
S. K. Y. HO,a N. KOVAČEVIĆ,b R. M. HENKELMAN,c A. BOYD,d T. PAWSONa AND J. T. HENDERSON**

aDepartment of Pharmaceutical Sciences, Leslie Dan Faculty of Pharmacy, University of Toronto, 144 College Street, Room 903, Toronto, ON, Canada M5S 3M2
bRotman Research Institute, Baycrest, 3560 Bathurst Street, Toronto, ON, Canada M6A 2E1
cMouse Imaging Centre, Hospital for Sick Children, Toronto Centre for Phenogenomics, 25 Orde Street, Toronto, ON, Canada M5T 3H7
dLeukemia Foundation Research Laboratory, the Queensland Institute of Medical Research, 300 Herston Road, Brisbane, Queensland 4029, Australia
eSamuel Lunenfeld Research Institute, Mount Sinai Hospital, 600 University Avenue, Toronto, ON, Canada MSG 1X5

Abstract—Previously, we have demonstrated that EphB2 activity is required for proper development of the posterior branch of the anterior commissure (ACpp) within the mammalian forebrain. In the present study, using magnetic resonance imaging (MRI), immunohistochemistry, and in vivo stereotactic fluorescence tracing of EphB2, B3, A4 and combinatorial Eph receptor mutants, we have developed a detailed three-dimensional model of how EphB-class receptors interact to regulate commissural formation within the forebrain. The results demonstrate that EphB2 and EphA4 each regulate distinct aspects of axon guidance within the ACcpp. Specifically, while EphB2 is required to retard ACcpp axons from projecting aberrantly into the ventral forebrain, EphA4 is required to restrict axons from entering the anterior branch of the anterior commissure (ACpa). Together, EphB2 and EphA4 act synergistically to prevent a subpopulation of axons within the anterior branch of the AC from mis-projecting caudally. Analysis of EphA4 null mice using high-resolution MRI reveals for the first time that, in addition to errors in midline guidance, loss of EphA4 results in aberrant lateral and ventral displacement of the ACpa tract. In addition, tracing studies in α-chimerin null mice reveal that EphA4-mediated effects are not regulated through this pathway. Taken together, the results demonstrate that each of the principal guidance decisions within both anterior and posterior tracts of the anterior commissure can be accounted for by the individual and combinatorial actions of EphB2/A4 receptors. © 2009 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: anterior commissure, axon guidance, Eph, MRI.

Proper assembly of the mammalian CNS is critically dependent upon the ability of projecting axons to appropriately innervate their targets (Dodd and Jessell, 1988). Over the past decade, several conserved families of molecules have been identified as important regulators of developmental CNS axon guidance, including the Robo/Slits family members (Brose et al., 1999; Dickson and Gilestro, 2006), netrins/semaphorins (Castellani and Rougon, 2002; Negishi et al., 2005) and Eph/ephrins (Wilkinson, 2000). The largest of these are the Eph family of receptor tyrosine kinases, of which 16 members have presently been identified in vertebrates (Pasquale, 2004). The Eph family can be divided into two broad subgroups based upon the nature of their ligands. EphA receptors primarily interact with ligands which are bound to the cell surface via glycosylphosphotidyl inositol linkages (ephrinAs), while EphB receptors typically interact with cell surface ligands containing both transmembrane and cytoplasmic domains (ephrinBs). The majority of EphB4 to interact with ephrinB2 and B3 (Gale et al., 1996). Similarly, it has also been demonstrated that the EphB2 receptor exhibits affinity toward ephrinA5 (Himanen et al., 2004). This overlap in binding specificity between subclasses suggests shared activities and potential functional redundancy among family members.

The majority of in vitro and in vivo studies on Eph receptor function point to the induction of repulsive interaction upon the binding of Eph or ephrin ligands (O'Leary and Wilkinson, 1999); although attractive interactions mediated by each group are known to exist (Davy and Robbins, 2000; Gao et al., 2000; Zhou et al., 2001; Hindges et al., 2002; Mann et al., 2002; Palmer et al., 2002; Davy and Soriano, 2005). Eph receptors are unique among receptor tyrosine kinases in that, in addition to interacting with a cell-bound ligand, significant activation of the receptor does not occur in the dimeric state; but instead requires higher order association (Day et al., 2005; Pabbisetty et al., 2007). Binding of ephrin ligands to the Eph receptor ectodomain, results in the induction of conformational changes which induce receptor activation. Upon activation, Eph receptors autophosphorylate key tyrosine residues within both kinase and juxtamembrane domains (Wybenga-Groot et al., 2001). Receptor phosphorylation induces a transient reduction in RasGAP activity, ultimately triggering local destabilization of the actin cytoskeleton resulting in neuritic retraction (Elowe et al., 2001). In addition to classical forward signaling through the Eph-expressing cell, Eph/ephrin binding can induce signal transduction via ephrin-expressing cell, known as the reverse...
signalng (Henkemeyer et al., 1996; Cowan and Henkemeyer, 2002).

Eph receptors have previously been shown to regulate several developmental processes which include cell migration (Wang and Anderson, 1997; Batlle et al., 2002), tissue patterning (Knoll et al., 2001; Davy and Soriano, 2007), angiogenesis (Gale et al., 2001) and axon guidance (Henkemeyer et al., 1996; Wilkinson, 2001; Palmer and Klein, 2003). Within the mammalian CNS, Eph receptors have been shown to play a role in the guidance of axons within the retinotectal (Frisen et al., 1998), corticospinal (Dottori et al., 1998), thalamocortical (Dufour et al., 2003), vomeronasal (Knoll et al., 2001), vestibular (Cowan et al., 2000), entorhino-hippocampal tracts (Stein et al., 1999; Yue et al., 2002; Chen et al., 2004; Otal et al., 2006) and the anterior commissure (AC) (Henkemeyer et al., 1996). The AC represents a primary inter-hemispheric tract for neurons within the rodent forebrain, and is required for bidirectional communication between components of the anterior aspects of the cerebral hemispheres. The AC is composed of two principal tracts: the anterior commissure pars anterior (ACpa), which communicates between the olfactory lobes, and the anterior commissure pars posterior (ACpp) which principally connects aspects of the temporal lobe. Previously, we have shown that loss of EphB2 results in a significant reduction of the pars posterior axons and that axon guidance was mediated by ephrinB-induced reverse signaling. EphA4 has also been shown to be expressed adjacent to the AC tract, and it has been suggested that mice lacking EphA4 exhibit reductions in AC axons (Dottori et al., 1998; Greferath et al., 2002); however the precise nature of these defects is unknown.

In the present study, we examined the nature of axon decision making in both single and combinatorial EphB2, B3 and A4 null mice and controls in several different genetic backgrounds. For the first time, we have analyzed and quantified axonal guidance defects within the forebrain of these Eph mutants in an interactive, non-destructive and three-dimensional manner. To determine the detailed three-dimensional structure of all axon tracts within the forebrain with respect to surrounding structures, we have performed high resolution magnetic resonance imaging (MRI) on whole brains. To identify the anatomic origin leading to aberrant AC projections, we have stereotactically labeled the relevant neural fields in vivo, via microinjection of fluorescent neural tracers. Using these methods, in combination with immunohistochemistry, a systematic morphologic analysis of the inter-hemispheric connections of the forebrain of each single and combinatorial mutant set was performed. The results obtained reveal the central role of EphB2 and A4 in regulating each of the axon guidance decisions required for neurons innervating both the anterior and posterior tracts of the AC.

**EXPERIMENTAL PROCEDURES**

**Animals**

Animals lacking EphB2, EphB3 and EphA4 genes were generated from the appropriate intercrosses and identified as previously described (Henkemeyer et al., 1996; Dottori et al., 1998). Mice homozygous for the null allele of EphB2 gene are designated EphB2−/−, whereas mice homozygous for a targeted mutation of EphB2 lacking the kinase, SAM and PDZ-binding motif are designated EphB2K2Q/H2, as previously described (Henkemeyer et al., 1996). Adult EphB2, EphB3, EphA4 and combinatorial lines were housed in our conventional animal colony at Mount Sinai Hospital in a controlled environment with a 12-h light/dark cycle. All procedures performed conformed to University of Toronto and the Canadian and Ontario Animal Care Guidelines and were conducted with the aim of minimizing the number of animals used and any acute discomfort.

**Stereotactic labeling**

Adult animals were anesthetized with 2.5% Avertin (180 μl/10 g body weight) via i.p. injection. Once anesthetized, the scalp was shaved and an incision was made along the dorsal midline. Murine heads were secured for stereotactic procedures using a standard Cunningham mouse stereotactic unit (Cunningham and McKay, 1993). Following proper alignment of the head, placements were made according to the coordinate system we have previously described, obtained from a three-dimensional stereotactic atlases we have developed (Chan et al., 2007) appropriated for the genetic background under study. At the appropriate location, a 500 μm diameter hole was made using a carbide-tipped dental drill. Based upon our cranial alignment scheme (Chan et al., 2007), the following coordinates were employed: (temporal cortex) X: 0.40 mm rostral from bregma, Y: +3.75 mm lateral from midline, Z: 3.77 mm ventral from the dura; (olfactory bulb) X: 4.15 mm rostral from bregma, Y: +0.84 mm lateral from midline, Z: 1.25 mm ventral from dura. Following interruption of the underlying dura, 100–150 nl of fluorescent tracers (Dil or Emerald Green—Molecular Probes) was injected into the target site (temporal cortex or olfactory bulb). To examine interaction of anterior and posterior axons of the AC, both single and dual (cortical/olfactory bulb) stereotactic placements were performed on the same side of the head. Dyes were delivered using a sealed oil microinjection system connected through a microelectrode housing to a borsilicate glass microcapillary with an outer diameter of 50 μm. Following delivery of the neural tracers, the capillary was held in place for 3 min, and then slowly retracted to avoid dye displacement. The incision was sutured closed, and the animals were allowed to recover for 72 h, at which time they were sacrificed and the brains were removed for analysis.

**Specimen and image preparation**

Animals were euthanized by an overdose of 2.5% Avertin (Miao et al., 2005). Upon the loss of deep tendon responses, mice were transcardially perfused with 0.1 M phosphate-buffered saline (pH 7.4; 0.9% NaCl) (PBS) to flush out vascular fluids, followed immediately by 4% paraformaldehyde in PBS at room temperature (25°C). Following perfusion, brains were excised and post-fixed in 4% paraformaldehyde in 0.1 M PBS overnight at 4°C. Specimens for MR imaging were removed from fixative, flushed, and placed into borosilicate tubes filled with a proton-free susceptibility-matching fluid (fluorinert FC-77, 3 M Corp, St. Paul, MN, USA) and imaged as indicated below. Specimens for histologic analyses were equilibrated in 30% sucrose and embedded in cryoprotectant OCT (Somagen, Torrance, CA, USA). Thirty micrometer frozen sections were obtained on a Leica, CM3050S cryostat and analyzed using a Nikon Eclipse E1000 motorized microscope equipped with a 270° rotating stage, a Hamamatsu C4742-95 camera, Nomarski contrast optics, and fluorescent excitation and emission filters appropriate for the detection of chromophores in the ranges of: DAPI, EGFP, FITC/Cy2, TRITC/Cy3, and Cy5 (excitation/emission filters used for data shown were EGFP: EX-470, EM-525 and TRITC/Cy3: EX-528, EM-600). Dual color fluo-
resonant images shown represent direct composites from the single channel images using Adobe Photoshop 7.0 software.

MR imaging
Specimens were imaged at the Mouse Imaging Centre (MICe), Hospital for Sick Children in a 7.0 T, 30 cm bore magnet (Magnex Scientific, Oxford, UK) with a 27 cm inner bore diameter gradient set (Tesla Engineering, Ltd., Storrington, Sussex, UK) and connected to a Unity INOVA console (Varian Instruments, Palo Alto, CA, USA). Two custom-built, 12 mm, non-uniform solenoid coils were used to image two brains in parallel. The parameters used in the brain scans were optimized for greatest contrast between grey matter and white matter in the mouse brain at 7.0 T (Guilfoyle et al., 2003): T₁-weighted, three-dimensional spin-echo sequence, with TR/TE=1600/35 ms, single average, field-of-view=12×12×24 mm and matrix size=200×200×400 giving an image with an isotropic resolution of 80 μm³. The total imaging time was 18.5 h.

Image registration and analysis
Image sets were registered as described previously (Kovacevic et al., 2005). Briefly, individual brain images in a given group were first registered and normalized to the average global size, shape and, MR intensity using a nine-parameter affine registration method (Woods et al., 1998), and the software package AIRS 5.2.2 (University of California). Subsequently, non-linear alignments were performed using the multi-resolution, multi-scale animal methodology initially developed at the Montreal Neurological Institute (MNI) (Kovacevic et al., 2005). Using this procedure, individual image matrices were compared (deformed into) the appropriate control average. Results of this process were recorded in terms of deformation field values, a vector field which described the magnitude and direction of the transformation required at any one point to match a particular region of the individual image to the averaged image (Kovacevic et al., 2005). Relative magnitudes of the spatial transformation are displayed referenced to a spectral color scale, with cool colors (purple) indicative of low levels of spatial variability, and warm colors (red) indicative of high spatial displacement.

3D image reconstruction of the AC
Following registration of the image sets, MRI-identifiable anatomical structures of the AC were automatically delineated as previously described (Kovacevic et al., 2005). Structures comprising the forebrain were additionally cross-checked through manual delineation in each of the three orthogonal planes; using the software package Display/Register (Montreal Neurological Institute, Montreal, Canada). Nomenclature of the structures herein was based upon the nomenclature of Franklin and Paxinos (1997) (Academic Press, San Diego, CA, USA). Two-dimensional visualizations of the results obtained were made using Display/Register (Montreal Neurological Institute). Three-dimensional surface renderings of the data were constructed using AMIRA (TGS, San Diego, CA, USA).

Immunostaining/histochemistry
Embryonic (E) or postnatal (P) brains of the indicated date were dissected and fixed overnight at 4 °C in 4% paraformaldehyde in PBS (0.1 M phosphate buffer, 0.9% NaCl, pH 7.4); then transferred to 30% sucrose, and embedded in OCT (Tissue-Tek). Thaw mount cryostat sections at 16 μm were then collected and allowed to dry at 4 °C overnight. Sections were washed in PBS and blocked in 5% serum and 0.2% tween-20 for 30 min prior to overnight incubation with the designated primary antisera at 4 °C. Antisera utilized for these studies were as follows: anti-ephrinA1, A2 (1:25; Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-EphA4 (1:20; R & D Systems, Minneapolis, MN, USA), anti-beta-galactosidase (1:200; Promega, Madison, WI, USA). Following washes, sections were incubated with fluorescent secondary antisera at a dilution of 1:200 for 2 h prior to visualization.

For lacZ staining, tissues were fixed for 20 min in 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl₂ in 0.1 M phosphate buffer, rinsed three times in wash buffer (2 mM MgCl₂, 0.02% NP-40 in PB) and incubated at 37 °C in lacZ staining buffer (wash buffer containing 1 mg/ml X-Gal (MBI Fermentas), 2.12 mg/ml potassium ferrocyanide, and 1.64 mg/ml potassium ferricyanide) until developed. Tissues were rinsed with 0.1 M NaCO₃ to stop lacZ development for 15 min. Tissues were postfixed in 4% paraformaldehyde in PBS.

RESULTS
MRI analysis of the CNS structures in Eph mutant mice
As a first step to characterize the nature and position of any anatomical anomalies within the brains of our Eph mutants, high resolution MRI was performed on both mutants and control littersmates. MRI examines the relative differences in local water and lipid content compared to surrounding cell soma (Neema et al., 2007). It is ideally suited for this study because it allows for non-destructive visualization of axon tracts in situ and detection of structural deviations observed within a given sample to be later examined histologically.

This method has been recently utilized to detect and quantify changes in axonal organization within the murine CNS that are due to genetic manipulation in mice (Mori and Zhang, 2006; Nessler et al., 2007; Park and Lee, 2007). For Eph mutants and controls, raw MRI data sets were collected at a resolution (voxel size) of 60 μm³. As an initial check of the fidelity and resolution of the system, we first examined our previously characterized EphB2⁺/⁻ mutant (Henkemeyer et al., 1996), in order to compare the MR results obtained with those determined histologically. Consistent with our previous findings, MR imaging of EphB2⁺/⁻ mutants revealed a substantial reduction in the extent of the ACpp (>85% P<0.001) (Fig. 1C, G: 3D representation) compared to EphB2⁺/⁺ controls (Fig. 1B, D: 3D representation). In conjunction with our previous MR atlases of the natural variation in murine strains (Chen et al., 2006), these results validate the utility of the MR parameters employed to examine axon tracts in situ; and provide an internal test of the true dimensional limits to which changes in fine tract structure could be determined.

As described previously (Kovacevic et al., 2005), MR-identifiable structures within the brain were compared between mutants, littermate, and atlas controls (Chen et al., 2006) with respect to two fundamental properties: (1) the total volume of the indicated structure (μm³), and (2) the position of the given neural structure relative to surrounding structures. Positional changes in CNS loci obtained from MR data are represented as the deformation magnitude (DM). DM captures the magnitude and direction of anatomical differences among individual CNS loci and translates the displacement in micrometers into a relative colorimetric scale (Fig. 1J). Together, these analyses
helped define the nature, extent, and significance of any structural deviations observed within mutant versus control comparisons. With the exception of the reductions seen in the ACpp, no additional structural anomalies were observed by MRI within the forebrain of EphB2 /−− mutants (n = 7). Analysis of MRI data from EphA4 /−− mice also demonstrated no significant differences compared to wild-type littermates or 129S1/SvImj atlas controls (n = 8 mice per genotype, data not shown). As shown in Fig. 1E, F, EphA4 /−− mice exhibit a morphologically normal AC, with little variation (<150 µm) among individuals with respect to either the AC or surrounding structures in comparison to the composite average (Fig. 1F) (n = 8). By contrast, all EphA4 /−− mice (n = 8) exhibited substantial variation in several regions of the AC (Fig. 1I) with respect to the relative position of the AC (indicated by DM up to 400 µm).

Fig. 1. Analysis of EphB2 and EphA4 mutants using high-resolution MRI. Horizontal views of the adult CNS at the level of the AC of EphB2 /−− (A, B, D), EphB2 /−− (C, G), EphA4 /−− (E, F), and EphA4 /−− (H, I) mice. An overview of the forebrain is shown in A. Box in A delineates the region of AC represented in the raw MR images and DM maps. The DM is a measure of the magnitude of positional changes within a CNS structure of mutant compared to controls MR images and represents the displacement (in µm) into a colorimetric scale. Areas with warm colors represent regions with large displacements compared to the natural variational average, while cooler colors denote regions with lower relative levels of displacements. (B) Average EphB2 /−− image showed normal AC tracts. (C) In contrast, average EphB2 /−− image displayed a significant loss of the ACpp, consistent with previous characterization of these mutants using histology (black arrow). 3D reconstruction of the AC for average EphB2 /−− (D) and average EphB2 /−− (G) MR images demonstrated similar defects in the ACpp as in histologic analyses (white arrowheads), confirming the fidelity and resolution of the MRI system. Raw average EphA4 /−− (E) and EphA4 /−− (H) MR images showed normal overall AC structure. DM from individual EphA4 /−− MR images (n = 8) against the control average image (F) and mutant MR images (n = 8) against the control average image (I) are illustrated as a color coded map. In these panels, the relative positions of the AC tract from the corresponding MR view are indicated by a dotted line. (I) Displacements of ~200–400 µm are evident in the ACpa EphA4 /−− compared to controls (grey arrows representing vectors of deformation). Note the apparent loss of ACpp density in the EphA4 /−− mice (arrowhead in H) compared to EphA4 /−− (E). (J) Spectral color scale for DM analyses with units in microns (F, I). The boxed region indicates the level of variability seen among EphA4 /−− controls ((heterozygotes compared against the heterozygous average) ±SEM at P < 0.05). Scale bars = 1 mm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
in Fig. 1I) to the surrounding CNS structures. However, the total volume of the ACpa was not significantly different (P=0.13) in EphA4+/− mice versus EphA4+/− controls (0.42±0.10 versus 0.5±0.05 mm³ respectively). Within the ACcpp, however, EphA4+/− mice exhibited a 43% reduction in tract volume compared to heterozygous controls (0.15±0.05 versus 0.27±0.04 mm³ respectively, P<0.01).

To better understand the nature of the morphologic defects induced by EphA4 deficiency, three-dimensional representations of the entire AC were generated for all EphA4+/− mutants and controls. In contrast to wild-type and EphA4+/− littermates (Fig. 2A, B), EphA4+/− mice (Fig. 2C, D) showed perturbations in both the distribution and axonal organization of the ACpa tract (Fig. 2C, D arrowheads). Tract perturbations reminiscent of de-fasciculation are a common feature in EphA4−/− mice along the caudal aspect of the ACpa tract (arrowhead). The full magnitude of these perturbations of the ACpa tract can be seen in the EphA4−/−/EphA4+/− overlays (Fig. 2E, F). To determine the relative morphologic significance of the increase in ACpa tract displacement from the midline seen in EphA4−/− mice, this distance was determined for EphB2 and A4 mutants and controls, together with CD1 (n=10), C57BL/6J (n=10) and 129 S1/SvImJ (n=10) wild-type (WT) mice. The ACpa in EphA4+/− mice exhibited a lateral displacement of greater than 400 μm from the midline compared to EphA4−/− mice (Fig. 2G). This lateral displacement of the ACpa from the CNS midline is fairly consistent across a number of genetically different comparator groups (Fig. 2G). This displacement is significantly increased in EphA4−/− mice compared to each of the comparison groups, consistent with the DM results for these mutants (Fig. 1I).

### Disruption of axon path finding in EphB-family mutants

As indicated above, the MRI parameters used provided a means to quantitatively analyze global positional and/or structural changes in MR-definable objects of sufficient magnitude. However, to understand the mechanisms by which these deviations arise, it was necessary to clearly determine the path of axons innervating each AC tract. We therefore performed simultaneous stereotactic microinjections of different fluorescent neural tracers into anesthetized wild-type and EphB family mutants into sites within the temporal cortex and olfactory bulb. Tracing was performed for a period of 4 days, at which time animals were sacrificed and serially sectioned in the horizontal (transverse) plane. Temporal and olfactory microinjections of EphA4−/− (Fig. 3A) and wild-type (data not shown) mice showed robust labeling and distinct axonal pathways by the ACpp and ACpa tracts, respectively. Tracing studies performed in EphB2−/− mice revealed a substantial reduction in ACpp axons (Fig. 3B), consistent with previous observations (Henkemeyer et al., 1996). Those axons which do arrive at the midline in EphB2−/− mice, however, persist in forming a discreet ACpp tract which crosses the midline properly (Fig. 3B). In contrast, ACpp axons in EphA4−/− mice exhibit aberrant axon guidance upon contact with ACpa axons (Fig. 3C–E) where the ACpp axons in these mice now freely intermingle with those of the ACpa tract. As a result, in EphA4−/− mice, approximately one-half of all ACpp axons become misdirected along the ACpa tract (Fig. 3D); while the remainder exit the midline and resume projection to the contralateral hemisphere. Interestingly, as shown in the progressively more ventral levels...
of the AC among different EphA4−/− mice (Fig. 3C–E), the character of ACpa and ACpp intermingling changes and becomes more stratified within the ventral AC. The aberrant behavior of ACpp axons in EphA4−/− mice as seen by in vivo neural tracing is consistent with both the MRI-based volumetric reduction of the ACpp tract, and the disappearance of MR contrast intensity along the AC midline seen in EphA4−/− mice (Fig. 1H). Despite this deviation, ACpp axons of EphA4−/− mice are still observed to project only along the well-defined tracts of the AC (ACpp, ACpa) rather than projecting randomly within a given zone, as observed for ACpp axons of EphB2−/− mice (Henkemeyer et al., 1996). In vivo tracing of axons also demonstrates that the apparent tract fasciculation events seen along the caudal aspect of the ACpa tract in EphA4−/− mice using MRI, actually represent bundles of mis-projecting ACpp axons which project through the ACpa tract (Fig. 3E). As shown in Fig. 3, aberrant axonal projection is not observed for ACpa axons in EphA4−/− mice, similar to EphB2−/− mice. With respect to other EphB family members known to be expressed within the forebrain, neural tracing experiments performed using EphB3−/−/− mice (Fig. 3F), and mice homozygous for a targeted deletion of the EphB2 intracellular domain (EphB2N2N2; Henkemeyer et al., 1996) (data not shown), show axonal tracing patterns identical to that seen in wild-type and heterozygous controls (Fig. 4A).

Recently, several groups have demonstrated that α-2 chimerin is a downstream target of EphA4, raising the possibility that α-chimerin-mediated signaling may also act to regulate AC formation (Beg et al., 2007). Histologic observation by the authors indicated no gross morphologic perturbations of the AC of α-2 chimerin null mutants. However, due to the nature of the targeting defects we ob-
erved in the EphA4 null mice (which would not be observed histologically), we performed in vivo neuro-anatomic tracing in mice homozygous for an inactivating point mutation in the GAP domain of the \( \alpha \)-chimerin gene (\( \alpha \)-1 and -2 chimerin null mutant), to determine whether \( \alpha \)-chimerin deletion results in similar axon guidance defects observed for EphA4 null mice (Iwasato et al., 2007). Analysis of serial sections from the AC from these animals demonstrated that both the anterior and posterior tracts of the AC were normal in \( \alpha \)-1 and -2 chimerin null mice (data not shown).

Synergistic regulation of axon guidance by Eph family members

As indicated above, genetic deletion of EphB2 and A4 (but not B3), exhibits unique effects on axon guidance within the AC. To examine potential interactions between Eph family members in this system, a series of combinatorial Eph mutants was generated (Fig. 4A). As indicated in the figure, with the exception of EphB2/A4 double knockouts (DKO), each of the combinatorial lines examined exhibited either a wild-type pattern of innervation, or recapitulated the phenotype of one of the single knockouts. EphB2/A4 double heterozygotes exhibited wild type patterns of axon guidance in both the ACpa and ACpp (Fig. 4B). While the ACpp axons in EphB2/A4 DKO exhibited an additive pattern of the axon guidance defects seen in EphB2\(^{+/−}\) and EphA4\(^{−/−}\) mice, the ACpp axons in DKO animals exhibited a unique pattern of aberrant axon guidance not seen in either of the single null mutants; as shown by axonal tracing. A subgroup of ACpp axons in EphB2/A4 DKO was redirected into the ACpp pathway (Fig. 4C–E). Similar to the behavior seen in EphA4\(^{−/−}\) mice, misdirected ACpp axons in EphB2/A4 DKO only project along the pathway defined by the ACpp. Thus, while EphB2 and EphA4 regulate distinct aspects of axon path finding in ACpp axons, both receptors participate in regulating the guidance of ACpp axons.

Expression of Eph receptors and ephrin ligands in the developing forebrain

Results obtained from MRI and stereotactic tracing analyses of Eph family mutants suggested a mechanism by which these Eph receptors interact to control axon guidance. To further define the nature of these interactions, we examined in three dimensions, the pattern of EphB2 and EphA4 and ephrin ligands expression within the developing forebrain at embryonic day (E) 15, a time at which ACpa and ACpp tracts are still forming. The stringent segregation between ACpp and ACpa axons at the AC junction was observed prior to E13.5–13.75. Neural tracings performed at E13–E14.5 suggested coincident or near coincident arrival of ACpp and ACpa axons within the components of the temporal lobe which gives rise to axons of the ACpp tract, we also observed ephrinB expression (Fig. 5D, arrowhead). Despite previous suggestions that EphB/ephrin signaling may assist in the guidance of cortical axons, none of the single or double mutant combinations examined (Fig. 4A), exhibited significant perturbations in the initial coalescence of the ACpp (or ACpa) tract.

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

**DISCUSSION**

Previous in vivo analyses of Eph/ephrin-mediated axon guidance within the AC by laboratories including our own have largely been confined to gross histologic examination. While such analyses provide a valuable overview of the structural morphology of the AC in genetic mutants, they do not allow the specific origin, innervation or path
taken by individual axons to be determined. To more clearly determine the mechanism by which EphB-type receptors act to control AC axon guidance, we have for the first time performed quantitative 3D MRI of EphB2, EphB3, EphA4 and combinatorial null mutants in concert with in vivo stereotactic tracing.

These results demonstrate that the loss of EphA4 activity results in the development of several previously unrecognized axon guidance defects within the AC; including ACpa, ACcpp axon segregation. Specifically, high-resolution MR imaging demonstrates for the first time that loss of EphA4 normally expressed in ACcpp axons, results in a stochastic intermingling of ACcpp axons with those of the ACpa tract. Thus, EphA4 normally regulates the midline segregation of ACcpp from ACpa axons during their coincident developmental arrival through a repulsive mechanism. We observe that this aberrant projection persists, continuing into the adult period. It is interesting to note that the degree of tract intermingling appears higher in the central and the dorsal aspect of the AC midline compared to ventral regions, suggesting the potential involvement of additional axon guidance molecules. Beyond the sagittal midline, in EphA4 null mice, mis-projecting ACcpp axons can continue to project along the ACpa tract. However, the converse projection (ACpa axon projection along the ACcpp tract beyond the midline) is prohibited. Our expression studies suggest that this difference in axon guidance may relate to the greater sensitivity of ACpa versus ACcpp axons.
In the absence of EphA4 expression beyond the midline segregation point for ACpp and ACpa axons, misprojecting ACpp axons either travel along the main body of the ACpa tract, or continue to proceed laterally until they encounter a region of elevated EphB2 expression (summarized in Fig. 6C). Upon reaching this point, these axons cannot project laterally or caudally due to the high local levels of EphB2. They, therefore, proceed anteriorly, frequently rejoining the ACpa tract at its rostral aspect. As such, the ACpa tract in EphA4 null mice appears to exhibit de-fasciculated bundles of the ACpa tract. Analysis of stereotactic tracing in such animals demonstrates however that these “de-fasciculated bundles” are in fact composed of mis-projecting ACpp axons. This also explains the true origin of the so-called U-turn projections seen in ACpa axons in EphA4 null mice, as described previously by Kullander (2001). The present study demonstrates that such projections are not ACpa tract element U-turns, but rather separate ACpp tract elements coalescing just lateral to the sagittal midline.

Taken together, both the genetic ablation and immunohistochemical expression studies support a model where ephrinB expressing ACpa axons are repelled through ventrolateral interaction with EphA4 expressing regions adjacent to ACpa tract (summarized in Fig. 6D). This model suggests that in the absence of rostral EphA4 expression, ACpa axons originate from the piriform cortex of olfactory bulb project ventral and lateral to their normal path, projecting caudally until they reach a zone of high EphB2 expression (Fig. 6D). At this point they are redirected toward the midline (Figs. 6D–4). The loss of this rostral EphA4 domain (Fig. 6C) also allows the ventrolateral deviation of mis-projecting ACpp axons from the midline (see below).

Interestingly, we observed concomitant expression of both EphA4 and ephrinBs expression within axons of the ACcpp. Consistent with this, previous reports have demonstrated ephrinB2 expression within the ACpp (Cowan et al., 2004). This poses an intriguing problem with regard to how ACpp axons respond to the dual expression of a given Eph receptors and its ligand within the same tract; as these axons do not appear to exhibit significant self-repulsion. Previous in vitro studies have provided evidence of down-regulation of Eph/ephrin signaling in cis (Carvalho et al., 2006). Another potential explanation involves mutual exclusivity of EphA4 and ephrinBs. An example of this is seen in the work of Marquardt et al. (2005) who examined EphA and ephrinAs co-expression in motor axons. It was proposed that EphA and ephrinAs were segregated into separate membrane domains and were capable of simultaneously binding their respective partners presented in trans (Marquardt et al., 2005). Whether internal repulsion arising from the relatively low levels of EphA4 in ACcpp axons (relative to adjacent domains such as the ACpa tract) is overridden by surrounding Eph-ephrin interactions or through the influence of additional signaling molecules remains to be determined. However recently, several laboratories have identified Rac-GTPase α2-chimerin as a key mediator of ephrinB3/EphA4 forward signaling in the

**Fig. 6.** Eph-mediated interactions within the developing forebrain. Schematic representations of the behavior of axons within the AC based upon the individual and cumulative affects of EphA4 and EphB2 during development. EphA4 (A4) and EphB2 (B2) expression domains and the nature of AC axonal projections within the forebrain of wild type mice are indicated (A). (B) As indicated previously (Henkemeyer et al., 1996), in the absence of EphB2, axons from temporal neurons comprising ACcpp tract fail to encounter EphB2-mediated repulsion as they approach the sagittal midline; resulting in aberrant projection to the floor of the forebrain (1). (C) In the absence of EphA4, several axonal deviations occur. Normally, the coincident developmental projection of the ACcpp and ACpa tracks results in segregation of ACcpp and ACpa axons. In the absence of EphA4, axons of the ACcpp exhibit a loss of repulsive contact between ACpa axons, resulting in free intermingling of ACcpp and ACpa axons (2). As a result of this intermingling, axons of the ACcpp stochastically mix with ACpa axons, ultimately resolving into either continued projections towards the contralateral temporal cortex, or aberrant projection along the ACpa tract (3). Aberrant ACcpp axons can either project within the displaced (see below) ACpa toward the rostral forebrain (3), or clusters of ACcpp axons can continue along a more lateral course from the midline, due to the absence of EphA4 expression in the region immediately rostral to the EphB2. In such instances, because of the more ventral position of the ACpa tract compared to the ACcpp, these axons continue until they encounter the rostral perimeter of the high EphB2 expressing domain. As these axons cannot project caudally due to high levels of EphB2, they turn in an anterior direction, typically rejoining the ACpa tract. (D) With respect to ACpa axons, the absence of EphA4 results in ventrolateral deviation of the ACpa tract (4) due to loss of the forebrain EphA4 domain indicated. In addition, while ACcpp and ACpa axons can freely intermingle at the midline, only ACcpp axons can project into ACpa territory in the absence of EphA4. ACpa axons are blocked from the converse behavior (ACcpp tract entry – 5), due to their apparently greater sensitivity to EphB2-mediated repulsion. (E) In the absence of both EphB2 and EphA4, each of the individual guidance errors described above occurs; however in addition, axons of the ACpa can now enter ACcpp territory (6) due to removal of the remaining EphB2 barrier. However, in contrast to the stochastic intermingling observed in ACcpp axons, only a minority of ACpa axons (– 20%) ultimately enter the ACcpp tract in the absence of EphB2 and A4.
development of motor circuits (Beg et al., 2007; Iwasato et al., 2007; Wegmeyer et al., 2007). Our tracing analysis of these mutants demonstrates that unlike the corticospinal tract, α-1 and -2 chimerin do not mediate the actions of EphA4 with respect to the AC. The fact that our axonal tracings in α-Chn null mice did not result in similar phenotype to that seen in EphA4 knockouts, supports evidence for a reverse signaling mechanism. Consistent with this, reverse signaling through ephrinB2 has previously been shown to mediate the repulsion of ACpp axons from the ventral regions (Cowan et al., 2004).

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

While we have demonstrated that EphA4 and EphB2 play distinct roles in the guidance of ACpp axons, our analysis of the EphB2/A4 DKO demonstrates for the first time, a synergistic effect for these receptors with respect to the guidance of a subpopulation of ACpa axons. In addition to exhibiting the guidance errors seen in each of the corresponding single knockouts, ~20% ±7% of the ACpa fibers in EphB2/A4 DKO now leave the ACpa tract and enter ACpp territory (Fig. 6D). In contrast to the stochastic behavior (50% of the axons) seen for ACpp axons in EphA4/−/− mice, the mis-projection seen in the DKO represents approximately half this number, suggesting that functional heterogeneity may exist within the ACpa population. The mis-projection of less than 50% of ACpa axons in EphB2/A4 DKOs suggests that additional guidance molecules may regulate this process. In this respect, it is interesting to note that like the EphA4 null mutant, mice lacking Sema3B also exhibit a ventral and lateral shift in the ACpa tract (Falk et al., 2005). In the absence of a comparable quantitative determination of ACpa tract morphology in Sema3B null mutants, it is difficult to determine with certainty whether the extent of these deviations is similar to that seen in EphA4 null mice. However, the ability of Sema3B ablation to phenocopy at least some of the features seen in EphA4 null mutants suggests that these disparate receptors ultimately act to affect common downstream mechanisms regulating axon guidance. With respect to Eph-mediated axon guidance, the altered projection of ACpa axons seen in the EphB2/A4 DKOs strongly implies that these receptors have compensatory roles in regulating the anterior–posterior guidance for at least some ACpa axons. However, it does not necessarily indicate that they share an identical mode of action, as suggested by their distinct pattern of expression and the differential effects on ACpa tract morphology. As shown in the model, we postulate that in the absence of EphB2, ACpa axons are restricted from entry into the AC through interaction with EphA4-expressing ACpp axons at the midline due to their expression of ephrinA3 (Kudo et al., 2005). By contrast, in the absence of EphA4 at the midline, ACpa axons are not formally repelled from entry into the ACpp tract. We demonstrate that at least a subgroup of these axons does not continue along the ACpp tract due to elevated levels of EphB2 expressed lateral and caudal to the midline.

While our analysis of Eph mutants identifies their role both alone, or in combination, in regulating the key steps involved in properly guiding forebrain axons to the contralateral hemisphere, these data also highlight the importance of additional regulators in controlling tract formation. In none of the single or combinatorial Eph receptor mutants examined was a complete loss of the ACpa or ACpp tract observed, suggesting the influence of axon guidance molecules in regulating initial projection of AC axons. Consistent with this, mice lacking netrin1 fail to extend axons to either the ACpa or ACpp (Serafini et al., 1996). In addition, the absence of the semaphorin ligand Sema3F (Sahay et al., 2003) or its principal receptor Npn2 (Chen et al., 2000; Giger et al., 2000), results in a substantial reduction in numbers of both ACpa and ACpp axons projecting to the AC. In contrast to the indicated EphB receptors, these additional guidance cues appear to exert a more generalize influence on axonal behavior within the CNS. Thus, while EphB-class receptors regulate discrete aspects of the projection of both ACpa and ACpp axons, as well as tract segregation, the process of commissure formation is influenced particularly with respect to initial projection by an array of additional guidance cues. In addition to the Eph null mutants indicated above, EphB3 and a series of additional combinatorial null mutants (Fig. 4) were examined with several different murine backgrounds (C57Bl/6J, 129 S1/SvImj, mixed). Within the forebrain EphB3 null mutants did not exhibit statistically significant defects in axon guidance with respect to the analyses performed. Similarly, none of the combinatorial mutants listed demonstrated additional axon guidance defects beyond that which we have described for the indicated single mutants. Taken together, these data provide a basis for understanding the mechanism by which EphB-class receptors regulate not only ACpa axon guidance, but the position and trajectory of the ACpa tract, as well as anterior–posterior tract segregation within the AC. Specifically, EphB2 and A4 exert both unique and synergistic activities with respect to inter-hemispheric connection of the forebrain.

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