin-B2 reverse signaling mutants demonstrate defects in tracheoesophageal septation, palatal shelf fusion, and abdominal body wall closure at the embryonic midline that likely implicates EphB:ephrin-B bidirectional signaling mediating adhesion along the midline (Dravis, submitted).

Axon Guidance

Development of proper neuronal circuitry is based on axons properly migrating to their target region, where upon their arrival, the axons must find their specific TZ to form synaptic connections. The initial step for axons to travel from their region of origin to their target region requires responding to guidance cues along the path, or guidepost cells, to navigate. Once they reach their target region, localization to their specific TZ requires either cell-cell recognition of a specific cell type or topographic mapping, the spatial projection of a group of axons to the target area. Evidence for Eph:ephrin signaling mediating these developmental processes is based on their expression patterns and targeted gene disruption of Eph and ephrin molecules.

The Ephs and ephrins were first documented to be axon guidance molecules, which remains to be a primary function. Axon guidance mediated by Eph and ephrin molecules is fundamental for establishing complex neuronal circuitry in a number of different systems. Early in the development of neuronal circuits, early axons, called pioneer axons, create axon tracts in order for follower axons to project later and fasciculate with the pioneering axon along the same path (Easter et al., 1994).

One of the first neuronal systems linked to Eph and ephrin signaling is the development of the anterior commissure. The anterior commissure is a major forebrain commissure that connects the two temporal cortical lobes. EphB2 is expressed in the region ventral to the anterior commissure, specifically the preoptic and hypothalamus regions (Henkemeyer et al., 1996). The deletion of EphB2, *EphB2*$^{-/-}$, resulted in anterior commissure defects in which the posterior tract of the anterior commissure projected into the ventral forebrain as a fasciculated bundle, and as a consequence did not reach the midline and crossover. *EphB2*$^{lacZ/lacZ}$ mutant mice, which activate reverse signaling, but not forward signaling, properly formed the anterior commissure indicating

that EphB2 is acting as a ligand to guide the formation of the posterior tract of the anterior commissure through the activation of ephrin reverse signaling.

Supporting the hypothesis that reverse signaling drives the formation of the anterior commissure is the expression of ephrin-B ligands in the anterior and posterior tracts of the anterior commissure axons (Cowan et al., 2004). Analysis of *ephrin-B2*$^{lacZ/lacZ}$ mutant mice, ephrin-B2 C-terminal truncated mutant mice able to activate forward, not reverse signaling, exhibited similar defects wherein the posterior tract anterior commissure axons failed to extend across the midline and form the commissure.

EphA4 is also expressed in the region between the anterior and posterior tracts of the anterior commissure and furthermore has been found to maintain the segregation between the anterior and posterior tracts of the anterior commissure (Ho et al., 2009). The posterior tract axons of the anterior commissure in *EphA4*$^{-/-}$ mutant mice misproject into the area dorsal to the posterior tract and intermingle with the anterior tract. The axons do not misproject ventral to the tract in *EphA4*$^{-/-}$ mutant mice most likely due to EphB2 inhibition. To test this hypothesis the *EphB2*$^{-/-}$;*EphA4*$^{-/-}$ mutant mice were examined in which the posterior tract misprojected similarly to the single mutants as well as had a subset of anterior tract axons that projected ventrally into the posterior tract. Their study proposed EphA4 activation of ephrin-B2 reverse signaling inhibits the intermingling between the anterior and posterior tracts of the anterior commissure. The development of the anterior commissure is a simple, but elegant example of how axons are able to pathfind and form the appropriate tract due to interaction with guidance cues expressed in the axon and surrounding tissue.

Promiscuous interactions between EphA4:ephrin-B can also lead to forward signaling dependent development as seen in the corticospinal tract (CST). For example, *EphA4*$^{-/-}$ and

ephrin-B3$^{-/-}$ mutants display a hopping behavior indicative of a spinal cord defect (Dottori et al., 1998; Kullander et al., 2001a; Yokoyama et al., 2001). EphA4 kinase dead mutants implicated EphA4 forward signaling was essential for CST formation (Kullander et al., 2001b). EphA4 expression in cortical motor neurons that form the CST and ephrin-B3 expression in along the spinal cord midline indicated the potential role these molecules may play in the targeting navigating motor neurons expressing EphA4. In *ephrin-B3*$^{-/-}$ mutants, the EphA4 expressing cells are uninhibited from crossing over the midline in developing spinal cords. However, *ephrin-B3*$^{lacZ/lacZ}$ mutant mice, expressing a C-terminal truncated ephrin-B3-β-gal fusion protein still able to traffic to the cell membrane, exhibited no defects indicating EphA4:ephrin-B3 interaction activates forward signaling to mediate proper CST formation.

Motor neurons in the spinal cord depend on guidance cues to direct their innervation into distinct limb muscle groups. Eph and ephrin expression patterns first indicated the involvement of Eph:ephrin bidirectional signaling. In chicks, EphA3 and EphA4 are expressed in the lateral motor column (LMC) neurons while ephrin-A2 and ephrin-A5 are expressed in the surrounding mesenchyme of the ventral limb bud (Iwamasa et al., 1999). Later, *EphA4* mutant mice showed Eph:ephrin signaling directs lateral LMC axons to the dorsal limb bud due to repulsion from the ventral limb bud (Coonan et al., 2003; Helmbacher et al., 2000; Kramer et al., 2006). In mice, EphB receptors are expressed on medial LMC neurons, while ephrin-B2 is expressed by cells of dorsal limb bud and after analysis of *EphB* and *ephrin-B* mutant mice, EphB1, EphB3, and ephrin-B2 were found critical for navigating medial LMC axons into the ventral limb bud due to repulsion from the dorsal limb bud (Luria et al., 2008). These data indicate repellent signaling activity via Eph:ephrin bidirectional signaling directs LMC axons to the correct limb bud.

Eph:ephrin bidirectional signaling mediates retinocollicular mapping

Eph receptors and ephrin ligands were the first family of guidance cues found to mediate retinotopic mapping and continue to be the chief molecules involved. Evidence for Eph/ephrin signaling mediating topographic mapping is based on complementary expression and binding gradients of these molecules (Figure 1.4) as well as topographic defects observed in Eph receptor and ephrin ligand mutant mice.

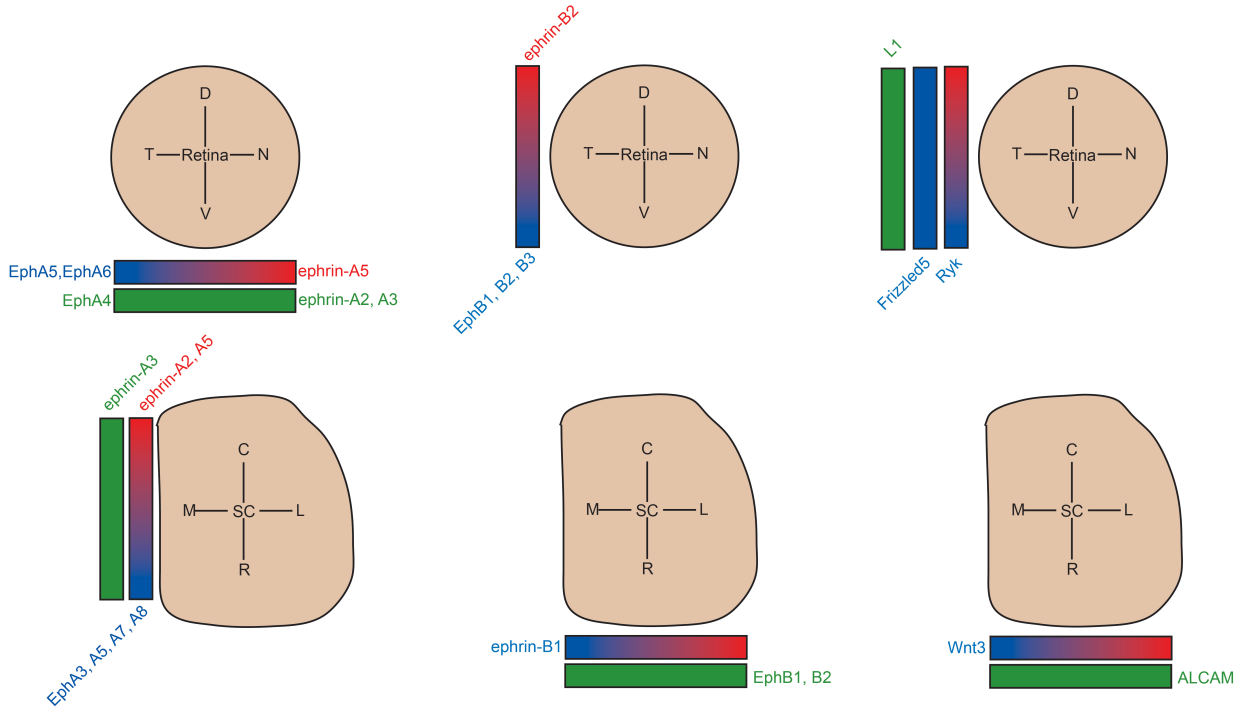


Figure 1.4 Schematic of complementary expression of retinocollicular mapping guidance molecules in mice.

A-subclass of Eph and ephrin molecules

While there are some differences between rodent and chick expression patterns of Eph and ephrin molecules, the underlying theme of complementary gradient expression of the projecting and target region remains the same (Figure 1.4). For this reason, I will focus on the rodent expression patterns revealed using RNA *in situ* hybridization (Brown et al., 2000; Feldheim et al., 2000; Frisen et al., 1998; Pfeiffenberger et al., 2006; Rashid et al., 2005). While *ephrin-A5* is expressed in a high nasal/low temporal gradient in the mouse retina, *ephrin-A2* and *ephrin-A3* are expressed uniformly. *EphA5* and *EphA6* are expressed in a low nasal/high temporal gradient while *EphA4* is expressed uniformly. In the SC, *ephrin-A2* and *ephrin-A5* are expressed in a low rostral/high caudal gradient and *ephrin-A3* is expressed at a low level uniformly. *EphA3, A4, A5, A7,* and *A8* are expressed in a high rostral/low caudal gradient.

Ephrin-A2 and ephrin-A5 were first discovered as chemical repellents that had a role in retinotopic mapping using *in vitro* and *in vivo* experiments in chicks (Monschau et al., 1997; Nakamoto et al., 1996). Studies in the mouse soon followed that demonstrated the distinct roles ephrin-A ligands have in mediating RGC axon termination along the rostral-caudal axis. The temporal RGC axons of *ephrin-A2*$^{-/-}$ and *ephrin-A5*$^{-/-}$ single knockout mice formed ectopic TZs caudal to the primary TZ whereas nasal RGC axons from only *ephrin-A5*$^{-/-}$ mutants formed ectopic TZs rostral to their primary TZ (Feldheim et al., 2000; Frisen et al., 1998). The temporal RGC axons of *ephrin-A5*$^{-/-}$ single knockout mice tended to overshoot caudally into the inferior colliculus. Differences between the *ephrin-A2* and *ephrin-A5* gradients in the SC attributed towards the difference between defects observed in *ephrin-A2*$^{-/-}$ and *ephrin-A5*$^{-/-}$ mutant mice. Ephrin-A2 reaches its peak expression in the mid-posterior SC and has a graded decline towards the rostral and most-posterior regions. On the other hand, *ephrin-A5* is robustly expressed in the

caudal most SC and declines in a much steeper gradient so that there is almost no detectable expression in the rostral SC. Based on the expression pattern and accumulation at the border in null mutants, ephrin-A5 likely maintains the border between the SC and inferior colliculus as well as direct retinotopy. Temporal and nasal RGC axons of *ephrin-A2*$^{-/-}$;*ephrin-A5*$^{-/-}$ compound mutants formed multiple ectopic TZs along the rostral-caudal axis and had a smaller primary TZ (Feldheim et al., 2000). While the double mutants had a severe defect not all the SC formed ectopic TZs leading to the analysis of ephrin-A3 mutants. In *ephrin-A2*$^{-/-}$; *ephrin-A3*$^{-/-}$; *ephrin-A5*$^{-/-}$ compound mutants, nasal and temporal RGC axons formed ectopic TZs in all the SC (Pfeiffenberger et al., 2006).

A recently developed method of Fourier imaging of intrinsic signals visualizes the functional maps in the SC as well as visual cortex to determine the relevance of the mapping errors seen at postnatal stages (Kalatsky and Stryker, 2003). An anesthetized mouse is exposed to a bar drifting across the monitor horizontally (stimulating the dorsoventral map) or vertically (stimulating nasotemporal map) for constant stimulation along the visual field. The contralateral SC or visual cortex is imaged just below the surface to measure the optical signal at the stimulation frequency and calculate the response magnitude and timing relative to the stimulus cycle to derived the topograhic map. Fourier imaging confirmed data derived from DiI tracing that retinocollicular maps, as well as cortical maps, were severely disrupted in *ephrin-A2*$^{-/-}$; *ephrin-A3*$^{-/-}$; *ephrin-A5*$^{-/-}$ compound mutants (Cang et al., 2008).These data demonstrated a new method to study mapping defects as well as how the mapping defects in postnatal pups are sustained into adulthood in *ephrin-A* mutants.

Temporal and nasal RGC axons from *EphA5*$^{lacZ/lacZ}$ mutant mice, C-terminal truncated proteins which have their intracellular domain replaced with β-gal and are still able to traffic to

the cell membrane, formed ectopic TZs caudal and rostral, respectively, relative to the primary TZ (Feldheim et al., 2004). Ventral RGC axons also demonstrated mapping errors in *EphA5*$^{lacZ/lacZ}$ mutants, forming rostral and caudal ectopic TZs relative to primary TZ. In the same study, an *in vitro* membrane stripe assay using WT or *EphA5*$^{lacZ/lacZ}$ retinal explants grown on WT or *EphA5*$^{lacZ/lacZ}$ SC cells demonstrated EphA5 expression in the retina is important for repellent activity mediated by EphA5 forward signaling (Feldheim et al., 2004).

One study examined a knockin of EphA3, not endogenously expressed in mouse retina or SC, in which roughly half of the RGCs expressed it thus elevating EphA expression throughout the RGC population in half the cells while the other half expressed EphA in the normal gradient (Brown et al., 2000). In half the retinal axon projections, the increased EphA expression resulted in RGC axons not projecting into the caudal SC as they normally would because of increased EphA forward signaling. On the other hand, RGC axons expressing WT levels of EphA were forced to project into the caudal SC perhaps due to competition. These data indicate increased EphA forward signaling inhibited RGC axons from moving up the ephrin-A gradient and instead localize to the rostral SC.

EphA and ephrin-A molecules not only establish the position of the RGC axon along the rostral-caudal axis, but they also oversee where the interstitial branches arise along the primary axon. While there is initially some branching all along the axon, there is greater branching and arborization in the position along the rostral-caudal axis that will later become the TZ (Yates et al., 2004; Yates et al., 2001). The current model for the regulation of interstitial branching along the rostral-caudal axis is that EphA mediated forward signaling inhibits caudal branching and ephrin-A reverse signaling inhibits rostral branching (Feldheim et al., 2000; Feldheim and O'Leary, 2010; Rashid et al., 2005; Yates et al., 2004; Yates et al., 2001). According to this

model, the point in the SC that has the lowest level of repulsive cues, where EphA forward signaling and ephrin-A reverse signaling are the weakest, an attractive guidance signal is present, perhaps expressed uniformly through the region, to promote axon extension.

B-subclass of Eph and ephrin molecules

In-depth characterization of the repulsive cues that mediate the formation of the TZ leads to the question of what is the attractive counterbalance to these molecules that are also required to direct RGC axon termination to the proper quadrant of the OT/SC. EphB and ephrin-B molecules are also expressed in the retina and OT/SC and provide a likely source for the attraction as first purposed after observing their expression patterns in the chick (Braisted et al., 1997).

While the expression patterns of EphB and ephrin-B molecules will be described in greater detail in Chapter 3 of this dissertation, described here is the general concept. In mice, *ephrin-B1* and *ephrin-B2* mRNA is expressed in a high dorsal/low ventral gradient in the retina and ephrin-B1 protein is expressed in the SC (Buhusi et al., 2009; Hindges et al., 2002). *EphB2* and *EphB3* are expressed in a low dorsal/high ventral gradient in the retina and a low medial/high lateral gradient in the SC (Hindges et al., 2002). The role of EphB:ephrin-B bidirectional signaling has also been studied in *Xenopus laevis* which has slightly different expression pattern although the general complementary expression between EphBs and ephrin-Bs still exists (Figure 1.4). RNA *in situ* hybridization showed *ephrin-B2* and *ephrin-B3* expressed in a high dorsal/low ventral gradient while *EphB2* is expressed in a low dorsal/high ventral gradient in the retina (Mann et al., 2002). In the OT, *ephrin-B1* and *ephrin-B2* are

expressed along the dorsal midline while *EphB1* is expressed in a low midline/high ventral gradient.

Experiments conducted in the *Xenopus laevis* demonstrated retinotopic mapping errors. Ectopic expression of EphB-ECD in the OT caused RGC axons to project further dorsally into the OT (Mann et al., 2002). Misexpression of dominant-negative truncated ephrin-B2 in the dorsal retina or WT ephrin-B2 expression in the ventral retina also resulted in misprojecting axons along the dorsoventral axis of the OT. In addition, *in vitro* experiments looking at how dorsal retinal explants responded to EphB-ECD demonstrated dorsal RGC axons expressing ephrin-B ligands are attracted to EphB-ECD. Therefore, EphB receptors expressed in the SC are likely acting as ligands to activate ephrin-B reverse signaling mediated attraction in the *Xenopus laevis*.

While EphB:ephrin-B bidirectional signaling may not navigate the primary RGC axon, evidence in the chick and mouse indicate it supports interstitial branch extension of RGC axons towards the proper TZ. The first and so far only published study of how EphB:ephrin-B bidirectional signaling mediates retinocollicular mapping analyzed *EphB2*$^{-/-}$;*EphB3*$^{-/-}$ and *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{-/-}$ mutant mice and showed ventral RGC axons formed ectopic TZs that tended to be lateral to the primary TZs (Hindges et al., 2002). Because the loss of EphB forward signaling caused RGC axons to move down the ephrin-B1 gradient, EphB:ephrin-B bidirectional signaling was proposed to promote attraction to the midline. The proposed ligand in SC is ephrin-B1, though its role has only been directly accessed in chicks by ectopic expression of ephrin-B1 in the OT (McLaughlin et al., 2003). These data demonstrated interstitial branches from RGC axons that were lateral to the correct TZ extended medially, or up the ephrin-B1 gradient, while interstitial branches from RGC axons that were medial to the correct TZ extended

laterally, or down the ephrin-B1 gradient. Therefore depending on the level of EphB and ephrin-B expressed on the RGC axons and OT/SC, the interstitial branches are able to extend towards and terminate in the appropriate quadrant of the region.

Other molecules that mediate retinocollicular mapping

Members of the Ig superfamily, L1 and ALCAM (activated leukocyte cell adhesion molecule), are expressed in the retina and SC and are known to mediate cell adhesion to actin cytoskeleton (Figure 1.4). Temporal and ventral-temporal RGC axons of L1 and ALCAM mutant mice had misoriented interstitial branching, formed ectopic TZs, and the primary TZ was often not correctly formed (Buhusi et al., 2009; Buhusi et al., 2008). L1 is expressed by the RGC axon entering the SC and ALCAM is expressed in SC. L1 and ALCAM interact directly or indirectly and a proposed co-receptor is EphB2 although studies are still in progress to determine any interaction.

In order for the interstitial branches to not overextend past their correct TZ along the mediolateral axis, another repellent guidance cue is required. Recent analysis in chicks demonstrated Ryk receptor is expressed in a low dorsal/high ventral gradient and Frizzled is expressed uniformly in the retina while Wnt3 ligand is expressed in the high medial (dorsal)/low lateral (ventral) gradient in the SC (OT) (Schmitt et al., 2006) (Figure 1.4). In chicks, dominant-negative Ryk in dorsal RGC axons and over-expression of Wnt3 in the lateral tectum resulted in ectopic TZs to form medial to their expected TZ thus demonstrating how Ryk:Wnt3 mediate interstitial branch repulsion to counterbalance EphB-ephrin-B mediated attraction. Determining the mapping roles of Ryk, Frizzled, and Wnt in mice would provide a complement to what is has already known about the mediators of interstitial branching.

Due to their gradient expression along the dorsoventral axis of the retina, EphB and ephrin-B molecules have been proposed to direct RGC presorting, which is the arrangement of RGC axon order in the optic tract to reflect the dorsoventral position of the RGCs. RGC axon presorting would as a result influence the RGC axon positioning across the mediolateral axis of the SC. RGC axon sorting has been shown to be mediated by Bone Morphogenetic Proteins (BMP) and Noggin and not EphB receptors (Plas et al., 2008). However, EphB:ephrin-B bidirectional signaling plays an important role in mediating RGC axon interstitial branching extension towards its correct TZ.

Motivations for my work

While the EphA:ephrin-A signaling role in retinocollicular mapping have been well characterized, the role EphB and ephrin-B molecules have in developing the retinocollicular map is poorly understood. Because of the variety of mutant mice generated in the Henkemeyer lab, I was able to conduct a comprehensive *in vivo* study of the necessity of EphB:ephrin-B bidirectional signaling in retinocollicular mapping. The ability for RGC axons to terminate in the SC relies on proper targeting through many different regions. While many factors along the optic nerve, optic chiasm, and optic tract serve to maintain axon bundles and inhibit axons from entering the surrounding tissue, I decided to focus my study on the beginning and end of the RGC axon journey. Chapter 2 describes how EphB:ephrin-B bidirectional signaling mediates RGC axon navigation towards the optic disc. Chapter 3 describes how EphB:ephrin-B bidirectional signaling mediates dorsoventral RGC axon retinocollicular mapping.

Chapter 2

Role of ephrin-B reverse signaling in dorsal RGC axon pathfinding to the optic nerve head

SUMMARY

The molecular mechanism underlying the precise manner in which RGC axons radially project into the center of the retina is not well understood. Eph and ephrin molecules have been shown to be critical axon guidance molecules in the CNS and have a robust gradient expression in the retina. EphB receptors are highly expressed in the ventral retina and the absence of the EphB receptor ECD results in dorsal RGC axons to aberrantly project into the ventral retina. These data indicate EphB receptors acting as ligands to inhibit dorsal RGC axons from entering the ventral retina. While ephrin-B ligands highly expressed by dorsal RGC axons are proposed to act as the receptor interacting with the ventral retina expressing EphB receptors, no direct analysis has tested this hypothesis. I characterized axon guidance defects in *ephrin-B1* and *ephrin-B2* single and compound mutants and found a mild axon guidance defect. EphB may interact with ephrin-A5 molecules expressed in a high nasal/low temporal gradient to mediate dorsal RGC axon guidance to the optic nerve head. These data indicate EphB:ephrin-B reverse signaling has a weak influence on RGC axon pathfinding to the optic disc, although their robust expression in the retina plays a major role in other aspects of visual development.

INTRODUCTION

Vision depends on complex neuronal circuitry that is received and integrated by photoreceptors, bipolar cells, amacrine cells, and horizontal cells and sent to regions of the brain involved with vision. Visual information from the eye is exported by RGC axons, the only output from the retina to make synaptic connections in the brain. Before RGC axons can travel the optic tract to its targets in the brain, the LGN and SC, RGC axons must project into the optic nerve head in a spatially defined manner. RGC axons radially project into the back of the retina to the optic disc where the optic nerve head connects to the retina. RGC axons from the peripheral retina fasciculate with RGC axons more proximal to the optic nerve head. L1 and neurolin have been found to support RGC axon fasciculation (Brittis et al., 1995; Leppert et al., 1999; Ott et al., 1998).

The fasciculated axon bundles require molecular repellents to inhibit aberrant axon projections into the peripheral retina and molecular attractants that encourage axon extension towards the optic nerve head. Chrondroitin sulfate proteogylcan, expressed in the peripheral retina (Brittis et al., 1992; Snow and Letourneau, 1992), and Slit, expressed in rat RGC axons (Ringstedt et al., 2000), inhibit RGC axon growth. In the absence of chrondroitin sulfate proteogylcan, RGC axons to do not radially project into the optic disc and instead project into the peripheral retina. No known chemical attractants important for RGC axon projection to the optic disc are currently known. However, once RGC axons arrive to the optic disc, Netrin-1, expressed in the optic nerve head, attracts RGC axons to enter and form the optic nerve (Deiner et al., 1997). Sema5A is purposed to inhibit RGC axons from projecting into the tissue surrounding the

optic nerve because it is expressed around the circumference of the optic nerve head and has been shown to promote growth cone collapse *in vitro* (Oster et al., 2003).

EphB and ephrin-B molecules are expressed in the retina in robust countergradients that indicates a potential role in RGC axon guidance to the optic nerve head. At E17.5 EphB1 is highly expressed in the ventral-temporal retina while EphB2 and EphB3 are expressed in a low dorsal/high ventral gradient (Birgbauer et al., 2000; Thakar et al., submitted; Williams et al., 2003). Ephrin-B1 and ephrin-B2 are expressed in a high dorsal/low ventral gradient while ephrin-B3 is uniformly expressed. In 20% of the *EphB2*$^{-/-}$;*EphB3*$^{-/-}$ retinas, a subset of DiI labeled dorsal RGC axons defasciculated away from the main axon bundle and aberrantly projected into the ventral retina (Birgbauer et al., 2000). To distinguish between forward and reverse signaling, an intracellular truncated mutant was used. The *EphB2*$^{lacZ/lacZ}$ mutant mice express a truncated EphB2-β-gal fusion protein that still traffics to the cell membrane to stimulate reverse signaling. Nearly 0% of the *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{-/-}$ retinas demonstrated axon guidance errors, therefore, forward signaling was not necessary for exiting the optic disc. While the role of ephrin-B reverse signaling was implied in the data, the role of ephrin-B molecules in RGC axon guidance to the optic nerve head has not been directly evaluated. Analysis of *ephrin-B1* null and *ephrin-B2* reverse signaling mutants showed ephrin-B reverse signaling has a minor role in RGC axon guidance to the optic disc.

RESULTS

Ephrin-B2 mutants exhibit pathfinding defects in the retina

RGC axons project their axons radially as tight fasciculated bundles into the optic disc and exit to form the optic nerve (Figure 2.1B). To examine intraretinal mapping to the optic disc, mouse embryos were isolated at E17.5 and fixed in 4% PFA overnight. The next day, heads were bisected, lens of the eye removed, and a DiI crystal was embedded in the dorsal region of each retina (Figure 2.1A). Heads were placed overnight in the 37°C incubator to allow DiI to travel the length of the axon. The following day, retinas were flat mounted and imaged using epifluorescence microscopy. Of the 114 *wild type* retinas analyzed, none of them exhibited aberrant axonal projections (Figure 2.1C, Table 2.1).

During the embryonic stages of retinal development, *ephrin-B1* and *ephrin-B2* mRNA are expressed in a high dorsal/low ventral gradient in the retina while *ephrin-B3* mRNA is expressed uniformly (Birgbauer et al., 2000). Ephrin-B2 protein is also expressed in a high dorsal/low ventral gradient at E17.5 (Thakar et al., submitted). The expression patterns of *ephrin-B1* and *ephrin-B2* indicate the potential role as the complementary guidance cue to EphB2 and EphB3, which are expressed in a low dorsal/high ventral gradient expression (Birgbauer et al., 2000; Thakar et al., submitted), in the embryonic retina. My study analyzed mutant mice that have the entire x-linked *ephrin-B1* coding region deleted in the germ-line, *ephrin-B1*$^{-/y}$ (Davy et al., 2004). Also analyzed, were mice expressing a mutant allele for a C-terminal truncated ephrin-B2-β-gal fusion protein able to reach the cell membrane, *ephrin-B2*^{lacZ} (Dravis et al., 2004), and an allele for a functional null ephrin-B2, *ephrin-B2*^{T} (Dravis et al., 2004). Of the 53 *ephrin-*

B2$^{lacZ/T}$ retinas analyzed, 3.8% exhibited aberrant axon projections (Table 2.1). Once they reached the optic disc, the RGC axons projected into the ventral retina as a fasciculated axon bundle. On the other hand, *ephrin-B1*$^{-/y}$ retinas did not exhibit any misprojecting axons (n=12, Table 2.1).

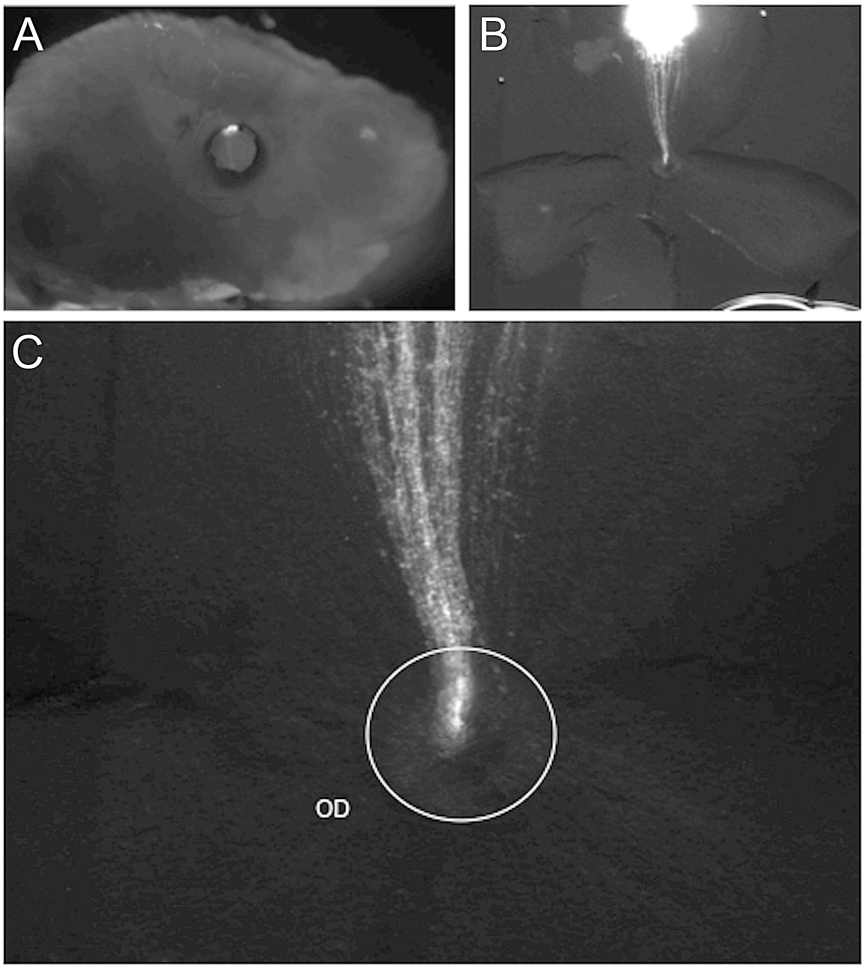


Figure 2.1 RGC axon guidance to the optic disc in *wild type*

(A) E17.5 embryos were fixed overnight and heads bisected. The next day, a DiI crystal was placed in the dorsal retina and heads were incubated in the 37° incubator to allow DiI to travel the length of the axons.

(B) The following day, retinas were flatmounted and imaged.

(C) *Wild type* retinas travel directly into the optic disc without aberrant axon projections. Optic disc (OD)

	Retinas with aberrant axon projections	Total retinas analyzed	% of retinas with guidance defects
Wild type	0	114	0%
ephrin-B1$^{-/y}$	0	12	0%
ephrin-B2$^{lacZ/T}$	2	53	3.8%
ephrin-B1$^{-/y}$;*ephrin-B2*$^{lacZ/T}$	2	25	8%

Table 2.1 *Wild type* and *ephrin-B* mutant retinas with aberrant axon projections.

***Ephrin-B* compound mutants exhibit pathfinding defects in the retina**

The overlapping mRNA expression of ephrin-B1 and ephrin-B2 indicates these molecules may potentially compensate for each other. For this reason *ephrin-B1*$^{-/y}$;*ephrin-B2*$^{lacZ/T}$ retinas were analyzed for axon guidance defects. Of the 25 retinas from compound mutants analyzed, 8% ($p=0.0356$) exhibited aberrant axon projections (Table 2.1). Similar to *ephrin-B2*$^{lacZ/T}$ single mutants, a subset of DiI labeled dorsal RGC axons did not enter the optic nerve head at the optic disc and instead projected into the periphery of the ventral retina (Figure 2.2A and B).

Due to the low frequency of the axon guidance defects, characterization of the defects observed in ephrin-B remains open for further study. However, the defects were distinct from those observed in EphB receptor null mutants which defasciculated as they approached the optic disc.

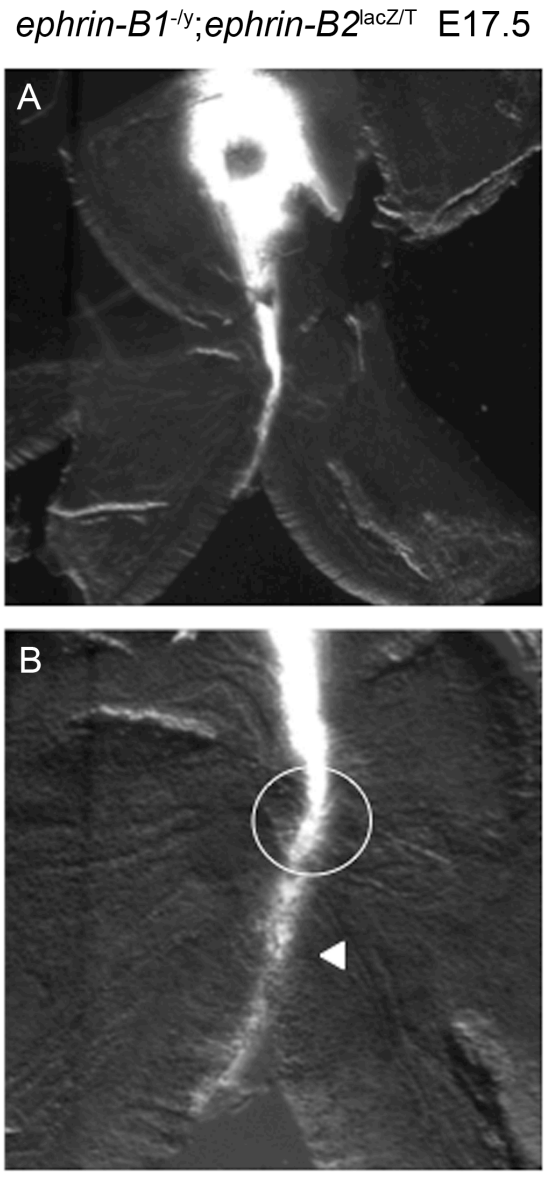


Figure 2.2 *Ephrin-B* mutants retinas with aberrant axon projections

(A) Example of a *ephrin-B1*$^{-/y}$;*ephrin-B2*$^{lacZ/T}$ retina that showed a subset of labeled RGC axons separate from the axon bundle and project into the ventral retina, rather than exit through the optic disc.

(B) Close up of the aberrant axon projection denoted by white arrowhead. Circle indicates the boundaries of the optic disc.

DISCUSSION

Analysis of *ephrin-B1* and *ephrin-B2* single and compound mutants showed ephrin-B reverse signaling is not critical for retinal axon guidance to the optic nerve head. RGC axon guidance to the optic disc is not well understood although it is presumed to require molecular repellents to inhibit aberrant axon projections from defasciculating and projecting into the periphery of the retinas as well as molecular attractants to promote RGC axon fasciculation and extension towards the optic nerve head. EphB:ephrin-B signaling was assumed to play a role because due to their robust expression in the retina. In addition, EphB ECD-Fc caused growth cone collapse thereby inhibiting RGC axon extension *in vitro* as well as in the absence of EphB receptors, dorsal RGC axons are uninhibited from entering the normally EphB enriched ventral retina (Birgbauer et al., 2000; Birgbauer et al., 2001). While not a highly penetrant phenotype, my study found dorsal RGC axons of *ephrin-B1*$^{-/y}$;*ephrin-B2*$^{lacZ/T}$ mutant mice showed axon guidance errors. A subset of DiI labeled dorsal RGC axons in these mutants projected into the ventral retina.

While these data indicate EphB:ephrin-B mediated reverse signaling has a role in RGC axon guidance to the optic nerve head, its influence is marginal as demonstrated by the low penetrance of the axon guidance defect. From E14 to E17.5, EphB1 mRNA and protein are highly expressed in the ventral-temporal retina and at lower levels in the dorsal retina (Thakar et al., submitted; Williams et al., 2003). Perhaps the EphB1 compensates in the absence of EphB2 and EphB3 therefore *EphB1*$^{-/-}$;*EphB2*$^{-/-}$;*EphB3*$^{-/-}$ mutants may exhibit a more penetrant phenotype. Ephrin-B3 mRNA is also expressed in the retina, although breeding of ephrin-B triple mutants proved difficult so I was unable to analyze RGC axon guidance to the optic disc in

the absence of all ephrin-B mediated reverse signaling. Complete lack of ephrin-B reverse signaling, (deletion of all EphB receptors or ephrin-B ligands expressed in the retina), would provide a definitive answer as to how critical ephrin-B reverse signaling is for intraretinal mapping. *Ephrin-A5* mRNA is expressed in a high nasal/low temporal gradient and interacts with EphB2 with a lower affinity compared to ephrin-B molecules. Its potential interaction with EphB2 indicates a potential role for ephrin-A5 reverse signaling for RGC axon guidance to the optic disc. If the analysis of RGC axon guidance defects in *ephrin-A5;ephrin-B1;ephrin-B2* or *EphB2;ephrin-A5* compound null mutants show a more penetrant or severe phenotype, then perhaps promiscuous interaction between EphB2:ephrin-A5 do regulate dorsal RGC axon guidance to the optic nerve head.

The method employed in this study allowed localized labeling of a subset of RGC axons that may have overlooked aberrantly projecting axons were not labeled with DiI. While it would be ideal to observe all the RGC axons, the density of RGC axons in the retina makes that nearly impossible. Selective labeling of a subset of RGC axons using a Thy-1-YFP reporter line (Feng et al., 2000) proved difficult to apply to this experiment because it would be hard to differentiate between aberrant axons and those projecting into the optic disc (data not shown). In addition, observing RGC axon mapping to the optic disc in embryos using this reporter lines is not possible because it does not turn on until postnatal week 2.

However, the data presented here suggests EphB:ephrin-B mediated reverse signaling is not essential for axon guidance to the optic disc. Perhaps, the robust expression of EphB and ephrin-B molecules in RGCs is required for navigating RGC axons along the optic tract and to its target regions in the brain. For example, ventral-temporal RGC axons of EphB1 mutants exhibit drastically reduced ipsilateral projections (Chenaux and Henkemeyer, unpublished; Williams et

al., 2003). The proposed model is that EphB1 expressing ventral-temporal RGC axons project ipsilaterally at the optic chiasm that expresses ephrin-B2 because of EphB1 forward signaling mediating repulsion. In addition, RGC axon expression of EphB and ephrin-B is required for dorsoventral retinocollicular mapping (Hindges et al., 2002; Thakar et al., submitted). The robust EphB and ephrin-B expression in the retina likely contributes towards the critical role EphB:ephrin-B bidirectional signaling plays in other aspects of visual development.

Chapter 3

Critical roles for EphB:ephrin-B bidirectional signaling in retinocollicular mapping

Work appearing in this chapter:

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

SUMMARY

The graded expression of EphB in the ventral retina and ephrin-B in the dorsal retina indicates a role for these bidirectional signaling molecules in mediolateral retinocollicular mapping. While previous studies have implicated EphB2 forward signaling in mice, the intracellular component of EphB2 essential for retinocollicular mapping is unknown as are the roles for EphB1, ephrin-B1, and ephrin-B2. Here, I show that EphB2 tyrosine kinase catalytic activity and EphB1 intracellular signaling are key mediators of ventral RGC axon retinocollicular mapping, by likely interacting with ephrin-B1 in the SC. I further elucidate roles for the ephrin-B2 intracellular domain in retinocollicular mapping and present the unexpected finding that both dorsal and ventral RGC axons utilize reverse signaling for mapping. These data demonstrate that both forward and reverse signaling initiated by EphB:ephrin-B interactions plays a major role in dorsoventral RGC axon termination along the mediolateral axis of the SC.

INTRODUCTION

Vision depends on specifically organized neuronal circuits formed during embryonic and postnatal development that are sustained into adulthood. The SC in mammals and OT in lower vertebrates are considered the visual reflex center of the brain, integrating visual, auditory, and motor inputs to direct head and eye movement in response to stimuli (Sprague and Meikle, 1965). The three most dorsal superficial layers of the SC receive input from the retina to mediate head and eye movement in response to visual stimuli. The axonal projections from the eye to this midbrain target provide an ideal system to investigate the development of topographic neuronal connections.

Retinocollicular mapping refers to the specific point-to-point localization of RGC axons of the eye to the SC. RGCs distributed along the dorsoventral axis extend axons that terminate along the mediolateral axis of the SC, while those distributed along the nasotemporal axis extend axons that terminate along the rostrocaudal axis of the SC. Thus, depending on their distinct dorsoventral and nasotemporal position in the retina, an individual RGC is able to project its axon to a precise retinotopic position in the SC. Topographic guidance receptor molecules expressed by the RGC axons and ligand cues in the SC target region are believed to mediate retinotopic mapping, leading to a highly precise two-dimensional representation of the surface of the eye onto the SC that becomes further refined by correlated retinal activity (Feldheim and O'Leary, 2010; Huberman et al., 2008).

Roger Sperry's chemoaffinity theory proposed nearly 50 years ago that topographic mapping depends on complementary gradients of guidance cues and receptors and this hypothesis still is the foundation for studies of retinocollicular mapping today (Sperry, 1963).

The first molecular confirmation of Sperry's theory came from the discovery that high caudal/low rostral expression of ephrin-A2 and ephrin-A5 in the SC functioned as chemical repellent ligands for EphA receptor tyrosine kinases expressed in high temporal/low nasal countergradient in RGC axons (Cheng and Flanagan, 1994; Cheng et al., 1995; Drescher et al., 1995; Nakamoto et al., 1996). While many follow up studies have further elucidated the guidance of nasotemporal RGC axon termination along the rostrocaudal axis of the SC, much less is known about dorsoventral RGC axon topographic mapping along the mediolateral axis (Feldheim and O'Leary, 2010; Luo and Flanagan, 2007; McLaughlin and O'Leary, 2005).

The few studies that have investigated dorsoventral RGC axon retinotopic mapping have determined it is different from what guides nasotemporal RGC axons because it depends on RGC axon sorting in the optic tract and interstitial branching. The arrangement of RGC axons along the mediolateral axis of the SC depends on the RGC axon order in the optic tract, which reflects the dorsoventral positioning in the retina. RGC axon sorting is mediated by Bone Morphogenetic Proteins (BMP) while EphB receptors did not affect the arrangement of RGC axons in the optic tract (Plas et al., 2008). The RGC axons that project along the rostrocaudal axis extend interstitial branches along the mediolateral axis of the SC that orient themselves towards their future TZ (Hindges et al., 2002; Nakamura and O'Leary, 1989; Simon and O'Leary, 1992b). The direction of the interstitial branches invokes both repulsion and attraction mechanisms to focus branch termination into the appropriate mediolateral region.

Recent analysis in the chick indicates Ryk:Wnt3 signaling is a probable candidate for the repulsion mechanism underlying mediolateral interstitial branch direction. The Ryk receptor is expressed in a high ventral/low dorsal gradient in the retina and expression of a dominant-negative form of this molecule in the dorsal retina resulted in a medially directed abnormal

growth of interstitial branches that formed ectopic TZs medial to the primary TZ (Schmitt et al., 2006). Forming a complementary gradient, its ligand Wnt3 is expressed in a high medial/low lateral gradient in the OT and overexpression of this molecule in the chick OT induced repulsion of the ventral RGC axons. While this data on Ryk:Wnt3 signaling demonstrates that repulsive cues can direct the formation of TZs along the mediolateral axis, the B-class Eph/ephrin molecules, which transduce bidirectional signals upon cell-cell or axon-cell contact, are thought to serve as an attractive counterbalance to these signals. EphB receptors are also expressed in a high ventral/low dorsal gradient in the RGC layer and the ligand ephrin-B1 is expressed in a high medial/low lateral gradient in the SC (Hindges et al., 2002). Ventral RGC axons of *EphB2*$^{-/-}$*;EphB3*$^{-/-}$ and *EphB2*$^{lacZ/lacZ}$*;EphB3*$^{-/-}$ compound mutants exhibited mis-projecting interstitial branches that formed lateral biased ectopic TZs in the SC demonstrating a role for EphB:ephrin-B forward signaling in retinocollicular mapping. However, many questions remain about the individual contribution of EphB2 and EphB3 forward signaling and potential roles for the related EphB1 receptor. Despite its robust ventral-temporal (VT) retinal expression and key role in mediating axonal repulsion at the optic chiasm to form the ipsilateral projections essential for binocular vision (Williams et al., 2003), nothing is known regarding how EphB1 participates in retinocollicular mapping. Likewise, despite the high dorsal/low ventral gradient of ephin-B2 in the retina and midline expression of ephrin-B1 in the SC (Birgbauer et al., 2000; Hindges et al., 2002), the *in vivo* functions of these two molecules are unknown.

Using a diverse array of protein-null, C-terminus truncation, as well as point mutations in the mouse, I report that EphB1 and EphB2 are the chief guidance molecules that mediate ventral RGC axon targeting to the medial SC and that mutation of EphB2 tyrosine kinase catalytic activity led to severe ventral RGC axon retinocollicular mapping defects. Genetic studies further

demonstrated ephrin-B1 is the primary ligand expressed in the SC for the mapping of ventral RGC axons expressing EphB receptors. Finally, I show that reverse signaling mediated by the high dorsal/low ventral expressed ephrin-B2 is unexpectedly important for retinocollicular mapping throughout the dorsoventral axis of the retina. My study thus establishes the essential roles for both EphB1/EphB2 forward signaling and ephrin-B2 reverse signaling in guiding RGC axons to their proper TZ in the SC.

RESULTS

EphB and ephrin-B expression patterns in the embryonic and postnatal retina and SC

EphB2 expression in coronal sections of the retina and SC was confirmed using X-gal stains of *EphB2*^{lacZ} mutant mice, which express a C-terminal truncated EphB2-β-gal fusion protein lacking the majority of the EphB2 intracellular domain including its tyrosine kinase catalytic domain, sterile alpha motif (SAM) domain, and PDZ binding motif (Henkemeyer et al., 1996). This protein correctly traffics to the cell membrane and can act like a ligand to bind ephrin-B molecules on adjacent cell surfaces to activate reverse signaling, but cannot transmit canonical forward signals into its own cell requiring the removed internal sequences. The EphB2-β-gal fusion protein also illustrates endogenous EphB2 protein expression in a high ventral/low dorsal gradient in the RGC layer at embryonic day 17.5 (E17.5) and postnatal days 1 (P1) and 8 (P8), although there is obvious expression in the dorsal retina as well (Figure 3.1A). The localization of EphB2-β-gal at these stages is highly enriched in the RGC axons funneling into the optic nerve head (arrowhead). Expression of EphB2 is also strong in the superficial and deeper layers of the SC at P1 and P8, but not in any visible gradient (Figure 3.1A).

To determine EphB1 expression in the retina and SC, two different EphB1 knock-in reporter lines expressing β-gal were used. In *EphB1*^{lacZ} mutant mice (Williams et al., 2003), homologous recombination was used to insert a sa-IRES-lacZ cassette into *EphB1* reading frame at exon 3 resulting in a protein-null mutation that also expresses a cytoplasmic β-gal reporter in cells that normally express *EphB1*. In *EphB1*$^{T\text{-}lacZ}$ mutant mice (Chenaux and Henkemeyer, *in preparation*), *lacZ* sequences were inserted in-frame into exon 9, directly after the transmembrane sequences, truncating the entire intracellular domain of the protein to express an

EphB1-β-gal fusion protein. Similar to the EphB2-β-gal fusion protein, the EphB1-β-gal fusion also traffics normally to the cell membrane. Both *EphB1*^{lacZ} and *EphB1*$^{T\text{-}lacZ}$ mutant reporters faithfully demonstrated robust VT expression of EphB1 in the RGC layer at E16.5 while the dorsal and ventral-nasal retina expressed EphB1 at lower levels (Figure 3.1B) (Williams et al., 2003). By the first postnatal week, EphB1 expression losses its gradient and becomes expressed uniformly as shown at P1 and P8 (Figure 3.1B). EphB1-β-gal fusion protein was expressed in the RGC axons exiting into the optic nerve (arrowhead). The *EphB1*^{lacZ} mutant reporter was also used to assess EphB1 expression in the SC as the expression of unconjugated β-gal labels primarily cell bodies of expressing cells and not their processes. Here again, like EphB2, strong *EphB1* expression was detected in the cells of the superficial and deeper layers of the SC at P1 and P8, and did not form a detectable gradient (Figure 3.1B).

Ephrin-B2 expression was determined using *ephrin-B2*^{lacZ} mutant mice, which express a C-terminal truncated ephrin-B2-β-gal fusion protein that lacks its intracellular segment (Dravis et al., 2004). The ephrin-B2-β-gal fusion protein is able to traffic to the cell membrane and bind Eph receptors on adjacent cells to activate forward signaling, but it is unable to transduce reverse signals into its own cell. Using *ephrin-B2*^{lacZ} mutant mice, a robust high dorsal/low ventral gradient expression of ephrin-B2-β-gal fusion protein was detected at E17.5, P0, and P8 in the RGC axons, which complements previous mRNA expression data (Birgbauer et al., 2000; Hindges et al., 2002). While ephrin-B2-β-gal fusion protein expression was observed to be most intense in the dorsal retina, ventral expression was also evident at E17.5 and this became more obvious during the first postnatal week as seen at P0 and P8 (Figure 3.1C). Ephrin-B2-β-gal expression is also evident in axons leaving the retina to form the optic nerve (arrowhead).

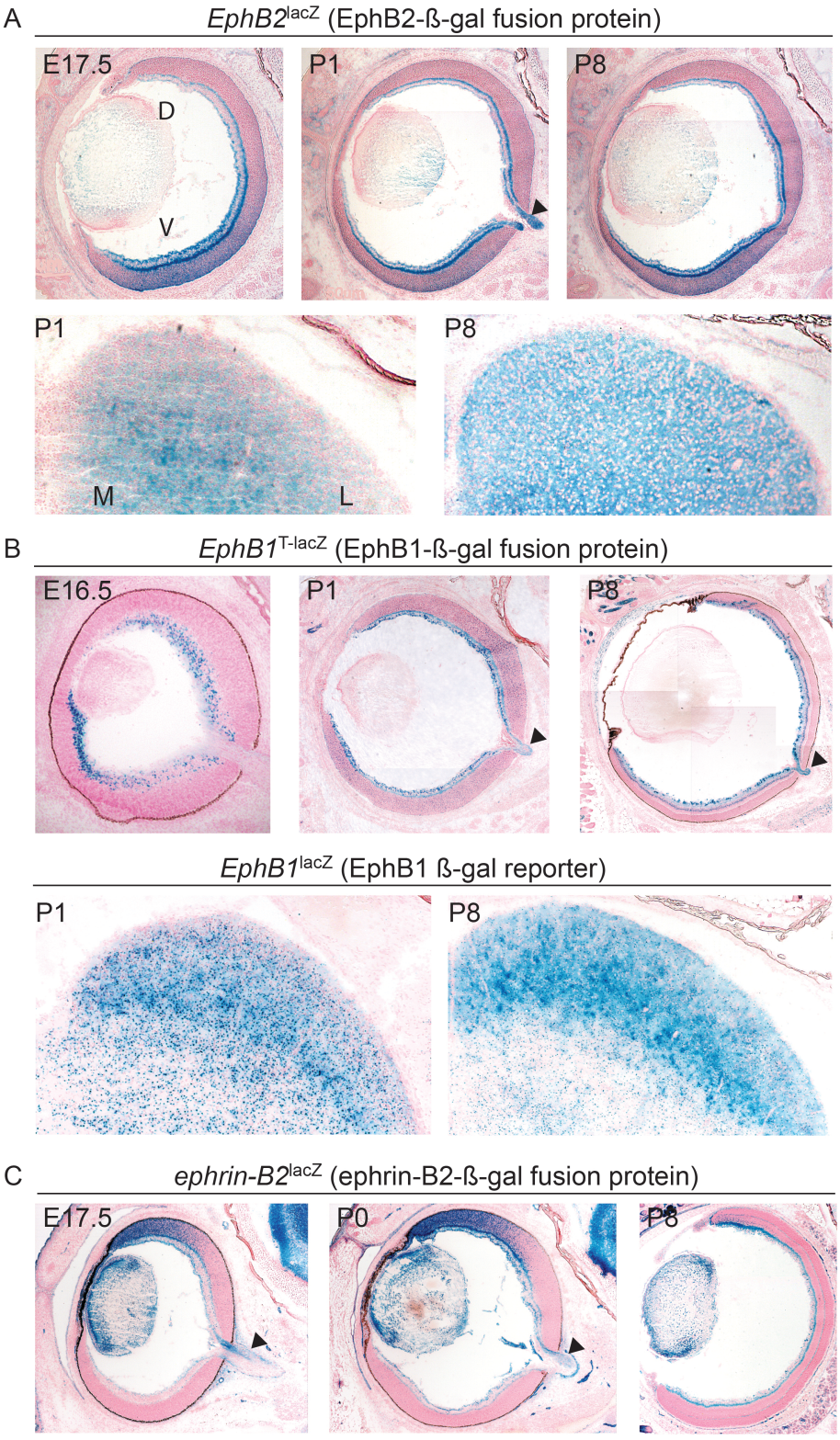

A
EphB2lacZ (EphB2-ß-gal fusion protein)
E17.5
D
V
P1
P8
P1
M
L
P8
B
EphB1T-lacZ (EphB1-ß-gal fusion protein)
E16.5
P1
P8
EphB1lacZ (EphB1 ß-gal reporter)
P1
P8
C
ephrin-B2lacZ (ephrin-B2-ß-gal fusion protein)
E17.5
P0
P8

Figure 3.1 EphB and ephrin-B are expressed in the embryonic and postnatal retina and SC

(A, B, and C) Selected coronal sections from E16.5 or E17.5 embryos and P1 and P8 pups from EphB1, EphB2, and ephrin-B2 β-gal reporter lines stained with X-gal stain to visualize β-galactosidase activity. Black arrowheads mark the optic nerve exiting the eye.

(A) EphB2-β-gal fusion protein is expressed in a low dorsal/high ventral gradient in the retina at E17.5. This steep gradient is maintained the first postnatal week. EphB2-β-gal fusion protein is expressed uniformly in the superior colliculus the first postnatal week.

(B) EphB1-β-gal fusion protein is expressed in the ventral-temporal retina at E16.5. EphB1-β-gal fusion protein expression becomes uniform the first postnatal week. EphB1 β-gal reporter expressed in the cytoplasm of EphB1 expressing cells shows EphB1 expressed uniformly in the superficial as well as deeper layers of the superior colliculus the first postnatal week.

(C) Ephrin-B2-β-gal fusion protein is expressed in a high dorsal/low ventral gradient at E17.5. While this gradient is maintained the first postnatal week, ephrin-B2 expression is increased in the ventral retina.

Embryonic day (E), Postnatal day (P), Dorsal (D), Ventral (V), Medial (M), Lateral (L)

EphB2 forward signaling alone is important for ventral RGC axon retinocollicular mapping

Hindges et al. (2002) previously reported significant retinocollicular mapping errors in *EphB2*$^{-/-}$*;EphB3*$^{-/-}$ and *EphB2*$^{lacZ/lacZ}$*;EphB3*$^{-/-}$ compound mutants, but did not analyze *EphB2* single mutants to determine the contribution of this receptor separately from EphB3. Therefore, I analyzed the formation of TZs at P8 by focal injection of DiI into the dorsal or ventral-temporal retina to anterograde label RGCs and their axonal projections as illustrated in the schematic (Figure 3.2A). Also shown is an example of how a focal injection into the dorsal retina labels a TZ in the lateral SC of a *wild type* (WT) mouse. Likewise, a focal injection into the VT retina illustrates the typical TZ size and medial-rostral location in a WT mouse, accompanied by the retinal flatmount to document the injection site (Figure 3.2B). Of a total of 109 WT mice injected in the VT retina in my study, only 3 (<3%) formed an ectopic TZ and of a total of 87 WT mice injected into the dorsal retina, only 1 (1%) of the SC formed an ectopic TZ (Figure 3.2E). In my analysis, the SC for each specimen that formed one or more ectopic TZ had their primary and secondary TZs outlined and then these images were superimposed according to genotype with the primary TZ positioned at the crosshairs of the compass as can be observed for the three WT ventral injected retinas that showed termination errors (Figure 3.2B). The schematic summary allows for the qualitative assessment of the localization of the ectopic TZs relative to the primary TZ.

EphB2$^{-/-}$ protein-null single mutants and *EphB2*$^{lacZ/lacZ}$ C-terminal truncation mutants (Henkemeyer et al., 1996) were used to determine the involvement of EphB2 and the contribution of its intracellular domain, respectively. Only 21% (p=0.0194, n=14) of VT injected *EphB2*$^{-/-}$ homozygotes and 6% (n=16) of the *EphB2*$^{+/-}$ heterozygotes formed 1-3 ectopic TZs

(Figure 3.2E). In contrast, 62% ($p<0.0001$, n=13) of the *EphB2*$^{lacZ/lacZ}$ homozygotes formed 1-4 ectopic TZs and even 32% ($p = 0.0049$, n=22) of the *EphB2*$^{lacZ/+}$ heterozygotes formed 1-3 ectopic TZs (Figure 3.2C, D, and E). While many ectopic TZs were lateral to the primary TZ, RGC axons also terminated caudal relative to the primary TZ in several cases. The increased frequency of mapping errors in the *EphB2*^{lacZ} mice indicates a dominant-negative effect due to expression of the C-terminal truncated EphB2-β-gal fusion protein (see also (Cowan et al., 2000; Hindges et al., 2002)). Together, these data show that disruption of EphB2 forward signaling hinders the formation of a properly localized, single TZ.

EphB2 mutant SC that received focal injections of DiI into the dorsal retina did not significantly form any ectopic TZs. Neither the *EphB2*$^{lacZ/+}$ (n=35) or *EphB2*$^{lacZ/lacZ}$ (n=25) forward signaling mutants formed any ectopic TZ and only 15% (n=13) of the *EphB2*$^{+/-}$ and 0% of the *EphB2*$^{-/-}$ (n=3) formed an ectopic TZ (Figure 3.2E) indicating EphB2 participates mainly in ventral RGC axon retinocollicular mapping.

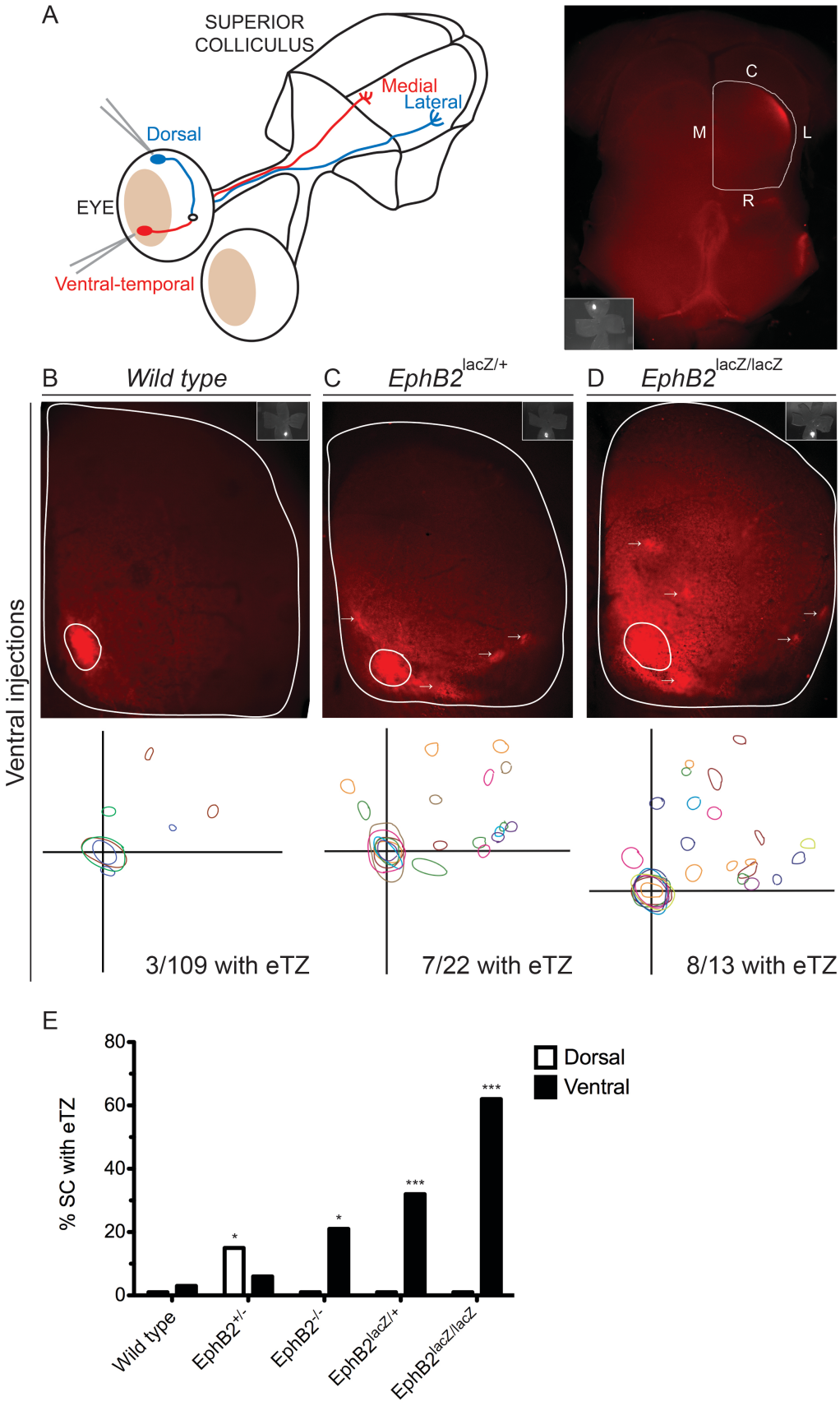

A
SUPERIOR COLLICULUS
Medial
Lateral
Dorsal
EYE
Ventral-temporal
C
M
L
R
B Wild type
C EphB2lacZ/+
D EphB2lacZ/lacZ
Ventral injections
3/109 with eTZ
7/22 with eTZ
8/13 with eTZ
E
% SC with eTZ
80
60
40
20
0
Dorsal
Ventral
*
*
***
***
Wild type
EphB2+/-
EphB2-/-
EphB2lacZ/+
EphB2lacZ/lacZ

Figure 3.2 EphB2 forward signaling alone is important for ventral RGC axon retinocollicular mapping

(A) Left, schematic of the site of DiI injection in the dorsal or ventral-temporal eye labeling RGCs and the expected TZ for the RGC axonal projection in the SC. Right, example of a retinal flatmount and midbrain whole-mount depicting its corresponding TZ in the lateral SC from a WT mouse injected in the dorsal retina.

(B, C, and D) Example of a retina flatmount and SC whole mount from *wild type*, *EphB2*$^{lacZ/+}$, and *EphB2*$^{lacZ/lacZ}$ mutant mice injected in the ventral retina. The boundary of the SC and the primary TZ is illustrated by the white outline. Ectopic TZs are marked with white arrows. SC for each genotype that formed ectopic TZs had their ectopic TZs and corresponding primary TZ outlined and then these images were superimposed on each other with the primary TZ positioned at the crosshairs of the compass as illustrated above.

(E) Summary of the percent of SC that formed ectopic TZs in mice injected in the dorsal or ventral retina. Fisher's two-tailed exact test compared mutant groups to wild-type mice. Statistical significance denoted by asterisks *$p \leq 0.05$, **$p < 0.01$, and ***$p < 0.001$. Dorsal DiI injected *Wild type* 1%, n=87. Ventral DiI injected *Wild type* 3%, n=109. Dorsal DiI injected *EphB2*$^{+/-}$ 15%, p=0.0437, n=13. Ventral DiI injected *EphB2*$^{+/-}$ 6%, n=16. Dorsal DiI injected *EphB2*$^{-/-}$ 0%, n=3. Ventral DiI injected *EphB2*$^{-/-}$ 21%, p=0.0194, n=14. Dorsal DiI injected *EphB2*$^{lacZ/+}$ 0%, n=35. Ventral DiI injected *EphB2*$^{lacZ/+}$ 32%, p=0.0001, n=22. Dorsal DiI injected *EphB2*$^{lacZ/lacZ}$ 0%, n=25. Ventral DiI injected *EphB2*$^{lacZ/lacZ}$ 62%, p<0.0001, n=13.

EphB2 tyrosine kinase catalytic activity is essential for ventral RGC axon retinocollicular mapping

The data above using the *EphB2*^{lacZ} allele indicated a critical role for EphB2 forward signaling in retinocollicular mapping, however it does not identify the specific intracellular signaling component responsible. Therefore a variety of point mutations were analyzed that disrupt either tyrosine kinase catalytic activity (*EphB2*^{K661R}), the ability to bind PDZ domain-containing proteins (*EphB2*$^{\Delta VEV}$), or both catalytic activity and PDZ binding (*EphB2*$^{K661R\Delta VEV}$) (Genander et al., 2009). VT RGC axons in 50% ($p<0.0001$, n=10) of the *EphB2*$^{K661R/K661R}$ homozygotes and 21% (p=0.0194, n=14) of the *EphB2*$^{K661R/+}$ heterozygotes formed 1-2 ectopic TZs lateral to the primary TZ (Figure 3.3B and E). The penetrance of ectopic TZs in the *EphB2*^{K661R} mice were not significantly different from corresponding *EphB2*^{lacZ} mice indicating the tyrosine kinase catalytic activity is the key component for EphB2 intracellular signaling required for VT axon retinocollicular mapping. The contribution of the EphB2 PDZ binding motif to retinocollicular mapping was examined in *EphB2*$^{\Delta VEV/\Delta VEV}$ mutant mice and was found not to participate in TZ formation and the *EphB2*$^{K661R\Delta VEV/K661R\Delta VEV}$ mutant showed no additional defects over what was found for the EphB2$^{K661R/K661R}$ kinase-dead mutant (Figure 3.4).

The involvement of EphB2 tyrosine kinase activity was also examined using the kinase overactive *EphB2*$^{F620D/F620D}$ mutant mice that contains a point mutation in the catalytic domain rendering EphB2 constitutively active, independent of ligand binding (Holmberg et al., 2006). While 16% (p=0.042, n=19) of VT injected *EphB2*$^{F620D/+}$ SC formed 1-3 lateral ectopic TZs, 29% (p=0.0011, n=17) of the VT injected *EphB2*$^{F620D/F620D}$ SC formed 1-2 lateral or caudal ectopic TZs (Figure 3.3C, D, and F). Of the specimens injected with DiI into the dorsal retina, 15% (p=0.0257, n=26) of the *EphB2*$^{F620D/+}$ mutants and 9% (n=22) of *EphB2*$^{F620D/F620D}$ mutants

formed 1-2 ectopic TZs medial to the primary TZ (Figure 3.3C, D, and F). Combined, these data suggest both ventral and dorsal RGC axons are somewhat affected by an overactive EphB2 catalytic domain, which is consistent with the low, although detectable, level of EphB2 expression in the dorsal retina.

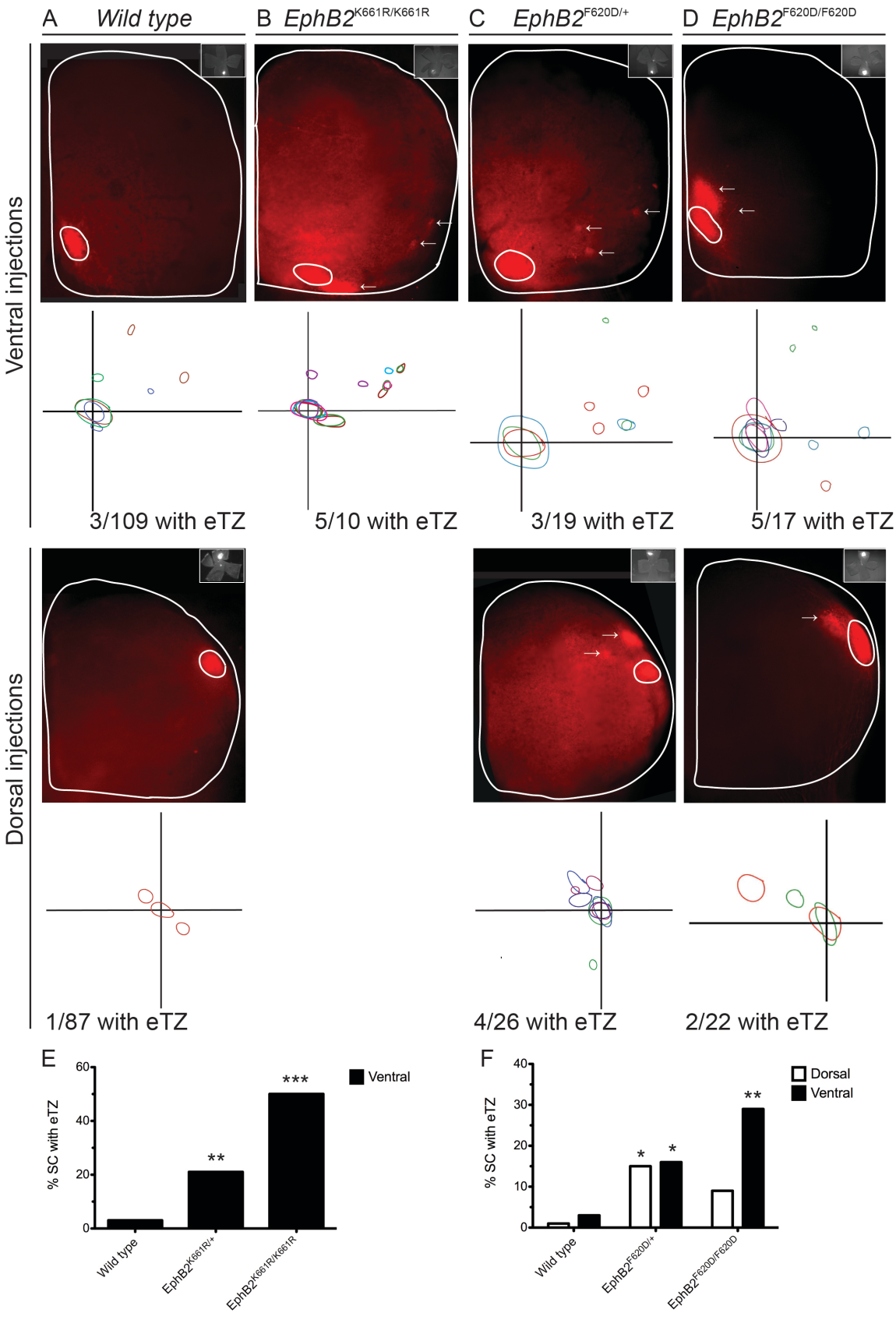

A Wild type
B EphB2K661R/K661R
C EphB2F620D/+
D EphB2F620D/F620D
Ventral injections
3/109 with eTZ
5/10 with eTZ
3/19 with eTZ
5/17 with eTZ
Dorsal injections
1/87 with eTZ
4/26 with eTZ
2/22 with eTZ
E
% SC with eTZ
Ventral
Wild type
EphB2K661R/+
EphB2K661R/K661R
F
% SC with eTZ
Dorsal
Ventral
Wild type
EphB2F620D/+
EphB2F620D/F620D

Figure 3.3 EphB2 tyrosine kinase catalytic activity is essential for ventral RGC axon retinocollicular mapping

(A, B, C, and D) Example of a retina flatmount, SC whole mount, and schematic of all the SC that formed ectopic TZs from *Wild type*, *EphB2*$^{K661R/K661R}$, *EphB2*$^{F620D/+}$, and *EphB2*$^{F620D/F620D}$ mutant mice injected in the ventral or dorsal retina. The boundary of the SC and the primary TZ is illustrated by the white outline. Ectopic TZs are marked with white arrows. SC for each genotype that formed ectopic TZs had their ectopic TZs and corresponding primary TZ outlined and then these images were superimposed on each other with the primary TZ positioned at the crosshairs of the compass as illustrated above.

(E and F) Summary of the percent of SC that formed ectopic TZs in mice injected in the dorsal or ventral retina. Fisher's two-tailed exact test compared mutant groups to wild-type mice. Statistical significance denoted by asterisks *$p \leq 0.05$, **$p < 0.01$, and ***$p < 0.001$. Dorsal DiI injected *Wild type* 1%, n=87. Ventral DiI injected *Wild type* 3%, n=109. Ventral DiI injected *EphB2*$^{K661R/+}$ mutants 21%, p=0.0194, n=14. Ventral DiI injected *EphB2*$^{K661R/K661R}$ mutants 50%, $p < 0.0001$, n=10. Dorsal DiI injected *EphB2*$^{F620D/+}$ mutants 15%, p=0.0257, n=26.

Ventral DiI injected *EphB2*$^{F620D/+}$ mutants 16%, p=0.042, n=19. Dorsal DiI injected *EphB2*$^{F620D/F620D}$ mutants 9%, n=22. Ventral DiI injected *EphB2*$^{F620D/F620D}$ mutants 29%, p=0.0011, n=17.

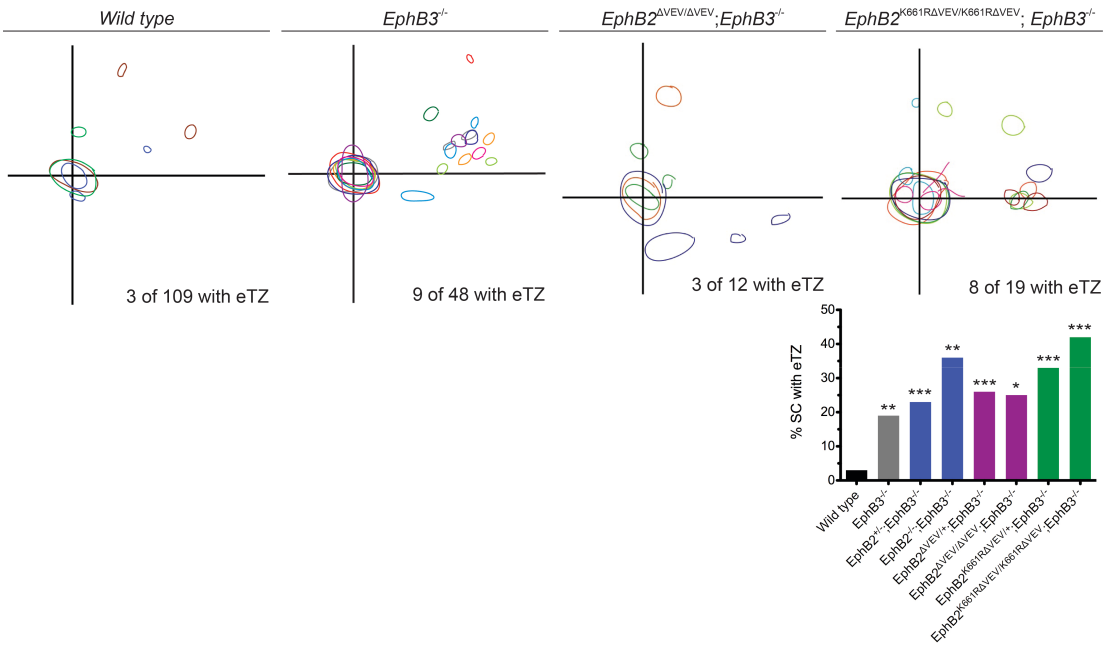


Figure 3.4 EphB2 PDZ binding motif does not regulate ventral RGC axon retinocollicular mapping

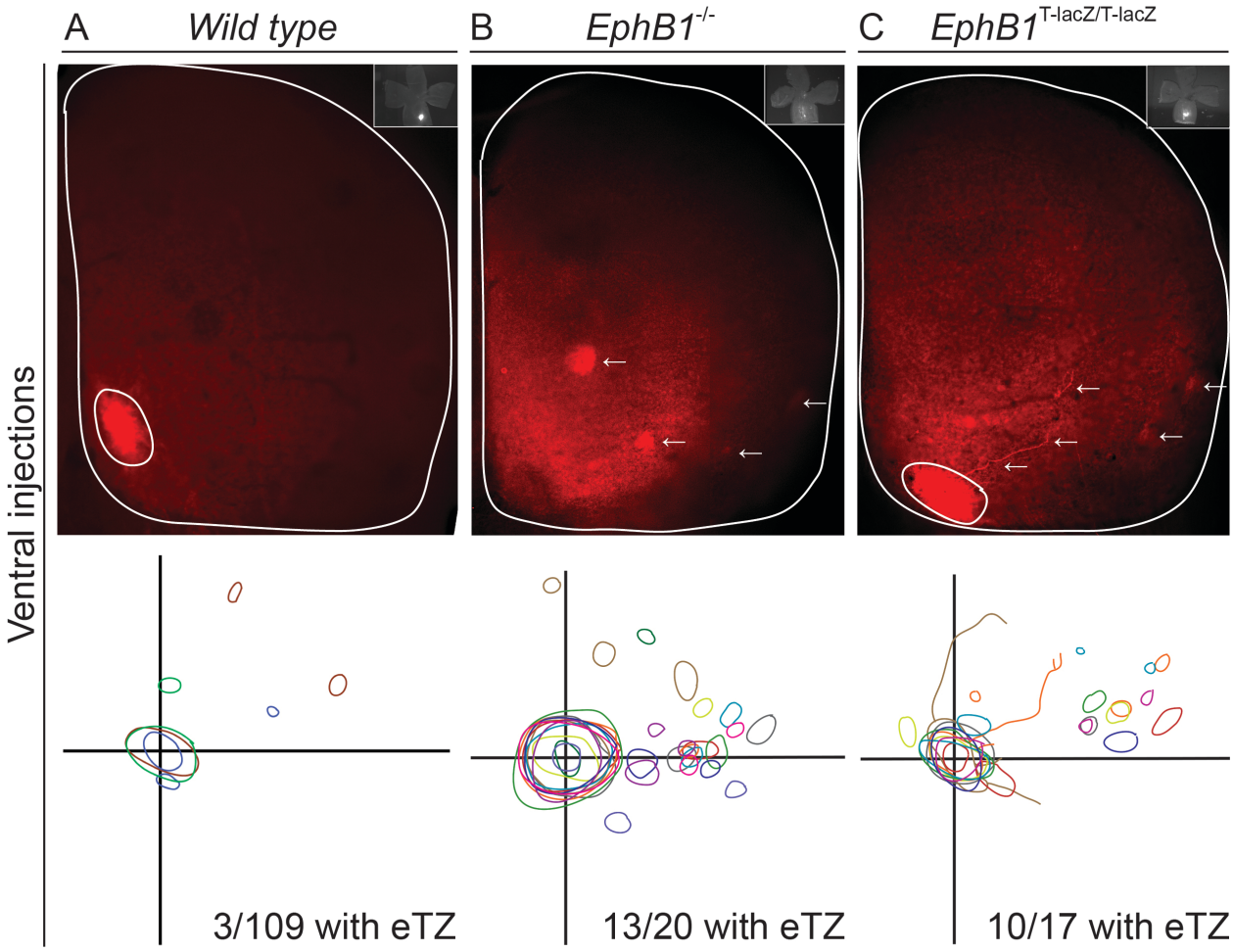
(A) Schematic of all the SC that formed ectopic TZs from *Wild type*, *EphB3*$^{-/-}$, *EphB2*$^{\Delta VEV/\Delta VEV}$*;EphB3*$^{-/-}$, and *EphB2*$^{K661R\Delta VEV/K661R\Delta VEV}$*;EphB3*$^{-/-}$ mutant mice injected in the ventral retina.

(B) Summary of the percent of SC that formed ectopic TZs in mice injected in the dorsal or ventral retina. Fisher's two-tailed exact test compared mutant groups to wild-type mice. Statistical significance denoted by asterisks *p≤0.05, **p<0.01, and ***p<0.001. Ventral DiI injected *Wild type* 3%, n=109. Ventral DiI injected *EphB3*$^{-/-}$ 19%, p=0.0013, n=48. Ventral DiI injected *EphB2*$^{+/-}$*;EphB3*$^{-/-}$ 23%, p=0.0001, n=48. Ventral DiI injected *EphB2*$^{-/-}$*;EphB3*$^{-/-}$ 36%, p=0.0012, n=11. Ventral DiI injected *EphB2*$^{\Delta VEV/+}$*;EphB3*$^{-/-}$ 26%, p=0.0009, n=23. Ventral DiI injected *EphB2*$^{\Delta VEV/\Delta VEV}$*;EphB3*$^{-/-}$ 25%, p=0.0128, n=12. Ventral DiI injected *EphB2*$^{K661R\Delta VEV/+}$*;EphB3*$^{-/-}$ 33%, p<0.0001, n=24. Ventral DiI injected *EphB2*$^{K661R\Delta VEV/K661R\Delta VEV}$*;EphB3*$^{-/-}$ 42%, p<0.0001, n=19.

EphB1 forward signaling is important for ventral RGC axon retinocollicular mapping

Previous studies have not looked at potential roles of the EphB1 receptor tyrosine kinase in retinocollicular mapping even though it is expressed in the visual system (Figure 3.1B) and has been documented to mediate the repulsion of VT RGC axons at the optic chiasm to form ipsilateral projections necessary for binocular vision (Williams et al., 2003). I therefore labeled dorsal and VT RGC axons with DiI to study retinocollicular mapping in *EphB1*$^{-/-}$ protein-null mutants. While dorsal RGC axons were found to terminate normally to a well-defined TZ, the VT axons in *EphB1*$^{-/-}$ mutant mice exhibited a highly penetrant mapping defect in which 65% ($p<0.0001$, n=20) of the SC showed 1-3 ectopic TZs (Figure 3.5B and D). Even 38% ($p=0.0003$, n=13) of the VT DiI labeled RGC axons of *EphB1*$^{+/-}$ heterozygotes formed 1-3 ectopic TZs, indicating a strong dosage effect (Figure 3.5). These ectopic TZs tended to form lateral relative to the primary TZ. Additionally, it was often noted that the primary TZ in the mutants was not compact like that observed in WT, and in one case a distinct primary TZ was not visible.

To determine whether forward signaling mediated by the EphB1 intracellular domain is important, *EphB1*$^{T\text{-}lacZ}$ mutant mice expressing the C-terminal truncated EphB1-β-gal fusion protein described above were analyzed. The data showed that 59% ($p<0.0001$, n=17) of the *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ homozygotes and 20% (p=ns, n=5) of the *EphB1*$^{T\text{-}lacZ/+}$ heterozygotes formed 1-3 ectopic TZs usually lateral to the primary TZ following DiI injection into the VT retina (Figure 3.5C and D). Thus, like EphB2, these results indicate forward signaling mediated by the EphB1 intracellular domain is required for normal retinocollicular mapping of ventral RGC axons.


A Wild type
B EphB1-/-
C EphB1T-lacZ/T-lacZ
Ventral injections
3/109 with eTZ
13/20 with eTZ
10/17 with eTZ

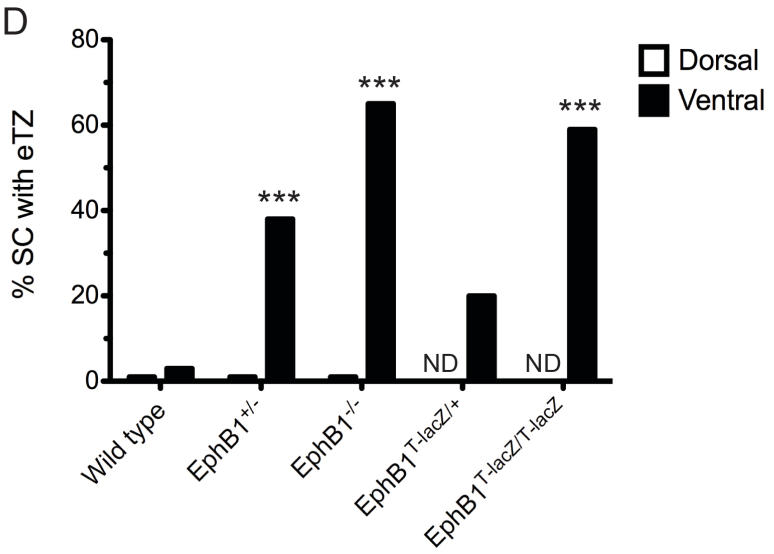

D
% SC with eTZ
80
60
40
20
0
Dorsal
Ventral
***
***
***
ND
ND
Wild type
EphB1+/-
EphB1-/-
EphB1T-lacZ/+
EphB1T-lacZ/T-lacZ

Figure 3.5 EphB1 forward signaling is important for ventral RGC axon retinocollicular mapping

(A, B, and C) Example of a retina flatmount, SC whole mount, and schematic of all the SC that formed ectopic TZs from *Wild type*, *EphB1*$^{-/-}$, and *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ mutant mice injected in the ventral retina. The boundary of the SC and the primary TZ is illustrated by the white outline. Ectopic TZs are marked with white arrows. SC for each genotype that formed ectopic TZs had their ectopic TZs and corresponding primary TZ outlined and then these images were superimposed on each other with the primary TZ positioned at the crosshairs of the compass as illustrated above.

(D) Summary of the percent of SC that formed ectopic TZs in mice injected in the dorsal or ventral retina. Fisher's two-tailed exact test compared mutant groups to wild-type mice. Statistical significance denoted by asterisks *$p \leq 0.05$, **$p < 0.01$, and ***$p < 0.001$. Dorsal DiI injected *Wild type* 1%, n=87. Ventral DiI injected *Wild type* 3%, n=109. Dorsal DiI injected *EphB1*$^{+/-}$ 0%, n=15. Ventral DiI injected *EphB1*$^{+/-}$ 38%, p=0.0003, n=13. Dorsal DiI injected *EphB1*$^{-/-}$ 0%, n=13. Ventral DiI injected *EphB1*$^{-/-}$ 65%, p<0.0001, n=20. Dorsal DiI injected *EphB1*$^{T\text{-}lacZ/+}$ 20%, n=5. Ventral DiI injected *EphB1*$^{T\text{-}lacZ/T\text{-}lacZ}$ 59%, p<0.0001, n=17.

Not Done (ND)

Synergistic role for both EphB1 and EphB2 in ventral RGC axon retinocollicular mapping

To determine if EphB1 and EphB2 work as a team to mediate retinocollicular mapping, DiI was used to trace RGC axons in *EphB1*;*EphB2* compound mutants. The *EphB1*$^{-}$ protein-null allele was combined with either *EphB2*$^{-}$ protein-null to eliminate both forward and reverse signaling or the *EphB2*^{lacZ} allele to eliminate only EphB2 forward signaling. Following labeling of VT RGC axons, 100% of the *EphB1*$^{-/-}$;*EphB2*$^{+/-}$ (n=12, $p<0.0001$) and *EphB1*$^{-/-}$;*EphB2*$^{-/-}$ (n=7, $p<0.0001$) SC analyzed exhibited 1-5 ectopic TZs that localized caudal, lateral, and lateral-caudal relative to the primary TZ (Figure 3.6A, B, and E). These mutants also exhibited an increase in stray axonal projections, and the primary TZ was often not fully compact, appearing diffused, compared to WT. The complete 100% penetrance and loss of a directional trend for the positioning of ectopic TZs emphasizes the impact the loss of both EphB1/EphB2 has on VT RGC retinocollicular mapping and how these molecules work synergistically.

Likewise, compound mutant mice carrying the *EphB1*$^{-}$ and *EphB2*^{lacZ} alleles had near full penetrance of mapping defects. Seventy-six percent (n=21, $p<0.0001$) of the *EphB1*$^{-/-}$;*EphB2*$^{lacZ/+}$ and 75% (n=8, $p<0.0001$) of the *EphB1*$^{-/-}$;*EphB2*$^{lacZ/lacZ}$ mutant SC formed 1-6 ectopic TZs that localized lateral, caudal, and lateral-caudal directions relative to the primary TZ (Figure 3.6C, D, and E). The primary TZs were also often not compact and exhibited stray axonal projections.

Dorsal RGC axons formed no ectopic TZs (n=7) in the *EphB1*$^{-/-}$;*EphB2*$^{-/-}$ compound nulls and only 1 (n=10) of the *EphB1*$^{-/-}$;*EphB2*$^{+/-}$ SC analyzed did (Figure 3.6E). Together, these data demonstrate the vital and specific role for EphB1/EphB2 mediated forward signaling in ventral RGC axon retinocollicular mapping.

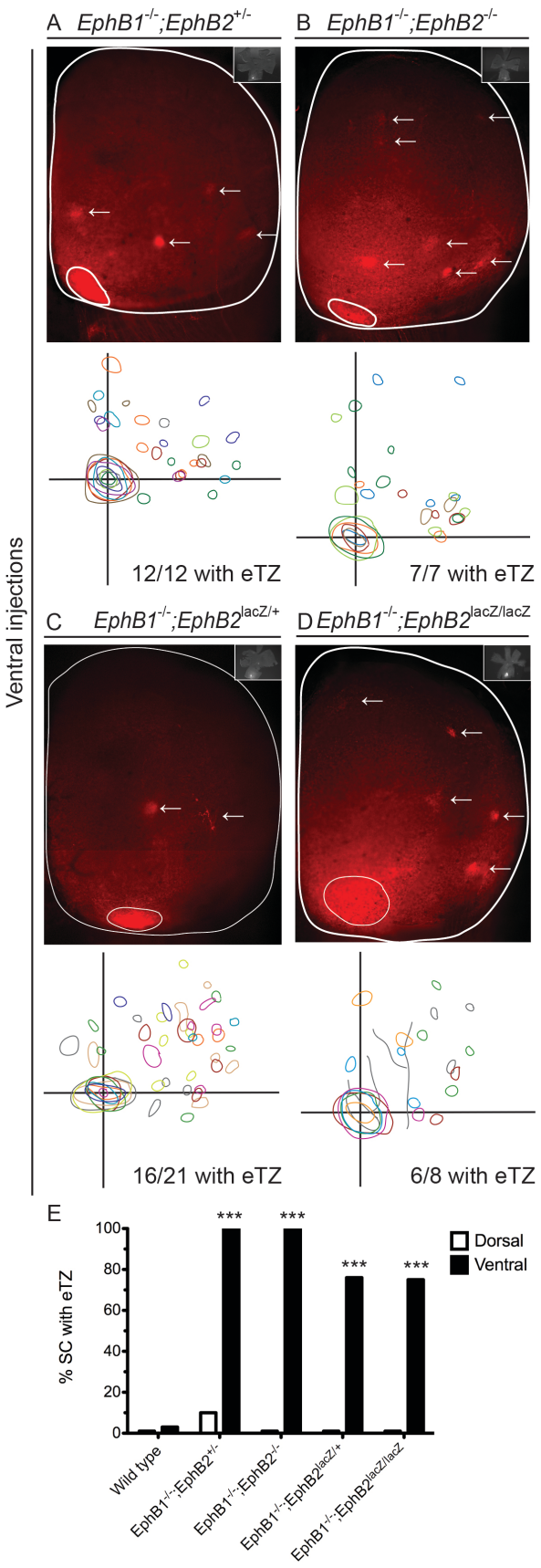

A EphB1-/-;EphB2+/-
B EphB1-/-;EphB2-/-
Ventral injections
12/12 with eTZ
7/7 with eTZ
C EphB1-/-;EphB2lacZ/+
D EphB1-/-;EphB2lacZ/lacZ
16/21 with eTZ
6/8 with eTZ
E
% SC with eTZ
100
80
60
40
20
0
***
***
***
***
Dorsal
Ventral
Wild type
EphB1-/-;EphB2+/-
EphB1-/-;EphB2-/-
EphB1-/-;EphB2lacZ/+
EphB1-/-;EphB2lacZ/lacZ

Figure 3.6 Synergistic role for both EphB1 and EphB2 in ventral RGC axon retinocollicular mapping

(A, B, C, and D) Example of a retina flatmount, SC whole mount, and schematic of all the SC that formed ectopic TZs from *Wild type*, *EphB1*$^{-/-}$; *EphB2*$^{+/-}$, *EphB1*$^{-/-}$; *EphB2*$^{-/-}$, *EphB1*$^{-/-}$; *EphB2*$^{lacZ/+}$ and *EphB1*$^{-/-}$; *EphB2*$^{lacZ/lacZ}$ mutant mice injected in the ventral retina. The boundary of the SC and the primary TZ is illustrated by the white outline. Ectopic TZs are marked with white arrows. SC for each genotype that formed ectopic TZs had their ectopic TZs and corresponding primary TZ outlined and then these images were superimposed on each other with the primary TZ positioned at the crosshairs of the compass as illustrated above.

(E) Summary of the percent of SC that formed ectopic TZs in mice injected in the dorsal or ventral retina. Fisher's two-tailed exact test compared mutant groups to wild-type mice. Statistical significance denoted by asterisks *$p \leq 0.05$, **$p < 0.01$, and ***$p < 0.001$. Dorsal DiI injected *Wild type* 1%, n=87. Ventral DiI injected *Wild type* 3%, n=109. Dorsal DiI injected *EphB1*$^{-/-}$;EphB2$^{+/-}$ 10% n=10. Ventral DiI injected *EphB1*$^{-/-}$;EphB2$^{+/-}$ 100%, $p < 0.0001$, n=12. Dorsal DiI injected *EphB1*$^{-/-}$;EphB2$^{-/-}$ 0%, n=7. Ventral DiI injected *EphB1*$^{-/-}$;EphB2$^{-/-}$ 100%, $p < 0.0001$, n=7. Dorsal DiI injected *EphB1*$^{-/-}$;*EphB1*$^{lacZ/+}$ 0%, n=11. Ventral DiI injected *EphB1*$^{-/-}$;*EphB1*$^{lacZ/+}$ 76%, $p < 0.0001$, n=21. Dorsal DiI injected *EphB1*$^{-/-}$;*EphB1*$^{lacZ/lacZ}$ 0%, n=9. Ventral DiI injected *EphB1*$^{-/-}$;*EphB1*$^{lacZ/lacZ}$ 75%, $p < 0.0001$, n=8.

Ephrin-B1 mediates ventral RGC axon retinocollicular mapping

Ephrin-B1 expression in the SC/OT midline suggests the potential role of ephrin-B1 acting as a ligand to mediate ventral RGC axon interstitial branching and termination in the medial SC (Braisted et al., 1997; Buhusi et al., 2009; Hindges et al., 2002; Mann et al., 2002; McLaughlin et al., 2003). My study analyzed mutant mice that have the entire x-linked *ephrin-B1* coding region deleted in the germ-line, *ephrin-B1*$^{-/y}$ (Davy et al., 2004), to determine if the absence of ephrin-B1 affects retinocollicular mapping.

While DiI labeled dorsal RGC axons from *ephrin-B1*$^{+/-}$ (n=4) and *ephrin-B1*$^{-/y}$ (n=17) mutant mice had no retinocollicular mapping errors, VT RGC axons formed 1 ectopic TZ in 25% ($p=0.0128$, n=12) of the *ephrin-B1*$^{-/y}$ hemizygous male mice that were small and lateral to the primary TZ (Figure 3.7A and C). Likewise, VT RGC axons in *ephrin-B1*$^{+/-}$ heterozygous female mice formed 1 ectopic TZ in 33% ($p=0.0213$, n=6) of the SC that were small and either caudal or lateral to the primary TZ (Figure 3.7B and C). As ephrin-B1 is weakly, if at all, expressed in the retina (data not shown), these data imply that ephrin-B1 expressed in the SC midline acts as ligand to mediate EphB1/EphB2 driven ventral RGC axon retinocollicular mapping, although the relatively mild effect suggests additional ephrin-B molecules likely participate.

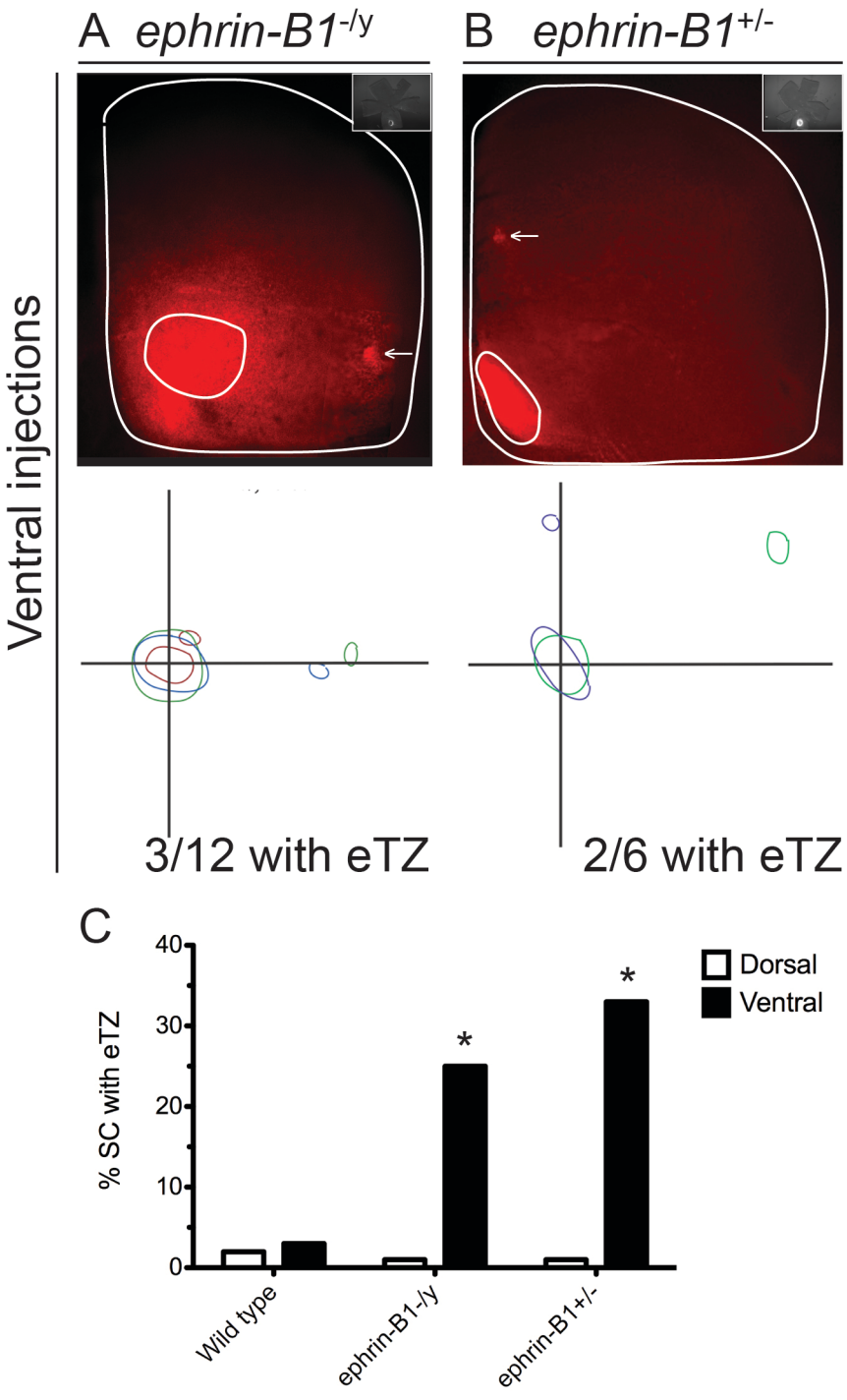

A ephrin-B1-/y
B ephrin-B1+/-
Ventral injections
3/12 with eTZ
2/6 with eTZ
C
% SC with eTZ
40
30
20
10
0
Dorsal
Ventral
*
*
Wild type
ephrin-B1-/y
ephrin-B1+/-

Figure 3.7 Ephrin-B1 mediates ventral RGC axon retinocollicular mapping

(A and B) Example of a retina flatmount, SC whole mount, and schematic of all the SC that formed ectopic TZs from *ephrin-B1*$^{+/-}$ and *ephrin-B1*$^{\Delta/y}$ mutant mice injected in the ventral retina. The boundary of the SC and the primary TZ is illustrated by the white outline. Ectopic TZs are marked with white arrows. SC for each genotype that formed ectopic TZs had their ectopic TZs and corresponding primary TZ outlined and then these images were superimposed on each other with the primary TZ positioned at the crosshairs of the compass as illustrated above.

(C) Summary of the percent of SC that formed ectopic TZs in mice injected in the dorsal or ventral retina. Fisher's two-tailed exact test compared mutant groups to wild-type mice. Statistical significance denoted by asterisks *$p \leq 0.05$, **$p < 0.01$, and ***$p < 0.001$. Dorsal DiI injected ephrin-B1$^{\Delta/+}$ 0%, n=4. Ventral DiI injected ephrin-B1$^{\Delta/+}$ 33%, p=0.0213, n=6. Dorsal DiI injected ephrin-B1$^{\Delta/y}$ 0%, n=17. Ventral DiI injected ephrin-B1$^{\Delta/y}$ 25%, p=0.0128, n=12.

Ephrin-B2 reverse signaling is necessary for both dorsal and ventral RGC axon retinocollicular mapping

To study how ephrin-B2 mediated reverse signaling may direct retinocollicular mapping, I analyzed the *ephrin-B2*^{lacZ} mutation that expresses a C-terminal truncated ephrin-B2-β-gal fusion protein, described above. While unable to transduce reverse signals, the ephrin-B2-β-gal fusion protein is still able to activate forward signaling. Because *ephrin-B2*$^{lacZ/lacZ}$ homozygotes do not survive after birth, this mutant allele was combined with a new mutant allele described here, *ephrin-B2*$^{6YF\Delta V}$. This *ephrin-B2*$^{6YF\Delta V}$ allele has point mutations in the exon that encodes the intracellular cytoplasmic tail that change the 6 tyrosine residues into phenylalanines and also deletes the C-terminal valine residue (Thakar et al., submitted). 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.

We created a new knock-in mutant mouse to disrupt the canonical reverse signaling of ephrin-B2. In order to stop all tyrosine phosphorylation and PDZ domain interactions, all 6 tyrosine residues within the intracellular domain of ephrin-B2 were replaced with phenylalanine and the C-terminal amino acid valine truncated, respectively. Previous studies have shown that similarly mutations of ephrin-B1 and ephrin-B3 lose their ability to induce canonical ephrin-B reverse signaling but maintain their ability to act as ligands to transduce forward signals in EphB expressing cells (Makinen et al., 2005; Xu and Henkemeyer, 2009).

The robust ephrin-B2 expression in the dorsal retina (Figure 3.1C) points towards a likely role for ephrin-B2 in dorsal RGC axon retinocollicular mapping. *Ephrin-B2*$^{lacZ/+}$ males were mated to *ephrin-B2*$^{6YF\Delta V/+}$ females and the resulting offspring were analyzed by DiI tracing of the dorsal retina, which revealed the formation of 1-2 ectopic TZs medial or medial-rostral to the

primary TZ in 11% (p=0.0442, n=28) of *ephrin-B2*$^{6YF\Delta V/+}$ SC, 8% (p=ns, 26) of the *ephrin-B2*$^{lacZ/+}$ SC, and 27% (p=0.0015, n=15) of the *ephrin-B2*$^{lacZ/6YF\Delta V}$ SC (Figure 3.8B, C, and D). This demonstrates a role for ephrin-B2 reverse signaling in dorsal RGC axon retinocollicular mapping.

While the gradient of ephrin-B2 expression is at an obviously lower level in the ventral retina compared to the dorsal retina, its ventral expression is evident at E17.5 and becomes more pronounced during the first postnatal week as observed at P0 and P8 (Figure 3.1C). This indicates ephrin-B2 may also participate in ventral RGC axon retinocollicular mapping. Indeed, DiI tracing of VT RGC axons indicated 1-2 ectopic TZs lateral or caudal to the primary TZ in 14% (p=0.0105, n=49) of the *ephrin-B2*$^{6YF\Delta V/+}$ SC and 36% (p<0.0001, n=22) of the *ephrin-B2*$^{lacZ/+}$ SC (Figure 3.8B and D). The severity of the mapping defect increased in *ephrin-B2*$^{lacZ/6YF\Delta V}$ SC in which 42% (p=0.0002, n=12) of the SC formed 1-3 ectopic TZs lateral, caudal, and rostral relative to the primary TZ (Figure 3.8C and D). These data indicate the unexpected role of ephrin-B2 reverse signaling in ventral RGC retinocollicular mapping.

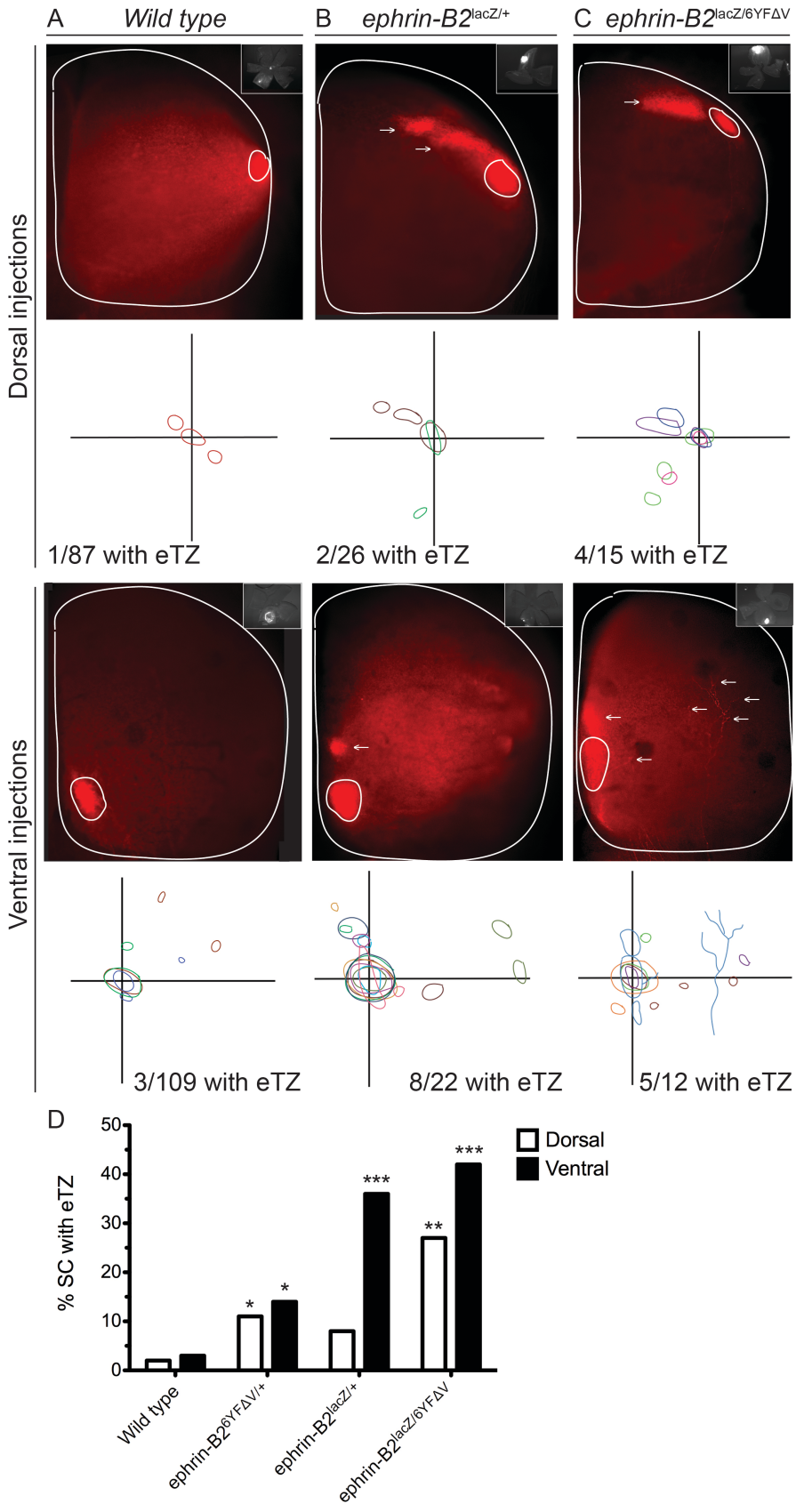

A Wild type
B ephrin-B2lacZ/+
C ephrin-B2lacZ/6YFΔV
Dorsal injections
1/87 with eTZ
2/26 with eTZ
4/15 with eTZ
Ventral injections
3/109 with eTZ
8/22 with eTZ
5/12 with eTZ
D
% SC with eTZ
Dorsal
Ventral
Wild type
ephrin-B26YFΔV/+
ephrin-B2lacZ/+
ephrin-B2lacZ/6YFΔV

Figure 3.8 Ephrin-B2 reverse signaling is necessary for both dorsal and ventral RGC axon retinocollicular mapping

(A, B, and C) Example of a retina flatmount, SC whole mount, and schematic of all the SC that formed ectopic TZs from *Wild type*, *ephrin-B2*$^{lacZ/+}$ and *ephrin-B1*$^{lacZ/6YF\Delta V}$ mutant mice injected in the ventral retina. The boundary of the SC and the primary TZ is illustrated by the white outline. Ectopic TZs are marked with white arrows. SC for each genotype that formed ectopic TZs had their ectopic TZs and corresponding primary TZ outlined and then these images were superimposed on each other with the primary TZ positioned at the crosshairs of the compass as illustrated above.

(D) Summary of the percent of SC that formed ectopic TZs in mice injected in the dorsal or ventral retina. Fisher's two-tailed exact test compared mutant groups to wild-type mice. Statistical significance denoted by asterisks *$p \leq 0.05$, **$p < 0.01$, and ***$p < 0.001$. Dorsal DiI injected *wild type* 1%, n=87. Ventral DiI injected *wild type* 3%, n=109. Dorsal injected *ephrin-B2*$^{6YF\Delta V/+}$11%, p=0.0442, n=28. Ventral DiI injected ephrin-B2$^{6YF\Delta V/+}$ 14%, p=0.0105, n=49. Dorsal DiI injected ephrin-B2$^{lacZ/+}$ 8%, n=26. Ventral DiI injected ephrin-B2$^{lacZ/+}$ 36%, p<0.0001, n=22. Dorsal DiI injected ephrin-B2$^{lacZ/6YF\Delta V}$ 27%, p=0.0015, n=15. Ventral DiI injected ephrin-B2$^{lacZ/6YF\Delta V}$ 42%, p= 0.0002, n=12.

DISCUSSION

My results show that EphB and ephrin-B signaling are key mediators of dorsoventral RGC axon retinocollicular mapping. Utilizing various mutant mice I determined EphB2 tyrosine kinase catalytic activity is critical for ventral RGC axon mapping, EphB1 forward signaling is required for ventral RGC axon retinocollicular mapping, ligand role for ephrin-B1 in ventral RGC axon retinocollicular mapping is important, and ephrin-B2 reverse signaling is essential for dorsal and ventral RGC axon mapping.

Over a majority of *EphB1*$^{-/-}$; *EphB1*$^{\text{T-lacZ/T-lacZ}}$; and *EphB2*$^{\text{lacZ/lacZ}}$ as well as half of the *EphB2*$^{\text{K661R/K661R}}$ mutant mice exhibited ventral RGC axons forming ectopic TZs in the SC and therefore demonstrating EphB1 and EphB2 alone are crucial for forwarding signaling mediated ventral RGC axon retinocollicular mapping. These EphB single mutants formed ectopic TZs that tended to be lateral, but also caudal to the primary TZ. Together EphB receptors mediate ventral RGC axon retinocollicular mapping, with EphB1 and EphB2 being the chief mediators as demonstrated by the 100% penetrance of the mapping defect exhibited in *EphB1*$^{-/-}$*;EphB2*$^{-/-}$ and *EphB1*$^{-/-}$*;EphB2*$^{+/-}$ mutants. These mutants exhibited completely disrupted retinocollicular maps as VT RGC axons terminated in multiple ectopic TZs throughout the SC in locations lateral, caudal, and caudal-lateral relative to the primary TZ. In addition, the primary TZs often formed loosely and projected stray axons.

While the experiments performed in this study perturbed intracellular signaling to determine its function, indirect evidence for the role of the EphB receptor extracellular domain is evident. While *EphB1*$^{-/-}$*;EphB2*$^{\text{lacZ/lacZ}}$ and *EphB1*$^{-/-}$*;EphB2*$^{\text{lacZ/+}}$ mutant mice exhibited mapping defects that were as severe as those observed in the compound null *EphB1;EphB2* mutants, the

penetrance of the retinocollicular mapping defects was decreased from 100% to 75%. The reduced penetrance may be due to the contribution of EphB2 extracellular domain still present in these mutants and able to activate reverse signaling.

While my study underscores that EphB1 intracellular and EphB2 kinase mediated intracellular signaling are critical for VT retinocollicular mapping, other studies have highlighted the role of EphB3 using *EphB2;EphB3* compound mutants. I found *EphB2*$^{-/-}$ and *EphB3*$^{-/-}$ single mutants formed ectopic TZs in about 20% of the SC while *EphB2*$^{-/-}$;*EphB3*$^{-/-}$ compound null mutants formed ectoptic TZs in about 36% of the SC (Figure 3.2; Figure 3.4). Though these data indicate EphB3 is important in ventral RGC axon retinocollicular mapping, the *EphB2*$^{-/-}$;*EphB3*$^{-/-}$ compound null mutants did not have the same severity and fully penetrant phenotype as the *EphB1*$^{-/-}$;*EphB2*$^{-/-}$ compound null mutants. Therefore, EphB1/EphB2 forward signaling are necessary for retinocollicular mapping while EphB3 plays a supportive role.

Ephrin-B1 was also found to have a role in VT RGC axon termination in the SC, but the deletion of ephrin-B1 did not strongly disrupt retinocollicular mapping as would be expected if it were the sole ligand in the SC. VT RGC axons expressing high levels of EphB receptors target into the medial-rostral SC that expresses ephrin-B1. The severity of the *EphB1*$^{-/-}$;*EphB2*$^{-/-}$ mutant mice mapping defect was not mirrored in the *ephrin-B1*$^{-/y}$ mutant mice suggesting another ligand, perhaps another ephrin-B, acting in a mutual role.

Ephrin-B2 reverse signaling mutants not only disrupted dorsal RGC axon TZ formation, as predicted by its robust dorsal expression pattern, but also VT RGC axons indicating the extensive range of ephrin-B2 receptor-like intracellular signaling has on retinocollicular mapping. Mediators of retinocollicular mapping for dorsal RGC axons have not been found previously, making this a significant finding. The role of ephrin-B2 as a ligand in the SC was not

examined because only *ephrin-B2* mutants with mutations in the cytoplasmic tail were used although other studies also suggest the role for ephrin-B2 reverse signaling to be important for retinotopic mapping. In *Xenopus laevis ephrin-B2* mRNA is expressed in a high dorsal/low ventral gradient in the retina and along the midline of the OT (Mann et al., 2002). Ectopic expression of a dominant-negative *ephrin-B2* cytoplasmic tail deleted mutant in either the dorsal or ventral retina resulted in RGC axon targeting errors in the *Xenopus* OT. While discovering ephrin-B2 reverse signaling is important for dorsoventral RGC axon retinocollicular mapping is a novel finding, the ligand that ephrin-B2 interacts with in the SC is unknown. Analysis of EphB1 and EphB2 single and compound null mutants showed no significant defect in dorsal RGC axon retinocollicular mapping indicating that these two molecules are not the principal ligands.

The Eph and ephrin molecules generally interact within their own class, however some interclass promiscuity exists, although at a lower affinity. For instance, both EphA4 and EphB2 bind to ephrin-B1/2/3 and ephrin-A5 as visualized in crystal structures of EphB2:ephrin-A5 and EphA4:ephrin-B2 (Chrencik et al., 2006; Himanen et al., 2004; Qin et al., 2010). Ephrin-A5 has a high caudal/low rostral gradient expression in the SC and is critical for axon pruning of the initial overshoot of the RGC axons past their proper TZ during the first postnatal week. Considering the established activation of EphB2 forward signaling by ephrin-A5 (Himanen et al., 2004), ventral RGC axons may be responding to the ephrin-A5 gradient similar to nasal-temporal RGC axons. In addition, ephrin-B2 may interact with EphA4, which is highly expressed in a shallow, if any, gradient in the mouse SC (Rashid et al., 2005). Supporting this hypothesis is the caudal and sometimes rostral position of the ectopic TZs relative to the primary TZ observed in ephrin-B2 mutant mice, especially the ventral RGC axon labeled mutants (Figure 8B and 8C).

These interactions may contribute to the formation of rostral and caudal ectopic TZs relative to the primary TZ in EphB and ephrin-B mutant SC as well as why the deletion of ephrin-B1 did not disrupt retinocollicular mapping throughout the SC.

Loss of EphB and ephrin-B signaling disrupts retinocollicular mapping, but if these topographic errors affect vision remains unknown. Fourier imaging of intrinsic signals can demonstrate the visual relevance as documented in ephrin-A mouse models (Cang et al., 2008). Questions also remain about how these molecules affect interstitial branching and laminar specification in the superior colliculus. Our study establishes how the B-subclass of Eph-ephrin molecules mediate topographic TZ formation in the SC and knowing the major and minor mediators of retinocollicular mapping allows these remaining questions to be better addressed.

Chapter 4

DISCUSSION AND FUTURE DIRECTIONS

What was known and what I contributed

"It is fair to say that, in general, no problems have been exhausted; instead, men have been exhausted by the problems."

~ Santiago Ramón y Cajal

(translated by Neely and Larry Swanson)

Ramón y Cajal, a pioneer in the study of neuronal circuitry, thought it was "*indolence masquerading as modesty*" to believe all the important questions have already been solved. For over a century, scientists have sought to understand how the neuronal connections of the visual system originate with such incredibly precision. My doctorate research has sought to expand our understanding of the molecular cues underlying the development of the visual system. In this dissertation, I have described a series of experiments in which a subset of RGC axons were DiI labeled to observe RGC axon guidance to the optic nerve head and termination in the SC in various *EphB* and *ephrin-B* mutant mice to further understand how EphB:ephrin-B bidirectional signaling participate in these developmental processes.

RGC axon guidance to the optic nerve head

When I first started my analysis, retinal development was known to require RGC axon fasciculation and pathfinding towards the optic nerve head. Once the RGC axons reach the optic disc, they must then exit the retina to form the optic nerve. The molecular basis directing axons towards the optic nerve head were not well understood although evidence pointed towards molecular repellents such as, chrondroitin sulfate proteogylcan (Brittis et al., 1992; Snow and Letourneau, 1992), Slits (Ringstedt et al., 2000), and EphBs (Birgbauer et al., 2000). EphBs and ephrin-Bs are expressed in a robust countergradient along the dorsoventral axis of the retina indicating their potential role in RGC axon pathfinding to the optic nerve head (summarized in Figure 4.1). The loss of EphB molecules in *EphB2*$^{-/-}$;*EphB3*$^{-/-}$ mutants caused a subset of dorsal RGC axons to defasciculate from the axon bundle and aberrantly project into the ventral retina (Birgbauer et al., 2000). On the other hand, the loss of forward signaling in *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{-/-}$ mutants showed no axon guidance defects in the retina. Although penetrance of the defect was very low, it indicated a role for ephrin-B reverse signaling and therefore in an attempt to better understand the role of EphB:ephrin-B signaling in RGC axon pathfinding, I analyzed *ephrin-B* mutants.

Because the study implicated EphB acting in a ligand-like role, I analyzed *ephrin-B1* null and *ephrin-B2* reverse signaling single and compound mutants and found an even lower percent of dorsal RGC axons with axon guidance defects. Interestingly, the subset of aberrantly projecting axons in the *ephrin-B* mutants did not defasciculate as they approached the optic disc as observed in the EphB mutants (summarized in Figure 4.1). Instead, the fasciculated bundle separated from the DiI labeled axon bundles funneling into the optic nerve head and projected into the ventral retina. These data indicate potentially distinct roles for EphB and ephrin-B in

axon fasciculation. Perhaps the additional deletion of ephrin-B3 would have amplified the defect, though *ephrin-B1*, *B2*, and *B3* triple mutants were hard to breed and homozygotes often did not survive. On the other hand, perhaps the low percent of axon guidance errors observed in the *EphB* and *ephrin-B* mutants indicates other guidance cues play a bigger role in directing RGC axons to the optic disc to exit and form the optic nerve. Conceivably, the primary role for the robust expression patterns of Eph and ephrin molecules in the retina is guiding RGC axon ipsilateral projections at the optic chiasm (Chenaux and Henkemeyer, unpublished; Williams et al., 2003) and retinotopic mapping to the OT/SC ((Thakar et al., submitted) reviewed in (Feldheim and O'Leary, 2010)).

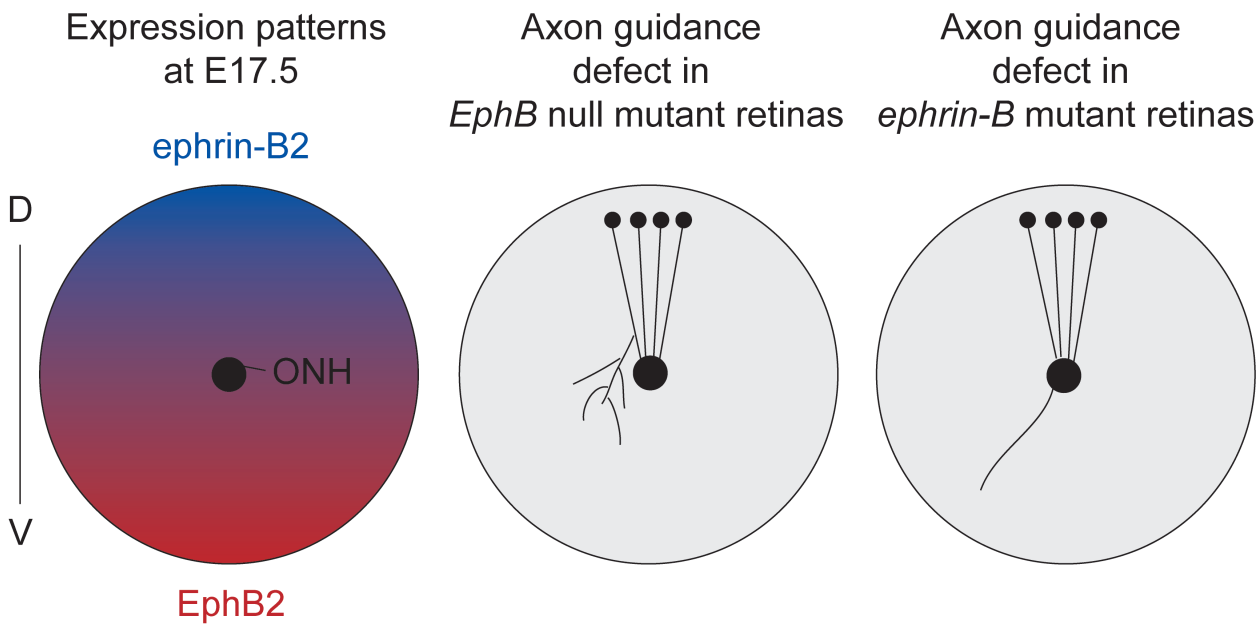


Figure 4.1 Summary of defects in axon guidance to the ONH in *EphB* and *ephrin-B* mutants

EphB2 and ephrin-B2 are expressed in steep complementary gradients during embryonic development. While 20% of the *EphB2*$^{-/-}$;*EphB3*$^{-/-}$ retinas exhibit a subset of DiI labeled dorsal RGC axons that defasciculated and projected incorrectly into the ventral retina, 8% of the *ephrin-B1*$^{-/y}$;*ephrin-B2*$^{lacZ/T}$ retinas showed a subset of DiI labeled dorsal RGC axons that projected a fasciculated axon bundle into the ventral retina.

Dorsal (D), Ventral (V), Optic nerve head (ONH)

RGC axon termination in the SC

The majority of my doctorate work has focused on how EphB:ephrin-B bidirectional signaling mediates dorsoventral retinocollicular mapping. RGC axons project to specific points in the superficial dorsal layers of the SC to maintain their spatial arrangement in order to project a 2D visual map onto the SC. Roger Sperry first proposed that topographic mapping is mediated by complimentary guidance cues expressed in the axons projecting and target regions. Confirmation for his chemoaffinity hypothesis arrived with the discovery of the role of ephrin-A ligands in retinotopic mapping in chicks (Drescher et al., 1995; Frisen et al., 1998). Retinocollicular mapping has been studied for over 50 years because it provides an ideal system to understand neuronal circuitry. It was not until the 1990's that the appropriate tools and molecular biology techniques were available to understand the molecular mechanism underlying this process. Ephs and ephrins have been found to be essential for retinotopic mapping although only EphA:ephrin-A signaling has been thoroughly examined (reviewed in (Feldheim and O'Leary, 2010)). The precise function of EphA:ephrin-A signaling in retinotopic mapping has been dissected although less is understood about the B-subclass, particularly in mammalian systems, until now. One study analyzed *EphB2*$^{-/-}$;*EphB3*$^{-/-}$ and *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{-/-}$ mutants and showed EphB forward signaling has a distinct role from EphA:ephrin-A signaling (Hindges et al., 2002). While the A-subclass primarily mediated RGC axon termination along the rostrocaudal axis of the SC, the B-subclass directs interstitial branching along the mediolateral axis. In order to gain a fuller understanding of EphB:ephrin-B bidirectional signaling mediated retinocollicular mapping, I wanted to undertake a comprehensive analysis by utilizing the various *EphB* and *ephrin-B* mutants generated in the Henkemeyer laboratory.

After confirming previous results in *EphB2;EphB3* compound null mutants, I examined *EphB* single null and signaling mutants to determine the contribution of the individual molecules towards forward signaling mediated retinocollicular mapping. Because ephrin-B1 has long been proposed as the primary ligand for ventral RGC axons in the SC, I analyzed *ephrin-B1* null mice. Ephrin-B2 has not been considered in the retinocollicular mapping previously and so I analyzed *ephrin-B2* reverse signaling mutants. The data presented in my dissertation (summarized in Figure 4.2): 1) demonstrates EphB2 receptor tyrosine kinase catalytic activity alone directs ventral RGC axon retinocollicular mapping 2) identifies a novel role for EphB1 forward signaling in ventral RGC axon retinocollicular mapping, 3) indicates EphB1/EphB2 forward signaling is the primary mediator of ventral RGC axon retinocollicular mapping, while EphB3 plays a supportive role, 3) identifies a ligand role for ephrin-B1 ligand in ventral RGC axon retinocollicular mapping, and 4) identifies a novel role for ephrin-B2 reverse signaling in both dorsal and ventral RGC axon retinocollicular mapping. While I have established the key regulators of dorsoventral RGC axon retinocollicular mapping are EphB and ephrin-B molecules, remaining questions linger.

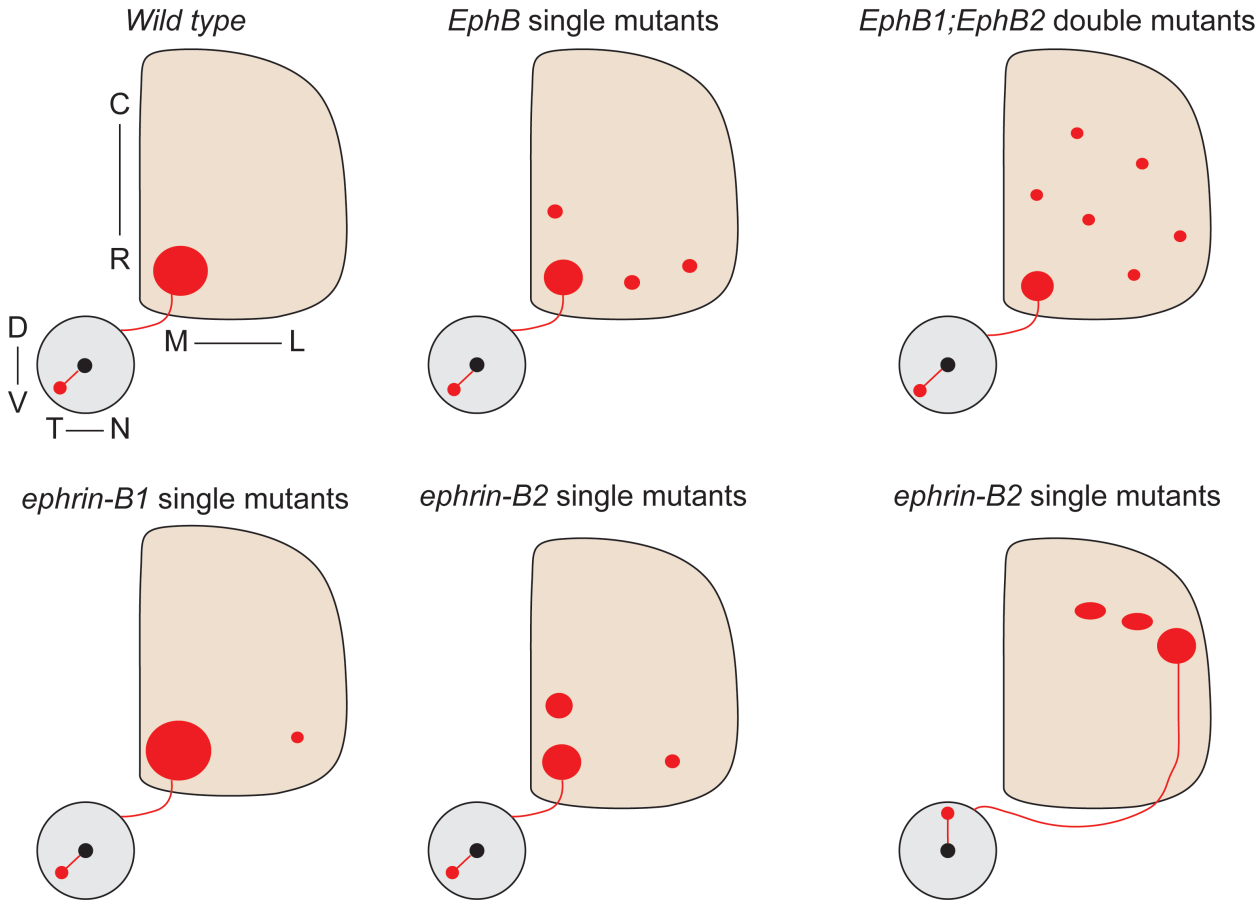


Figure 4.2 Summary of retinocollicular mapping defects in *EphB* and *ephrin-B* mutants

Wild type RGC axons form compact TZs in the SC by P8. Ventral-temporal RGC axons of *EphB* single mutants form 1-3 ectopic TZs lateral and sometimes caudal to the primary TZ. *EphB1;EphB2* compound mutants have a severe mapping defect in which ventral-temporal RGC axons form multiple ectopic TZs throughout the SC lateral, caudal, and caudal-lateral relative to the primary TZ. Ventral-temporal RGC axons of ephrin-B1 mutants form 1 ectopic TZ, while ephrin-B2 mutants generally formed multiple ectopic TZs that were caudal or lateral to the primary TZ. Dorsal RGC axons of ephrin-B2 mutants generally form 2 ectopic TZs medial to the primary TZ.

Dorsal (D), Ventral (V), Temporal (T), Nasal (N), Caudal (C), Rostral (R), Medial (M), Lateral (L)

What are the ligands for EphB and ephrin-B?

An immediate question is what ligand(s) are the RGC axons interacting with in the SC. The data presented here indicates ephrin-B1 expressed in the SC attracts ventral RGC axons highly expressing EphB receptors to terminate in the medial SC, although interestingly it is not the sole ligand because *ephrin-B1* deleted mutants do not disrupt retinocollicular mapping for all RGC axons. In addition, both the dorsal and ventral RGC axons of *ephrin-B2* reverse signaling mutants exhibited mapping defects signifying ephrin-B2 is interacting with an unidentified ligand in the SC. It has been recently theorized that a uniformly expressed attractant cue in the target region, such as the SC, serves to counterbalance gradient expressed repellent cues in the SC (Feldheim and O'Leary, 2010). I found EphB1 and EphB2 to be expressed uniformly in the SC indicating its potential role as a ligand. Because of the low expression of EphB receptors in the dorsal retina, an EphB ligand-like role would be less confounded by retinal expression if dorsal RGC axons were evaluated for defects. We found dorsal RGC axons of *EphB1* and *EphB2* single and compound mutants did not exhibit retinotopic mapping defects suggesting another ligand(s) is responsible. One interpretation is that EphB receptors and ephrin-B ligands mediate dorsoventral RGC axon retinocollicular mapping primarily by their receptor-like signaling with the exception of ephrin-B1 and the additional ligand(s) is not yet revealed. Another possibility is that the lower affinity interaction with ephrin-A5, which is also expressed in the SC, provides the necessary ligand for the RGC axons expressing EphB receptors and ephrin-B2 ligand. One method to indirectly test this theory would be to determine if *ephrin-A5*$^{-/-}$ mice have misdirected interstitial branching. Looking at high magnification images of the SC at P2-3 after a focal injection of DiI would allow analysis of interstitial branching patterns. While no published data on interstitial branching is available for these mutants, *ephrin-A2*$^{-/-}$;*ephrin-A5*$^{-/-}$ compound

mutants exhibit ectopic TZs that are not only along the rostrocaudal axis, but also medial and lateral to where the primary TZ should be. If *ephrin-A5*$^{-/-}$ SC exhibit interstitial branches that are misoriented along the mediolateral axis, then ephrin-A5 does mediate interstitial branching. The next step would be to determine if ephrin-A5 mediates interstitial branching through EphB interaction starting by analysis of *EphB2;ephrin-A5* compound mutants.

What other molecules regulate retinocollicular mapping?

If EphB:ephrin-B bidirectional signaling promotes interstitial branching extension towards the midline, what counterbalances its signaling in the mouse SC? In the chick and mouse, *Ryk* and *Wnt3* mRNA are expressed in matching gradient expression patterns as *EphB* and *ephrin-B*, respectively, while *Frizzled* is expressed uniformly in the retina (Schmitt et al., 2006). In the chick, Ryk:Wnt3 signaling was found to inhibit interstitial branch extension medially, which perhaps counterbalances the EphB:ephrin-B signaling mediated interstitial branching attraction towards the midline. Based on their expression pattern, Frizzled5:Wnt3 signaling is also proposed to promote attraction to the midline although Wnt3 has greater affinity for Ryk compared to Frizzled5. While the roles of Ryk, Wnt, and Frizzled in retinocollicular mapping have not been investigated in the mouse, *Ryk*, *Wnt* and *Frizzled* mutant mice have recently been generated to examine this question. It stands to reason that Ryk:Wnt would also counterbalance EphB:ephrin-B to mediate interstitial branch direction as it does in chicks.

What are the downstream targets of EphB:ephrin-B signaling?

The adhesion molecules L1 and ALCAM, transmembrane Ig superfamily molecule and its recognition molecule, also mediate interstitial branch direction towards the midline (Buhusi et

al., 2009; Buhusi et al., 2008). Mutant mice lacking L1 and ALCAM showed retinocollicular mapping defects along the mediolateral axis. Analysis of their interstitial branching indicated L1 and ALCAM, known for their role as adhesion molecules, likely promote adhesion/attraction of interstitial branches to the medial SC. It is unknown if L1 and ALCAM interaction operates downstream or independent of EphB:ephrin-B signaling although it has been suggested that EphB:ephrin-B signaling may initiate their association based on overlapping expression patterns and similarity of retinocollicular mapping defects (Buhusi et al., 2009). Perhaps demonstrating that EphB dependent tyrosine phosphorylation of L1 regulates L1/ALCAM association and cytoskeleton rearrangement would support the hypothesis of L1/ALCAM being downstream of EphB signaling.

The Rho family of GTPases are common downstream targets of Eph:ephrin signaling and are known for their role in cytoskeleton rearrangement. While Cdc42, Rac1, and RhoA regulate RGC axon pathfinding and growth cone collapse in the *Xenopus* (Ruchhoeft et al., 1999), their role in retinocollicular mapping has not been closely examined. The Vav family of GEFs are a likely regulator of retinocollicular mapping based on evidence for Vav mediating RGC axon guidance at the optic chiasm and endocytosis of the Eph:ephrin complex (Cowan et al., 2005). After Eph receptor autophosphorylation and activation, Vav2 is recruited to the juxatamembrane region by its SH2 domain. In $Vav2^{-/-}$;$Vav3^{-/-}$ mutants, the ipsilateral projections are reduced and also appears to be more dispersed within the LGN. In addition, $Vav2^{-/-}$;$Vav3^{-/-}$ RGCs in a dissociated cell culture fail to switch cell-cell adhesion mediated by Eph:ephrin interaction at the cell surface to cell-cell repulsion initiated by endocytosis of the Eph:ephrin complex. According to the *in vitro* results, Vav appears to mediate cell repulsion that may play a role in EphA expressing RGC axons being repulsed by the caudal SC cells expressing ephrin-A to mediate

RGC axon targeting along the rostrocaudal axis. On the other hand, EphB:ephrin-B signaling may regulate Vav to promote cell adhesion/attraction. The potential role of Vav in retinocollicular mapping could be examined by focal injection of DiI into the four quadrants of the *Vav* mutant retina to determine mapping defects along the rostrocaudal or mediolateral axis.

How do EphB and ephrin-B molecules direct interstitial branching?

RGC axons projecting along the rostrocaudal axis extend interstitial branches along the mediolateral axis of the SC and their direction is oriented towards their future TZ. As a result, RGC axons medial to the proper TZ extend their interstitial branches laterally and RGC axons lateral to the correct TZ extend their interstitial branches medially (Hindges et al., 2002; McLaughlin et al., 2003). *EphB2*$^{-/-}$;*EphB3*$^{-/-}$ and *EphB2*$^{lacZ/lacZ}$;*EphB3*$^{-/-}$ mutant mice exhibited RGC axons that extended interstitial branching only in the lateral direction, therefore indicating a loss of a signaling directing branch extension towards the midline (Hindges et al., 2002). We would predict that *EphB1*, *ephrin-B1*, and *ephrin-B2* mutants to have the same defect although the interstitial branching was not examined in these mutants. Looking at interstitial branching at P2 in these mutants could either confirm predictions or even define a novel role that would explain why some of the mutants form ectopic TZs along the rostrocaudal axis rather than only the mediolateral axis to the primary TZ.

How do EphB and ephrin-B molecules influence layer specification in the SC?

Guidance cues mediating RGC axon targeting to the chick inner plexius layer (IPL) have been studied, although far less is understood about what mediates RGC axon guidance to the correct layer of the OT/SC (Huberman et al., 2010; Yamagata and Sanes, 2008; Yamagata et al.,

2002). While *EphA* and *ephrin-A* null mutant mice do not seem to exhibit defects in layer specification, *EphB* and *ephrin-B* mutants may demonstrate a novel role for EphB:ephrin-B signaling in this process. EphB1 and EphB2 are expressed not only in the dorsal three superficial layers of the SC, but also within the intermediate and deep layers of the SC (Figure 3.1). While the superficial layer primarily receives input from the retina, the intermediate and deeper layers receive input from motor and sensory neurons. Disruption of layer specification in the SC would interrupt information integration of the visual, motor, and sensory systems in the SC. The influence EphB:ephrin-B have on layer specification could be determined by focal DiI injection into *EphB* and *ephrin-B* mutant retina and then sectioning through the SC to determine if RGC axons incorrectly pathfind into the deeper layers of the SC.

What is the functional significance of the retinocollicular mapping defects observed in EphB:ephrin-B bidirectional signaling mutants?

Retinocollicular maps are refined by spontaneous retinal activity before eye opening and the critical period after eye opening by eliminating aberrant axons and arborizations (Bansal et al., 2000; Demas et al., 2003). While *EphB* and *ephrin-B* mutants form ectopic TZs in the postnatal SC, how these misprojections influence the visual experience is unknown. One method that has been developed to examine this question is Fourier imaging of intrinsic signals. This method creates a functional map by correlating a point in the visual field to a region in the SC (Cang et al., 2008; Kalatsky and Stryker, 2003). The SC of mice under anesthesia are imaged as their visual field is stimulated. *Ephrin-A2*$^{-/-}$*;ephrin-A3*$^{-/-}$*;ephrin-A5*$^{-/-}$ mutant mice stimulated relative to the nasotemporal, though not dorsoventral, axis showed discontinuous maps with patches that correspond to ectopic TZs (Cang et al., 2008). These data demonstrate the adult SC

maintains the mapping defects observed during postnatal development. Fourier imaging could also show if mutations in *EphB* and *ephrin-B* result in sustained mapping errors that perturb the adult visual experience. If the retinocollicular mapping errors exhibited in postnatal *EphB* and *ephrin-B* mutants (Thakar et al., submitted) are sustained into adult, then Fourier imaging would also show discontinuous and patchy maps along the dorsoventral axis. As hypothesized in my dissertation, EphB and ephrin-B may interact with EphA and ephrin-A in the SC and as a result also disrupt the map of the nasotemporal axis. Fourier imaging would not only confirm results described in my dissertation, but also emphasize the relevance these molecules have in visual experience.

Can EphB and ephrin-B molecules promote axon growth and innervations in adults after injury?

Frogs and fish are able to regenerate their central nervous system (CNS), which initially made them ideal systems to study topography in cell ablation and severed nerve experiments. Although the rodent and human peripheral nervous system (PNS) as well as the developing CNS can regenerate, the complicated neuronal circuitry of adult rodents and humans are unable to regenerate their neurons or CNS axons once injured. As we expand our understanding of the roles of Eph and ephrin molecules in axon guidance, perhaps we will gain greater insight into how these molecules regulate neuronal regeneration and reinnervation into their appropriate target region.

Certain Ephs and ephrins are up regulated after CNS injury in adults and have a role in axon sprouting, cellular remodeling, and scar formation. For example, *EphB3* mRNA expression increases two days after optic nerve injury in macrophages that are recruited in response to the

injury while *EphB1* and *EphB2* were not present before or after injury (Liu et al., 2006). *Ephrin-B3* mRNA expression in RGCs and ephrin-B3 protein expression in the optic nerve is present before injury and is increased after injury while *ephrin-B1* and *ephrin-B2* are not expressed before or after injury. In addition, *EphB3* null mice exhibited decreased axon re-extension compared to injured wild type mice and *in vitro* experiments demonstrated RGC axons turning towards EphB3-Fc indicating a potential role in promoting axon extension. These data support the model that macrophages expressing EphB3 promote RGC axon extension via interaction with ephrin-B3 expressed by the RGC axons.

To recover from an optic nerve lesion, RGC axons must reproject through the optic tract and reestablish topographic mapping in the SC. Since Ephs and ephrins are primarily responsible for retinocollicular mapping, they would need to be expressed in the adult SC although mRNA levels of EphA and ephrin-A molecules are downregulated or not present in the retina and SC compared to neonates (Knoll et al., 2001). Perhaps establishing their expression patterns to embryonic/neonatal levels in the retina and SC would promote axon reinnervation into correct topographic locations. Further analysis is necessary before we are able to employ Ephs and ephrins as therapeutic agents, though the potential exists.

The immediate question to answer is if RGC axons in adult *ephrin-B3*$^{-/-}$ mutants fail to recover from an acute injury such as optic nerve crush. Initially after optic nerve crush in *wild type* mice, RGC axon projections at the optic nerve head are reduced. Then after day four, there is an increase in RGC axon projection through the optic nerve head (Liu et al., 2006). The delayed response is likely attributed to the arrival of EphB3 macrophages that encourage ephrin-B3 expressing RGC axons to extend their axons. *Ephrin-B3*$^{-/-}$ mutants exhibiting no change in neuronal plasticity after 4 days would show direct evidence for ephrin-B3 having an important

role in recovering from neuronal injury. Increased neuronal plasticity does not ensure correct innervation. Expression analysis of topographic guidance molecules in the adult brain indicates the change in expression patterns compared to the developing brain. Perhaps ectopic expression of guidance molecules like Eph and ephrin could promote guidance to correct targets, although controlling the level of ectopic expression is difficult and there is usually overexpression of the molecule of interest. In addition, recreating the entire molecular environment of the developing brain would be nearly impossible. Therefore, initial research should go into determining the molecules that inhibit axon extension. Perhaps turning off these signaling molecules during the period axons are recovering from injury would promote axon extension, although it would still not ensure targeting into appropriate regions. For example, deletion of myelin inhibitors such as Nogo, MAG, and OMgp, increases axonal sprouting, but do not improve innervation to appropriate targets and physical recovery from spinal cord injury in mice (Cafferty et al., 2010; Lee et al., 2010). Perhaps examining *Eph* and *ephrin* mutants in the context of optic nerve injury or spinal cord injury could provide a tool for understanding how guidance molecules inhibit or promote axon regeneration.

METHODS

Mice

Generation of CD1 background EphB and ephrin-B mutant mice and genotyping by PCR have been described previously.

EphB1^{-} (Williams et al., 2003), ***EphB1***^{lacZ} (Williams et al., 2003), ***EphB1***$^{T\text{-}lacZ}$ (Chenaux and Henkemeyer, unpublished), ***EphB2***$^{-}$ (Henkemeyer et al., 1996), ***EphB2***^{lacZ} (Henkemeyer et al., 1996), ***EphB2***$^{\Delta PDZ}$ (Genander et al., 2009), ***EphB2***^{K661R} (Genander et al., 2009), **EphB2**$^{K661R\Delta PDZ}$ (Genander et al., 2009), ***EphB3***$^{-}$ (Orioli et al., 1996), ***ephrin-B1***$^{-/y}$ (Davy et al., 2004), ***ephrin-B2***^{lacZ} (Dravis et al., 2004), ***ephrin-B2***$^{6YF\Delta V}$ (Thakar et al., submitted).

Histology and X-gal staining

Embryos and postnatal pups taken at E17.5, P1, and P8 were anesthetized, decapitated, washed in phosphate buffer, embedded in OCT and frozen in a slurry of dry ice and ethanol. Blocks were stored at -80 °C until cryosectioned at 14 μm onto Superfrost Plus (Fisher) slides. Sections were fixed in lacZ fixation solution (0.2% gluteraldehyde, 5mM EGTA pH 7.3, 2mM $MgCl_2$ in 0.1M phosphate buffer) for 10 minutes followed by three 5 minutes washes in lacZ wash buffer (8mM $MgCl_2$, 0.08% Nonidet-P40 in 0.1 phosphate buffer). Sections were then stained in X-gal stain (2mL of 25 mg/mL X-gal, 0.106 g potassium ferrocyanide, 0.082 g potassium ferricyanide in 50 mL wash buffer) overnight at 37°C. The next day, slides were washed twice for 5 minutes in lacZ wash buffer and fixed in 4% formalin for 5 minutes. After one wash for 5 minutes with PBS and then water, slides were immersed in Nuclear Fast Red for 45 seconds to label cell bodies.

Slides were then washed for 10 minutes followed by ethanol dehydration, xylene clearing and slide cover mounting.

DiI labeling of RGC axon guidance to the optic nerve head

Mouse embryos were isolated at E17.5 and fixed in 4% PFA overnight. The next day, heads were bisected, lens of the eye removed, and a DiI crystal (Molecular Probes) was embedded in the dorsal region of the retina overnight at 37°C. The next day, retinas were flat mounted and imaged using epifluorescence microscopy.

DiI labeling of RGC axon guidance to the SC and analysis

Mice were anesthetized by hypothermia and DiI (Molecular Probes) solution (DiI crystals dissolved 5% in dimethylformamide) was injected into the dorsal or ventral peripheral retina at P6 as described previously (Buhusi et al., 2009; Hindges et al., 2002; Simon and O'Leary, 1992a). P8 pups were sacrificed, brains isolated, cortex removed, and contralateral SC whole mounts were analyzed blind to genotype. Retinas from pups with DiI labeled TZs in the SC were flatmounted to assess the injection site. Injection site was evaluated for position in relation to the 4 major eye muscles and containment of the DiI to the focal dorsal or ventral region desired.

Flatmounted retinas and whole mount SC (boundaries determined by characteristic shape and location) were visualized using epifluorescence microscopy, CCD camera, and MetaVue software. DiI labeling of the optic tract path to the SC was observed at 2x and then the SC was visualized at 4x to evaluate the termination zone(s) labeled.

The primary TZ was defined as the larger TZ formed in the expected region corresponding to the focal injection in the retina. Ectopic TZs were defined as DiI labeled axons that were not localized in the expected position of the primary TZ.

All results were analyzed using the Fisher's exact two-tail test comparing the mutant to the wild type group. Data was reported as distinct levels of significance (* $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$).

REFERENCES

Abdul-Aziz, N.M., Turmaine, M., Greene, N.D., and Copp, A.J. (2009). EphrinA-EphA receptor interactions in mouse spinal neurulation: implications for neural fold fusion. Int J Dev Biol *53*, 559-568.

Adams, R.H., Wilkinson, G.A., Weiss, C., Diella, F., Gale, N.W., Deutsch, U., Risau, W., and Klein, R. (1999). Roles of ephrinB ligands and EphB receptors in cardiovascular development: demarcation of arterial/venous domains, vascular morphogenesis, and sprouting angiogenesis. Genes Dev *13*, 295-306.

Attardi, D.G., and Sperry, R.W. (1963). Preferential selection of central pathways by regenerating optic fibers. Exp Neurol *7*, 46-64.

Bansal, A., Singer, J.H., Hwang, B.J., Xu, W., Beaudet, A., and Feller, M.B. (2000). Mice lacking specific nicotinic acetylcholine receptor subunits exhibit dramatically altered spontaneous activity patterns and reveal a limited role for retinal waves in forming ON and OFF circuits in the inner retina. J Neurosci *20*, 7672-7681.

Bartley, T.D., Hunt, R.W., Welcher, A.A., Boyle, W.J., Parker, V.P., Lindberg, R.A., Lu, H.S., Colombero, A.M., Elliott, R.L., Guthrie, B.A.*, et al.* (1994). B61 is a ligand for the ECK receptor protein-tyrosine kinase. Nature *368*, 558-560.

Beckmann, M.P., Cerretti, D.P., Baum, P., Vanden Bos, T., James, L., Farrah, T., Kozlosky, C., Hollingsworth, T., Shilling, H., Maraskovsky, E.*, et al.* (1994). Molecular characterization of a family of ligands for eph-related tyrosine kinase receptors. EMBO J *13*, 3757-3762.

Bennett, B.D., Zeigler, F.C., Gu, Q., Fendly, B., Goddard, A.D., Gillett, N., and Matthews, W. (1995). Molecular cloning of a ligand for the EPH-related receptor protein-tyrosine kinase Htk. Proc Natl Acad Sci U S A *92*, 1866-1870.

Bergemann, A.D., Cheng, H.J., Brambilla, R., Klein, R., and Flanagan, J.G. (1995). ELF-2, a new member of the Eph ligand family, is segmentally expressed in mouse embryos in the region of the hindbrain and newly forming somites. Mol Cell Biol *15*, 4921-4929.

Binns, K.L., Taylor, P.P., Sicheri, F., Pawson, T., and Holland, S.J. (2000). Phosphorylation of tyrosine residues in the kinase domain and juxtamembrane region regulates the biological and catalytic activities of Eph receptors. Mol Cell Biol *20*, 4791-4805.

Birgbauer, E., Cowan, C.A., Sretavan, D.W., and Henkemeyer, M. (2000). Kinase independent function of EphB receptors in retinal axon pathfinding to the optic disc from dorsal but not ventral retina. Development *127*, 1231-1241.

Birgbauer, E., Oster, S.F., Severin, C.G., and Sretavan, D.W. (2001). Retinal axon growth cones respond to EphB extracellular domains as inhibitory axon guidance cues. Development *128*, 3041-3048.

Braisted, J.E., McLaughlin, T., Wang, H.U., Friedman, G.C., Anderson, D.J., and O'Leary D, D. (1997). Graded and lamina-specific distributions of ligands of EphB receptor tyrosine kinases in the developing retinotectal system. Dev Biol *191*, 14-28.

Brittis, P.A., Canning, D.R., and Silver, J. (1992). Chondroitin sulfate as a regulator of neuronal patterning in the retina. Science *255*, 733-736.

Brittis, P.A., Lemmon, V., Rutishauser, U., and Silver, J. (1995). Unique changes of ganglion cell growth cone behavior following cell adhesion molecule perturbations: a time-lapse study of the living retina. Mol Cell Neurosci *6*, 433-449.

Brown, A., Yates, P.A., Burrola, P., Ortuno, D., Vaidya, A., Jessell, T.M., Pfaff, S.L., O'Leary, D.D., and Lemke, G. (2000). Topographic mapping from the retina to the midbrain is controlled by relative but not absolute levels of EphA receptor signaling. Cell *102*, 77-88.

Bruckner, K., Pablo Labrador, J., Scheiffele, P., Herb, A., Seeburg, P.H., and Klein, R. (1999). EphrinB ligands recruit GRIP family PDZ adaptor proteins into raft membrane microdomains. Neuron *22*, 511-524.

Bruckner, K., Pasquale, E.B., and Klein, R. (1997). Tyrosine phosphorylation of transmembrane ligands for Eph receptors. Science *275*, 1640-1643.

Buhusi, M., Demyanenko, G.P., Jannie, K.M., Dalal, J., Darnell, E.P., Weiner, J.A., and Maness, P.F. (2009). ALCAM regulates mediolateral retinotopic mapping in the superior colliculus. J Neurosci *29*, 15630-15641.

Buhusi, M., Schlatter, M.C., Demyanenko, G.P., Thresher, R., and Maness, P.F. (2008). L1 interaction with ankyrin regulates mediolateral topography in the retinocollicular projection. J Neurosci *28*, 177-188.

Cafferty, W.B., Duffy, P., Huebner, E., and Strittmatter, S.M. (2010). MAG and OMgp synergize with Nogo-A to restrict axonal growth and neurological recovery after spinal cord trauma. J Neurosci *30*, 6825-6837.

Cang, J., Wang, L., Stryker, M.P., and Feldheim, D.A. (2008). Roles of ephrin-as and structured activity in the development of functional maps in the superior colliculus. J Neurosci *28*, 11015-11023.

Cerretti, D.P., Vanden Bos, T., Nelson, N., Kozlosky, C.J., Reddy, P., Maraskovsky, E., Park, L.S., Lyman, S.D., Copeland, N.G., Gilbert, D.J.*, et al.* (1995). Isolation of LERK-5: a ligand of the eph-related receptor tyrosine kinases. Mol Immunol *32*, 1197-1205.

Chan, J., and Watt, V.M. (1991). eek and erk, new members of the eph subclass of receptor protein-tyrosine kinases. Oncogene *6*, 1057-1061.

Chenaux, G., and Henkemeyer, M. (unpublished).

Cheng, H.J., and Flanagan, J.G. (1994). Identification and cloning of ELF-1, a developmentally expressed ligand for the Mek4 and Sek receptor tyrosine kinases. Cell *79*, 157-168.

Cheng, H.J., Nakamoto, M., Bergemann, A.D., and Flanagan, J.G. (1995). Complementary gradients in expression and binding of ELF-1 and Mek4 in development of the topographic retinotectal projection map. Cell *82*, 371-381.

Chrencik, J.E., Brooun, A., Kraus, M.L., Recht, M.I., Kolatkar, A.R., Han, G.W., Seifert, J.M., Widmer, H., Auer, M., and Kuhn, P. (2006). Structural and biophysical characterization of the EphB4*ephrinB2 protein-protein interaction and receptor specificity. J Biol Chem *281*, 28185-28192.

Coonan, J.R., Bartlett, P.F., and Galea, M.P. (2003). Role of EphA4 in defining the position of a motoneuron pool within the spinal cord. J Comp Neurol *458*, 98-111.

Cowan, C.A., and Henkemeyer, M. (2001). The SH2/SH3 adaptor Grb4 transduces B-ephrin reverse signals. Nature *413*, 174-179.

Cowan, C.A., and Henkemeyer, M. (2002). Ephrins in reverse, park and drive. Trends Cell Biol *12*, 339-346.

Cowan, C.A., Yokoyama, N., Bianchi, L.M., Henkemeyer, M., and Fritzsch, B. (2000). EphB2 guides axons at the midline and is necessary for normal vestibular function. Neuron *26*, 417-430.

Cowan, C.A., Yokoyama, N., Saxena, A., Chumley, M.J., Silvany, R.E., Baker, L.A., Srivastava, D., and Henkemeyer, M. (2004). Ephrin-B2 reverse signaling is required for axon pathfinding and cardiac valve formation but not early vascular development. Dev Biol *271*, 263-271.

Cowan, C.W., Shao, Y.R., Sahin, M., Shamah, S.M., Lin, M.Z., Greer, P.L., Gao, S., Griffith, E.C., Brugge, J.S., and Greenberg, M.E. (2005). Vav family GEFs link activated Ephs to endocytosis and axon guidance. Neuron *46*, 205-217.

Dail, M., Richter, M., Godement, P., and Pasquale, E.B. (2006). Eph receptors inactivate R-Ras through different mechanisms to achieve cell repulsion. J Cell Sci *119*, 1244-1254.

Davis, S., Gale, N.W., Aldrich, T.H., Maisonpierre, P.C., Lhotak, V., Pawson, T., Goldfarb, M., and Yancopoulos, G.D. (1994). Ligands for EPH-related receptor tyrosine kinases that require membrane attachment or clustering for activity. Science *266*, 816-819.

Davy, A., Aubin, J., and Soriano, P. (2004). Ephrin-B1 forward and reverse signaling are required during mouse development. Genes Dev *18*, 572-583.

Deiner, M.S., Kennedy, T.E., Fazeli, A., Serafini, T., Tessier-Lavigne, M., and Sretavan, D.W. (1997). Netrin-1 and DCC mediate axon guidance locally at the optic disc: loss of function leads to optic nerve hypoplasia. Neuron *19*, 575-589.

Demas, J., Eglen, S.J., and Wong, R.O. (2003). Developmental loss of synchronous spontaneous activity in the mouse retina is independent of visual experience. J Neurosci *23*, 2851-2860.

Dottori, M., Hartley, L., Galea, M., Paxinos, G., Polizzotto, M., Kilpatrick, T., Bartlett, P.F., Murphy, M., Kontgen, F., and Boyd, A.W. (1998). EphA4 (Sek1) receptor tyrosine kinase is required for the development of the corticospinal tract. Proc Natl Acad Sci U S A *95*, 13248-13253.

Dravis, C., Yokoyama, N., Chumley, M.J., Cowan, C.A., Silvany, R.E., Shay, J., Baker, L.A., and Henkemeyer, M. (2004). Bidirectional signaling mediated by ephrin-B2 and EphB2 controls urorectal development. Dev Biol *271*, 272-290.

Drescher, U., Kremoser, C., Handwerker, C., Loschinger, J., Noda, M., and Bonhoeffer, F. (1995). In vitro guidance of retinal ganglion cell axons by RAGS, a 25 kDa tectal protein related to ligands for Eph receptor tyrosine kinases. Cell *82*, 359-370.

Easter, S.S., Jr., Burrill, J., Marcus, R.C., Ross, L.S., Taylor, J.S., and Wilson, S.W. (1994). Initial tract formation in the vertebrate brain. Prog Brain Res *102*, 79-93.

Feldheim, D.A., Kim, Y.I., Bergemann, A.D., Frisen, J., Barbacid, M., and Flanagan, J.G. (2000). Genetic analysis of ephrin-A2 and ephrin-A5 shows their requirement in multiple aspects of retinocollicular mapping. Neuron *25*, 563-574.

Feldheim, D.A., Nakamoto, M., Osterfield, M., Gale, N.W., DeChiara, T.M., Rohatgi, R., Yancopoulos, G.D., and Flanagan, J.G. (2004). Loss-of-function analysis of EphA receptors in retinotectal mapping. J Neurosci *24*, 2542-2550.

Feldheim, D.A., and O'Leary, D.D. (2010). Visual Map Development: Bidirectional Signaling, Bifunctional Guidance Molecules, and Competition. Cold Spring Harb Perspect Biol.

Feng, G., Mellor, R.H., Bernstein, M., Keller-Peck, C., Nguyen, Q.T., Wallace, M., Nerbonne, J.M., Lichtman, J.W., and Sanes, J.R. (2000). Imaging neuronal subsets in transgenic mice expressing multiple spectral variants of GFP. Neuron *28*, 41-51.

Frisen, J., Yates, P.A., McLaughlin, T., Friedman, G.C., O'Leary, D.D., and Barbacid, M. (1998). Ephrin-A5 (AL-1/RAGS) is essential for proper retinal axon guidance and topographic mapping in the mammalian visual system. Neuron *20*, 235-243.

Fujisawa, H. (1987). Axonal growth in developing and regenerating amphibian retinotectal projection. Prog Brain Res *71*, 75-88.

Gale, N.W., Flenniken, A., Compton, D.C., Jenkins, N., Copeland, N.G., Gilbert, D.J., Davis, S., Wilkinson, D.G., and Yancopoulos, G.D. (1996). Elk-L3, a novel transmembrane ligand for the

Eph family of receptor tyrosine kinases, expressed in embryonic floor plate, roof plate and hindbrain segments. Oncogene *13*, 1343-1352.

Genander, M., Halford, M.M., Xu, N.J., Eriksson, M., Yu, Z., Qiu, Z., Martling, A., Greicius, G., Thakar, S., Catchpole, T.*, et al.* (2009). Dissociation of EphB2 signaling pathways mediating progenitor cell proliferation and tumor suppression. Cell *139*, 679-692.

Gerety, S.S., Wang, H.U., Chen, Z.F., and Anderson, D.J. (1999). Symmetrical mutant phenotypes of the receptor EphB4 and its specific transmembrane ligand ephrin-B2 in cardiovascular development. Mol Cell *4*, 403-414.

Gilardi-Hebenstreit, P., Nieto, M.A., Frain, M., Mattei, M.G., Chestier, A., Wilkinson, D.G., and Charnay, P. (1992). An Eph-related receptor protein tyrosine kinase gene segmentally expressed in the developing mouse hindbrain. Oncogene *7*, 2499-2506.

Harris, W.A., Holt, C.E., and Bonhoeffer, F. (1987). Retinal axons with and without their somata, growing to and arborizing in the tectum of Xenopus embryos: a time-lapse video study of single fibres in vivo. Development *101*, 123-133.

Hatta, K., and Takeichi, M. (1986). Expression of N-cadherin adhesion molecules associated with early morphogenetic events in chick development. Nature *320*, 447-449.

Hattori, M., Osterfield, M., and Flanagan, J.G. (2000). Regulated cleavage of a contact-mediated axon repellent. Science *289*, 1360-1365.

Helmbacher, F., Schneider-Maunoury, S., Topilko, P., Tiret, L., and Charnay, P. (2000). Targeting of the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb motor axons. Development *127*, 3313-3324.

Henkemeyer, M., Marengere, L.E., McGlade, J., Olivier, J.P., Conlon, R.A., Holmyard, D.P., Letwin, K., and Pawson, T. (1994). Immunolocalization of the Nuk receptor tyrosine kinase suggests roles in segmental patterning of the brain and axonogenesis. Oncogene *9*, 1001-1014.

Henkemeyer, M., Orioli, D., Henderson, J.T., Saxton, T.M., Roder, J., Pawson, T., and Klein, R. (1996). Nuk controls pathfinding of commissural axons in the mammalian central nervous system. Cell *86*, 35-46.

Henkemeyer, M., Rossi, D.J., Holmyard, D.P., Puri, M.C., Mbamalu, G., Harpal, K., Shih, T.S., Jacks, T., and Pawson, T. (1995). Vascular system defects and neuronal apoptosis in mice lacking ras GTPase-activating protein. Nature *377*, 695-701.

Herbert, S.P., Huisken, J., Kim, T.N., Feldman, M.E., Houseman, B.T., Wang, R.A., Shokat, K.M., and Stainier, D.Y. (2009). Arterial-venous segregation by selective cell sprouting: an alternative mode of blood vessel formation. Science *326*, 294-298.

Himanen, J.P., Chumley, M.J., Lackmann, M., Li, C., Barton, W.A., Jeffrey, P.D., Vearing, C., Geleick, D., Feldheim, D.A., Boyd, A.W.*, et al.* (2004). Repelling class discrimination: ephrin-A5 binds to and activates EphB2 receptor signaling. Nat Neurosci *7*, 501-509.

Himanen, J.P., and Nikolov, D.B. (2003). Eph receptors and ephrins. Int J Biochem Cell Biol *35*, 130-134.

Himanen, J.P., Rajashankar, K.R., Lackmann, M., Cowan, C.A., Henkemeyer, M., and Nikolov, D.B. (2001). Crystal structure of an Eph receptor-ephrin complex. Nature *414*, 933-938.

Himanen, J.P., Saha, N., and Nikolov, D.B. (2007). Cell-cell signaling via Eph receptors and ephrins. Curr Opin Cell Biol *19*, 534-542.

Hindges, R., McLaughlin, T., Genoud, N., Henkemeyer, M., and O'Leary, D.D. (2002). EphB forward signaling controls directional branch extension and arborization required for dorsal-ventral retinotopic mapping. Neuron *35*, 475-487.

Hirai, H., Maru, Y., Hagiwara, K., Nishida, J., and Takaku, F. (1987). A novel putative tyrosine kinase receptor encoded by the eph gene. Science *238*, 1717-1720.

Ho, S.K., Kovacevic, N., Henkelman, R.M., Boyd, A., Pawson, T., and Henderson, J.T. (2009). EphB2 and EphA4 receptors regulate formation of the principal inter-hemispheric tracts of the mammalian forebrain. Neuroscience *160*, 784-795.

Hock, B., Bohme, B., Karn, T., Yamamoto, T., Kaibuchi, K., Holtrich, U., Holland, S., Pawson, T., Rubsamen-Waigmann, H., and Strebhardt, K. (1998). PDZ-domain-mediated interaction of the Eph-related receptor tyrosine kinase EphB3 and the ras-binding protein AF6 depends on the kinase activity of the receptor. Proc Natl Acad Sci U S A *95*, 9779-9784.

Holland, S.J., Gale, N.W., Gish, G.D., Roth, R.A., Songyang, Z., Cantley, L.C., Henkemeyer, M., Yancopoulos, G.D., and Pawson, T. (1997). Juxtamembrane tyrosine residues couple the Eph family receptor EphB2/Nuk to specific SH2 domain proteins in neuronal cells. EMBO J *16*, 3877-3888.

Holland, S.J., Gale, N.W., Mbamalu, G., Yancopoulos, G.D., Henkemeyer, M., and Pawson, T. (1996). Bidirectional signalling through the EPH-family receptor Nuk and its transmembrane ligands. Nature *383*, 722-725.

Holmberg, J., Genander, M., Halford, M.M., Anneren, C., Sondell, M., Chumley, M.J., Silvany, R.E., Henkemeyer, M., and Frisen, J. (2006). EphB receptors coordinate migration and proliferation in the intestinal stem cell niche. Cell *125*, 1151-1163.

Hubel, D.H., and Wiesel, T.N. (1963). Receptive Fields of Cells in Striate Cortex of Very Young, Visually Inexperienced Kittens. J Neurophysiol *26*, 994-1002.

Huberman, A.D., Clandinin, T.R., and Baier, H. (2010). Molecular and cellular mechanisms of lamina-specific axon targeting. Cold Spring Harb Perspect Biol *2*, a001743.

Huberman, A.D., Feller, M.B., and Chapman, B. (2008). Mechanisms underlying development of visual maps and receptive fields. Annu Rev Neurosci *31*, 479-509.

Huse, M., and Kuriyan, J. (2002). The conformational plasticity of protein kinases. Cell *109*, 275-282.

Irie, F., and Yamaguchi, Y. (2002). EphB receptors regulate dendritic spine development via intersectin, Cdc42 and N-WASP. Nat Neurosci *5*, 1117-1118.

Iwamasa, H., Ohta, K., Yamada, T., Ushijima, K., Terasaki, H., and Tanaka, H. (1999). Expression of Eph receptor tyrosine kinases and their ligands in chick embryonic motor neurons and hindlimb muscles. Dev Growth Differ *41*, 685-698.

Janes, P.W., Saha, N., Barton, W.A., Kolev, M.V., Wimmer-Kleikamp, S.H., Nievergall, E., Blobel, C.P., Himanen, J.P., Lackmann, M., and Nikolov, D.B. (2005). Adam meets Eph: an ADAM substrate recognition module acts as a molecular switch for ephrin cleavage in trans. Cell *123*, 291-304.

Kaethner, R.J., and Stuermer, C.A. (1992). Dynamics of terminal arbor formation and target approach of retinotectal axons in living zebrafish embryos: a time-lapse study of single axons. J Neurosci *12*, 3257-3271.

Kalatsky, V.A., and Stryker, M.P. (2003). New paradigm for optical imaging: temporally encoded maps of intrinsic signal. Neuron *38*, 529-545.

Kalo, M.S., Yu, H.H., and Pasquale, E.B. (2001). In vivo tyrosine phosphorylation sites of activated ephrin-B1 and ephB2 from neural tissue. J Biol Chem *276*, 38940-38948.

Knoll, B., Isenmann, S., Kilic, E., Walkenhorst, J., Engel, S., Wehinger, J., Bahr, M., and Drescher, U. (2001). Graded expression patterns of ephrin-As in the superior colliculus after lesion of the adult mouse optic nerve. Mech Dev *106*, 119-127.

Kozlosky, C.J., Maraskovsky, E., McGrew, J.T., VandenBos, T., Teepe, M., Lyman, S.D., Srinivasan, S., Fletcher, F.A., Gayle, R.B., 3rd, Cerretti, D.P.*, et al.* (1995). Ligands for the receptor tyrosine kinases hek and elk: isolation of cDNAs encoding a family of proteins. Oncogene *10*, 299-306.

Kramer, E.R., Knott, L., Su, F., Dessaud, E., Krull, C.E., Helmbacher, F., and Klein, R. (2006). Cooperation between GDNF/Ret and ephrinA/EphA4 signals for motor-axon pathway selection in the limb. Neuron *50*, 35-47.

Kullander, K., Croll, S.D., Zimmer, M., Pan, L., McClain, J., Hughes, V., Zabski, S., DeChiara, T.M., Klein, R., Yancopoulos, G.D.*, et al.* (2001a). Ephrin-B3 is the midline barrier that prevents corticospinal tract axons from recrossing, allowing for unilateral motor control. Genes Dev *15*, 877-888.

Kullander, K., Mather, N.K., Diella, F., Dottori, M., Boyd, A.W., and Klein, R. (2001b). Kinase-dependent and kinase-independent functions of EphA4 receptors in major axon tract formation in vivo. Neuron *29*, 73-84.

Lai, C., and Lemke, G. (1991). An extended family of protein-tyrosine kinase genes differentially expressed in the vertebrate nervous system. Neuron *6*, 691-704.

Lee, J.K., Geoffroy, C.G., Chan, A.F., Tolentino, K.E., Crawford, M.J., Leal, M.A., Kang, B., and Zheng, B. (2010). Assessing spinal axon regeneration and sprouting in Nogo-, MAG-, and OMgp-deficient mice. Neuron *66*, 663-670.

Leppert, C.A., Diekmann, H., Paul, C., Laessing, U., Marx, M., Bastmeyer, M., and Stuermer, C.A. (1999). Neurolin Ig domain 2 participates in retinal axon guidance and Ig domains 1 and 3 in fasciculation. J Cell Biol *144*, 339-349.

Letwin, K., Yee, S.P., and Pawson, T. (1988). Novel protein-tyrosine kinase cDNAs related to fps/fes and eph cloned using anti-phosphotyrosine antibody. Oncogene *3*, 621-627.

Lindberg, R.A., and Hunter, T. (1990). cDNA cloning and characterization of eck, an epithelial cell receptor protein-tyrosine kinase in the eph/elk family of protein kinases. Mol Cell Biol *10*, 6316-6324.

Liu, X., Hawkes, E., Ishimaru, T., Tran, T., and Sretavan, D.W. (2006). EphB3: an endogenous mediator of adult axonal plasticity and regrowth after CNS injury. J Neurosci *26*, 3087-3101.

Lu, Q., Sun, E.E., Klein, R.S., and Flanagan, J.G. (2001). Ephrin-B reverse signaling is mediated by a novel PDZ-RGS protein and selectively inhibits G protein-coupled chemoattraction. Cell *105*, 69-79.

Luo, L., and Flanagan, J.G. (2007). Development of continuous and discrete neural maps. Neuron *56*, 284-300.

Luria, V., Krawchuk, D., Jessell, T.M., Laufer, E., and Kania, A. (2008). Specification of motor axon trajectory by ephrin-B:EphB signaling: symmetrical control of axonal patterning in the developing limb. Neuron *60*, 1039-1053.

Makinen, T., Adams, R.H., Bailey, J., Lu, Q., Ziemiecki, A., Alitalo, K., Klein, R., and Wilkinson, G.A. (2005). PDZ interaction site in ephrinB2 is required for the remodeling of lymphatic vasculature. Genes Dev *19*, 397-410.

Mann, F., Ray, S., Harris, W., and Holt, C. (2002). Topographic mapping in dorsoventral axis of the Xenopus retinotectal system depends on signaling through ephrin-B ligands. Neuron *35*, 461-473.

Marston, D.J., Dickinson, S., and Nobes, C.D. (2003). Rac-dependent trans-endocytosis of ephrinBs regulates Eph-ephrin contact repulsion. Nat Cell Biol *5*, 879-888.

Matsunaga, M., Hatta, K., and Takeichi, M. (1988). Role of N-cadherin cell adhesion molecules in the histogenesis of neural retina. Neuron *1*, 289-295.

McLaughlin, T., Hindges, R., Yates, P.A., and O'Leary, D.D. (2003). Bifunctional action of ephrin-B1 as a repellent and attractant to control bidirectional branch extension in dorsal-ventral retinotopic mapping. Development *130*, 2407-2418.

McLaughlin, T., and O'Leary, D.D. (2005). Molecular gradients and development of retinotopic maps. Annu Rev Neurosci *28*, 327-355.

Monschau, B., Kremoser, C., Ohta, K., Tanaka, H., Kaneko, T., Yamada, T., Handwerker, C., Hornberger, M.R., Loschinger, J., Pasquale, E.B.*, et al.* (1997). Shared and distinct functions of RAGS and ELF-1 in guiding retinal axons. EMBO J *16*, 1258-1267.

Nakamoto, M., Cheng, H.J., Friedman, G.C., McLaughlin, T., Hansen, M.J., Yoon, C.H., O'Leary, D.D., and Flanagan, J.G. (1996). Topographically specific effects of ELF-1 on retinal axon guidance in vitro and retinal axon mapping in vivo. Cell *86*, 755-766.

Nakamura, H., and O'Leary, D.D. (1989). Inaccuracies in initial growth and arborization of chick retinotectal axons followed by course corrections and axon remodeling to develop topographic order. J Neurosci *9*, 3776-3795.

Nicola, N.A., Viney, E., Hilton, D.J., Roberts, B., and Willson, T. (1996). Molecular cloning of two novel transmembrane ligands for Eph-related kinases (LERKS) that are related to LERK-2. Growth Factors *13*, 141-149.

Nikolaev, A., McLaughlin, T., O'Leary, D.D., and Tessier-Lavigne, M. (2009). APP binds DR6 to trigger axon pruning and neuron death via distinct caspases. Nature *457*, 981-989.

Nobes, C.D., and Hall, A. (1995). Rho, rac, and cdc42 GTPases regulate the assembly of multimolecular focal complexes associated with actin stress fibers, lamellipodia, and filopodia. Cell *81*, 53-62.

Noren, N.K., and Pasquale, E.B. (2004). Eph receptor-ephrin bidirectional signals that target Ras and Rho proteins. Cell Signal *16*, 655-666.

O'Rourke, N.A., and Fraser, S.E. (1990). Dynamic changes in optic fiber terminal arbors lead to retinotopic map formation: an in vivo confocal microscopic study. Neuron *5*, 159-171.

Ogita, H., Kunimoto, S., Kamioka, Y., Sawa, H., Masuda, M., and Mochizuki, N. (2003). EphA4-mediated Rho activation via Vsm-RhoGEF expressed specifically in vascular smooth muscle cells. Circ Res *93*, 23-31.

Orioli, D., Henkemeyer, M., Lemke, G., Klein, R., and Pawson, T. (1996). Sek4 and Nuk receptors cooperate in guidance of commissural axons and in palate formation. EMBO J *15*, 6035-6049.

Oster, S.F., Bodeker, M.O., He, F., and Sretavan, D.W. (2003). Invariant Sema5A inhibition serves an ensheathing function during optic nerve development. Development *130*, 775-784.

Oster, S.F., Deiner, M., Birgbauer, E., and Sretavan, D.W. (2004). Ganglion cell axon pathfinding in the retina and optic nerve. Semin Cell Dev Biol *15*, 125-136.

Ott, H., Bastmeyer, M., and Stuermer, C.A. (1998). Neurolin, the goldfish homolog of DM-GRASP, is involved in retinal axon pathfinding to the optic disk. J Neurosci *18*, 3363-3372.

Palmer, A., Zimmer, M., Erdmann, K.S., Eulenburg, V., Porthin, A., Heumann, R., Deutsch, U., and Klein, R. (2002). EphrinB phosphorylation and reverse signaling: regulation by Src kinases and PTP-BL phosphatase. Mol Cell *9*, 725-737.

Pasquale, E.B. (1991). Identification of chicken embryo kinase 5, a developmentally regulated receptor-type tyrosine kinase of the Eph family. Cell Regul *2*, 523-534.

Pasquale, E.B., and Singer, S.J. (1989). Identification of a developmentally regulated protein-tyrosine kinase by using anti-phosphotyrosine antibodies to screen a cDNA expression library. Proc Natl Acad Sci U S A *86*, 5449-5453.

Penzes, P., Beeser, A., Chernoff, J., Schiller, M.R., Eipper, B.A., Mains, R.E., and Huganir, R.L. (2003). Rapid induction of dendritic spine morphogenesis by trans-synaptic ephrinB-EphB receptor activation of the Rho-GEF kalirin. Neuron *37*, 263-274.

Pfeiffenberger, C., Yamada, J., and Feldheim, D.A. (2006). Ephrin-As and patterned retinal activity act together in the development of topographic maps in the primary visual system. J Neurosci *26*, 12873-12884.

Plas, D.T., Dhande, O.S., Lopez, J.E., Murali, D., Thaller, C., Henkemeyer, M., Furuta, Y., Overbeek, P., and Crair, M.C. (2008). Bone morphogenetic proteins, eye patterning, and retinocollicular map formation in the mouse. J Neurosci *28*, 7057-7067.

Puschmann, T.B., and Turnley, A.M. (2010). Eph receptor tyrosine kinases regulate astrocyte cytoskeletal rearrangement and focal adhesion formation. J Neurochem *113*, 881-894.

Qin, H., Noberini, R., Huan, X., Shi, J., Pasquale, E.B., and Song, J. (2010). Structural characterization of the EphA4-Ephrin-B2 complex reveals new features enabling Eph-ephrin binding promiscuity. J Biol Chem *285*, 644-654.

Rashid, T., Upton, A.L., Blentic, A., Ciossek, T., Knoll, B., Thompson, I.D., and Drescher, U. (2005). Opposing gradients of ephrin-As and EphA7 in the superior colliculus are essential for topographic mapping in the mammalian visual system. Neuron *47*, 57-69.

Reichardt, L.F., Bossy, B., de Curtis, I., Neugebauer, K.M., Venstrom, K., and Sretavan, D. (1992). Adhesive interactions that regulate development of the retina and primary visual projection. Cold Spring Harb Symp Quant Biol *57*, 419-429.

Riehl, R., Johnson, K., Bradley, R., Grunwald, G.B., Cornel, E., Lilienbaum, A., and Holt, C.E. (1996). Cadherin function is required for axon outgrowth in retinal ganglion cells in vivo. Neuron *17*, 837-848.

Ringstedt, T., Braisted, J.E., Brose, K., Kidd, T., Goodman, C., Tessier-Lavigne, M., and O'Leary, D.D. (2000). Slit inhibition of retinal axon growth and its role in retinal axon pathfinding and innervation patterns in the diencephalon. J Neurosci *20*, 4983-4991.

Ruchhoeft, M.L., Ohnuma, S., McNeill, L., Holt, C.E., and Harris, W.A. (1999). The neuronal architecture of Xenopus retinal ganglion cells is sculpted by rho-family GTPases in vivo. J Neurosci *19*, 8454-8463.

Sahin, M., Greer, P.L., Lin, M.Z., Poucher, H., Eberhart, J., Schmidt, S., Wright, T.M., Shamah, S.M., O'Connell, S., Cowan, C.W.*, et al.* (2005). Eph-dependent tyrosine phosphorylation of ephexin1 modulates growth cone collapse. Neuron *46*, 191-204.

Sajjadi, F.G., Pasquale, E.B., and Subramani, S. (1991). Identification of a new eph-related receptor tyrosine kinase gene from mouse and chicken that is developmentally regulated and encodes at least two forms of the receptor. New Biol *3*, 769-778.

Schmitt, A.M., Shi, J., Wolf, A.M., Lu, C.C., King, L.A., and Zou, Y. (2006). Wnt-Ryk signalling mediates medial-lateral retinotectal topographic mapping. Nature *439*, 31-37.

Shao, H., Lou, L., Pandey, A., Pasquale, E.B., and Dixit, V.M. (1994). cDNA cloning and characterization of a ligand for the Cek5 receptor protein-tyrosine kinase. J Biol Chem *269*, 26606-26609.

Shintani, T., Ihara, M., Sakuta, H., Takahashi, H., Watakabe, I., and Noda, M. (2006). Eph receptors are negatively controlled by protein tyrosine phosphatase receptor type O. Nat Neurosci *9*, 761-769.

Simon, D.K., and O'Leary, D.D. (1992a). Development of topographic order in the mammalian retinocollicular projection. J Neurosci *12*, 1212-1232.

Simon, D.K., and O'Leary, D.D. (1992b). Responses of retinal axons in vivo and in vitro to position-encoding molecules in the embryonic superior colliculus. Neuron *9*, 977-989.

Simons, K., and Ikonen, E. (1997). Functional rafts in cell membranes. Nature *387*, 569-572.

Snow, D.M., and Letourneau, P.C. (1992). Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). J Neurobiol *23*, 322-336.

Sperry, R.W. (1943). Effect of 180 degree rotation of the retinal field on visuomotor coordination. J Expl Zool *92*, 263-279.

Sperry, R.W. (1944). Optic nerve regeneration with return of vision in anurans. J Neurophysiol *7*, 57-69.

Sperry, R.W. (1945). Restoration of vision after crossing of optic nerves and after contralateral transposition of the eye. J Neurophysiol *8*, 15-28.

Sperry, R.W. (1963). Chemoaffinity in the Orderly Growth of Nerve Fiber Patterns and Connections. Proc Natl Acad Sci U S A *50*, 703-710.

Sprague, J.M., and Meikle, T.H., Jr. (1965). The Role of the Superior Colliculus in Visually Guided Behavior. Exp Neurol *11*, 115-146.

Sung, C.H., and Chuang, J.Z. (2010). The cell biology of vision. J Cell Biol *190*, 953-963.

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

Tolias, K.F., Bikoff, J.B., Kane, C.G., Tolias, C.S., Hu, L., and Greenberg, M.E. (2007). The Rac1 guanine nucleotide exchange factor Tiam1 mediates EphB receptor-dependent dendritic spine development. Proc Natl Acad Sci U S A *104*, 7265-7270.

Tomita, T., Tanaka, S., Morohashi, Y., and Iwatsubo, T. (2006). Presenilin-dependent intramembrane cleavage of ephrin-B1. Mol Neurodegener *1*, 2.

Torres, R., Firestein, B.L., Dong, H., Staudinger, J., Olson, E.N., Huganir, R.L., Bredt, D.S., Gale, N.W., and Yancopoulos, G.D. (1998). PDZ proteins bind, cluster, and synaptically colocalize with Eph receptors and their ephrin ligands. Neuron *21*, 1453-1463.

Wang, H.U., Chen, Z.F., and Anderson, D.J. (1998). Molecular distinction and angiogenic interaction between embryonic arteries and veins revealed by ephrin-B2 and its receptor Eph-B4. Cell *93*, 741-753.

Wiesel, T.N., and Hubel, D.H. (1963a). Effects of Visual Deprivation on Morphology and Physiology of Cells in the Cats Lateral Geniculate Body. J Neurophysiol *26*, 978-993.

Wiesel, T.N., and Hubel, D.H. (1963b). Single-Cell Responses in Striate Cortex of Kittens Deprived of Vision in One Eye. J Neurophysiol *26*, 1003-1017.

Wiesner, S., Wybenga-Groot, L.E., Warner, N., Lin, H., Pawson, T., Forman-Kay, J.D., and Sicheri, F. (2006). A change in conformational dynamics underlies the activation of Eph receptor tyrosine kinases. EMBO J *25*, 4686-4696.

Williams, S.E., Mann, F., Erskine, L., Sakurai, T., Wei, S., Rossi, D.J., Gale, N.W., Holt, C.E., Mason, C.A., and Henkemeyer, M. (2003). Ephrin-B2 and EphB1 mediate retinal axon divergence at the optic chiasm. Neuron *39*, 919-935.

Winslow, J.W., Moran, P., Valverde, J., Shih, A., Yuan, J.Q., Wong, S.C., Tsai, S.P., Goddard, A., Henzel, W.J., Hefti, F*., et al.* (1995). Cloning of AL-1, a ligand for an Eph-related tyrosine kinase receptor involved in axon bundle formation. Neuron *14*, 973-981.

Wybenga-Groot, L.E., Baskin, B., Ong, S.H., Tong, J., Pawson, T., and Sicheri, F. (2001). Structural basis for autoinhibition of the Ephb2 receptor tyrosine kinase by the unphosphorylated juxtamembrane region. Cell *106*, 745-757.

Xu, N.J., and Henkemeyer, M. (2009). Ephrin-B3 reverse signaling through Grb4 and cytoskeletal regulators mediates axon pruning. Nat Neurosci *12*, 268-276.

Xu, Q., Alldus, G., Holder, N., and Wilkinson, D.G. (1995). Expression of truncated Sek-1 receptor tyrosine kinase disrupts the segmental restriction of gene expression in the Xenopus and zebrafish hindbrain. Development *121*, 4005-4016.

Xu, Q., Alldus, G., Macdonald, R., Wilkinson, D.G., and Holder, N. (1996). Function of the Eph-related kinase rtk1 in patterning of the zebrafish forebrain. Nature *381*, 319-322.

Yamagata, M., and Sanes, J.R. (2008). Dscam and Sidekick proteins direct lamina-specific synaptic connections in vertebrate retina. Nature *451*, 465-469.

Yamagata, M., Weiner, J.A., and Sanes, J.R. (2002). Sidekicks: synaptic adhesion molecules that promote lamina-specific connectivity in the retina. Cell *110*, 649-660.

Yates, P.A., Holub, A.D., McLaughlin, T., Sejnowski, T.J., and O'Leary, D.D. (2004). Computational modeling of retinotopic map development to define contributions of EphA-ephrinA gradients, axon-axon interactions, and patterned activity. J Neurobiol *59*, 95-113.

Yates, P.A., Roskies, A.L., McLaughlin, T., and O'Leary, D.D. (2001). Topographic-specific axon branching controlled by ephrin-As is the critical event in retinotectal map development. J Neurosci *21*, 8548-8563.

Yokoyama, N., Romero, M.I., Cowan, C.A., Galvan, P., Helmbacher, F., Charnay, P., Parada, L.F., and Henkemeyer, M. (2001). Forward signaling mediated by ephrin-B3 prevents contralateral corticospinal axons from recrossing the spinal cord midline. Neuron *29*, 85-97.

Zhou, R., Copeland, T.D., Kromer, L.F., and Schulz, N.T. (1994). Isolation and characterization of Bsk, a growth factor receptor-like tyrosine kinase associated with the limbic system. J Neurosci Res *37*, 129-143.

Zimmer, M., Palmer, A., Kohler, J., and Klein, R. (2003). EphB-ephrinB bi-directional endocytosis terminates adhesion allowing contact mediated repulsion. Nat Cell Biol *5*, 869-878.