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EphB1 and EphB2 Intracellular Domains Regulate the Formation of the Corpus Callosum and Anterior Commissure

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Summary

The two cortical hemispheres of the mammalian forebrain are interconnected by major white matter tracts, including the corpus callosum (CC) and the posterior branch of the anterior commissure (ACp), that bridge the telencephalic midline. We show here that the intracellular signaling domains of the EphB1 and EphB2 receptors are critical for formation of both the ACp and CC. We observe partial and complete agenesis of the corpus callosum (AgCC), as well as highly-penetrant ACp misprojection phenotypes in truncated EphB1/2 mice that lack intracellular signaling domains. Consistent with the roles for these receptors in formation of the CC and ACp, we detect expression of these receptors in multiple brain regions associated with the formation of these forebrain structures. Taken together, our findings suggest that a combination of forward and reverse EphB1/2 receptor-mediated signaling contribute to ACp and CC axon guidance.

Introduction

The Eph receptors are a large family of axon guidance receptor molecules that interact with their high-affinity binding partners, the ephrins, and function to mediate many developmental processes in the central nervous system (CNS), including axon guidance,

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M.A.R., G.C., M.H. and C.W.C. conceived and designed the ACp and CC studies. M.A.R. and G.C. performed the experiments. H.-Y.H., M.J.S., and M.E.G. contributed to the AS-Eph studies. Data analysis was performed by all authors. M.A.R., G.C. and C.W.C. wrote the manuscript.

synapse formation and synapse plasticity (Egea and Klein, 2007; Himanen et al., 2004; Pasquale, 2005; Shen and Cowan, 2010). The Eph receptors are classified as either A- or B-class Eph receptors, based on homology and binding affinity for ephrin-A proteins (glycophosphatidylinositol-anchored membrane proteins) or the ephrin-B proteins (single-pass transmembrane proteins), respectively. During axon guidance, the binding of Ephs to ephrins initiates bidirectional signaling events that often results in repulsive axon growth cone turning. Depending on the context, forward signaling mediated by the Eph receptor intracellular domains and/or reverse signaling through the ephrin interaction domains are required for axon guidance *in vivo* (Cowan and Henkemeyer, 2002; Egea and Klein, 2007; Holland et al., 1996). The intracellular domains of EphB1 and EphB2 contain several highly-conserved functional regions, including: (1) juxtamembrane tyrosines that mediate SH2-containing protein binding, (2) a tyrosine kinase domain, and (3) a sterol alpha motif (SAM) protein-protein interaction domain (Egea and Klein, 2007). EphBs and ephrin-Bs are key regulators of axon guidance during the development of multiple white matter tracts in the mammalian CNS. These include *in vivo* studies of the optic nerve tract, the corpus callosum (CC), the posterior branch of the anterior commissure (ACp), thalamocortical projections (TC), corticothalamic projections (CT) and the corticospinal tract (CST) (Chenau and Henkemeyer, 2011; Henkemeyer et al., 1996; Kadison et al., 2006; Kullander et al., 2001; Mendes et al., 2006; Orioli et al., 1996; Robichaux et al., 2014; Williams et al., 2003).

The CC is an interhemispheric tract that connects layer II/III and layer V cortical neurons, and is essential for the integration of motor, sensory and cognitive circuits between the cortical hemispheres (Innocenti et al., 1995). The formation of the CC occurs between embryonic day 15 (E15) to E18 and is a complex process involving multiple axon guidance factors functioning around the midline (Mendes et al., 2006; Ozaki and Wahlsten, 1992; Rash and Richards, 2001; Serafini et al., 1996; Shu and Richards, 2001). EphB1 and EphB2 receptors are both expressed in the cortical plate near the dorsal region where the CC axons eventually cross the midline (Mendes et al., 2006; North et al., 2009), and the EphB2 receptor, in particular, is expressed in both CC axons as well as midline glial structures (Mendes et al., 2006). Single *EphB1*^{-/-} or *EphB2*^{-/-} mutant mice each display agenesis of the CC (AgCC), but the phenotype displays partial penetrance (Mendes et al., 2006; Orioli et al., 1996). The EphB receptor tyrosine kinase domain appears to be critical for normal CC formation since inhibition of EphB1–3 receptor kinase activity *in vivo* results in a partial AgCC phenotype (Soskis et al., 2012). However, the relative contribution of each EphB receptor's kinase activity for normal CC formation remains unclear.

The AC is another large, interhemispheric white matter tract of the mammalian forebrain that connects the temporal cortical regions of the basal telencephalon. The AC tract is a bipartite axon bundle with anterior and posterior branches (abb. ACa & ACp) that converge with the stria terminalis axon bundle into a single, large fascicle at the ventral telencephalic midline (Jouandet and Hartenstein, 1983). In rodents, the ACa comprises axons from the olfactory nuclei and anterior piriform cortex, whereas the ACp comprises cortical axons from the posterior piriform cortex and other temporal cortices along with axons from the lateral nucleus of the amygdala. Posterior ACp fibers initially migrate together along the rostral-caudal external capsule before converging with other ACp axons and turning toward

the midline (Cummings et al., 1997; Jouandet and Hartenstein, 1983; Pires-Neto and Lent, 1993). Both the EphB1 and EphB2 receptors have previously been implicated in ACp axon tract development. *EphB2*^{-/-} and *EphB2*^{-/-};*EphB3*^{-/-} mutants display a severe ACp phenotype. Specifically, a major fraction of ACp tract axons fail to cross the ventral midline and is misplaced to the floor of the ventral telencephalon (Henkemeyer et al., 1996; Ho et al., 2009; Orioli et al., 1996), while ipsilateral projections from the somatosensory cortex aberrantly cross the ventral midline at the AC in *EphB1*^{-/-} mutants (Lodato et al., 2014). Still, it remains unclear how EphB1 is directly involved in ACp formation and guidance, nor is it clear whether the EphB1 and EphB2 role in ACp formation requires their forward and/or reverse signaling capacities.

In this study, we find that both *EphB1* and *EphB2* are required for proper formation of the CC and the ACp, and that these genes will partially compensate for the loss of the other. We also document essential roles for EphB1 and EphB2 receptor intracellular signaling domains in the formation of both the CC and ACp, suggesting that both forward and reverse signaling events are involved in these guidance events *in vivo*. In *EphB1/B2 DKO* mice we also observed ACp axon guidance errors from a discrete cortical subregion that does not normally project axons to the ACp. Similar to our recent study on EphB1/B2 forward signaling in CT and corticofugal axon guidance (Robichaux et al., 2014), our current findings suggest an important role for *EphB1* and *EphB2*, either directly or indirectly, in the proper navigation of a subpopulation of cortical axons into the internal capsule and away from the ACp.

Results

EphB Receptor intracellular domain function is required for the formation of the corpus callosum

To examine the roles of EphB1 and EphB2 receptors, and more specifically the function of their intracellular domains, in cortical axon guidance *in vivo*, we analyzed the CC of total null (*EphB1*^{-/-}) or truncated EphB1 or EphB2 receptor mutant knock-in mice (*EphB1*^{Tr/Tr} or *EphB2*^{Tr/Tr}). The *EphB1*^{Tr/Tr} and *EphB2*^{Tr/Tr} mice possess knock-in mutations that replace their respective EphB intracellular domains with the β -galactosidase coding region. As such, these mutant mice produce a surface-expressed EphB1 or EphB2 receptor that is capable of supporting reverse signaling (Henkemeyer et al., 1996; Mendes et al., 2006; Chenaux and Henkemeyer, 2011), but lacking the intracellular kinase domain that is essential for forward signaling.

We analyzed various combinations of the *EphB1* and *EphB2* single and compound mutant mice serial coronal sections (Figure 1A–D), and quantified the extent of partial or complete agenesis of the CC (AgCC) under experimenter-blinded conditions. The hippocampal commissure was chosen as the structural landmark that divided the rostral and caudal domains of the CC since we observed several distinct phenotypes. Partial AgCC was divided into three distinct categories: (1) agenesis of the rostral region only, (2) agenesis of the caudal region only, or (3) agenesis of both the rostral and caudal regions, but with some CC evident over the hippocampal commissure (Figure 1B and Table 1). Complete AgCC was

defined as a complete absence of crossing fibers, whereas partial AgCC was considered as any of the three distinct subcategories described above (Figure 1C).

Wild-type mice display very little spontaneous partial or complete AgCC (1 in 27, Figure 1D), whereas 58% of *EphB1*^{-/-} mutant mice displayed evidence of AgCC, with a majority of mice displaying partial AgCC (Figure 1D). In comparison, 27% of the *EphB1*^{Tr/Tr} truncated mutant mice had evidence of AgCC (Figure 1D), suggesting that EphB1 receptors contribute to CC formation via both forward and reverse signaling mechanisms. *EphB2*^{Tr/Tr} mutant mice displayed an even higher proportion of mice with AgCC, although, these animals displayed mostly partial AgCC confined to the caudal region (Table 1). A previous study reported no evidence of AgCC in these mice (Mendes et al., 2006), but since the agenesis is only observed in the caudal CC, this phenotype might have been overlooked previously. Interestingly, reducing one EphB1 truncated allele (*EphB1*^{Tr/+}:*EphB2*^{Tr/Tr}) increased the EphB2 truncated mutant AgCC phenotype to a greater extent (75% vs 43% AgCC, respectively), suggesting that these receptors might function in the same genetic pathway. Similarly, removing one truncated EphB2 allele (*EphB1*^{Tr/Tr}:*EphB2*^{Tr/+}) increased the EphB1 truncated mutant phenotype (57% vs. 27%, respectively, Figure 1D). *EphB1*^{Tr/+} heterozygous mutant mice did not display any AgCC (Sup. Figure 1). When both EphB1 and EphB2 receptor intracellular domains were removed (*EphB1*^{Tr/Tr}:*EphB2*^{Tr/Tr}), 91% of the mice had partial or complete AgCC (Figure 1D). Taken together, these analyses indicate that: (1) EphB1 and EphB2 receptor intracellular domains contribute essential functions during proper formation of the corpus callosum *in vivo*, (2) there is partial functional redundancy between EphB1 and EphB2 in this process, and (3) there is a partial role for the extracellular domain of EphB1 and EphB2 in CC formation, likely as a ligand for ephrin-mediated reverse signaling. Additionally, mice lacking *ephrin-B1* do not display either partial or complete AgCC (Sup. Figure 1); however, the severity or presence of AgCC phenotypes is background strain-dependent in *ephrin-B1* mutant mice (Bush and Soriano, 2009). Previous studies documented the expression of EphB2 in the cortical axon tracts of the corpus callosum (Mendes et al., 2006), but the expression of EphB1 Receptors during CC formation has not previously been explored. Since the *EphB1*^{Tr/+} mutant mice express a fusion protein of EphB1 and β -galactosidase (β -gal), we monitored the expression of the EphB1 β -gal protein at embryonic day 16. (E16.5) – a time when callosal axons are approaching the forebrain midline. EphB1- β -gal expression was observed in the cingulate cortex and extended to an area matching the path of axons stemming from this region during callosal axon pathfinding (Figure 1E). EphB1 receptor was also expressed in key glial structures, the indusium griseum (Ig) and the dorsal portion of the glial wedge (GW) (Figure 1E), and within the ventral midline zipper glia. As such, EphB1 receptors are expressed in locations that enable them to function as potential repellant ligands (glial structures) or to mediate EphB1 receptor repulsive signaling in navigating cortical axons.

EphB1 and EphB2 are required for the formation of the anterior commissure

We previously observed that the EphB1 and EphB2 intracellular signaling domains serve partially redundant roles in reciprocal thalamocortical and corticothalamic axon guidance, suggesting that there might be similar functional redundancies for formation of the ACp. Using L1-CAM immunostaining to visualize the ACp white matter tracts that traverse the

ventral midline in WT mice at Bregma 3.4–3.5 mm in coronal sections (Paxinos, 2007), we compared brains from wild-type, *EphB1*^{-/-}, *EphB2*^{-/-}, and *EphB1/2 DKO* (*EphB1*^{-/-};*EphB2*^{-/-}) double knockout mice at postnatal day 0 (P0). Compared to WT (Figure 2A), *EphB1*^{-/-} and *EphB2*^{-/-} single knockout mice displayed moderate-to-severe ACp axon guidance errors within these regions (Figure 2B–C and Sup. Figure 2), although with only partial penetrance (Figure 3C). L1+ fibers emerged from the EC, but instead of projecting toward the midline, many axons misprojected toward the ventral brain floor (Figure 2B, 2C & 3C, orange arrows). In contrast to single mutants, the *EphB1/2 DKO* mutants displayed fully penetrant, severe ACp axon guidance errors, typically resulting in the complete absence of midline-directed ACp fibers (Figure 2D & 3C, yellow arrows). These ACp misprojections are distinct from the misprojected corticothalamic fibers that also misproject toward the ventral floor of the brain in *EphB1/2 DKO* mutants (Robichaux et al., 2014), and can be seen in adjacent coronal sections (Figure 2B and 2D, yellow triangles at 3.6 mm Bregma).

To confirm that the misprojected ACp axons originate from the deep-layer cortex, we bred the *EphB1/2 DKO* mice together with transgenic mice carrying alleles for *Fezf2-GFP*, which expresses GFP in cortical subplate and cortical layer V under control of the Fezf2 promoter (Kwan et al., 2008), or *Golli-tau-GFP*, which expresses tau-GFP in cortical layer VI under control of the *Golli* promoter (Jacobs et al., 2007; Kwan et al., 2008; Robichaux et al., 2014). There was extensive overlap between GFP and L1+ axons in the ACp in both WT and *EphB1/2 DKO* mice (Supp. Figure 3 and data not shown). In addition, the ventrally misprojected ACp tract in the *EphB1/2 DKO* mice also co-localized with GFP+ axons, indicating that many, if not all, of the misprojected axons originate from deep-layer cortical layers.

EphB1/2 receptor intracellular domains are required for formation of the ACp

To test whether the intracellular domains of EphB1/2 are involved in ACp formation, we again analyzed this cortical tract in *EphB1*^{Tr/Tr} and *EphB2*^{Tr/Tr} mutant knock-in mice. Unlike *EphB1*^{-/-} mice, which had a partially-penetrant ACp formation defect, the *EphB1*^{Tr/Tr} mice displayed no abnormalities in the ACp (Figure 3A, 3C). Similar to previous reports (Henkemeyer et al., 1996; Mendes et al., 2006), *EphB2*^{Tr/Tr} mice displayed largely normal development of the ACp (data not shown, and Figure 3C). In contrast to the single truncated EphB mutants, *EphB1*^{Tr/Tr}*EphB2*^{Tr/Tr} double mutant mice displayed a highly penetrant, and mostly severe, ACp misprojection phenotype reminiscent of the *EphB1/2 DKO* phenotype (Figure 3B, 3C, yellow arrows). Together, these findings suggest that: (1) EphB1/2 intracellular domains are required for ACp formation, (2) EphB1 and EphB2 intracellular domain share near complete functional redundancy, and (3) the extracellular domains of EphB1 and EphB2 are also required for ACp formation, likely as ligands for ephrin-B reverse signaling events and/or as mediators of cell adhesion events necessary for ACp formation (Gale et al., 1996; Henkemeyer et al., 1996; Cowan et al., 2004; Mendes et al., 2006).

To test whether EphB receptor kinase domain activity, *per se*, is required for normal ACp formation, we analyzed the analog-sensitive EphB receptor (*AS-EphB*) mice (Robichaux et

al., 2014; Soskis et al., 2012). Subtle knock-in mutations in the kinase domains of the EphB1, EphB2, and EphB3 receptors render these receptors sensitive to reversible kinase inhibition by the ATP analog, 1-NA-PP1 *in vivo* (Robichaux et al., 2014; Soskis et al., 2012). Timed-pregnant WT or *AS-EphB* mice were treated daily with vehicle or 1-NA-PP1 (80 mg/kg; SC) from E12.5 to E19.0 to inhibit EphB kinase activity during the period of ACp formation *in utero* (Figure 4A). At E19.5 we observed that *AS-EphB* embryos treated with 1-NA-PP1 displayed a fully-penetrant ACp formation phenotype, that was characterized by numerous ventrally-misprojected L1-CAM⁺ axons and a partial thinning of the ACp (Figure 4C vs. 4E). We also observed that 1-NA-PP1 administered to WT mice caused a mild, partially-penetrant ACp phenotype (Figure 4D, 4F, pink arrow), but this effect did not quite reach statistical significance. In contrast, the vehicle-treated WT or *AS-EphB* mice developed normal ACp structures (Figure 4B and 4C). Interestingly, unlike those seen in the truncated *EphB1/2* double knock-in mutant mice (Fig. 3B), the *AS-EphB* mice treated with 1-NA-PP1 did not display any severe ACp guidance phenotypes, suggesting that the EphB intracellular regions serves kinase-dependent and kinase-independent roles in ACp development *in vivo*.

EphB1/2 receptors influence the guidance of corticothalamic axons away from the ACp

ACp axons of cortical origin are derived exclusively from the lateral-most (temporal) regions of the rodent neocortex (Cummings et al., 1997; Jouandet and Hartenstein, 1983; Pires-Neto and Lent, 1993). Axons from these temporal cortical regions converge along with axons from the amygdala into a tight bundle to form the ACp near the rostral end of the forebrain (Bregma 3.4mm at P0). In *EphB1/2* DKO mutants, the ACp bundle is misprojected toward the ventral floor (and away from the midline), and we sought to determine whether these misguided axons originate from lateral cortical regions that normally comprise the ACp tract. Using an unbiased axon labeling approach (Figure 5A–B), we found in WT mice that dye placement in lateral, but not dorsomedial, cortical regions resulted in dye-labeled axons that co-localize with L1+ ACp axons, as expected (Figure 5C). In *EphB1/2* DKO mouse brains at P0, we also observed that dye placement in lateral cortical regions labeled the ACp fibers in the VTel (Figure 5D). However, in the *EphB1/2* mutant mice, we also found that dye labeling of a discreet dorsomedial cortical region (within the presumptive somatosensory cortex regions) also projected axons into the aberrant ACp tract (Figure 5D). In WT mice, no fibers from this region are observed navigating along the ACp (Figure 5C), suggesting that EphB1/2 receptors, either directly or indirectly, are critical for proper navigation of axons from this specific cortical subregion, consistent with our recent findings (Robichaux et al., 2014). Thus, in the absence of EphB1 and EphB2, labeled axons from this cortical subregion make two distinct axon guidance errors: (1) misprojection into the ACp tract (Figure 5B) and (2) misprojections within the ventral telencephalon (Robichaux et al., 2014).

EphB1/2 direct the early development of anterior commissure axons at embryonic stages

The anterior commissure is a well-documented axon tract in neonatal and adult mouse brains (Jouandet and Hartenstein, 1983; Katz et al., 1983; Silver et al., 1982). In the mouse brain, the ACp tract has been observed as early as embryonic age E14.5. At this age, EphB2 protein expression was not detected in ACp axons themselves, but was localized to ventral

forebrain regions surrounding ACp axons as they approach the midline (Henkemeyer et al., 1996). Similar to EphB2, we first analyzed EphB1 protein expression at E14.5 using an *EphB1^{lacZ}* reporter mouse, which expresses cytosolic β -galactosidase (β -gal) under control of the EphB1 promoter (Williams et al., 2003). Similar to EphB2, we detected strong lacZ expression in the tissues surrounding the ACp axons, but not obviously within the ACp axons themselves (Figure 6A). However, we detected clear EphB1-lacZ expression in the cortical plate, including the lateral-most regions that project to the ACp (Figure 6A), suggesting that the lack of β -gal staining might reflect the lack of β -gal diffusion into ACp axons. As such, EphB1 appears to be expressed in both cortical neurons and in the subpallial regions where ACp axons are navigating toward the VTel midline.

We next analyzed WT and *EphB1/2 DKO* embryonic brains at ages E12.5 – E14.5 to determine if L1+ ACp misprojections are observed at very early stages of ACp axon growth in the ventral telencephalon. In WT mouse brains analyzed at E12.5 and E13.5, no L1+ ACp axons were detected near regions of the ventrolateral cortical intermediate zone, the ventral telencephalon (VTel) or around the ventral midline (data not shown). However, at E14.5, the emerging ACp tract becomes clearly identifiable (Figure 6B), suggesting that initial L1+ ACp cortical axons enter the VTel as midline-projecting fibers around ~E13.5–E14.5. Interestingly in EphB1/2 mutant mice (*EphB1^{-/-}·EphB2^{Tr/Tr}*) at E14.5, we observed ACp axons misprojecting toward the ventral brain floor (Fig. 6C), suggesting that a major role for EphB1/2 receptors in ACp axon guidance occurs at or before this developmental stage *in vivo*.

Discussion

Commissural agenesis is a common and prevalent form of CNS malformation in the developing human brain (Raybaud and Girard, 2005) with 50 known congenital human syndromes leading to instances of AgCC (Richards et al., 2004). Notably, commissural agenesis observed in human patients is characterized as either complete or partial, and, cases of complete CC agenesis are often accompanied with the loss of the AC (Raybaud and Girard, 2005). In this study, we assessed EphB1 and EphB2 receptor intracellular domain function during the development of the CC and ACp, two major telencephalic commissures in the mammalian brain. First, we analyzed partial CC agenesis (AgCC) phenotypes in truncated EphB mutants, and demonstrated that the EphB1 and EphB2 intracellular domains are critical for the complete formation of the CC fiber tract. We also analyzed the ACp tract, which is also abnormal in mice lacking EphB1/2 genes, lacking EphB1/2 intracellular domains, or lacking embryonic EphB kinase activity. In EphB1/2 mutant mice, we detected ACp guidance errors as early as E14.5 when L1+ cortical axons are typically navigating toward the ventral midline, suggesting an early role for EphB1/2 receptors in proper ACp axon guidance decisions. In *EphB1/2 DKO* mice, we also observed misprojected ACp axons that are derived from a neocortical region that does not normally project to the ACp, suggesting that EphB1/2 receptors play an important role, directly or indirectly, in the proper navigation of these cortical subregion-specific fibers away from the ACp in a wild-type brain.

Together with previous published work (Bush and Soriano, 2009; Henkemeyer et al., 1996; Hu et al., 2003; Mendes et al., 2006; Orioli et al., 1996; Soskis et al., 2012), our results indicate that a combination of EphB1 and EphB2 forward and reverse signaling contributes to the proper development of the CC and ACp. Specifically, EphB1 and EphB2 are expressed within the midline cortical plate at embryonic stages (Figure 1, Mendes et al., 2006; North et al., 2009), where they likely function as both guideposts and as axon guidance receptors for pioneer CC axons. In both instances, EphB1/2 might mediate either forward or reverse signaling upon ephrin-B binding (discussed further below). Our ACp results expand upon previous work that established a role for the EphB1 and EphB2 receptors in ACp formation with knockout animals (Henkemeyer et al., 1996; Orioli et al., 1996; Lodato et al., 2014). Our detailed analysis of compound EphB knockout and knock-in mutant mice has revealed more subtle AgCC and ACp defects that indicate a novel role for the EphB1 and EphB2 intracellular domains in the formation of these axon tracts.

Unlike previous EphB studies that did not consider partial phenotypes (Mendes et al., 2006; Orioli et al., 1996), our analysis in EphB1/2 mutant mice focused on both complete and partial AgCC phenotypes, and we assessed rostral-caudal region AgCC effects. We found that truncated EphB1 and EphB2 mutants displayed partially penetrant AgCC phenotypes that becomes near fully penetrant in the *EphB1^{Tr/Tr}EphB2^{Tr/Tr}* double mutant mice, indicating that the EphB1 and EphB2 intracellular signaling domains are essential for proper CC formation. In addition, we performed a limited rostral-region analysis of AgCC phenotypes in a subset of the P0 mice included in our ACp studies and find the incidence of rostral AgCC generally consistent with our adult AgCC results (Supp. Figure 1). These results confirm the role of the EphB1 and EphB2 intracellular domains in CC formation, but also show how each protein may function in a more positional- and temporal-specific manner. For example, EphB2 seems to have greater function in caudal CC development compared to EphB1.

We previously reported an important role for EphB receptor kinase activity in CC formation (Soskis et al., 2012). In the current study, we observed full penetrance of a partial AgCC phenotype in the 1-NA-PP1-treated *AS-EphB* mice (Supp. Table 1). The reason for the partial AgCC is not clear, particularly since the EphB1/2 truncated mutant mice displayed a slightly more severe AgCC phenotype. It remains possible that the partial AgCC phenotype in the AS-EphB mice is due to incomplete inhibition of EphB kinase activity *in utero* and/or that kinase-independent EphB intracellular regions contribute to proper CC formation *in vivo*.

In contrast to the additive nature of EphB1 and EphB2 intracellular domain function in CC formation, loss of these domains in EphB1 or EphB2 alone did not result in significant ACp guidance errors. However, the double mutant (*EphB1^{Tr/Tr}EphB2^{Tr/Tr}*) mice displayed highly penetrant, severe ACp misprojections, indicating that the EphB1 and EphB2 intracellular domains are functionally redundant for proper ACp formation. However, *EphB1 KO* and *EphB2 KO* mice each have a low penetrance of ACp axon guidance errors, confirming an important role for reverse signaling in this process. The *EphB1/2 DKO* mice or *EphB1^{-/-}EphB2^{Tr/Tr}* mutant mice each display fully penetrant, severe ACp formation, but the *EphB1^{Tr/Tr}EphB2^{Tr/Tr}* mutant phenotypes are not as complete and severe, suggesting

important roles for the intracellular domain and ephrin-B reverse signaling in ACp formation. Since EphB1 and EphB2 receptor expression is not readily observed in the cortical axons of the ACp, it seems likely that the observed ACp guidance errors are an indirect effect of EphB receptor intracellular domain truncation. This could be due to inappropriate localization of the truncated receptors that disrupts the ability of the EphB receptors to function as ligands for reverse signaling or could reflect a role for EphB1/2 in early axon guidance events that influence ACp formation indirectly (see discussion below). While the ACp phenotypes were fully penetrant in the 1-NA-PP1-treated *AS-EphB* mice, these guidance errors were only moderate in nature, suggesting that only partial EphB kinase inhibition was achieved *in vivo* or that other domains in the intracellular regions contribute to EphB1/2-mediated axon guidance. Interestingly, we observed some mild ACp guidance errors in one-third of the WT mice treated with 1-NA-PP1 (Figure 4D), suggesting possible weak inhibition of endogenous EphB receptors. However, we failed to detect inhibition of WT EphB2 even at concentrations that completely inhibit AS-EphB2 (data not shown), suggesting that the minor ACp guidance errors that we observed in WT mice are likely due to low-level, off-target drug effects.

To explore the role of ephrin-B genes that might mediate reverse signaling events in ACp formation, we analyzed postnatal brains at P0 from *ephrin-B1*^{-/-} or *ephrin-B3*^{-/-} mice and observed normal ACp development (Sup. Figure 4). Due to the embryonic lethality (at ~E9) of *ephrin-B2*^{-/-} mice (Cowan et al., 2004), we were unable to test its contribution to ACp development. However, ephrin-B2 protein is highly expressed in the ACp of postnatal brains, and the ACp tract of *ephrin-B2*^{lacZ} truncated mutants (which are reverse signaling mutants) is significantly reduced (Cowan et al., 2004), suggesting that ephrin-B2 reverse signaling is likely involved in ACp axon guidance. Future analysis of conditional ephrin-B2 loss-of-function mutants, and compound *ephrin-B* mutants will be required to determine which of these genes, or gene combinations, contribute to development of forebrain commissures.

We performed cortical axon tracing experiments in WT brains, which essentially confirmed previous tracing studies in rodents documenting the origin of ACp fibers to the lateral-most regions of the cortex (*i.e.* posterior piriform cortex, temporal cortices and dorsal amygdalar nucleus) (Jouandet and Hartenstein, 1983; Silver et al., 1982). Analysis of cortical axon tracing in the *EphB1/2* DKO mice revealed that axons from these lateral-most cortical regions are misprojected to the base of the ventral telencephalon (Figure 5D), and that axons from a more medial cortical region, which appears to correspond roughly to the presumptive somatosensory cortex which do not normally project into the ACp, are observed co-migrating in the mistargeted ACp structures. Interestingly, axons from this same cortical region were also observed to be misprojected along with thalamocortical projections in the internal capsule (Robichaux et al., 2014), suggesting that *EphB1* and *EphB2* play critical roles in the proper targeting of descending axons from this cortical subregion. Our findings here are in general agreement with a recent study that demonstrated that *Fezf2*-specified corticospinal motor neurons, which have ipsilateral projections through the internal capsule, are misprojected toward the ACp in *EphB1*^{-/-} mutants (Lodato et al., 2014). Future studies will be required to determine whether EphB1 is acting in cortical cell autonomous fashion to guide these descending cortical axons, or whether this is an indirect effect of the

misprojected caudal thalamic axons in the ventral telencephalon observed in *EphB1 KO* and *EphB1/2 DKO* mice (Robichaux et al., 2014).

Our results also suggest that the EphB1/2 receptors regulate early stages of ACp formation. In E14.5 brains of EphB mutant mice, we observe L1-CAM-positive misprojected axons (Figure 6) located in the ventral telencephalon where we also observed misprojected thalamocortical axons (Robichaux et al., 2014). In EphB1/2 mutants, deep-layer corticothalamic axons co-mingle with the misprojected ascending thalamocortical axons later in development, suggesting the possibility that the cortical axon guidance errors could be an indirect consequence of cofasciculation with misprojected thalamic axons. As such, we speculate that misprojected ACp axons in the EphB1/2 intracellular domain mutants might misproject along the aberrant thalamocortical axon projections that are clearly present in the ventral half of the VTel in *EphB1/2* truncated mutants at age E14.5 (Robichaux et al., 2014), the same age when we first observed the developing ACp cortical axons emerging from the prenatal cortex (Figure 6B,C). This cortical cell non-autonomous mechanism could potentially explain why ACp axons do not appear to express EphB1/2 receptors (Figure 6A, (Henkemeyer et al., 1996)), but still require EphB intracellular domain functions for proper ACp axon guidance.

While our current findings reveal important roles for both forward and reverse EphB1/2 receptor-dependent signaling events in the proper formation of major forebrain commissures, they also highlight the subtleties inherent in EphB1 and EphB2 signaling, and how these two receptors have largely redundant functions *in vivo*. Future studies exploring cell autonomous manipulations of *EphB1* and *EphB2* will be critical for understanding the cellular and molecular mechanisms by which EphB1/2 signaling regulates proper formation of the ACp, the internal capsule, and the corpus callosum.

Materials and Methods

Animals

The EphB receptor knockout and knock-in mice used in this study, including *EphB1^{-/-}*, *EphB1^{Tr}* (previously annotated as *EphB1^{T-lacZ}*), *EphB1^{lacZ} EphB2^{-/-}*, *EphB2^{Tr}* (previously annotated as *EphB2^{lacZ}*), *EphB3^{-/-}* mice, *ephrin-B1^{-/-}*, and *ephrin-B3^{-/-}* were previously described (Chenau and Henkemeyer, 2011; Davy et al., 2004; Genander et al., 2009; Henkemeyer et al., 1996; Orioli et al., 1996; Williams et al., 2003; Yokoyama et al., 2001) and maintained in a CD1 background. *EphB1^{Tr}* and *EphB2^{Tr}* mice express C-terminal truncated fusion proteins containing the extracellular and transmembrane domains of EphB1 or EphB2 in frame with β -galactosidase (Chenau and Henkemeyer, 2011; Henkemeyer et al., 1996; Yokoyama et al., 2001). Notably, the *EphB2^{Tr}* mice still retain the EphB2 juxtamembrane domain region that contains two critical tyrosine residues (Y604 & Y610). The generation of *EphB1^{T697}EphB2^{T699A}EphB3^{T706A}* analog sensitive EphB triple knockin mice (*AS-EphB*) has been reported (Soskis et al., 2012). The *Fzef2-GFP* reporter line was previously described, and expresses GFP overwhelmingly in mature layer V cortical neurons and cortical pioneer axons (Kwan et al., 2008). The *Golli-tau-GFP* reporter mouse expresses tau-GFP protein exclusively in mature layer VI cortical neurons and pioneer cortical axons during early developmental stages (Jacobs et al., 2007). The GFP reporter mice were bred

together with EphB mutant mice to generate the compound mutants used in this study. For PCR genotyping, DNA was extracted from tails samples of dissected mice embryos with 100% isopropanol after an intervening lysis step. PCR was performed using the extracted DNA in a total volume of 25 μ l using primer sets described previously (Chenau and Henkemeyer, 2011; Genander et al., 2009; Henkemeyer et al., 1996; Orioli et al., 1996; Williams et al., 2003). All animal procedures were in accordance with the UTSW and McLean Hospital Institutional Animal Care and Use (IACUC) guidelines.

Tissue Processing

Embryonic brains used in this study were obtained at appropriate time points from timed-pregnant dams, where embryonic day 0 (E0) is defined as midnight prior to the morning a vaginal plug is observed. Pregnant dams were killed by CO₂ asphyxiation, after which embryos were immediately harvested for processing. Postnatal P0 pups were killed via quick decapitation. For E14.5 embryos, heads were removed and immediately post-fixed in 3.7% formaldehyde. For P0 postnatal pups, brains were dissected away before post fixation in 3.7% formaldehyde. All post-fixed tissue was fixed at 4°C for 48 hours before rinsing with phosphate-buffered saline (PBS) and storage at 4°C in PBS + 0.02% (w/v) Na-Azide.

L1-CAM Immunohistochemistry

Immunohistochemical staining of free floating brain sections was performed on 70 μ m coronal brain sections obtained from fixed brains embedded in a 1.5% (w/v) mix of 0.75% Low-Melting Point Agarose (Promega) and 0.75% standard Agarose (Fisher) and cut on a Leica VT 1000P vibratome. Sections were immersed in blocking solution (5% normal donkey serum, 1% BSA, 0.2% glycine, 0.2% lysine with 0.3% TritonX-100 in PBS) and incubated on a rotating shaker for 1 hour at room temperature. Sections were next incubated overnight at 4°C with rat anti-L1-CAM, 1:200 (Millipore, MAB5272MI) diluted in blocking solution. Sections were washed three times with PBS before fluorescent secondary antibody incubation with Cy3-conjugated donkey anti-rat IgG (Jackson). Sections were counterstained with 50 μ g/ml Hoechst 33342 nuclear stain (Life Tech. #H21492) diluted in 1xPBS for 5 minutes at room temperature and serially mounted onto glass slides with Aquamount.

Axon Tracing

Anterograde dye tracing of deep layer cortical axons was performed using focal application of NeuroVue® Jade or NeuroVue® Red carbocyanine dye filters (source: MTTI) placed directly into the neocortex of fixed P0 brains. Dye-implanted P0 brains were incubated 2 weeks at 37°C in PBS for adequate anterograde axon tracing before sectioning. All dye-labeled brains were embedded in 1.5% agarose before 70 μ m coronal vibratome sectioning. Sections were serially mounted onto glass slides with Aquamount (Thermo Scientific) and counterstained with Hoechst 33342 (Life Tech. - 50 μ g/ml). Focal application points of the NeuroVue dye filter within the neocortex were mapped and diagramed using a P0 developing mouse brain atlas (Paxinos, 2007).

In Vivo 1-NA-PP1 Delivery

1-NA-PP1 was synthesized as described previously (Blethrow et al., 2004; Soskis et al., 2012). Timed-pregnant female WT or *AS-EphB* mice were injected subcutaneously twice daily with 80 mg/kg 1-NA-PP1 dissolved in 10% (v/v) DMSO, 20% Cremaphor-EL, and 70% sterile saline for the embryonic timepoints required. At E19.0–19.5 dams were euthanized by CO₂ asphyxiation and the embryonic brains were harvested and processed as described above.

X-Gal Staining

To stain cryosections, unfixed brain tissue was quickly harvested and frozen under OCT medium or else cryopreserved in 30% sucrose in 0.1M PO₄ buffer pH 7.3 prior to freezing. Tissue was then sectioned on a cryostat (15–25 microns), immediately mounted on charged slides, and allowed to air dry. Sections were then washed in wash buffer (2 mM MgCl₂ and 0.02% (v/v) Nonidet P-40 in 0.1 M PO₄ buffer pH 7.3) and placed in X-gal solution (2% gluteraldehyde, 0.02% (v/v) Nonidet P-40, 2 mM MgCl₂, 5 mM EGTA, 1 mg/mL X-gal (Roche), 2.12 mg/mL K₄[Fe(CN)₆]•3H₂O, 1.64 mg/mL K₃[Fe(CN)₆] (Sigma) in 0.1 M PO₄ buffer, pH 7.3) O/N at 37 °C. Slides were washed, post-fixed in 4% (w/v) PFA, and counterstained with nuclear fast red.

Luxol Blue Staining

Adult wild-type and mutant mice were perfused with 1x PBS followed by 4% PFA. Brains were removed and post-fixed in 4% PFA overnight at 4°C, and were then serial sectioned to 40 micron thickness on vibratome and postfixed in 1% PFA overnight at 4°C. Sections were dehydrated for one hour in 70% EtOH followed by overnight incubation with agitation in 100% EtOH at room temperature. Sections were transferred to luxol blue staining solution (0.1% Luxol fast blue and 0.5 Acetic acid in 95% EtOH), which was preheated to 60 °C, and were stained overnight. Sections were washed 3x in 95% EtOH at 60°C, rehydrated in deionized water, and differentiated in 0.05% lithium carbonate for one to five minutes. Slides were washed in deionized water, mounted on charged slides, dehydrated in ethanol and then xylene, sealed with cyto seal (ThermoScientific) and glass coverslips, and imaged with an Olympus BX50 microscope.

Analysis of ACp Misguidance Phenotypes

L1-CAM immunolabeled brain sections, dye-labeled sections, and counterstained sections were imaged on an epifluorescent microscope using standard Cy2, Cy3, and UV filters. Photomicrograph images were produced using an Olympus DP70 CCD digital camera, and images were prepared using MS PowerPoint and Adobe Photoshop. Pseudo-coloring and adjustments to image brightness, contrast, and color balance were made for image clarity. ACp “misprojections” were scored as L1-CAM-positive axon fibers from the AC bundle that turned in a ventral fashion toward the brain floor. The moderate ACp misprojection phenotype was classified by the observation of significant axon bundles that became mistargeted toward the ventral floor, whereas the severe ACp misprojection phenotype was classified by the complete misplacement of the entire ACp fiber bundle. For analysis of mutant phenotypes in 1-NA-PP1 administered *AS-EphB* mutants, the mild ACp

misprojection phenotype was distinguished by the presence of only a few mistargeted ACp axons. All scoring analyses were experimenter blinded.

Data Analysis

AgCC and ACp misprojection phenotype scores in EphB Receptor mutants were assessed for statistical significance using Fisher's exact test (FET) to directly compare mutant scores to wild-type brains where brains were scored as either AgCC positive or negative. Statistics were compiled and graphs were designed using GraphPad Prism software.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The EphB1 and EphB2 intracellular domains mediate the formation of the corpus callosum

(A) Luxol blue stains of serial coronal sections through a WT adult (>P14) mouse with a normal corpus callosum (CC). (B) An *EphB1^{Tr/Tr}* homozygous mutant mouse brain with partial rostral and caudal AgCC with an intact corpus callosum above the hippocampal commissure (HC). (C) Another *EphB1^{Tr/Tr}* mutant example with complete AgCC rostral to and throughout the cortical region dorsal to the hippocampus (H). (D) Data graph displaying the percentage of each mouse genotype that displayed partial (grey) or complete (black) agensis of the corpus callosum (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Fisher's exact test; scale bare = 1mm). (E) X-gal stained E16.5 *EphB1^{Tr/+}* mutant mouse brains sectioned coronally through the region of the developing corpus callosum. Representative images from rostral to more caudal portions of the developing corpus callosum are presented. β -gal expression (blue coloring) is evident in the indusium griseum (IGG), the glial wedge (GW), and the ventral midline zipper glia (vMZG). Higher magnification of X-Gal staining ventral to the cingulate cortex and dorsal to the ventral portion of the glial wedge is shown below. (Counterstained with nuclear fast red, scale bars are 200 microns.)

Figure 2. EphB1 and EphB2 are necessary for complete ACp tract development

(A) Coronal sections of WT mice at age P0 from Bregma 3.4mm – 3.6mm (Paxinos, 2007) stained with anti-L1-CAM. The normal ACp bundle (asterisk) and external capsule (EC) are indicated. Matched L1-CAM sections from (B) *EphB1*^{-/-}, (C) *EphB2*^{-/-} and (D) *EphB1*^{-/-}*EphB2*^{-/-} knockout mutants (P0). Moderate ACp misprojections (orange arrows) and severe ACp misprojections (yellow arrows) are indicated. Other misprojected CTA fibers from the VTel are also indicated at Bregma 3.6mm in *EphB1*^{-/-} and *EphB1*^{-/-}*EphB2*^{-/-} examples (arrowheads).

Figure 3. The EphB1 and EphB2 intracellular domains mediate proper ACp formation
 (A) Coronal sections of *EphB1^{Tr/Tr}* mutant mice at age P0 from Bregma 3.4mm – 3.6mm stained with anti-L1-CAM. The ACp bundle is indicated (asterisk). (B) Matched L1-CAM sections from *EphB1^{Tr/Tr}EphB2^{Tr/Tr}* doubled truncated mutants (P0). Severe ACp misprojections are indicated (yellow arrows). Misprojected CTA fibers from the VTel are also indicated for both genotypes (arrowhead). (C) Data graph of mutant ACp phenotype scores from L1-CAM stained EphB knockout and truncated mutants analyzed in this study. (*p<0.05, **p<0.01, ****p<0.0001, Fisher's exact test compared to wild-type).

Figure 4. EphB receptor tyrosine kinase activity is necessary for complete ACp formation
 (A) Drug injection schedule for subcutaneous 1-NA-PP1 delivery to timed-pregnant WT and *AS-EphB* mutant mice. Pregnant dams were administered either vehicle or 1-NA-PP1 (80 mg/kg body weight) twice-daily between the embryonic stages of E12.5 and E19.0. Mice were sacrificed and embryos were harvested at stage E19.5 (asterisk). Coronal sections of L1-CAM stained (B)(C) WT and *AS-EphB* embryos brains treated with vehicle, and (D)(E) WT and *AS-EphB* embryo brains treated with 1-NA-PP1 (as outlined in (A)). Mild ACp misprojections (pink arrow) and moderate ACp misprojections (orange arrows) are indicated. (F) Data graph of ACp misprojection scores for WT and *AS-EphB* brains that underwent vehicle or 1-NA-PP1 treatment. (***) $p < 0.001$, Fisher's exact test compared to *AS-EphB* + vehicle).

Figure 5. Non-temporal cortical axons are misprojected into the ACp of EphB mutants
 (A) Diagram outlining the neocortical anterograde tracing experiments in P0 brains with the NeuroVue-Jade lipophilic tracer. (B) Example coronal sections of dye-labeled WT and *EphB1^{-/-}EphB2^{-/-}* mutant brains with dye placement in a similar temporal cortical region. Dye-labeled ACp axons in WT brains and misprojected ACp axons *EphB1/2 DKO* mutants are enlarged below. Sections were counterstained with Hoechst. (C)(D) Neocortical dye placement diagram with indicator points for dye placement origins in WT (n=31) and *EphB1/2 DKO* (n=48) labeling experiments. In the left hemispheres of the diagrams, open circles indicate cortical regions that project axons that traverse the striatum and do not develop along the ACp bundle. Purple shaded circles indicate regions that project along the ACp. In the right hemispheres, functional cortical subregions are outlined and the neocortical regions that encompass the origin points for ACp-projecting axons are outlined with a dashed line in both WT and *EphB1/B2 DKO* diagrams. A - auditory, Cg - cingulate, M - motor, RS - retrosplenial, S - somatosensory, V - visual.

Figure 6. EphB1 and EphB2 are required for early embryonic formation of the ACp
 (A) X-gal stained E14.5 *EphB1^{lacZ/+}* mutant mouse brain sectioned coronally through the region of the developing ACp. The temporal region of the developing cortical plate is indicated (arrowhead). (B) Coronal sections of E14.5 WT brains stained with anti-L1-CAM and ordered from the rostral-caudal position. The developing ACp bundle is apparent in all example sections. (B) Matched sections of L1-CAM stained *EphB1^{-/-}EphB2^{Tr/Tr}* compound mutants at E14.5. Misprojection of the early ACp is indicated (yellow arrows). Aberrant L1+ CTA fibers are also indicated (arrowhead). Th - thalamus, hy - hypothalamus.

Table 1**Quantification of AgCC phenotypes in EphB mutants**

A table showing the penetrance of AgCC phenotypes of adult (>P14) single and compound EphB1/2 mutant mice. Columns show the total number of mice, the number of mice with no visible AgCC, rostral only, caudal only, rostral and caudal, or complete AgCC. This is followed by percentage of mice with no AgCC and any type of AgCC. P-values are Fisher's exact test individually comparing each mutant genotype against wild-type controls.

Genotype	Total mice	Region Specific Aggenesis				Merged Analysis		p-value to WT
		No AgCC	Rostral Only	Caudal Only	Rostral & Caudal	Complete	% No AgCC	% Any AgCC
Wild-type	27	26	0	1	0	0	96%	3.7%
EphB1 $-/-$	12	5	1	1	2	3	42%	58%
EphB1 Tr/Tr	33	24	1	0	3	5	73%	27%
EphB2Tr/Tr	7	4	0	3	0	0	57%	43%
EphB1 Tr/+;EphB2 Tr/Tr	8	2	0	4	1	1	25%	75%
EphB1 Tr/Tr; EphB2Tr/+	7	3	0	2	0	2	43%	57%
EphB1 Tr/Tr; EphB2 Tr/Tr	22	2	0	12	2	6	9.1%	91%
EphB1 $-/-$; EphB2 Tr/Tr	9	0	0	5	0	4	0.00%	100%