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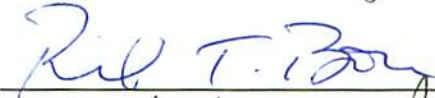
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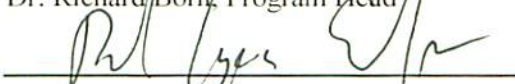
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**A chemical-genetic study of EphB receptor tyrosine kinase signaling in the
developing nervous system**

A dissertation presented

by

Michael Jefferson Soskis

to

The Division of Medical Sciences

in partial fulfillment of the requirements

for the degree of

Doctor of Philosophy

in the subject of

Neurobiology

Harvard University

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**A chemical-genetic study of EphB receptor tyrosine kinase signaling in
the developing nervous system**

Abstract

EphB receptor tyrosine kinases regulate cell-cell contacts throughout nervous system development, mediating processes as diverse as axon guidance, topographic mapping, neuronal migration and synapse formation. EphBs bind to a group of ligands, ephrin-Bs, which span the plasma membrane, thus allowing for bidirectional signaling between cells. Since EphBs are capable of multiple modes of signaling, and since they regulate numerous interdependent stages of development, it has been challenging to define which signaling functions of EphBs mediate particular developmental events.

To overcome this hurdle, we developed an approach combining chemical biology with genetic engineering to reversibly inhibit EphB receptors *in vivo*. By mutating a residue in the receptor's ATP-binding pocket, we rendered its kinase activity sensitive to reversible inhibition by PP1 analogs that do not inhibit wild type receptors. We engineered triple knockin mice bearing this mutation in which the kinase activity of EphB1, EphB2, and EphB3 can be rapidly, reversibly, and specifically blocked. Since we are able to block the kinase activity of EphBs while leaving their scaffolding and reverse signaling capabilities intact, we can precisely isolate the role of the kinase domain. In addition, acute inhibition can circumvent the developmental compensation that may

occur after genetic mutations and can even allow the controlled study of EphBs in the mature brain and in disease models.

Using these mice, termed analog-sensitive EphB triple knockin (AS-EphB TKI) mice, we demonstrate a requirement for the kinase-dependent signaling of EphBs in the collapse of retinal ganglion cell growth cones *in vitro* and the guidance of retinal axons at the optic chiasm *in vivo*. In addition, we show that the formation of several cortical axon tracts, including the corpus callosum, requires EphB tyrosine kinase signaling. In contrast, we find that steps in synapse development that are thought to be EphB-dependent occur normally when the kinase activity of EphBs is inhibited. We conclude that a cardinal *in vivo* function of EphB signaling, the ability to mediate axon guidance via growth cone repulsion, requires the tyrosine kinase activity of EphBs, while the development of functional excitatory synapses is independent of EphB tyrosine kinase activity.

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Attributions

The EphB-AS TKI mice were originally generated by Henry Ho. Chao Zhang and Kevan Shokat provided the intellectual foundation for the analog-sensitive kinase allele strategy. All further characterization and experimentation was performed by Michael Soskis under the guidance of Michael Greenberg with the following exceptions:

Figure 1.2 uses publicly available data generated by the Greenberg lab.

Figures 2.2 and 2.5 were generated by Henry Ho.

Figure 2.6 is based on data from Henry Ho, who also generated panels A and B.

Figure 2.7B is based on data collected by Bulent Ataman.

Figures 3.4 and 3.5 are based on data from a collaboration between the laboratories of Michael Greenberg and Chris Cowan at the University of Texas Southwestern.

Experiments were performed by Michael Soskis, Henry Ho, and Michael Robichaux.

Chapter 1

Introduction

The human brain may well be the most complex structure in the known universe. For it to spring forth fully formed would be incredible; but to self-assemble in the womb as it does millions of times a year is even more astounding.

The question of how the nervous system arises out of a set of genetic instructions underlies the major pursuits of developmental neurobiology. Piecing together these processes can have profound implications for understanding how the brain works, how humans evolved, how diseases lead to nervous system dysfunction, and how these disorders can be treated.

Unfortunately, “if the human brain were so simple that we could understand it, we would be so simple that we couldn't (Pugh, 1977).” It is for this reason, in addition to practical and ethical considerations, that we study model organisms such as the worm, fly, zebrafish, chicken, and mouse. Decades of work in embryology and developmental biology have elucidated the intricate processes that lead from a fertilized egg to an adult organism and how the nervous system takes shape. Since the molecular revolution, core pathways have been identified underlying these processes. The bottom of the well has not yet been reached, as new layers of complexity continue to emerge.

Much of development hinges on the precise communication between cells. Receptor tyrosine kinases (RTKs) have proven vital to this process in a variety of contexts. Understanding how they function in the development of the nervous system and how their dysregulation causes disease has been an area of active research, with a Nobel prize already awarded and new directions of promising research on the horizon.

1.1 Receptor tyrosine kinases in nervous system development

The History of RTKs

Growth factors orchestrate many of the central processes in development. In classic experiments conducted by Rita Levi-Montalcini and Stanley Cohen, nerve growth factor (NGF), the first growth factor to be isolated, was shown to dramatically affect the survival, differentiation, and morphology of cells (Levi-Montalcini, 1987). However, despite the importance of growth factors, several decades elapsed until the first relevant receptor was identified. This receptor for epidermal growth factor, termed EGFR, launched decades of study into how receptors mediate growth factor signals (Carpenter et al., 1978). The discovery soon after that EGFR could catalyze the transfer of phosphate onto tyrosine residues in proteins made it the prototypical receptor tyrosine kinase, or RTK (Ushiro and Cohen, 1980).

Since the initial characterization of RTKs, 20 subfamilies have been identified in humans, with most having known ligands (Lemmon and Schlessinger, 2010). The study of RTKs has progressed enormously over the last three decades and at all times been closely linked to both normal development and cancerous growth. Many of the early RTKs were implicated in tumors, such as the EGFR gene *her2* in breast cancer. A great deal of work is currently focused on understanding how to develop therapeutics that target RTKs as potential cancer therapies (Sawyers, 2002).

In the nervous system, RTKs have proven to play a key role in many stages of development. The ErbB receptors and their ligands, the neuregulins, are particularly important for glial development (Mei and Xiong, 2008). Musk and its ligand agrin coordinate the assembly of the neuromuscular junction (Sanes and Lichtman, 2001). The

neurotrophin receptor family controls a number of processes including the survival and determination of electrophysiological properties in neurons (Lu et al., 2005; Luther and Birren, 2009). Finally, the Eph receptors and their ligands, the ephrins, control numerous cell-cell interactions, including those required for retinotopic mapping (Kullander and Klein, 2002).

Signaling by RTKs

The basic structure of an RTK consists of an extracellular region containing a ligand binding motif and an intracellular region containing a catalytic kinase domain, in addition to various regulatory regions (Lemmon and Schlessinger, 2010). The fundamental principles of RTK signaling were worked out in classic experiments from the 1980s. Ligand binding leads to dimerization of the receptor, either as heterodimers or homodimers (Schlessinger, 1988). This dimerization occurs through several different mechanisms depending on the particular subclass of RTK (Bennasroune et al., 2004). Once the dimerized receptors are brought into close proximity, autophosphorylation of the receptor greatly enhances its catalytic kinase function (Hubbard and Till, 2000).

RTK autophosphorylation has two major effects on signaling. First, once the kinase domain of the receptor has been activated, it can catalyze the phosphorylation of cellular substrates. Second, autophosphorylated residues on the RTK can serve as docking sites for adaptor proteins within the cell containing specialized domains, such as the Src homology 2 (SH2) domain (Sadowski et al., 1986) or the phosphotyrosine binding (PTB) domain (Kavanaugh et al., 1995). In the EGF pathway, receptor activation leads to Grb2/Sos binding to phosphotyrosines on EGFR and the activation of Sos, which then

activates Ras family small-GTPases that modulate MAPK transcriptional signaling (Lowenstein et al., 1992; Rozakis-Adcock et al., 1993).

Atypical RTK signaling

After the initial characterization of receptor tyrosine kinases, it became apparent that other modes of signaling apart from tyrosine kinase activity and SH2-binding domain interactions were possible. This was supported by the following lines of evidence:

First, a surprising finding in kinase research has been the identification of proteins in the kinase family that lack catalytic kinase activity. Ten percent of putative kinases lack a conserved amino acid in their kinase domain and are therefore thought to be pseudokinases (Boudeau et al., 2006). These pseudokinases signal completely independently of their deficient kinase domains. Among RTKs, ErbB3 and PTK7, both pseudokinases, have been implicated in tumor cell proliferation and cell polarity, respectively, despite their inability to phosphorylate protein targets (Holbro et al., 2003; Lu et al., 2004). In the nervous system, the catalytically-inactive RTK RYK has been shown to mediate WNT signaling in a kinase-independent manner (Bovolenta et al., 2006).

Second, several RTKs contain additional signaling domains such as the Src Homology 3 (SH3), PSD-95, Dlg, Zo-1 (PDZ), and WW domains. Each of these domains regulates protein-protein interactions, suggesting a possible scaffolding role for some RTKs that may be independent of their tyrosine kinase activity (Reimand et al., 2012). Alternatively, these domains could function in the regulation of receptor kinase activity.

Third, several RTKs have been shown to signal through their ligands in a form of reciprocal signaling termed reverse signaling. For example, binding of the erbB receptor to its ligand, neuregulin-1, can cause proteolytic cleavage of the intracellular region of neuregulin, which may then translocate to the nucleus to engage a transcriptional pathway (Bao et al., 2003). Thus, erbB, and possibly other RTKs, can function as a ligand independent of its kinase activity.

These pieces of evidence support a role for atypical modes of RTK signaling. Of all the subfamilies of RTKs, none has the complexity and sheer signaling potential of the Eph receptors, to which all three of the arguments above can apply.

1.2 The Eph family of receptors

The Eph subclass of RTKs

The Eph receptors constitute the largest subclass of RTKs. Eph receptors were initially isolated from an Erythropoietin-producing human hepatocellular carcinoma cell line (Hirai et al., 1987). During normal development, Ephs regulate diverse processes including the formation of the nervous, skeletal, and circulatory systems (Pasquale, 2008). However, their misexpression and altered signaling can lead to pathologies and have been linked to several tumors including breast and colon cancers (Genander and Frisen, 2010).

The identification of the Eph receptor ligands, the ephrins, required seven more years and was reported independently by several researchers: Bartley et al. screened Chinese hamster ovary (CHO) cell supernatants for binding to immobilized Eph receptors using surface plasmon resonance to identify a protein which they were able to sequence (Bartley et al., 1994). Flanagan and colleagues used soluble alkaline phosphatase receptor

fusions (AP-Eph) to identify regions in the embryo expressing the ligand (the midbrain and anterior hindbrain). They then prepared cDNA libraries from those regions, expressed them in pools in COS cells, and identified appropriate clones that produced proteins which could bind AP-Eph (Cheng and Flanagan, 1994). Remarkably, the identified ligands (ephrins) were predicted to be membrane-bound proteins, as opposed to soluble growth factors.

The size of the Eph/ephrin family continued to grow as related ligands and receptors were discovered. Currently, Ephs are divided into two classes based on their ligand binding (Lemke, 1997). The mammalian EphA family is comprised of 9 receptors (EphA1-EphA8, EphA10) and binds a set of glycosylphosphatidylinositol (GPI)-linked ligands, the ephrin-As (ephrinA1-A5) (Klein, 2009). The EphB class (EphB1-4, EphB6) preferentially binds a separate set of ligands, transmembrane proteins called ephrin-Bs (ephrin-B1-3) (Klein, 2009). These binding rules have some exceptions: EphA4 binds to ephrin-B2 and ephrin-B3 in addition to ephrin-As (Gale et al., 1996) and EphB2 binds to ephrin-A5 in addition to ephrin-Bs (Himanen et al., 2004). There might even be greater promiscuity in vivo, since most measurements to date have been made using soluble ephrins, which bind to the receptor in a three-dimensional reaction, whereas ligand-receptor binding in vivo is essentially two-dimensional (Poliakov et al., 2004).

The large Eph family of receptors shows a striking increase in complexity over evolution: *C. elegans* contain one Eph homologue, VAB-1 (George et al., 1998), as do *Drosophila*, Dek (Scully et al., 1999). It is reasonable to postulate that Ephs play a part in the evolution of the more complex mammalian nervous system and that the generic

structure of family members represents duplications throughout evolution (Drescher, 2002).

Since their ligands are membrane bound, Ephs are particularly well suited among RTKs to mediate short-range signaling and cell-cell contacts. This allows them to exert more localized influence than other RTKs (Toth et al., 2001). Ephs also have the ability to coordinate reciprocal communication between cells as both EphAs and EphBs can conduct bidirectional signaling by serving as ligands (to ephrins) in addition to serving as receptors (Holland et al., 1996; Lim et al., 2008b).

Ephs are expressed broadly during development and in the adult (Gale et al., 1996). However, classically they were studied for their role in retinotectal mapping in the chick, where they mediate the projection of axons from the retina to the superior colliculus or tectum. A gradient of EphA expression in the retina causes pathfinding axons to locate the correct target area in the tectum based on a local repellent gradient of ephrin-As (Flanagan and Vanderhaeghen, 1998).

The Structure of Ephs

To achieve their many functions in nervous system development and beyond, Ephs have a complex domain structure that allows them to signal in multiple modalities (**Figure 1.1**). The extracellular region of Ephs consists of three major domains: the globular domain which is sufficient for ephrin binding, a cysteine rich region, and two fibronectin type III repeat domains which mediate protein-protein interactions (Labrador et al., 1997).

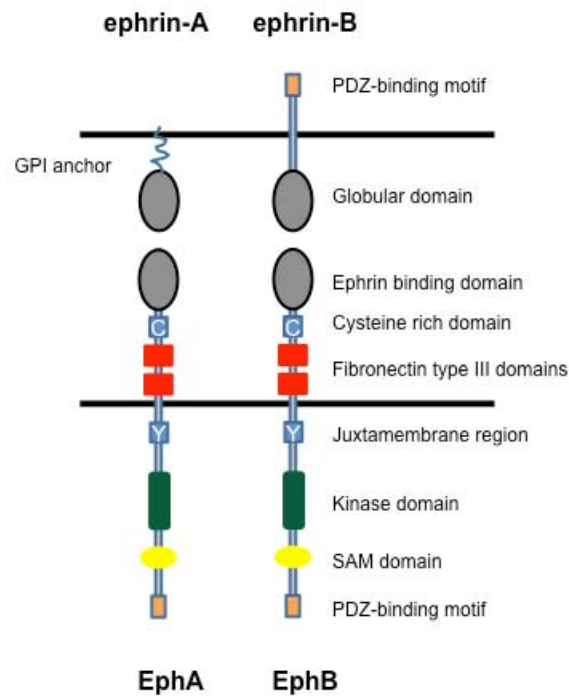


Figure 1.1: Structure of EphBs and their ligands ephrin-Bs

The extracellular region of EphAs and EphBs contains a globular ephrin binding domain, a cysteine rich region, and two fibronectin type III repeat domains. The intracellular region of Ephs contains a juxtamembrane region, a kinase domain, a sterile-alpha motif (SAM) domain, and a PDZ-binding motif at the C-terminus.

Both ephrin-As and ephrin-Bs contain a globular domain in their extracellular region. Ephrin-As are tethered to the plasma membrane via a GPI-linkage while ephrin-Bs span the membrane and contain an intracellular domain with a PDZ-binding motif at the C-terminus.

Intracellularly, Ephs have three major regions: the juxtamembrane region is essential for regulating receptor activation (Wybenga-Groot et al., 2001). The kinase domain is responsible for catalytic activity. A sterile-alpha motif (SAM) domain regulates oligomerization (Thanos et al., 1999). Finally, a PDZ-binding motif at the C-terminus mediates interactions with PDZ-domain containing proteins.

The structure of ephrins is consistent with their ability to signal through multiple modalities as well. Ephrins have a globular domain which mediates ligand dimerization, as determined by crystal structures of an ephrin ectodomain (Toth et al., 2001). Ephrin-As are tethered to the membrane via a GPI anchor. Ephrin-Bs have a short cytoplasmic region containing 5 conserved tyrosines and a C-terminal PDZ-binding motif (Kullander and Klein, 2002).

Structural studies of ephrin binding to Eph show that the ligand-receptor interaction promotes higher order clustering (Himanen et al., 2001). Biochemical studies have demonstrated previously that only clustered ephrins activate Ephs (Davis et al., 1994). In addition, a stoichiometry of 1:1 is predicted based on ephrin-A-EphA binding experiments (Lackmann et al., 1997). Thus ephrin/Eph signaling consists of the formation of large clusters on both the ephrin- and Eph-expressing cells that can serve as signaling centers at the plasma membrane. Researchers have taken advantage of this finding by generating ephrin or Eph peptides fused to the FC-region of IgG antibodies that can be easily clustered by addition of a secondary antibody.

Cell biology of Ephs

Ephs regulate several key cellular processes. Broadly speaking, their activities fall into two opposite classes, adhesion and repulsion. This paradoxical duality of purpose seems to depend upon exact context. The question of what mediates this switch has stimulated several hypotheses, with groups postulating the essential determinant to be the density of ligand clustering (Poliakov et al., 2004), regulated endocytosis (Marston et al., 2003; Zimmer et al., 2003), alternative splicing (Holmberg et al., 2000), and proteolytic cleavage (Hattori et al., 2000).

During different phases of neuronal development, the cellular compartment of Eph expression varies, from generally axonal to majority dendritic (Henderson et al., 2001). In the hippocampus, this contrast is seen most clearly with EphBs postsynaptic at Schaffer collateral synapses and presynaptic at mossy fiber synapses (Klein, 2009).

The EphB family

Of the two subclasses of Ephs, the EphBs present the most elaborate array of signaling capabilities due to the relative complexity of their ligands, the ephrin-Bs. EphBs play an important role in numerous aspects of nervous system development and have been studied extensively in this context. Of the five EphB receptors, EphB1-3 and EphB6 are expressed in neurons while EphB4 is expressed in blood vessels (Palmer and Klein, 2003). EphB6, a pseudokinase, has no catalytic kinase activity but can still engage in signaling, as it is required for proper T cell responses in vivo (Luo et al., 2004).

EphB1, EphB2, and EphB3 are considered the catalytically-active EphB receptors in the brain and will be referred to as EphBs in this dissertation (**Figure 1.2**). They

mediate processes as diverse as axon guidance, topographic mapping, neuronal migration, and synapse formation (Genander and Frisen, 2010; Klein, 2004; Lai and Ip, 2009).

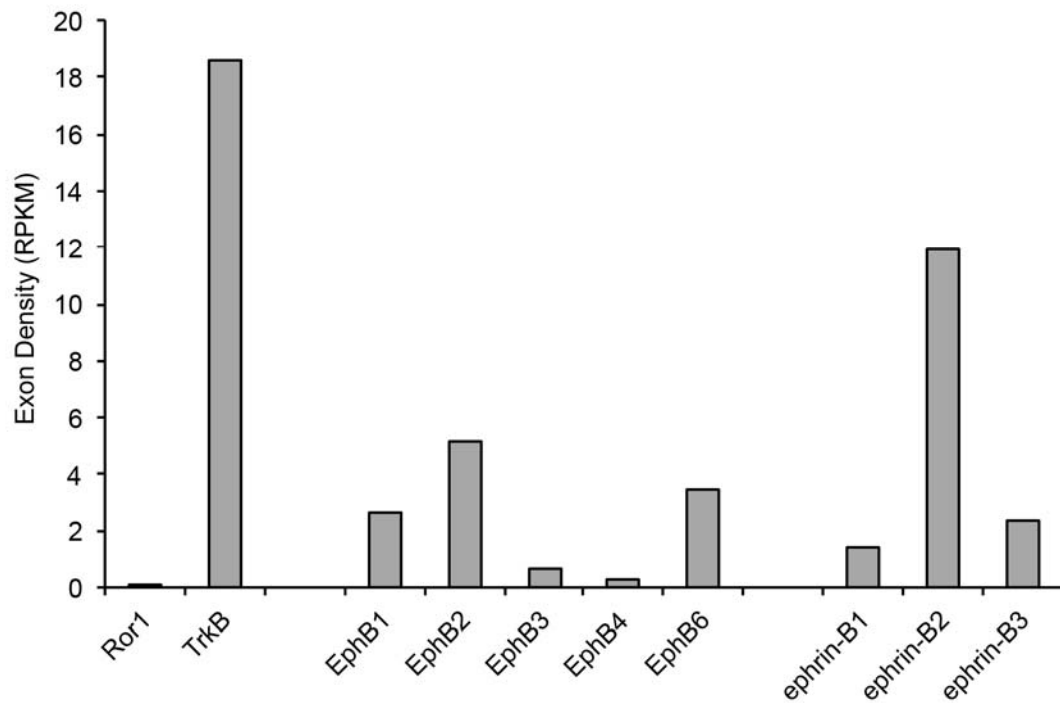


Figure 1.2: Expression of EphBs and ephrin-Bs in cortical neurons

Expression data from RNA sequencing experiments from 7 day in vitro (DIV) cultured cortical neurons. Figures were assembled from publicly available data published previously (Kim et al., 2010). Comparison of mRNA expression between genes is possible since quantification does not depend upon hybridization of probes as in qPCR or *in situ*. EphBs are expressed at moderate levels well above background (ROR1), but below the highly-expressed TrkB receptor. All 3 ephrin-Bs are expressed in cortical cultures. RPKM = reads per kilobase of exon model per million mapped reads.

In addition to these developmental roles, the *ephb2* gene has been linked to autism spectrum disorder (Sanders et al., 2012) and EphB dysfunction in the mature organism is thought to contribute to pathologies such as Alzheimer's Disease (Cisse et al., 2011).

EphBs are highly pleiotropic and have been implicated in almost every step of nervous system development. Because of the interlocking nature of developmental stages, isolating the specific role of EphBs in individual processes has been exceedingly challenging.

1.3 EphB receptor function in the developing nervous system

EphBs and ephrin-Bs are often expressed in intricate patterns: one particularly prescient study investigated expression of EphB2 in the developing embryo and noted complementary/edge expression compared to ephrin-B2. The authors correctly hypothesized a role for EphBs in boundary formation (Gale et al., 1996). A later comprehensive in situ study in neonatal and adult mice revealed broad expression of EphBs and ephrin-Bs throughout the nervous system, including in the cortex, basal ganglia, hippocampus, hypothalamus, and midbrain (Liebl et al., 2003). This broad expression pattern is consistent with the numerous developmental roles assigned to the EphB receptors throughout the literature.

Early patterning and differentiation

EphBs have been linked to even the earliest stages of neural development. After the establishment of germ layers in the embryo, the nervous system arises out of the

ectoderm. Cells within the ectoderm are induced to form the neural plate (which will later give rise to the nervous system) by a group of mesodermal cells forming a transient structure called the notochord. Disruption of ephrin-B/EphB signaling by overexpression of dominant negative constructs in Zebrafish leads to defects in notochord development (Chan et al., 2001).

During the process of neurulation, the neural plate folds upon itself giving rise to the neural tube (which will form the central nervous system) and the outlying neural crest (which will form the peripheral nervous system). Even as early as embryonic day 10.5 (E10.5), EphB2/EphB3 knockout mice have defects in neural tube formation, with an expanded ventral midline composed of cells destined to become the midbrain (Altick et al., 2005).

The neural tube goes on to differentiate into disparate regions of the brain and spinal cord, while cells in the neural crest migrate extensively to the periphery. In EphB2 knockout mice, this migration of neural crest cells is perturbed (Wang and Anderson, 1997). Studies in the chick support this role (Krull et al., 1997; Santiago and Erickson, 2002).

After basic patterning, neural progenitors give rise to the cells that will populate the nervous system. Perturbing ephrin-B1 signaling causes premature differentiation of neural progenitors in the developing mouse embryo (Qiu et al., 2008), suggesting a role for EphB signaling in restricting this process. These newborn neurons then migrate to the correct cortical layers. In vitro studies and mouse developmental expression data suggest that EphBs are particularly important for the migration of cerebellar granule cells (Lu et al., 2001). Taken together, these reports of EphB involvement in the most basic processes

of neural development suggest that perturbation of EphBs in the embryo might have far reaching consequences in the juvenile or adult organism that originate from aberrant embryonic development.

Neurite structure

Once a neuron has been born and migrated, it soon polarizes and sprouts two types of processes: dendrites, which generally receive inputs, and axons, which transmit action potentials and ultimately release neurotransmitter. EphBs have been implicated in regulating the development of both of these fundamental structures.

As dendrites grow, they often form elaborate branches on which to receive inputs. Cultured hippocampal neurons from EphB1/EphB2/EphB3 triple knockout (TKO) mice display decreased total dendritic length and complexity (Hoogenraad et al., 2005). In addition, a recent report found an increase in the number of primary dendrites in pyramidal neurons in slices from EphB1/EphB2 knockouts, consistent with a defect in dendritic pruning (Xu et al., 2011).

Since the original identification of ephrin-A5 as a potent activator of growth cone collapse (Drescher et al., 1995), Ephs have been shown to mediate several functions in the growth cone. Ephrin-B2 binding to EphBs causes collapse of retinal ganglion cell (RGC) axon growth cones (Petros et al., 2010). In addition, EphBs regulate axon fasciculation, as EphB2/EphB3 knockout mice display abnormal bundling in the habenular-interpeduncular tract (Orioli et al., 1996) and hippocampal axon tracts (Chen et al., 2004). EphB TKO mice also exhibit defects in axon pruning, as hippocampal fibers

from these mice display increased infra-pyramidal bundle length of the mossy fiber, indicating the failure of some fibers to be properly eliminated (Xu and Henkemeyer, 2009).

Axon guidance

In order for the intricate connections of the nervous system to form, neurons must send axons to specific target regions, sometimes up to distances of one meter in humans. Axon guidance throughout the nervous system, particularly across the midline, is heavily regulated by EphBs. The formation of the two main tracts that carry information between the hemispheres of the brain is controlled by EphB signaling: anterior commissure defects were observed in the original EphB2 knockout mouse (Henkemeyer et al., 1996). Additionally, corpus callosum defects were identified in an EphB2/EphB3 knockout mouse (Orioli et al., 1996).

Several other regions display EphB-dependent axon guidance. In the spinal cord, commissural axons express EphBs and collapse in response to ephrin-Bs (Imondi et al., 2000). EphB TKO mice have spinal cord commissural axon routing defects (Kadison et al., 2006), suggesting a role for EphBs in spinal midline guidance. EphBs have also been implicated in fine-tuning the guidance of lower motor neuron axons to their targets in the limbs (Luria et al., 2008).

EphBs are important mediators of axon guidance in many of the sensory systems as well. In the visual system, studies using EphB1 and ephrin-B1/ephrin-B2 knockout mice show a role in forming the ipsilateral retinal projection to the lateral geniculate

nucleus (LGN) and superior colliculus (SC) (Chenaux and Henkemeyer, 2011; Williams et al., 2003). In the vestibular system, EphB2 knockout mice exhibit strain-specific circling behavior and have delayed midline crossing by inner ear efferents (Cowan et al., 2000). In the auditory system, EphB1 and EphB3 knockout mice have defective hearing, consistent with a perturbation of axon guidance in the cochlea (Howard et al., 2003). These studies demonstrate the vital role for EphBs in axon guidance throughout the nervous system.

Topographic mapping

After axons have reached the proper brain region, they terminate in specific target areas. Many of the connections between brain regions preserve a map, often representing a sensory space. Thus, points that are close in space are represented by neurons that are in proximity. In the visual system, mapping of spaces in the visual field is achieved at several relay centers from the retina to the cortex. To achieve this retinotopy, comparable areas in different brain regions are often matched by gradients of guidance factors. There is a dorso-ventral gradient of EphB2 in the chick retina (Holash et al., 1997) and ephrin-B2 in the chick tectum (Braisted et al., 1997) suggesting a role in retinotectal mapping. In *Xenopus*, perturbing ephrin-B/EphB signaling in vivo leads to defects in dorso-ventral topography of retinotectal axons (Mann et al., 2002). In mice, EphB2 and EphB3 are expressed in a dorso-ventral gradient in the retina while ephrin-B1 is expressed along a lateral-medial gradient in the superior colliculus (Hindges et al., 2002). Consequently, EphB2/EphB3 knockouts display defective retinocollicular mapping along this axis (Hindges et al., 2002).

Synapse formation

As axons home in on their proper targeting field, they make contact with dendrites and begin the process of synaptogenesis. Electron microscopy studies have localized EphBs at synapses, and thus implicated them in the formation or function of synapses (Buchert et al., 1999). Early dominant negative data confirmed this role, as perturbing EphB2 function in cultured neurons led to fewer glutamate receptors on dendrites (Dalva et al., 2000) and alterations in the morphology and number of dendritic spines, the sites of excitatory synapses (Ethell et al., 2001).

While dominant negative experiments can be difficult to interpret due to possible off-target effects, the report of an EphB1/EphB2/EphB3 triple knockout (TKO) mouse provided firm evidence that EphBs regulate synapse and spine development either directly or secondarily (Henkemeyer et al., 2003). Hippocampal neurons cultured from EphB TKO embryos display a striking lack of dendritic spines at 21 days in vitro (DIV). Instead, they exhibit long, thin filopodia on their dendrites. Remarkably, these neurons also display a complete absence of glutamate receptors at synapses as assessed by both western blot and immunocytochemistry for synaptic markers. In acute hippocampal slices from these mice, dendritic spine density and morphology are severely perturbed, although not as dramatically as in culture. Analysis of various genetic combinations identified EphB2 as the most relevant family member in synaptogenesis, but EphB1 and EphB3 contribute to the phenotypes as well. Indeed, the phenotypes of any double knockout combination are less severe than the triple knockout. While these findings supported the idea that EphBs are key regulators of synapse and spine development, the pleiotropic

nature of EphBs makes it difficult to isolate these defects as primarily synaptic, since earlier processes vital to cellular development may have been perturbed.

The question of the timing of EphB involvement in synaptogenesis was further addressed using RNAi (Kayser et al., 2008). Even though EphB2 knockouts lack synaptic defects, RNAi of EphB2 in cultured neurons appears to cause significant synaptic defects. Studies of varying time points identified 3-14 days in vitro (DIV) as the key window of synapse regulation by EphB2, as RNAi from 0-7 DIV and 14-21 DIV produced no loss of synapses as measured by immunocytochemistry of PSD-95 and vGlut colocalization.

Although EphB2 knockout mice do not display abnormalities in basal synaptic transmission, RNAi of EphB2 at 0 DIV leads to a large decrease in miniature excitatory postsynaptic current (EPSC) frequency, but not amplitude, at 10 DIV, suggesting that EphBs regulate the formation of functional synapses (Kayser et al., 2006). However, this experiment did not contain a rescue or discussion of recording parameters, and cell health may have been perturbed in the RNAi, but not empty vector control condition.

The role of EphBs in promoting dendritic spine formation was supported by studies in which exogenous ephrin addition led to spine formation in cultured hippocampal neurons (Henkemeyer et al., 2003; Tolia et al., 2007). Sometimes, this effect required overexpression of EphB2 (Penzes et al., 2003). Surprisingly, application of clustered EphB2 also led to spine formation (Segura et al., 2007) suggesting that either multiple forms of ephrin-B/EphB signaling may regulate spine formation or that the application of clustered proteins may cause non-physiological spine formation under certain culture conditions.

Development in the adult

Many of the early processes that shape the nervous system reappear in repeated motifs at later stages in development and in the adult. This fascinating recapitulation of developmental pathways in the adult is challenging to study. Knockout mice may have defects in the mature animal that seem to be bona fide phenotypes, but are actually relics of earlier problems in development. EphBs have been implicated in a number of such processes in the adult that are reminiscent of their developmental roles.

Pathways involved in the formation of synapses and spines may be reactivated during plasticity. *C. Elegans* Eph mutants show defects in synaptic homeostasis in the neuromuscular junction (NMJ) (Frank et al., 2009). In *Xenopus*, retinotectal Long Term Potentiation (LTP) is perturbed when soluble unclustered ephrin-B and EphB are bath applied (Lim et al., 2008a).

In mammals, EphB mutants have been studied in the context of LTP and Long term depression (LTD) in the hippocampus. EphB2 knockout mice display defects in LTP and LTD at the Schaffer Collateral (Grunwald et al., 2001; Henderson et al., 2001) and perforant pathway synapses (Henderson et al., 2001). Mossy fiber LTP was defective when EphB signaling was perturbed acutely using soluble blocking peptides, however the specificity of these peptides is difficult to assess (Contractor et al., 2002).

Studies of ephrin-B knockout mice support this role in plasticity. EphrinB2 and ephrinB3 knockouts have defects in Schaffer Collateral LTP and LTD (Grunwald et al., 2004). Ephrin-B3 mice have defects in mossy fiber LTP as well (Armstrong et al., 2006). These results suggest that multiple forms of hippocampal plasticity are compromised when ephrin-B/EphB signaling is perturbed. However, given the extensive role of EphBs

in regulating the development of the hippocampus, it is not clear whether these defects are primary, or rather a secondary result of defective cellular migration or morphology.

An even more striking example of developmental processes recapitulated in the adult is the proliferation of stem cells. Just as EphBs have been implicated in neurogenesis in the developing embryos, they have also been linked to neurogenesis of adult stem cells in the hippocampus. Perturbing ephrin-B signaling in the adult by perfusing soluble unclustered ephrin-B and EphB increases cell proliferation in the subventricular zone (SVZ) (Conover et al., 2000). In support of this, ephrin-B3 knockout mice display an increase in SVZ cell proliferation in the adult (Ricard et al., 2006) and EphB1/EphB2 knockouts exhibit a decrease in the number of neural progenitors in the adult hippocampus (Chumley et al., 2007).

Sometimes developmental pathways are reactivated in response to cellular trauma. EphB2 has been linked to Schwann cell migration after nerve injury. Consequently, mice in which soluble blocking EphB2 peptides have been applied show impaired recovery from peripheral nerve injury (Parrinello et al., 2010). Similarly, EphB3 has been implicated in the recovery from retinal nerve injury (Liu et al., 2006).

1.4 EphB receptor signaling

In order to mediate these disparate processes, EphBs utilize a number of signaling capacities including their tyrosine kinase activity, clustering of cellular proteins, scaffolding, and reverse signaling.

Kinase signaling of EphBs

Like most RTKs, EphB1, EphB2, and EphB3 can engage in conventional forward signaling through their kinase domain. In their basal state, the catalytic domain of EphBs is autoinhibited by a highly conserved juxtamembrane region (Wybenga-Groot et al., 2001). The binding of ephrin-B ligands to EphBs leads to receptor clustering and intermolecular autophosphorylation of conserved tyrosine residues in the juxtamembrane region and the activation segment of the kinase domain (Binns et al., 2000); the initial autophosphorylation is thought to be possible due to low-level intrinsic kinase activity of the receptor (Himanen et al., 2007). This phosphorylation event acts as a switch to relieve catalytic autoinhibition and greatly enhances the kinase activity of EphBs. In addition, phosphorylation of these juxtamembrane tyrosines can create an SH2-binding site which recruits SH2 domain containing proteins (Himanen et al., 2007; Holland et al., 1996).

Once activated, EphBs can phosphorylate a number of downstream targets. Identification of these substrates can help elucidate the pathways mediating the effects of EphBs in the cell. A number of these substrates have been discovered using various mass-spectrometry techniques. One group used stable isotope labeling by amino acids in cell culture (SILAC) to look for changes in phospho-tyrosines after ephrin-B1 stimulation (Zhang et al., 2008). While this approach cannot differentiate between direct and indirect targets of EphBs, it was able to identify many of the previously studied targets and suggest hundreds more.

A separate mass spectrometry approach also yielded a number of putative EphB targets spanning several cell biological families (Jorgensen et al., 2009). Inspection of the putative EphB substrates from these studies shows that they are often involved in

pathways regulating cytoplasmic kinases and monomeric GTP-ases. This finding is consistent with the role for EphBs in regulating the actin cytoskeleton.

Other modes of forward signaling

Engagement of ephrin-B with EphB can lead to separate modes of signaling in the EphB-expressing cell that are not directly linked to its kinase function. The extracellular domain of EphBs can recruit binding partners including subunits of the NMDA subtype of glutamate receptor (Dalva et al., 2000). This interaction can lead to modulation of calcium influx through the NMDA receptor and can alter the induction of genes in the nucleus (Takasu et al., 2002). Since the initial discovery of this NR1 interaction, EphBs have been implicated in more extensive interactions with excitatory amino acid receptors and their regulatory machinery (Calo et al., 2006).

In their cytoplasmic regions, EphBs contain a PDZ-domain binding motif. This allows them to bind and recruit a number of synaptic proteins that contain PDZ domains, such as GRIP1 (Torres et al., 1998) and Pick1 (Zhuang et al., 2010). Given their ability to form high-order multimers, EphBs have the potential to rapidly cluster PDZ proteins and coordinate synaptic functions.

Reverse signaling

A remarkable feature of EphBs is their ability to engage in reciprocal signaling with their ligands, the ephrin-Bs. The surprising discovery that exposure of ephrin-B1 to EphB2 could cause phosphorylation of ephrin-B1 opened the door to a new model of signaling (Holland et al., 1996). The conserved tail of ephrin-Bs contains 5 tyrosines, 3 of

which have been shown to be phosphorylated in vivo (Kalo et al., 2001). Phosphorylation of the cytoplasmic tail of ephrin-Bs results in the recruitment of SH2-domain containing proteins like Grb4 (Cowan and Henkemeyer, 2001; Palmer et al., 2002). Ephrin-Bs also contain a PDZ-binding motif at their C-terminus (Lin et al., 1999) and can recruit PDZ-domain containing proteins like GRIP-1 (Bruckner et al., 1999).

Reverse signaling remains mysterious in many regards. While there is controversy as to what terminates ephrin/Eph signaling, the less discussed question of what initiates this signaling is perhaps even more unclear. In some ways, the initiation of signaling is a chicken and egg scenario, since one cell must be activated first. For the case where two cells come into contact, such as during axon guidance, there is a clear time at which signaling might begin. However, more research is required to understand ephrin-B/EphB signaling initiation in other contexts where cells are already in stable contact. The interdependency of forward and reverse signaling also makes experimental manipulations challenging, since many perturbations intended to isolate one mode of signaling may affect other modes as well.

1.5 Previous approaches to studying EphB signaling

Experimental biology depends upon observation followed by gain- and loss-of-function experiments with appropriate controls. The study of EphBs has progressed as a result of all three approaches, each with advantages and limitations. In the case of EphBs, these strategies sometimes produce conflicting results; it is therefore worthwhile considering the merits of each approach separately.

Observation

In developmental biology, identifying where and when a protein is expressed can often indicate a large part of the protein's function. One early study investigating the localization of ephrins and Ephs noted their reciprocal expression patterns and correctly postulated their role in patterning and boundary formation (Gale et al., 1996). The creation of a mouse expressing a lacZ-EphB2 fusion protein made more detailed study of EphB expression possible and further implicated EphBs in a variety of developmental contexts (Henkemeyer et al., 1996). In particular, the gradients of ephrins and Ephs in the visual system indicated a role for mapping of retinal axons, as postulated by Sperry in his chemoaffinity hypothesis (Flanagan and Vanderhaeghen, 1998). Finally, certain stimuli such as seizure have been shown to regulate the expression of EphBs in the adult brain (Bruckner et al., 1999).

Relying on expression patterns, however, may be misleading. Many developmental proteins continue their expression in the adult nervous system, yet it is uncertain whether they serve a function there. Rather, they may represent developmental relics whose downregulation has not been selected for by evolution.

A more complex and perhaps significant observation would be to identify the patterns of activation of EphBs in various contexts. While one study noted that EphBs can be activated in the adult brain, and thus may indeed serve a role there, more careful analysis is warranted (Grunwald et al., 2001). This might be accomplished by probing brain sections with an antibody that specifically recognizes activated or clustered EphBs.

Finally, careful observation of cellular processes can indicate the mechanism by which EphBs function. For example, live imaging studies of pathfinding growth cones

lent important insights into how retinal axons identify guidance cues and make decisions (Mason and Wang, 1997). Live-imaging studies in cultured neurons have similarly attempted to understand how EphBs mediate synaptogenesis (Kayser et al., 2008).

Gain-of-function

Several gain-of-function approaches have been used to study EphBs. The addition of soluble clustered ephrins can activate EphBs and lead to biological effects. For example, the addition of clustered ephrin leads to the clustering of the NMDA Receptor subunit NR1 in dissociated cultures of cortical neurons (Dalva et al., 2000). This approach has even been used in vivo, as intrathecal injection of dimeric ephrin-B2 can lead to changes in thermal sensation in mice (Battaglia et al., 2003).

Similarly, overexpression of EphBs in heterologous cells has been shown to promote synaptogenesis (Kayser et al., 2006). This is supported by overexpression experiments in neurons that show an increase in synapse number as assessed by colocalization of PSD-95 and synapsin (Margolis et al., 2010). However, the overexpression of PDZ-binding motif containing proteins like EphBs may be difficult to interpret, since at high levels they may recruit synaptic proteins with which they do not normally interact.

Finally, EphB gain-of-function experiments can be performed using tissue/stripe assays to isolate EphB activity. For example, the response of EphB-expressing axons to different cues can be measured by allowing the axons to grow between stripes of substrates (Bush and Soriano, 2009). This approach has been vital for understanding many features of Eph-dependent axon guidance.

Genetic loss-of-function

The earliest EphB loss-of-function experiments utilized knockout technology to genetically excise the EphB2 gene (Henkemeyer et al., 1996). Study of these knockout mice immediately implicated EphBs in axon guidance and several other processes. These conclusions were supported by the later generation of ephrin-B knockout mice (Davy et al., 2004; Twigg et al., 2004; Wieland et al., 2004). Since the knockout approach should completely remove the protein and have no off-target effects, it is especially useful. However, knockout mice have several shortcomings: they are hampered by compensation and pleiotropism, so that in some cases other genes may compensate for the loss of EphBs, and in other cases observed defects may be secondary to an earlier developmental defect caused by loss of EphBs (Henkemeyer et al., 2003). In addition, loss of EphBs can be lethal in several backgrounds, making these mice difficult to study. Finally, these knockouts are all-or-none and do not distinguish between different modes of EphB signaling.

Some of these deficiencies are solved by knockin mice in which EphB or ephrin-B mutants are engineered to replace genes at their endogenous loci. The EphB2 lacZ mouse expresses a fusion protein of a cytoplasmically-truncated EphB2 fused to lacZ. This mouse has been reported to lack EphB2 forward signaling, however its juxtamembrane tyrosines still become phosphorylated (Grunwald et al., 2001). Similar ephrin-B lacZ mice exist as well. Although these mice can help with domain analysis and have indeed been the main evidence for reverse signaling, they still have the major problem of compensation. In addition, the EphB2 lacZ mouse fails to distinguish kinase

activity from other modes of cytoplasmic signaling (Henkemeyer et al., 2003). In particular, since ephrin-B binding to EphBs induces the formation of EphB plasma membrane aggregates, it remains a likely possibility that EphB aggregation and scaffolding, in the absence of induction of EphB tyrosine kinase activity, mediates some of the biological effects of EphBs (Himanen et al., 2007).

Transient loss-of-function

The problems of compensation and pleiotropism in knockouts can be solved by using approaches with better temporal control. Dominant negative mutant proteins are one such approach. Mutant or truncated proteins are overexpressed so that they will inactivate endogenous protein signaling, often by sequestering endogenous signaling partners or preventing the normal trafficking or folding of the target protein. A kinase-inactive form of EphB2 has been successfully overexpressed as a dominant negative. This presumably heterodimerizes with endogenous EphB2s and blocks their autophosphorylation. However, as with most dominant negatives, the precise mechanism is often unclear and may involve binding to PDZ-domain containing proteins. In addition, EphB2 dominant negative experiments require overexpression at levels far higher than those at which the endogenous proteins are expressed (Dalva et al., 2000; Ethell et al., 2001). While this dominant negative approach can assist the identification of possible functions of EphBs, its use in rigorous experiments designed to test endogenous signaling is limited.

While RNA interference (RNAi) constructs have been extensively used throughout neuroscience, they have not been widely used in the EphB field (Kayser et al.,

2006; Kayser et al., 2008; Parrinello et al., 2010). When used appropriately, RNAi can allow acute knockdown of genes. However, in the case of EphBs, knockdown is difficult to rescue since EphB overexpression has a synaptic phenotype. In addition, off-target effects, especially with sensitive assays measuring synapses, are a major problem. One study found that introduction of shRNA into neurons can trigger the loss of excitatory synapses and dendritic spines in a manner dependent upon the hairpin sequence, and not upon the target (Alvarez et al., 2006). Perhaps most confounding is the fact that shRNAs might have cell-health effects that are difficult to measure. We have performed preliminary studies using a published EphB2 RNAi (Kayser et al., 2006) that suggest this may indeed be the case.

One final acute loss-of-function approach is the use of unclustered ephrin ligands and Eph extracellular domains to block endogenous ephrin-B/EphB signaling (Contractor et al., 2002). The approach is based upon the idea that these unclustered proteins can soak up ephrins and Ephs on the cell surface and prevent their multimerization, thus rendering them inactive. As a control, unclustered FC proteins are often applied. While convenient, these soluble proteins may affect other cell surface proteins, thus leading to off-target effects that are difficult to measure. In addition, unclustered ephrin-B binding to EphB may not only block reverse signaling, but may also engage the receptor in unnatural modes of forward signaling.

Pharmacology

Considering the limitations of these previous approaches, an ideal strategy would be the design of inhibitors that could acutely and selectively inhibit EphB signaling in

vivo. Several groups have screened potential inhibitors using a variety of lead compounds, but with limited success. In targeting RTKs, the major strategies are usually to block one of the following processes: ligand binding; receptor dimerization; autophosphorylation; recruitment of adaptor proteins; signaling cascades; internalization and nuclear translocation; and biosynthesis (Bennasroune et al., 2004).

One group has developed the SNEW peptide, a 12-mer, which binds in the ligand-binding site of EphB2 (Koolpe et al., 2005). The peptide blocks the binding of ephrin-B2 to EphB2 at an IC₅₀ of 15 μ M (Chrencik et al., 2007) and blocks EphB activation at an IC₅₀ of 100 μ M (Noberini et al., 2012). As the IC₅₀s suggest, this peptide has limited potency.

Gray and colleagues have used a different approach to screen and identify more potent inhibitors based on the lead compound nilotinib that targets the kinase domain of EphBs. Several of their identified compounds inhibit EphB autophosphorylation at an IC₅₀ of 40 nM (Choi et al., 2009). However, these compounds are not sufficiently selective and inhibit a range of other kinases including Frk, Kit, and Lck.

One more group developed an assay to rapidly screen molecules that inhibit the kinase activity of EphB3 in vitro. An EphB3 peptide was incubated with the biotinylated tyrosine peptide substrate (BTK), biotin-AGAGLKKVVALY*DYMPM, where Y* denotes phosphorylation. After screening through a library of 120,000 small molecules for their ability to block BTK phosphorylation, they identified several potent inhibitors of EphB3 (Qiao et al., 2009). However, as with previous approaches, these were not sufficiently selective for mechanistic studies.

1.6 Summary of dissertation

While EphBs have been implicated in almost every stage of neural development, for the most part it remains to be determined which cellular processes require EphB receptor tyrosine kinase activity, and which cellular responses are mediated by EphB tyrosine kinase-independent signaling events. Since the EphB family of receptors has been shown to regulate a large number of developmental processes, it has been particularly challenging to determine the specific functions of EphBs at defined times during brain development. The presence of at least three partially redundant EphB family members expressed in the nervous system complicates experiments further. For example, the EphB1, EphB2, and EphB3 single and compound knockout mice display defects in a number of processes including stem cell proliferation, axon guidance, filopodial motility, dendritic spine formation, synapse development, and long-term potentiation (LTP), but it is unclear which of these interdependent phenotypes are primary and which are secondary to the disruption of EphB signaling (Grunwald et al., 2001; Henderson et al., 2001; Henkemeyer et al., 1996; Kayser et al., 2008).

Thus, new ways of selectively inhibiting specific functions of EphBs are needed to clarify the kinase-dependent and kinase-independent mechanisms by which EphBs control specific developmental events such as synapse formation and axon guidance.

In chapter 2, we describe the development of a chemical-genetic approach to selectively and acutely inhibit EphB tyrosine kinase activity. We generated knockin mice and demonstrate the utility of this approach in neurons.

In chapter 3, we use these knockin mice to investigate the function of EphB tyrosine kinase activity in axon guidance. We inhibit kinase signaling in several contexts and demonstrate a requirement for EphB tyrosine kinase activity in retinal and cortical axon guidance *in vivo*.

In chapter 4, we address the role of EphB tyrosine kinase signaling in synapse and dendritic spine development. Using dissociated neurons and organotypic slices from the knockin mice, we find that EphB tyrosine kinase signaling is not required for synaptic development.

Chapter 2

A chemical-genetic approach to studying EphB signaling

2.1 Background

Chemical-genetics

Despite the great interest in targeting protein kinases, the development of potent and selective inhibitors has proven challenging (Bishop et al., 1998; Blethrow et al., 2004). For small molecules which do successfully inhibit a kinase, there is no straightforward way to assess specificity in cell-based systems and in vivo. Indeed, many successful drugs in the clinic affect numerous targets in humans and their relevant mechanisms remain unclear. While this ambiguity may be acceptable in medicine, it is not suitable in the scientific investigation of basic biology. Even if selective inhibitors can be developed for in vitro use, their pharmacokinetic properties must be acceptable for use in vivo.

To overcome these hurdles, a chemical-genetic strategy has been developed combining the advantages of pharmacology and genetics. Using this approach, drug-sensitivity can be engineered into a protein, allowing for more controlled experimentation. This chemical-genetic technique was pioneered by Kevan Shokat and colleagues of the University of California, San Francisco.

Analogue-sensitive kinase alleles (ASKA)

In the 1990s, the laboratory of Kevan Shokat developed new methods of protein engineering to identify kinases that could accept unnatural ATP analogs. This approach aimed to uniquely label the substrates of a specific kinase and thus gain new insights into cell signaling. Based on available crystal structures of serine/threonine kinases, Shokat

and colleagues mutated Src at two sites and identified a mutation at residue 338 in the ATP-binding pocket that allowed the mutant Src to accept the ATP analog N⁶-(cyclopentyl)-ATP (Shah et al., 1997). While these mutant kinases could utilize new unnatural nucleotides (and thus facilitate labeling), they were also made sensitive to new synthetic inhibitors (Bishop et al., 1998). In fact, the investigators discovered that it was residue 338 that conferred the structural basis of Src inhibition by the previously used drug 4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]-pyrimidine (PP1) (**Figure 2.1B**) (Liu et al., 1999).

This approach was soon generalized. The ATP-binding pocket of all kinases contains a bulky hydrophobic “gatekeeper” residue, amino acid 338 in Src. When mutated to an alanine or a glycine, the kinase can be rendered sensitive to inhibition by bulky PP1 analogs that cannot effectively enter the wild type ATP-binding pocket (Alaimo et al., 2001; Bishop et al., 1998). These are known as analog-sensitive (AS) kinases (**Figure 2.1A, B**). Importantly, these mutant kinases can still accept ATP in the absence of PP1 analogs.

After its initial conception, this strategy was successfully used in heterologous cells (Bishop et al., 1998) and soon after in a budding yeast model (Bishop et al., 2000). Investigators were able to acutely inhibit cdc28 kinase function and isolate its roles in the cell cycle, altering time and dose to parse its function in vivo.

In 2005, an analog-sensitive knockin mouse was generated to study neurotrophin signaling in vivo (Chen et al., 2005). TrkA, TrkB, and TrkC were all successfully targeted and specific inhibition was achieved in vivo, as assessed through survival assays of the superior cervical ganglia (TrkA), nodose ganglia (TrkB), and dorsal root ganglia

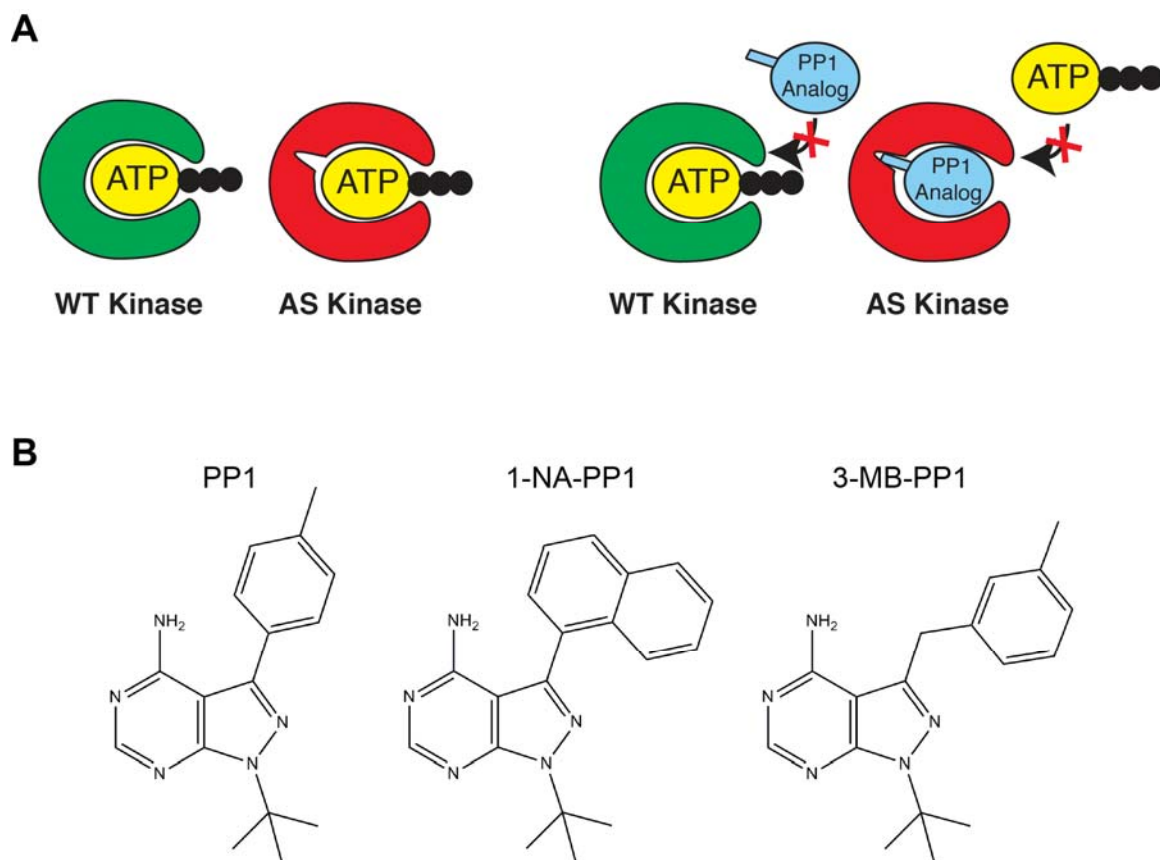


Figure 2.1: Analog-sensitive kinases alleles (ASKA)

(A) Schematic demonstrating the chemical-genetic strategy of targeted kinase inhibition. Analog-sensitive (AS) kinases contain a mutation in the ATP-binding pocket which does not alter normal kinase function (left). However, this mutation renders the AS kinases sensitive to competitive inhibition by PP1 analogs that cannot efficiently enter the ATP-binding pocket of WT kinases (right).

(B) Chemical structures of:

4-Amino-5-(4-methylphenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (**PP1**)

4-amino-1-*tert*-butyl-3-(1'naphthyl)pyrazolo[3,4-*d*]pyrimidine (**1-NA-PP1**)

1-(*tert*-Butyl)-3-(3-methylbenzyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (**3-MB-PP1**)

(TrkC). These mice were later used to address new questions in neurotrophin signaling, such as in isolating the spatial requirements of TrkA signaling on cell/dendrites as opposed to axons in regulating synaptic assembly in sympathetic neurons (Sharma et al., 2010).

We reasoned that this technology would be especially appropriate for the EphBs because of their pleiotropism and multiple signaling domains.

2.2 EphB analog-sensitive kinases

Generation of AS-EphBs kinases

To determine the feasibility of generating AS-EphBs, we compared the amino acid sequence of kinase domains of EphBs with those of related tyrosine kinases for which AS versions had been successfully generated (Bishop et al., 2000; Chen et al., 2005). This analysis revealed a conserved gatekeeper threonine residue in the ATP-binding site of mouse EphB1, EphB2, and EphB3. Using DNA plasmids in the pcs2+ vector, we mutated these gatekeeper residues to either alanine or glycine to generate EphB1^{T697G}, EphB2^{T699A}, and EphB3^{T706A} AS receptors (**Figure 2.2**).

Validation in heterologous cells

To verify that the mutations introduced into the EphB ATP-binding sites did not affect the kinase activity of EphBs in the absence of the PP1 analogs, we assessed the activity of these kinases using a heterologous cell culture system. We previously

avSrc	328	VVSEE-PIYIV I EYMSKGSLLDFLKG	AS Mutation:
mTrkB	605	VCVEGDPLIMV F EYMKHGDLNKFLRA	
mEphB1	686	VVTKSRPVMII T EFMENGALDSFLRQ	T697G
mEphB2	688	VVTKSTPVMII T EFMENGSLDSFLRQ	T699A
mEphB3	685	VVTKSRPVMIL T EFMENCALDSFLRL	T706A

Figure 2.2: Sequence alignment of EphB kinase domains

Amino acid alignment of kinase domains of mouse EphBs with those of Src and TrkB. The gatekeeper residue is highlighted in red and the drug-sensitive mutation (AS) in EphBs is shown on the right.

generated an antibody that specifically recognizes the phosphorylated form of these juxtamembrane tyrosine residues for all EphBs and used these anti-phospho-EphB antibodies as a readout for receptor kinase activity (Dalva et al., 2000). In order to study EphB autophosphorylation without the need for ligand application, we overexpressed the receptors in Human Embryonic Kidney (HEK) 293 cells where EphBs cluster spontaneously and are activated even in the absence of ligand.

We probed lysates from cells overexpressing AS-EphBs or WT EphBs with the anti-phospho-EphB antibody, and found that WT and AS-EphB receptors are phosphorylated at their juxtamembrane tyrosines to a similar extent (**Figure 2.3A**). This

indicates that the AS mutation does not affect the ability of EphBs to activate their kinase domains in the absence of PP1 analogs.

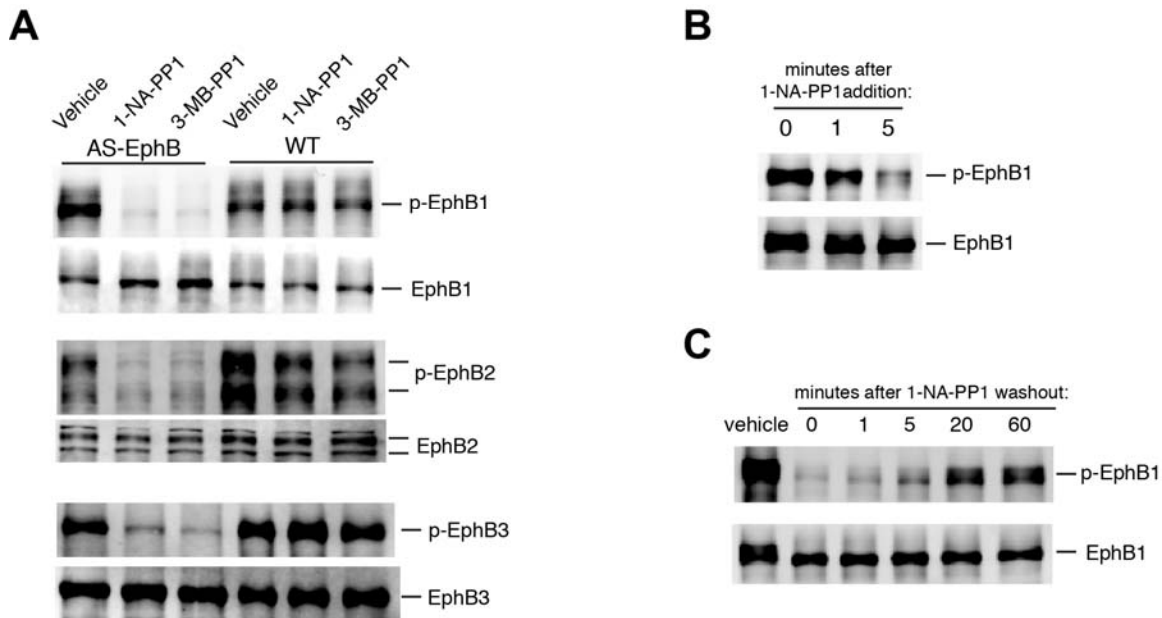


Figure 2.3: Rapid, reversible, and selective inhibition of AS EphBs

(A) Inhibition of the kinase function of heterologously expressed EphB1, EphB2, and EphB3 in HEK 293 cells. EphB-expressing cells were incubated in 1-NA-PP1 (250 nM) or 3-MB-PP1 (1 μ M) for 1 hour before lysis in SDS sample buffer, followed by western blotting for total EphB levels or auto-phosphorylated EphBs to assess kinase activation.

(B) Time course of EphB1 inhibition in minutes after 1-NA-PP1 (250 nM) addition to EphB1-expressing HEK 293 cells.

(C) Time course of recovery of EphB1 autophosphorylation in minutes after 1-NA-PP1 (250 nM) washout from EphB1-expressing HEK 293 cells. The zero time point reflects the removal of 1-NA-PP1 after an initial 1 hour incubation.

To test the ability of PP1 analogs to inhibit AS-EphBs, we treated cells overexpressing AS-EphBs or wild type EphBs with the bulky PP1 analog 4-amino-1-*tert*-butyl-3-(1'naphthyl)pyrazolo[3,4-*d*]pyrimidine (1-NA-PP1) (**Figure 2.1B**) and assessed EphB tyrosine autophosphorylation. We found that incubation with 1-NA-PP1 (250nM) blocked the phosphorylation of AS-EphBs but not the phosphorylation of WT EphBs (**Figure 2.3A**). Subsequent screening through a range of other inhibitors identified the PP1 analog 1-(*tert*-Butyl)-3-(3-methylbenzyl)-1H-pyrazolo[3,4-*d*]pyrimidin-4-amine (3-MB-PP1) (**Figure 2.1B**) as another potent and selective inhibitor of AS-EphBs (**Figure 2.3A**).

The PP1-derivates were designed for cell permeability. To test how rapidly we could inhibit EphB autophosphorylation, we added inhibitor for increasing lengths of time before lysing cells. We found that inhibition of kinase activity of AS-EphBs was acute, occurring within minutes (**Figure 2.3B**). Inhibition was rapidly reversible upon removal of 1-NA-PP1, with autophosphorylation resuming within minutes (**Figure 2.3C**). These properties make the inhibitors ideal for studying dynamic processes in cells.

To quantify the potency and specificity of 1-NA-PP1 and 3-MB-PP1 with respect to inhibition of each EphB, we generated dose-response curves ranging four orders of magnitude from 5nM to 21uM (**Figure 2.4A, B**). Western blots were quantified and plots were fitted with a standard Hill coefficient of -1. IC50s were calculated and revealed a preference for inhibiting AS kinases (9-48 nM) over WT kinases (0.6-8.9 μ M) by two orders of magnitude. (**Figure 2.4A, B**). These results indicate that the kinase activity of AS-EphBs is selectively inhibited by PP1 analogs.

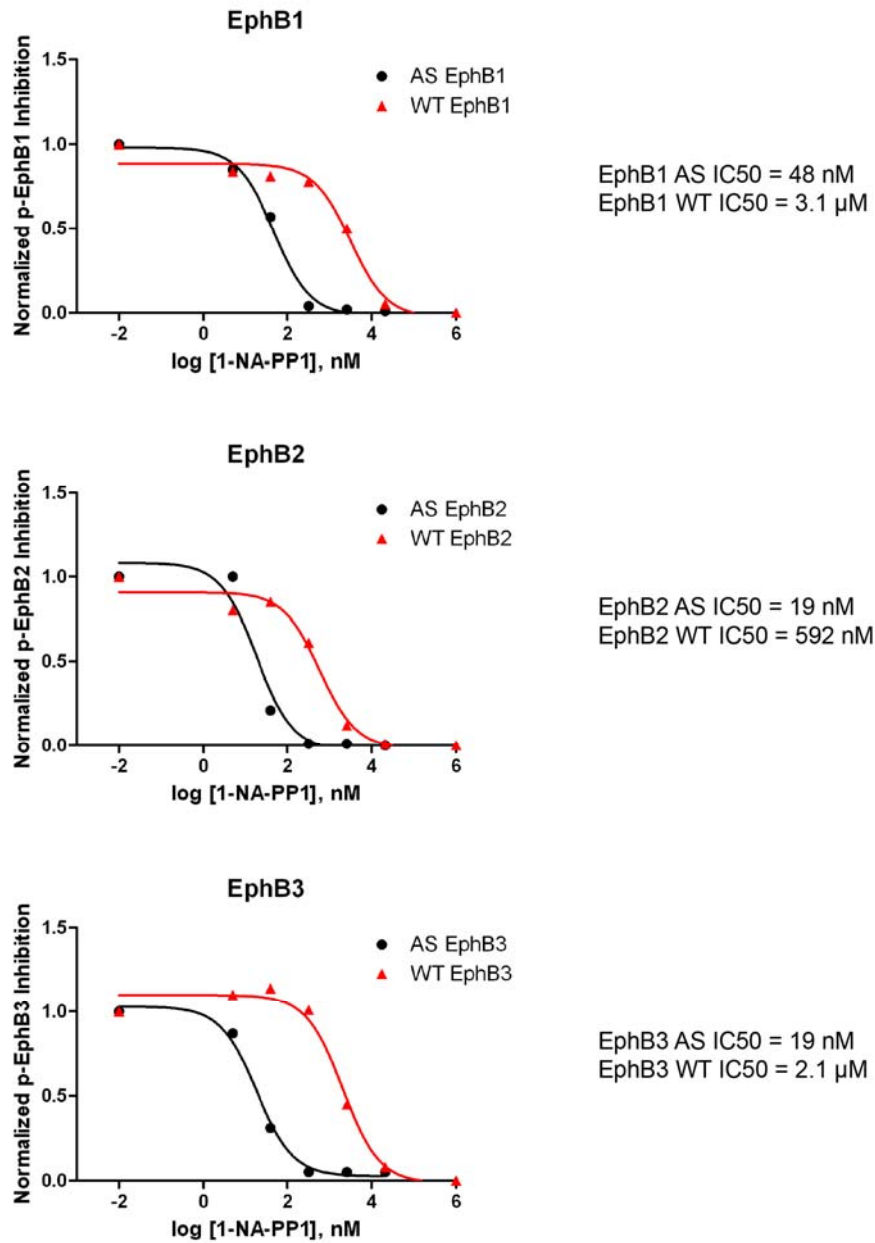
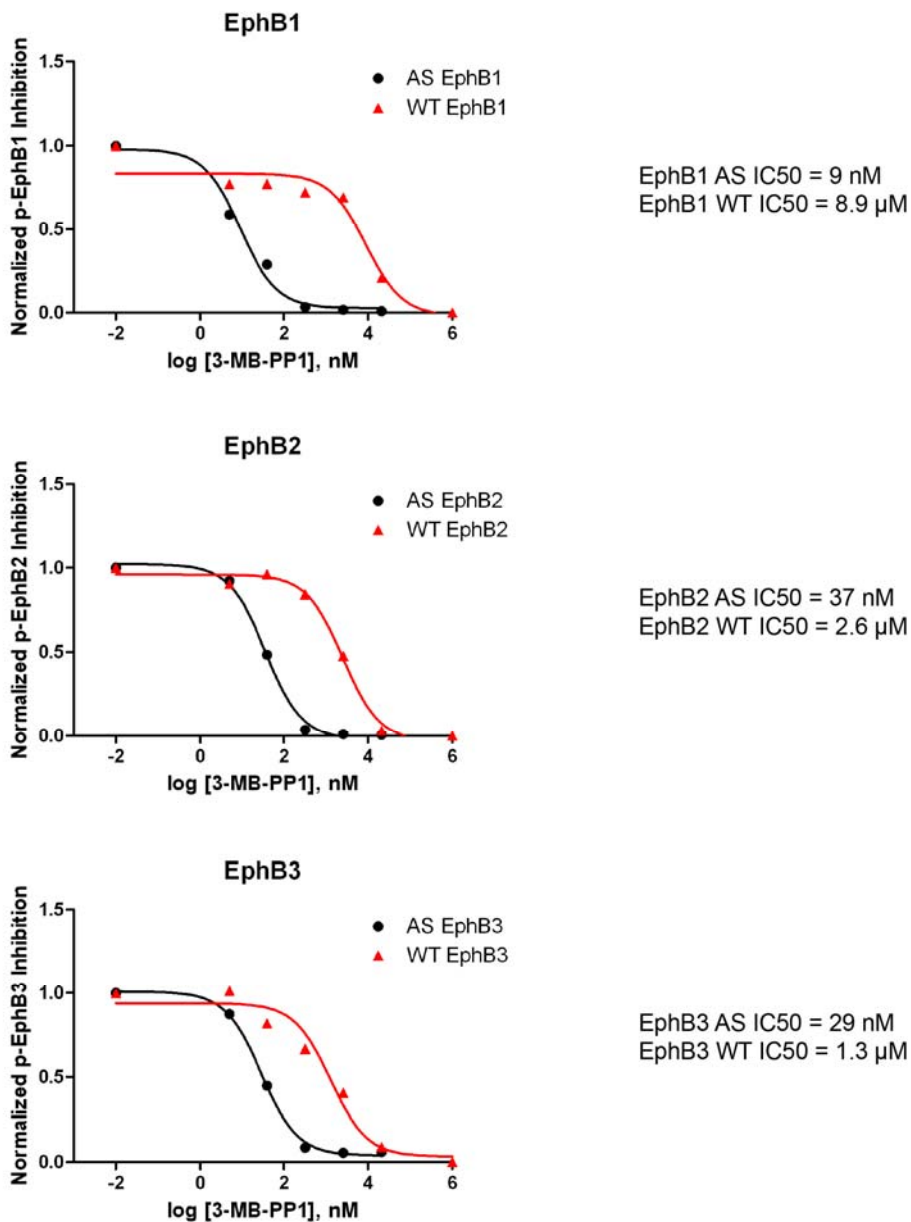
A

Figure 2.4: Dose-response curves of EphB inhibition after treatment with PP1 derivatives

(A) Dose response curves from inhibition of EphBs with 1-NA-PP1 in HEK 293 cells. Curves were fitting from 6-7 quantitative western blot data points using the least-squares method with a Hill coefficient of -1. The following R^2 values describe the fitting to derive IC₅₀ values: EphB1 AS (0.99), EphB1 WT (0.98), EphB2 AS (0.96), EphB2 WT (0.98), EphB3 AS (0.99), EphB3 WT (0.99).

B**Figure 2.4 (continued)**

(B) Dose response curves from inhibition of EphBs with 3-MB-PP1 in HEK 293 cells. The following R^2 values describe the least-squares fitting to derive IC₅₀ values: EphB1 AS (0.99), EphB1 WT (0.94), EphB2 AS (0.996), EphB2 WT (0.99), EphB3 AS (0.998), EphB3 WT (0.97).

2.3 Generation of AS-EphB TKI mice

Mouse genetics

Based on these initial experiments, we designed knockin mice harboring the gatekeeper point mutation in EphB1 (T697G), EphB2 (T699A), and EphB3 (T706A), the three neuronally expressed EphB receptor tyrosine kinases. Targeting vectors were designed against exon 11 of chromosome 9 for EphB1 (**Figure 2.5A**), exon 11 of chromosome 4 for EphB2 (**Figure 2.5B**), and exon 11 of chromosome 16 for EphB3 (**Figure 2.5C**). Blastocysts injections yielded chimeras which were mated to C57Bl/6 WT mice. Subsequent breeding of knockin mice was validated by direct sequencing for the AS mutation (**Figure 2.6A**) and by PCR amplification to detect the presence of the loxP site (**Figure 2.6B**).

Previous studies investigating neural progenitor proliferation and dendritic spine development have demonstrated the redundancy of EphBs in several contexts (Chumley et al., 2007; Henkemeyer et al., 2003). To overcome potential functional compensation by different EphB family members, heterozygote F1 offspring of EphB1^{T697G}, EphB2^{T699A}, and EphB3^{T706A} single mutant mice were intercrossed for several generations to produce homozygous triple knockin mice, designated EphB-analog sensitive triple knockin (AS-EphB TKI) mice (**Figure 2.6C**).

Whereas EphB1/EphB2/EphB3 triple knockout (TKO) mice suffer from profound developmental defects including numerous morphological abnormalities (Risley et al., 2009; Yucel et al., 2007), AS-EphB TKI mice develop normally into healthy, fertile adults, indistinguishable from WT mice.

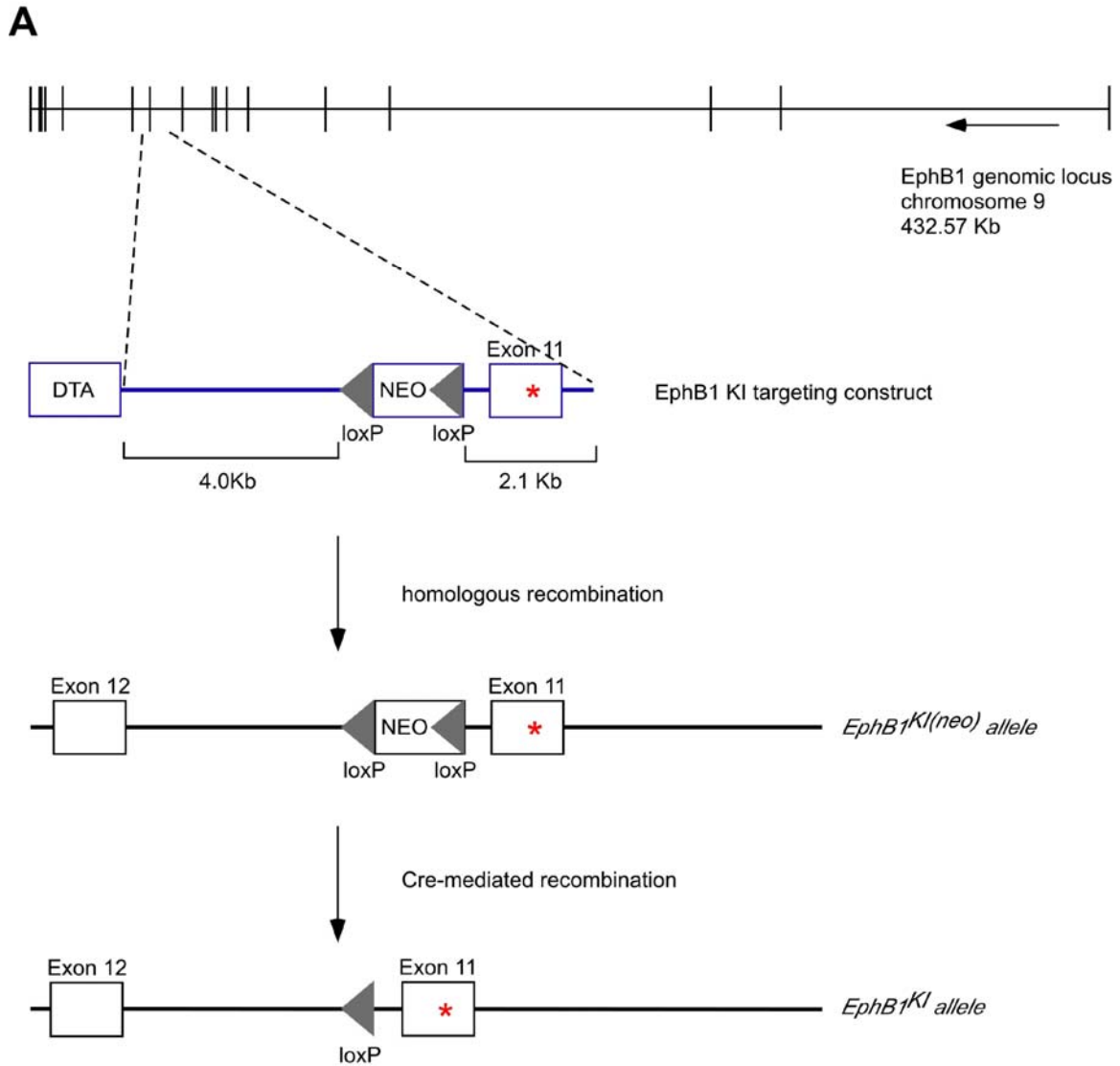


Figure 2.5: AS-EphB KI mouse targeting strategy

(A) Targeting strategy for generation of the analog-sensitive (AS) mutation in exon 11 of the *ephb1* gene located on chromosome 9.

* denotes the AS mutation

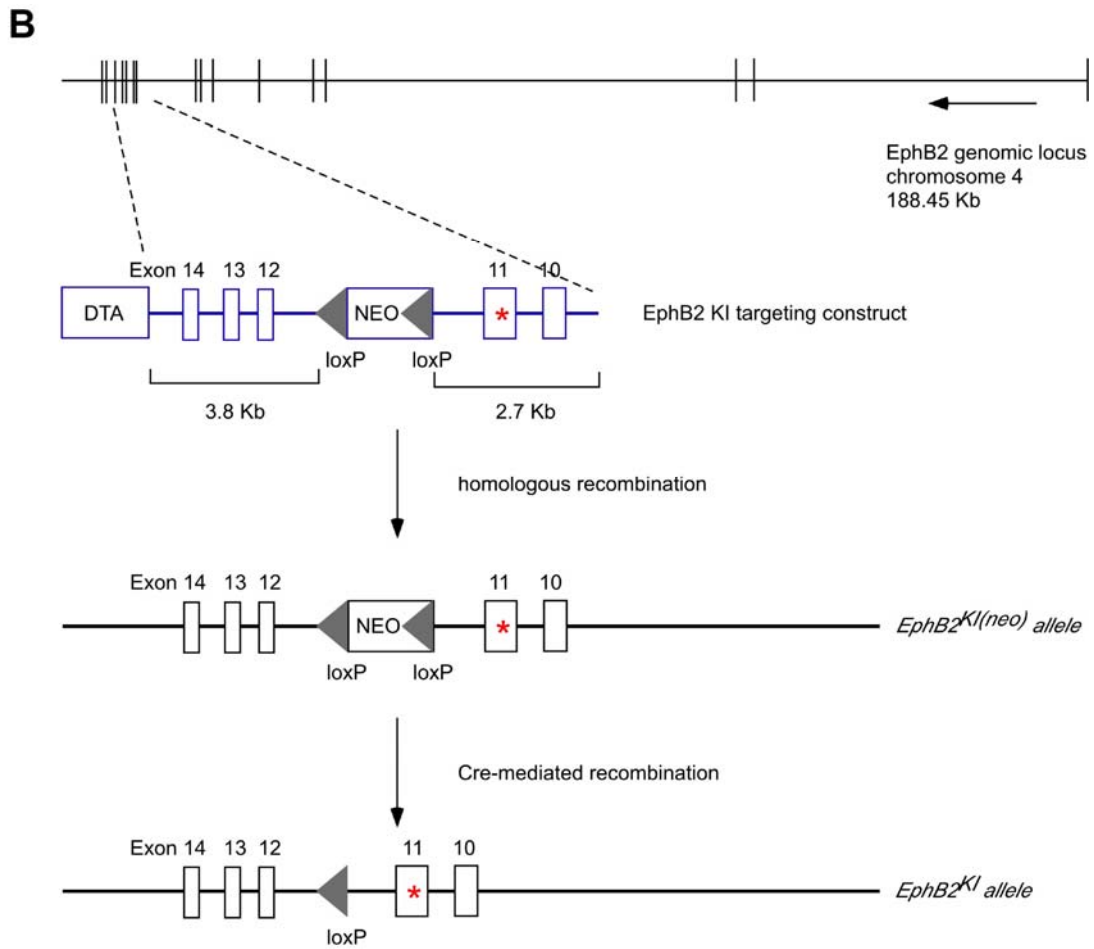


Figure 2.5 (continued)

(B) Targeting strategy for generation of the analog-sensitive (AS) mutation in exon 11 of the *ephb2* gene located on chromosome 4.

* denotes the AS mutation

C

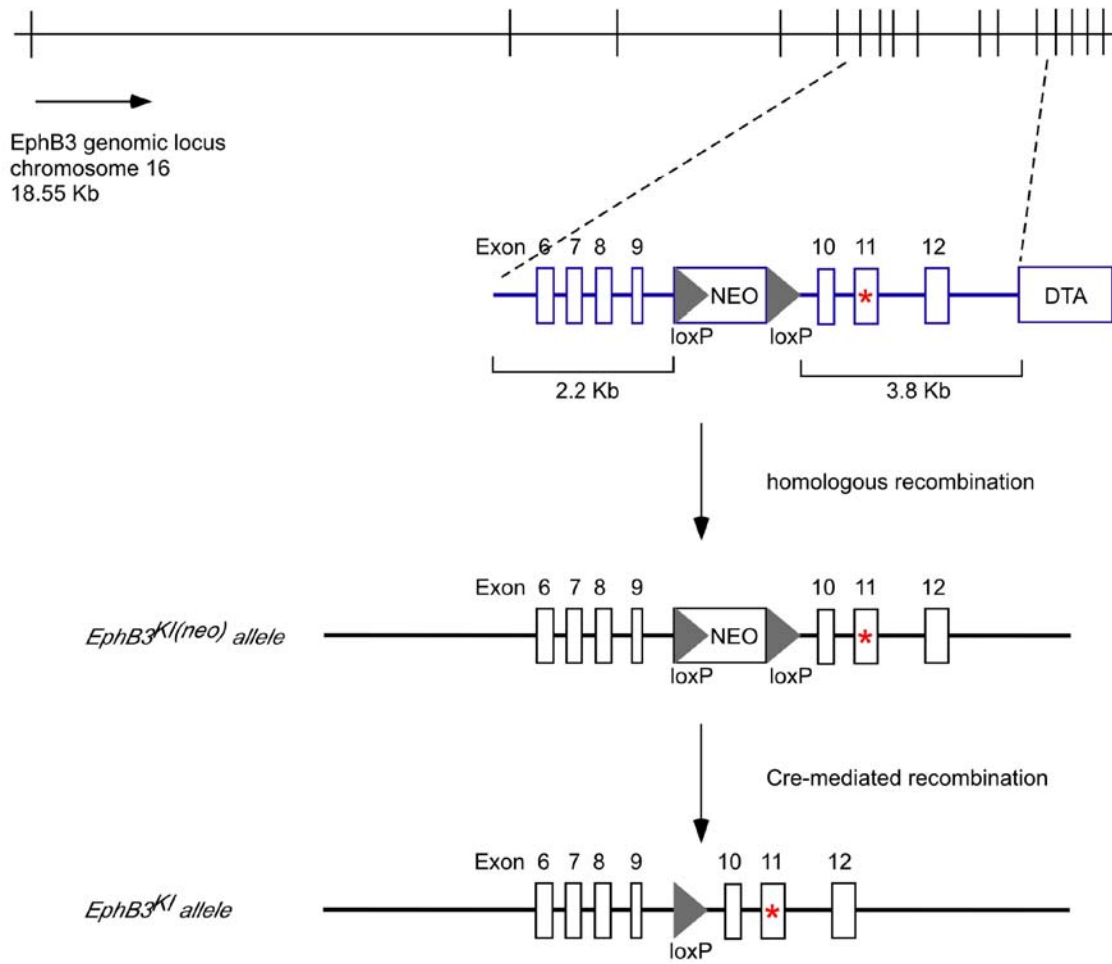


Figure 2.5 (continued)

(C) Targeting strategy for generation of the analog-sensitive (AS) mutation in exon 11 of the *ephb3* gene located on chromosome 16.

* denotes the AS mutation

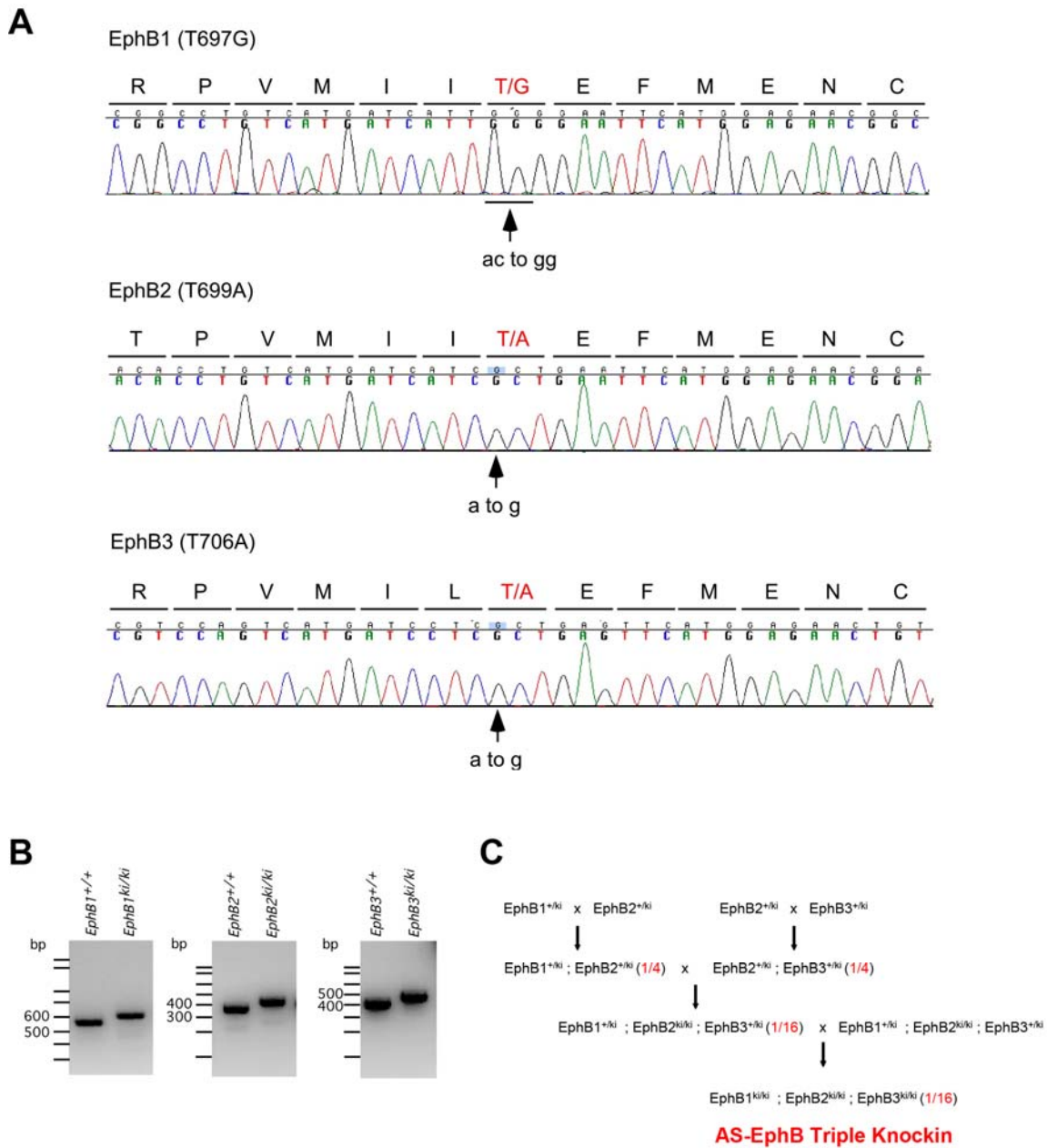


Figure 2.6: Validation of the AS mutation in AS-EphB TKI mice

(A) Sequencing reads from EphB-AS TKI mice showing the gatekeeper (AS) mutation in the *ephb1*, *ephb2*, and *ephb3* genes.

(B) Genotyping PCR showing the presence of a novel band in DNA from AS-EphB TKI mice reflecting the insertion of a loxP site.

(C) Breeding strategy to obtain AS-EphB TKI mice from single heterozygote mutants.

For example, anogenital defects in EphB TKOs are not observed in AS-EphB TKI mice. AS-EphB TKI brains exhibit normal morphology and are of equal size to WT mouse brains. In vitro assays measuring axon guidance, neuronal morphology, and synaptic development revealed no differences between WT and AS-EphB TKI mice (shown below), suggesting that AS-EphBs function normally in the absence of PP1 analogs.

EphB-AS TKI mice are normal in the absence of PP1 derivatives

Critical to the interpretation of experiments comparing wild-type and AS-EphB TKI mice is evidence that EphB mRNA expression and ligand-mediated receptor activation occur normally in AS-EphB TKI neurons in the absence of PP1 analogues. To assess whether EphB gene expression was affected by the gatekeeper residue mutations or the residual loxP sites that were introduced during generation of the mice, we performed quantitative PCR (qPCR) to measure expression of EphB1, EphB2, and EphB3 in neurons from WT and AS-EphB TKI mice. We found that the mutant alleles were expressed at the same levels as WT (**Figure 2.7A**). We conclude that the gene targeting did not affect the levels of EphB1, EphB2, or EphB3 mRNA expression in AS-EphB TKI neurons.

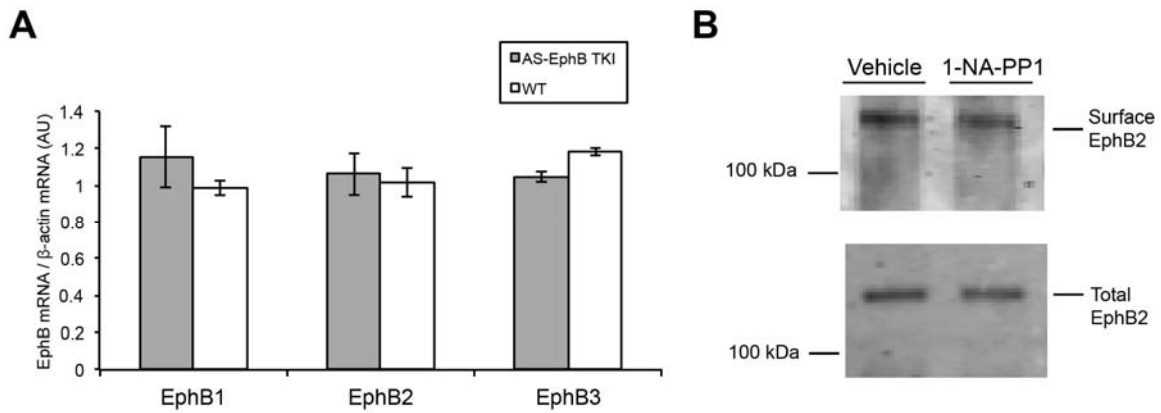


Figure 2.7: Normal EphB expression and surface localization in AS-EphB TKI mice

(A) Relative mRNA expression of EphB1, EphB2, and EphB3 normalized to β -actin in AS-EphB TKI vs. WT cultured cortical neurons at 7DIV. Data are mean \pm SEM.

(B) Surface expression of EphB2 at 12 DIV in cultured cortical neurons after treatment with vehicle or 1 μ M 1-NA-PP1 from 6-12 DIV. Surface EphB2 was isolated by treating cells with sulfo-NHS-biotin, immunoprecipitating EphB2, and probing eluates with fluorescent streptavidin.

We reasoned that receptor trafficking and internalization might be perturbed by the AS mutation or the inhibition of EphB tyrosine kinase activity. We performed surface biotin labeling for the EphB2 receptor after chronic inhibition from 6-12 days in vitro (DIV) in cortical neurons. We found equivalent levels of labeled EphB2 in vehicle and 1-NA-PP1 (1 μ M)-treated cells (**Figure 2.7B**). This result indicates that EphBs can properly traffic to the cell surface in AS-EphB TKI neurons in the presence of PP1 analogs.

The analog-sensitive kinase approach is designed to block EphB tyrosine kinase function and leave other signaling modalities intact. The clustering of EphBs allows them to serve as scaffolds and is thought to be kinase-independent. To test whether

inhibiting the tyrosine kinase activity of EphBs affected their higher-ordered clustering, cultured cortical neurons were stimulated with ephrin-B1, fixed, and stained with antibodies for EphB2. Large puncta were observable by 30 minutes and increased in number by 60 minutes. These were not seen after stimulation with clustered FC or after stimulation with ephrin-B1 and replacement of anti-EphB2 with a rabbit IgG. After inhibition with 1-NA-PP1 (250 nM) or 3-MB-PP1 (1 μ M), EphB2 still formed large clusters, suggesting that high-order receptor oligomerization does not rely on the kinase signaling of EphBs (**Figure 2.8**). We therefore conclude that EphBs should be able to cluster cytoplasmic proteins via kinase-independent mechanisms.

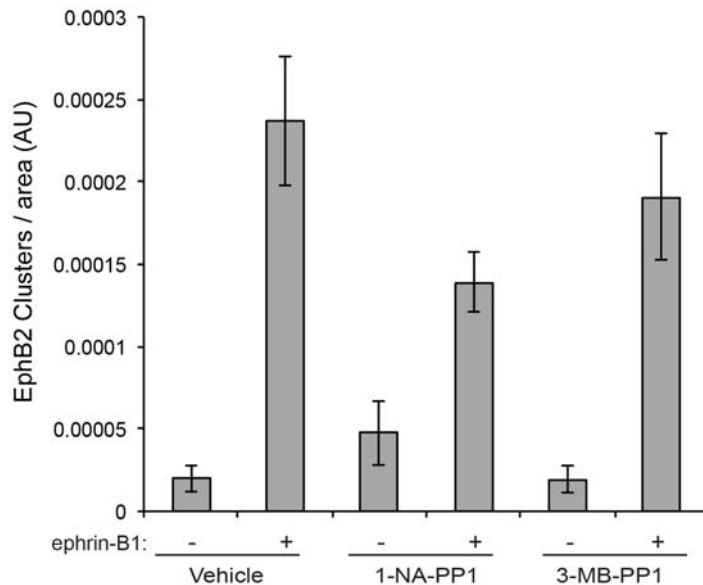


Figure 2.8: EphB clustering persists in the absence of EphB tyrosine kinase activity

1 DIV cortical neurons were treated with vehicle, 250 nM 1-NA-PP1, or 1 μ M 3-MB-PP1 for 1 hour before a 60 minute ephrin-B1 stimulation. Cells were fixed, blocked, and stained for MAP-2 and EphB2. Data represent the number of EphB2 clusters per unit area. N=11-16 fields of view (63x) per condition.

We next asked whether neurons from AS-EphB TKI mice could engage in normal ephrin ligand-mediated tyrosine kinase signaling in the absence of PP1 derivatives. We cultured dissociated cortical neurons from embryonic AS-EphB TKI or WT mice and stimulated the neurons with clustered ephrin-B1 for 30 minutes. Western blotting of AS-EphB TKI or WT lysates with phospho-EphB antibodies revealed that both WT and AS-EphB TKI neurons exhibited robust EphB activation to similar levels, thus indicating that AS EphBs are fully competent to engage in kinase signaling in the absence of PP1 analogs (**Figure 2.9A**).

EphB tyrosine kinase signaling is blocked by PP1 derivatives in EphB-AS TKI mice

To test whether the kinase function of EphBs from AS-EphB TKI mice is potently and selectively inhibited by PP1 analogs, we pretreated cultured neurons with vehicle (DMSO) or PP1 analogs for 1 hour before ephrin-B1 stimulation. Treatment with 250 nM 1-NA-PP1 or 1 μ M 3-MB-PP1 completely blocked ephrin-B induced EphB activation in the AS-EphB TKI, but not WT neurons (**Figure 2.9A**), thus demonstrating the efficacy and selectivity of these PP1 analogs in AS-EphB TKI neurons.

To more rigorously test the selectivity of these inhibitors, we assessed the effect of inhibitors on EphA signaling. Cortical neurons from WT mice were pretreated with 250 nM 1-NA-PP1 or 1 μ M 3-MB-PP1 and stimulated with ephrin-A1 for 30 minutes. Lysates were immunoprecipitated with antibodies against EphA4 and probed for phospho-Eph or total EphA4. We found no inhibition of EphA4 autophosphorylation at doses of PP1 derivatives that fully block EphB tyrosine kinase function (**Figure 2.9B**),

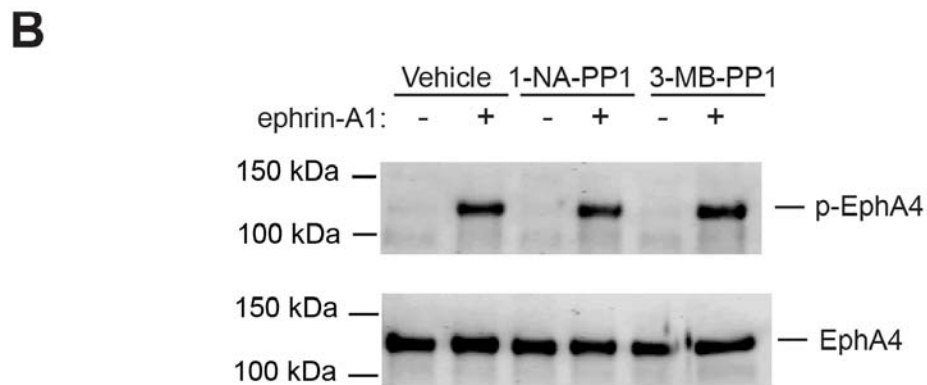
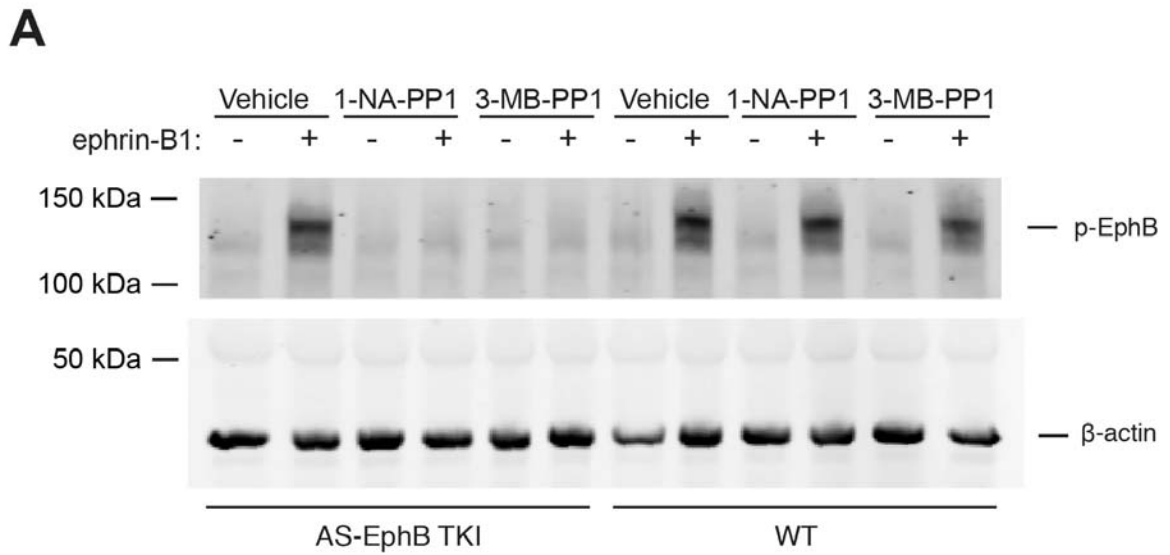


Figure 2.9: Selective inhibition of EphBs, but not EphAs, in AS-EphB TKI neurons

(A) 15 DIV cortical neurons were treated with vehicle, 250 nM 1-NA-PP1, or 1 μ M 3-MB-PP1 for 1 hour before a 30 minute ephrin-B1 stimulation. Lysates were blotted for phospho-EphB or β -actin.

(B) 4 DIV cortical neurons were treated with vehicle, 250 nM 1-NA-PP1, or 1 μ M 3-MB-PP1 for 1 hour before a 30 minute ephrin-A1 stimulation. Lysates were immunoprecipitated with anti-EphA4 antibodies and blotted for EphA4 and phospho-Eph.

indicating that EphA tyrosine kinase signaling remains unperturbed even when EphB tyrosine kinase signaling is blocked. The specificity of AS inhibition between Eph family members contrasts with previous attempts at designing selective inhibitors for EphBs.

Kinase cascades can amplify signals greatly, making inhibition of downstream pathways difficult. To test whether inhibition of the kinase activity of EphBs effectively blocks the phosphorylation of substrates, we studied phosphorylation of the well-characterized Eph substrate Vav2, a Rho family guanine nucleotide exchange factor (GEF) that mediates growth cone collapse (Cowan et al., 2005). WT or AS-EphB TKI neurons were incubated in 1-NA-PP1 (250 nM) or 3-MB-PP1 (1 μ M) and stimulated with ephrin-B1 for 30 minutes to induce Vav2 tyrosine phosphorylation. Immunoprecipitation of Vav2 followed by western blotting with a pan-phosphotyrosine antibody showed a 2- to 3-fold increase in tyrosine phosphorylation of Vav2 after ephrin-B1 stimulation (**Figure 2.10A, B**). Treatment with 1-NA-PP1 (250 nM) or 3-MB-PP1 (1 μ M) selectively blocked the increase in p-Vav2 in AS-EphB TKI cells, but had no effect in WT cells (**Figure 2.10A, B**). We conclude that PP1 analogs can selectively block the kinase signaling of EphBs and the phosphorylation of their substrates in AS-EphB TKI cells.

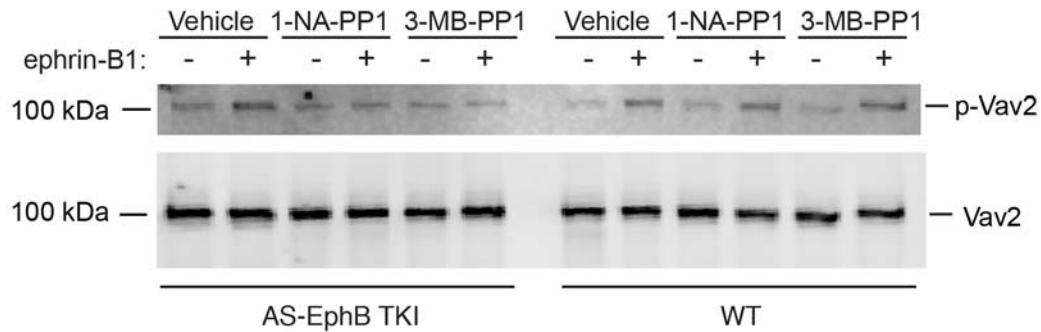
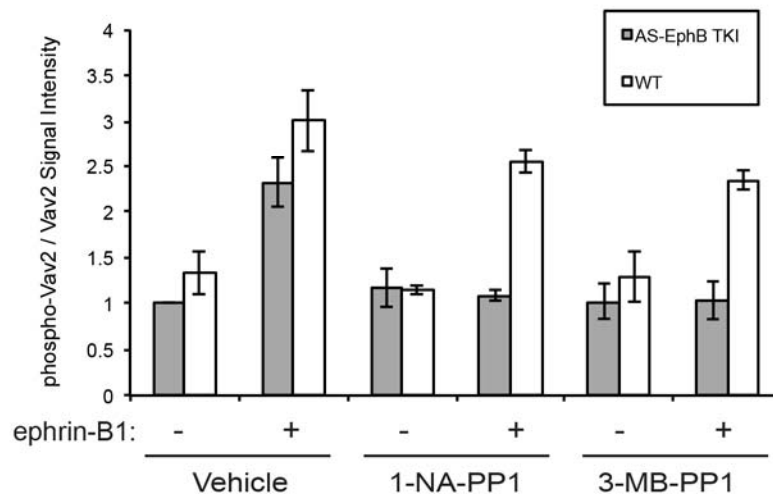
A**B**

Figure 2.10: PP1 derivatives block the phosphorylation of EphB substrates in AS-EphB TKI neurons

(A) 4 DIV cortical neurons were treated with vehicle, 250 nM 1-NA-PP1, or 1 μ M 3-MB-PP1 for 1 hour before a 30 minute ephrin-B1 stimulation. Lysates were immunoprecipitated with anti-Vav2 antibodies, and blotted with pan-phosphotyrosine (py99) or total Vav2 antibodies.

(B) Quantification of phospho-Vav2 / Vav2 band intensity from quantitative western blots in (A). Graph represents mean \pm SEM (n=3). Values were normalized to the unstimulated AS-EphB TKI vehicle-treated condition.

2.4 Discussion

We have generated the first mice in which it is possible to conditionally block EphB tyrosine kinase signaling. Our chemical-genetic approach offers several advantages over conventional genetic loss-of-function studies (Knight and Shokat, 2007); these advantages are particularly relevant for studying EphBs given the multitude of mechanisms used by EphBs to regulate nervous system development. Since we are able to block the kinase activity of EphBs while leaving their scaffolding and reverse signaling capabilities intact, it is possible to dissect the role of the kinase activity of EphBs *in vivo* under conditions where EphBs are expressed at their normal levels. In addition, acute AS-EphB inhibition can circumvent the problem of developmental compensation that sometimes occurs after genetic lesions. As there are no conditional EphB1/EphB2/EphB3 mice yet available, the AS-EphB TKI mice will provide an appealing and in some ways more informative alternative for many experiments. Additionally, the reversibility of AS EphB inhibition should allow for temporal control in experiments investigating synaptic plasticity and behavior.

The analog sensitivity of AS-EphB TKI mice should make it possible to determine in greater detail which of the many defects observed in EphB triple knockout mice are a direct consequence of the loss of the tyrosine kinase activity of EphBs and which defects are due to a loss of kinase-independent signaling. Finally, unlike conventional pharmacology, the AS-EphB knockin approach makes it possible to control for off-target effects of the PP1 analogs: each experiment can compare the effects of the inhibitors in AS-EphB TKI mice with those seen in WT mice which are identical except for the gatekeeper mutations within the ATP-binding sites of EphBs.

This chemical-genetic approach also has some disadvantages. Certain *in vivo* systems may be difficult to access with the inhibitors, as methods of drug delivery to the nervous system are often not trivial. In addition, the specificity for inhibiting tyrosine kinase signaling is useful, but it cannot distinguish other modes of signaling, such as reverse or scaffolding, from each other. Finally, a problem inherent to all EphB loss-of-function approaches is that perturbing the kinase signaling may affect other aspects of EphBs. While this cannot be completely determined, since effects may be small or difficult to measure, it is important to keep in mind when drawing conclusions.

Our experiments indicate that the doses of 1-NA-PP1 (250 nM) and 3-MB-PP1 (1 μ M) that we are using are relatively specific. Although it is not practical to screen through all kinases for off-target effects in our AS-EphB TKI mice, a study by Bain et al. has used an *in vitro* system to test the specificity of 1-NA-PP1 on other kinases using robotic screens. They found only two kinases, RIP2 and CKI, with IC₅₀s for 1-NA-PP1 below 250 nM; however, both IC₅₀s were still significantly higher than for the AS-EphB kinases (Bain et al., 2007). Although these data suggest that 1-NA-PP1 is highly selective for EphBs in AS-EphB TKI cells, WT controls can still be used in every experiment to control for off-target effects.

Even with WT controls, it is possible that 1-NA-PP1 and 3-MB-PP1 inhibit other kinases to a small degree, thus creating a sensitizing background for certain readouts (Bain et al., 2007). Thus, it is not strictly possible to conclude that inhibiting EphB tyrosine kinase function is sufficient to cause any phenotypes detected (since dozens of other kinases may be slightly inhibited and promote a phenotype), but it is possible to conclude that EphB tyrosine kinase signaling is required. These limitations are well

within the scope of what might be expected for a novel technique, and still offer significant advantages over previous approaches.

2.5 Materials and methods

Generation of AS-EphB TKI mice

EphB1^{T697G}, EphB2^{T699A}, and EphB3^{T706A} single mutants were generated individually by homologous recombination in mouse embryonic stem (ES) cells. Mice harboring each of the KI mutations were then bred together through > 3 generations to obtain the triple homozygous AS-EphB TKI mice (**Figure 2.6C**).

To facilitate the construction of three targeting constructs for modifying each of EphB1, EphB2 and EphB3 loci, a general purpose knockin vector (pKSNeoDTAIII) was first built from pKSNeoDTA (originally constructed in the lab of Philippe Soriano) to contain the following features: a first multiple cloning site containing NotI, FseI and HindIII sites (for inserting the 5' homologous arm), a loxP-PGK-Neo-loxP cassette for positive selection of ES cell clones, followed by a second multiple cloning site containing AscI, PacI, EcoRV and EcoRI sites (for inserting the 3' homologous arm), and finally a diphtheria toxin A negative-selection cassette (DTA). The pKSNeoDTAIII vector was used to construct all three EphB KI targeting vectors (**Figure 2.6A**).

To generate the EphB1 targeting construct, the 2.1 Kb 5' arm was PCR amplified from J1 ES cell genomic DNA using a two-step PCR procedure. In the first step, primers EphB1-5outer-F and EphB1-5outer-R (**Table 2.1**) were used to enrich the target sequence using the Expand Long Template PCR system (Roche) for 15 cycles. The low number of cycles was designed to minimize the introduction of mutations. The product from this

first-step PCR, which was in an insufficient quantity to be visualized on an ethidium bromide-stained agarose gel, was column purified and used as template in the second-step, nested PCR (primers EphB1-5F-Not and EphB1-5R-Fse) using the high fidelity DNA polymerase PFU for 25 cycles. The product was then subcloned into the first multiple cloning site of pKSNeoDTAIII through the NotI and FseI sites. The 4 Kb 3' arm was similarly amplified using primers EphB1-3outer-F and EphB1-3outer-R in the first-step PCR and primers EphB1-3F-Asc and EphB1-3R-Pac in the second nested PCR reaction. The 3' arm was subcloned into pKSNeoDTAIII through the AscI and PacI sites. The EphB1 gatekeeper mutation was introduced by QuickChange (Stratagene) site-directed mutagenesis. The final targeting construct was confirmed by direct sequencing.

PRIMER NAME	PRIMER SEQUENCE	PURPOSE
EphB1-5outer-F	GCATCCTTATGTGTGGGTGGG	1st step PCR for EphB1 5' arm
EphB1-5outer-R	TCATTTACTGTGGCCCTCCCC	1st step PCR for EphB1 5' arm
EphB1-3outer-F	TAAGAGCCACAGGGTTGTGGG	1st step PCR for EphB1 3' arm
EphB1-3outer-R	CCCACTAAAGCCCTCACTCAC	1st step PCR for EphB1 3' arm
EphB1-5F-Not	GATCGCGCCGCTTTGGGTGACACAGTGTGAC GGTC	2nd step, nested PCR of EphB1 5' 'arm
EphB1-5R-Fse	GATCGGCCCGCCTCATGGTGGCAGGGAGGGAG TTCC	2nd step, nested PCR of EphB1 5' arm
EphB1-3F-Asc	GATCGGCGCGCCGACATTCCCTCTTCCTTGTTA GCG	2nd step, nested PCR of EphB1 3' arm
EphB1-3R-Pac	GATCTTAATTAATAGACCAGGTATCGCTATAG CTTCTGGG	2nd step, nested PCR of EphB1 3' arm
EphB2-3581	CAGAAGCAGGCAGATCTCTGG	1st step PCR for EphB2 5' arm
EphB2-2R	GTCTCTTAGGGCTGCTCTGAC	1st step PCR for EphB2 5' arm
EphB2-2F	CCCCAAACCCCAATGTTGGG	1st step PCR for EphB2 3' arm
EphB2-6R	CTGGTGTAGTCCGGTATCGTG	1st step PCR for EphB2 3' arm
EphB2-5armU-Not	GCGGCCGCTCCAGCTTCCAGCTTCCCTGGGATG	2nd step, nested PCR of EphB2 5' arm
EphB2-5armL-Hind	GAAGCTTCCAGGAACACATTGAGAAGAAC	2nd step, nested PCR of EphB2 5' arm
EphB2-3armU-Asc-ex	GAGGCGCGCCGAAACCTGAGCATTTCCTCAGG G	2nd step, nested PCR of EphB2 3' 'arm
EphB2-3armL-Pac-ex	GATCTTAATTAATGAGGTGCAGCAGGGAGAAG	2nd step, nested PCR of EphB2 3' arm
EphB3-5outer-F	TCTTCCTGTCCTCGCCTCTG	1st step PCR for EphB3 5' arm
EphB3-5outer-R	ATGCAGGGACCAGCCAGATTG	1st step PCR for EphB3 5' arm
EphB3-3outer-F	GGAGGGTCTTAAACAGCAGG	1st step PCR for EphB3 3' arm
EphB3-3outer-R	TTGGTGAAGGGACCCAGGAG	1st step PCR for EphB3 3' arm
EphB3-5F-Not	AGGTGCGGCCGCGAACAGGAGTCGTGTTGCC	2nd step, nested PCR of EphB3 5' arm
EphB3-5R-Fse	AGGTGGCCCGCCCCGTGTTCTGGAACAATA TGTC	2nd step, nested PCR of EphB3 5' arm
EphB3-3F-Asc	AGGTGGCGCGCCGAGCTGGGTGAGAAAAGT GCAGAG	2nd step, nested PCR of EphB3 3' arm
EphB3-3R-Pac	AGGTTTAATTAAGTCCCGCTTCTGTTTGTGG	2nd step, nested PCR of EphB3 3' arm
EphB1-2398	CATCATTCGCCTGGAGGGTGTGCG	Genotyping of EphB1 KI allele
EphB1gt-5R-1	CCTCCCTGCATTCTCACTC	Genotyping of EphB1 KI allele
EphB2cre_GT_F1	CAGTTCGACCACCCCAATGTC	Genotyping of EphB2 KI allele
EphB2cre_GT_R1	GCACCTCAGGCAGCATAACC	Genotyping of EphB2 KI allele
EphB3-3outer-F	GGAGGGTCTTAAACAGCAGG	Genotyping of EphB3 KI allele
EphB3-5outer-R	ATGCAGGGACCAGCCAGATTG	Genotyping of EphB3 KI allele

Table 2.1: Primers used in the generation of AS-EphB TKI mice.

To target the EphB1 locus in ES cells, the targeting construct was linearized with NotI restriction enzyme and electroporated into J1 ES cells, which were subsequently selected with G418. Surviving colonies were picked and genotyped for correct recombination of both 5' and 3' arms by PCR. The correct targeting events were further confirmed by Southern analysis using probes that anneal to EphB1 genomic regions outside of the homologous arms, and by direct sequencing of PCR products amplified from the mutated allele. The positive clones were karyotyped for gross chromosomal abnormality. The Neo cassette was removed by electroporating targeted ES cells with a Cre expression plasmid. Individual clones were picked again, genotyped for the loss of the Neo cassette and karyotyped. The final selected ES clones were microinjected into C57BL/6 blastocysts to generate chimeric mice. Male chimeric animals with high-percentage aguti coat color were mated to C57BL/6 wild-type females, and aguti offsprings were genotyped for germline transmission of the targeted allele.

The EphB2 and EphB3 KI mice were similarly generated (**Figure 2.6B, C**). The primers used in the two-step PCR reactions to subclone the targeting arms of EphB2KI and EphB3KI targeting vectors are listed in **Table 2.1**. The ES cell targeting efficiencies were as follows: EphB1 KI (17/192 = 9%), EphB2 KI (3/186 = 2%), EphB3 KI (12/96 = 13%).

Mice were maintained as homozygotes in a mixed 129/C57BL/6 background. Unless noted, wild-type (WT) mice were F1 offspring of a C57BL/6 x129sv cross.

All experiments with mice were approved by the Animal Care and Use Committee of Harvard Medical School. Embryonic day 0 (E0) was defined as midnight preceding the morning a vaginal plug was found.

HEK 293 cell culture and transfection

HEK 293 cells were maintained in DMEM supplemented with 10% fetal bovine serum (Gibco), 2mM glutamine (Gibco), and penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively; Gibco). HEK 293 cells were transfected for 24 hours using the calcium phosphate method as previously described (Lois et al., 2002). For experiments using overexpressed EphB receptors, 0.15ug of pcs2+-based EphB plasmids were transfected into 2×10^5 cells in one well of a 24-well plate.

Western blotting

Lysates were prepared in 1x SDS-sample buffer (50 mM Tris pH 6.8, 2% SDS, 10% glycerol, 0.1% Bromphenol Blue, 5% β-mercaptoethanol). Western blots were probed with the following antibodies: rabbit anti-EphB1 (H-80, Santa Cruz); rabbit anti-EphB3 (H-85, Santa Cruz); rabbit anti-EphB2 (generated in the Greenberg lab and described previously (Dalva et al., 2000)); rabbit anti-pan-phospho-Eph (generated in the Greenberg lab and described previously (Dalva et al., 2000)). The anti-pan-phospho-Eph was generated from the peptide CLR^{Y*}VDPHRY^{*}EDPTQ of EphA3, where Y^{*} denotes phosphorylation. The phospho-specificity of the antibody was validated in HEK 293 cells for EphB1, EphB2, and EphB3 individually. For probing lysates in neurons or for

immunocytochemistry, rabbit anti-phospho-EphB was generated against the peptide CMKIY*TFPFTY*EDPNE, where Y* denotes phosphorylation, and affinity purified on two peptide columns. Mouse anti- β -actin (Abcam) was used as a loading control where appropriate.

All Western Blots were performed with the quantitative Odyssey Infrared Imaging System (Licor) using fluorescently labeled secondary antibodies (Rockland Immunochemicals). To quantify band intensity, median fluorescence intensity within a two pixel border was subtracted as background.

Immunoprecipitation

For immunoprecipitations, neurons were collected and homogenized in RIPA buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.5% Sodium Deoxycholate, 0.1% SDS, 10 mM NaF, complete protease inhibitor cocktail tablet (Roche), 1 mM sodium orthovanadate, and Phosphatase Inhibitor cocktails 1 and 2 (1x; Sigma)). After clearing lysates by centrifugation for 15 minutes at 14,000 x g, supernatants were incubated with appropriate antibody for 1-2 hour at 4°C, followed by addition of Protein-A Fastflow Agarose beads (Sigma) for 1 hour. Beads were washed in lysis buffer or PBS three times and eluted in 2x SDS sample buffer followed by boiling.

The following antibodies were used for immunoprecipitation experiments: rabbit anti-Vav2 (generated in the Greenberg lab and described previously (Cowan et al., 2005)); mouse anti-pan-phosphotyrosine (py99) (Santa Cruz); rabbit anti-EphA4 (generated in the Greenberg lab against a GST-fusion of the intracellular region of EphA4).

Neuronal cell culture

Cortical and hippocampal neurons were prepared from E15-E17 mouse embryos. Embryos were removed from the uterus and placed in ice-cold dissection media (10 mM MgCl₂, 10 mM HEPES, 1 mM kynurenic acid in HBSS). Cortices or hippocampi were dissected, digested with papain (Worthington), rinsed three times with ovomucoid trypsin inhibitor (Worthington), and gently triturated. Cultured neurons were maintained in Neurobasal Medium (Invitrogen) supplemented with 2% B27 (Invitrogen), penicillin/streptomycin (100 U/mL and 100 µg/mL, respectively, Gibco), and 2 mM glutamine (Gibco). For biochemistry, neurons were seeded at a density of 2×10^6 neurons/well of a 6-well plate coated with polyornithine (Sigma). For electrophysiology and imaging, neurons were seeded at a density of 7.5×10^4 neurons/well of a 24-well plate on a glial monolayer on glass coverslips coated with polyornithine and laminin (Invitrogen) or at 1.25×10^5 neurons/well on laminin/polyornithine coverslips for imaging at 1 DIV.

Ephrin stimulation

For ephrin stimulations in dissociated cultured neurons and retinal explants, mouse ephrin-B1-FC or ephrin-B2-FC (1 µg/ul; R&D Systems) was pre-clustered for 50 minutes with goat anti-human IgG FC (1.3 µg/µl; Jackson Immunoresearch) at room temperature in PBS at a molar ratio of 1:1 prior to stimulation. Pre-clustered ephrin-B1-FC or ephrin-B2-FC was added to the appropriate medium at a final concentration of 2.5

µg/mL. As a control, clustered human FC in media was applied to neurons where specified.

Surface labeling

Labeling of surface proteins was performed using the Pierce Cell Surface Protein Isolation Kit (Thermo Scientific). After chronic treatment with vehicle or 1 µM 1-NA-PP1, cultured cortical neurons were incubated with EZ-link biotin for 30 minutes at room temperature, washed with PBS, and lysed in RIPA buffer. Lysates were immunoprecipitated with antibodies to EphB2 (or control IgG), and probed with anti-EphB2 or fluorescently labeled streptavidin (Invitrogen).

Pharmacology

1-NA-PP1 was synthesized as described previously and dissolved in DMSO (Wang et al., 2003). 3-MB-PP1 was synthesized using a similar procedure and dissolved in DMSO. Dose response curves using 1-NA-PP1 and 3-MB-PP1 were calculated on GraphPad Prism using the least-squares method. The vehicle dose was calculated as two orders of magnitude below the lowest dose (=0.05 nM). For WT EphBs, 100% inhibition was defined at 1 mM.

EphB clustering assay

1 DIV dissociated cortical neurons were stimulated for 1 hour with clustered ephrin-B1 or FC as in Takasu et al., 2002 (Takasu et al., 2002). Neurons were fixed in

4% PFA/2% sucrose in PBS for 8 minutes, blocked in 5% goat serum, 0.1% gelatin, 0.3% Triton-X 100, 0.01% sodium azide in PBS for 2 hours, stained with rabbit anti-EphB2 (Santa Cruz; 2 µg/mL) and chicken anti-MAP2 (Abcam, 3.8 µg/mL) and mounted on slides using Fluoromount-G (Southern Biotech). Five confocal z-stacks of 1 µm thickness were acquired on a laser scanning Zeiss Pascal microscope using a 63× objective and maximum intensity z-projections were constructed. Metamorph image analysis software was used to identify the number of EphB2 clusters per area of MAP2-stained neuronal dendrite.

Quantitative PCR

Total RNA was isolated from mouse 7 DIV cortical cultures using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Isolated RNA was treated with DNaseI Amplification Grade (Invitrogen) and a cDNA library was synthesized by cDNA High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The cDNA was the source of input for quantitative PCR, using a Step One Plus Real-Time PCR Instrument and SYBR Green reagents (Applied Biosystems). The relative expression plot was generated using concentration values that were normalized to corresponding β-actin concentrations. The following qPCR primer pairs were used:

EphB1-F- ACTGCAGAGTTGGGATGGAC

EphB1-R- CATCATAGCCACTGACTTCTTCC

EphB2-F- TTCATGGAGAACGGATCTCTG

EphB2-R- GACTGTGAACTGCCCATCG

EphB3-F- CCCTGGACTCCTTTCTACGG

EphB3-R- GCAATGCCTCGTAACATGC

Chapter 3

EphB receptor tyrosine kinase signaling in axon guidance

3.1 Background

The unique power and enormous computing capabilities of the brain derive from its extensive wiring. These synaptic connections are made possible by the proper guidance of billions of axons to their targets. Axon navigation in the nervous system is mediated by several families of guidance molecules using attractive and repulsive cues to direct the axonal growth cone.

Early evidence suggested that EphBs plays a critical role in some of these axon guidance decisions (Henkemeyer et al., 1996). However, it was not clear whether this function was mediated by the kinase activity of EphBs or other modes of EphB signaling such as through PDZ-domain interactions, receptor aggregation, reverse signaling through ephrin-Bs, or EphB extracellular domain interactions. In general, the mechanisms by which axon guidance receptors signal growth cone attraction or repulsion have been challenging to identify. It is noteworthy that most axon guidance receptors that have been studied to date appear not to signal by activating protein kinases (O'Donnell et al., 2009). Indeed, many of the experiments studying EphBs suggest a role for kinase-independent signaling. However, these studies faced significant technical limitations that make their findings inconclusive.

Commissural axons of the cortex

While EphB2 knockouts display a defective anterior commissure (AC), EphB2-lacZ knockin mice do not share this phenotype (Henkemeyer et al., 1996). This finding has led investigators to conclude that reverse signaling is responsible. The kinase-

independent role was later supported by a study showing first, that ephrinBs are expressed on AC axons, and second, that ephrinB lacZ mice have an AC defect (Cowan et al., 2004). The authors reasoned that since the intracellular domain of ephrin-B2 was required for AC formation, then the role of EphBs must be as ligands and not receptors. However, this conclusion does not consider the fact that ephrin clustering and forward signaling could also be defective in the ephrin-lacZ mice.

A more convincing experiment was performed while studying the role of the related EphA family of receptors in forming the anterior commissure. While EphA4 knockouts have an AC defect, an EphA4 kinase inactive point mutation knockin has no effect on AC development, thus arguing for a kinase-independent role (Kullander et al., 2001). While this experiment improves upon those using the lacZ mutants in the EphB field, the conclusions may not apply to the EphB family.

Analysis of another major commissural tract, the corpus callosum (CC), has yielded similar results. Several mouse mutants have defective CCs, since many molecules orchestrate CC formation during mouse embryonic development (Donahoo and Richards, 2009). Of interest, EphB2/EphB3 knockout mice display agenesis of the corpus callosum (Orioli et al., 1996). However, this defect is not seen in mice in which the intracellular region of EphB2 is replaced by lacZ, thus arguing for a reverse signaling mechanism (Mendes et al., 2006).

This conclusion is supported by data from studies of ephrin-B mouse mutants. Ephrin-B1 knockout mice have a CC defect, as do ephrin-B1 knockin mutants where the PDZ-binding motif has been destroyed (Bush and Soriano, 2009). In addition, in vitro experiments suggest that ephrin-B1 is expressed on axons that form the corpus callosum,

while EphB2 is expressed on the midline guidance glia (Bush and Soriano, 2009). Thus, the available evidence points to a kinase-independent role for EphBs in commissural axon guidance in the cortex.

Retinal axon guidance

Much of what is currently known about the role of EphB signaling during axon guidance in vivo comes from studies of the guidance of retinal ganglion cell (RGC) axons to their targets in the lateral geniculate nucleus (LGN) and superior colliculus (SC) (Feldheim and O'Leary, 2010; Flanagan, 2006; Petros et al., 2008). During the course of visual system development, an RGC axon encounters a number of choice points as it travels to the optic disc, across the midline, along the optic tract to the SC or LGN, and finally to its target zone of innervation (Birgbauer et al., 2000; Hindges et al., 2002). Even the first step in the process, guidance to the optic disc, is defective in EphB2/EphB3 knockouts. The authors in this study argue for a role for reverse signaling based on the finding that EphB2lacZ/EphB3 mice had more minor pathfinding defects than double knockouts (Birgbauer et al., 2000).

One particularly important choice for RGC axons occurs at the midline. RGC axons from the ventrotemporal (VT) region of the retina express EphB1. At the same time, guide cells in the optic chiasm display a highly specific expression pattern for ephrin-B2 (Nakagawa et al., 2000; Williams et al., 2003). Studies using EphB1 and ephrin-B1/ephrin-B2 knockout mice revealed that the EphB1-ephrin-B2 interaction is responsible for the strong repulsion of EphB-expressing axons at the midline and subsequently leads to the development of the ipsilateral retinal projection in the LGN and

SC (Chenau and Henkemeyer, 2011; Williams et al., 2003). In contrast to EphB1-expressing axons, retinal axons that lack EphB1 pass through the chiasm and create the larger contralateral retinal projection. Previous studies by Carol Mason and colleagues show that overexpressed EphB1 is sufficient to drive retinal axons to ectopically project ipsilaterally, and that this function requires an intact EphB1 kinase domain (Petros et al., 2009). In addition, a recent study in which a knockin mouse was made with the intracellular region of EphB1 replaced with lacZ showed a loss of the ipsilateral retinal projection, suggesting a requirement for forward EphB signaling in the formation of the ipsilateral retinal projection (Chenau and Henkemeyer, 2011). Notably, it was not shown in these previous in vivo studies if the axon guidance function of endogenous EphBs requires tyrosine kinase signaling.

In addition to the optic chiasm, forward signaling of EphBs has been implicated in the guidance of spinal commissural axons (Kadison et al., 2006). This conclusion was based on the presence of guidance errors in ephrin-B3 knockout mice, but not in ephrin-B3 lacZ mice.

The conclusion from these studies is that a mix of forward and reverse signaling determines various axon decisions mediated by EphBs. However, these techniques are all indirect and are therefore not conclusive. For example, the EphB2 lacZ fusion protein can be phosphorylated at juxtamembrane residues and may be able to engage in some modes of signaling, or might inhibit others (Grunwald et al., 2001). We sought to directly test these questions using the As-EphB TKI mice.

3.2 EphB tyrosine kinase activity in growth cone collapse

To determine if the kinase activity of EphBs is required for growth cone collapse and axon guidance, we began by examining the effect of inhibiting the kinase activity of EphBs on ephrin-B2-dependent growth cone collapse in actively growing retinal ganglion cell (RGC) axons. We prepared explants from embryonic day 14 (E14) ventrotemporal retinae, a time and region where EphBs are highly expressed (Williams et al., 2004). Growth cones were visualized with fluorescent phalloidin (to label F-actin) and axons were identified by staining with anti-neurofilament antibodies (**Figure 3.1A**). In these explants RGCs extend axons with broad, fan-shaped growth cones over a laminin substrate. We measured the state of growth cones by two methods: first, growth cones were scored as collapsed if they exhibited rod-like morphology and lacked visible lamellipodia. This method allowed us to quantify the percentage of growth cones that were collapsed in each explant. Second, we measured the maximum axial width of each growth cone and calculated the average axial width of the growth cones in each explant.

To test whether treatment of explants with 1-NA-PP1 and 3-MB-PP1 led to inhibition of ephrinB-dependent EphB tyrosine phosphorylation, we stained explants with an antibody to phosphorylated EphBs. In the absence of stimulation, no phospho-EphB staining was observed in growth cones from WT or AS-EphB TKI explants (**Figure 3.1A**). Upon ephrin-B2 stimulation, punctate patterns of anti-phospho-EphB antibody staining were seen in the growth cone and along the axon (**Figure 3.1A**). This staining was blocked in 1-NA-PP1 (250 nM) or 3-MB-PP1 (1 μ M)-treated AS-EphB TKI explants, but not in WT explants, indicating that PP1 analogs selectively inhibit the kinase activity of EphBs in AS-EphB TKI RGC explants (**Figure 3.1A**).

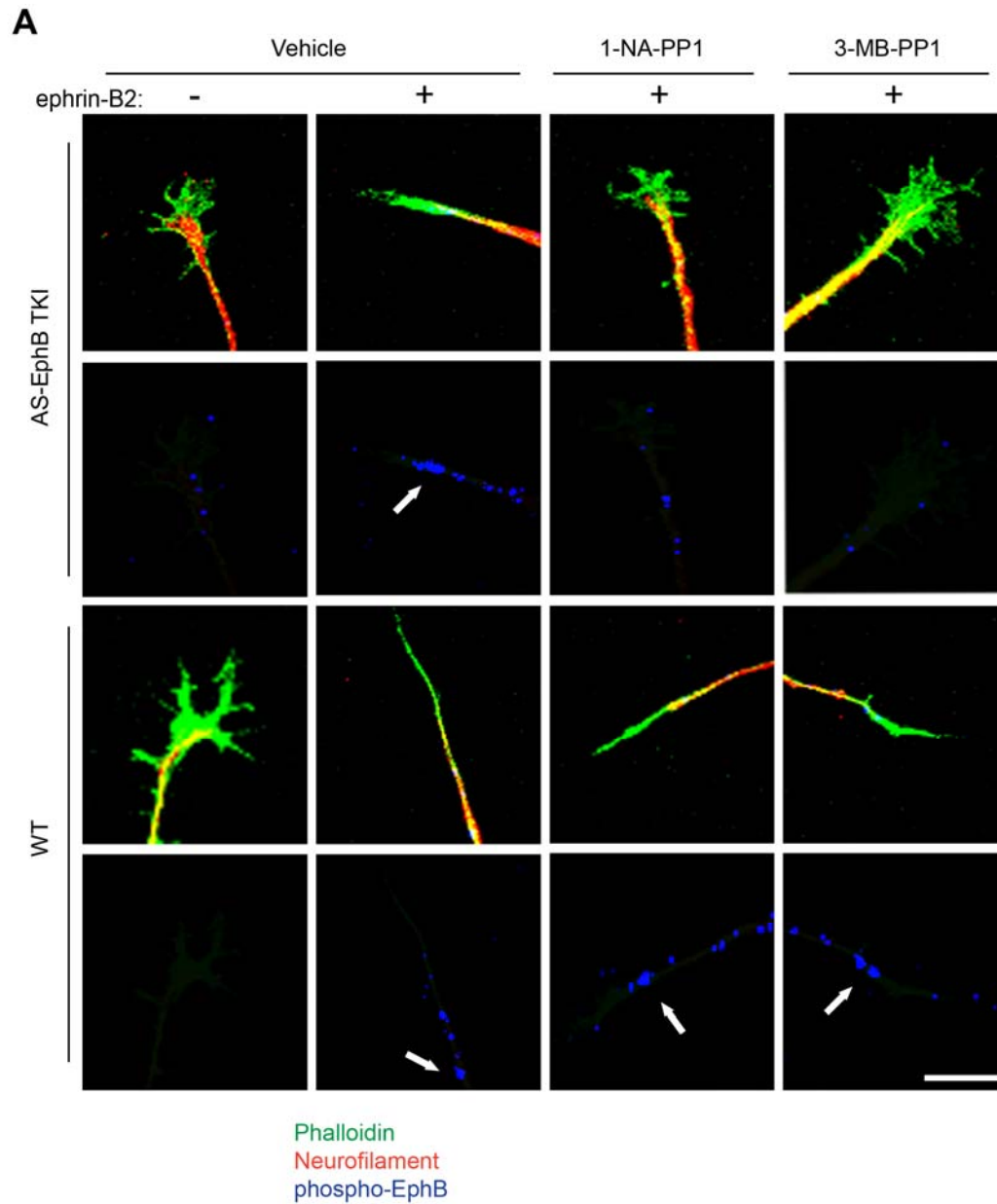


Figure 3.1: EphB-dependent growth cone collapse in retinal explants

(A) Embryonic day 14 (E14) VT retinal explants were preincubated for 1 hour with vehicle, 250 nM 1-NA-PP1, or 1 μ M 3-MB-PP1 before a 30 minute ephrin-B2 stimulation. Explants were fixed and stained with neurofilament (red), phospho-EphB (blue) and labeled with phalloidin (green).

White arrows denote clusters of phospho-EphB staining. Scale bar represents 10 μ m.

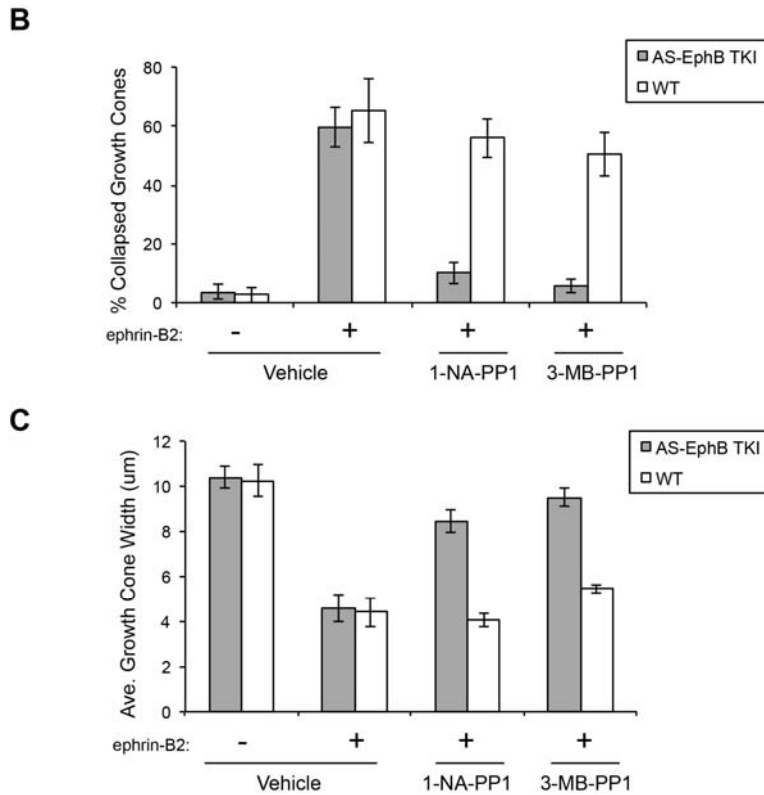


Figure 3.1 (continued)

(B) Quantification of the percentage of collapsed growth cones per explant from (A). A 2-way ANOVA revealed a significant interaction between genotype and inhibitor treatment in each of the conditions, indicating that PP1 analog treatment effects were significantly greater in AS-EphB TKI neurons than in WT neurons:

% collapsed growth cones, vehicle vs. 1-NA-PP1: $F_{(1,20)}=6.66$, $p=.018$

% collapsed growth cones, vehicle vs. 3-MB-PP1: $F_{(1,22)}=7.41$, $p=.012$

Data are mean \pm SEM. N=4-8 explants per condition.

(C) Quantification of the average growth cone width per explant from (A). A 2-way ANOVA revealed a significant interaction between genotype and inhibitor treatment in each of the conditions:

Average growth cone width, vehicle vs. 1-NA-PP1: $F_{(1,20)}=11.75$, $p=.0027$

Average growth cone width, vehicle vs. 3-MB-PP1: $F_{(1,22)}=11.49$, $p=.0026$

Data are mean \pm SEM. N=4-8 explants per condition.

When treated with clustered ephrin-B2, VT growth cones from AS-EphB TKI explants collapse robustly (from $4 \pm 2\%$ of growth cones collapsed in the unstimulated condition to $60 \pm 7\%$ collapsed in the stimulated condition) (**Figure 3.1A, B**). Treatment of AS-EphB TKI explants with 1-NA-PP1 (250 nM) or 3-MB-PP1 (1 μ M) led to a dramatic decrease in the percentage of collapsed growth cones upon ephrin-B treatment ($10 \pm 4\%$ and $6 \pm 2\%$ of growth cones, respectively) (**Figure 3.1A, B**). In addition, AS-EphB TKI explants showed an ephrin-B2-induced decrease in growth cone width from $10.4 \pm 0.5 \mu\text{m}$ to $4.6 \pm 0.6 \mu\text{m}$ in vehicle-treated explants, but only a slight reduction to $8.4 \pm 0.5 \mu\text{m}$ and $9.5 \pm 0.4 \mu\text{m}$ in explants exposed to 1-NA-PP1 or 3-MB-PP1, respectively (**Figure 3.1A, C**).

As an important control, this drug-dependent blockade of growth cone collapse was not seen in WT explants: WT cells showed a robust collapse regardless of the presence of inhibitors ($56 \pm 7\%$ of growth cones collapsed in 1-NA-PP1 and $51 \pm 7\%$ in 3-MB-PP1 compared to $65 \pm 11\%$ in the absence of drug) as well as a significant reduction in average growth cone width after stimulation ($4.1 \pm 0.3 \mu\text{m}$ and $5.4 \pm 0.2 \mu\text{m}$, respectively, compared to an average width of $10.2 \pm 0.7 \mu\text{m}$ in explants not exposed to ephrin-B2) (**Figure 3.1A, B**).

To rule out the possibility that inhibiting EphBs leads to general changes in cell health within the retinal explants, we washed out 1-NA-PP1 (250 nM) midway through a 30 minute ephrin-B2 stimulation in AS-EphB TKI explants. Whereas in the presence of 1-NA-PP1, ephrin-B-stimulated growth cones were uncollapsed ($3 \pm 2\%$ of growth cones), after washout growth cones rapidly collapsed ($54 \pm 10\%$ of growth cones were collapsed) (**Figure 3.2A, B**). This finding demonstrates the reversibility of PP1 analog

inhibition of the kinase activity of EphBs in retinal explants and rules out cell death as a cause of reduced growth cone collapse. We conclude that the kinase activity of EphBs is required for ephrin-B-induced growth cone collapse.

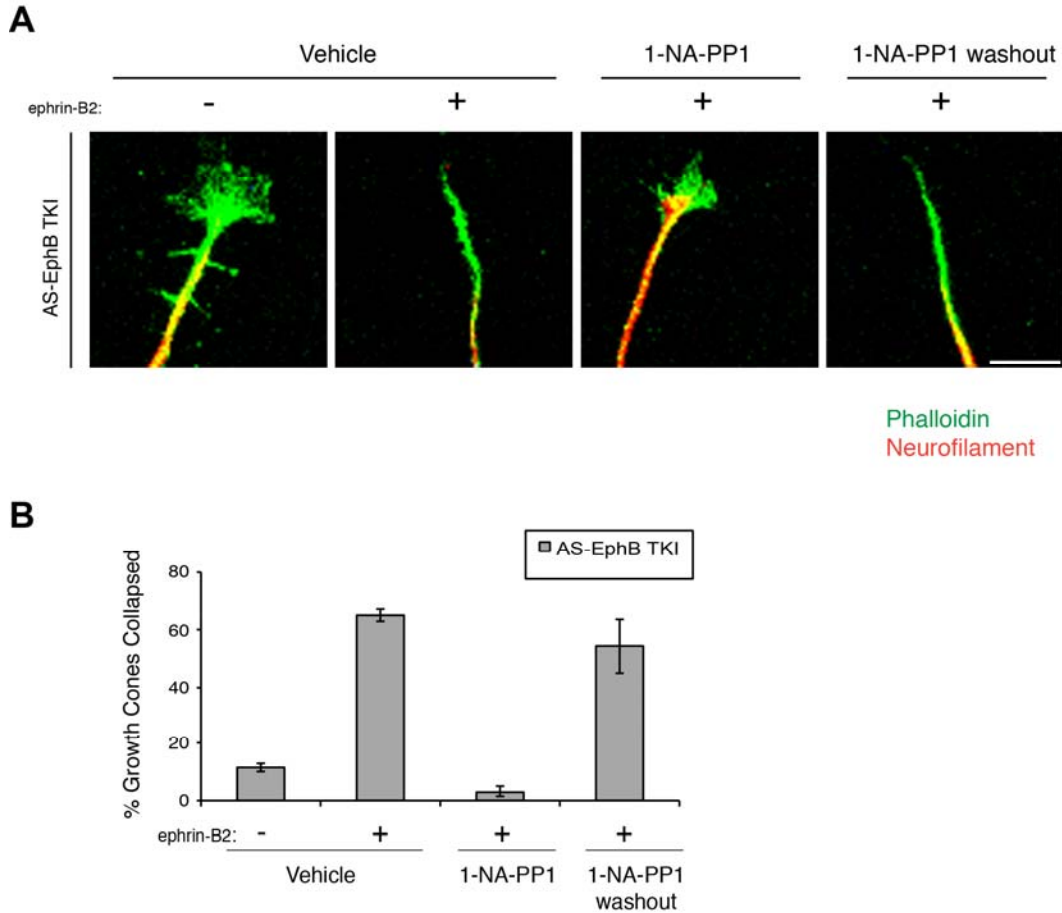


Figure 3.2: Reversible blockade of growth cone collapse in AS-EphB TKI explants

(A) E14 VT retinal explants were treated with 1-NA-PP1 (250 nM) as in Figure 3.1, but 15 minutes into a 30 minute ephrin-B2 stimulation, media was removed and replaced with vehicle. Scale bar represents 10 μ m.

(B) Quantification of the percentage of collapsed growth cones per explant from (A).

Data are mean \pm SEM. N=4 explants per condition.

3.3 The kinase activity of EphBs is required for axon guidance at the optic chiasm

Axon guidance *in vivo* is considerably more complex than the *in vitro* process of growth cone collapse. For example, the saltatory motion of growth cones *in vivo* involves more than simple extension and collapse, and often includes intermediate movements (Mason and Wang, 1997). To determine whether the kinase activity of EphBs is required for the repulsive guidance of axons *in vivo*, we examined the effect of inhibiting the kinase activity of EphBs on axon repulsion at the optic chiasm.

In vivo EphB tyrosine kinase inhibition

In determining how to treat AS-EphB TKI mice *in vivo*, we considered several approaches. An earlier study with AS-Trk mutants used oral administration and booster intraperitoneal (IP) injections (Chen et al., 2005). While oral administration is convenient, the effective concentration may not be high enough and it would require large amounts of inhibitor that need to be synthesized. IP injections provide a high effective dose, but they are cleared rapidly and might require frequent injections to see a phenotype. Based on unpublished data from Zach Knight and Chao Zhang of the Shokat lab, we decided to try subcutaneous injections since the clearance rate is substantially slower than for IP injections.

Determining the effective dose of inhibitor in the nervous system is challenging. A radiolabeling assay measuring tritium-labeled 1-NM-PP1, another PP1 analog, in the brain suggests that PP1 analogs cross the blood brain barrier (Wang et al., 2003).

However, this study showed that 1-NM-PP1 levels in the brain after IP injections only lasted for one hour. Also, there is the caveat that these studies using brain homogenates from mice may contain contaminating tissue such as blood vessels.

For our assays, we used twice daily injections of 80mg/kg 1-NA-PP1 dissolved in 10% DMSO, 20% Cremaphor-EL, 70% Saline.

Formation of the ipsilateral retinal projection

To test whether the tyrosine kinase activity of EphBs is required for formation of the ipsilateral retinal projection, we treated pregnant female mice with 1-NA-PP1 from E13.5-16.5, the time when RGC axons encounter the optic chiasm. By E16, much of the ipsilateral projection has already formed. To label the retinal projections at E16, we fixed embryos and applied DiI crystals to the optic disc to allow selective anterograde labeling of the retinal projections from one eye (**Figure 3.3A**).

To assess the degree of ipsilateral vs. contralateral retinal projection, we measured the fluorescence intensity of a rectangular region in the ipsilateral projection and divided this by the total fluorescence intensity in the combined ipsilateral and contralateral projections to derive the Ipsilateral Index (Williams et al., 2003). In 1-NA-PP1-treated AS-EphB TKI embryos, we found that the ipsilateral retinal projection was strongly reduced (by 42%) compared to untreated AS-EphB TKI embryos (**Figure 3.3B, C**). In many of the AS-EphB TKI embryos treated with 1-NA-PP1, the ipsilateral projection was completely absent. By contrast, 1-NA-PP1 treatment had no effect on the ipsilateral

retinal projection in WT embryos, indicating that the observed guidance deficit was due to specific inhibition of EphBs (**Figure 3.3B, C**).

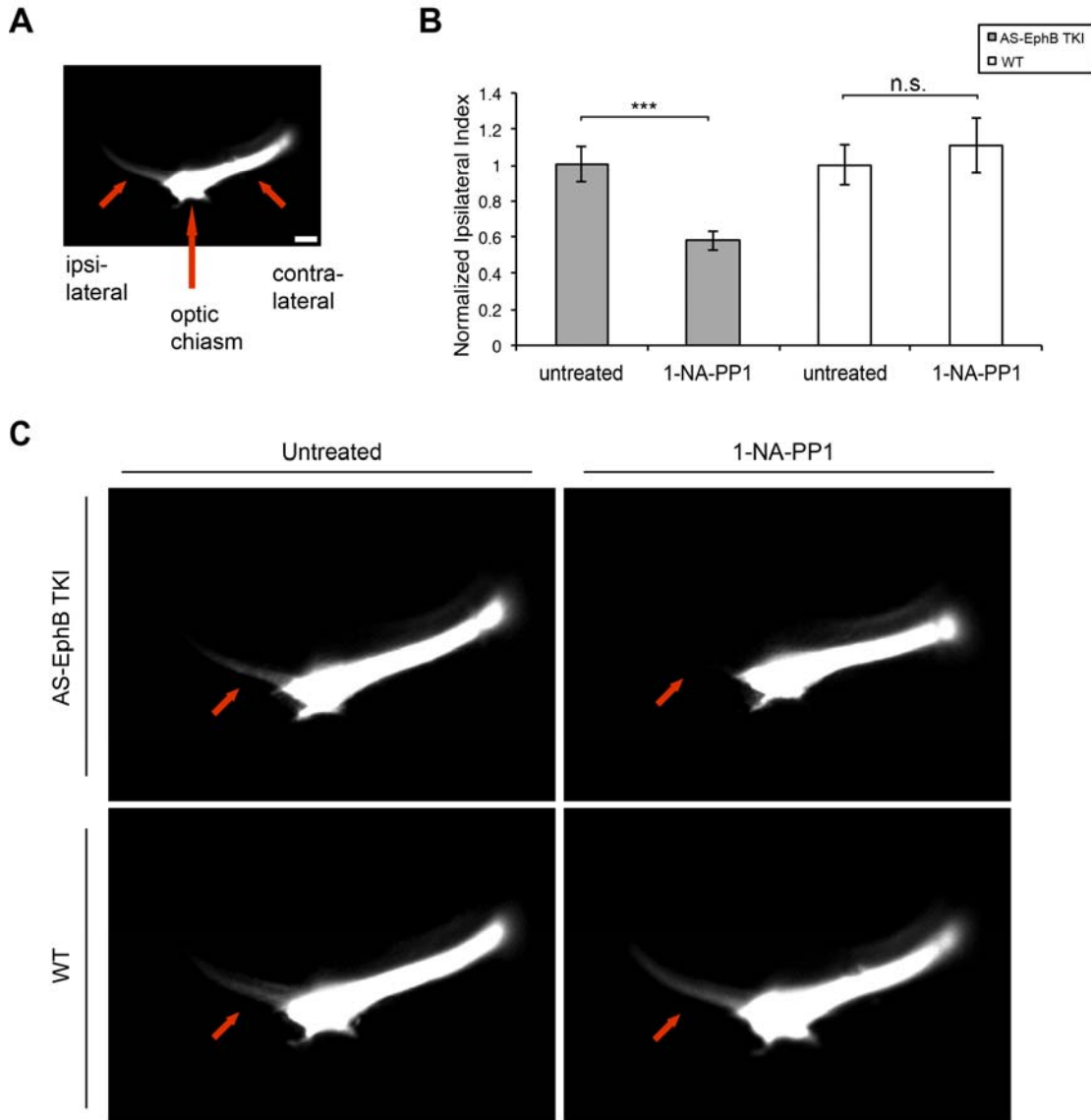


Figure 3.3: The formation of the ipsilateral retinal projection requires EphB tyrosine kinase signaling

Figure 3.3 (continued)

(A) Representative image demonstrating the orientation of the ipsilateral and contralateral retinal projections of the optic tract with respect to the optic chiasm as visualized by DiI labeling. Scale bar represents 100 μm .

(B) Twice-daily subcutaneous injections of 80 mg/kg 1-NA-PP1 were administered to pregnant mice from E13.5-E16.5. Graph depicts quantification of the Ipsilateral Index normalized for each genotype. The number of embryos examined was as follows: Untreated AS-EphB TKI (n=30), 1-NA-PP1-treated AS-EphB TKI (n=30), Untreated WT (n=15), 1-NA-PP1-treated WT (n=17).

***, $p < .001$; n.s., not significant by Student's t-test. A 2-way ANOVA revealed a significant interaction between genotype and inhibitor treatment, indicating that 1-NA-PP1 treatment effects were significantly greater in AS-EphB TKI embryos than in WT embryos ($F_{(1,88)}=6.5$, $p=.0125$). Data are mean \pm SEM.

(C) Representative images of DiI-filled retinal projections at E16.5. C57BL/6 WT mice in untreated condition show a larger ipsilateral component than untreated AS-EphB TKI mice, but display no response to 1-NA-PP1 treatment.

We conclude a requirement for the tyrosine kinase activity of EphBs in mediating the guidance decision at the optic chiasm. It is worth noting that this phenotype is often strain-specific and exact background matches cannot be obtained for the AS-EphB TKI mice. The baseline level of ipsilateral projection size for the highly inbred, mixed-strain AS-EphB TKI mice was lower than for WT mice (**Figure 3.3C**). Thus, the formal possibility remains that the AS-EphB TKI mice are hypomorphic in this regard. However, no other assays revealed any evidence of hypomorphism.

3.4 EphB tyrosine kinase signaling regulates the formation of cortical tracts

Based on the finding that EphB tyrosine kinase activity mediates axon guidance at the optic chiasm, we next asked about its role in other commissural axon tracts in the brain. EphB knockout mice have defects in the corpus callosum and anterior commissure that are thought to be kinase-independent, although it has not been possible to test this directly. We treated pregnant mice with 1-NA-PP1 from E12.5-19.0, harvested embryos, then fixed and stained the brains with an antibody to L1-CAM to visualize major axon tracts. Based on studies of the EphB knockouts, there are three major tracts of interest: the corpus callosum, the anterior commissure, and the corticofugal tracts.

Untreated AS-EphB TKI mice had normal corpus callosa (0/6 agenesis) (**Table 3.1**) (**Figure 3.4**). However, AS-EphB TKI mice treated with 1-NA-PP1 all had partial corpus callosum agenesis with a gap in the dorsal midline region (11/11) (**Table 3.1**) (**Figure 3.4**). While fully penetrant, the phenotype is not as severe as in the EphB TKO, which may be explained by incomplete pharmacological inhibition. WT mice treated with

1-NA-PP1 had a normal corpus callosum (0/6), demonstrating that this phenotype was specific to inhibition of EphBs (**Table 3.1**) (**Figure 3.4**). These data suggest that the tyrosine kinase activity of EphBs is required for proper formation of the corpus callosum, in contrast to earlier studies (Mendes et al., 2006).

CONDITION	N	CORPUS CALLOSUM (PARTIAL AGENESIS)	ANTERIOR COMMISURE MISPROJECTION (+)	ANTERIOR COMMISURE MISPROJECTION (+++)	CORTICOFUGAL MISPROJECTION
AS-EphB TKI untreated	6	0% (0)	0% (0)	0% (0)	0% (0)
AS-EphB TKI + 1-NA-PP1	11	100% (11)	0% (0)	100% (11)	64% (7)
WT untreated	6	0% (0)	16% (1)	0% (0)	0% (0)
WT + 1-NA-PP1	6	0% (0)	50% (3)	0% (0)	0% (0)

Table 3.1: Summary of cortical phenotypes in AS-EphB TKI and WT mice.

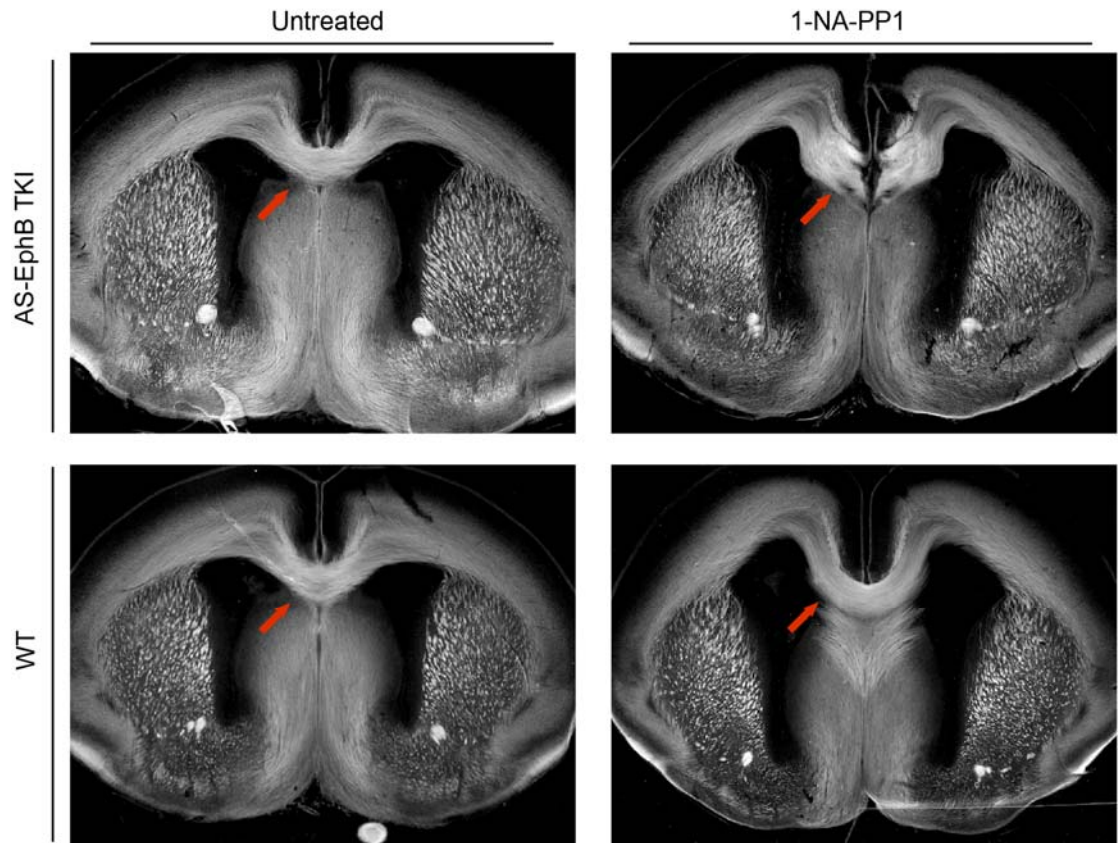


Figure 3.4: EphB tyrosine kinase activity is required for formation of the corpus callosum

Twice-daily subcutaneous injections of 80 mg/kg 1-NA-PP1 were administered to pregnant mice from E12.5-E19.0. Brain sections from E19.0 embryos were stained with L1-CAM antibody to visualize axon tracts. Red arrows denote the corpus callosum.

Further details are described in Table 3.1.

After noting this highly penetrant corpus callosum defect, we next examined the anterior commissure. Untreated AS-EphB TKI mice had normal anterior commissures (0/6 misprojected) (**Table 3.1**) (**Figure 3.5**). However, mice treated with 1-NA-PP1 all had partially misprojected anterior commissure tracts (11/11) (**Table 3.1**) (**Figure 3.5**). In these mice, the posterior branch of the anterior commissure (ACp) contained large branches that failed to join the primary ACp white matter tract toward the ventral midline. Untreated WT mice were generally normal (0/6), but displayed some minor misprojections of the AC with a few misguided fibers (1/6) (**Table 3.1**) (**Figure 3.5**). WT mice treated with 1-NA-PP1 also displayed similarly minor misprojections (3/6), but did not have any significantly misguided ACs (0/6) (**Table 3.1**) (**Figure 3.5**). The small number of partially misguided ACs in WT may demonstrate a partial off-target effect, but this phenotype is still much less severe than that seen in the AS-EphB TKI mice. Thus, we can conclude that the tyrosine kinase activity of EphBs is required for proper formation of the anterior commissure, in contrast to previous studies (Cowan et al., 2004; Henkemeyer et al., 1996).

To better quantify this finding, we measured thickness of the posterior branch of the anterior commissure in sections where ACp fiber bundles visibly crossed the midline. While untreated AS-EphB TKI mice had ACp widths of $162 \pm 11 \mu\text{m}$, 1-NA-PP1 treated mice had significantly thinner tracts of $94 \pm 5 \mu\text{m}$ (**Figure 3.5B**). WT mice did not display a significant reduction (from $159 \pm 10 \mu\text{m}$ to $138 \pm 7 \mu\text{m}$) (**Figure 3.5B**).

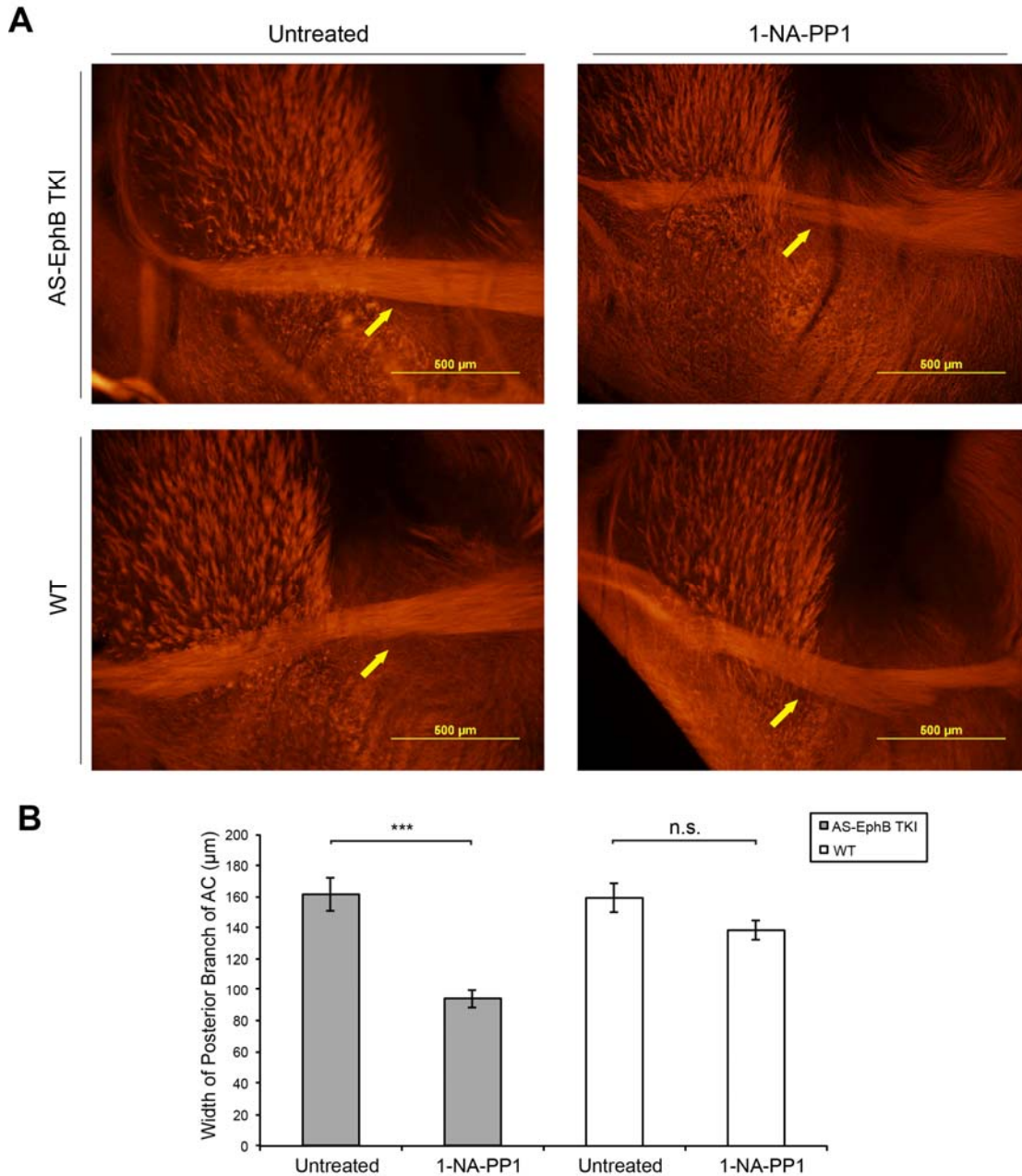


Figure 3.5: The proper formation of the anterior commissure requires EphB tyrosine kinase function

(A) Twice-daily subcutaneous injections of 80 mg/kg 1-NA-PP1 were administered to pregnant mice from E12.5-E19.0. Brain sections from E19.0 embryos were stained with L1-CAM antibody to visualize axon tracts. Yellow arrows denote the posterior branch of the anterior commissure.

Figure 3.5 (continued)

(B) Quantification of tract width from (A).

***, $p < .001$; n.s., not significant by Student's t-test. A 2-way ANOVA revealed a significant interaction between genotype and inhibitor treatment, indicating that 1-NA-PP1 treatment effects were significantly greater in AS-EphB TKI embryos than in WT embryos ($F_{(1,25)}=8.7$, $p=.007$). Data are mean \pm SEM. Further details are described in Table 3.1.

Recent unpublished experiments from Michael Robichaux in the laboratory of Chris Cowan at the University of Texas Southwestern have identified corticofugal (CF) tract defects in EphB KO mice. We examined these tracts in AS-EphB TKI mice. Untreated mice were normal (0/6), but 1-NA-PP1 treated mice had pronounced defects in the corticofugal tract (7/11) (**Table 3.1**). CF axon bundles often failed to integrate into the internal capsule and instead grew aberrantly through the ventral striatum. Untreated WT mice displayed normal CFs (0/6) as did 1-NA-PP1-treated WT mice (0/6) (**Table 3.1**). We conclude that the tyrosine kinase activity of EphBs is required for proper formation of corticofugal tracts.

3.5 Discussion

Kinase-dependence of axon guidance

Taken together, these data demonstrate for the first time a requirement for the kinase activity of EphBs in axon guidance in vivo. In fact, all four of the axon tracts we

examined required EphB tyrosine kinase signaling. These experiments highlight the ability of the chemical-genetic approach to inhibit the kinase signaling of EphBs at defined times in vivo during embryonic development and should make it possible to examine the importance of the kinase activity of EphBs in other axon guidance decisions.

Our data disagree with previously reported findings using lacZ fusion proteins (Henkemeyer et al., 1996; Mendes et al., 2006). As discussed previously, these fusion proteins may lead to functional compensation or may still engage in unexpected modes of signaling. Our results suggest that a second look at assays using ephrin-B/EphB lacZ fusion proteins would be critical. In addition, previous studies that blocked ephrin-B reverse signaling may have perturbed EphB tyrosine kinase activity as well, thus leading to defects in axon guidance.

Mechanism of EphB-mediated axon repulsion

Signaling pathways at the growth cone often converge on regulators of the actin cytoskeleton. However, very little is known about the mechanism of EphB-kinase-dependent growth cone collapse. In fact, little is known about the mechanism of growth cone collapse in most major guidance systems. Studies of the related EphA family of RTKs suggest a possible role for the Ephexin and Vav Rho family GEFs in this process (Cowan et al., 2005; Sahin et al., 2005; Shamah et al., 2001). While Rho family GTPases are good candidates to bridge RTKs to the actin cytoskeleton, we find that RhoA inhibitors do not affect EphB-dependent growth cone collapse in retinal explants. Consistent with this finding, growth cone collapse still occurs in explants from Ephexin5 knockout mice (M.J.S., unpublished). Recent studies show that a key component of the

RhoA pathway, Rho associated protein kinase (ROCK), is also not required for ephrin-B-induced growth cone collapse, although it is required for axon retraction (Petros et al., 2010).

As our data suggest that the tyrosine kinase activity of EphBs is required for growth cone collapse, the identification of substrates of EphBs in the growth cone that are not involved in RhoA signaling may help to elucidate further the mechanism of EphB-dependent growth cone collapse. With the development of orthologous ATP-analogues that are selectively used by AS kinases, it is possible to directly label substrates of an AS kinase (Banko et al., 2011). Thus, the AS-EphB mice may facilitate the identification of EphB substrates that help mediate the effects of ephrin-B/EphB signaling on axon guidance.

Based on the available data, we propose a model where Vav2/3 may act downstream of EphBs in mediating guidance at the optic chiasm. We have demonstrated that Vav2 is phosphorylated downstream of EphBs, and it will be important to test this in retinal explants. A previous study investigating Vav2/Vav3 knockout mice demonstrated defects in the ipsilateral retinal projection to the LGN (Cowan et al., 2005). This process was thought to be dependent on Rac1, which may mediate endocytosis in the growth cone (Jurney et al., 2002). As effectors of EphB-mediated axon guidance in cortical tracts are yet to be identified, it will be worthwhile to investigate whether the signaling processes there are similar to those in the retina.

3.6 Materials and methods

Retinal explants

Ventrotemporal (VT) segments of retina were microdissected from E14.5 mouse embryos and cultured as previously described (Petros et al., 2010). Embryos were removed from the uterus and decapitated, and heads were placed in ice-cold DMEM/F12 (Gibco). VT sections of the retina were excised and placed on glass coverslips coated with polyornithine and laminin. Explants were maintained in serum free medium (10 mg/mL BSA (Sigma), 1% ITS supplement (Sigma), Pen/Strep (20 U/mL and 20 µg/mL, respectively, Gibco) in DMEM/F12) supplemented with 0.2% methyl cellulose (Sigma) to increase media viscosity and minimize explant movement. All experiments were conducted 18-24 hour after initial plating.

Immunocytochemistry and growth cone collapse assay

For experiments with drug treatment, explants were pretreated with vehicle or PP1 derivatives for 1 hour, followed by a 30 minute ephrin-B2 stimulation, with variations as described in the text. Following stimulation, retinal explants were fixed for 20 minutes at 25°C with 4% paraformaldehyde/2% sucrose in PBS. Explants were then blocked in 10% Goat Serum, 0.2% Tween-20 in PBS for 1 hour, followed by incubation with neurofilament antibody or phospho-EphB antibody in 50% blocking solution overnight. Mouse anti-neurofilament, (2H3) originally derived in the laboratory of Thomas Jessell, was obtained from the Developmental Studies Hybridoma Bank. Rabbit

anti-phospho-EphB was generated against the peptide CMKIY*TFPFTY*EDPNE, where Y* denotes phosphorylation, and affinity purified on phospho-Eph peptide columns.

After PBS washes, explants were incubated in Alexa Fluor-conjugated secondary antibodies (Invitrogen) and Alexa Fluor 488-conjugated phalloidin (Invitrogen). Explants on coverslips were mounted on glass slides using Fluoromount-G (Southern Biotech). Neurons were imaged using a laser scanning Zeiss Pascal microscope using a 40× objective with sequential acquisition settings at 1024 × 1024 pixel resolution. All imaging and image analysis were performed blind to the genotype and treatment condition of the samples. At least 10 growth cones were analyzed per explant.

In Vivo 1-NA-PP1 delivery

Pregnant female WT or AS-EphB TKI mice were injected subcutaneously twice daily with 80mg/kg 1-NA-PP1 dissolved in 10%DMSO, 20% Cremaphor-EL, 70% saline from E13.5 to E16.5 for optic tract experiments or from E12.5-E19.0 for cortical tract experiments.

DiI labeling of the optic tract

DiI labeling was performed as previously described by Plump et al. (Plump et al., 2002). E16.5 embryo heads were fixed in 4%PFA /2% sucrose in PBS overnight and then washed with PBS. The lens and retina were removed from the left eye and a small crystal of DiI (Invitrogen) was placed in the optic disc. The retina was then replaced securely and the heads were stored in PBS+0.1% azide at room temperature for 12 days. After

labeling, brains were removed and fluorescent optic tracts were imaged on a Leica MZ16F fluorescent stereomicroscope. Images were captured using Spot Advanced software. Labeling was quantified using Metamorph software by drawing rectangular regions of interest around the ipsilateral and contralateral tracts, subtracting background, and calculating the ipsilateral index based on integrated intensity of fluorescence:

$$\text{ipsilateral index} = \text{ipsilateral} / (\text{ipsilateral} + \text{contralateral}).$$

To compare WT and AS-EphB TKI responses with respect to 1-NA-PP1 treatment, each genotype was normalized to its untreated condition, producing a normalized ipsilateral index.

Labeling of cortical tracts

E19.0 embryos were decapitated and fixed in 4% PFA/2% sucrose in PBS for 2 days, then stored in PBS + .02% sodium azide at 4°C. Brains were removed and vibratome sectioned to 70 µm. Sections were blocked in 5% normal donkey serum, 1% BSA, 0.2% glycine, 0.2% lysine with 0.3% TritonX-100 in PBS at room temperature for 1 hour. To stain axon tracts, sections were incubated with rat anti-L1-CAM (Millipore, MAB5272MI) 1:200 in blocking solution at 4°C overnight. Sections were washed three times with PBS and incubated with Cy3-conjugated donkey anti-rat IgG (Jackson) for 1.5 hours. Sections were washed 7-8 times with PBS and mounted with Aqua-mount (Lerner laboratories). Sections were imaged on an Olympus Bx51 epifluorescent microscope at 4x magnification.

Scoring of cortical phenotypes

All in vivo experiments included data from at least two separate litters of embryos per condition. Corpus callosum (CC) partial agenesis was scored as the apparent failure of CC axons to cross the midline in a specific region along the rostral-caudal brain axis, and was evident by a gap in the dorsal midline region.

Misprojections of the posterior branch of the anterior commissure (ACp) were scored by the observation of ACp axon fibers that failed to join the primary ACp white matter tract toward the ventral midline. These misprojections often occurred as the primary ACp tract turns medially toward the midline. The severity of the ACp misprojections was scored based on the apparent number of misguided axons. Large bundles of misprojected axons are represented as (+++), while a few misguided fibers are represented as (+).

For ACp measurements, images were acquired under 10x magnification and sections were chosen where the ACp fiber bundle visibly crossed the midline. Measurements were made using the ImageJ line tool to quantify the width of the AC fiber path in the caudate putamen region (to isolate the posterior component).

Corticofugal (CF) misprojections were scored by the observation of CF axon bundles that failed to integrate into the internal capsule and instead grew aberrantly through the ventral striatum toward the brain floor.

Statistical analysis:

T-tests and 2-way ANOVA analyses were conducted using GraphPad Prism software. All error bars represent SEM.

Chapter 4

EphB receptor tyrosine kinase signaling in synapse development

4.1 Background

Molecular mechanisms of synaptogenesis

EphBs were one of the first members of a growing class of molecules implicated in the formation of excitatory synapses (Waites et al., 2005). Most excitatory synapses in the brain form onto dendritic spines, small bulb-like structures that can compartmentalize signaling (Yuste and Denk, 1995). The development of synapses and the development of spines are intimately related, although still quite separate phenomena (Yuste and Bonhoeffer, 2004). EphBs have been repeatedly implicated in spine development as well as synaptogenesis.

There have been conflicting reports as to the timing of pre- vs. postsynaptic phases of synaptogenesis, but it appears that these confusions reflect a heterogeneity of synapses in terms of developmental patterns (McAllister, 2007). Similarly, spine development depends on sex, species, and brain region (Zhang and Benson, 2000). Thus, descriptions of spine and synapse formation must be considered in their appropriate developmental contexts. Still, excitatory synapse formation in the CNS follows several general trends.

After the guidance of an axon to its proper target field, nascent synapses begin to form on dendritic shafts and filopodia, thin, highly motile structures (Ziv and Smith, 1996). Interactions between pre- and postsynaptic structures are mediated by cell adhesion molecules that allow a reciprocal induction of the presynaptic terminal and the postsynaptic density (Waites et al., 2005). These adhesive interactions involve several families of molecules and are thought to underlie a large part of the specificity of synaptic connections. It is at this point that EphBs are thought to act since they can signal bidirectionally at the synapse (Kayser et al., 2008). However, studies of neuroligin have

shown that synapse maturation and stabilization can be equally important for determining synapse specificity (Chubykin et al., 2007).

There are several opposing models of how synaptogenesis and spinogenesis relate in pyramidal neurons. In one model, filopodia actively seek out axonal partners, stabilizing along preferred sites of the axon, perhaps where glutamate is released (McAllister, 2007). The filopodia recruit synapses to the shaft, where spines then develop. This is consistent with the finding that only after the first two weeks postnatally does shaft synapse number start to decrease appreciably until the vast majority are spine synapses (Fiala et al., 1998).

Another model proposes that synapses initially form onto filopodia, which directly morph into spines. Indeed, published observations of this phenomenon support the theory (Parnass et al., 2000). The notion that filopodia develop directly into spines is countered by the observation that aspiny neurons have filopodia during development, yet never develop spines (Portera-Cailliau et al., 2003). However, the filopodia on these aspiny neurons may lead to new dendritic branches, so they may be fundamentally different from some filopodia observed on spiny neurons.

A final model suggests that filopodia and spines of various morphologies are in a constant state of flux. Filopodia are merely highly motile, very narrow spines and should be considered nascent spines (Nimchinsky et al., 2002).

The underlying molecular mechanisms of spinogenesis have been investigated in great detail. The development of the mature spine involves Rho GTPases and rearrangements of the actin cytoskeleton. Generally, Rac activation promotes spine formation and RhoA activation prevents spine formation (Tashiro et al., 2000).

After formation of nascent synapses, maturation begins to occur. During this step, receptor subunit compositions are often altered, significant changes in neurotransmitter release take place, and the density of synaptic proteins is dramatically increased (Sanes and Lichtman, 2001). Some synapses, however, are permanently destabilized and eliminated. Several signaling pathways have been implicated in this maturation phase, including EphBs (Penzes et al., 2003). Even for the spines that remain, there is a great deal of dynamism. Further experiments that measure the movement of spines in genetic mutants are important for shedding light on the mechanism of these dynamic events. In the case of EphBs, recent studies suggest that dendritic filopodia from EphB TKO mice have decreased motility (Kayser et al., 2008).

EphBs in synapse and spine development

As discussed in Chapter 1, the evidence for EphB involvement in synapse and spine development is based on four independent approaches: soluble ephrin stimulation, overexpression of dominant negatives, RNAi, and transgenic mice.

Gain-of-function experiments, such as adding clustered soluble ephrin, can suggest the sufficiency of EphBs in certain processes, but cannot be used to test a requirement for EphB tyrosine kinase signaling in synaptogenesis.

Dominant negative EphB2 overexpression leads to decreases in synapse and spine number (Ethell et al., 2001). While the authors of this study conclude a kinase-dependent role for EphBs, the use of dominant negatives is hard to interpret because the mechanism of perturbation is not fully understood. In addition, this approach cannot test the requirement of EphB tyrosine kinase activity in endogenous proteins.

An alternative approach that solves this problem is the use of RNAi. Kayser et al. have demonstrated that knockdown of EphB2 alone is sufficient to cause decreases in synapse density, spine density, and filopodial motility in cultured cortical neurons (Kayser et al., 2008). This is true even when RNAi treatments lead to only a 50% reduction in EphB2 staining (Kayser et al., 2008). Considering that multiple EphBs need to be genetically excised to see defects in hippocampal synaptogenesis and spine development, this result is surprising (Henkemeyer et al., 2003). Kayser et al. went on to show that re-expression of an EphB2 kinase inactive mutant failed to rescue synapse density, thus suggesting a requirement for EphB tyrosine kinase activity in synaptogenesis. However, this technique also relies upon overexpressed protein which may not be relevant to endogenous signaling.

Transgenic mice have also been used to address the kinase dependence of EphBs. The EphB2 lacZ mouse (in EphB1/EphB3 KO background) has a dendritic spine phenotype intermediate between WT and knockout mice (Henkemeyer et al., 2003). Given the full penetrance of the knockin procedure, this observation is difficult to interpret. Even still, these experiments do not directly address the role of kinase signaling.

Thus, none of these former studies has directly tested the requirement of the kinase activity of EphBs in the regulation of synapse number or function under conditions where ephrin-Bs and EphBs are expressed at physiological levels. This leaves open the possibility that aspects of EphB-dependent synapse development may be kinase-independent.

4.2 EphB tyrosine kinase signaling is dispensable for synapse and spine formation

EphB tyrosine kinase signaling in synaptogenesis

To test whether synaptogenesis requires the kinase activity of EphBs or whether it might be mediated by a kinase-independent function of EphBs, we measured miniature excitatory postsynaptic currents (mEPSCs) in dissociated cortical neurons from AS-EphB TKI mice in the presence or absence of 1-NA-PP1. In previous studies, mEPSC frequency was significantly reduced in dissociated cortical neurons in which EphB2 expression is knocked down with RNAi; this is thought to signify a reduction in the number of excitatory synapses (Kayser et al., 2006). We treated cultures with 1 μ M 1-NA-PP1 beginning at 3 days in vitro (DIV) until 10-12 DIV and performed whole cell electrophysiological recordings. Surprisingly, we found no difference in mEPSC frequency between AS-EphB TKI neurons treated with vehicle or 1-NA-PP1 (1.1 ± 0.3 Hz in vehicle-treated vs. 1.1 ± 0.3 Hz in 1-NA-PP1-treated) (**Figure 4.1A, B**). There was also no significant difference in mEPSC amplitude (16.4 ± 2.5 pA in vehicle-treated vs. 15.6 ± 1.3 pA in 1-NA-PP1-treated) (**Figure 4.1A, B**). To confirm that 1-NA-PP1 inhibits EphB tyrosine phosphorylation in these cultures, we performed a western blot on concurrent cultures which showed clear inhibition of all ephrin-B-induced EphB autophosphorylation in the presence of 1 μ M 1-NA-PP1 (**Figure 4.1C**).

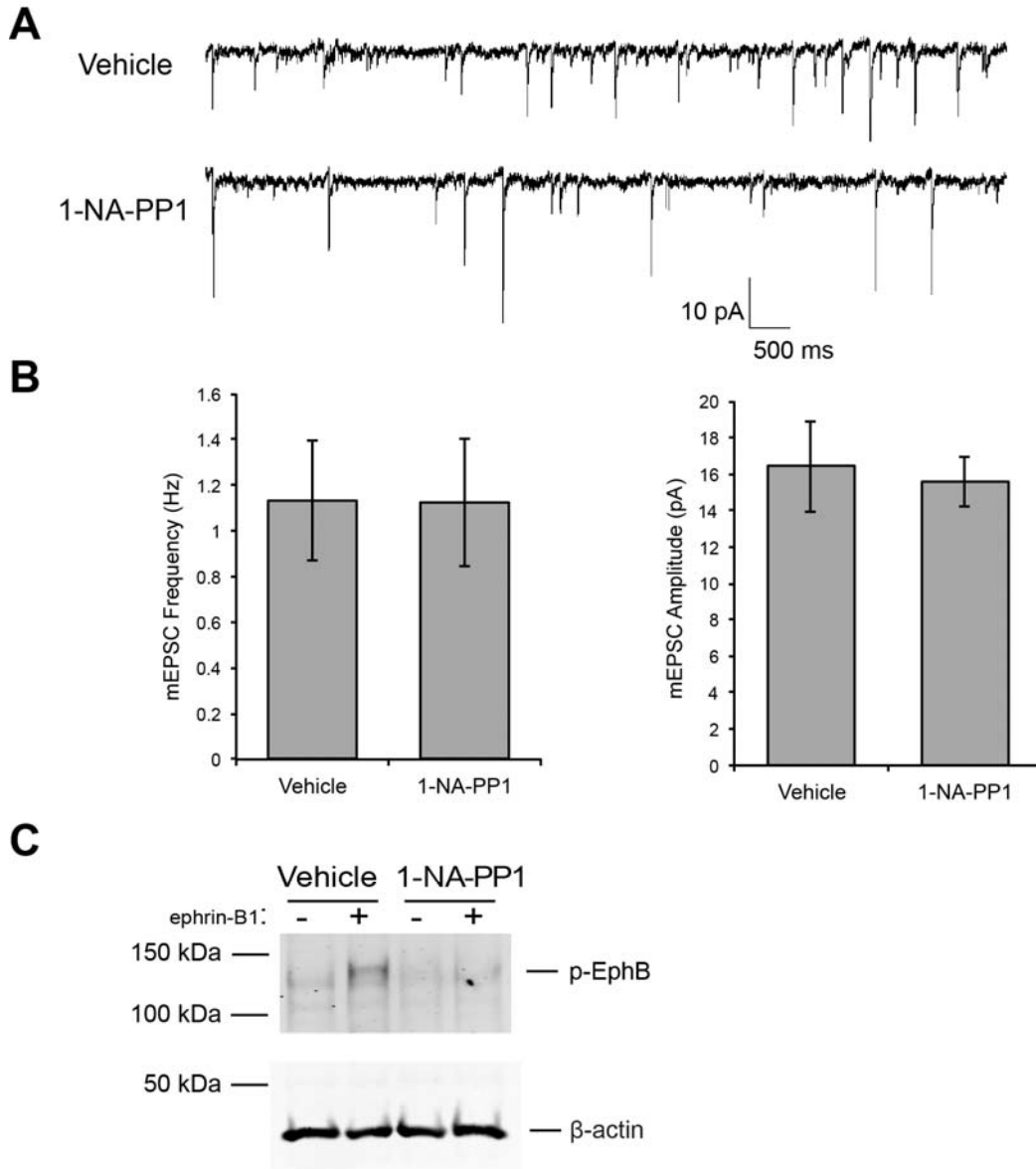


Figure 4.1: EphB tyrosine kinase activity is dispensable for the formation of functional excitatory synapses in vitro

(A) Representative traces from recordings from dissociated cortical neurons from AS-EphB TKI embryos at 10-12 days in vitro (DIV). Cultures were treated with vehicle (DMSO) or 1-NA-PP1 (1 μ M) from 3 DIV until the time of recording.

(B) Quantification of frequency and amplitude of miniature excitatory postsynaptic currents (mEPSCs) from vehicle and 1-NA-PP1 treated neurons. Data are mean \pm SEM. N=13 cells per condition.

Figure 4.1 (continued)

(C) Concurrent cultures treated with identical drug conditions were stimulated for 30 minutes with ephrin-B1 at the time of recording (10 DIV) and subsequently lysed and processed for western blotting with antibodies to phospho-EphB and β -actin.

Our finding that inhibition of EphB tyrosine kinase activity does not affect mEPSC frequency in cultures, taken together with many previous studies that have implicated EphBs in excitatory synapse formation (Dalva et al., 2000; Henkemeyer et al., 2003; Kayser et al., 2008), suggests the possibility that kinase-independent EphB signaling mediates early synaptogenesis. To test the requirement of EphB tyrosine kinase signaling for synapse development using an independent method, we treated cultured hippocampal neurons with vehicle or 1-NA-PP1 (250 nM-5 μ M) from 10-14 DIV, the peak of synaptogenesis. To quantify the number of excitatory synapses, we stained cultures with antibodies against synapsin and PSD-95, markers for pre- and post-synaptic specializations, respectively, and counted their overlap. Consistent with our electrophysiological results, we found that inhibiting EphB tyrosine kinase activity at a range of doses had no effect on the number of excitatory synapses as measured by the apposition of pre- and post-synaptic specializations (**Figure 4.2**). Taken together, we conclude that the tyrosine kinase activity of EphBs is not required for excitatory synapse formation and that EphBs may signal through a kinase-independent mechanism to regulate synaptic development.

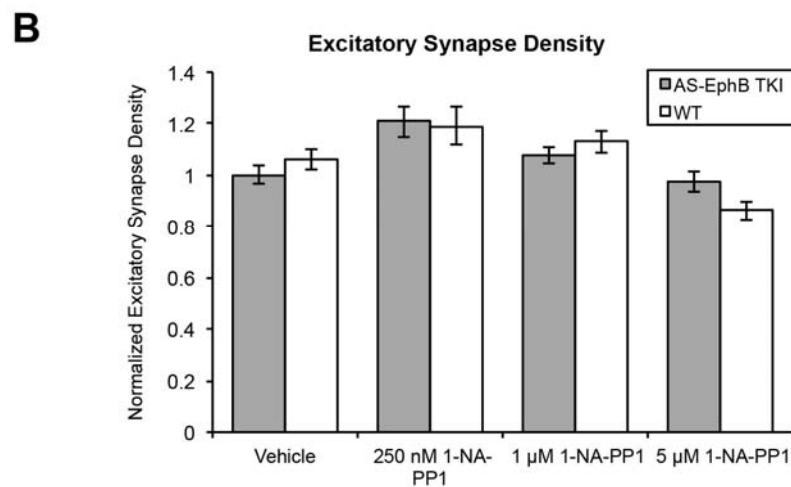
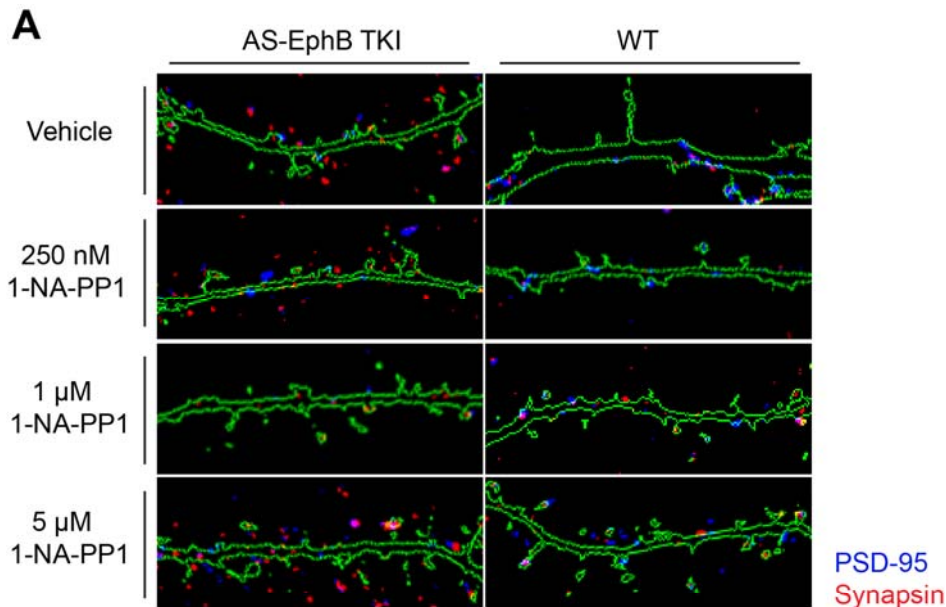


Figure 4.2: The assembly of synapses does not require EphB tyrosine kinase activity

Quantification of synaptic puncta density in dissociated hippocampal neuronal cultures sparsely transfected with GFP and treated with vehicle (DMSO) or 1-NA-PP1 (250 nM - 5 μM) from 10-14 DIV. Neurons were fixed and stained at 14 DIV and excitatory synaptic puncta were defined as discrete regions of overlap between PSD-95, synapsin, and GFP. Data are mean ± SEM. N (neurons) = WT Vehicle (112), WT 250 nM 1-NA-PP1 (31), WT 1 μM 1-NA-PP1 (67), WT 5 μM 1-NA-PP1 (49), AS-EphB TKI Vehicle (114), AS-EphB TKI 250 nM 1-NA-PP1 (31), AS-EphB TKI 1 μM 1-NA-PP1 (66), AS-EphB TKI 5 μM 1-NA-PP1 (49).

EphB tyrosine kinase signaling in spinogenesis

Even more dramatic than the loss of synapses in EphB triple knockouts is the dysfunction in dendritic spine development (Henkemeyer et al., 2003). We reasoned that EphB tyrosine kinase signaling might regulate this morphological aspect of neural development, which reaches its peak between 10-21 DIV in dissociated neuronal cultures. Several groups have reported significant defects in spinogenesis after perturbing EphB function during this period (Ethell et al., 2001; Kayser et al., 2008). To block the kinase activity of EphBs over this long period of time, we treated cortical neuronal cultures with 1 μ M 1-NA-PP1 from 10-21 DIV and exchanged media completely every three to four days. To image fine dendritic structures, we transfected neurons with GFP at 10 DIV and subsequently fixed and stained them with antibodies to GFP at 21 DIV to enhance the fluorescent signal. To assess spine density, we counted spines per unit dendritic length over multiple segments of dendrite totaling more than 50 μ m. We found no significant change in the number of spines or their morphology in AS-EphB TKI or WT neurons after treatment with 1-NA-PP1 (**Figure 4.3A, B**). We conclude that EphB tyrosine kinase activity does not regulate dendritic spine development in dissociated cortical neurons.

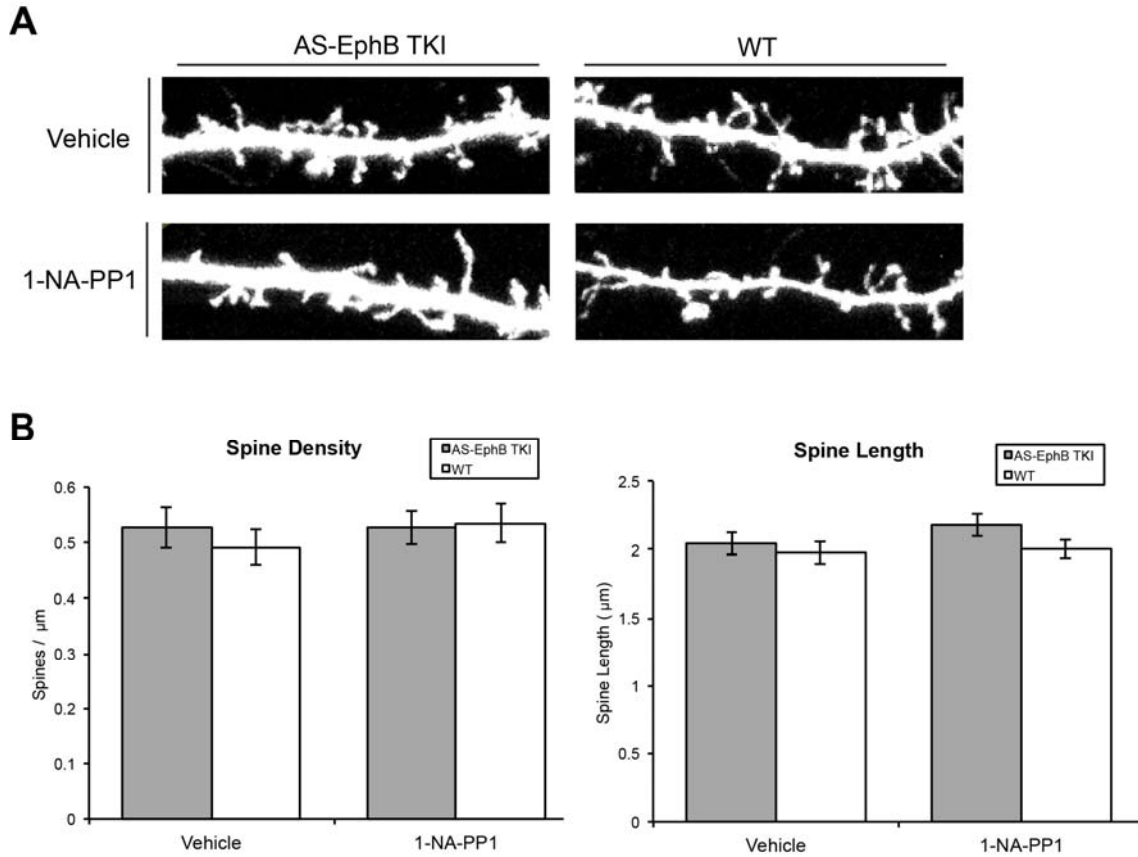


Figure 4.3: EphB tyrosine kinase activity is not required for dendritic spine development in culture

(A) Representative images of dendritic spines from cultured cortical neurons treated with vehicle or 1 μM 1-NA-PP1 from 10-21 DIV. Neurons were transfected at 10 DIV with GFP and stained against GFP after fixation to allow visualization of fine dendritic processes.

(B) Quantification of spine density and spine length from (A). Data are mean \pm SEM. N = 25-33 neurons / condition.

The use of dissociated neurons has limitations, as these neurons are not able to form connections in a circuit as they would in vivo. In addition, dissociated cultures are a heterogeneous mix of several cell populations; therefore, changes in one subset of neurons may be masked by a surrounding class of neurons. To investigate the role of EphB tyrosine kinase signaling in a more physiological context, we studied spinogenesis in an intact circuit where the architecture of the hippocampus is preserved. We prepared organotypic slices of the hippocampus from postnatal day 6 or 7 (P6/7) WT or AS-EphB TKI pups. To visualize fine dendritic morphology, neurons were biolistically transfected using gold bullets containing a plasmid encoding GFP at 2 DIV.

We incubated AS-EphB TKI or WT slices in vehicle or 1-NA-PP1 (250 nM-5 μ M) for 8 DIV during the peak of spinogenesis and measured spine density and length in both apical and basal dendrites from pyramidal neurons. Similar to results in dissociated cultures, we found no change in the density or length of spines in apical (**Figure 4.4A, B**) or basal (**Figure 4.4C, D**) dendrites. High doses of 1-NA-PP1 (5 μ M) led to small, but statistically insignificant reductions in spine density for both AS-EphB TKI and WT neurons, suggesting a possible off-target effect at this dose (**Figure 4.4B**). There was also a small increase in length that was specific to AS-EphB TKI neurons, but this trend was not significant as assessed by two-way ANOVA ($F_{(1,62)}=1.35$, $p=0.25$). Our data therefore support the conclusion that the tyrosine kinase activity of EphBs is not required for the development of dendritic spines in hippocampal slices.

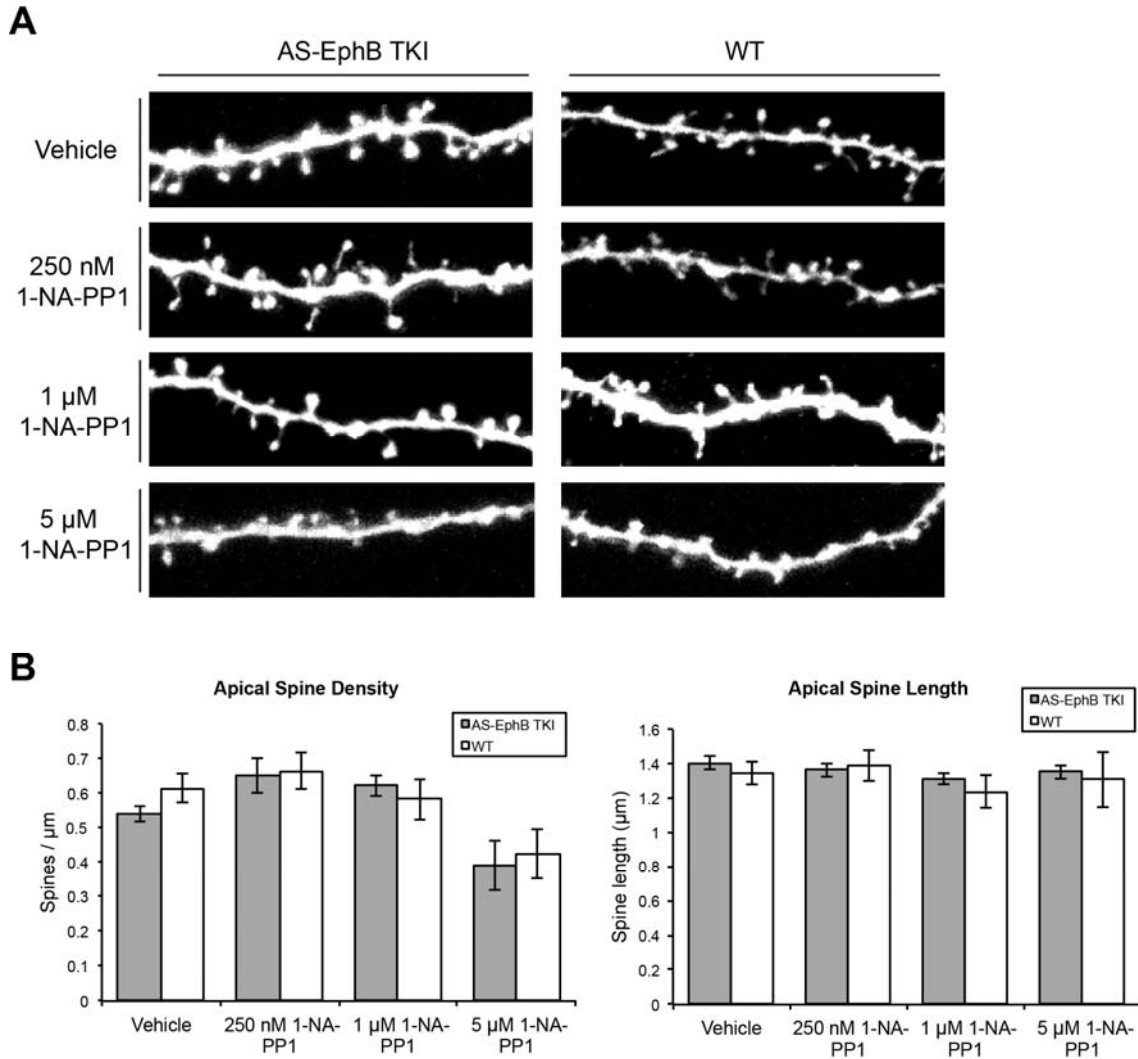


Figure 4.4: EphB tyrosine kinase signaling is not required for dendritic spine development in hippocampal slices

(A) Organotypic slices from P6/7 mouse pups were treated with vehicle or 1-NA-PP1 (1 μM) from 0-8DIV and biolistically transfected with GFP at 2-3 DIV. Representative images of apical spines.

(B) Quantification of apical spine density and spine length. Data are mean \pm SEM. N (neurons) = WT Vehicle (21), WT 250 nM 1-NA-PP1 (14), WT 1 μM 1-NA-PP1 (11), WT 5 μM 1-NA-PP1 (7), AS-EphB TKI Vehicle (30), AS-EphB TKI 250 nM 1-NA-PP1 (18), AS-EphB TKI 1 μM 1-NA-PP1 (17), AS-EphB TKI 5 μM 1-NA-PP1 (10).

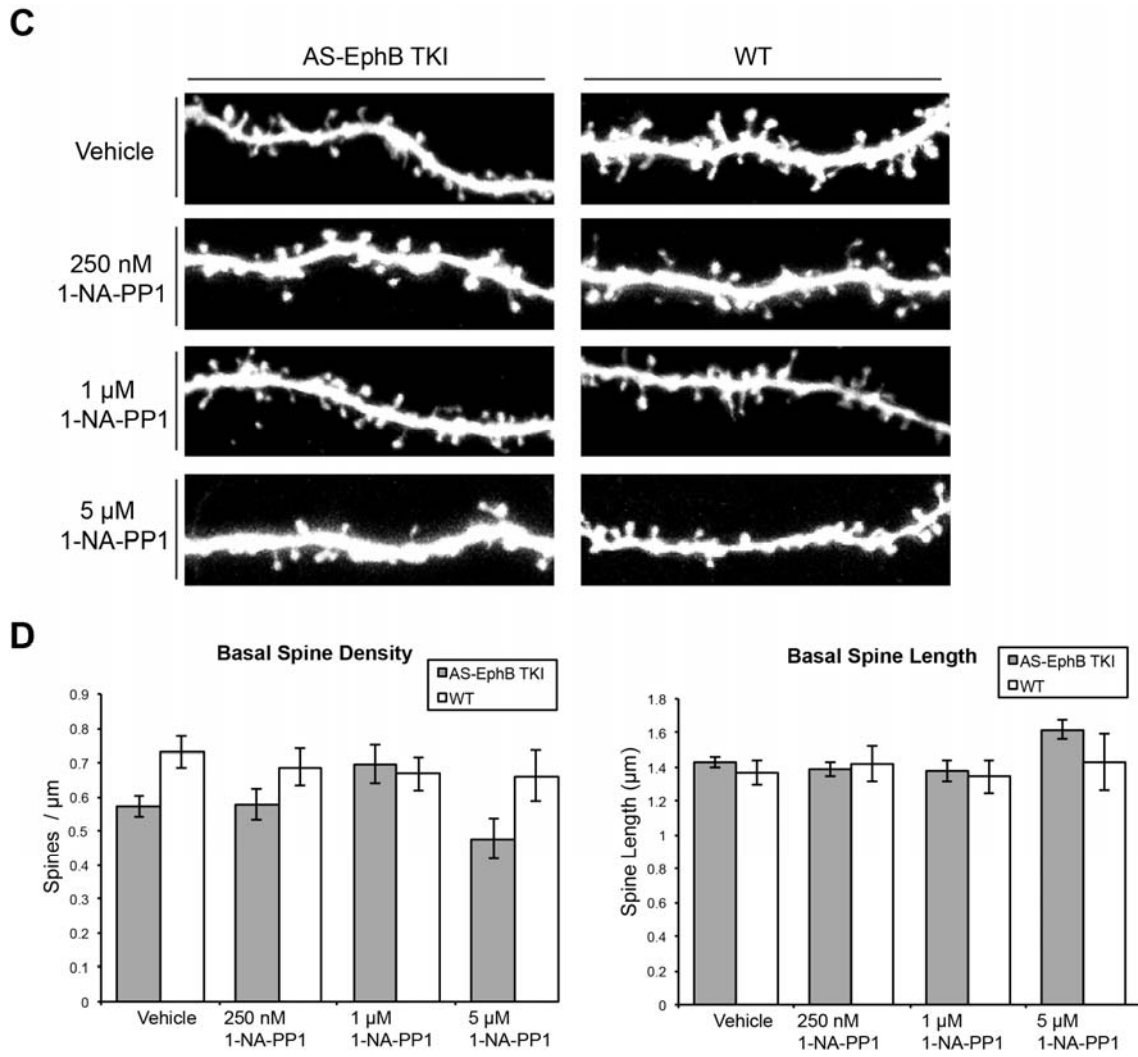


Figure 4.4 (continued)

(C) Representative images of basal spines.

(D) Quantification of basal spine density and spine length.

To compare our findings to acute loss-of function of EphB2 in the same context, we attempted RNAi knockdown of EphB2 using a vector (psuper) based strategy to express previously published shRNA hairpins (Kayser et al., 2006; Kayser et al., 2008). We selected a dose of EphB2 hairpin routinely used in our laboratory that did not affect the viability of cells. As a control, we used a scrambled hairpin previously generated in our lab that has no known targets. Cells were biolistically transfected with the control or EphB2 shRNA from 2-8 DIV. We found no difference in spine density or morphology between control and EphB2 hairpin-transfected cells, indicating that EphB2 alone is not required for spinogenesis in this context (**Figure 4.5A, B**). As an important caveat, we were not able to verify the level of EphB2 knockdown in the individual neurons that were analyzed, so it is possible that residual EphB2 was sufficient to drive spinogenesis. Knockdown of multiple EphBs is more challenging, and carries more risk of cell toxicity, but we are currently generating multiple hairpins that may be used to revisit this question.

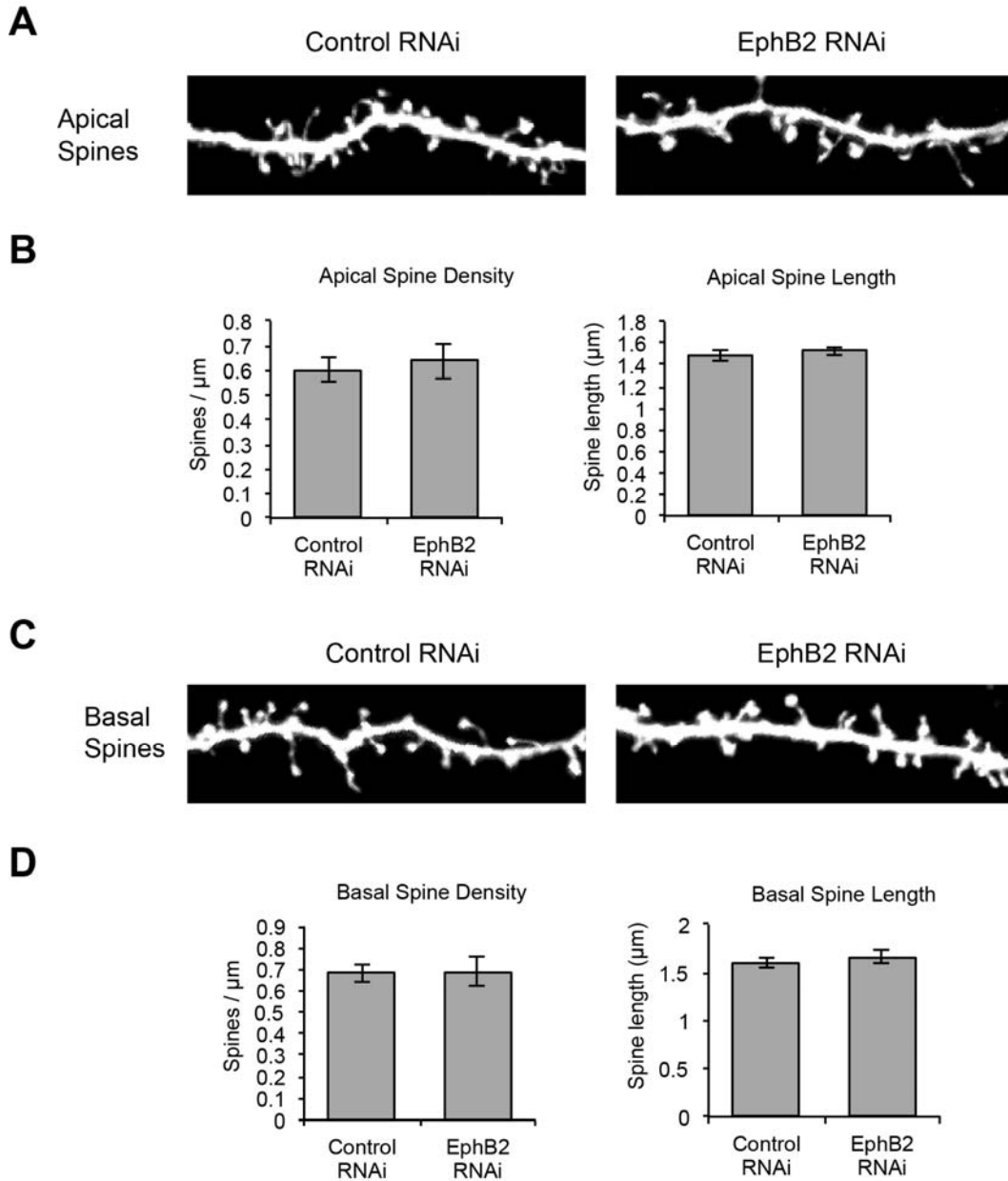


Figure 4.5: Analysis of dendritic spines in EphB2 shRNA-transfected neurons

(A) Organotypic slices from P6/7 mouse pups were transfected with control RNAi or EphB2 RNAi from 2-8DIV. Representative images of apical spines.

(B) Quantification of apical spine density and spine length. Data are mean \pm SEM. N = 10-16 neurons / condition.

(C) Representative images of basal spines.

(D) Quantification of basal spine density and spine length.

4.3 Discussion

We have demonstrated that in several developmental contexts EphB tyrosine kinase function is not required for synapse or spine formation. Blocking the kinase signaling of EphBs in both culture and slice had no effect on synapse number by immunocytochemistry or electrophysiology and no effect on spine number or morphology in culture or slice.

Consistent with these results, we find very low or even absent levels of endogenous EphB phosphorylation in cultures under a number of conditions at multiple timepoints, as assessed through both western blot and immunocytochemistry. This is true even at times when EphBs (and ephrin-Bs) are highly expressed, indicating that the ratio of activated EphBs to total EphBs is incredibly low (**Figure 1.2**). While it is possible that EphB tyrosine kinase function is rarely activated (and if so, only for a short time), such as when a dendritic filopodium comes in contact with an axon, it is also possible that EphBs are not activated in these contexts and are signaling through kinase-independent mechanisms.

Our data are consistent with a role for different aspects of EphB signaling, such as PDZ interactions, in mediating spine development. The clustering of PDZ-domain containing synaptic proteins or the recruitment of NMDA receptors via the EphB extracellular region may initiate or stabilize the formation of excitatory synapses during synaptogenesis (Dalva et al., 2000).

Importantly, since our experiments studying synaptogenesis all yielded negative results, it is still possible that inhibition of EphB tyrosine kinase function would have

some effect in another experimental context where EphBs play a more active role. However, we have chosen timepoints and conditions that encompass the majority of spine formation and synaptogenesis and still see no effect of EphB tyrosine kinase inhibition.

Another possibility is that most acute manipulations target individual cells and that our manipulations affect every cell. Thus, competition between cells for the relative amount of EphB might be more important than the absolute value of EphB activation. However, we find this unlikely given the strong phenotypes reported in EphB TKO mice where EphBs are absent from all cells, not just a subset (Henkemeyer et al., 2003).

Alternatively, it remains possible that EphBs are not involved in synaptogenesis or spine formation directly. Ideally, an acute control to test EphB involvement at a certain timepoint would address this possibility. However, this goal is not trivial. Indeed, this challenge was the basis for our aim of designing AS-EphB TKI mice. Given the limitations of RNAi, especially for three separate targets, a loxP-based conditional knockin would be the best approach. The *ephB1*, *ephB2*, and *ephB3* genes could be acutely deleted by CRE-mediated excision. CRE- transfected WT mice or mock-transfected conditional mice would serve as excellent controls for toxicity effects. A number of molecules have been identified as mediators of synapse development, usually by RNAi, and our results may confirm that synaptogenesis and spine formation are particularly sensitive to these manipulations. The conditional knockout approach would overcome this hurdle and greatly clarify this question.

Since EphB tyrosine kinase activity is not required for the development of excitatory synapses, it will be important to test whether the kinase activity of EphBs

functions instead in the plasticity of synapses, as multiple reports have identified a role for EphBs in long-term potentiation (LTP) in the hippocampus (Grunwald et al., 2001; Henderson et al., 2001).

4.4 Materials and methods

Electrophysiology:

Whole-cell voltage clamp recordings were obtained using an Axopatch 200B amplifier at 25°C. During recordings, neurons were perfused with artificial cerebrospinal fluid containing 127 mM NaCl, 25 mM NaHCO₃, 1.25 mM Na₂HPO₄, 2.5 mM KCl, 2 mM CaCl₂, 1 mM MgCl₂, 25 mM D-glucose, and saturated with 95% O₂, 5% CO₂. Vehicle or 1-NA-PP1 treatment was begun at 3 DIV and continued throughout recordings. The internal solution for mEPSC analysis contained 120 mM cesium methane sulfonate, 10 mM HEPES, 4 mM MgCl₂, 4 mM Na₂ATP, 0.4 mM Na₂GTP, 10 mM sodium phosphocreatine and 1 mM EGTA. Osmolarity and pH were adjusted to 310 mOsm and 7.3 with Millipore water and CsOH, respectively.

mEPSCs were isolated by exposing neurons to 0.5 μM tetrodotoxin, 50 μM picrotoxin, and 10 μM cyclothiazide (all from Tocris Bioscience). Cells were allowed a 5 minute equilibration period before recordings began. Cells with series resistance larger than 25 MΩ during the recordings were discarded. Data were analyzed in IgorPro (Wavemetrics) using custom-written macros. For each trace, the event threshold was set at 1.5 times the root-mean-square current. Currents were counted as events if they crossed the event threshold, had a rapid rise time (1.5 pA/ms) and had an exponential decay ($\tau < 50$ ms for mEPSC).

As a control for inhibition of EphBs, concurrent plates of neurons were treated with inhibitor and at the time of recording were stimulated with ephrin-B1 for 30 minutes. Neurons were lysed in 1x SDS-sample buffer, run on western blot and probed with rabbit anti-phospho-Eph and mouse anti- β -actin (Abcam).

Synaptic puncta colocalization assay

Hippocampal neurons were cultured as described in Chapter 2. Neurons were transfected with GFP at 10 DIV using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Neurons were fixed in 4% PFA / 2% sucrose in PBS and stained with mouse anti-PSD-95 (Pierce) and rabbit anti-synapsin (Millipore) in 0.1% gelatin, 0.3% Triton-X 100, 0.01% sodium azide in PBS. Images were obtained on a Zeiss Pascal confocal microscope, using a 63x objective with sequential acquisition settings at 1024 x 1024 pixel resolution. 5 stacks of 0.5 μ m thickness were acquired for each neuron. Synapse density was calculated using custom-written MATLAB software. Unbiased image thresholds for synaptic marker channels were set at the mean pixel intensity plus two standard deviations for each channel. Thresholded images were segmented and objects of less than 4 pixels were removed. Synapses were defined as areas of triple overlap between the GFP, pre-synaptic channel (synapsin), and post-synaptic channel (PSD-95) greater than 3 pixels, and synapse density was determined by dividing the number of synapses by the area of the thresholded GFP dendrites.

Organotypic slices

Hippocampal organotypic slices were prepared in ice cold dissection media (1 mM CaCl₂, 5 mM MgCl₂, 10mM D-glucose, 4 mM KCl, 26 mM NaHCO₃, 218 mM sucrose, 1.3 mM sodium phosphate, and 30 mM HEPES, pH 7.4). Brains were isolated from P6/7 pups, and hippocampi were excised and chopped into 400 µm sections. Slices were cultured on Millicell cell culture inserts (Millipore) in media containing 20% horse serum, 1 mM L-glutamine, 0.0012% ascorbic acid, 1 µg/mL insulin, 1 mM CaCl₂, 2 mM MgCl₂, 2.3 mg/mL glucose, 0.44 mg/mL NaHCO₃, 7.16 mg/mL HEPES in MEM.

Analysis of dendritic spines

For dissociated neuron experiments, cortical neurons were cultured as described in Chapter 2 and grown on a monolayer of astrocytes on glass coverslips. Neurons were treated with vehicle or PP1 derivatives at 10 DIV and media was changed entirely every 3-4 days (using neuronally preconditioned media). Neurons were transfected with GFP at 10 DIV using Lipofectamine 2000 and fixed with 4%PFA/2% sucrose in PBS at 21 DIV. Coverslips were stained for GFP and mounted on slides using Fluoromount-G.

Neurons were imaged on a Zeiss Pascal confocal microscope, using a 63x objective, and maximal z-projections were analyzed using Metamorph software. Multiple sections of dendrite totaling > 50 µM were counted for each neuron.

For experiments in slice, hippocampal slices were treated with vehicle or PP1 derivatives at 2 DIV and media/drug were fully replaced every 2-3 days. At 2-3 DIV, GFP (or GFP + hairpins) was biolistically transfected using a Helios gene gun. DNA bullets were prepared from 1.6 µm gold microcarrier particles (Biorad). After 8 DIV,

slices were fixed in 2.5% PFA / 4% sucrose in PBS for 1 hour and stained with chicken anti-GFP (Aves Labs) and rabbit anti-NeuN (Millipore) antibodies to visualize the structure of hippocampal fields. Basal and apical dendrites were analyzed separately and sections of dendrite totaling $> 50 \mu\text{M}$ were counted for each neuron.

shRNA constructs

Published shRNA constructs were obtained from the laboratory of Matthew Dalva (Kayser et al., 2006; Kayser et al., 2008). The EphB2 hairpin sequence was: AGCCGGCGCCATCTATGTC. As a control, we used a hairpin that originally targeted Mef2 but contained two mutations: GTATCTCTCTGGTCGCTCC (Flavell et al., 2006). DNA bullets consisted of 10 μg shRNA + 25 μg GFP + 15 μg pcs2+ empty vector per 12.5 mg gold particles.

Chapter 5

Conclusion

EphBs play a vital role in the development of the nervous system. In an attempt to understand their function, numerous studies have catalogued the deficits observed when EphBs are perturbed. However, these experiments have suffered from severe constraints: they cannot isolate the specific signaling modalities of EphBs at precise stages of development. As a result, the EphB field has produced an abundance of data and a dearth of firm conclusions.

At the initiation of our project, we identified four major hurdles to studying EphBs. First, since EphBs are involved at almost every stage of development, only acute manipulations can identify specific phenomena as primarily controlled by EphBs. Second, the multiple domains of EphBs make it difficult to isolate functions of specific signaling modalities. Third, the presence of three partially redundant EphB receptors in the brain makes loss-of-function experiments challenging. Finally, most perturbations of EphB signaling do not contain suitable controls to ensure that the observed phenomena are a result of EphB manipulation.

We set out to overcome these hurdles and devise a novel approach that would facilitate well-controlled and temporally precise experiments. We have developed a chemical-genetic strategy to acutely inhibit the kinase signaling of EphB receptors *in vitro* and *in vivo*. We have demonstrated the specificity and effectiveness of inhibition in our analog-sensitive EphB triple knockin mice and studied these mutant kinases in the context of axon guidance and synaptogenesis.

Using the AS-EphB TKI mice, we have definitively shown a requirement for EphB tyrosine kinase activity in the guidance of ventrotemporal retinal ganglion cell axons at the optic chiasm. Furthermore, we have resolved a major controversy in the field

of Eph research by identifying EphB tyrosine kinase signaling as vital for the formation of the corpus callosum, the anterior commissure, and corticofugal tracts. Our experiments provide clear mechanistic data about the nature of EphB signaling in a field replete with confusing studies. Indeed, our results call into question previous experiments that have relied on overexpression paradigms and mutant mice.

While we have focused on axon guidance and synaptogenesis, the experiments detailed in this thesis demonstrate the utility of the chemical-genetic approach for studying EphB signaling in general. The AS-EphB TKI mice should lay the groundwork for further studies of EphBs that will greatly clarify their signaling and may identify new functions in the developing and mature organism. Additionally, the acute and reversible nature of our system make it ideal to study dynamic cell biological processes involving EphBs, such as receptor trafficking, internalization, and movements along the membrane.

The determination that kinase activity is required for axon guidance still leaves a major question: does the relevant signaling pathway stem from autophosphorylation and recruitment of docking proteins or from the phosphorylation of protein substrates. AS kinases accept orthologous ATP analogs that can directly label the targets of the kinase. We should also be able to use the AS-EphB TKI mice to identify direct targets for the first time (Banko et al., 2011). The disadvantage of this technique is that the current labeling ATP orthologues require permeabilizing conditions to enter the cell. Nevertheless, given the scarcity of known axon guidance pathways, determination of EphB substrates in the relevant neurons could be a powerful approach.

Model for EphB receptor tyrosine kinase signaling in nervous system development

Considering the data available before our studies, a reasonable model in the context of axon guidance would be as follows: when EphBs are present in the growth cone, the kinase activity is essential, but when ephrins are acting at the growth cone, EphB tyrosine kinase activity is not required for guidance. This hypothesis could be falsified by finding axon guidance decisions that are EphB-dependent, where ephrin-Bs are predominantly expressed on the growth cone.

Surprisingly, based on current expression data, anterior commissure and corpus callosum fibers appear to be such an example where EphBs are not highly expressed on the axons (Michael Robichaux and Chris Cowan, unpublished). How would the kinase activity of EphBs mediate repulsion of an ephrin-B-expressing growth cone? As Bush et al. have demonstrated, the extracellular domain of Ephs can cause this collapse (Bush and Soriano, 2009). It is possible that the adhesive nature of the ephrin/Eph interaction dominates *in vivo* unless the EphB-expressing cell can disrupt this interaction (through a kinase-dependent process). Our studies provide some evidence that this is unlikely to be mediated by surface expression, but further study is warranted. Alternatively, EphBs could lead to morphological changes in the guidepost cell that cause it to retract from the growth cone, thus freeing the axon from the adhesive interaction. Live-imaging of these two interacting cell types could begin to test this hypothesis.

Based on our data, we propose a model in which the tyrosine kinase activity of EphBs is required for repulsive interactions such as axon guidance, but is not required for adhesive interactions such as synapse formation. This hypothesis is consistent with a theory proposed by Holmberg et al. in which the amount of kinase activation predicts the

strength of repulsion (Holmberg and Frisen, 2002). Furthermore, inhibiting kinase-activity in a normally repulsive context (such as axon guidance) may lead to unnatural adhesion. Thus, it will be interesting to study the misprojected axons we observed in the optic tract, corpus callosum, anterior commissure, and corticofugal tracts. It will also be important to search for any counterexamples to our model, such as an adhesive interaction that is kinase-dependent.

Missing from our study is a clear EphB loss-of-function phenotype in the context of our AS-EphB TKI experiments. Thus, it is still formally possible that all EphB signaling, at least all forward signaling, is kinase dependent. An appropriate solution to this deficiency is to generate specific hairpins to all three neuronal EphBs, an approach our lab is currently taking.

Outstanding questions

Based on our findings, it is likely that the use of lacZ mutants and dominant negatives can lead to unreliable results. Therefore, it will be worthwhile to revisit a number of other developmental processes that rely on EphBs and determine whether they are kinase-dependent. Using the reversibility of the inhibitors will also allow the ability to pinpoint a specific temporal window when these events are occurring. Notably, all of our experiments have been conducted with triple knockin mice. In the future, it may be worth separating AS-EphB1, AS-EphB2, and AS-EphB3 in order to investigate whether their kinase functions play differing roles in development.

In our *in vivo* experiments, we generally saw partial reductions in axon tracts, suggesting the possibility that EphB tyrosine kinase activity was not fully inhibited. To

this point, we have used subcutaneous injections of 1-NA-PP1, but developing an oral delivery method or a releasable capsule could improve future experiments. In addition, new PP1 derivatives are being developed, and testing their potency on our AS mutants could yield more suitable inhibitors.

Isolation of EphB signaling in the adult has proven extremely challenging. With our chemical-genetic approach, previously inaccessible questions now become tractable. This exploration of how EphBs might mediate the maintenance, plasticity, and regeneration of the adult nervous system beckons as the next frontier in the field of Eph biology.

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