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Drosophila Eph receptor guides specific axon branches of mushroom body neurons

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Abstract

The conserved Eph receptors and their Ephrin ligands regulate a number of developmental processes, including axon guidance. In contrast to the large vertebrate Eph/Ephrin family, *Drosophila* has a single Eph receptor and a single Ephrin ligand, both of which are expressed within the developing nervous system. Here, we show that Eph and Ephrin can act as a functional receptor-ligand pair in vivo. Surprisingly, and in contrast to previous results using RNA-interference techniques, embryos completely lacking Eph function show no obvious axon guidance defects. However, Eph/Ephrin signaling is required for proper development of the mushroom body. In wild type, mushroom body neurons bifurcate and extend distinct branches to different target areas. In *Eph* mutants, these neurons bifurcate normally, but in many cases the dorsal branch fails to project to its appropriate target area. Thus, Eph/Ephrin signaling acts to guide a subset of mushroom body branches to their correct synaptic targets.

Keywords

Drosophila; Eph; Axon branching; Mushroom body

INTRODUCTION

Developing neurons reach their targets through the directed growth of their axons, the growth cones of which recognize and respond to guidance cues present in their environment. Neurons often make synaptic contacts with multiple remote targets by extending distinct axon branches that grow in different directions. A notable example of this is the development of neurons within the mushroom body (MB), the olfactory learning and memory center of insects (Heisenberg, 2003). MB neurons bifurcate and extend axons into two distinct areas of the brain, ultimately synapsing with different targets and giving the MB its characteristic bi-lobed appearance. The apparent synchrony of the growth of the two branches suggests that the growth cones from individual branching MB neurons may differentially respond to guidance cues (Wang et al., 2002).

A class of molecules known to play key roles in the organization of many tissues, including axon guidance in the nervous system, is the Eph receptor tyrosine kinase (RTK) family members and their ligands, the Ephrins. Members of these large, phylogenetically conserved families of receptors and ligands are found throughout the animal kingdom, but the families have undergone a considerable expansion within vertebrates, resulting in a total of 14–16 Eph

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receptors and eight to nine Ephrin ligands depending upon the species. Eph receptors are divided into two subclasses based on sequence and their preferential binding affinity for Ephrin ligands: EphA receptors bind to GPI linked Ephrin A ligands, while EphB receptors bind to transmembrane Ephrin B ligands. Ephs and Ephrins are expressed in many developing tissues and mediate a variety of cell-cell contact-dependent signaling events, including axon attraction and repulsion within the nervous system.

The diverse roles played by Ephs and Ephrins are reflected in the complexity of their signaling. Both the receptor and ligand are membrane bound molecules capable of transducing intracellular signals within a cell. Thus, signaling through both the receptor (forward) and the ligand (reverse) are possible. In some cases, such as rhombomere boundary formation within the hindbrain, activation of Eph/Ephrin signaling occurs when Eph-expressing cells encounter domains of Ephrin expression (Poliakov et al., 2004). However, in many cases Eph/Ephrin signaling occurs within cells expressing both receptor and ligand. The consequence of such ligand/receptor co-expression appears to be context dependent. For example, within the retinotectal system Eph/Ephrin co-expression by retinal ganglion cells may result in the Ephrin ligand masking the Eph receptor, blocking receptor activation by ligand expressed in trans (Hornberger et al., 1999). By contrast, within developing motoneurons, co-expressed ligand and receptor do not appear to interact, thereby allowing both receptor and ligand in principal to signal independently within the same cell (Marquardt et al., 2005). Consistent with this idea, within such co-expressing motoneurons the Eph receptor mediates growth cone collapse and repulsion, while the Ephrin mediates axon growth and attraction (Marquardt et al., 2005).

Eph/Ephrin signaling in axon guidance is perhaps best illustrated by its role in the topographic mapping of retinal ganglion cells in the superior colliculus (SC) (Poliakov et al., 2004). Retinal ganglion cell axons initially overshoot their targets within the superior colliculus and subsequently contact their correct termination zones through the extension of collateral branches. Along the anteroposterior (AP) axis of the SC, retinal ganglion cell targeting relies in part on EphA/Ephrin A signaling mediating repulsion (Brown et al., 2000; Nakamoto et al., 1996; Yates et al., 2001). By contrast, along the mediolateral axis of the SC, opposing gradients of EphB receptors on retinal ganglion cells and Ephrin B ligands on SC cells act to control the direction of branch extension and arborization by mediating either attraction or repulsion, depending upon the position of the branch relative to its termination zone (Hindges et al., 2002; Mann et al., 2002; McLaughlin et al., 2003).

In contrast to animals that have multiple Eph receptors and/or ligands, *Drosophila* has a single Eph and a single Ephrin. The *Drosophila* Eph receptor shows equal similarity to both the A and B subclasses (Dearborn, Jr et al., 2002; Scully et al., 1999), while the *Drosophila* Ephrin ligand is most similar to vertebrate Ephrin B ligands. Like other Ephrin B ligands, *Drosophila* Ephrin contains a transmembrane domain and a conserved tyrosine phosphorylation site (Bossing and Brand, 2002).

Both *Drosophila Eph* and *Ephrin* are expressed within the embryonic CNS at a time when neurons are extending axons towards their targets (Bossing and Brand, 2002; Scully et al., 1999). Two previous studies have suggested a role for *Drosophila* Eph/Ephrin signaling in neuronal development using RNA interference (RNAi) technology (Bossing and Brand, 2002; Dearborn et al., 2002). Here, we describe the generation of a null mutation in *Eph*, plus our analysis of Eph/Ephrin function within the *Drosophila* CNS in individuals lacking all Eph function. We show that *Drosophila* Eph and Ephrin can act as a functional receptor ligand pair in vivo to mediate axon repulsion. Despite this, we fail to detect axon guidance defects in the embryonic CNS of *Eph* mutant embryos. However, later in development Eph/Ephrin signaling plays a crucial role in the developing MB by guiding the projection of specific axon branches of individual MB neurons.

MATERIALS AND METHODS

Fly stocks and genetics

39c-18 (Wallrath and Elgin, 1995) is a lethal *white+* (*w+*) P element insertion on chromosome IV that was mapped to the *bent* locus by sequencing fragments generated by inverse PCR (Dalby et al., 1995). 39c-18 was mobilized using *Δ2,3* (Robertson et al., 1988). Six-hundred *w;CyO,Δ2,3/+;39c-18/CiD* males were tested for chromosomes with reversion of 39c-18 lethality by singly crossing them to *w–* females carrying a lethal allele of *bent* (*39c-18$^{LR\#56}$/CiD*) generated by excision of 39c-18. Five-hundred and eighty *w+* non-*CyO,Δ2,3* flies were isolated, 57 of which mapped to chromosome IV. Insertion sites for 54 lines were determined by inverse PCR; flanking sequences from three lines did not map to a single site and were discarded.

P114 excisions were generated by crossing *w;CyO, Δ2,3/+;P114/CiD* males to *w;ey^{D}/CiD* females. One-thousand three-hundred *w-* excision lines were isolated, 2.5% of which were homozygous lethal. Southern blot analysis demonstrated that none of the lethal lines deleted sequences within the *Eph* genomic region. Seven out of 16 lethal lines did show rearrangements within the *onecut* genomic region, including the *onecut^{x122}* and *onecut^{x49}* alleles. Eight additional *onecut* alleles were identified by non-complementation with *onecut^{x122}* and *onecut^{x49}*.

Viable lines were screened for rearrangements within *Eph* by PCR using primers that amplify the first three exons of the *Eph* gene (Scully et al., 1999). After additional southern blot analysis of *Eph^{x652}* DNA indicated a deletion of the first three exons, breakpoints were determined by sequencing a PCR fragment generated from *Eph^{x652}* DNA using primers predicted to bracket the excised region. The 5′ breakpoint of *Eph^{x652}* maps to genomic position 627320 (BDGP release 4), 368 bp upstream of the *onecut* translation start site. The 3′ breakpoint lies within the third intron of *Eph*, at genomic position 632468.

Immunohistochemistry and in situ hybridization

Immunohistochemistry on dissected embryos was preformed as previously described (Callahan and Thomas, 1994). Larval and adult brains were dissected and fixed as described by Hummel et al. (Hummel et al., 2003) with a total fixation time was 60 minutes.

Primary antibodies used: monoclonal antibody (mAb) anti-BP102 (1:20 dilution) (Seeger et al., 1993); mAb anti-Fas2 (1:30) (Lin et al., 1994); mAb 9E10 anti-c-myc (Dm – FlyBase) (1:50) (Developmental Studies Hybridoma Bank); Cy3-conjugated anti-HRP (1:500) (Jackson Immunoresearch); rabbit anti-β-Gal antibody (1:1000) (Cappel); and rabbit anti-GFP (1:5000) (Molecular Probes).

Ephrin-Fc was produced as described (Kaneko and Nighorn, 2003). Samples were incubated in either purified Ephrin-Fc (1:500 dilution in PBS) or straight supernatant, followed by incubation with either rabbit or mAb anti-human IgG, Fcγ (1:500) (Jackson ImmunoResearch). For fluorescence immunostaining, Alexa Fluor 488-conjugated anti-rabbit (1:500) (Molecular Probes) and Cy3-conjugated anti-mouse (1:500) (Jackson ImmunoResearch) were used. In situ hybridization was carried out as described (Dougan and DiNardo, 1992) using probes generated with fragments corresponding to ~1 kb of 5′ sequences of the *Eph* cDNA (Scully et al., 1999) or the *Ephrin* cDNA.

Constructs

pUAS-Ephrin:myc was constructed from the full-length cDNA clone RE46807 tagged in-frame to six copies of the c-myc epitope in the *pUAS* vector (Brand and Perrimon, 1993). For

construction of pUAS-dephrinmyc$^{\Delta IC}$, a PCR fragment was generated that deleted intracellular sequences from amino acids 611 to 652. This was subcloned as an *Eco*R1/*Age*I fragment into *pUAS-Ephrin:myc*, replacing the wild-type sequence. For each *UAS* transgene, multiple lines were generated by P element transformation (Spradling and Rubin, 1982). Lines with the strongest anti-myc staining were used.

Genetic mosaics

The following flies were generated for MARCM analysis of MB neurons: *hsp70-FLP,elav-Gal4,UAS-mCD8GFP*/+ or Y;*FRT^{G13}*/*FRT^{G13},TubP-Gal80;Eph^{x652}*/*CiD* and *hsp70-FLP,elav-Gal4,UAS-mCD8GFP*/+ or Y;*FRT^{G13}*/*FRT^{G13},TubP-Gal80;Eph^{x652}*/*Eph^{x652}*. Clones within the α/β lobes were generated by heat shocking pupae at 38°C, as described (Lee and Luo, 2001). Eighty adult *Eph^{x652}* individuals were examined for marked MB clones. Branching patterns of 41 unambiguously labeled *Eph^{x652}* mutant MBs clones were examined.

RESULTS

Generation of *Eph* mutants

Both *Eph* and *Ephrin* genes map within 33 kb of one another on the 4th chromosome. To generate mutations in the two genes, we mobilized a P element, 39c-18 (Wallrath and Elgin, 1995), located ~145 kb away from *Eph* and inserted in the *bent* locus (see Materials and methods). Fifty-five independent 4th chromosome insertion lines were generated. Insertion sites for all 55 were determined by inverse PCR and represent a unique collection of 4th chromosome P-element insertion lines which will be made available from the Bloomington *Drosophila* Stock Center. One line, P114, maps within 3 kb of the *Eph* transcription start site and ~1 kb upstream of *onecut*, a gene transcribed on the opposite strand encoding a homeodomain class transcription factor (Nguyen et al., 2000). As both *Eph* and *onecut* expression are unaffected in homozygous P114 flies (data not shown), we used this line to generate *Eph* deletions by imprecise excision. A total of 1300 excision lines were generated, 33 of which, or 2.5%, were found to be homozygous lethal. Both viable and lethal excision lines were assayed by PCR and southern blot analysis for rearrangements within the *Eph* genomic region. A single viable excision line, *Eph^{x652}*, was isolated that removes *Eph* genomic sequences (Fig. 1A). This excision deletes the first three exons of *Eph*, thus removing the *Eph* transcriptional start site and the first 79 amino acids of the coding region. The absence of detectable *Eph* mRNA and protein expression in *Eph^{x652}* homozygous individuals argues that it acts as a null mutation (Fig. 1C; see below).

Sequencing across the breakpoint of *Eph^{x652}* revealed that this excision also removes 5Δ sequences of the *onecut* locus, including its transcription start site. It does not, however, remove any *onecut* coding sequence. Among the excision lines, we identified 15 that extend in the direction of *onecut*, removing *onecut*-coding sequence but not removing sequence in the direction of or within the *Eph* gene (Fig. 1A). All of these excision lines are lethal as homozygotes. In addition, intra-allelic combinations of the excisions, such as *onecut^{lx122}*/*onecut^{lx49}*, are lethal, demonstrating an essential function for the *onecut* locus. In contrast to the lethality of the *onecut^{lx122}* and *onecut^{lx49}* alleles, homozygous *Eph^{x652}* flies are viable and fertile, and have no obvious morphological defects, suggesting that any effect of *Eph^{x652}* on *onecut* function must be partial. Consistent with this, *Eph^{x652}* complements the lethality of the *onecut* alleles, as *Eph^{x652}*/*onecut^{lx122}* and *Eph^{x652}*/*onecut^{lx49}* flies are viable and fertile.

The single *Drosophila Ephrin* gene contains a P element insertion, KG09118, generated by the Berkeley Gene Disruption Project, that lies ~20 bp downstream of the *Ephrin* transcription start site (Bellen et al., 2004; Roseman et al., 1995). In situ hybridization of *Ephrin* antisense RNA probes to homozygous *Ephrin^{KG09118}* embryos reveals that this insertion severely

reduces *Ephrin* mRNA expression (Fig. 1F), indicating that this insertion is an *Ephrin* allele. Like Eph^{x652}, homozygous $Ephrin^{KG09118}$ individuals are viable and fertile.

Drosophila Eph is expressed on embryonic CNS axons during pathfinding

Previous in situ hybridization studies showed that *Eph* is expressed within the CNS at a time when neurons extend axons towards their targets and is expressed by most, if not all, CNS neurons at this stage (Scully et al., 1999). To visualize the distribution of *Drosophila* Eph receptor, we used Ephrin-Fc, a soluble probe consisting of the extracellular domain of *Manduca* Ephrin fused to the human immunoglobulin Fc fragment (Kaneko and Nighorn, 2003). The major axon tracts within the CNS, the bilaterally symmetric longitudinal connectives and the two commissures connecting each hemisegment, can be visualized with the panaxonal BP102 antibody (Seeger et al., 1993). Incubation of unfixed, dissected embryos with BP102 and Ephrin-Fc reveals that the Eph receptor is expressed on both longitudinal and commissural axons within the CNS in a pattern similar to that of BP102 staining (Fig. 2A–C). Thus, the Eph receptor is expressed by most, if not all embryonic CNS neurons and is targeted to axons.

Binding of Ephrin-Fc is abolished in homozygous Eph^{x652} mutants (Fig. 2D,E), demonstrating the specificity of Ephrin-Fc binding to the Eph receptor and confirming that Eph^{x652} is null for *Eph* expression. By contrast, Ephrin-Fc binding is unaffected in embryos homozygous for the *onecut* alleles $onecut^{lx122}$ and $onecut^{lx49}$ (Fig. 2F), further demonstrating that the lethality associated with the *onecut* alleles is not due to compromised *Eph* function.

Ephrin mRNA, like *Eph*, is widely expressed throughout the embryonic CNS, as assayed by in situ hybridization (Fig. 1F) and Ephrin protein is expressed by most if not all CNS neurons, but in contrast to Eph has been reported to be localized to cell bodies rather than axons (Bossing and Brand, 2002). Consistent with this, we have found that when expressed by neurons using the GAL4/UAS transactivation system (Brand and Perrimon, 1993), a myc-tagged version of Ephrin is localized to cell bodies (Fig. 2G–I). Therefore, both *Drosophila* Eph and Ephrin are present at a time and place that would allow them to control axon pathfinding, but each appears to reside within a distinct compartment of the neuron.

Eph mediates repulsion of axons by Ephrin

In vertebrates, Eph activation by Ephrin often leads to axon repulsion. To test for repellent activity of Eph/Ephrin signaling, we misexpressed Ephrin in midline glia and assayed the effects on commissural axons that normally cross the midline. For these experiments, we used *sim-Gal4* plus *UAS-Ephrin:myc* to drive expression of myc-tagged Ephrin exclusively within midline glia. In these embryos, many commissural axons fail to cross the midline and thus fail to form normal commissures, similar to what has been reported previously (Fig. 3B) (Bossing and Brand, 2002). These defects are consistent with Ephrin acting as an axonal repellent, preventing axons from crossing the ectopic source of Ephrin at the midline. Similar axon guidance defects are observed when an Ephrin transgene lacking all intracellular sequences, $UAS\text{-}Ephrin^{\Delta IC}:myc$, is expressed by *sim-Gal4* (Fig. 3C). This result, combined with the finding that misexpression of Eph by midline glia has no effect on commissural axons (data not shown), confirms that it is forward signaling that causes axons to be repelled and that reverse signaling plays no role in this repulsion. To test whether the repellent activity of Ephrin is mediated by Eph, we misexpressed Ephrin in midline glia using the same combination of transgenes as above, but in an Eph^{x652} mutant background. In these embryos, the ability of Ephrin to repel commissural axons is abolished, restoring the commissural tracts to wild type in their appearance (Fig. 3D). Thus, Eph and Ephrin are able act as a receptor/ligand pair in vivo and, at least in this assay, Ephrin acts solely through the Eph receptor.

Eph function is not essential for embryonic axon guidance

The expression of Eph and Ephrin within the embryonic CNS and the severe guidance defects observed when Eph/Ephrin signaling is ectopically activated suggest a role for these signaling molecules in axon guidance during normal development. To assess this, we first examined the overall architecture of the embryonic CNS of homozygous Eph^{x652} mutant embryos using the BP102 antibody to label all axons (Seeger et al., 1993). As homozygous Eph^{x652} flies are viable and fertile, we took embryo collections from a homozygous mutant stock, thus eliminating both zygotic and maternal Eph. Homozygous Eph^{x652} mutant embryos show no obvious defects in overall axon tract organization compared with wild-type embryos (Fig. 4A,B).

We next assayed axon pathfinding in greater detail using markers that label the axonal projections of subsets of neurons. First, we stained embryos for Fasciclin 2 (Fas2), a cell-adhesion molecule expressed on five distinct bilaterally symmetric bundles of axons that run along the anteroposterior axis of the CNS (Lin et al., 1994). We detected no obvious difference in the Fas2 axon bundles between wild-type and Eph^{x652} mutant embryos, although the bundles in Eph^{x652} mutants appear slightly less fasciculated (Fig. 4C,D). We next examined the axon trajectories of the Apterous (Ap) neurons, three neurons per abdominal hemisegment that extend axons anteriorly as a tightly fasciculated bundle along the most medial Fas2 bundle (Lundgren et al., 1995; Simpson et al., 2000). The axonal projections of the Ap neurons in Eph^{x652} mutant embryos are indistinguishable from wild type (Fig. 4E,F). In addition to the Ap neurons, we examined the trajectories of other subsets of neurons such as the Eagle neurons (Bonkowsky et al., 1999), the VUM neurons (Callahan and Thomas, 1994) and the MP1 neurons (Hidalgo and Brand, 1997). In all cases, we could detect no axon guidance defects in Eph^{x652} mutant embryos (data not shown).

We conclude from these results that although activation of Eph signaling by ectopic Ephrin expression results in axon repulsion, Eph signaling plays only a limited role, if any, in embryonic axon guidance. Consistent with this, we found no obvious axon pathfinding defects in $Ephrin^{KG09118}$ mutant embryos (data not shown).

Eph is expressed by mushroom body neurons throughout development

Studies in vertebrates have implicated a role for Eph/Ephrin signaling in the development of the olfactory system. In *Drosophila*, olfactory neurons within the antennae and maxillary palp project to glomeruli within the antennal lobe. Projection neurons, which form dendritic connections with antennal glomeruli, in turn project their axons to the dendritic region of the bilaterally symmetric mushroom bodies (MB) where olfactory information is processed (Crittenden et al., 1998; Heisenberg, 2003; Jefferis et al., 2002).

The neurons giving rise to the distinct lobes of the MB are clonally related (Ito et al., 1997; Lee et al., 1999). Four neuroblasts set aside early in development generate a pool of MB neurons in three distinct mitotic waves, giving rise in sequence to the larval born γ neurons, the early pupal α′/β′ neurons and finally the later born α/β neurons (Fig. 5A). The initial axonal projections of γ neurons prefigure those of the later born α′/β′ and α/β neurons. They send axons anteriorly as a tightly packed bundle within a structure known as the peduncle. Axons then bifurcate forming a dorsal and medial branch seen in late embryo/early 1st instar through 3rd instar larval stages. These axonal processes undergo degeneration during metamorphosis, only to re-extend a single axonal projection medially during puparation to give rise to the adult structure (Jefferis et al., 2002).

The second group of neurons, the α′/β′ neurons are generated during late 3rd instar. Like γ neurons, α′/β′ neurons extend an axon through the peduncle, and bifurcate, sending a distinct projection medially, along with a second collateral extension dorsally, generating the α′ and β

′ lobes. Finally, similar projections by the third group of MB neurons, the α/β neurons, result in the formation of an additional, distinct dorsal and medial projection, forming the α and β lobes. The α and β lobes are further characterized by strong expression of Fas2 (Crittenden et al., 1998; Noveen et al., 2000).

As revealed by Ephrin-Fc staining, we found that Eph is expressed within the MB, specifically within MB neurons and is targeted to their axons throughout MB development. At late embryo/early 1st instar larval stages, when MB γ neurons already display their characteristic bifurcated appearance, we detect Eph expression by most neurons of the brain, including the developing MB γ neurons (Fig. 5B–D). As in the embryonic CNS, Eph is targeted to axons. By 3rd instar larval stages, Eph staining within the brain becomes restricted to the bifurcated γ neurons (Fig. 5E–G). At early pupal stages, levels of Eph expression within the MB increase and the receptor is present on α′/β′ axons (Fig. 5H–J). Eph receptor is present throughout the MB peduncle, as well as on both dorsal and medial lobe projections at pupal stages. Expression persists as development proceeds, but becomes limited primarily to the α/β neurons at late pupal stages (Fig. 5K–M) and the adult (Fig. 5N–P), as revealed by the colocalization with Fas2. Interestingly, in the adult MB, Eph levels are higher on the dorsal projecting α lobes, particularly within their terminal regions (Fig. 5O, arrow). Thus, Eph receptor is restricted to specific lobes within the MB in the adult and its localization within a single α/β neuron is tightly regulated.

We have examined *Ephrin* expression by in situ hybridization on larval brains at a time when MB neurons bifurcate, forming dorsal and medial lobes. *Ephrin* expression within the brain at this time is detectable in many subsets of neurons including MB neurons (Fig. 5S, arrows). These results demonstrate that MB neurons, like embryonic neurons, co-express both ligand and receptor during MB development. Furthermore, as in embryonic neurons, Ephrin:myc is localized to MB cell bodies when expressed by *OK107-Gal4* (Fig. 5Q,R), a Gal4 driver expressed specifically by the MB throughout its development (Connolly et al., 1996;Kurusu et al., 2002). Therefore, both *Drosophila* Eph and Ephrin are present within developing MB neurons, but each appears to reside within distinct neuronal compartments.

Eph/Ephrin function is required for dorsal lobe formation within the MB

To examine the overall structure of the adult MB in both wild type and *Eph* mutants, we used *elav-Gal4* plus *UAS-mCD8:GFP*. This combination of transgenes labels all five lobes of the adult MB (Fig. 6A,B); the α and β lobes can be specifically visualized by double labeling with Fas2. We found that Eph^{x652} adult flies have severe MB defects (n=50). The most dramatic phenotype is reduced or absent α′ and α lobes, often with an associated increase in the thickness of β′ and β lobes (Fig. 6B,K). This phenotype is present in 60% of adult flies, and primarily exhibits itself unilaterally within the MB. Rarely do we detect severe bilateral defects, although in these cases similar reduced or absent dorsal lobe projections are observed. The severity of MB α lobe projection defects covers a continuous range, from lobes completely missing to lobes that appear normal. Defects in α lobe projections are always associated with defects in the earlier forming α′ lobe, suggesting that α′/β′ neurons may act as pioneers for the later born α/β projections as has been proposed (Wang et al., 2002). MB development in Eph^{x652}/$onecut^{x122}$ and Eph^{x652}/$onecut^{x49}$ heterozygotes (n=26) is indistinguishable from wild type (Fig. 6I), indicating that the phenotypes we observe within the MB of Eph^{x652} mutants are specifically due to loss of *Eph*.

Defects in dorsal lobe development of *Eph* mutants are evident at all stages of MB development. Defects are detected in developing α neuron dorsal projections in early pupae (6C,D), as well as in dorsal projecting branches of the earlier born γ neurons (Fig. 6E–H). By the 3rd instar larval stage, wild-type γ neurons have acquired a bifurcated appearance, generating a dorsal and medial projection (Fig. 6E). In ~60% of Eph^{x652} mutant 3rd instar larvae, we see either

reduced or absent dorsal lobe projections (Fig. 6F). Many of these same MBs show obviously thicker medial lobes in association with dorsal lobe defects (Fig. 6F, arrowhead). Similar defects in dorsal lobe projections are apparent within developing late embryonic/early 1st instar larval MBs, at a time when γ neurons are first pathfinding (6G–H). Thus, Eph function is required at all stages of MB development.

The role for Eph signaling in MB development is further supported by the phenotypes we observe in *Ephrin* mutants. In 40% of homozygous $Ephrin^{KG9118}$ adult flies (n=30), we see defects in α lobe projections identical in nature to those of Eph^{x652} individuals (Fig. 6J). Taken together, these results indicate a requirement for Eph/Ephrin signaling in the development of dorsal lobe projections within the MB.

Targeted overexpression of Eph disrupts MB development

To confirm that lack of Eph expression and function in MBs results in the dorsal lobe defects, we attempted to rescue the MB defects of Eph^{x652} homozygous adult flies by replacing Eph expression using the Gal4/UAS system. For these experiments, we used *elav-Gal4*, which is expressed in all postmitotic neurons throughout development, and the MB-specific *OK107-Gal4* driver. Expression of Eph within the MB using either of these drivers fails to rescue the α/α′ lobe defects. In fact, expression of Eph within the MB of either wild type or Eph^{x652} mutants results in a phenotype similar to the *Eph* loss of function phenotype. In these individuals, there is a high frequency and often bilateral loss of α lobes and a concomitant increase in β lobe thickness (Fig. 6K). In ~50% of these MBs, β lobes appear fused at the midline (arrowhead), signifying that medially projecting branches now fail to respect the midline boundary. Such failure to respect the midline boundary is also observed in 10% of Eph^{x652} mutant MBs (arrowhead in Fig. 6L). These results argue that MB neurons are sensitive to levels or timing of Eph expression.

Eph is required for guidance of dorsal branches of MB neurons

MB neurons may require Eph/Ephrin signaling to bifurcate and generate both medial and dorsal lobe projections. Alternatively, MB neurons may bifurcate normally in the absence of Eph function, but fail to extend a branch into the dorsal lobe and instead extend both branches in the medial lobe. To distinguish between these possibilities, we visualized individually marked mutant neurons within the MB.

We visualized individual neurons and their projections using the MARCM system (Lee and Luo, 1999), in which clones of cells are generated by mitotic recombination and identified by expression of *UAS-mCD8:GFP*. We randomly generated marked clones in wild type and in homozygous Eph^{x652} mutants at late pupal stages, thus labeling the trajectories of α/β neurons. Ideally, we would have generated Eph^{x652} mutant neurons in an otherwise phenotypically wild-type animal, thus determining the autonomous function of Eph within MB neurons. However, owing to the difficulties in generating clones by recombination of the 4th chromosome, we were limited to examining the fate of individual MB neurons within homozygous Eph^{x652} mutant individuals.

In wild type, individual α/β neurons extend a single axonal projection into the peduncle that eventually bifurcates, one branch extending medially in the β lobe, and the other dorsally in the α lobe (Fig. 7A,B). Little or no additional branching is observed along the length of each projection, although limited arborization at the end of each lobe can occur. Individual α/β neurons in Eph^{x652} adults project a single axon along the length of the peduncle as in wild type (Fig. 7C, inset) and at the base of the peduncle mutant neurons bifurcate normally. However, instead of projecting one branch dorsally along the α lobe, both branches extend medially along distinct paths within the β lobe (Fig. 7C,D). In addition, supernumerary branches along the

lengths of the β lobe projections are present in *Eph*^{x652} mutants (arrowheads in Fig. 7D). Thus, Eph/Ephrin signaling is not required for branch formation, but for correct pathfinding of dorsal lobe branches within the developing MB.

In *Eph*^{x652} MBs that completely lack the dorsal lobe, all of the neurons in a clone show projection defects (*n*=15). In *Eph*^{x652} mutant MBs where the α lobe is present but reduced, marked MB neurons either project normally or show α lobe projection defects (*n*=8). In larger clones within such MBs, we observe more labeled neurons projecting axons within the β lobe than the α lobe (Fig. 7E,F), indicating that some neurons within a single *Eph*^{x652} mutant MB can extend branches normally within dorsal and medial lobes, while other neurons do not, instead extending both branches within the medial β lobe. These results argue that individual branches of MB neurons make independent pathfinding decisions. Furthermore, these results are consistent with a continual requirement of Eph signaling within MB neurons for the correct guidance of individual branches.

In *Eph*^{x652} mutants in which the overall MB structure appears normal, individually marked neurons within all clones examined extend branches normally in α and β lobes (Fig. 7G,H; *n*=18). In these MBs, individually marked neurons also have a normal unbranched appearance, suggesting that the ectopic branching observed in phenotypically mutant MBs may be a consequence of incorrect pathfinding and target selection of the α dorsal branch. It is possible that within these overall normal-looking MBs there are neurons with abnormal projections, but their numbers are sufficiently small that the probability of one being included in a marked clone is very low.

DISCUSSION

Vertebrate Eph RTKs and their Ephrin ligands have been shown to play roles in many aspects of axon guidance, including midline crossing at the optic chiasm, retinotopic map formation and motoneuron innervation of target muscles within the limb (Klein, 2004; Palmer and Klein, 2003; Poliakov et al., 2004). Given the simplicity of *Drosophila* Eph/Ephrin, with its single Eph receptor and single Ephrin ligand, each sharing similarities to both A and B vertebrate subclasses, one might have expected the role of this prototypical Eph/Ephrin complex to reflect the most basic axon guidance functions of this family of proteins. Surprisingly, our analysis of Eph mutants shows that Eph/Ephrin signaling has a very specific requirement in MB development rather than a general role in axon guidance. Although the receptor/ligand pair is expressed in the embryonic CNS and Eph is capable of signaling when ectopically activated, this signaling appears to have a limited role in the guidance of axons. Similarly, although *Drosophila* Eph is expressed within the developing visual system, and RNAi knockdown experiments previously suggested a role in retinotopic map formation (Dearborn et al., 2002), we detect no defects in the overall targeting of photoreceptor cells in the lamina and medulla in the absence of *Eph* function (not shown). Thus, in these developing systems, Eph/Ephrin signaling either acts with other guidance molecules or functions in processes other than axon guidance.

Genetic loss of function versus RNAi

Our analysis of the embryonic CNS in Eph-null mutants shows that Eph signaling plays little or no role in axon guidance. Although we have not examined all guidance events, and in fact subtle guidance and targeting defects may yet be uncovered, the lack of any obvious defects is in marked contrast to a previous study using RNAi to knock down Eph expression (Bossing and Brand, 2002). RNAi is a widely used method to suppress gene expression in many organisms, including *Drosophila*. Although it is generally accepted that RNAi acts specifically to downregulate target gene expression because of its requirement for near perfect sequence identity with the target transcript, there is evidence that interference can occur with sequences

carrying only limited sequence similarity (Jackson et al., 2003). The Eph receptor contains regions of sequence similarity, such as the fibronectin type III repeats, to other proteins expressed within the nervous system (Scully et al., 1999). Perhaps the disparity between our results and the results using RNAi is due to expression changes in genes other than *Eph* that contain sequences capable of binding the *Eph* RNAi.

Eph signaling in *Drosophila* MB axon guidance

Our analysis of *Eph* mutants reveals a specific role for Eph signaling in regulating the guidance of individual axon branches within the MB. As in wild type, growing axons of MB neurons in *Eph* mutants bifurcate at the base of the peduncle, forming two branches. However, these two branches often fail to choose divergent paths and instead both extend medially. This is the first evidence for receptor signaling in the guidance of individual branches of MB neurons. Other receptors such as the Down syndrome cell-adhesion molecule (Dscam) and signaling molecules such as the Rho family of GTPases appear to have broader roles in regulating MB axon fasciculation, branch formation, extension and segregation (Schmucker et al., 2000; Wang et al., 2002; Zhan et al., 2004). For example, Dscam is required for MB neuron branch segregation (Wang et al., 2002; Zhan et al., 2004) through a mechanism thought to involve the mutual repulsion of sister branches (Wang et al., 2002; Wojtowicz et al., 2004). In the absence of Dscam, when branches form, they extend randomly in either dorsal or medial directions and can be fasciculated. By contrast, Eph has a more specific role in the guidance of dorsal projections and sister branches do not tightly associate with each other even when both project medially. Therefore, the mechanisms that ensure the formation and maintenance of distinct axon branches are unaffected in *Eph* mutants and we would predict that Dscam functions normally in *Eph* mutant MB neurons.

How branch formation occurs in the *Drosophila* MB is unclear, as branching MB neurons have yet to be followed in situ and in real time. Individual MB growth cones might respond to specific cues by splitting, resulting in formation of two growth cones that project independently, a possibility supported by the relative synchrony of branch formation (Wang et al., 2002). Alternatively, branches might arise as collateral extensions off existing axons (Noveen et al., 2000). Regardless of the precise mechanism of branching within the MB, sister branches must extend away from each other, one in the dorsal lobe and one in the medial lobe. Although Eph is clearly required for the proper guidance of dorsal branches, the mechanism by which it acts is unknown. In one model, Eph receptor signaling, in response to a localized Ephrin source, acts as an attractive signal to guide the extension of one sister branch in a dorsal direction. In the absence of this attractive cue, both branches extend in the ‘default’, or medial, direction. Alternatively, Eph/Ephrin signaling may act as a repellent guidance signal, in which case Eph-mediated repulsion could steer the future dorsal branch away from the midline, allowing its extension dorsally. If branching does occur by growth cone splitting, both of these models call for the differential localization or activation of the Eph receptor within a single branch, and that this spatially localized signaling leads to the guidance of this branch dorsally. In this regard, we have not detected any clear differences in Eph receptor distribution between dorsal and medial lobes at a time when MB neurons are in the process of projecting axons. However, like branch initiation itself, differential Eph expression could be a transient event and difficult to visualize by examining the entire collection of bifurcating MB neurons.

Co-expression of Eph and Ephrin

At present, the precise localization of the Ephrin ligand during branch formation is not known. Our studies of *Ephrin* RNA expression, however, indicate that, as in the embryonic CNS, MB neurons co-express both receptor and ligand. Therefore, the means by which Eph/Ephrin signaling regulates branch extension dorsally may be more complicated than Eph-expressing MB neurons simply responding to Ephrin in their environment. There are now a growing

number of instances where Eph receptors and their corresponding ligands are co-expressed within a given tissue (Knoll and Drescher, 2002; Marquardt et al., 2005). This co-expression allows, at least in theory, the possibility for both trans and cis regulation of the Eph/Ephrin signaling complex, as well as for both forward and reverse signaling in the same cell (Santiago and Erickson, 2002). The ultimate path that a growing axon takes therefore, may depend on the balance between these various signals.

Eph/Ephrin signaling in axon branch guidance

The requirement for Eph and Ephrin in guiding branches within the developing *Drosophila* MB is reminiscent of the role of Eph/Ephrin signaling in the retinotopic mapping of retinal ganglion cells within the midbrain of vertebrates (Hindges et al., 2002; McLaughlin et al., 2003; Poliakov et al., 2004) and layer-specific branching of thalamic axons within the cortex (Mann et al., 2002). In the retinotectal system, initial imprecise axon extensions of retinal ganglion cells into the midbrain are refined by extension of specific collateral branches that resolve into synaptic arbors along appropriate retinotopic locations along both the anteroposterior and dorsoventral axes. EphA/Ephrin A signaling appears to control, in part, the point where branches form along the AP axis of the midbrain, while graded EphB/Ephrin B cues guide branch extension to appropriate locations along the DV axis. Here, as in the MB of *Drosophila*, Eph/Ephrin signaling controls not the process of branching itself, but the guidance of branches to appropriate target areas, suggesting a conserved function for Eph/Ephrin in guiding branch extensions. *Drosophila* MB neurons, with their stereotyped branch formation and extension may provide a system for understanding the mechanisms whereby Eph/Ephrin signaling activates intracellular signaling cascades that ultimately lead to the guidance of individual branches to appropriate targets.

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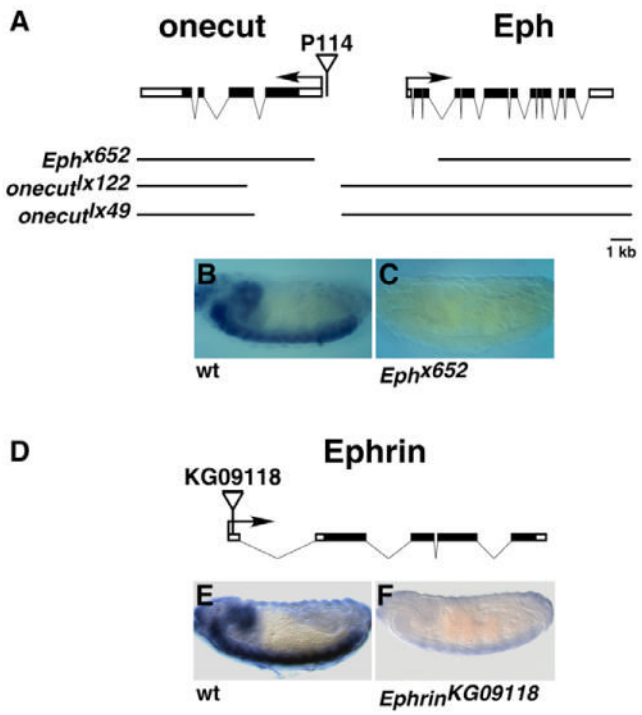

Fig. 1. Generation of the *Eph* mutant

(**A**) The P114 P element lies 3 kb upstream of the *Eph* transcription start site and ~300 bp upstream of the *onecut* transcription start site. Below are shown the extents of three deletions generated using P114. Eph^{x652} removes the first three exons of *Eph*, including the transcription and translation start sites; it also removes 5′ sequences from the *onecut* transcription unit, but does not remove the *onecut* translation initiation site. Also shown are two deletions extending solely in the direction of the *onecut* transcription unit, $onecut^{lx122}$ and $onecut^{lx49}$. Both break within the *onecut*-coding region. (**B**) In situ hybridization of *Eph* antisense RNA to a stage 15 wild-type embryo. *Eph* expression is restricted to the embryonic CNS and is detected in most, if not all, neurons. (**C**) *Eph* expression is eliminated in similarly staged homozygous Eph^{x652} embryos. (**D**) The $Ephrin^{KG09118}$ P element insertion is inserted just downstream of the *Ephrin* transcription start site (Bellen et al., 2004). (**E**) In situ hybridization of *Ephrin* antisense RNA to a stage 15 wild-type embryo. *Ephrin* expression is detected in most if not all neurons within the CNS. (**F**) *Ephrin* expression is severely reduced in homozygous $Ephrin^{KG09118}$ embryos, suggesting that $Ephrin^{KG09118}$ is a hypomorphic loss-of-function mutation. Anterior is leftwards, dorsal is upwards.

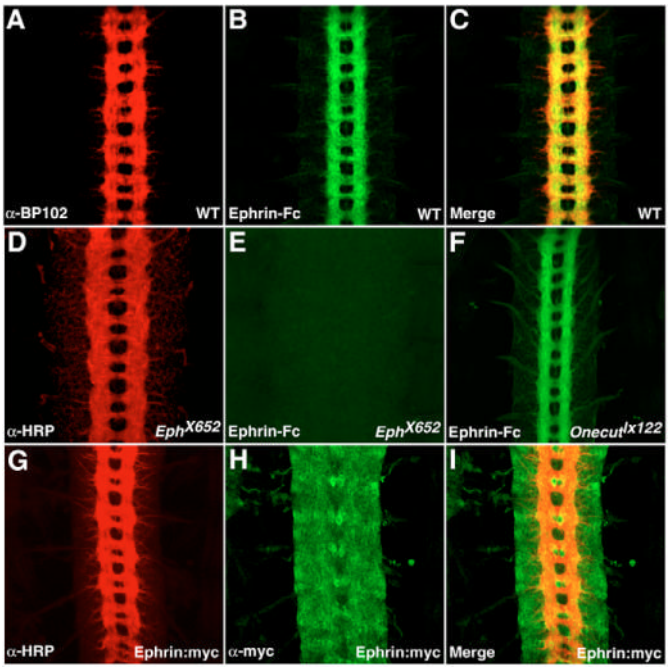

Fig. 2. Eph and Ephrin are targeted to distinct neuronal compartments

(**A–C**) View of approximately five segments of the ventral nerve cord of an unfixed dissected stage 16 embryo double-labeled with the panaxonal BP102 antibody (A) and Ephrin-Fc (B). Ephrin-Fc binds to axons in a pattern that essentially overlaps that of BP102. (**D**,**E**) Homozygous *Eph*^{x652} embryo double-labeled with the panaxonal anti-HRP antibody (D) and Ephrin-Fc (E). Homozygous *Eph*^{x652} embryos lack all Ephrin-Fc binding, confirming the specificity of Ephrin-Fc binding and that *Eph*^{x652} is null for *Eph* function. (**F**) Ephrin-Fc binding is unaffected in homozygous *onecut*^{lx122} embryos. (**G–I**) A *scrt-Gal4/+;UAS-Ephrin:myc/+* embryo double labeled with the panaxonal anti-HRP antibody (F) and anti-myc antibody(G). *scrt-Gal4* drives expression of Ephrin:myc in all neurons where it is targeted to cell bodies. Anterior is upwards.

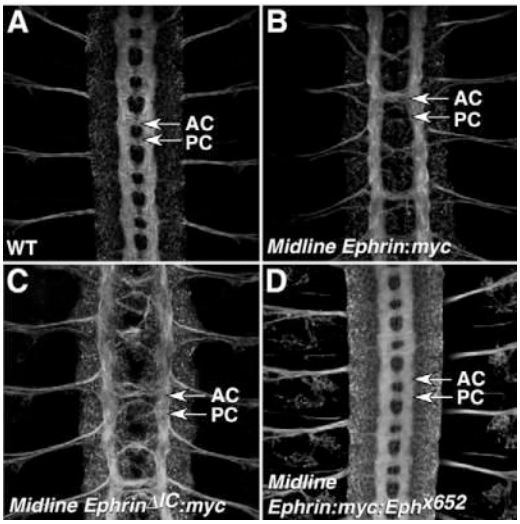

Fig. 3. Eph and Ephrin can act as a functional receptor/ligand pair in vivo

View of dissected stage 15–16 embryos labeled with anti-HRP to visualize the axon tracts within the CNS, including the anterior (AC) and posterior (PC) commissures. (**A**) Wild type. In *sim-Gal4/+;UAS-Ephrin:myc/+* embryos (**B**) where Ephrin:myc is ectopically expressed by midline glia, commissural axon tracts are disrupted. In addition, the CNS often appears less compact. This is consistent with an axon repellent activity for Ephrin. (**C**) Similar disruptions in midline axon crossing are seen in *sim-Gal4/+;UAS-Ephrin$^{\Delta IC}$:myc/+* embryos expressing an Ephrin:myc variant deleted for intracellular sequences, demonstrating that forward signaling through Eph is responsible for producing the guidance defects. (**D**) Ectopic Ephrin expression in *Eph* mutant embryos (genotype=*sim-Gal4/+;UAS-Ephrin:myc/+;Eph^{x652}*) does not disrupt commissural axons, demonstrating the Ephrin repellent activity is mediated by Eph.

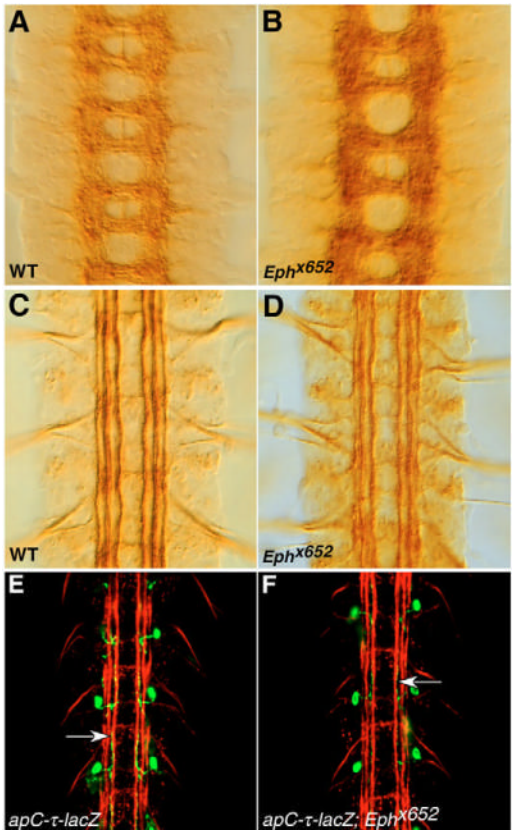

Fig. 4. *Eph*^{x652} mutant embryos lack obvious axon guidance defects in the embryonic CNS

View of the ventral nerve cord of stage 15–16 embryos in wild type (A,C,E) and *Eph*^{x652} mutants (B,D,F). (**A**,**B**) Embryos labeled with BP102 to visualize all axons. No obvious defects in axon guidance are detected in *Eph*^{x652} mutant embryos (B). (**C**,**D**) Staining with anti-Fas2 antibody, which labels five distinct axon bundles running anteroposteriorly on each side of the midline, three of which are in the plane of focus, is normal in *Eph*^{x652} mutants (D). (**E**,**F**) Embryos double-labeled for Fas2 (red) and for β-galactosidase (green) driven by the expression of *apC-tau-lacZ* to visualize axonal projections of Ap neurons. Ap neurons extend axons (arrows) normally along the medial Fas2 bundle in *Eph*^{x652} mutants (F). Genotypes: (E) *apC-tau-lacZ*/+; *Eph*^{x652}/+; (F) *apC-tau-lacZ*/+;*Eph*^{x652}/*Eph*^{x652}. Anterior is upwards for all panels.

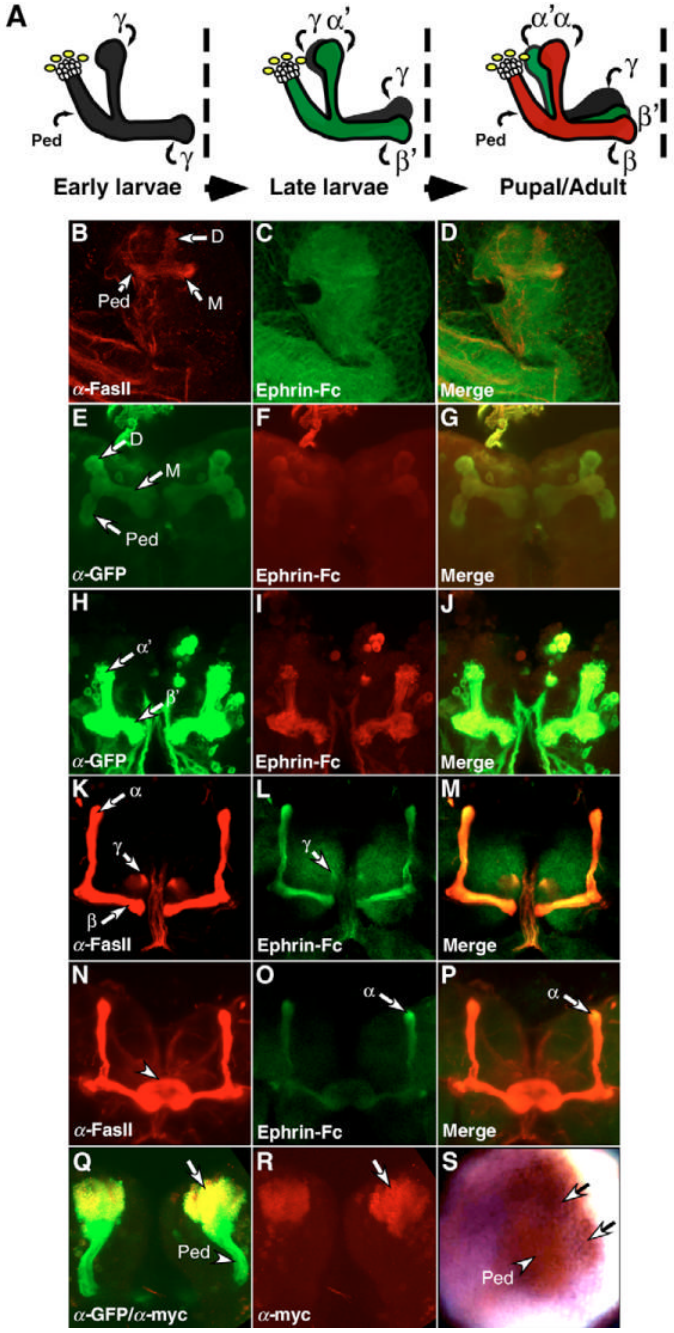

Fig. 5. Eph is expressed on axons of the developing and adult MB

(**A**) Diagram of MB development, showing the left hemisphere. Broken line indicates the midline. MB neuroblasts are shown in yellow, the axonal projections of γ neurons in black, α′/β′ neurons in green and α/β neurons in red (see text for details). (**B–P**) Eph expression, as assayed by Ephrin-Fc binding, during MB development. A high-power view of the left hemisphere of a late embryonic/early 1st instar larval brain (B–D), stained with anti-Fas2 (B) and Ephrin-Fc binding (C). Eph is present throughout the neuropil at this stage, including the MB γ neurons and their bifurcated axons (D, dorsal lobe; M, medial lobe; Ped, penduncle). (E–G) A 3rd instar larval MB from an individual carrying the γ neuron driver *201Y-Gal4* driving the expression of *UAS-mCD8:GFP* stained for GFP (E) and Ephrin-Fc binding (F).

Eph is present at low levels throughout the γ neurons and their bifurcated axons. (H–J) An early pupal MB from an individual carrying the MB-specific driver *OK107-Gal4* driving the expression of *UAS-mCD8:GFP* stained with anti-GFP (H) and Ephrin-Fc binding (I). Eph is expressed throughout the MB, including α′/β′ neurons (arrows). (K–M) Late pupal stage MB stained for Fas2 (K) and Ephrin-Fc binding (L). Eph expression is restricted to primarily α/β lobes, here identified by double labeling with Fas2, with only low level Ephrin-Fc staining present within the terminal region of the γ lobe (arrow in I). (N–P) An adult MB double stained for Fas2 (N) and Ephrin-Fc (O). Eph expression is maintained within the α/β lobes, with the highest levels of staining restricted to the terminal regions of the γ lobe (arrow in O). Fas2 expression at this stage also labels the fan-shaped body (arrowhead in N) lying directly beneath the medial lobes. (**Q**,**R**) A pupal MB from an individual carrying the *OK107-Gal4* driver plus *UAS-mCD8:GFP* and *UAS-Ephrin:myc* stained for GFP (green) and myc (red). Ephrin-myc is concentrated within MB cell bodies (arrows) and absent from axons within the peduncle (Ped, arrowhead in N). (**S**) One hemisphere of a late 3rd instar larval brain double labeled to visualize *Ephrin* mRNA expression (blue) by in situ hybridization and anti-GFP (brown) to detect mCD8:GFP driven by the MB-specific *OK107-Gal4* driver. *Ephrin* RNA expression is widespread within the brain at this time, but is detected within the MB neuron cell bodies, two clusters of which are in the focal plane (arrows). Co-labeled MB cell bodies project axons within the peduncle (arrowhead indicates the middle of peduncle), here labeled by mCD8:GFP. Genotypes: (E–G) *201Y-Gal4*/+; *UASmCD8:GFP*/+; (H-J,S) *OK107-Gal4/+;UAS-mCD8:GFP*/+; (Q,R) *OK107-Gal4/+; UASmCD8:GFP/UAS-Ephrin:myc*.

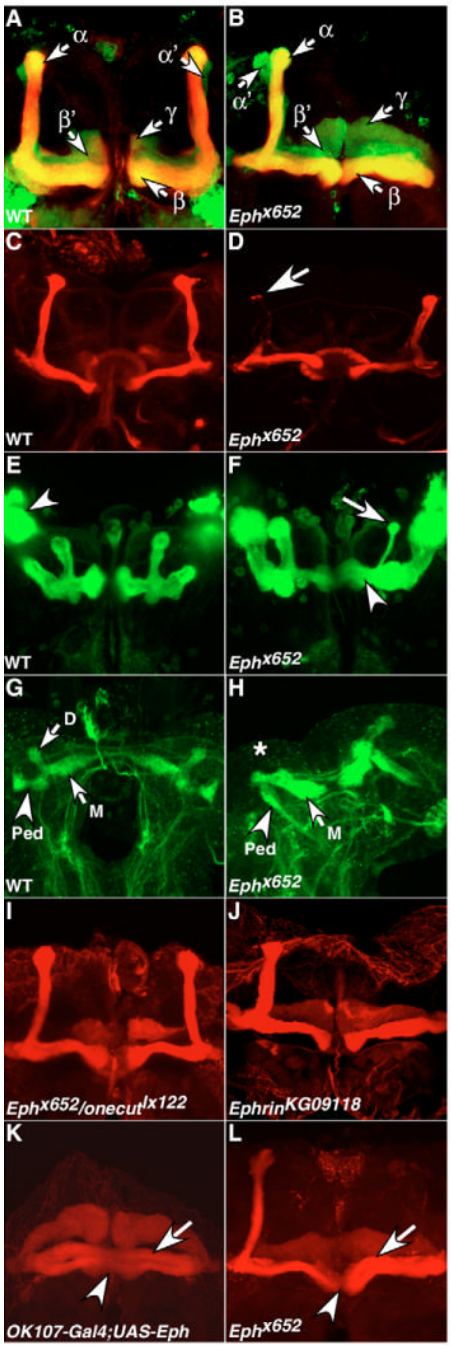

Fig. 6. *Eph*^{x652} mutants exhibit defects in dorsal lobe formation throughout MB development

(**A**,**B**) Adult MBs from wild-type (A) and *Eph*^{x652} (B) individuals carrying *UAS-mCD8:GFP* driven by *elav-Gal4* double-labeled with anti-GFP (green) and anti-Fas2 (red). *Eph*^{x652} mutant has missing dorsal projections of both α′/β′ (green) and α/β (yellow) MB neurons. (**C**,**D**) Pupal MBs from wild type (C) and *Eph*^{x652} (D) stained with anti-Fas2 showing severely reduced dorsal lobe projections (arrow) in the *Eph*^{x652} mutant. (**E**,**F**) Third instar MB from wild-type (E) and *Eph*^{x652} (F) individuals carrying *201Y-Gal4* driving *UAS-mCD8:GFP* (green), which labels cell bodies of γ neurons (arrowhead in E) and their dorsal and medial axonal projections. γ Neurons show reduced dorsal lobe projections (arrow) in the *Eph*^{x652} mutant (F). Reduction of dorsal lobe projections is accompanied by an increase in

medial lobe projections (arrowhead in F). (**G**,**H**) Late stage embryos stained with anti-Fas2. In wild type (G), the developing MB γ neurons and their dorsal and medial projections are labeled (D, dorsal lobe; M, medial lobe; Ped, penduncle). (H) *Eph*^{x652} mutant showing loss of dorsal lobe projections (asterisk). (**I**) MB of an *Eph*^{x652}/*onecut*^{lx122} adult stained with anti-Fas2. *Eph*^{x652}/*onecut*^{lx122} MBs are indistinguishable from wild type, showing no dorsal lobe defects. (**J**) MB of an *Ephrin*^{KG09118} adult stained with anti-Fas2, showing defects in dorsal branch formation similar to *Eph*^{x652}. (**K**) Anti-Fas2 staining of an adult MB overexpressing Eph using *OK107-Gal4* driving a *UAS-Eph* transgene. Similar to *Eph*^{x652} loss-of-function mutants, dorsal lobes are absent, and medial β projections are thicker (arrows), often appearing fused at the midline (arrowhead). (**L**) MB from an *Eph*^{x652} adult stained with anti-Fas2 showing loss of dorsal α lobes and thicker β medial lobes (arrow), which are fused at the midline (arrowhead). Genotypes: (A) *elav-Gal4* (X)/+ or Y;*UAS-mCD8GFP*/+; *Eph*^{x652}/*CiD*; (B) *elav-Gal4* (X)/+ or Y;*UAS-mCD8GFP*/+;*Eph*^{x652}/*Eph*^{x652}; (C,G) *Eph*^{x652}/*CiD*; (D,H,K) *Eph*^{x652}/*Eph*^{x652}; (E) *201Y-Gal4*/*UAS-mCD8GFP;Eph*^{x652}/*CiD*; (F) *201Y-Gal4*/*UAS-mCD8GFP;Eph*^{x652}/*Eph*^{x652}; (I) *Eph*^{x652}/*onecut*^{lx122}; (J) *Ephrin*^{KG09118}/*Ephrin*^{KG09118}; (K) *OK107-Gal4*/+;*UAS-Eph*/+.

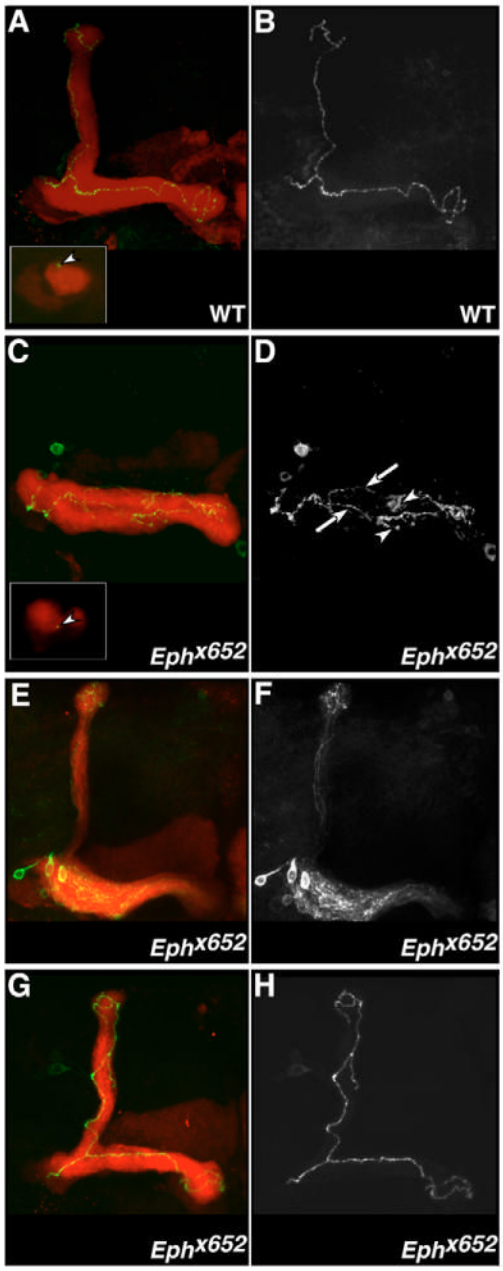

Fig. 7. Eph is not required for branch formation but for guidance of axon branches within the dorsal lobes of the MB

Axonal projections of labeled α/β neurons generated by MARCM (Lee and Luo, 2001) labeled with *UAS-mCD8:GFP*. Images of one half of the MB, double-labeled with anti-GFP (green in A,C,E,G and shown alone in B,D,F,H) and anti-Fas2 (red in A,C,E,G) to visualize the projections of α/β neurons. (**A**,**B**) In wild type, a single-labeled α/β neuron bifurcates, sending one projection dorsally in the α lobe and one medially in the β lobe. Inset in A shows the axon projection (arrowhead) within the peduncle of this singly marked clone. (**C**,**D**) In Eph^{x652} mutant MBs where the dorsal α lobe is absent, a single-labeled α/β neuron bifurcates as in wild type, but both projections extend along the medial projecting β lobe (arrows in D). Unlike wild type, extra branches can be seen along the length of both branches in the mutant (arrowheads in D). Inset in C shows the axon projection (arrowhead) within the peduncle of this singly

marked clone. (**E**,**F**) In *Eph*^{x652} mutant MBs where the dorsal α lobe is present, but reduced multiple labeled α/β neurons show fewer axons within the α lobe compared with the medial β lobe. (**G**,**H**) A single-labeled α/β neuron in an *Eph*^{x652}mutant where the overall morphology of the MB is unaffected, showing normal bifurcation and branching. Genotypes: (A,B) *elav-Gal4*, *UAS-mCD8GFP*, *hs-FLIP* (X)/+ or Y;*FRT*^{G13}/*FRT*^{G13} *Gal80;Eph*^{x652}/*CiD*; (C–H) *elav-Gal4*, *UAS-mCD8GFP*, *hs-FLIP* (X)/+ or Y;*FRT*^{G13}/*FRT*^{G13} *Gal80;Eph*^{x652}/*Eph*^{x652}.