Ptpmeg is required for the proper establishment and maintenance of axon projections in the central brain of *Drosophila*

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Ptpmeg is a cytoplasmic tyrosine phosphatase containing FERM and PDZ domains. *Drosophila* Ptpmeg and its vertebrate homologs PTPN3 and PTPN4 are expressed in the nervous system, but their developmental functions have been unknown. We found that *ptpmeg* is involved in neuronal circuit formation in the *Drosophila* central brain, regulating both the establishment and the stabilization of axonal projection patterns. In *ptpmeg* mutants, mushroom body (MB) axon branches are elaborated normally, but the projection patterns in many hemispheres become progressively abnormal as the animals reach adulthood. The two branches of MB α/β neurons are affected by *ptpmeg* in different ways; *ptpmeg* activity inhibits α lobe branch retraction while preventing β lobe branch overextension. The phosphatase activity of Ptpmeg is essential for both α and β lobe formation, but the FERM domain is required only for preventing α lobe retraction, suggesting that Ptpmeg has distinct roles in regulating the formation of α and β lobes. *ptpmeg* is also important for the formation of the ellipsoid body (EB), where it influences the pathfinding of EB axons. *ptpmeg* function in neurons is sufficient to support normal wiring of both the EB and MB. However, *ptpmeg* does not act in either MB or EB neurons, implicating *ptpmeg* in the regulation of cell-cell signaling events that control the behavior of these axons.

KEY WORDS: Axon branch, Retraction, Tyrosine phosphatase, FERM, PDZ, Mushroom body, *Drosophila*

INTRODUCTION

Neuronal wiring patterns are crucial determinants of brain function. During development, axons navigate to reach their appropriate targets in response to guidance information in their environment (Dickson, 2002). Once established, axonal projection patterns must be appropriately refined and maintained as the nervous system matures and ages. The maintenance of axonal projections is an active process important for nervous system development, with the selective retention of axonal input sculpting patterns of neuronal connectivity (Katz and Shatz, 1996; Lichtman and Colman, 2000). Disruptions in the maintenance of axonal projections are also implicated in human disease, and axonal atrophy is observed in several common neurological disorders, including Alzheimer's, Parkinson's, and Huntington's diseases (Luo and O'Leary, 2005). Understanding the molecular mechanisms that control the establishment and maintenance of neuronal connectivity patterns is therefore critical for understanding how the brain's wiring pattern arises during development and how it is maintained in healthy adults.

Cell-cell communication is critical for establishing and maintaining neuronal wiring patterns. The initial pathfinding of axons is modulated by extracellular guidance cues that bind guidance receptors on the axon surface and act to repel or attract the growth cone at the axon tip (Dickson, 2002). During the maturation of the nervous system, patterns of axon branch retention and pruning are also strongly influenced by environmental signals. Some signals

that control the maintenance of neuronal wiring act systemically, as in *Drosophila* where the hormone ecdysone modulates neuronal remodeling throughout the nervous system (Lee et al., 2000; Levine et al., 1995). Other signals that affect the maintenance of neuronal wiring act more locally, as in the mammalian forebrain where semaphorin proteins trigger the pruning of axon branches (Bagri et al., 2003).

Like pruning, the long-term retention of axon branches is an active process involving cell-cell communication. In the *Drosophila* mushroom bodies, the maintenance of axon branches requires the inhibition of axon branch retraction by RhoGAP, a negative regulator of Rho (Billuart et al., 2001) and related pathways are proposed to act in mice, where focal adhesion kinase negatively regulates axon branch stabilization *via* Rho GTPases (Rico et al., 2004). Despite the importance of axon branch maintenance to the function of neural circuits, little is known about the molecular mechanisms of long-term axon branch maintenance.

Tyrosine phosphatases have important roles in the establishment of neuronal connectivity. In *Drosophila*, the neuronally expressed receptor tyrosine phosphatases LAR, PTP10D, PTP52F, PTP69D, and PTP99A contribute to axon guidance decisions (Johnson and Van Vactor, 2003), and LAR regulates synaptogenesis at the neuromuscular junction (Kaufmann et al., 2002). In vertebrates, LAR also regulates the formation and maintenance of synapses (Dunah et al., 2005). *Drosophila ptpmeg* encodes an evolutionarily conserved cytoplasmic protein tyrosine phosphatase that is characterized by the presence of an N-terminal FERM domain followed by a single PDZ domain. FERM domains are multi-functional protein and lipid binding domains commonly found in membrane-associated signaling and cytoskeletal proteins (Bretscher et al., 2002). PDZ domains are protein-binding motifs often found in scaffolding proteins (Kim and Sheng, 2004).

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DEVELOPMENT

Orthologs of Ptpmeg are present in animals from flies to humans (Bretscher et al., 2002). There are two mammalian homologs of Ptpmeg: PTPN3, which acts as a colon cancer tumor suppressor gene in humans (Wang et al., 2004), and PTPN4. Both PTPN3 and PTPN4, as well as the *C. elegans* ortholog PTP-1/PTP-FERM, are neuronally expressed (Hironaka et al., 2000; Sahin et al., 1995; Takeuchi et al., 1994; Uchida et al., 2002), but the developmental functions of these proteins have not been examined. PTPN4 has been detected in post-synaptic density fractions and physically associates with two predominantly post-synaptic proteins, NMDAR2B [also known as glutamate receptor, ionotropic, *N*-methyl D-aspartate 2B (GRIN2B); gene ID: 14812], a glutamate receptor subunit, and GRID2 (glutamate receptor, ionotropic, delta 2 MG1:95813), a glutamate-receptor related protein (Hironaka et al., 2000). Both NMDAR2B and GRID2 are important for brain development and function, although the contribution of PTPN4 to their activities is unknown. Among other functions, both NMDAR2B and GRID2 regulate the branching of axons, acting in target cells to control the behavior of innervating axons (Cline and Constantine-Paton, 1989; Debski and Cline, 2002; Kurihara et al., 1997; Kutsuwada et al., 1996; Lee et al., 2005; Takeuchi et al., 2005).

Here, we characterize the function of *ptpmeg* in *Drosophila* by analyzing the effects of *ptpmeg* mutations on neuronal development. We find that *ptpmeg* is required to stabilize patterns of mushroom body (MB) axon branching as animals reach adulthood and for axon pathfinding in the developing ellipsoid body (EB). Structure-function studies indicate Ptpmeg phosphatase activity is required for normal MB and EB axon patterning, whereas the FERM domain appears specifically required for stabilizing a particular subset of MB axon branches. Together these data substantiate a role for Ptpmeg in the establishment and maintenance of neuronal wiring patterns.

MATERIALS AND METHODS
Genetics and molecular biology
ptpmeg[1] was generated via homologous recombination-mediated gene replacement (Rong et al., 2002). The *ptpmeg[1]* mutation was confirmed by sequencing of genomic DNA and Southern blot. *ptpmeg[2]* (pGATB-NP4498) was obtained from the *Drosophila* Genomic Resource Center (DGRC) (Hayashi et al., 2002). GenBank submission AAL55993 refers to Ptpmeg as *split central complex* (*scc*) (Boquet et al., 2000b). *scc[P]* mutants produced full-length Ptpmeg protein and complemented EB and MB phenotypes of *ptpmeg[1]* (*n*=21) and *ptpmeg[1]/Df(3L)ED201* (*n*=25), suggesting that *scc* involves additional genes.

The wild-type Ptpmeg construct was created using cDNA LD22982, and ΔFERM was created using an alternatively spliced cDNA (LD27491) lacking the amino-terminal 365 amino. Point mutations were introduced using GeneTailor (Invitrogen). *Elav-Gal4* (*P{GAL4-elav.L}3*) (Luo et al., 1994) is expressed in neurons but not glia and is more restricted than *c155-Gal4*. Mosaic analysis with a repressible cell marker (MARCM) was performed as described previously (Lee and Luo, 1999).

Histology and immunoblotting
Antisera against amino acids (aa) 337-592 of Ptpmeg, including PDZ domain (aa 480-569), were raised in guinea pigs by Covance and used at a dilution of 1:750 for histology and 1:2500 for immunoblotting. Antibodies used were: mouse anti-FasII (4D1), at 1:50; rat anti-Elav, at 1:2000 for histology and 1:1000 for immunoblotting; HRP-conjugated goat anti-guinea pig, at 1:5000; HRP-conjugated goat anti-rat, at 1:5000; donkey anti-guinea pig (mouse subtracted) Cy5, at 1:200; donkey anti-mouse (guinea pig subtracted) FITC, at 1:200; goat anti-mouse Cy3, at 1:400. Fluorescent and HRP-conjugated antibodies were obtained from Jackson Immunoresearch (West Grove, PA, USA). Other antibodies were obtained from DSHB (University of Iowa, IA, USA). Immunoblotting and immunohistochemistry were as described previously (Sears et al., 2003; Whited et al., 2004).

RESULTS
ptpmeg mutants
To study the function of *ptpmeg*, we introduced a four base-pair insertion into the locus using homologous recombination-mediated gene replacement, creating *ptpmeg[1]*. The 4 bp insertion in *ptpmeg[1]* is predicted to introduce a translational frameshift, truncating Ptpmeg within the PDZ domain (Fig. 1A-C). As predicted, *ptpmeg[1]* mutants did not express full-length Ptpmeg protein (Fig. 1D). In addition to *ptpmeg[1]*, we obtained two additional disruptions of the *ptpmeg* locus from publicly available collections. *ptpmeg[2]* (*pGATB-NP4498*) contains a transposable element insertion upstream of the Ptpmeg open reading frame and expresses reduced levels of Ptpmeg protein (Fig. 1D). Genetic data below suggests *ptpmeg[2]* is a weak loss-of-function allele. In addition, we obtained *Df(3L)ED201*, which contains an ~224 kb chromosomal deletion that disrupts the *ptpmeg* locus and is predicted to delete ~43 additional protein coding genes (Ryder et al., 2004).

ptpmeg is required for proper mushroom body axon projection patterns
Homozygous *ptpmeg[1]* adults were viable and fertile, but often became trapped alive in their food when cultured under normal conditions (J.L.W. and P.A.G., unpublished observations). This phenotype was rescued by expression of a Ptpmeg cDNA in the nervous system using *elav-Gal4*, raising the possibility of nervous system disruptions in *ptpmeg* mutants. We examined connectivity patterns in the adult brain and observed significant disruptions of mushroom body (MB) axon projections.

The MBs of the adult fly are a higher order brain structure involved in multiple behaviors including olfactory memory and sleep (Joiner et al., 2006; Zars, 2000). MB neurons each extend an axon that bifurcates to send one branch dorsally and one branch medially (Lee et al., 1999) (Fig. 2A,B). Each α′/β′ neuron extends one axon branch dorsally, into the α′ lobe, and one branch medially, into the β′ lobe. Similarly, each α/β neuron extends one axon branch dorsally, into the α lobe, and one branch medially, into the β lobe. In *ptpmeg* mutant adults, the dorsally projecting MB lobes were often reduced in thickness and/or length (Fig. 2C). Meanwhile, the medially projecting MB lobes were often overextended, with the medial lobes of one hemisphere reaching the midline and sometimes fusing with the medial lobes from the contralateral hemisphere (Fig. 2C). By contrast, the cell body and dendritic regions of MB neurons appeared normal in *ptpmeg* mutants. Thus, *ptpmeg* is important for MB axon branch development.

We focused our analysis of *ptpmeg* function on the axons of α/β neurons, selectively visualizing these axons in the adult using antibodies directed against the cell adhesion molecule Fasciclin II. In wild-type animals, the α and β lobes had a highly regular morphology (Fig. 2D, and quantified in Fig. 3A). By contrast, *ptpmeg* mutant α lobes were frequently reduced, and were often short, thin or absent (Fig. 2E-G, Fig. 3A). In some instances, the tip of the α lobe lost its knob-like appearance, creating a 'thin tip'. Expression of a wild-type ptpmeg cDNA in neurons rescued α lobe reduction in *ptpmeg[1]* mutants and *ptpmeg[1]/Df(3L)ED201* animals (Fig. 2H, Fig. 3A). Animals homozygous mutant for the partial reduction of expression allele *ptpmeg[2]* did not exhibit MB defects. However, ~20% of α lobes were defective in *ptpmeg[2]/Df(3L)ED201* animals (Fig. 3A). Together these data suggest *ptpmeg* acts in neurons to regulate α lobe patterning.

In addition to exhibiting α lobe defects, *ptpmeg* mutants also had disrupted β lobes. In wild-type animals and in *ptpmeg[1]* heterozygotes, the β lobe terminated before reaching the midline of the brain (Fig. 2J,
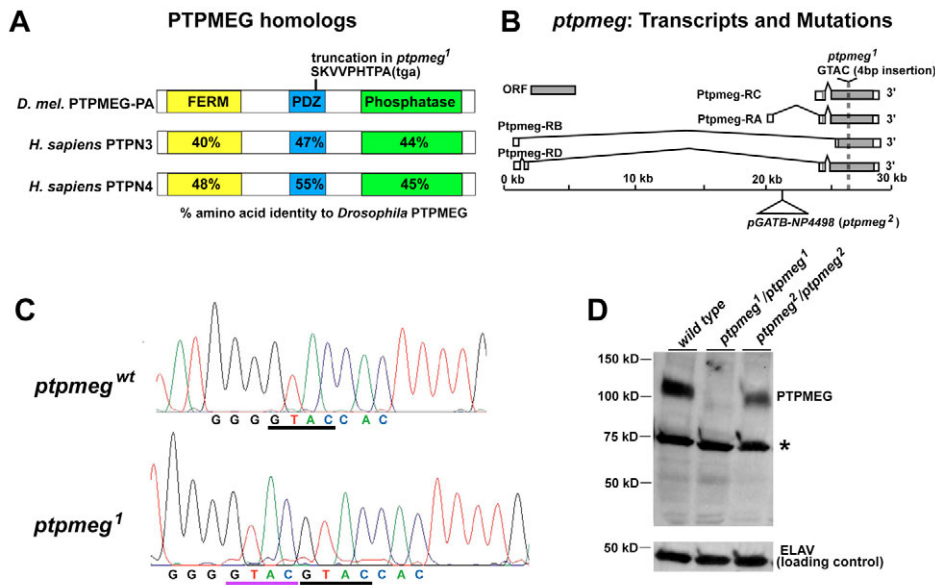
Fig. 1. Ptpmeg homologs, locus structure and mutations.
(**A**) *Drosophila* and human Ptpmeg family proteins. The *ptpmeg¹* mutation is indicated. (**B**) *ptpmeg* locus with the major transcripts, *ptpmeg²* insertion and *ptpmeg¹* mutation indicated.
(**C**) Sequence trace showing *ptpmeg¹* mutation. (**D**) *ptpmeg¹* produces no detectable full-length Ptpmeg, whereas *ptpmeg²* reduces Ptpmeg expression. Asterisk indicates background band. Elav is loading control.

and quantified in Fig. 3B). In *ptpmeg¹* mutants, the β lobes often touched the midline (Fig. 2K) and in some cases completely fused with the contralateral β lobe (Fig. 2L). Similar to the α lobe defects, the β lobe defects were rescued by the expression of wild-type *ptpmeg* in neurons (Fig. 2M, Fig. 3B). Thus, *ptpmeg* regulates both α and β lobe patterning. Analysis of β lobe development in *Df(3L)ED201* animals was not included in this analysis of *ptpmeg* function, as *Df(3L)ED201* caused dominant β lobe defects that were not rescued by *ptpmeg* expression, suggesting that disruption of genes in addition to *ptpmeg* contributed to *Df(3L)ED201*-derived β lobe defects (J.L.W. and P.A.G., unpublished data).

To determine whether *ptpmeg* acts in the MB neurons, we generated marked clones of homozygous mutant *ptpmeg¹* neurons in otherwise heterozygous animals using the MARCM system (Lee and Luo, 1999). Mutant clones of varying sizes were generated, including small clones containing one to 10 mutant α/β cells (*n*=14) and medium clones containing ~10-50 mutant α/β cells (*n*=11), but in no cases did we observe MB axon defects. Larger MB-restricted clones were generated in which nearly all α/β neurons along with some α′/β′ and γ neurons were mutant (*n*=16), but MB axon branches still appeared normal (Fig. 2I). Clones containing substantial amounts of mutant brain tissue outside the MBs did confer phenotypes (J.L.W. and P.A.G., unpublished data), but did not permit the identification of the critical cell populations in which *ptpmeg* was required. These data suggest that Ptpmeg acts in neurons to control MB axon patterning, but does not act in the MB neurons themselves.

ptpmeg is required to maintain proper mushroom body axon projections

The MB axon defects observed in *ptpmeg* mutant adults could arise in at least two different ways. In the first scenario, *ptpmeg* mutants could be defective in the initial pathfinding or elaboration of MB axon branches. Alternatively, *ptpmeg* MB axons might initially pathfind and elaborate normally, but become progressively abnormal at later times. To distinguish these possibilities, we examined MB axon development in *ptpmeg* mutants, initially focusing on the α/β neurons, which are born early during pupariation (Lee et al., 1999). By 18 hours post-pupal formation (PPF), α/β dorsal and medial axon branches can be detected and by 48 hours PPF their branching

is well established. In *ptpmeg¹* animals, dorsal branches appeared normal at both 18 hours PPF (*n*=26) and 48 hours PPF (*n*=36), whereas medial lobe branches were normal in all hemispheres at 18 hours (*n*=26) and in 33 of 36 hemispheres at 48 hours. The large increase in MB defects observed between 48 hours PPF and adult – from 0% to ~55% of dorsal lobes defective and from ~10% to ~80% of medial lobes defective – indicates that α/β axon branching defects are detected only after the α/β axon projections are well-established. This suggests that *ptpmeg* is not essential for branching or pathfinding of α/β axons, but is rather required for these branches to be maintained into the adult.

We also followed the onset of MB axon projection defects in the dorsal lobes of *ptpmeg¹/Df(3L)ED201* animals, using a marker that labels all subsets of MB neurons throughout development (Fig. 4A). In early third instar larvae, the MB lobes of wild type and *ptpmeg* mutants were indistinguishable (Fig. 4B,H). As larval MB lobes are composed of largely of γ axons with some α′/β′ axons, the initial extension of these axons thus appeared normal (Fig. 5). Between third instar and 18 hours PPF in wild-type animals, branches from additional α′/β′ neurons and from α/β neurons enter the dorsal MB region. However, the overall innervation of dorsal MB regions temporarily decreases due to the pruning of the dorsal branches of γ neurons (Fig. 4C). As the dorsal lobes of 18 hours PPF wild-type and *ptpmeg* mutants were indistinguishable, this stage of development also appears to proceed normally in *ptpmeg* mutants (Fig. 4I, Fig. 5). The dorsally projecting MB lobes thicken during pupation as additional α/β neurons send branches into this region. At 24 hours PPF, the dorsal lobes of *ptpmeg* mutant MBs remained essentially indistinguishable from wild type, as only 1 of 24 hemispheres exhibited defects (Fig. 4D,J, Fig. 5). At later times, however, MB defects became common: ~15-20% of dorsal MB lobes exhibited defects at 36 hours and 48 hours PPF (4 of 24 and 5 of 27 hemispheres defective, respectively), increasing to nearly 50% by the first day of adulthood (29 of 62) (Fig. 4E-G,K-M, Fig. 5).

The morphology of the dorsal lobes in *ptpmeg* mutants was also informative. Not only did the loss of *ptpmeg* cause a preferential reduction in the distal region of dorsal lobes, dots of axonal material were frequently observed near the regions where dorsal lobes were reduced (Fig. 4L, inset). Together these data are consistent with the loss of *ptpmeg* causing axon retraction in the dorsal lobe.
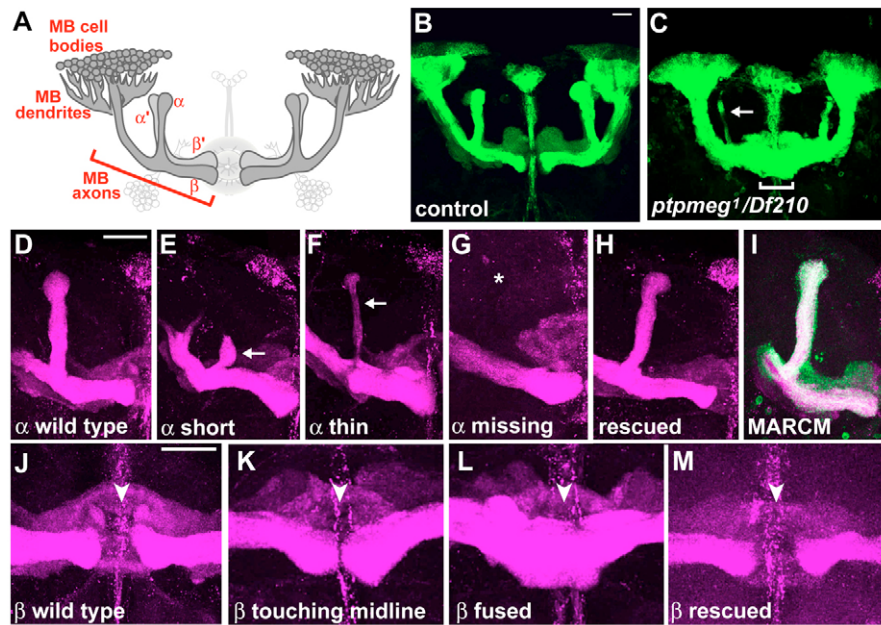
Fig. 2. *ptpmeg* is required for mushroom body (MB) axon patterning. (**A**) Diagram of the mushroom body (MB) with the neurons labeled in bold type. The locations of α, α', β, and β' lobes are indicated. (**B,C**) MB neurons visualized with *OK107-Gal4;UAS-mCD8:GFP*. In normal adults (B), dorsally projecting α and α' lobes are of similar thickness as medially projecting β and β' lobes. β and β' lobes project towards the midline but do not touch or cross it. In *ptpmeg* mutants (C), α and α' lobes are reduced, appearing thin and/or short (arrow), and β and β' lobes overgrow, touching the midline or fusing with the contralateral MB (bracket). (**D-I**) α and β lobe axons from adult left brain hemispheres labeled with anti-Fasciclin II. E-G highlight the α lobe defects of *ptpmeg* mutants, including short (E, arrow), thin (F, arrow), and missing (G, asterisk) lobes. (**H**) α lobe reduction in *ptpmeg* mutants is rescued by expression of Ptpmeg in neurons using *Elav-Gal4*. (I) Animals with large clones of homozygous *ptpmeg¹* mutant MB tissue (marked in green) in otherwise heterozygous animals do not exhibit MB defects. (**J**) Normal spacing of β lobe termini near midline. (**K,L**) β lobes touch midline (K) or fuse with contralateral β lobe (L) in *ptpmeg* mutants. (**M**) β lobe defects in *ptpmeg* mutants are rescued by expression of wild-type Ptpmeg in neurons using *Elav-Gal4*. The midline is marked with arrowhead in J-M.

Taken together, the *ptpmeg¹* and *ptpmeg¹/Df(3L)ED201* time-course data provide a consistent picture in which MB axon elaboration is initially normal, but becomes aberrant over time (summarized in Fig. 5). As the majority of defects are detected only after the initial elaboration of MB axons is completed, these data suggest that *ptpmeg* is required for a later stage in MB development. The finding that the onset of dorsal lobe defects is slightly earlier in *ptpmeg¹/Df(3L)ED201* animals than in *ptpmeg¹/ptpmeg¹* animals raises the possibility that *ptpmeg¹* might not be a null allele. Such residual *ptpmeg* activity could also explain the partial penetrance of
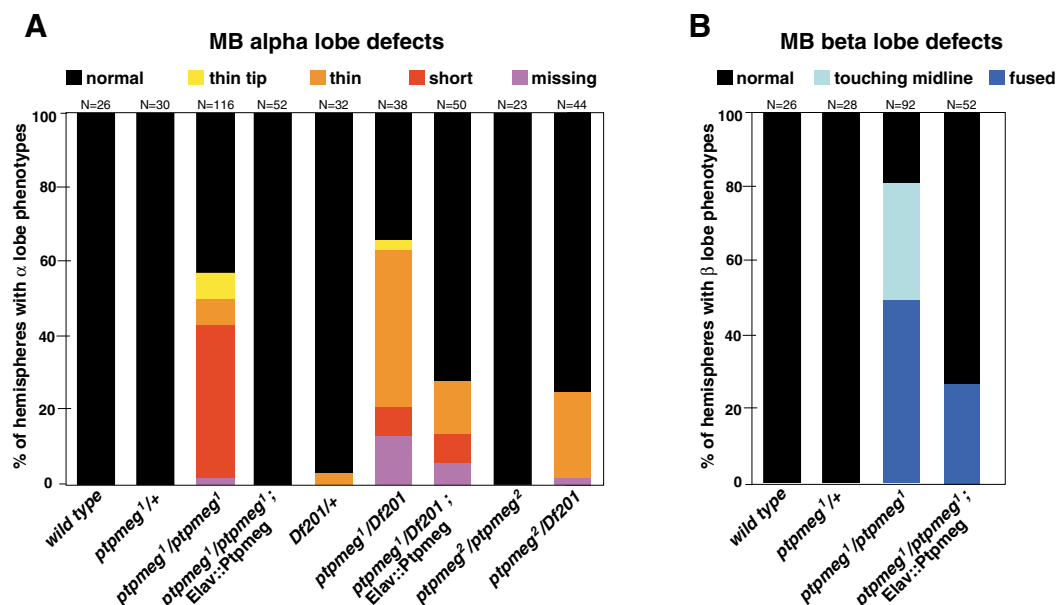

Fig. 3. Quantification of MB axon defects in *ptpmeg* mutant animals. (**A,B**) Frequency of α and β lobe defects in adults of the indicated genotypes. Df201 denotes *Df(3L)ED201*, which disrupts *ptpmeg* locus. *ptpmeg¹/TM6b* and *Df201/TM6c* are heterozygous for *ptpmeg*.
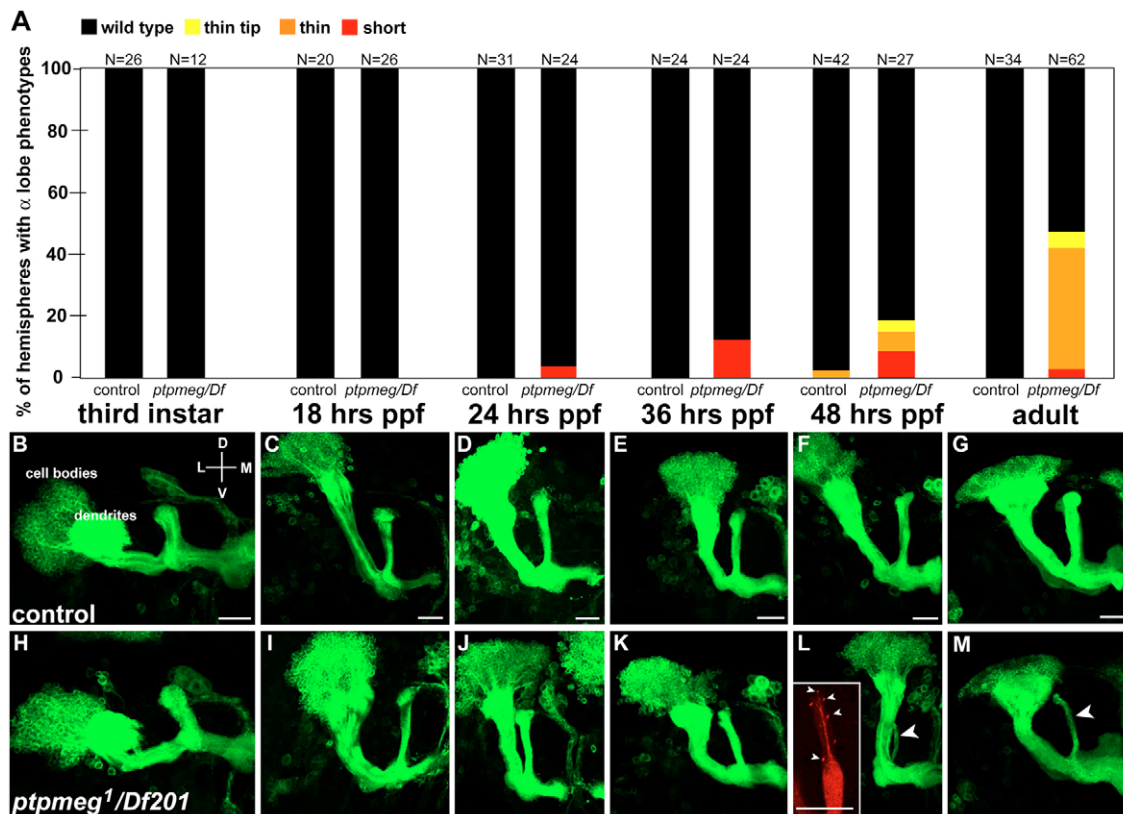
Fig. 4. *ptpmeg* is required to prevent the retraction of MB dorsal lobe axons. (**A**) Dorsal MB lobe defects in control and *ptpmeg[1]/Df(3L)ED201* animals over developmental time. (**B-M**) Representative left hemispheres. MB neurons visualized with *OK107-Gal4;UAS-mCD8:GFP*. Control (B-G) and *ptpmeg[1]/Df(3L)ED201* mutant (H-M) are shown for each age. Arrowheads in L and M indicate thin dorsal lobe in *ptpmeg*. Inset in L is a high magnification view of an α lobe (visualized using FasII) that has partially withdrawn. Small arrowheads denote the concentrations of material that are occasionally observed.

MB defects observed here. Alternatively, partial penetrance could reflect the ability of Ptpmeg-independent pathways to maintain apparently normal patterns of MB axon branches in some hemispheres.

Ptpmeg protein is expressed by neurons in the developing and adult brain

We examined Ptpmeg protein expression and found that Ptpmeg was enriched along fiber tracts in the brain at all stages examined from third instar into adulthood, including the periods when MB axons begin to exhibit defects (Fig. 6A,D,F). As expected, *ptpmeg[1]/Df(3L)ED201* mutants exhibited little or no Ptpmeg expression (Fig. 6B). Consistent with the mosaic analyses presented above indicating that *ptpmeg* did not act in the MB neurons, Ptpmeg expression was not detected on MB axons (Fig. 6C-E), However, Ptpmeg was expressed by many neurons in the central brain and the developing visual system (Fig. 6F-H, see Fig. S1 in the supplementary material). In the embryonic CNS, Ptpmeg expression was also detected in midline glial cells (data not shown), indicating that expression of Ptpmeg was not entirely restricted to neurons.

Although Ptpmeg was expressed on neuronal processes, it was largely excluded from synapse-rich neuropil regions in the central brain (Fig. 6A and J.L.W. and P.A.G., unpublished data). The subcellular localization of Ptpmeg in the central brain was examined in greater detail using a set of highly polarized neurons in the ellipsoid body (EB) that strongly express Ptpmeg. EB neurons are located in two clusters, one in each brain hemisphere. Each EB neuron extends a neurite which branches to form a dendritic tuft and an axon ring. Ptpmeg was concentrated on the cell bodies and proximal neurites of EB neurons (Fig. 6I-K). Ptpmeg expression was also detected in EB dendritic regions, but it did not extend into the axon terminals (Fig. 6I-K). Even when overexpressed using an EB-specific promoter (*EB1-Gal4*), Ptpmeg could not be detected in the axon ring (Fig. 6L-N). Rather, Ptpmeg accumulated to increased levels in the EB cell bodies (particularly near the cell surface) and on the proximal neurites and the dendrites. These data demonstrate that Ptpmeg can localize to discrete regions within a neuron.

ptpmeg is required for the establishment of ellipsoid body axon projections

Having demonstrated a requirement for *ptpmeg* in the maintenance of MB axon branches, we wondered whether *ptpmeg* was exclusively required for later stages of development or whether *ptpmeg* might be needed for the initial pathfinding of other axons in the brain. We addressed this question by examining the role of *ptpmeg* in the formation of the ellipsoid body (EB) (Fig. 7A). EB axons normally grow to reach the midline and then extend ventrally to form a closed ring, which is completed by 48 hours PPF (Zheng et al., 2006) (Fig. 7B, n=15). In *ptpmeg[1]* mutants, EB axons reached the midline, but their extension toward ventral regions halted prematurely, leaving an omega-shaped EB open along its ventral aspect (Fig. 7C, n=19). EB axon defects persisted into the adult, as *ptpmeg[1]* mutant adults displayed a ventral cleft in the EB ring (85%, n=46) (Fig. 7E). *ptpmeg[1]/Df(3L)ED201* adults showed similar defects (100%, n=10)

wild-type   ***ptpmeg* mutants**

A   early third instar

B   late third instar

C   beginning of pupation
     γ neurons are pruned

D   24 hrs PPF

E   48 hrs PPF

F   72 hrs PPF

G   adult

● γ neurons
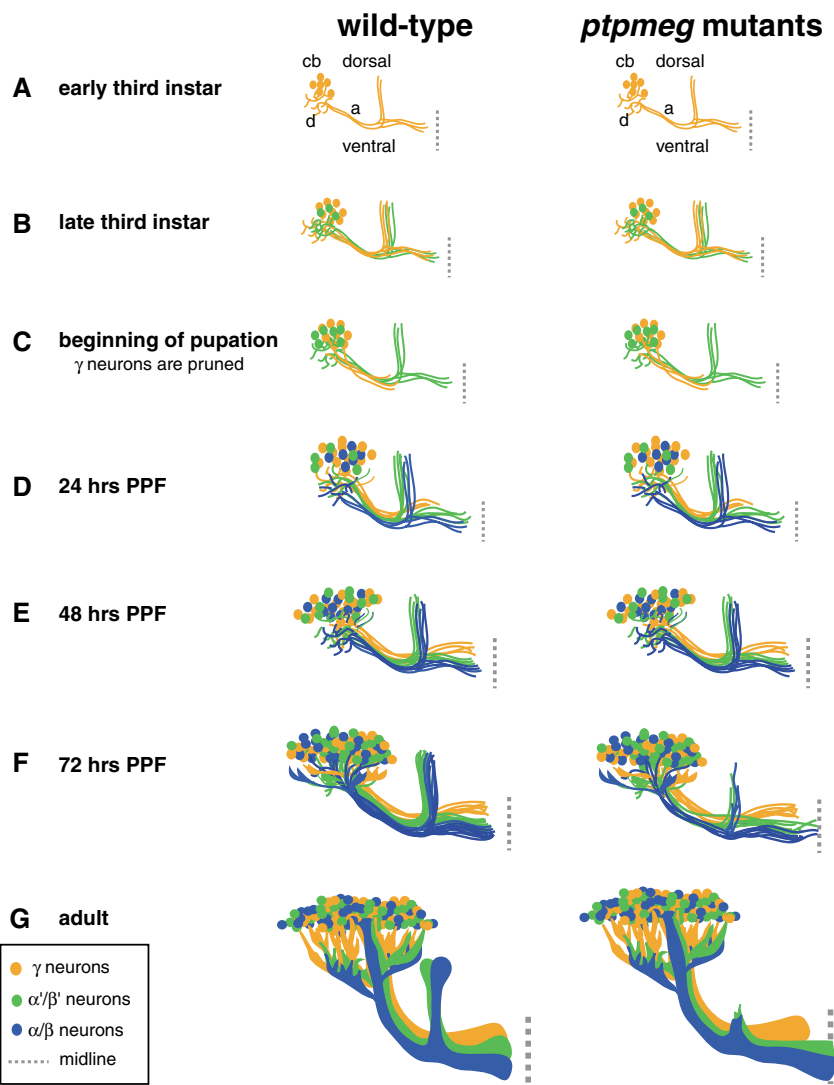● α'/β' neurons
● α/β neurons
⋯⋯ midline

Fig. 5. Summary of mushroom body development in wild type versus *ptpmeg* mutants. (**A-G**) Left column depicts developmental time course of mushroom bodies in wild-type animals [adapted from Lee et al. (Lee et al., 1999)]. Right column depicts most commonly observed defects in *ptpmeg* mutants. See text for details.

(Fig. 7F). In contrast to the MB, which was established normally in *ptpmeg* mutants but became increasingly aberrant over time, the EB appeared never to form normally and the axonal projections defects in the *ptpmeg* mutant EB did not become more severe at later time points. Taken together these data suggest that *ptpmeg* is critical for the initial pathfinding of EB axons. The EB projection defects appeared restricted to axons, as the dendritic tufts and cell bodies of EB neurons appeared normal in *ptpmeg* mutants (Fig. 7H,I).

The identity of the cells in which *ptpmeg* acts to control EB axon patterning was examined through tissue-specific rescue and genetic mosaic experiments. Expression of a wild-type Ptpmeg cDNA in neurons using *Elav-GAL4* rescued the EB defects of *ptpmeg* mutants (Fig. 7G, n=35/35 for *ptpmeg¹/ptpmeg¹*; n=23/23 for *ptpmeg¹/Df(3L)ED201*), indicating that *ptpmeg* was required in neurons to correctly pattern the EB axonal ring. To determine if *ptpmeg* was required within the EB neurons, marked clones of homozygous mutant *ptpmeg¹* EB neurons were examined in otherwise heterozygous animals using the MARCM system. Animals containing *ptpmeg¹* mutant EB neurons were analyzed (n>13), including animals in which essentially all EB neurons were mutant. In no case did we observe defects in EB axon projections (Fig. 7H). Furthermore, EB-specific expression of a wild-type Ptpmeg cDNA using *EB1-Gal4* failed to rescue the EB defect (0 of

17 animals were rescued). Thus, whereas EB neurons express Ptpmeg, they do not require *ptpmeg* to control the trajectories of their axons, suggesting that *ptpmeg* acts in other neurons to control EB axonal projections.

Phosphatase activity is essential for multiple aspects of Ptpmeg function, whereas the FERM domain is specifically required for α lobe maintenance

The Ptpmeg subfamily of tyrosine phosphatases is characterized by the presence of FERM, PDZ and PTP domains (Bretscher et al., 2002) (Fig. 1A), and we examined the requirements for these domains in brain development. We examined the role of the FERM domain on *ptpmeg* function using a naturally occurring splice variant that encodes a Ptpmeg without the FERM domain (see Materials and methods for details). When expressed in neurons, the ΔFERM variant of Ptpmeg strongly rescued the EB defect (Fig. 8A). In the MBs, the ΔFERM variant rescued the β lobe overextension phenotype of *ptpmeg¹* (P<0.002 compared to mutant; Fischer exact test), and any differences between ΔFERM and wild-type *ptpmeg* transgene rescue of the β lobe defect were not of statistical significance (P>0.1). By contrast, the ΔFERM variant did not significantly rescue the α lobe reduction of *ptpmeg¹* (P>0.15), and there was a highly significant

difference between ΔFERM and wild-type transgenes for α lobe rescue (*P*<0.0001). These data suggest that the FERM domain is important for Ptpmeg's role in α lobe maintenance, but not essential for β lobe maintenance and EB pathfinding.

The function of the Ptpmeg PDZ domain was examined by mutating residues in the GLGF motif that forms part of the substrate-binding pocket of other PDZ domains (Kim and Sheng, 2004). As a GF to AA mutation in the GLGF motif of the PDZ domain protein Enigma disrupts its ability to bind ligand (Guy et al., 1999), we mutated these amino acids in Ptpmeg, creating Ptpmeg[G494A,F495A]. Ptpmeg[G494A,F495A] rescued the EB defects of *ptpmeg[1]* mutants as effectively as a wild-type Ptpmeg cDNA (Fig. 8A). Ptpmeg[G494A,F495A] also rescued both the MB α and β lobe defects (*P*<0.001 compared to *ptpmeg* mutant). However, the ability of Ptpmeg[G494A,F495A] to rescue the MB defects was reduced compared to wild-type Ptpmeg for both the α lobe and β lobe (*P*<0.04 and *P*<0.01 compared to wild-type rescue, respectively), suggesting that the PDZ domain contributes to the effectiveness of Ptpmeg in maintenance of the MBs.

We examined the importance of catalytic activity for *ptpmeg* function by creating three forms of Ptpmeg in which residues crucial for phosphatase function were mutated. Both Ptpmeg[C877S] and Ptpmeg[Y650F,D787A] contained mutations that disrupt catalysis (Flint et al., 1997; Zhang et al., 1999), whereas Ptpmeg[R883M] contained a mutation predicted to disrupt substrate binding (Flint et al., 1997). Whereas expression of a wild-type Ptpmeg cDNA in neurons completely rescued the EB axon defects of *ptpmeg* mutants, none of the three phosphatase domain mutants significantly rescued EB defects (Fig. 8A). Similarly, none of the phosphatase mutants rescued either the α lobe or β lobe defects in the MB (Fig. 8B). In no case did expression of a mutant form of Ptpmeg cause a dominant EB axon or MB axon phenotype in an otherwise normal animal. Control experiments demonstrated that each mutant protein was expressed at a level comparable to wild-type transgenic protein as detected by western blot (J.L.W. and P.A.G., data not shown). These results demonstrate that the phosphatase activity of Ptpmeg is crucial for all of the *ptpmeg* functions observed here, including EB axon pathfinding and the stabilization of MB axon branching, where Ptpmeg inhibits retraction of dorsal lobe branches and prevents overextension of medial lobe branches.

DISCUSSION

We have found that the evolutionarily conserved cytoplasmic tyrosine phosphatase Ptpmeg contributes to the establishment and the maintenance of axonal projections in the *Drosophila* central brain. *ptpmeg* is required for the proper establishment of axon projections in the ellipsoid body (EB), where formation of the EB axon ring is not completed in the absence of *ptpmeg*. *ptpmeg* is also required for the formation of normal patterns of axonal projections in the adult mushroom body (MB), but in this case *ptpmeg* is required to stabilize MB axon projection patterns that have already formed. In the MB, *ptpmeg* promotes the retention of the dorsally directed α and α′ axon branches and inhibits the overgrowth of the medially-directed β and β′ axon branches. The FERM domain of Ptpmeg is required for MB dorsal branch retention, but is dispensable for preventing medial branch overgrowth, suggesting *ptpmeg* functions via distinct molecular pathways in dorsal and medial MB axon branch stabilization. Members of the Ptpmeg family of tyrosine phosphatase are neuronally expressed in animals from worms to flies to mice. The present work provides the first evidence that a member of the Ptpmeg family is important for neuronal connectivity.
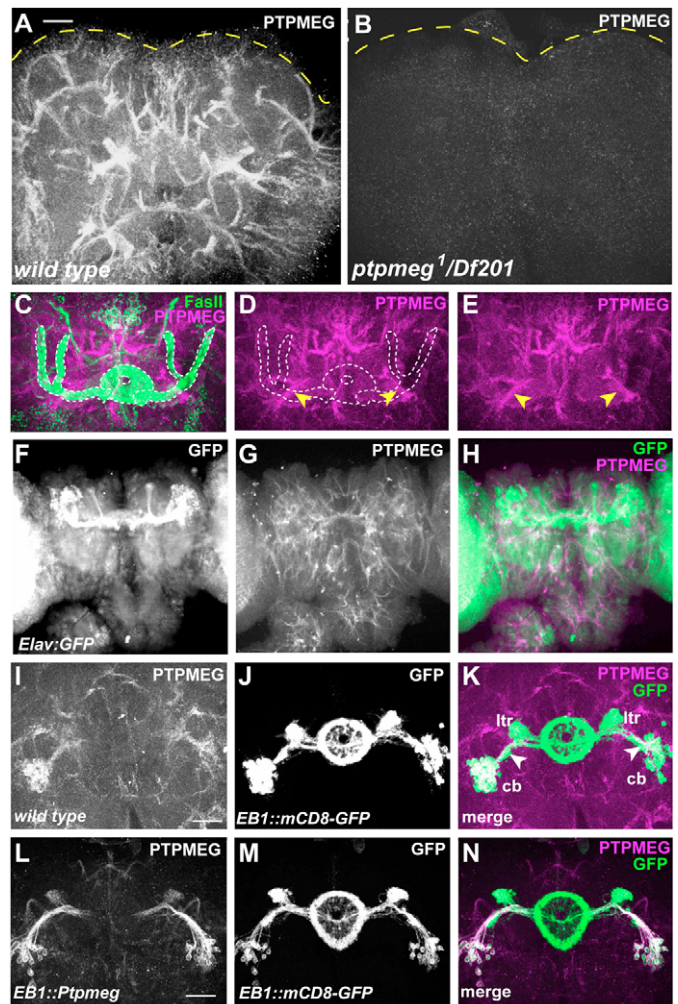

Fig. 6. Ptpmeg protein expression in the developing and adult brain. (**A**) Ptpmeg expression in wild-type brain at 72 h PPF. (**B**) Ptpmeg expression is absent in *ptpmeg[1]/Df(3L)ED201*. (**C-E**) 48h PPF brain. Ptpmeg is not detectably expressed on mushroom body axons. (C) α and β lobe axons and ellipsoid body ring axons are labeled with anti-FasII (green). Ptpmeg expression is magenta. (D-E) Ptpmeg expression (magenta), with outline of α and β lobes and ellipsoid body superimposed in panel D. Ptpmeg is strongly expressed on ellipsoid body neurites (yellow arrowheads). (**F-H**) *Elav-GAL4;UAS-mCD8:GFP* brain at ~42 hours PPF. (F) mCD8:GFP; (G) Ptpmeg expression; (H) merge. Ptpmeg (magenta) is present on neurites, indicated by overlap with mCD8:GFP (green). Ptpmeg is also expressed in mCD8:GFP-negative regions. (**I-K**) Ptpmeg expression on ellipsoid body neuron cell bodies (cb) and neurites (arrowheads in K). Ptpmeg expression extends to dendritic region (ltr), but not the ellipsoid body ring. (I) Ptpmeg expression in adult. (J) Ellipsoid body neurons visualized using *EB1-Gal4;UAS-mCD8:GFP*. (K) Merge. (**L-N**) Ptpmeg expression in *EB1-Gal4;UAS-mCD8:GFP;UAS-ptpmeg* animal overexpressing Ptpmeg in the ellipsoid body. (L) Ptpmeg expression. (M) Ellipsoid body neurons visualized using *EB1-Gal4;UAS-mCD8:GFP*. (N) Merge. Note overexpressed Ptpmeg outlines cell bodies, appearing to accumulate close to the surface of the cell.

Ptpmeg affects mushroom body dorsal and medial axon branch stability in distinct fashions

The loss of *ptpmeg* function has different effects on dorsal and medial MB axon branches. In one model, *ptpmeg* would primarily affect one set of MB axon branches, with the other set of branches
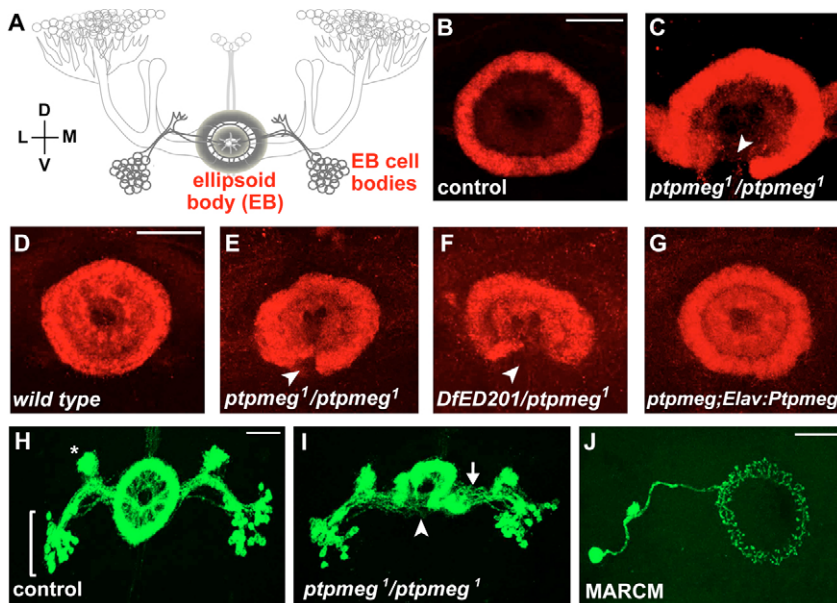
Fig. 7. *ptpmeg* is required for normal ellipsoid body development. (**A**) Ellipsoid body (EB; darker part of diagram). EB axon termini form a ring labeled with anti-Fasciclin II in B-G. (**B**) 48h PPF control EB axon ring. (**C**) 48 hours PPF *ptpmeg* mutant EB rings are often open ventrally (arrowhead). (**D**) EB axons in control adult. (**E,F**) In *ptpmeg* mutant adults, EB ring is often open (arrowheads). (**G**) Ptpmeg expression in neurons rescues *ptpmeg* EB defects. (**H,I**) EB neurons visualized using *EB1-Gal4;UAS-mCD8:GFP*. (H) Control. EB cell bodies (bracket) extend a fascicle that branches to a dendritic region (asterisk) and to axons that form the ring. (I) *ptpmeg* mutant. EB axons are slightly defasciculated (arrow) and do not elaborate a normal ring (arrowhead). (**J**) *ptpmeg* is not required in the EB cells. Homozygous *ptpmeg¹* mutant EB neuron (marked in green) in otherwise heterozygous animal.

affected secondarily. Alternatively, *ptpmeg* could affect dorsal and medial branches separately. Both structure-function and phenotypic analyses suggest *ptpmeg* affects dorsal and medial branches separately. The FERM domain of Ptpmeg was not required to stabilize the medial β lobes, but was essential for stabilizing the dorsal α lobes. Furthermore, in *ptpmeg¹* animals with fused β lobes, ~25% of α lobes appeared normal (13 of 52 hemispheres). Similarly, in *ptpmeg¹* hemispheres with reduced α lobes, ~15% of β lobes appeared normal (9 of 66). Therefore, β lobe overextension does not always accompany α lobe reduction in *ptpmeg* mutants and vice versa. Taken together, these data suggest Ptpmeg affects α and β lobes separately, acting to inhibit α lobe retraction and β lobe overextension. As *ptpmeg* is not required within MB neurons, this suggests that Ptpmeg acts in cells that communicate to dorsal branches and in cells that communicate to medial branches.

Ptpmeg stabilizes mushroom body dorsal lobe axons by preventing retraction

The ability of *ptpmeg* to promote the retention of α and α′ axon branches could reflect the inhibition of either axon degeneration or retraction by *ptpmeg*. Degenerating and retracting axons often exhibit distinct morphologies. For example, in degenerating axons, such as the axons of MB γ neurons that degenerate during *Drosophila* metamorphosis, the entire axon branch often appears to degenerate simultaneously (Watts et al., 2003). However, retracting axons often exhibit preferential reductions in thickness at the distal end of the axon branch, with small dots of axonal material left behind (Bernstein and Lichtman, 1999; Bishop et al., 2004). The withdrawal of α lobe axons in *ptpmeg* mutants does not resemble previously characterized axon branch degeneration, but rather resembles axon retraction, as α lobe reduction appears to proceed in a distal to proximal fashion. In addition, the distal tip of the withdrawing branch is often pointed and small dots of axonal material often lie nearby. Similar morphologies are also associated with branch retraction in other systems (Bernstein and Lichtman, 1999; Bishop et al., 2004), and so we propose Ptpmeg inhibits axon retraction pathways in the dorsal lobes.

Previous evidence indicates that the persistent inhibition of axon retraction pathways is important for long-term maintenance of α and α′ dorsal lobes. Reductions in the expression of *Drosophila*

RhoGAP, which is proposed to act by inhibiting a Rho-dependent axon retraction pathway, cause dorsal lobe retraction resembling that in *ptpmeg* mutants (Billuart et al., 2001). However, there are significant differences between *ptpmeg* and *RhoGAP* mutant phenotypes. *RhoGAP* inhibition causes medial lobe retraction whereas *ptpmeg* mutation cause medial lobe overextension. Furthermore, defects are detected earlier in *RhoGAP* than in *ptpmeg* mutants, with ~50% of RhoGAP RNAi hemispheres exhibiting dorsal lobe reduction by 18 hours PPF, increasing to ~95% by 36 hours PPF. Finally, *RhoGAP* is required in the MB neurons, but *ptpmeg* is not. Therefore, we suggest Ptpmeg participates in additional mechanisms that maintain mushroom body axon branches.

In contrast to the retraction of dorsal MB lobes, there is limited precedent for mechanisms that underlie overextension of medial lobe MB axons across the midline. Although several mutants with MB midline crossing defects have been described (Boquet et al., 2000b; Moreau-Fauvarque et al., 1998; Simon et al., 1998), a detailed time-course that could distinguish pathfinding defects from later onset defects has been reported only for *fmr1* mutants, defective in the *Drosophila* homolog of the fragile X mental retardation gene (Michel et al., 2004). In *fmr1* mutants, α/β axons extend branches across the midline by 24 hours PPF and medial lobe fusion appears complete by 48 hours PPF, consistent with a defect in initial outgrowth (Michel et al., 2004). By contrast, *ptpmeg¹* mutants exhibit no midline crossing defects at 48 hours PPF, suggesting most β lobe axons initially terminate extension, but reinitiate growth at later stages to cross the midline. Alternatively, midline crossing could be restricted to just the subset of β axon branches that arrive after 48 hours PPF and might reflect the failure of these axons to stop their initial extension. However, the severity of MB fusion observed in many *ptpmeg* adults suggests a large proportion of β lobe axons contribute to the phenotype, consistent with the former explanation.

How Ptpmeg might influence 'maintenance' of axon projections after initial extension remains to be determined. MB neurons show no evidence of degeneration in *ptpmeg* mutants, as both their cell body and dendritic regions appear normal. One possible source of MB defects is that Ptpmeg could act in synaptic partners of MB neurons and affect axon target recognition or synaptogenesis. A potentially similar scenario has been observed in the cerebellum of mice mutant
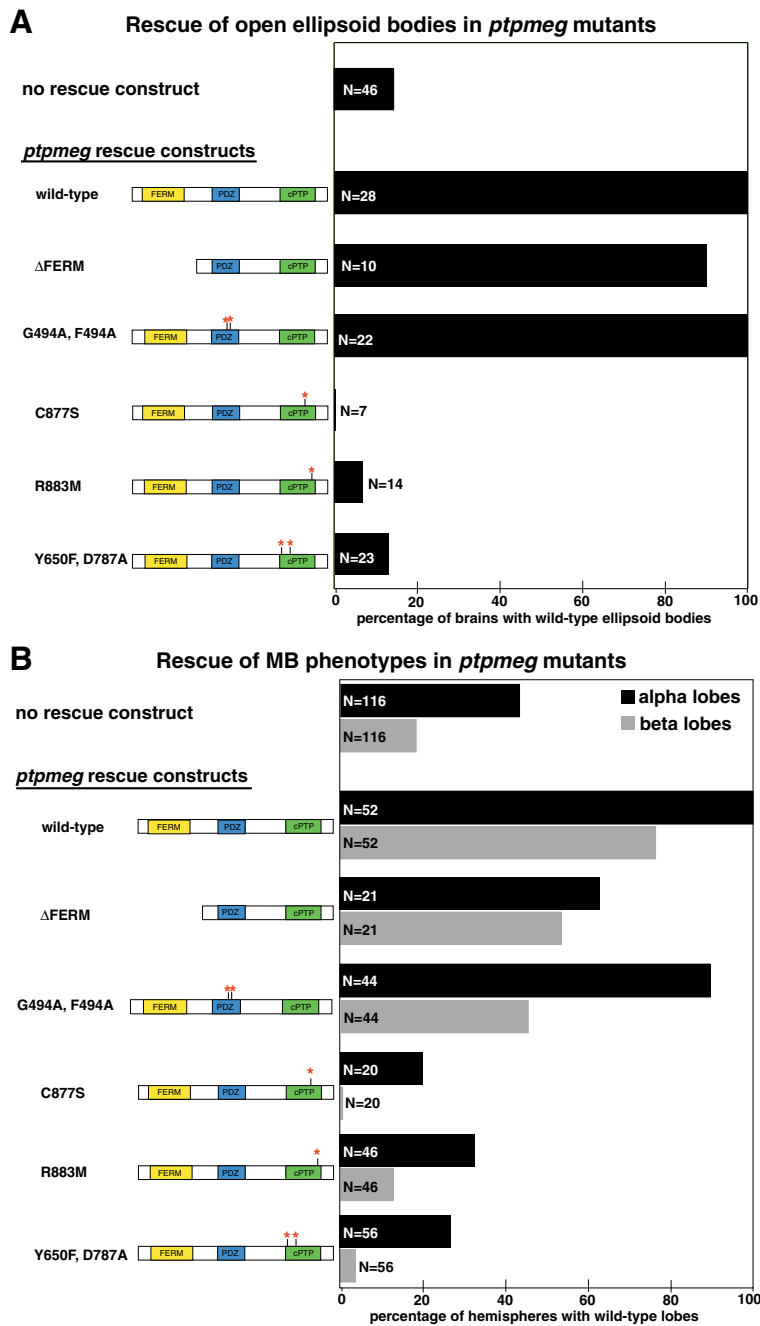
A   Rescue of open ellipsoid bodies in *ptpmeg* mutants

no rescue construct   N=46

***ptpmeg* rescue constructs**

wild-type   N=28

ΔFERM   N=10

G494A, F494A   N=22

C877S   N=7

R883M   N=14

Y650F, D787A   N=23

percentage of brains with wild-type ellipsoid bodies

Fig. 8. Ptpmeg structure-function analysis. (**A,B**) Rescue of *ptpmeg[1]* phenotypes by expression of Ptpmeg transgenes using *Elav-Gal4*. (A) Rescue of EB defects. (B) Rescue of MB defects.

B   Rescue of MB phenotypes in *ptpmeg* mutants

no rescue construct   N=116 / N=116

■ alpha lobes   ■ beta lobes

***ptpmeg* rescue constructs**

wild-type   N=52 / N=52

ΔFERM   N=21 / N=21

G494A, F494A   N=44 / N=44

C877S   N=20 / N=20

R883M   N=46 / N=46

Y650F, D787A   N=56 / N=56

percentage of hemispheres with wild-type lobes

for GRID2, a PTPN4-interacting protein (Takeuchi et al., 2005). Alternatively, Ptpmeg could control the production of structures or signals that influence MB axon behavior more indirectly. Identifying the critical cell populations and molecular pathways through which Ptpmeg modulates MB axon behavior will help determine the basis of these defects. Interestingly, ectopic expression of Ptpmeg in the eye and wing antagonizes the effects of insulin receptor signaling; however, such interactions have been observed only in the context of misexpression (J.L.W. and P.A.G., unpublished).

Ptpmeg is required to complete the ellipsoid body axonal ring

ptpmeg is critical for formation of the EB, a higher order brain region implicated in the control of locomotion (Strauss, 2002). The EB contains axons that travel to the midline and extend ventrally to form

a complete ring. In *ptpmeg* mutants, the EB axons fail to fully extend ventrally, leaving a ventral opening in the EB. These defects appear to result from a defect in EB axon pathfinding rather than axon maintenance. In contrast to the MB, which formed normally but became increasingly abnormal with time, the EB axon ring never completely formed and the defect did not become more severe with time. Similar defects in EB formation have been observed in other central complex mutants (Ilius et al., 1994; Strauss and Heisenberg, 1993), including *ciboulet*, which encodes a regulator of actin dynamics (Boquet et al., 2000a). In *ciboulet* mutants, the defect in EB ring closure was proposed to result from a failure of EB axon extension caused by a defect in actin assembly in the EB axon (Boquet et al., 2000a). As *ptpmeg* is not required in the EB neurons, *ptpmeg* likely affects the production of a structure or signal that influences the ventral extension of EB axons, rather than interacting

with *ciboulet* directly. Interestingly, Ptpmeg is expressed on fibers that cross the midline near the developing central complex, which could potentially affect EB axon pathfinding (Fig. 6G).

The ventral region of the EB lies adjacent to the β and β′ lobes of the MBs, raising the possibility that EB and MB defects are interrelated. We believe this unlikely as expression of a wild-type Ptpmeg cDNA in a *ptpmeg* mutant created many animals in which the EB ring was complete, but the medial lobes remained defective. Thus, restoration of the EB ring did not eliminate medial lobe defects and the presence of medial lobe defects were not always accompanied by EB ring defects, suggesting these defects can arise separately during development.

Ptpmeg structure, function and localization studies

In addition to its phosphatase domains, Ptpmeg also contains FERM and PDZ domains, protein interaction motifs that could facilitate the assembly of Ptpmeg into signaling complexes and the binding of substrates. Our analysis indicates that the ability of Ptpmeg to bind and dephosphorylate substrates is essential for the function of Ptpmeg, and that the FERM and PDZ domains also contribute to Ptpmeg function. Complete elimination of the FERM domain disrupts the ability of Ptpmeg to prevent α lobe retraction, while other activities supported by Ptpmeg appear largely normal. In the case of the PDZ domain, mutation of conserved residues in the GLGF motif partially reduced the ability of Ptpmeg to support MB formation, but had not effect on EB development. Given the partial effects of the FERM and PDZ mutations on Ptpmeg function, it will be of interest to perform further mutational analyses of Ptpmeg to determine whether the FERM and PDZ domains might have redundant roles or whether the phosphatase domain can perform many of the major functions of Ptpmeg by itself.

The presence of PDZ and FERM domains in Ptpmeg raised the possibility that Ptpmeg could act as a scaffolding protein. In the mouse brain, the Ptpmeg homolog PTPN4 binds the glutamate receptor subunit NMDAR2B and the glutamate-receptor related protein GRID2 (GluRδ2) (Hironaka et al., 2000), indicating Ptpmeg family members can interact with synaptic receptors. Several PDZ domain containing proteins are important modulators of receptor complex localization and activity at the growth cone tip and synapse (Kim and Sheng, 2004), while other PDZ domain proteins regulate neurite morphogenesis by acting more proximal to the cell body through the control of receptor trafficking (Hoogenraad et al., 2005). We find Ptpmeg is strongly expressed on fibers in the developing and adult brain, but that synapse-rich neuropil regions of the central brain are largely devoid of Ptpmeg. When examined specifically within EB neurons, Ptpmeg expression is restricted to the cell body and the regions of the neurite proximal to the cell body and is not present on axons. Such localization of Ptpmeg to axonal regions near the cell body and its absence from synaptic regions suggest Ptpmeg could act in cell body-proximal regions to influence neurite behavior.

Functional significance of Ptpmeg-mediated regulation of axon branch stability

Our studies demonstrate a role for Ptpmeg in the stabilization of neuronal connectivity patterns in the fly mushroom body. As the mushroom bodies are critical for olfactory learning and memory, molecular pathways that can elicit structural changes in mushroom body axons, such as the pathways in which Ptpmeg participates, are interesting candidates for mediating structural plasticity in this region. More generally, this work shows that Ptpmeg activity is necessary to prevent a progressive loss of the fly's normal wiring

pattern as it matures, inhibiting distal-to-proximal retraction of dorsal lobe MB axon branches and inhibiting delayed overextension of medial lobe MB axon branches. Progressive distal-to-proximal disruptions in axonal branching are commonly observed in CNS neurodegenerative diseases such as Alzheimer's and Parkinson's as well as neuropathies associated with diabetes, alcoholism and AIDS (Luo and O'Leary, 2005; Raff et al., 2002). Understanding the kinds of genetic lesions that can destabilize axon branches and the mechanisms that modulate axon branch maintenance could provide useful insights into the mechanisms that contribute to neurological disorders in humans.

Supplementary material
Supplementary material for this article is available at
http://dev.biologists.org/cgi/content/full/134/1/43/DC1

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