Compartmentalization and Axon Guidance in the *Drosophila* Brain

by

Timothy D. Tayler

B.A., Biology
Earlham College

Submitted to the Department of Biology
in Partial Fulfillment of the Requirements for the Degree of
Doctor of Philosophy in Biology

at the

Massachusetts Institute of Technology

February 2005

Signature of Author.....
Department of Biology
December 17, 2004

Certified by.....
Paul A. Garrity
Assistant Professor of Biology
Thesis Supervisor

Accepted by.....
Stephen P. Bell
Professor of Biology
Co-Chair, Biology Graduate Committee

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ABSTRACT

The *Drosophila* brain is composed of many morphologically and functionally distinct processing centers and brain morphogenesis depends on the creation and maintenance of distinct boundaries between adjacent regions to prevent cells from mixing. In the *Drosophila* visual system, I have found that Slit and Roundabout (Robo) proteins function to prevent cells from adjacent compartments from mixing. I have defined a boundary between two distinct compartments, the lamina and lobula, and find that the secreted ligand Slit is present in the lamina, while the Robo receptors (Robo, Robo2 and Robo3) are expressed on lobula neurons. I examined the function of theses proteins by identifying a tissue-specific allele of *slit* and creating transgenic RNAi flies that inhibit the expression of the Robo proteins. Loss of Slit or all three Robo proteins in the visual system results in the invasion of lobula neurons into the lamina. Mixing of cells at the lamina/lobula boundary results in glial cell mispositioning and aberrant photoreceptor axon targeting. Thus, Slit and Robo proteins are required to restrict movement of cells across the lamina/lobula boundary. Additionally, I have characterized Ptpmeg, a highly conserved protein tyrosine phosphatase (PTP). In addition to the C-terminus PTP domain, Ptpmeg contains a central PDZ domain and an N-terminus FERM domain. The *in vivo* role of this family of proteins is unknown. To explore the function of Ptpmeg in flies, mutants were generated by targeted gene disruption. Examination of the adult nervous system of *Ptpmeg* mutants reveals a defect in the mushroom bodies (MB), brain structures required for olfactory learning and memory. In mutant animals, the MB lobes are disorganized and fail to elaborate their characteristic structure. I find that Ptpmeg is expressed on MB axons and targeted knockdown of Ptpmeg in the MB results in similar defects as seen in homozygous mutants. Thus, the MB neurons appear to require Ptpmeg for proper formation.

Thesis Supervisor: Paul Garrity
Title: Assistant Professor of Biology

CHAPTER ONE

Introduction

The human brain contains more than 10^{12} neurons, and each neuron can make more than 1000 connections (Kandel, 2000). Each connection is precisely wired, with presynaptic and postsynaptic partners finding each other among the milieu of many other cells. How do axons navigate, often over long distances, to find their precise targets and how are complex structures within the brain generated? The vertebrate brain contains many morphologically and functionally distinct compartments, but less complex brains, such as those of insects, are similarly compartmentalized. In this thesis, I have used the model system of *Drosophila melanogaster*, which contains ~10^5 neurons, to understand how the separation of distinct regions of the brain is maintained and how complex neuronal wiring is achieved.

This thesis is comprised of two parts. The first is concerned with the role of four well characterized regulators of axon guidance and cell migration, Slit and the Roundabout (Robo) family of proteins, in the developing visual system. The second part of this thesis describes the first characterization of *Drosophila* Ptpmeg, a protein tyrosine phosphatase (PTP) that appears to regulate axon guidance in another important region of the brain, the mushroom bodies. How neurons reach their appropriate targets within the brain, and how regions of the brain become compartmentalized are fundamental questions in developmental neurobiology, and my work contributes towards an understanding of these topics.

Here I will introduce the regions of the fly brain I have chosen to work on, the visual system and the mushroom bodies, the molecules I have found to be important in the development of each of these structures, Slit, Robo, and Ptpmeg, and the processes of neural development I have studied, axon guidance and compartmentalization.

Nervous System Development in *Drosophila*

The impressive architecture of the brain is a product of precisely followed genetic instructions and complex cellular interactions that begin in the earliest stages of

development. The *Drosophila* embryonic CNS develops from progenitor cells called neuroblasts (Lu et al., 2000). These neural precursors are singled out from a layer of undifferentiated epithelial cells through complex signaling mechanisms that result in the separation (delamination) of a neuroblast from the epithelial sheet into the embryo (Campos-Ortega, 1988). Neuroblasts divide asymmetrically to give rise to two different daughter cells, a ganglion mother cell (GMC) and another neuroblast (Jan and Jan, 1998; Jan and Jan, 1999). The GMC will divide once more to produce two postmitotic neurons or a postmitotic neuron and a glial cell. Before the neuroblast divides, certain molecules are unequally distributed in the cell, resulting in molecularly distinct daughter cells after division (Hirata et al., 1995; Knoblich et al., 1995; Spana and Doe, 1995; Spana et al., 1995). Asymmetric cell division helps drive the process of differentiation. Neuroblast divisions are repeated over and over again to generate a great diversity of neuronal and glial cell types (Goodman, 1993). Some populations of neural progenitors are set aside for later stages of development. They remain mitotically quiescent until reactivation signals cause them to begin dividing again to generate mature neurons (Lu et al., 2000).

After the decision to become a neuron is ensured, axons and dendrites begin the process of nervous system wiring. Sensory structures at the tip of neurites, termed growth cones, guide axons and dendrites to specific targets (Goodman and Shatz, 1993). Once the growth cone reaches the target cell or cells, connections are formed that give rise to functional synapses. Although the cell bodies of many neurons will remain near their place of differentiation, a large number of neurons migrate to different locations using many of the same conserved molecules that are used by navigating axons and dendrites (Guan and Rao, 2003). Some of these molecules will be discussed later in this chapter. My graduate work has focused primarily on a window of neural development that begins after cell fate has been specified and before synapses form.

Introduction to the Brain Regions

The *Drosophila* Visual System

The *Drosophila* visual system has emerged as an important model for neural development and brain wiring (Clandinin and Zipursky, 2002; Tayler and Garrity, 2003;

Wolff, 1997). *Drosophila* vision is mediated through two compound eyes that each consist of precisely arranged ommatidia, the individual facets of the eye (Wolff and Ready, 1993). The ommatidial lenses focus incoming light onto underlying photoreceptors that contain light-absorbing visual pigment and through the phototransduction process, the absorbed photons are transduced into membrane potentials which are then converted into signals that are passed on to other neurons in the brain (Yarfitz and Hurley, 1994). The visual ganglia, which will be discussed below, are the image processing centers of the brain.

The *Drosophila* eye and brain provide exceptionally accessible systems to study the mechanisms of axon guidance and compartmentalization. There are a large number of molecular tools that allow disruptions in these highly ordered structures to be identified. In addition, early cellular events that pattern the eye are well understood and the eye is amenable to genetic manipulation, as it is dispensable for the viability of the fly (Dickson and Hafen, 1993; Pappu, 2002). The adult eye is composed of ~800 repeated ommatidial units (Wolff and Ready, 1993). Each ommatidium contains 8 uniquely identifiable photoreceptor neurons (or R-cell for Retinula cell). There are three subtypes of photoreceptors, R1-R6, R7 and R8. In the adult, each subtype expresses a different photosensitive opsin and responds to different wavelengths of light (Hardie, 1985). R-cells differentiate in the developing eye disc and send projections through the optic stalk into distinct layers of the brain during larval and pupal development (Meinertzhagen, 1993). R1-R6 project axons into the most superficial layer of the optic ganglion, the lamina, while R7 and R8 send axons past the lamina, into two deeper layers of the brain in the medulla. The developing lamina and medulla are separated from another region of the visual system, the lobula, by a distinct boundary (Tayler et al., 2004). The R-cell axonal projections and the developing target region can be visualized after dissection and antibody staining. Therefore, mutations that disrupt R-cell targeting or target region formation can be readily identified.

The *Drosophila* eye disc arises from a small group of cells (~20) during early embryogenesis (Garcia-Bellido and Merriam, 1969; Wieschaus and Gehring, 1976). The distinct morphology of the young eye disc is easily visible after hatching and the eye disc rapidly grows in size and cell number. From the end of the first larval instar to the

beginning of the third larval instar, cell numbers increase from approximately 130 to more than 1300 (Becker, 1957; Wolff and Ready, 1993). The adult eye begins to take shape at the beginning of the third larval instar. The differentiation of cell clusters that will compose ommatidia begins at the posterior margin of the eye imaginal disc. The line of newly differentiated cells is marked by the morphogenetic furrow (MF), which sweeps across the eye disc toward the anterior margin (Wolff and Ready, 1993). The MF represents the constriction and contraction of cells just prior to differentiation and appears as an indentation in the eye disc (Tomlinson, 1985). Photoreceptor neurons differentiate in a sequential manner: R8, followed by R2 and R5, R3 and R4, R1 and R6, and finally R7. As the photoreceptors differentiate they secrete Hedgehog protein, a morphogen required in many developmental pathways, which triggers the differentiation of more anterior cells resulting in the progression of the MF (Heberlein et al., 1993; Lum and Beachy, 2004; Ma et al., 1993). Photoreceptor differentiation begins in the posterior margin of the eye disc at the dorsal-ventral midline. R8 is the founder cell of each ommatidium and organizes the developmental events that follow (Wolff and Ready, 1993). Newly differentiated photoreceptor neurons begin sending out axons that travel through the optic stalk into the brain. The axons converge on the optic stalk and then spread out again as they enter the brain, maintaining the same position relative to their neighbor in the eye disc and thus generating retinotopy in the brain. In the adult brain, there are four visual processing centers: the lamina, medulla, lobula and lobula plate. The lamina and medulla receive input directly from the retina, while the lobula and lobula plate connect visual system neurons to higher processing centers of the brain (Meinertzhagen, 1993).

A considerable amount of effort has been devoted to mapping the structure and neuronal connections of the adult optic lobe (Bausenwein et al., 1992; Dittrich and Fischbach, 1989; Meinertzhagen, 1993). However, the cellular and molecular mechanisms that generate the precise organization of optic lobes are not well understood. The adult optic lobes are located beneath the compound eye and consist of the four neuropil regions mentioned above. Each neuropil contains of an orderly array of columns that reflect the organization of the overlying eye (Meinertzhagen, 1993). The photoreceptor axons from a single ommatidium travel into the brain together as a tightly

fasciculated bundle. Hence, the number of columns formed in the lamina and medulla optic lobe neuropil is nearly the same as the number of ommatdia in the eye (~800) (Meinertzhagen, 1993). Stacks of neuronal projections running perpendicular to the columns innervate specific layers within the optic lobes and have been postulated to have an integrative function. In addition, the visual centers of each brain hemisphere are connected by large tangential neurons and provide further integration of visual information (Dittrich and Fischbach, 1989).

The adult optic lobes are derived from neuroblast proliferation centers (anlagen) that arise during embryonic development. Two crescent-shaped neuroblast proliferation centers create most of the optic lobe, the inner optic anlagen (IOA) and the outer optic anlagen (OOA) (Hofbauer and Campos-Ortega, 1990). The lamina (the R1-R6 target region) and distal medulla (R7/R8 target region) are derived from cells of the OOA. The IOA generates neurons of the lobula, lobula plate and proximal medulla. As a result of cell division and cell migration, the spatial relationship of the two proliferation centers changes during the third larval instar stage, yet the cells formed remain separate and distinct (Hofbauer and Campos-Ortega, 1990; Meinertzhagen, 1993).

Of the optic lobe structures, the lamina has received the most attention. The differentiation of lamina precursors is triggered by photoreceptor innervation and therefore lamina development is completely dependent on ingrowth of photoreceptor axons (Power, 1943; Selleck et al., 1992; Selleck and Steller, 1991). The medulla, lobula and lobula plate are less dependent on photoreceptor innervation, as the structures are still present (although reduced in size) even when photoreceptors axons have been prevented from entering the brain (Fischbach and Heisenberg, 1981; Power, 1943).

Although it had long been observed that the development of the optic lobes depended on photoreceptor innervation (Power, 1943), the mechanisms governing the induction process were unknown. Shortly after photoreceptor differentiation begins, the first photoreceptor axons travel through the optic stalk and enter the brain. Photoreceptor axon entry into the brain triggers a series of developmental events. Work from many groups has shown that the migrating photoreceptor axons are primarily engaged in two concurrent tasks: establishing the patterning of their final targets and identifying their intermediate targets (Clandinin and Zipursky, 2002).

The organization of the lamina target field is accomplished, in part, by the delivery of secrected cues to precursor cells in or near the target region. Elegant studies by Kunes and coworkers have shown that Hedgehog (Hh) protein is expressed in photoreceptors and transported down their axons. Delivery of Hedgehog to the lamina induces the final cell division of lamina precursor cells (LPCs), the eventual R1-R6 synaptic targets (Huang and Kunes, 1996; Huang and Kunes, 1998). Later, they showed that Spitz, a member of the EGF family, was the factor that promoted the final differentiation step of the lamina neurons (Huang et al., 1998).

The observation that R1-R6 growth cones terminate precisely between rows of glia, suggested that glial cells provided the signal(s) for the R1-R6 growth cones to stop in lamina, effectively functioning as the intermediate target of the R1-R6 axons (Perez and Steller, 1996; Winberg et al., 1992). The lamina glia also depend on R-cell axons for migration and these anterograde signals are essential for proper visual system development (Poeck et al., 2001; Suh et al., 2002; Winberg et al., 1992). It has recently been shown that retrograde signals from these glia must also be required for the R-cells to make the correct target layer selection. In mutants in which glial cells do not migrate into the lamina target region, R-cells fail to stop in the lamina and extend into the underlying medulla (Poeck et al., 2001; Suh et al., 2002).

The *Drosophila* Mushroom Bodies

The mushroom bodies are lobed nervous system structures found in many marine annelids and all arthropod groups except crustaceans (Strausfeld et al., 1998). Although the basic structure is the same, the size and complexity varies widely among species and sometimes even within species. The mushroom bodies were first described in 1850 by the French biologist Felix Dujardin. He termed the structures "corps peloncules" (stalk-like bodies), as their shape appeared similar to the fruiting bodies of lichens (Dujardin, 1850). Experiments that revealed a role for the mushroom bodies in learning and memory followed the early descriptive studies of Dujardin. Ants have the ability to navigate relatively complex mazes using specific olfactory cues and cockroaches have a keen ability to recall the location of food sources (place memory). These abilities are perturbed after lesioning the mushroom bodies (Mizunami, 1993; Vowles, 1964).

Elegant genetic experiments in *Drosophila* later revealed a number of genes that were required in the mushroom bodies for olfactory learning and memory (Dudai et al., 1976; Livingstone et al., 1984; Quinn et al., 1974; Quinn et al., 1979). These experiments importantly established that fruit flies could indeed learn and that single genes could affect the process. Further anatomical, electrophysiological and genetic studies over many years have contributed to the view that the mushroom bodies play a major role in olfactory processing and the integration of other sensory modalities, such as vision, touch and hearing (Strausfeld et al., 1998).

The *Drosophila* mushroom bodies (MBs) have been used as a model to study various aspects of neuronal morphogenesis. The development of MBs is well described and mutagenesis screens have uncovered genes that are important in regulating cell size, neuroblast proliferation, axonal transport and axon and dendrite morphogenesis (Lee et al., 1999; Ng et al., 2002; Reuter et al., 2003). A combination of Golgi staining and genetic mosaic analysis has formed a comprehensive picture of mushroom body structure and development (Kenyon, 1896a; Kenyon, 1896b; (Lee et al., 1999; Strausfeld, 1976). There are three classes of mushroom body neurons (also called Kenyon cells), all of which originate from four neuroblasts in each hemisphere of the embryonic brain. The neuroblasts begin dividing during mid-embryogenesis (stage 9) and continue into the pupal stages (Ito et al., 1997; Ito and Hotta, 1992). Densely packed Kenyon cell bodies (~2500 in each hemisphere) are located near the dorsal-anterior surface of the brain and are designated according to which lobes their axons innervate. γ neurons are generated in the first instar larval stage, α' and β' neurons are generated between the third instar larval stage and puparian formation, and lastly, the α and β neurons are generated after puparian formation. As the newly generated mushroom body neurons extend axons to form the α and β lobes, the γ neurons partially retract their axons and then re-extend toward the midline, to form the γ lobe (Lee et al., 1999). Sequential expression of transcription factors appears to underlie the temporal development of the mushroom bodies (Isshiki et al., 2001).

After Kenyon cells elaborate dendritic processes within the mushroom body calyx, just beneath their cell bodies, the axons fasciculate, forming tightly bundled parallel axon fibers called a peduncle (Strausfeld et al., 1998). As the axons exit the

peduncle, they bifurcate and enter dorsal and medial lobes. An interesting feature of the mushroom bodies is the concentric development of the axons. Younger axons are found in the interior, while the older axons are found on the exterior (Verkhusha et al., 2001).

Screens for defects in organization of the adult mushroom bodies have revealed a number of genes that regulate MB neuroblast proliferation: the histone acetyltransferase (enok), the p21-activated kinase-like protein serine/threonine kinase (mbt), and a coiled-coil protein (mud) (Heisenberg, 1980; Melzig et al., 1998) (Guan et al., 2000; Scott et al., 2001). Recent investigations have used mosaic analysis to study more specific aspects mushroom body development such as axon growth, guidance and branching (Ng et al., 2002; Reuter et al., 2003).

INTRODUCTION TO THE MOLECULES

In this thesis I have studied molecules required for the formation of the visual system and mushroom bodies, and will provide some background on these molecules below.

Slit and Roundabout

Mutations in *slit* were originally recovered by Nusslein-Volhard and Wiechaus in a screen for genes controlling pattern formation in *Drosophila* (Nusslein-Volhard et al., 1984). A few years later *slit* was cloned by Artavanis-Tsakonas and coworkers after being identified in a homology based screen (Rothberg et al., 1988). The *slit* embryonic phenotype was similar to a previously characterized gene called *single-minded* (*sim*) (Crews et al., 1988; Nusslein-Volhard et al., 1984; Thomas et al., 1988). Sim protein is expressed by glia and neurons of the embryonic CNS midline. Loss of Sim expression results in the collapse of the ladder-like structure of embryonic nerve cord, resulting from the loss of midline cells. Slit protein was expressed in a similar pattern and the loss-of-function phenotype was nearly identical to that of *sim* mutants. Therefore, it was concluded that the longitudinal axon collapse phenotype seen in *slit* mutants, likely resulted from loss or improper positioning of midline cells, in particular the midline glia that had been shown to play an important role in the formation of axon commissures at the midline (Thomas et al., 1988). More than a decade after its initial characterization, three groups independently demonstrated the role of Slit in axon guidance decisions

(Brose et al., 1999; Kidd et al., 1999; Li et al., 1999), axon branching (Wang et al., 1999), and neuronal migration (Wu et al., 1999). Furthermore, these groups showed that Slit controlled these processes through a previously characterized guidance receptor called Roundabout (Robo).

Goodman and colleagues (Seeger et al., 1993) performed a screen for mutations that disrupted the development of axon pathways in the developing CNS of *Drosophila* embryos. The first two mutant lines to be molecularly characterized were the previously unidentified genes *roundabout* (*robo*) and *commissureless* (*comm*). Mutations in *robo* resulted in axons aberrantly crossing and recrossing the midline. Mutations in *comm* caused the absence of nearly all commissures, as axons rarely approached or crossed the midline. The *robo* gene was later shown to encode a highly conserved transmembrane protein, expressed on the growth cones of axons that never cross the midline and on axons that have recently crossed the midline (Kidd et al., 1998a; Seeger et al., 1993). It became the founding member of a novel family of axon guidance receptors. Ectopic expression of Robo resulted in a *comm*-like phenotype, where axons never entered the midline. Comm was shown to regulate Robo by preventing Robo protein from being expressed on the growth cone surface (Georgiou and Tear, 2003; Keleman et al., 2002). This suggested a model in which axons that never crossed the midline express high levels Robo protein on their growth cones, whereas axons destined to cross the midline express little or no Robo. After crossing the midline, Robo expression is upregulated, preventing axons from re-crossing (Kidd et al., 1998a; Kidd et al., 1998b).

The characterization of Robo as a transmembrane receptor led to the search for the unknown ligand. In *Drosophila*, the midline mutagenesis screen that had identified *robo* and *comm*, apparently covered much of the genome. However, the corresponding *robo* phenotype that would be predicted for the ligand, had not been found (Seeger et al., 1993). A closer look at other mutants recovered from the initial screen finally revealed Slit as the missing ligand (Kidd et al., 1999). *slit* had initially been overlooked for two reasons. First, the *slit* mutant phenotype did not fully resemble the *robo* mutant phenotype. In *robo* mutants, too many axons cross and recross the midline, resulting in thicker commissures and thin or missing longitudinal axons. The phenotype in *slit* mutants is more dramatic: axons converge on the midline and do not reemerge. This

issue was resolved later when two more Robo-family members were identified. It was shown that the Robo proteins function redundantly and loss of Robo and Robo2 proteins results in a *slit*-like phenotype (Simpson et al., 2000a; Simpson et al., 2000b). Second, the similarity of the *slit* and *sim* mutant phenotypes misled researchers to believe that the CNS collapse was due to loss or mispositioning of midline cell rather than errors in axon guidance. Better molecular markers revealed that the midline cells of slit mutants appeared normal (Kidd et al., 1999).

While there are multiple forms of Slit in vertebrates, there is only a single *slit* gene in *Drosophila.* Slit is a secreted protein, containing four leucine rich repeats (LRRs), seven EGF repeats, a laminin G domain, an Agrin-Laminin-Perlecan-Slit (ALPS) spacer domain, and a cysteine-rich repeat. It has been shown to be expressed in both glia and neurons (Guan and Rao, 2003; Wong et al., 2002). The binding of Slit to Robo requires the LRRs but not the EGF repeats (Battye et al., 2001; Chen et al., 2001). Robo receptors belong to a novel subfamily of immunoglobulin (Ig) superfamily proteins. The extracellular portion of Robo receptors contains five Ig repeats and three fibronectin type III (FNIII) repeats. The intracellular region contains four conserved motifs, or CC domains, that share little homology to intracellular domains of other transmembrane receptors. Elimination of each CC domain reduces but does not abolish Robo function, suggesting built in redundancy of these motifs (Bashaw et al., 2000). The Robo family consists of three members, Robo, Robo2 and Robo3. In *Drosophila*, differences in the CC domain composition likely mediate differential responses to Slit (Simpson et al., 2000a; Simpson et al., 2000b). Both Slit and Robos can be proteolytically cleaved, but the significance of the cleavage is unclear (Wong et al., 2002).

A growing list of molecules has been identified to be important in Robo signal transduction. Through biochemical studies, Enabled (a member of the Ena/Vasp family) and Abelson tyrosine kinase (Abl) have been identified as binding partners of Robo (Bashaw et al., 2000). Such interactions may be responsible for the mechanism by which the cellular signaling events downstream of Robo are induced. Bashaw et al (2000) showed that both Abl and Ena are able to bind to one or more of the conserved cytoplasmic motifs found within Robo-family members. Genetic interactions suggested that Ena functions to promote Robo repulsion while Abl, possibly through

phosphorylation, acts to reduce Robo repulsion (Bashaw et al., 2000). Dreadlocks (Dock), another established regulator of axon guidance events, also physically interacts with Robo (Fan et al., 2003; Garrity et al., 1996). Previous studies had shown that Dock binds to p21activated kinase (Pak), an evolutionarily conserved regulator of the actin cytoskeleton (Bokoch et al., 1996; Hing et al., 1999; Leeuw et al., 1998; Sells et al., 1997). Upon Slit stimulation, it appears that Dock and Pak are recruited to the Robo receptor, resulting in the apparent stimulation Rac GTPase activity (Fan et al., 2003).

Slit and Robo are also expressed outside of the nervous system, where they have been found to control the movements of cell types as diverse as trachea, muscle, and leukocytes (Englund et al., 2002; Kramer et al., 2001; Wong et al., 2002). *Drosophila* mesodermal and tracheal cell migration is directed by Slit through both repulsive and attractive mechanisms (Englund et al., 2002; Kramer et al., 2001). This suggests that there are conserved mechanisms that control movements in both immune system and nervous system.

PTPMEG

Ptpmeg belongs to a class of highly conserved proteins, that contain an N-terminal FERM domain, an internal PDZ-binding motif, and a C-terminal protein tyrosine phosphatase domain (PTP) (Edwards et al., 2001; Gu et al., 1991; Uchida et al., 2002; Yang and Tonks, 1991). Ptpmeg has been shown to be expressed in the nervous system of both *C. elegans* and vertebrates (Hironaka et al., 2000; Uchida et al., 2002). The vertebrate homologs have also been shown to be expressed in cancer cell lines, immune cells and testis (Gjorloff-Wingren et al., 2000; Sahin et al., 1995). However, there are no published *in vivo* loss-of-function phenotypes in any species. In Chapter 3, I present the work that other members of the lab and I completed on *Drosophila* Ptpmeg. Here, I will briefly introduce two of the domains contained within Ptpmeg that likely regulate the protein localization and binding partners of Ptpmeg.

PDZ domains

PDZ domains were originally identified as conserved elements in the postsynaptic density protein PSD-95/SAP90, the *Drosophila* septate junction protein Discs Large and

the tight junction protein ZO-1 (Mitic and Anderson, 1998; Sheng and Sala, 2001; Woods and Bryant, 1991). PDZ domains commonly function as protein-protein interaction motifs and are found in a diverse set of proteins (Kim and Sheng, 2004). PDZ domains are known to bind to the C-terminal of target proteins and several PDZ domains have been shown to bind the plasma membrane lipid PIP_2 or short internal protein sequences (Sheng and Sala, 2001; Zimmermann et al., 2002).

The wide range of PDZ domain targets include transmembrane receptors, ion channels and other PDZ domain proteins. These interactions suggest that PDZ domain-containing proteins are capable of functioning in a variety of cellular processes. Proteins with multiple PDZ domains can serve as scaffolding proteins, by assembling well-ordered, multi-protein complexes (Sheng and Sala, 2001; Tsunoda et al., 1998). However, Ptpmeg contains a single PDZ domain and is therefore unlikely to serve as a typical scaffolding protein, although it could serve a signaling function within a scaffolding protein to which it is bound.

PDZ domains also exhibit significant sequence variation, which may underlie their ability to bind to a diverse set of ligands. Importantly, each PDZ domain appears to bind a small number of ligands and show a high degree of target sequence specificity (Sheng and Sala, 2001). Subcellular localization of PDZ-containing proteins suggests that they participate in the formation of cell junctions, receptor/channel clustering and intracellular signaling pathways (Kim and Sheng, 2004; Ponting et al., 1997).

FERM domains

Ptpmeg is a member of the FERM superfamily. FERM domains, named for prominent family members 4.1 protein, Ezrin, Radixin, and Moesin, are found in a variety of cytoplasmic proteins that are involved in the control of cell adhesion, cell motility, cell shape and signal transduction (Bretscher et al., 2002). FERM domains participate in localizing proteins to the plasma membrane and have been shown to localize to the cytoplasmic surface of the plasma membrane and bind to PIP_2 (phosphatidylinositol 4,5-bisphosphate) and phosphatidylserine (Chishti et al., 1998). A number of FERM proteins, such as Ezrin, Radixin and Moesin, serve as cytoskeletal-membrane linkers by binding cell surface proteins and connecting them to the cytoskeleton through their actin-

binding domain (Chishti et al., 1998). Unlike many other FERM domain-containing proteins, Ptpmeg does not appear to contain an actin-binding motif.

In addition to binding to the plasma membrane, FERM domains also associate with transmembrane proteins and PDZ domain-containing proteins (Chishti et al., 1998). For example, the FERM domain of the protein Talin, interacts with the cytoplasmic tail of β integrin, a transmembrane protein, and is required for integrin function (Brown et al., 2002; Calderwood et al., 1999; Cram et al., 2003; Hynes, 2002). Although direct protein or plasma membrane interactions of Ptpmeg are unknown, the domains contained within Ptpmeg suggest several mechanisms by which Ptpmeg could be localized to the plasma membrane to regulate phosphotyrosine signaling.

INTRODUCTION TO THE DEVELOPMENTAL PROCESSES

Axon Guidance

Precise neuronal wiring is fundamental to proper nervous system function. During development, axons navigate to their appropriate targets and establish synaptic connections (Goodman and Shatz, 1993; Tessier-Lavigne and Goodman, 1996). Axons must be able to correctly navigate through an extracellular environment of multiple signals and cell types and distinguish their target from an array of potential targets. Growth cones at the tip of extending axons are largely responsible for the decisions made by navigating axons (Bentley and O'Connor, 1994). Growth cones use dynamic, actin-rich structures, filopodia and lamellipodia, to probe the environment. Filopodia and lamellipodia extend and retract in response to directional cues (O'Connor et al., 1990). The growth cone was initially described by Ramon y Cajal (1890), who observed Golgi-stained preparations of embryonic chick spinal cord neurons (Ramon y Cajal, 1893). From these observations he proposed that axons could be attracted by diffusible signals emanating from a target.

It is now clear that both attractive and repulsive forces guide axons to their destination (Guan and Rao, 2003; Mueller, 1999). For example, in the mouse embryonic spinal cord, commissural neurons differentiate near the roof plate in the dorsal region of the spinal cord and extend axons ventrally toward the floor plate where they cross the

midline (Augsburger et al., 1999; Colamarino and Tessier-Lavigne, 1995). The roof plate secretes diffusible factors that repel the commissural axons while the floor plate secretes attractive proteins, such as Netrin, to guide the axons to the ventral midline (Kennedy et al., 1994). The Slit protein is also secreted by the floorplate and guides Robo-expressing axons (Long et al., 2004; Sabatier et al., 2004; Yuan et al., 1999).

Guidance cues can be diffusible, associated with the ECM, or cell-surface bound. They often function as ligands for receptors located on the cell surface of growth cones. It has been shown that an individual cue can be both an attractant and a repellent. For example, *C. elegans* UNC-6, a Netrin homolog, is expressed in ventral regions of the embryonic ectoderm and controls both ventral and dorsal migration of certain axons and cells, suggesting that it can function as both an attractant and a repellent (Culotti and Merz, 1998; Hedgecock et al., 1990; Wadsworth et al., 1996). Netrin-family proteins act through two receptor families, the Deleted in Colorectal Cancer (DCC) and UNC-5 families. In *C. elegans*, the attractive response to UNC-6 requires the DCC homolog, UNC-40. The repulsive responses involve both UNC-40 and the UNC-5 receptor, suggesting that the combination of receptors determines the nature of the response (Kim et al., 1999). Additional work on the *Drosophila* homolog of DCC (Frazzled) and UNC-5 homolog suggests that Frazzled is required attractive as well as long-range repulsive effects and that UNC-5 is required for short-range and long-range repulsive effects (Keleman and Dickson, 2001; Kolodziej et al., 1996). These examples serve to illustrate that the same cues and receptors can be used to achieve numerous effects.

The response of the growth cone to a given cue can also be affected by the presence of other guidance cues. In *Xenopus* spinal neuron cultures, axons will migrate toward a pipette dispensing Netrin (Ming et al., 1997; Song et al., 1997). However, when the ECM component laminin is added to the substrate, axons will steer away from the Netrin-1 source. cAMP levels in the growth cone normally increase upon Netrin-1 exposure in this system. However, laminin alters signaling by lowering cAMP levels in the growth cone. If cAMP levels are artificially elevated in the presence of laminin, Netrin-1 once again becomes attractive (Hopker et al., 1999).

In developing organisms, axons are guided by a combination of attractive and repulsive signals that, in effect, work to push, pull, and hem in growing axons (Guan and

Rao, 2003; Mueller, 1999). These forces, acting together, guide axons to their appropriate targets. Dynamic remodeling of the cytoskeleton is the driving force behind growth cone motility. Actin filament assembly and disassembly along with retrograde flow of F-actin appear to control the rapid protrusions and retractions of the highly motile growth cone (Lin and Forscher, 1995). Actin filaments predominate at the leading edge of the growth cone in the lamellipodia and filopodia. Microtubules serve as the major structural component of the axon body, but occasionally enter the leading edge (Dent et al., 1999).

The Rho-family of small GTPases, including Rho, Rac, Cdc42 and others, are important for axon guidance (Guan and Rao, 2003). GTPases cycle between an active GTP-bound state and an inactive GDP-bound state. Transitions between the two states are influenced by guanine nucleotide exchange factors (GEFs), dissociation inhibitors (GDIs), and activating proteins (GAPs) (Kozma et al., 1997). In fibroblast injection studies it was shown that activated Rho GTPase resulted in the assembly of stress fibers and focal adhesion complexes, while activated Rac and Cdc42 appeared to stimulate formation of lamellipodia and filopodia, respectively (Hall, 1998). GTPase interactors include proteins that are involved in regulating actin dynamics. Several of these proteins are characterized regulators of axon guidance, including UNC-73/Trio (a Rac and Rho GEF) (Awasaki et al., 2000; Debant et al., 1996), p21-activated serine/threonine kinase (Pak) (Hing et al., 1999) and the cell adhesion molecule N-WASP (Higgs and Pollard, 1999).

Compartments and Boundaries

The ability to keep discrete cell populations from mixing is critical for proper animal development. One commonly used strategy to maintain the separation of adjacent cell populations is compartmentalization (Herrup and Kuemerle, 1997; Irvine and Rauskolb, 2001). Compartments are groups of adjacent but non-intermingling cells that often arise from distinct progenitors (Garcia-Bellido et al., 1973; Irvine and Rauskolb, 2001). A prevailing model is that cells from different compartments have distinct adhesive properties that prevent them from intermingling across defined boundary regions and that differential adhesion is conferred through a developmental program controlled by

transcriptional regulators and signaling pathways (Irvine and Rauskolb, 2001; Lumsden, 2004).

The landmark studies of Garcia-Bellido in the early 1970s firmly established the concept of compartmentalization. Examination of mosaic animals revealed distinct compartments in the *Drosophila* wing that were not marked by any visible morphological boundary (Garcia-Bellido et al., 1973). Clonal analysis experiments revealed that cells on either side of the boundary were restricted by their lineage. For example, along the anterior-posterior (A/P) boundary, clonal populations would stay within the same anterior or posterior region and if they approached the adjacent region they would spread out, forming a smooth line, but never cross the other side. Regions on either side of the A/P boundary were termed "compartments" (Garcia-Bellido et al., 1973; Garcia-Bellido et al., 1976). Distinct "cell affinities" were proposed to keep adjacent cell compartments separate (Garcia-Bellido, 1975).

The A/P boundaries of the *Drosophila* wing are established by *engrailed* gene expression (Lawrence and Morata, 1976). Posterior cells express Engrailed which directs expression of Hedgehog (Hh) and prevents expression of Cubiutus interruptus (Ci), a component of Hedgehog signal transduction (Basler and Struhl, 1994). Anterior cells do not express *engrailed*, thus only anterior cells of the wing respond to the Hedgehog signal, permitting the expression of Hh target genes such as *patched* (a Hedgehog receptor) and *dpp* (a BMP homolog) (Chen and Struhl, 1996). As a result of regulation from both anterior and posterior cells, *dpp* expression is confined to the A/P boundary (Dahmann and Basler, 2000). It is hypothesized that these and other signaling pathways are then used to generate distinct compartments and confer differential cell adhesion properties to adjacent compartments by regulating the expression of cell adhesion molecules, ultimately through the regulation of transcription factors (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001).

As mentioned above, Hedgehog signaling plays an important role in A/P boundary formation in the *Drosophila* wing. The Notch signaling pathway has also been shown to be important in this process. Notch signaling is involved in many developmental processes, including cell specification and lateral inhibition of neurogenesis (Artavanis-Tsakonas et al., 1999; Kimble and Simpson, 1997). Notch is

also important in the formation of compartment boundaries and specialized boundary cells (Irvine, 1999; Tepass et al., 2002). The restriction of membrane-bound ligands Delta and Serrate and the ligand affinity factor Fringe to distinct cell populations increase Notch signaling specificity (Bruckner et al., 2000; Moloney et al., 2000; Munro and Freeman, 2000; Panin et al., 1997). Consistent with previous studies that implicated Notch signaling as an important regulator of boundary formation, Lunatic fringe (L-fng) was found to be expressed at compartment borders and ectopic expression disrupted border formation in the chick brain (Zeltser et al., 2001).

In some cases, specialized boundary cells are formed between adjacent compartments. It has been postulated that these cells promote the refinement of borders and act as a specialized signaling center that affect further patterning events (Irvine and Rauskolb, 2001). For example, border cells along the D/V compartments in the *Drosophila* wing produce specialized bristle cells (de Celis and Garcia-Bellido, 1994; Kim et al., 1995; Rulifson et al., 1996). The establishment of these specialized cells along the D/V border requires Notch signaling. In the vertebrate midbrain-hidbrain junction, cells along the border help direct further cell specification events (Liu and Joyner, 2001).

The most studied and best understood vertebrate compartments are the rhombomeres of the developing hindbrain. As in the *Drosophila* wing, the partitioning of rhombomeres into compartments is hypothesized to be driven, in part, by differential adhesion that arises from differential expression of regulatory genes. (Dahmann and Basler, 1999; Fraser et al., 1990; Lumsden, 2004). In the vertebrate hindbrain the neuroepitelium is subdivided along its A/P axis. After neural tube closure, visible bulges in the presumptive hindbrain begin to form. As rhombomere boundaries begin to form, the movement of cells within a delineated rhombomere is restricted (Fraser et al., 1990). Rhombomeres are arranged in a segmentally repeating pattern and distinct expression domains are generated in an alternating pattern (Guthrie and Lumsden, 1991; Wizenmann and Lumsden, 1997b). For example, even-numbered rhombomers (r2, r4, r6) express the transmembrane ligand ephrin-B. Odd-numbered rhombomers (r3 and r5) express three Eph receptors: EphA4, EphB2, EphB3. Eph receptor tyrosine kinases and their ligands, Ephrins, are cell-cell signaling molecules that are key regulators of attractive and

repulsive cell migration events and cell adhesion (Poliakov et al., 2004). Interactions between this ligand-receptor pair appear to prevent cell mixing and sharpen compartment boundaries, implicating repulsive signaling in the process of compartmentalization (Gilardi-Hebenstreit et al., 1992; Nieto et al., 1992; O'Leary and Wilkinson, 1999).

A number of transcription factors have been shown to be crucial for hindbrain compartment formation and their downstream control of genes such as Eph receptors and ephrins is assumed to be important for the establishment of compartments and maintenance of compartment boundaries (Lumsden, 2004). The zinc-finger transcription factor Krox20 is expressed in two stripes of the neural plate before neurulation. These regions later become rhombomeres r3 and r5 (Wilkinson et al., 1989). In Krox20 mutants, r3 and r5 are absent, resulting in partial fusion of the even-numbered rhombomeres r2, r4 and r6. Importantly, Krox20 was found to directly regulate EphA4 expression (Theil et al., 1998). These studies established Krox20 as one of the major upstream regulators of rhombomere development. Another transcription factor discovered to be important in compartment formation in the hindbrain is Kreisler, a leucine zipper containing protein. Kreisler is expressed in r5 and r6 (Manzanares et al., 1999; Moens et al., 1998), both of which are missing in the mutant animal (McKay et al., 1994). The Hox family of homeobox genes influence the early steps of brain patterning in flies and vertebrates (Hirth et al., 1998). Hox gene expression occurs early in development, before the rhombomeres have begun to form. Rhombomere identity appears to be controlled through the combined expression of a number of Hox genes (Keynes and Krumlauf, 1994; Krumlauf, 1994). It has been suggested that one role of compartments is to stably maintain homeotic gene expression within a given boundary (Struhl, 1984). Importantly, Krox-20 controls the expression of a number of Hox genes (Nonchev et al., 1996).

As mentioned above, Eph receptors and their Ephrin ligands have been implicated in the cell-cell signaling processes that restricts intermingling (Mellitzer et al., 1999; Xu et al., 1999). They have been shown to mediate repulsive and attractive interactions in axon guidance and cell migration (Wilkinson, 2001). In cell culture they were shown to restrict cell intermingling (Mellitzer et al., 1999) and expression of a presumed dominant-negative form of EphA4 and Ephrin-B disrupted boundary formation (Xu et al., 1999).

Although these studies are consistent with the idea that Eph/Ephrin signaling restricts cell mixing, loss-of-function mutations in genes encoding Ephrins and Eph receptors have not revealed compartment boundary disruptions (Adams et al., 2001; Chen et al., 1996; Helmbacher et al., 2000).

Differential cell adhesion likely contributes to many of the complex morphogenetic movements that underlie nervous system development. Classical cell aggregation experiments showed that when cells of the ectoderm and neural tube were dissociated from amphibian embryos and then mixed together, two populations of cells consequently separated over time, forming distinct aggregates (Townes, 1955). This implied that cells had adhesive properties located on their surfaces that permitted associations with cells having the same adhesive properties and excluded associations with cells that had different adhesive properties. Differential chemoaffinity properties of the developing hindbrain have been tested by mixing cells of dissociated rhombomeres. Cell from even-numbered rhombomeres and odd-numbered rhombomeres do not mix and only form aggregates with cells from the same rhombomere. The segregation was abolished when Ca^{2+} was removed, suggesting that Ca^{2+}-depedent cell adhesion molecules, such as the cadherins, would be required for rhombomere development (Wizenmann and Lumsden, 1997a).

Cadherins are a class of cell surface glycoproteins that play an important role in tissue morphogenesis and cell-cell adhesion (Gumbiner et al., 1988; Takeichi, 1988). Cells expressing cadherin proteins are able to bind to other cells expressing the same cadherin. Homotypic binding is mediated through the extracellular domain and the presence of calcium is required for adhesion (Nakagawa et al., 1998; Redies, 2000). There are multiple cadherin proteins, therefore combinatorial expression of different cadherin proteins can potentially generate numerous variations of adhesive specificity (Redies, 2000). A family of cytoplasmic proteins, the catenins, interacts with the intracellular domain of the cadherins. This association links the cadherins to the actin cytoskeleton (Gooding et al., 2004). Cadherins are often restricted to discrete domains and segments of the developing nervous system (Redies, 2000). For example, in the developing mouse brain, cadherin-6 is expressed in the lateral ganglionic eminence of the

telencephalon (Inoue et al., 1997; Matsunami and Takeichi, 1995). However *cadherin-6* knockout mice showed no telencephalic compartment phenotypes (Inoue et al., 2001).

Although members of both the cadherin family and Eph/Ephrin family have been implicated as molecules that restrict cell mixing across compartment boundaries within the developing brain, loss-of-function analysis has not yet demonstrated the precise *in vivo* role that these proteins play in compartmentalization (Cooke and Moens, 2002; Inoue et al., 2001).

Concluding Remarks

In my work, I have sought to identify and characterize molecules that are required for nervous system development. Using the developing visual system of *Drosophila* as a model, I uncovered a role for the secreted guidance cue Slit and the Robo-family receptors in visual system morphogenesis. These molecules work together to promote compartmentalization of the visual centers in the *Drosophila* brain. My work provides insight into the process of compartmentalization by identifying Slit and Robo-family proteins as molecules that restrict cell mixing between compartments in the brain. It will be interesting to see if the signaling pathways that are used by Slit and Robo-family proteins in the processes of cell migration and axon guidance are also used to control compartmentalization. It will also be of interest to determine whether other molecules that are known to guide migrating cells and neurites also participate in compartmentalization within the brain and other developing tissues.

Precise neuronal wiring is fundamental to proper nervous system function. During development, axons and dendrites navigate to their appropriate targets and establish synaptic connections. A growing list of cues and receptors has been identified to be required for the formation of neuronal connections, although the intracellular signaling pathways that link cell surface detection of cues to directed rearrangements of the cytoskeleton remain less clear. Unlike Slit and Robo-family proteins, very little is known about the *in vivo* function of the protein tyrosine phosphatase Ptpmeg. The protein domains and protein localization patterns suggest that Ptpmeg could serve as a regulator of nervous system development and my initial characterization of *Ptpmeg* mutants likely indicates that this protein plays a role in axon branching in the mushroom

bodies of the *Drosophila* brain. Ptpmeg is broadly expressed in neurons and it will be interesting to determine whether Ptpmeg plays additional roles in nervous system development. Additional work will be needed to identify Ptpmeg binding partners and substrates and further characterization of Ptpmeg could shed light on how phosphotyrosine signaling is used to control aspects of neural development, such as axon guidance and branching.

References

Adams, R. H., Diella, F., Hennig, S., Helmbacher, F., Deutsch, U. and Klein, R. (2001). The cytoplasmic domain of the ligand ephrinB2 is required for vascular morphogenesis but not cranial neural crest migration. *Cell* **104**, 57-69.

Artavanis-Tsakonas, S., Rand, M. D. and Lake, R. J. (1999). Notch signaling: cell fate control and signal integration in development. *Science* **284**, 770-6.

Augsburger, A., Schuchardt, A., Hoskins, S., Dodd, J. and Butler, S. (1999). BMPs as mediators of roof plate repulsion of commissural neurons. *Neuron* **24**, 127-41.

Awasaki, T., Saito, M., Sone, M., Suzuki, E., Sakai, R., Ito, K. and Hama, C. (2000). The Drosophila trio plays an essential role in patterning of axons by regulating their directional extension. *Neuron* **26**, 119-31.

Bashaw, G. J., Kidd, T., Murray, D., Pawson, T. and Goodman, C. S. (2000). Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. *Cell* **101**, 703-15.

Basler, K. and Struhl, G. (1994). Compartment boundaries and the control of Drosophila limb pattern by hedgehog protein. *Nature* **368**, 208-14.

Battye, R., Stevens, A., Perry, R. L. and Jacobs, J. R. (2001). Repellent signaling by Slit requires the leucine-rich repeats. *J Neurosci* **21**, 4290-8.

Bausenwein, B., Dittrich, A. P. and Fischbach, K. F. (1992). The optic lobe of Drosophila melanogaster. II. Sorting of retinotopic pathways in the medulla. *Cell Tissue Res* **267**, 17-28.

Becker, H. J. (1957). [Roentgen mosaic spots & defective mutations at the eye of Drosophila & the evolutive physiology of the eye.]. *Z Indukt Abstamm Vererbungsl* **88**, 333-73.

Bentley, D. and O'Connor, T. P. (1994). Cytoskeletal events in growth cone steering. *Curr Opin Neurobiol* **4**, 43-8.

Bokoch, G. M., Wang, Y., Bohl, B. P., Sells, M. A., Quilliam, L. A. and Knaus, U. G. (1996). Interaction of the Nck adapter protein with p21-activated kinase (PAK1). *J Biol Chem* **271**, 25746-9.

Bretscher, A., Edwards, K. and Fehon, R. G. (2002). ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol* **3**, 586-99.

Brose, K., Bland, K. S., Wang, K. H., Arnott, D., Henzel, W., Goodman, C. S., Tessier-Lavigne, M. and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* **96**, 795-806.

Brown, N. H., Gregory, S. L., Rickoll, W. L., Fessler, L. I., Prout, M., White, R. A. and Fristrom, J. W. (2002). Talin is essential for integrin function in Drosophila. *Dev Cell* **3**, 569-79.

Bruckner, K., Perez, L., Clausen, H. and Cohen, S. (2000). Glycosyltransferase activity of Fringe modulates Notch-Delta interactions. *Nature* **406**, 411-5.

Calderwood, D. A., Zent, R., Grant, R., Rees, D. J., Hynes, R. O. and Ginsberg, M. H. (1999). The Talin head domain binds to integrin beta subunit cytoplasmic tails and regulates integrin activation. *J Biol Chem* **274**, 28071-4.

Campos-Ortega, J. A. (1988). Cellular interactions during early neurogenesis of Drosophila melanogaster. *Trends Neurosci* **11**, 400-5.

Chen, J., Nachabah, A., Scherer, C., Ganju, P., Reith, A., Bronson, R. and Ruley, H. E. (1996). Germ-line inactivation of the murine Eck receptor tyrosine kinase by gene trap retroviral insertion. *Oncogene* **12**, 979-88.

Chen, J. H., Wen, L., Dupuis, S., Wu, J. Y. and Rao, Y. (2001). The N-terminal leucine-rich regions in Slit are sufficient to repel olfactory bulb axons and subventricular zone neurons. *J Neurosci* **21**, 1548-56.

Chen, Y. and Struhl, G. (1996). Dual roles for patched in sequestering and transducing Hedgehog. *Cell* **87**, 553-63.

Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N. et al. (1998). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends Biochem Sci* **23**, 281-2.

Clandinin, T. R. and Zipursky, S. L. (2002). Making connections in the fly visual system. *Neuron* **35**, 827-41.

Colamarino, S. A. and Tessier-Lavigne, M. (1995). The role of the floor plate in axon guidance. *Annu Rev Neurosci* **18**, 497-529.

Cooke, J. E. and Moens, C. B. (2002). Boundary formation in the hindbrain: Eph only it were simple. *Trends Neurosci* **25**, 260-7.

Cram, E. J., Clark, S. G. and Schwarzbauer, J. E. (2003). Talin loss-of-function uncovers roles in cell contractility and migration in C. elegans. *J Cell Sci* **116**, 3871-8.

Crews, S. T., Thomas, J. B. and Goodman, C. S. (1988). The Drosophila single-minded gene encodes a nuclear protein with sequence similarity to the per gene product. *Cell* **52**, 143-51.

Culotti, J. G. and Merz, D. C. (1998). DCC and netrins. *Curr Opin Cell Biol* **10**, 609-13.

Dahmann, C. and Basler, K. (1999). Compartment boundaries: at the edge of development. *Trends Genet* **15**, 320-6.

Dahmann, C. and Basler, K. (2000). Opposing transcriptional outputs of Hedgehog signaling and engrailed control compartmental cell sorting at the Drosophila A/P boundary. *Cell* **100**, 411-22.

de Celis, J. F. and Garcia-Bellido, A. (1994). Roles of the Notch gene in Drosophila wing morphogenesis. *Mech Dev* **46**, 109-22.

Debant, A., Serra-Pages, C., Seipel, K., O'Brien, S., Tang, M., Park, S. H. and Streuli, M. (1996). The multidomain protein Trio binds the LAR transmembrane tyrosine phosphatase, contains a protein kinase domain, and has separate rac-specific and rho-specific guanine nucleotide exchange factor domains. *Proc Natl Acad Sci U S A* **93**, 5466-71.

Dent, E. W., Callaway, J. L., Szebenyi, G., Baas, P. W. and Kalil, K. (1999). Reorganization and movement of microtubules in axonal growth cones and developing interstitial branches. *J Neurosci* **19**, 8894-908.

Dickson, B. and Hafen, E. (1993). Genetic dissection of eye development in *Drosophila.* Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Dittrich, A. P. and Fischbach, K. F. (1989). The optic lobe of Drosophila melanogaster. Part I. A Golgi analysis of wild-type structure. *Cell Tissue Res* **258**, 441-475.

Dudai, Y., Jan, Y. N., Byers, D., Quinn, W. G. and Benzer, S. (1976). dunce, a mutant of Drosophila deficient in learning. *Proc Natl Acad Sci U S A* **73**, 1684-8.

Dujardin, F. (1850). Memoire sur le systeme nerveux des insectes. *Ann. Sci. Nat. Zool.* **14**, 195-206.

Edwards, K., Davis, T., Marcey, D., Kurihara, J. and Yamamoto, D. (2001). Comparative analysis of the Band 4.1/ezrin-related protein tyrosine phosphatase Pez from two Drosophila species: implications for structure and function. *Gene* **275**, 195-205.

Englund, C., Steneberg, P., Falileeva, L., Xylourgidis, N. and Samakovlis, C. (2002). Attractive and repulsive functions of Slit are mediated by different receptors in the Drosophila trachea. *Development* **129**, 4941-51.

Fan, X., Labrador, J. P., Hing, H. and Bashaw, G. J. (2003). Slit stimulation recruits Dock and Pak to the roundabout receptor and increases Rac activity to regulate axon repulsion at the CNS midline. *Neuron* **40**, 113-27.

Fischbach, K. F. and Heisenberg, M. (1981). Structural brain mutant of Drosophila melanogaster with reduced cell number in the medulla cortex and with normal yaw optomoter response. *Proc Natl Acad Sci U S A* **78**, 1105-1109.

Fraser, S., Keynes, R. and Lumsden, A. (1990). Segmentation in the chick embryo hindbrain is defined by cell lineage restrictions. *Nature* **344**, 431-5.

Garcia-Bellido, A. (1975). Genetic control of wing disc development in Drosophila. *Ciba Found Symp* **0**, 161-82.

Garcia-Bellido, A. and Merriam, J. R. (1969). Cell lineage of the imaginal discs in Drosophila gynandromorphs. *J Exp Zool* **170**, 61-75.

Garcia-Bellido, A., Ripoll, P. and Morata, G. (1973). Developmental compartmentalisation of the wing disk of Drosophila. *Nat New Biol* **245**, 251-3.

Garcia-Bellido, A., Ripoll, P. and Morata, G. (1976). Developmental compartmentalization in the dorsal mesothoracic disc of Drosophila. *Dev Biol* **48**, 132-47.

Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T. and Zipursky, S. L. (1996). Drosophila photoreceptor axon guidance and targeting requires the dreadlocks SH2/SH3 adapter protein. *Cell* **85**, 639-50.

Georgiou, M. and Tear, G. (2003). The N-terminal and transmembrane domains of Commissureless are necessary for its function and trafficking within neurons. *Mech Dev* **120**, 1009-19.

Gilardi-Hebenstreit, P., Nieto, M. A., Frain, M., Mattei, M. G., Chestier, A., Wilkinson, D. G. and Charnay, P. (1992). An Eph-related receptor protein tyrosine kinase gene segmentally expressed in the developing mouse hindbrain. *Oncogene* **7**, 2499-506.

Gjorloff-Wingren, A., Saxena, M., Han, S., Wang, X., Alonso, A., Renedo, M., Oh, P., Williams, S., Schnitzer, J. and Mustelin, T. (2000). Subcellular localization of intracellular protein tyrosine phosphatases in T cells. *Eur J Immunol* **30**, 2412-21.

Gooding, J. M., Yap, K. L. and Ikura, M. (2004). The cadherin-catenin complex as a focal point of cell adhesion and signalling: new insights from three-dimensional structures. *Bioessays* **26**, 497-511.

Goodman, C. S. and Shatz, C. J. (1993). Developmental mechanisms that generate precise patterns of neuronal connectivity. *Cell* **72 Suppl**, 77-98.

Goodman, C. S. a. D., C. Q. (1993). Embryonic development of the *Drosophila* central nervous system. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Gu, M. X., York, J. D., Warshawsky, I. and Majerus, P. W. (1991). Identification, cloning, and expression of a cytosolic megakaryocyte protein-tyrosine-phosphatase with sequence homology to cytoskeletal protein 4.1. *Proc Natl Acad Sci U S A* **88**, 5867-71.

Guan, K. L. and Rao, Y. (2003). Signalling mechanisms mediating neuronal responses to guidance cues. *Nat Rev Neurosci* **4**, 941-56.

Guan, Z., Prado, A., Melzig, J., Heisenberg, M., Nash, H. A. and Raabe, T. (2000). Mushroom body defect, a gene involved in the control of neuroblast proliferation in Drosophila, encodes a coiled-coil protein. *Proc Natl Acad Sci U S A* **97**, 8122-7.

Gumbiner, B., Stevenson, B. and Grimaldi, A. (1988). The role of the cell adhesion molecule uvomorulin in the formation and maintenance of the epithelial junctional complex. *J Cell Biol* **107**, 1575-87.

Guthrie, S. and Lumsden, A. (1991). Formation and regeneration of rhombomere boundaries in the developing chick hindbrain. *Development* **112**, 221-9.

Hall, A. (1998). Rho GTPases and the actin cytoskeleton. *Science* **279**, 509-14.

Hardie, R. C. (1985). Functional organization of the fly retina. Berlin: Springer.

Heberlein, U., Wolff, T. and Rubin, G. M. (1993). The TGF beta homolog dpp and the segment polarity gene hedgehog are required for propagation of a morphogenetic wave in the Drosophila retina. *Cell* **75**, 913-26.

Hedgecock, E. M., Culotti, J. G. and Hall, D. H. (1990). The unc-5, unc-6, and unc-40 genes guide circumferential migrations of pioneer axons and mesodermal cells on the epidermis in C. elegans. *Neuron* **4**, 61-85.

Heisenberg, M. (1980). Mutants of brain structure and function: what is the significance of the mushroom bodies for behavior? *Basic Life Sci* **16**, 373-90.

Helmbacher, F., Schneider-Maunoury, S., Topilko, P., Tiret, L. and Charnay, P. (2000). Targeting of the EphA4 tyrosine kinase receptor affects dorsal/ventral pathfinding of limb motor axons. *Development* **127**, 3313-24.

Herrup, K. and Kuemerle, B. (1997). The compartmentalization of the cerebellum. *Annu Rev Neurosci* **20**, 61-90.

Higgs, H. N. and Pollard, T. D. (1999). Regulation of actin polymerization by Arp2/3 complex and WASp/Scar proteins. *J Biol Chem* **274**, 32531-4.

Hing, H., Xiao, J., Harden, N., Lim, L. and Zipursky, S. L. (1999). Pak functions downstream of Dock to regulate photoreceptor axon guidance in Drosophila. *Cell* **97**, 853-63.

Hirata, J., Nakagoshi, H., Nabeshima, Y. and Matsuzaki, F. (1995). Asymmetric segregation of the homeodomain protein Prospero during Drosophila development. *Nature* **377**, 627-30.

Hironaka, K., Umemori, H., Tezuka, T., Mishina, M. and Yamamoto, T. (2000). The protein-tyrosine phosphatase PTPMEG interacts with glutamate receptor delta 2 and epsilon subunits. *J Biol Chem* **275**, 16167-73.

Hirth, F., Hartmann, B. and Reichert, H. (1998). Homeotic gene action in embryonic brain development of Drosophila. *Development* **125**, 1579-89.

Hofbauer, A. and Campos-Ortega, J. A. (1990). Proliferation pattern and early differentiation of the optic lobes in Drosophila melanogaster. *Roux's Arch Dev Biol* **198**, 264-274.

Hopker, V. H., Shewan, D., Tessier-Lavigne, M., Poo, M. and Holt, C. (1999). Growth-cone attraction to netrin-1 is converted to repulsion by laminin-1. *Nature* **401**, 69-73.

Huang, Z. and Kunes, S. (1996). Hedgehog, transmitted along retinal axons, triggers neurogenesis in the developing visual centers of the Drosophila brain. *Cell* **86**, 411-22.

Huang, Z. and Kunes, S. (1998). Signals transmitted along retinal axons in Drosophila: Hedgehog signal reception and the cell circuitry of lamina cartridge assembly. *Development* **125**, 3753-64.

Huang, Z., Shilo, B. Z. and Kunes, S. (1998). A retinal axon fascicle uses spitz, an EGF receptor ligand, to construct a synaptic cartridge in the brain of Drosophila. *Cell* **95**, 693-703.

Hynes, R. O. (2002). Integrins: bidirectional, allosteric signaling machines. *Cell* **110**, 673-87.

Inoue, T., Chisaka, O., Matsunami, H. and Takeichi, M. (1997). Cadherin-6 expression transiently delineates specific rhombomeres, other neural tube subdivisions, and neural crest subpopulations in mouse embryos. *Dev Biol* **183**, 183-94.

Inoue, T., Tanaka, T., Takeichi, M., Chisaka, O., Nakamura, S. and Osumi, N. (2001). Role of cadherins in maintaining the compartment boundary between the cortex and striatum during development. *Development* **128**, 561-9.

Irvine, K. D. (1999). Fringe, Notch, and making developmental boundaries. *Curr Opin Genet Dev* **9**, 434-41.

Irvine, K. D. and Rauskolb, C. (2001). Boundaries in development: formation and function. *Annu Rev Cell Dev Biol* **17**, 189-214.

Isshiki, T., Pearson, B., Holbrook, S. and Doe, C. Q. (2001). Drosophila neuroblasts sequentially express transcription factors which specify the temporal identity of their neuronal progeny. *Cell* **106**, 511-21.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y. and Yamamoto, D. (1997). The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* **124**, 761-71.

Ito, K. and Hotta, Y. (1992). Proliferation pattern of postembryonic neuroblasts in the brain of Drosophila melanogaster. *Dev Biol* **149**, 134-48.

Jan, Y. N. and Jan, L. Y. (1998). Asymmetric cell division. *Nature* **392**, 775-8.

Jan, Y. N. and Jan, L. Y. (1999). Asymmetry across species. *Nat Cell Biol* **1**, E42-4.

Kandel, S. J., and Jessel TM. (2000). Principles of Neural Science. New York: McGraw Hill.

Keleman, K. and Dickson, B. J. (2001). Short- and long-range repulsion by the Drosophila Unc5 netrin receptor. *Neuron* **32**, 605-17.

Keleman, K., Rajagopalan, S., Cleppien, D., Teis, D., Paiha, K., Huber, L. A., Technau, G. M. and Dickson, B. J. (2002). Comm sorts robo to control axon guidance at the Drosophila midline. *Cell* **110**, 415-27.

Kennedy, T. E., Serafini, T., de la Torre, J. R. and Tessier-Lavigne, M. (1994). Netrins are diffusible chemotropic factors for commissural axons in the embryonic spinal cord. *Cell* **78**, 425-35.

Keynes, R. and Krumlauf, R. (1994). Hox genes and regionalization of the nervous system. *Annu Rev Neurosci* **17**, 109-32.

Kidd, T., Bland, K. S. and Goodman, C. S. (1999). Slit is the midline repellent for the robo receptor in Drosophila. *Cell* **96**, 785-94.

Kidd, T., Brose, K., Mitchell, K. J., Fetter, R. D., Tessier-Lavigne, M., Goodman, C. S. and Tear, G. (1998a). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* **92**, 205-15.

Kidd, T., Russell, C., Goodman, C. S. and Tear, G. (1998b). Dosage-sensitive and complementary functions of roundabout and commissureless control axon crossing of the CNS midline. *Neuron* **20**, 25-33.

Kim, E. and Sheng, M. (2004). PDZ domain proteins of synapses. *Nat Rev Neurosci* **5**, 771-81.

Kim, J., Irvine, K. D. and Carroll, S. B. (1995). Cell recognition, signal induction, and symmetrical gene activation at the dorsal-ventral boundary of the developing Drosophila wing. *Cell* **82**, 795-802.

Kim, S., Ren, X. C., Fox, E. and Wadsworth, W. G. (1999). SDQR migrations in Caenorhabditis elegans are controlled by multiple guidance cues and changing responses to netrin UNC-6. *Development* **126**, 3881-90.

Kimble, J. and Simpson, P. (1997). The LIN-12/Notch signaling pathway and its regulation. *Annu Rev Cell Dev Biol* **13**, 333-61.

Knoblich, J. A., Jan, L. Y. and Jan, Y. N. (1995). Asymmetric segregation of Numb and Prospero during cell division. *Nature* **377**, 624-7.

Kolodziej, P. A., Timpe, L. C., Mitchell, K. J., Fried, S. R., Goodman, C. S., Jan, L. Y. and Jan, Y. N. (1996). frazzled encodes a Drosophila member of the DCC immunoglobulin subfamily and is required for CNS and motor axon guidance. *Cell* **87**, 197-204.

Kozma, R., Sarner, S., Ahmed, S. and Lim, L. (1997). Rho family GTPases and neuronal growth cone remodelling: relationship between increased complexity induced by Cdc42Hs, Rac1, and acetylcholine and collapse induced by RhoA and lysophosphatidic acid. *Mol Cell Biol* **17**, 1201-11.

Kramer, S. G., Kidd, T., Simpson, J. H. and Goodman, C. S. (2001). Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration. *Science* **292**, 737-40.

Krumlauf, R. (1994). Hox genes in vertebrate development. *Cell* **78**, 191-201.

Lawrence, P. A. and Morata, G. (1976). Compartments in the wing of Drosophila: a study of the engrailed gene. *Dev Biol* **50**, 321-37.

Lee, T., Lee, A. and Luo, L. (1999). Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development* **126**, 4065-76.

Leeuw, T., Wu, C., Schrag, J. D., Whiteway, M., Thomas, D. Y. and Leberer, E. (1998). Interaction of a G-protein beta-subunit with a conserved sequence in Ste20/PAK family protein kinases. *Nature* **391**, 191-5.

Li, H. S., Chen, J. H., Wu, W., Fagaly, T., Zhou, L., Yuan, W., Dupuis, S., Jiang, Z. H., Nash, W., Gick, C. et al. (1999). Vertebrate slit, a secreted ligand for the transmembrane protein roundabout, is a repellent for olfactory bulb axons. *Cell* **96**, 807-18.

Lin, C. H. and Forscher, P. (1995). Growth cone advance is inversely proportional to retrograde F-actin flow. *Neuron* **14**, 763-71.

Liu, A. and Joyner, A. L. (2001). Early anterior/posterior patterning of the midbrain and cerebellum. *Annu Rev Neurosci* **24**, 869-96.

Livingstone, M. S., Sziber, P. P. and Quinn, W. G. (1984). Loss of calcium/calmodulin responsiveness in adenylate cyclase of rutabaga, a Drosophila learning mutant. *Cell* **37**, 205-15.

Long, H., Sabatier, C., Ma, L., Plump, A., Yuan, W., Ornitz, D. M., Tamada, A., Murakami, F., Goodman, C. S. and Tessier-Lavigne, M. (2004). Conserved roles for Slit and Robo proteins in midline commissural axon guidance. *Neuron* **42**, 213-23.

Lu, B., Jan, L. and Jan, Y. N. (2000). Control of cell divisions in the nervous system: symmetry and asymmetry. *Annu Rev Neurosci* **23**, 531-56.

Lum, L. and Beachy, P. A. (2004). The Hedgehog response network: sensors, switches, and routers. *Science* **304**, 1755-9.

Lumsden, A. (2004). Segmentation and compartition in the early avian hindbrain. *Mech Dev* **121**, 1081-8.

Ma, C., Zhou, Y., Beachy, P. A. and Moses, K. (1993). The segment polarity gene hedgehog is required for progression of the morphogenetic furrow in the developing Drosophila eye. *Cell* **75**, 927-38.

Manzanares, M., Trainor, P. A., Nonchev, S., Ariza-McNaughton, L., Brodie, J., Gould, A., Marshall, H., Morrison, A., Kwan, C. T., Sham, M. H. et al. (1999). The role of kreisler in segmentation during hindbrain development. *Dev Biol* **211**, 220-37.

Matsunami, H. and Takeichi, M. (1995). Fetal brain subdivisions defined by R- and E-cadherin expressions: evidence for the role of cadherin activity in region-specific, cell-cell adhesion. *Dev Biol* **172**, 466-78.

McKay, I. J., Muchamore, I., Krumlauf, R., Maden, M., Lumsden, A. and Lewis, J. (1994). The kreisler mouse: a hindbrain segmentation mutant that lacks two rhombomeres. *Development* **120**, 2199-211.

Meinertzhagen, I. A. a. H. T. E. (1993). The development of the optic lobe. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Mellitzer, G., Xu, Q. and Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.

Melzig, J., Rein, K. H., Schafer, U., Pfister, H., Jackle, H., Heisenberg, M. and Raabe, T. (1998). A protein related to p21-activated kinase (PAK) that is involved in neurogenesis in the Drosophila adult central nervous system. *Curr Biol* **8**, 1223-6.

Ming, G. L., Song, H. J., Berninger, B., Holt, C. E., Tessier-Lavigne, M. and Poo, M. M. (1997). cAMP-dependent growth cone guidance by netrin-1. *Neuron* **19**, 1225-35.

Mitic, L. L. and Anderson, J. M. (1998). Molecular architecture of tight junctions. *Annu Rev Physiol* **60**, 121-42.

Mizunami, M., Weibrecht, J. M., and Strausfeld, N. J. (1993). A new role for the insect mushroom body: Place memory and motor control. Cambridge, MA: Academic Press.

Moens, C. B., Cordes, S. P., Giorgianni, M. W., Barsh, G. S. and Kimmel, C. B. (1998). Equivalence in the genetic control of hindbrain segmentation in fish and mouse. *Development* **125**, 381-91.

Moloney, D. J., Panin, V. M., Johnston, S. H., Chen, J., Shao, L., Wilson, R., Wang, Y., Stanley, P., Irvine, K. D., Haltiwanger, R. S. et al. (2000). Fringe is a glycosyltransferase that modifies Notch. *Nature* **406**, 369-75.

Mueller, B. K. (1999). Growth cone guidance: first steps towards a deeper understanding. *Annu Rev Neurosci* **22**, 351-88.

Munro, S. and Freeman, M. (2000). The notch signalling regulator fringe acts in the Golgi apparatus and requires the glycosyltransferase signature motif DXD. *Curr Biol* **10**, 813-20.

Nakagawa, S., Matsunami, H. and Takeichi, M. (1998). Selective aggregation assays for embryonic brain cells and cell lines. *Curr Top Dev Biol* **36**, 197-210.

Ng, J., Nardine, T., Harms, M., Tzu, J., Goldstein, A., Sun, Y., Dietzl, G., Dickson, B. J. and Luo, L. (2002). Rac GTPases control axon growth, guidance and branching. *Nature* **416**, 442-7.

Nieto, M. A., Gilardi-Hebenstreit, P., Charnay, P. and Wilkinson, D. G. (1992). A receptor protein tyrosine kinase implicated in the segmental patterning of the hindbrain and mesoderm. *Development* **116**, 1137-50.

Nonchev, S., Maconochie, M., Vesque, C., Aparicio, S., Ariza-McNaughton, L., Manzanares, M., Maruthainar, K., Kuroiwa, A., Brenner, S., Charnay, P. et al. (1996). The conserved role of Krox-20 in directing Hox gene expression during vertebrate hindbrain segmentation. *Proc Natl Acad Sci U S A* **93**, 9339-45.

Nusslein-Volhard, C., Wieschaus, E. and Kluding, H. (1984). Mutations affecting the pattern of the larval cuticle in Drosophila melanogaster. I. Zygotic loci on the second chromosome. *Roux's Arch Dev Biol* **193**, 267-282.

O'Connor, T. P., Duerr, J. S. and Bentley, D. (1990). Pioneer growth cone steering decisions mediated by single filopodial contacts in situ. *J Neurosci* **10**, 3935-46.

O'Leary, D. D. and Wilkinson, D. G. (1999). Eph receptors and ephrins in neural development. *Curr Opin Neurobiol* **9**, 65-73.

Panin, V. M., Papayannopoulos, V., Wilson, R. and Irvine, K. D. (1997). Fringe modulates Notch-ligand interactions. *Nature* **387**, 908-12.

Pappu, K. a. M., G. (2002). Retinal specification and determination in *Drosophila*. Berlin: Springer.

Perez, S. E. and Steller, H. (1996). Migration of glial cells into retinal axon target field in Drosophila melanogaster. *J Neurobiol* **30**, 359-73.

Poeck, B., Fischer, S., Gunning, D., Zipursky, S. L. and Salecker, I. (2001). Glial cells mediate target layer selection of retinal axons in the developing visual system of Drosophila. *Neuron* **29**, 99-113.

Poliakov, A., Cotrina, M. and Wilkinson, D. G. (2004). Diverse roles of eph receptors and ephrins in the regulation of cell migration and tissue assembly. *Dev Cell* **7**, 465-80.

Ponting, C. P., Phillips, C., Davies, K. E. and Blake, D. J. (1997). PDZ domains: targeting signalling molecules to sub-membranous sites. *Bioessays* **19**, 469-79.

Power, M. E. (1943). The effect of reduction in numbers of ommatidia upon the brain of Drosophila melanogaster. *J. Exp. Zool* **94**, 33-71.

Quinn, W. G., Harris, W. A. and Benzer, S. (1974). Conditioned behavior in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **71**, 708-12.

Quinn, W. G., Sziber, P. P. and Booker, R. (1979). The Drosophila memory mutant amnesiac. *Nature* **277**, 212-4.

Ramon y Cajal, S. (1893). La retine des vertebres. *La Cellule* **9**, 119-258.

Redies, C. (2000). Cadherins in the central nervous system. *Prog Neurobiol* **61**, 611-48.

Reuter, J. E., Nardine, T. M., Penton, A., Billuart, P., Scott, E. K., Usui, T., Uemura, T. and Luo, L. (2003). A mosaic genetic screen for genes necessary for Drosophila mushroom body neuronal morphogenesis. *Development* **130**, 1203-13.

Rothberg, J. M., Hartley, D. A., Walther, Z. and Artavanis-Tsakonas, S. (1988). slit: an EGF-homologous locus of D. melanogaster involved in the development of the embryonic central nervous system. *Cell* **55**, 1047-59.

Rulifson, E. J., Micchelli, C. A., Axelrod, J. D., Perrimon, N. and Blair, S. S. (1996). wingless refines its own expression domain on the Drosophila wing margin. *Nature* **384**, 72-4.

Sabatier, C., Plump, A. S., Le, M., Brose, K., Tamada, A., Murakami, F., Lee, E. Y. and Tessier-Lavigne, M. (2004). The divergent Robo family protein rig-1/Robo3 is a negative regulator of slit responsiveness required for midline crossing by commissural axons. *Cell* **117**, 157-69.

Sahin, M., Slaugenhaupt, S. A., Gusella, J. F. and Hockfield, S. (1995). Expression of PTPH1, a rat protein tyrosine phosphatase, is restricted to the derivatives of a specific diencephalic segment. *Proc Natl Acad Sci U S A* **92**, 7859-63.

Scott, E. K., Lee, T. and Luo, L. (2001). enok encodes a Drosophila putative histone acetyltransferase required for mushroom body neuroblast proliferation. *Curr Biol* **11**, 99-104.

Seeger, M., Tear, G., Ferres-Marco, D. and Goodman, C. S. (1993). Mutations affecting growth cone guidance in Drosophila: genes necessary for guidance toward or away from the midline. *Neuron* **10**, 409-26.

Selleck, S. B., Gonzalez, C., Glover, D. M. and White, K. (1992). Regulation of the G1-S transition in postembryonic neuronal precursors by axon ingrowth. *Nature* **355**, 253-5.

Selleck, S. B. and Steller, H. (1991). The influence of retinal innervation on neurogenesis in the first optic ganglion of Drosophila. *Neuron* **6**, 83-99.

Sells, M. A., Knaus, U. G., Bagrodia, S., Ambrose, D. M., Bokoch, G. M. and Chernoff, J. (1997). Human p21-activated kinase (Pak1) regulates actin organization in mammalian cells. *Curr Biol* **7**, 202-10.

Sheng, M. and Sala, C. (2001). PDZ domains and the organization of supramolecular complexes. *Annu Rev Neurosci* **24**, 1-29.

Simpson, J. H., Bland, K. S., Fetter, R. D. and Goodman, C. S. (2000a). Short-range and long-range guidance by Slit and its Robo receptors: a combinatorial code of Robo receptors controls lateral position. *Cell* **103**, 1019-32.

Simpson, J. H., Kidd, T., Bland, K. S. and Goodman, C. S. (2000b). Short-range and long-range guidance by slit and its Robo receptors. Robo and Robo2 play distinct roles in midline guidance. *Neuron* **28**, 753-66.

Song, H. J., Ming, G. L. and Poo, M. M. (1997). cAMP-induced switching in turning direction of nerve growth cones. *Nature* **388**, 275-9.

Spana, E. P. and Doe, C. Q. (1995). The prospero transcription factor is asymmetrically localized to the cell cortex during neuroblast mitosis in Drosophila. *Development* **121**, 3187-95.

Spana, E. P., Kopczynski, C., Goodman, C. S. and Doe, C. Q. (1995). Asymmetric localization of numb autonomously determines sibling neuron identity in the Drosophila CNS. *Development* **121**, 3489-94.

Strausfeld, N. J. (1976). Atlas of an insect brain. Heidelberg: Springer.

Strausfeld, N. J., Hansen, L., Li, Y., Gomez, R. S. and Ito, K. (1998). Evolution, discovery, and interpretations of arthropod mushroom bodies. *Learn Mem* **5**, 11-37.

Struhl, G. (1984). A universal genetic key to body plan? *Nature* **310**, 10-1.

Suh, G. S., Poeck, B., Chouard, T., Oron, E., Segal, D., Chamovitz, D. A. and Zipursky, S. L. (2002). Drosophila JAB1/CSN5 acts in photoreceptor cells to induce glial cells. *Neuron* **33**, 35-46.

Takeichi, M. (1988). The cadherins: cell-cell adhesion molecules controlling animal morphogenesis. *Development* **102**, 639-55.

Tayler, T. D. and Garrity, P. A. (2003). Axon targeting in the Drosophila visual system. *Curr Opin Neurobiol* **13**, 90-5.

Tayler, T. D., Robichaux, M. B. and Garrity, P. A. (2004). Compartmentalization of visual centers in the Drosophila brain requires Slit and Robo proteins. *Development* **131**, 5935-45.

Tepass, U., Godt, D. and Winklbauer, R. (2002). Cell sorting in animal development: signalling and adhesive mechanisms in the formation of tissue boundaries. *Curr Opin Genet Dev* **12**, 572-82.

Tessier-Lavigne, M. and Goodman, C. S. (1996). The molecular biology of axon guidance. *Science* **274**, 1123-33.

Theil, T., Frain, M., Gilardi-Hebenstreit, P., Flenniken, A., Charnay, P. and Wilkinson, D. G. (1998). Segmental expression of the EphA4 (Sek-1) receptor tyrosine kinase in the hindbrain is under direct transcriptional control of Krox-20. *Development* **125**, 443-52.

Thomas, J. B., Crews, S. T. and Goodman, C. S. (1988). Molecular genetics of the single-minded locus: a gene involved in the development of the Drosophila nervous system. *Cell* **52**, 133-41.

Tomlinson, A. (1985). The cellular dynamics of pattern formation in the eye of Drosophila. *J Embryol Exp Morphol* **89**, 313-31.

Townes, P. L. a. H., J. (1955). Directed movements and selective adhesions of embryonic amphibian cells. *J. Exp. Zool* **128**, 53-120.

Tsunoda, S., Sierralta, J. and Zuker, C. S. (1998). Specificity in signaling pathways: assembly into multimolecular signaling complexes. *Curr Opin Genet Dev* **8**, 419-22.

Uchida, Y., Ogata, M., Mori, Y., Oh-hora, M., Hatano, N. and Hamaoka, T. (2002). Localization of PTP-FERM in nerve processes through its FERM domain. *Biochem Biophys Res Commun* **292**, 13-9.

Verkhusha, V. V., Otsuna, H., Awasaki, T., Oda, H., Tsukita, S. and Ito, K. (2001). An enhanced mutant of red fluorescent protein DsRed for double labeling and developmental timer of neural fiber bundle formation. *J Biol Chem* **276**, 29621-4.

Vowles, D. M. (1964). Olfactory Learning and Brain Lesions in the Wood Ant (Formica Rufa). *J Comp Physiol Psychol* **58**, 105-11.

Wadsworth, W. G., Bhatt, H. and Hedgecock, E. M. (1996). Neuroglia and pioneer neurons express UNC-6 to provide global and local netrin cues for guiding migrations in C. elegans. *Neuron* **16**, 35-46.

Wang, K. H., Brose, K., Arnott, D., Kidd, T., Goodman, C. S., Henzel, W. and Tessier-Lavigne, M. (1999). Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. *Cell* **96**, 771-84.

Wieschaus, E. and Gehring, W. (1976). Clonal analysis of primordial disc cells in the early embryo of Drosophila melanogaster. *Dev Biol* **50**, 249-63.

Wilkinson, D. G. (2001). Multiple roles of EPH receptors and ephrins in neural development. *Nat Rev Neurosci* **2**, 155-64.

Wilkinson, D. G., Bhatt, S., Cook, M., Boncinelli, E. and Krumlauf, R. (1989). Segmental expression of Hox-2 homoeobox-containing genes in the developing mouse hindbrain. *Nature* **341**, 405-9.

Winberg, M. L., Perez, S. E. and Steller, H. (1992). Generation and early differentiation of glial cells in the first optic ganglion of Drosophila melanogaster. *Development* **115**, 903-11.

Wizenmann, A. and Lumsden, A. (1997a). Segregation of Rhombomeres by Differential Chemoaffinity. *Mol Cell Neurosci* **9**, 448-59.

Wizenmann, A. and Lumsden, A. (1997b). Segregation of rhombomeres by differential chemoaffinity. *Mol Cell Neurosci* **9**, 448-59.

Wolff, T., Martin, K. A., Rubin, G. M., and Zipursky, S. L. (1997). The development of the *Drosophila* visual system. New York: Oxford University Press.

Wolff, T. and Ready, D. F. (1993). Pattern formation in the *Drosophila* retina. Cold Spring Harbor: Cold Spring Harbor Laboratory Press.

Wong, K., Park, H. T., Wu, J. Y. and Rao, Y. (2002). Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr Opin Genet Dev* **12**, 583-91.

Woods, D. F. and Bryant, P. J. (1991). The discs-large tumor suppressor gene of Drosophila encodes a guanylate kinase homolog localized at septate junctions. *Cell* **66**, 451-64.

Wu, W., Wong, K., Chen, J., Jiang, Z., Dupuis, S., Wu, J. Y. and Rao, Y. (1999). Directional guidance of neuronal migration in the olfactory system by the protein Slit. *Nature* **400**, 331-6.

Xu, Q., Mellitzer, G., Robinson, V. and Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**, 267-71.

Yang, Q. and Tonks, N. K. (1991). Isolation of a cDNA clone encoding a human protein-tyrosine phosphatase with homology to the cytoskeletal-associated proteins band 4.1, ezrin, and talin. *Proc Natl Acad Sci U S A* **88**, 5949-53.

Yarfitz, S. and Hurley, J. B. (1994). Transduction mechanisms of vertebrate and invertebrate photoreceptors. *J Biol Chem* **269**, 14329-32.

Yuan, W., Zhou, L., Chen, J. H., Wu, J. Y., Rao, Y. and Ornitz, D. M. (1999). The mouse SLIT family: secreted ligands for ROBO expressed in patterns that suggest a role in morphogenesis and axon guidance. *Dev Biol* **212**, 290-306.

Zeltser, L. M., Larsen, C. W. and Lumsden, A. (2001). A new developmental compartment in the forebrain regulated by Lunatic fringe. *Nat Neurosci* **4**, 683-4.

Zimmermann, P., Meerschaert, K., Reekmans, G., Leenaerts, I., Small, J. V., Vandekerckhove, J., David, G. and Gettemans, J. (2002). PIP(2)-PDZ domain binding controls the association of syntenin with the plasma membrane. *Mol Cell* **9**, 1215-25.

CHAPTER TWO

Compartmentalization of visual centers in the *Drosophila* brain requires Slit and Robo proteins

Chapter 2 is a previously published manuscript entitled, "Compartmentalization of visual centers in the *Drosophila* brain requires Slit and Robo proteins" by Timothy D. Tayler, Myles B. Robichaux, and Paul A. Garrity. This manuscript appears in the journal Development [2004 Dec:131 (23): 5935-45]. Myles Robichaux provided excellent technical assistance and constructed the Robo-RNAi transgenes. Timothy Tayler completed all other experiments.

SUMMARY

Brain morphogenesis depends on the maintenance of boundaries between populations of non-intermingling cells. We have used molecular markers to characterize a boundary within the optic lobe of the *Drosophila* brain and find that Slit and the Robo family of receptors, well-known regulators of axon guidance and neuronal migration, inhibit the mixing of adjacent cell populations in the developing optic lobe. Our data suggest that Slit is needed in the lamina to prevent inappropriate invasion of Robo-expressing neurons from the lobula cortex. We show that Slit protein surrounds lamina glia, while the distal cell neurons in the lobula cortex express all three *Drosophila* Robos. We examine the function of these proteins in the visual system by isolating a novel allele of *slit* that preferentially disrupts visual system expression of Slit and by creating transgenic RNAi flies to inhibit the function of each *Drosophila* Robo in a tissue-specific fashion. We find that loss of Slit or simultaneous knockdown of Robo, Robo2 and Robo3 cause distal cell neurons to invade the lamina, resulting in cell mixing across the lamina/lobula cortex boundary. This boundary disruption appears to lead to alterations in patterns of axon navigation in the visual system. We propose that Slit and Robo-family proteins act to maintain the distinct cellular composition of the lamina and the lobula cortex.

INTRODUCTION

The establishment of compartments, groups of adjacent but non-intermingling cells, is a common method for creating organization during development and the mechanisms underlying the generation of cellular compartments has been extensively studied (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; McNeill, 2000; Vegh and Basler, 2003). Compartmentalization plays a critical role in the development of the nervous system and multiple compartments have been defined in the developing vertebrate forebrain, midbrain and hindbrain (Irvine and Rauskolb, 2001; Larsen et al., 2001; Lumsden and Krumlauf, 1996; Redies and Puelles, 2001; Zeltser et al., 2001). Transcription factors, such as Kreisler (Cordes and Barsh, 1994) and Krox-20 (Schneider-Maunoury et al., 1997), and cell-cell signaling proteins, such as Notch and regulators of Notch signaling (Cheng et al., 2004; Zeltser et al., 2001), have been identified that have critical roles in establishing compartment boundaries during nervous system development. These proteins appear to affect cell-mixing between compartments by regulating the expression or activity of factors that confer distinct affinities upon cells of different compartments (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; McNeill, 2000; Vegh and Basler, 2003).

Mechanisms that have been proposed to restrain cell mixing between compartments include preferential adhesion among cells within a compartment, preferential adhesion between cells of different compartments at the compartment boundary, and mutual repulsion between cells of different compartments (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; McNeill, 2000; Milan et al., 2001). Members of the Cadherin family of adhesion molecules and the Eph/Ephrin family of repellant signaling proteins have been implicated in regulating cell mixing between compartments in the developing vertebrate nervous system (Cooke and Moens, 2002; Inoue et al., 2001; Redies, 2000; Xu et al., 2000). *In vitro* reconstitution experiments have shown that differential Cadherin expression or Eph/Ephrin signaling is sufficient to create groups of non-intermingling cells (Mellitzer et al., 1999; Nose et al., 1988), while ectopic expression and dominant-negative studies have shown that these proteins can alter cell sorting in vivo (Cooke and Moens, 2002; Inoue et al., 2001; Xu et al., 1999). However, loss-of-function analysis has not yet demonstrated a requirement for either Cadherin

expression or Eph/Ephrin signaling in restricting cell movement between compartments of the developing brain (Cooke and Moens, 2002; Inoue et al., 2001).

The developing *Drosophila melanogaster* brain, like the vertebrate brain, contains multiple compartments that give rise to multiple, anatomically distinct processing centers, and recent work has begun to comprehensively detail the morphogenetic events of fly brain development (Dumstrei et al., 2003; Hartenstein et al., 1998; Meinertzhagen et al., 1998; Nassif et al., 2003; Younossi-Hartenstein et al., 2003). The visual centers of the fly brain, the optic lobes, contain four ganglia (the lamina, medulla, lobula and lobula plate), which are derived from two distinct populations of progenitor cells, the outer and inner optic anlagen (Hofbauer and Campos-Ortega, 1990; Meinertzhagen and Hanson, 1993; Younossi-Hartenstein et al., 1996). Progeny of the outer optic anlagen contribute to the lamina and outer medulla, while progeny of the inner optic anlagen contribute to the inner medulla, lobula and lobula plate. Descendents of these different anlagen lie adjacent to one another during development without intermingling and act as distinct developmental compartments within the brain. For example, the neurons and glia of the developing lamina, derived from the outer optic anlagen (Dearborn and Kunes, 2004; Meinertzhagen and Hanson, 1993), lie immediately adjacent to the neurons of the developing lobula cortex, which are derived from the inner optic anlagen (Hofbauer and Campos-Ortega, 1990; Meinertzhagen and Hanson, 1993), but the two cell populations remain distinct. How these cell populations are prevented from intermingling is unknown.

The Slit and Robo protein families are essential for axon guidance and cell migration in worms, flies, fish and mice (Brose and Tessier-Lavigne, 2000; Wong et al., 2002). Slits are secreted proteins that can act as either attractive or repulsive guidance cues (Englund et al., 2002; Kramer et al., 2001), while members of the Robo family encode transmembrane receptors for Slits (Brose et al., 1999; Rajagopalan et al., 2000b; Simpson et al., 2000b). *Drosophila* has a single Slit and three Robo receptors (Robo, Robo2 and Robo3) (Kidd et al., 1999; Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b). The recent identification of mutations in human Robo3 (Rig1) in patients with Horizontal Gaze Palsy and Progressive

Scoliosis (HGPPS) with hindbrain dysplasia demonstrates that Robo-receptor function is also important for human brain development (Jen et al., 2004).

In this work, we identify Slit and Robo-family members as key factors that limit cell mixing between two adjacent cell populations in the *Drosophila* brain, the lamina glia and the distal cell neurons of the lobula cortex. We characterize a set of molecular markers that permit us to examine the behavior of cells at the boundary between the lamina and the lobula cortex. We find that Slit protein surrounds the lamina glia, while the distal cell neurons of the lobula cortex express multiple Robo-family receptors. We show that either loss of Slit or the tissue-specific knockdown of multiple Robo family members causes distal cell neurons to intermingle with the lamina glia, disrupting the boundary between the lamina and lobula cortex. We propose that Slit and Robo family proteins prevent cell mixing at the lamina/lobula interface, enforcing a boundary between adjacent compartments of the developing *Drosophila* brain that is essential for morphogenesis of the visual system.

RESULTS

Organization of the developing optic lobe

The optic lobes are comprised of four processing centers derived from two distinct populations of precursor cells. In several regions of the optic lobe, cells derived from these different sets of progenitors lie immediately adjacent to one another, but do not intermingle. This type of organization is found at the interface of the lamina and the lobula cortex, which are derived from the outer and inner optic anlagen, respectively. As shown in the horizontal section in Fig. 1A and depicted in Fig. 1C, distal cell neurons form the anterior edge of the lobula cortex and are located immediately adjacent to the posterior face of the lamina (dotted yellow line denotes anterior edge of lobula cortex, Fig. 1A). The close apposition of distal cell neurons to the glia at the posterior edge of the developing lamina is visible in the lateral section in Fig. 1B and depicted in Fig. 1D. In this work, we examine the mechanisms that prevent the distal cell neurons of the lobula cortex from intermingling with the lamina glia.

Slit is required for optic lobe morphogenesis

Our examination of the lamina/lobula cortex boundary initiated with the identification from a genetic screen of a novel allele of *slit*, $slit^{dui}$ *(dui, disrupted innervation)*, that severely disrupted photoreceptor axon innervation of the optic lobe (Fig. 2A,B). We found that $slit^{dui}$ was caused by insertion of a transposable element 29,404 bases upstream of the 5' end of the Slit transcript (see Methods for details). Similar photoreceptor connectivity defects were obtained when $slit^{dui}$ was examined in combination with other *slit* loss-of-function alleles, including the transposon insertion alleles $slit^{l(2)k05248}$ (described in greater detail below) and $slit^{E158}$ (Battye et al., 2001) (Fig. 2C,D). In particular, the photoreceptor connectivity phenotypes of $slit^{dui}/slit^{2}$ animals ($slit^{2}$ is a previously characterized null, Kidd et al., 1999) were indistinguishable from those of $slit^{dui}/slit^{dui}$ animals indicating that $slit^{dui}$ behaved as a recessive strong loss-of-function allele in the visual system (Fig. 2E). However, unlike previously described strong alleles of *slit*, which die prior to the development of the adult optic lobe, $slit^{dui}$ mutants were homozygous viable, greatly facilitating analysis of *slit* function in the visual system. As

shown below, *slit*^{dui} significantly reduced Slit expression in the optic lobes without completely eliminating Slit expression in other regions. The *slit* photoreceptor connectivity defect could be rescued by expression of a Slit cDNA in the visual system under the control of Omb-Gal4 (Fig. 2F), which drives expression broadly in optic lobe glia and in a subset of optic lobe neurons (Dearborn and Kunes, 2004; Rangarajan et al., 1999) and restores expression of Slit in the optic lobe neuropils (data not shown). Examination of the photoreceptor axon target region in *slit* mutants showed that the lamina glia, intermediate targets of R1-R6 photoreceptor axons, were also disrupted (Fig. 2G,H), and that regions of photoreceptor axon mistargeting correlated with areas of lamina glial disruption (Fig. 2I,J). This raised the possibility that the photoreceptor axon targeting defects in *slit* mutants could be a secondary consequence of disruptions in optic lobe development.

Slit prevents distal cell neurons from entering the lamina

The disrupted positioning of lamina glia in *slit* mutants prompted us to examine whether the adjacent distal cell neurons might be disorganized as well. In Fig. 3, post-mitotic neurons were visualized with the nuclear marker Elav (blue), while the distal cell neurons and their IPC neuroblast progenitors were visualized using the cell-surface marker Fasciclin III (red), and the lamina was visualized using the photoreceptor axon marker GMR:GFP (green). In wild type, the distal cell neurons never entered the lamina (Fig. 3A). However, in *slit* mutants, many distal cell neurons entered the base of the lamina (arrow, Fig. 3B) and some distal cell neurons invaded the lamina neuropil, disrupting photoreceptor innervation (asterisk, Fig. 3B). A lateral cross-section near the base of the lamina further demonstrated that the normally precise boundary between distal cell neurons and the lamina neuropil (Fig. 3C) was disrupted in *slit* mutants, with large numbers of distal cell neurons invading the lamina neuropil (arrow and arrowhead in Fig. 3D). These data demonstrated that in the absence of *slit*, distal cell neurons invaded the developing lamina.

Slit protein concentrates in the lamina

To further investigate Slit function in visual system development, the pattern of Slit expression was examined in third instar larvae. In the visual system, Slit protein expression was detected in the medulla neuropil (medn) and at the base of the lamina (arrow) (Fig. 4A). Consistent with genetic evidence that $slit^{dui}$ is a strong loss of function allele in the visual system, Slit expression in the optic lobe was greatly reduced in $slit^{dui}$ mutants (Fig. 4B). Slit was also expressed within the midline of the ventral ganglion (vg) and in the mushroom bodies (mb). Slit expression was partially reduced in the ventral ganglion in $slit^{dui}$ mutants, but not visibly altered in the mushroom bodies (Fig. 4B). Such residual Slit expression may explain why $slit^{dui}$ mutants were viable and did not show the midline axon guidance defects observed in lethal alleles of *slit* (T.D.T. and P.A.G., unpublished data). Taken together, these data demonstrated that Slit was expressed in the optic lobe and that $slit^{dui}$ reduced optic lobe expression of Slit.

The expression of Slit was examined in greater detail. Slit mRNA production was detected within the optic lobes, with strongest expression near the medulla neuropil (Fig. 4C). Simultaneous staining for the glial-specific nuclear protein Repo (green) and Slit protein (magenta) demonstrated that Slit was concentrated throughout the medulla neuropil within the region demarcated by the medulla neuropil glia (mng) (Fig. 4D). Slit protein was also present near the base of the lamina. Three layers of glial cells, epithelial glia (ep), marginal glia (ma), and medulla glia (mg), reside in this region and Slit protein concentrated around these glia (Fig. 4E). When Slit protein was observed in the absence of Repo staining, Slit localization around these glia gave the base of the lamina a honeycomb appearance (Fig. 4F). A horizontal section demonstrated that Slit was present immediately adjacent to the distal cell neurons (Fig. 4G). Thus, Slit protein was found in a relatively continuous fashion from the lamina neuropil into the medulla neuropil (depicted in Fig. 4H). The region of Slit protein concentration was immediately adjacent to the distal cell neurons, demonstrating that Slit protein was present at the appropriate time and place to control the behavior of distal cell neurons.

As the ingrowth of photoreceptor axons induces many developmental events in the optic lobe, we tested whether Slit production depended upon photoreceptor axon innervation. Slit protein was still present in the optic lobe of *eyes absent* (*eya*) mutant

animals that had no photoreceptor neurons, indicating that photoreceptor axon innervation was not essential for Slit production (Fig. 4I).

To begin to further characterize the identity of the cells producing Slit protein in the optic lobe, we examined optic lobe expression of an enhancer trap transposon insertion in the Slit locus. The *slit*$^{l(2)k05248}$ insertion is located 30,258 bases upstream of the Slit mRNA start site, 1,853 bases from the *slit*^{dui} insertion site, and behaves as a loss of function *slit* allele in the visual system (see Fig. 2E). Expression of the LacZ enhancer trap in *slit*$^{l(2)k05248}$ resembled the Slit RNA in situ pattern, with strong expression in the optic lobe and in the midline of the ventral ganglion, and is referred to here as Slit:LacZ. Slit:LacZ was expressed at the base of the lamina by the medulla glia, the most basal of the three layers of lamina glia (Fig. 4J). Slit:LacZ was also expressed by cells in the medulla cortex (Fig. 4J). These cells lay immediately adjacent to the glia that surround the medulla neuropil (Fig. 4K) and appear to be differentiating neurons of the medulla cortex as they express varying levels of the neuronal marker Elav (Fig. 4L). Medulla cortex neurons are known to project axons into the medulla neuropil and could thus provide Slit protein to the medulla neuropil region. These Slit:LacZ enhancer trap data combine with the Slit protein and RNA in situ data to provide a consistent picture where expression of Slit, a diffusible protein, by cells at the base of the lamina and at the periphery of the medulla generate a region of Slit expression extending from the lamina into the medulla.

Distal cell neurons express Robo family proteins

Since Robo-family receptors commonly mediate responses to Slit proteins, we characterized the distribution of the three *Drosophila* Robo proteins in the developing visual system. Robo, Robo2 and Robo3 were all expressed within the developing optic lobes (Fig. 5A,D,G). More detailed analysis of Robo and Robo2 expression showed that both proteins were expressed by IPC neuroblasts and distal cell neurons (Fig. 5B,C,E,F). Robo3 protein was not detected on IPC neuroblasts, but was present on distal cell neurons (Fig. 5H,I). Thus, all three Robo receptors were expressed within the developing lobula cortex in partially overlapping patterns, consistent with Robo-family receptors mediating responses to Slit in this region of the visual system.

Inhibition of Robo family protein expression using transgenic RNAi

The expression patterns of Robo proteins suggested they could mediate the effects of Slit on distal cell neurons. To begin to address this question, we examined existing loss-of-function mutations in *robo*, *robo2*, and *robo3*. Animals homozygous for the previously described strong loss-of-function alleles *robo2*^{x123} or *robo3*^{1} had no detectable defect in distal cell neuron positioning. Distal cell neuron positioning could not be examined in animals homozygous for null alleles of *robo*, as they died before the third instar larval stage. Large marked clones homozygous mutant for the *robo*^{5} null allele were generated in the visual system, but no defects were detected.

As *robo*, *robo2*, and *robo3* have partially redundant functions in the embyronic CNS (Rajagopalan et al., 2000a; Rajagopalan et al., 2000b; Simpson et al., 2000a; Simpson et al., 2000b), we wanted to examine the effect of simultaneous disruption of multiple Robo-family proteins in the visual system. However, analysis of Robo-family function using existing alleles proved insufficient. First, marked clones of *robo*^{5} mutant tissue were generated in a homozygous *robo3*^{1} background, but no defects were observed (T.D.T. and P.A.G., unpublished data). Second, *robo,robo2* double mutant mosaic analysis could not be performed because the necessary animals did not survive to form adult visual systems and the proximity of the *robo2* and *robo3* genes (87 kb (Simpson et al., 2000b)) prevented the creation of a *robo2,robo3* recombinant. Third, we determined that the only existing mutant allele of *robo3* (*robo3*^{1}), characterized as a strong loss-of-function or null allele in the embryo (Rajagopalan et al., 2000b), produced substantial quantities of full-length Robo3 protein and increased levels of a lower molecular weight form of Robo3 in the adult head (Fig. 4A). Significant amounts of Robo3 immunostaining were also observed in the developing visual system of *robo3*^{1} animals (T.D.T. and P.A.G., unpublished data), suggesting *robo3*^{1} is not a null in the visual system. Therefore, a different strategy was needed to achieve simultaneous inhibition of Robo, Robo2 and Robo3 in the visual system.

Tissue-specific transgenic RNA interference was used to inhibit expression of each of the Robos. *UAS-RoboRNAi*, *UAS-Robo2RNAi* and *UAS-Robo3RNAi* transgenic flies were generated and the transgenes proved effective inhibitors of their targets as

assessed using a combination of western blot analysis and tissue staining (Fig. 6B,C,D). As shown in Fig. 6E-M, expression of *UAS-RoboRNAi*, *UAS-Robo2RNAi* or *UAS-Robo3RNAi* under the control of Gal4 substantially reduced expression of the corresponding Robo-family protein without detectably affecting expression of other Robo-family members. Thus, these transgenic RNAi constructs permitted inducible knockdown of each Robo-family protein.

Inhibition of Robo family expression causes distal cell neurons to enter the lamina

We examined the function of Robo receptors in the visual system by expressing our UAS-RNAi transgenes under the control of a variety of different Gal4 sources. The nervous system-specific *c155-Gal4* was used to drive expression of transgenic RNAi in optic lobe neuroblasts and neurons. Expression of a single copy of *UAS-RoboRNAi, UAS-Robo2RNAi,* or*UAS-Robo3RNAi* under the control of *c155-Gal4* had no effect on visual system development. However, simultaneous inhibition of all three Robos in *c155-Gal4,UAS-GFP,UAS-RoboRNAi,UAS-Robo2RNAi,UAS-Robo3RNAi* animals had strong visual system phenotypes (Fig. 7A,B). As in *slit* mutants, distal cell neurons invaded the developing lamina in *c155-Gal4,UAS-GFP,UAS-RoboRNAi,UAS-Robo2RNAi,UAS-Robo3RNAi* animals (arrow and arrowhead, Fig. 7B). Thus, Robo-family proteins act within the nervous system to prevent distal cell neurons from invading the lamina.

Distal cell neuron defects were also induced by expressing UAS-RNAi transgenes under the control of *Sca-Gal4*, which drives expression in a smaller subset of neuroblasts and neurons than *c155-Gal4*. Expression of a single copy of *UAS-RoboRNAi, UAS-Robo2RNAi,* or*UAS-Robo3RNAi* or simple pairwise combinations of these transgenes under the control of *Sca-Gal4* caused no phenotypes. However, simultaneous inhibition of all three Robos in *Sca-Gal4,UAS-GFP,UAS-RoboRNAi,UAS-Robo2RNAi,UAS-Robo3RNAi* animals caused distal cell neurons to invade the developing lamina (Fig. 7C,D). Simultaneous visualization of lamina glia and distal cell neurons in Robo-knockdown animals further demonstrated the intermingling of distal cell neurons and lamina glia in these animals (Fig. 7E,F, compare with Fig. 1B). These observations

indicate that all three Robo family members contribute to preventing distal cells neurons from intermingling with the lamina glia.

As the role of Slit in visual system development was initially identified through its effect on photoreceptor axon targeting, we examined whether photoreceptor axon targeting was similarly dependent upon Robo-family receptors. Indeed, generalized inhibition of all three Robo receptors under the control of *tubulin-Gal4* in *tubulin-Gal4,UAS-RoboRNAi,UAS-Robo2RNAi,UAS-Robo3RNAi* animals disrupted photoreceptor axon targeting in a fashion similar to that observed in *slit* mutants (Fig. 8A,B). Interestingly, simultaneous expression of *UAS-RoboRNAi,UAS-Robo2RNAi,*and *UAS-Robo3RNAi* under the control of the eye-specific Gal4 source *GMR-Gal4* generated no defects in photoreceptor axon targeting (Fig. 8C), while inhibition of Robo-family expression using *Sca-Gal4* did disrupt photoreceptor axon targeting (Fig. 8D,E,F). In fact, regions of photoreceptor mistargeting corresponded to regions where *Sca-Gal4* cells (distal cell neurons) entered the lamina (Fig. 8D,E,F). While these knockdown experiments do not preclude a role for Robo-family receptors in the photoreceptors, they nonetheless suggest that the misplacement of distal cell neurons contributes to photoreceptor axon mistargeting (Fig. 8G). These data also further emphasize the similarity of the effects of knockdown of Robo-family receptors and reductions in Slit expression on optic lobe morphogenesis.

Discussion

The construction of anatomically-distinct processing centers in the brain is a complex morphogenetic task that requires segregation of adjacent groups of cells. Despite the extensive study of how cells are segregated into distinct groups, the identities of the molecules that prevent intermingling between adjacent groups remain largely unknown (Dahmann and Basler, 1999; Irvine and Rauskolb, 2001; McNeill, 2000; Vegh and Basler, 2003). Here we have identified a novel role for Slit and the Robo receptors as key factors that prevent mixing between adjacent groups of cells in the fly brain. We have focused on the effect of Slit and Robo family proteins on the boundary between the glia at the posterior edge of the lamina and the neurons at the anterior edge of the lobula cortex. We have found that the secreted protein Slit surrounds the lamina glia on one side of the boundary while Robo-family proteins (receptors for Slit) are expressed by the distal cell neurons on the other side of the boundary. We show that loss of Slit expression or tissue-specific inhibition of Robo-family expression in distal cell neurons cause the intermingling of lamina glia and distal cell neurons. We propose that Slit protein in the lamina keeps Robo-expressing neurons within the normal confines of the lobula cortex, establishing the sharp boundary between these two regions. Given the conservation of Slit and Robo signaling in axon guidance throughout evolution, Slit and Robo family members may also regulate boundary formation in the brains of other animals. Interestingly, humans with mutations in Robo3 exhibit defects in hindbrain morphology, although the underlying developmental defect in the human patients is not known (Jen et al., 2004).

Slit and Robo-family proteins are regulators of boundary maintenance

Compartmentalization is important throughout nervous system development (Pasini and Wilkinson, 2002) and structural compartmentalization underlies functional compartmentalization in the adult brain. The adult vertebrate brain contains many distinct compartments, such as Brodmann's areas of the cerebral cortex and the brainstem nuclei, and anatomical studies point to similar compartmentalization in the *Drosophila* brain (Younossi-Hartenstein et al., 2003). As noted above, several molecules that regulate cell adhesion or cell repulsion have been implicated in restricting cell mixing

between compartments in the developing nervous system, but loss of these proteins has not been shown to cause intermingling between compartments. Here we have shown that Slit and the Robos are required to prevent cell intermingling across a boundary in the optic lobe.

We determined that knockdown of Robo-family protein expression in the optic lobe using the *Sca-Gal4* driver caused robust defects in distal cell neuron positioning. In addition to driving gene expression in IPC neuroblasts and distal cell neurons, *Sca-Gal4* also drives expression in R8 photoreceptor axons and neuroblasts of the Outer Proliferation Center and neurons of the medulla cortex. As noted above, inhibition of Robo-family expression only in the photoreceptors caused no detectable defects. In addition, knockdown of all three Robo-family proteins in the medulla cortex using *apterous-Gal4* had no effect on distal cell neuron behavior, and no defects in medulla neuron movement or axon targeting were identified in either *slit* mutants or Robo-family knockdowns (T.D.T. and P.A.G., unpublished data). Taken together with Robo-family protein expression data, the Robo-family knockdown analysis strongly supports a requirement for Robo-family receptors in distal cell neurons to prevent them from invading the lamina neuropil.

Slit and Robo-family protein expression in the optic lobe

In the *Drosophila* visual system, Slit protein is present in a continuous zone from the base of the lamina extending into the underlying medulla neuropil. Although Slit mRNA is detected within the optic lobe and Slit:LacZ expression is detected in medulla glia at the base of the lamina and in medulla cortex neurons, the optic lobe does not appear highly sensitive to the precise source or level of Slit. Attempts to use mosaic analysis to define the cells in which *slit* function was required were unsuccessful as no phenotypes were observed despite the generation of large marked patches of $slit^2$ mutant tissue in the visual system and the use of the Minute technique to maximize mutant clone size (T.D.T. and P.A.G., unpublished data). We suspect that the diffusibility of Slit protein combined with the large number of Slit-expressing cells in the optic lobe permitted the remaining heterozygous and wild-type cells in the mosaic animals to provide sufficient Slit to support proper optic lobe development. In addition, expression of Slit in photoreceptors

under the control of *GMR-Gal4* rescued the photoreceptor projection phenotype of *slit* mutants as effectively as more general expression of Slit in the optic lobe using *Omb-Gal4*. Thus, delivery of Slit to these neuropil regions may be sufficient to restore the boundary between the lobula cortex and the lamina.

We also examined the effects of overexpression and ectopic expression of Slit and Robo proteins in the optic lobe. Overexpression of Slit in the optic lobe using *GMR*-Gal4, *Sca-Gal4, Omb-Gal4* or the more ubiquitously expressed *Tubulin-Gal4* did not generate detectable phenotypes in the optic lobe (T.D.T. and P.A.G., unpublished data). The failure to generate strong overexpression phenotypes could reflect the increased Slit expression within the lamina that accompanied overexpression in other regions using these Gal4 drivers. However, overexpression of Robo2 under the control of *Sca-Gal4* dramatically distorted the shape of the lobula cortex, causing the distal cell neurons to move around the ventral and dorsal edges of the lamina (T.D.T. and P.A.G., unpublished data). As distal cell neurons normally encounter Slit protein at the posterior face of the lamina, this redistribution could reflect repulsion from regions of Slit expression. Overexpression of Robo or Robo3 caused no detectable defects.

Robo-family proteins appear to localize around the cell body periphery of newly differentiated distal cell neurons. This cell body-associated expression contrasts with the predominantly axonal expression of Robo-family proteins by more mature lobula cortex neurons. Whether this reflects a regulated shift in the subcellular localization of Robo proteins or simply the availability of axonal processes in more mature neurons is unknown. However, as Slit and Robo-family proteins control both neuronal migration and axon navigation (Wong et al., 2002), such a change in Robo-family protein distribution could alter a neuron's response to Slit from one involving the cell body to one involving just the axon. We have not detected obvious misprojections of the axons of the distal cell neurons in our mutants (T.D.T. and P.A.G., unpublished data), although subtle defects in targeting of these axons would not be detected using available markers.

Regulation of cell mixing at boundaries in the developing brain

Boundaries are commonly encountered during development and several mechanisms have been proposed to prevent mixing between compartments. Our observations provide

evidence for a signal associated with one cell population preventing the invasion of a neighboring cell population expressing receptors for this signal. Interestingly, even when the distal cell neurons invade the lamina in *slit* mutants or Robo-family knockdown animals, they do not disperse evenly among the lamina glia. Rather the distal cell neurons remain preferentially associated with one another, suggesting the persistence of differential adhesion when the Slit signal is absent. Thus, multiple parallel mechanisms, possibly involving both repulsion and differential adhesion, are potentially involved in maintaining the normally precise distinction between lamina and lobula cortex. Combinations of adhesion and repulsion may act at other boundaries, providing robustness as well as functional redundancy to the molecular mechanisms of compartment maintenance.

MATERIALS AND METHODS

Genetics and Fly Stocks

The *slit*^{dui} phenotype was originally identified in *l(2)k04807* (Karpen and Spradling, 1992; Torok et al., 1993). *l(2)k04807* contains pLacW P-element transposon insertions located at 52D and 53C which were separated by meiotic recombination. *slit*^{dui} was associated with the 52D P-element which was inserted between bases 10,983,983 and 10,983984 on 2R with LacZ coding region oriented toward *slit* locus. (Slit transcript extends from bases 10,954,579 to 10,936,369.) Precise transposon excision reverted *slit*^{dui} phenotype in all 11 lines tested. *slit*$^{l(2)k05248}$ (Karpen and Spradling, 1992; Torok et al., 1993) contains pLacW transposon inserted between bases 10,985,837 and 10,985,838 on 2R, with LacZ coding region oriented away from *slit* locus. MARCM analysis performed as described (Lee and Luo, 1999). Fly stocks *slit*^{2}, *slit*$^{l(2)k05248}$, Df(2R)WMG, *Df(2R)Jp1*, omb-Gal4, repo-LacZ, and c155-Gal4 were obtained from the Bloomington stock center. *slit*^{E158}, *slit*^{2}; UAS-Slit, *loco*$^{3-109}$, *robo*^{5} and robo2^{x123} were provided by J. Simpson, G. Bashaw and C. Goodman, *robo3*^{1} (Rajagopalan et. al., 2000) by B. Dickson, *ro-tauLacZ* by U. Gaul and *eya*^{2} by I. Rebay.

Immunohistochemistry and In Situ Hybridization

Third instar whole mounts performed as described (Garrity et al., 1996). Distal cell neuron positioning, glial positioning and photoreceptor axon targeting defects were observed in all *slit* and triple Robo-family RNAi animals examined and greater than 20 hemispheres were examined for each genotype. The following primary antibodies were obtained from the Developmental Studies Hybridoma Bank and used at the following concentrations: 24B10 mAb (1:200), Slit C555.6D (1:200), Robo mAb 13C9 (1:200), Robo3 mAb 14C9 (1:200), FasII 1D4 (1:200), FasIII 7G10 (1:50), Repo 8D12 (1:200), Elav 7E8A10 (1:20), and beta-galactosidase 40-1A (1:200). Robo2 polyclonal antisera (1:750) (Rajagopalan et al., 2000b; Simpson et al., 2000a) were provided by C. Goodman and by B. Dickson and Repo polyclonal (1:1000) (Campbell et al., 1994) by A. Tomlinson. Anti-phospho-Histone H3 (1:200) was purchased from Upstate Biotechnology. Secondary antibodies were obtained from Jackson Laboratories and used

at the following concentrations: goat-anti-mouse hrp-conjugated (1:200), goat-anti-mouse Cy3-conjugated (1:500),goat-rat-mouse Cy5-conjugated (1:400). Fluorescent samples were visualized using a Nikon PCM2000 confocal microscope. In situ hybridization performed as described (Wolff, 2000).

Molecular Biology

Genomic DNA flanking the *slit*^{dui} P-element was isolated by plasmid rescue and sequenced to identify the insertion site as described (Garrity et al., 1996). Western Blot analysis used the following antibodies: Robo mAb 13C9 (1:2000), Robo3 mAb 14C9 (1:1000), Elav 7E8A10 (1:1000), anti-hrp-conjugated secondary antibody (1:5000). Robo-family RNAi constructs were generated using the strategy described (Kalidas and Smith, 2002). Fragments for creating the RNAi constructs were generated by PCR (Expand Hi-Fidelity, Roche) and cloned into pUASt (Brand and Perrimon, 1993). PCR primers used to create UAS-RoboRNAi were: genomic fragment 5'-ACCGGGCAGCTGATCCTAGC and 5'-ATACTAGTCTGTCGAATAATAAGAAGATATAAAATGATTC; cDNA fragment 5'-TGTCAGTCGCACCAGCATTAGTC and 5'-ATACTAGTCATCTTCATAGGTGAGGGCTGTC. PCR primers used to create UAS-Robo2RNAi were: genomic fragment 5'-GTTCCCTCTGAGGCACCATATG and 5'-ATACTAGTGTGTGATTGCCTGCAGGTGAG; cDNA fragment 5'-GTTCCCTCTGAGGCACCATATG and 5'-ATACTAGTCCACGCATTGTATTTAGGGCCG. PCR primers used to create UAS-Robo3RNAi were: genomic fragment 5'-TATATCGCAGTGGCGGCTGCC and 5'-ATAGATCTCTGCAATTGGAGGGGATGAAATCAG; cDNA fragment 5'-TATATCGCAGTGGCGGCTGCC and 5'-ATAGATCTCTCTCGTAATCGGGTAGCAGC.

Acknowledgments

We thank C. Goodman, J. Simpson, R. Jacobs, B. Dickson, G. Bashaw for generously providing many antibodies and fly stocks. We thank I. Rebay and Garrity lab members for helpful discussions and L. Huang and J. Whited for comments on the manuscript.

This work was supported by grants to P.A.G. from the National Eye Institute, the Raymond and Beverly Sackler Foundation, and the McKnight Foundation. T.D.T.was supported by an NIH Predoctoral Training Grant.

REFERENCES

Battye, R., Stevens, A., Perry, R. L. and Jacobs, J. R. (2001). Repellent signaling by Slit requires the leucine-rich repeats. *J Neurosci* **21**, 4290-8.

Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401-415.

Brose, K., Bland, K. S., Wang, K. H., Arnott, D., Henzel, W., Goodman, C. S., Tessier-Lavigne, M. and Kidd, T. (1999). Slit proteins bind Robo receptors and have an evolutionarily conserved role in repulsive axon guidance. *Cell* **96**, 795-806.

Brose, K. and Tessier-Lavigne, M. (2000). Slit proteins: key regulators of axon guidance, axonal branching, and cell migration. *Curr Opin Neurobiol* **10**, 95-102.

Campbell, G., Goring, H., Lin, T., Spana, E., Andersson, S., Doe, C. Q. and Tomlinson, A. (1994). RK2, a glial-specific homeodomain protein required for embryonic nerve cord condensation and viability in Drosophila. *Development* **120**, 2957-66.

Cheng, Y. C., Amoyel, M., Qiu, X., Jiang, Y. J., Xu, Q. and Wilkinson, D. G. (2004). Notch activation regulates the segregation and differentiation of rhombomere boundary cells in the zebrafish hindbrain. *Dev Cell* **6**, 539-50.

Cooke, J. E. and Moens, C. B. (2002). Boundary formation in the hindbrain: Eph only it were simple. *Trends Neurosci* **25**, 260-7.

Cordes, S. P. and Barsh, G. S. (1994). The mouse segmentation gene kr encodes a novel basic domain-leucine zipper transcription factor. *Cell* **79**, 1025-34.

Dahmann, C. and Basler, K. (1999). Compartment boundaries: at the edge of development. *Trends Genet* **15**, 320-6.

Dearborn, R., Jr. and Kunes, S. (2004). An axon scaffold induced by retinal axons directs glia to destinations in the Drosophila optic lobe. *Development* **131**, 2291-303.

Dumstrei, K., Wang, F., Nassif, C. and Hartenstein, V. (2003). Early development of the Drosophila brain: V. Pattern of postembryonic neuronal lineages expressing DE-cadherin. *J Comp Neurol* **455**, 451-62.

Englund, C., Steneberg, P., Falileeva, L., Xylourgidis, N. and Samakovlis, C. (2002). Attractive and repulsive functions of Slit are mediated by different receptors in the Drosophila trachea. *Development* **129**, 4941-51.

Garrity, P. A., Rao, Y., Salecker, I., McGlade, J., Pawson, T. and Zipursky, S. L. (1996). Drosophila photoreceptor axon guidance and targeting requires the Dreadlocks SH2/SH3 adapter protein. *Cell* **85**, 639-650.

Hartenstein, V., Nassif, C. and Lekven, A. (1998). Embryonic development of the Drosophila brain. II. Pattern of glial cells. *J Comp Neurol* **402**, 32-47.

Hofbauer, A. and Campos-Ortega, J. A. (1990). Proliferation pattern and early differentiation of the optic lobes in Drosophila melanogaster. *Roux's Archives of Developmental Biology* **198**, 264-274.

Inoue, T., Tanaka, T., Takeichi, M., Chisaka, O., Nakamura, S. and Osumi, N. (2001). Role of cadherins in maintaining the compartment boundary between the cortex and striatum during development. *Development* **128**, 561-9.

Irvine, K. D. and Rauskolb, C. (2001). Boundaries in development: formation and function. *Annu Rev Cell Dev Biol* **17**, 189-214.

Jen, J. C., Chan, W. M., Bosley, T. M., Wan, J., Carr, J. R., Rub, U., Shattuck, D., Salamon, G., Kudo, L. C., Ou, J. et al. (2004). Mutations in a human ROBO gene disrupt hindbrain axon pathway crossing and morphogenesis. *Science* **304**, 1509-13.

Kalidas, S. and Smith, D. P. (2002). Novel genomic cDNA hybrids produce effective RNA interference in adult Drosophila. *Neuron* **33**, 177-84.

Kidd, T., Bland, K. S. and Goodman, C. S. (1999). Slit is the midline repellent for the robo receptor in Drosophila. *Cell* **96**, 785-94.

Kramer, S. G., Kidd, T., Simpson, J. H. and Goodman, C. S. (2001). Switching repulsion to attraction: changing responses to slit during transition in mesoderm migration. *Science* **292**, 737-40.

Larsen, C. W., Zeltser, L. M. and Lumsden, A. (2001). Boundary formation and compartition in the avian diencephalon. *J Neurosci* **21**, 4699-711.

Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-61.

Lumsden, A. and Krumlauf, R. (1996). Patterning the vertebrate neuraxis. *Science* **274**, 1109-15.

McNeill, H. (2000). Sticking together and sorting things out: adhesion as a force in development. *Nat Rev Genet* **1**, 100-8.

Meinertzhagen, I. A., Emsley, J. G. and Sun, X. J. (1998). Developmental anatomy of the Drosophila brain: neuroanatomy is gene expression. *J Comp Neurol* **402**, 1-9.

Meinertzhagen, I. A. and Hanson, T. E. (1993). The development of the optic lobe. In *The development of Drosophila melanogaster.*, (ed. M. Bate and A. Martinez-Arias), pp. 1363-1491. Cold Spring Harbor, New York: Cold Spring Harbor Press.

Mellitzer, G., Xu, Q. and Wilkinson, D. G. (1999). Eph receptors and ephrins restrict cell intermingling and communication. *Nature* **400**, 77-81.

Milan, M., Weihe, U., Perez, L. and Cohen, S. M. (2001). The LRR proteins capricious and Tartan mediate cell interactions during DV boundary formation in the Drosophila wing. *Cell* **106**, 785-94.

Nassif, C., Noveen, A. and Hartenstein, V. (2003). Early development of the Drosophila brain: III. The pattern of neuropile founder tracts during the larval period. *J Comp Neurol* **455**, 417-34.

Nose, A., Nagafuchi, A. and Takeichi, M. (1988). Expressed recombinant cadherins mediate cell sorting in model systems. *Cell* **54**, 993-1001.

Pasini, A. and Wilkinson, D. G. (2002). Stabilizing the regionalisation of the developing vertebrate central nervous system. *Bioessays* **24**, 427-38.

Rajagopalan, S., Nicolas, E., Vivancos, V., Berger, J. and Dickson, B. J. (2000a). Crossing the midline: Roles and regulation of Robo receptors. *Neuron* **28**, 767-777.

Rajagopalan, S., Vivancos, V., Nicolas, E. and Dickson, B. J. (2000b). Selecting a longitudinal pathway: Robo receptors specify the lateral position of axons in the Drosophila CNS. *Cell* **103**, 1033-1045.

Rangarajan, R., Gong, Q. and Gaul, U. (1999). Migration and function of glia in the developing Drosophila eye. *Development* **126**, 3285-92.

Redies, C. (2000). Cadherins in the central nervous system. *Prog Neurobiol* **61**, 611-48.

Redies, C. and Puelles, L. (2001). Modularity in vertebrate brain development and evolution. *Bioessays* **23**, 1100-11.

Schneider-Maunoury, S., Seitanidou, T., Charnay, P. and Lumsden, A. (1997). Segmental and neuronal architecture of the hindbrain of Krox-20 mouse mutants. *Development* **124**, 1215-26.

Simpson, J. H., Bland, K. S., Fetter, R. D. and Goodman, C. S. (2000a). short-range and long-range guidance by Slit and it Robo receptors: a combinatorial code of Robo receptors controls lateral position. *Cell* **103**, 1019-1032.

Simpson, J. H., Kidd, T., Bland, K. S. and Goodman, C. S. (2000b). Short-range and long-range guidance by Slit and its Robo receptors: Robo and Robo2 play distinct roles in midline guidance. *Neuron* **28**.
Vegh, M. and Basler, K. (2003). A genetic screen for hedgehog targets involved in the maintenance of the Drosophila anteroposterior compartment boundary. *Genetics* **163**, 1427-38.

Wolff, T. (2000). Histological Techniques for the Drosophila Eye. Part II: Adult. In *Drosophila Protocols*, (ed. W. Sullivan M. Ashburner and R. S. Hawley), pp. 224-243. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Wong, K., Park, H. T., Wu, J. Y. and Rao, Y. (2002). Slit proteins: molecular guidance cues for cells ranging from neurons to leukocytes. *Curr Opin Genet Dev* **12**, 583-91.

Xu, Q., Mellitzer, G., Robinson, V. and Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* **399**, 267-71.

Xu, Q., Mellitzer, G. and Wilkinson, D. G. (2000). Roles of Eph receptors and ephrins in segmental patterning. *Philos Trans R Soc Lond B Biol Sci* **355**, 993-1002.

Younossi-Hartenstein, A., Nassif, C., Green, P. and Hartenstein, V. (1996). Early neurogenesis of the Drosophila brain. *J Comp Neurol* **370**, 313-29.

Younossi-Hartenstein, A., Salvaterra, P. M. and Hartenstein, V. (2003). Early development of the Drosophila brain: IV. Larval neuropile compartments defined by glial septa. *J Comp Neurol* **455**, 435-50.

Zeltser, L. M., Larsen, C. W. and Lumsden, A. (2001). A new developmental compartment in the forebrain regulated by Lunatic fringe. *Nat Neurosci* **4**, 683-4.

FIGURE LEGENDS

Fig. 1. Developing *Drosophila* visual system.

(A) Horizontal view of wild type third instar visual system (anterior to left) of animal expressing CD8:GFP under the control of *Sca-Gal4* (Sca:GFP). GFP is expressed in the outer proliferation center (OPC), inner proliferation center (IPC), medulla cortex (medc) and portions of the lobula cortex (lobc). Sca:GFP (green). Neuronal nuclei are visualized using anti-Elav (blue); photoreceptor axons, lamina monopolar axons, and axons from neurons of the lobula cortex (a subset of which contact the medulla neuropil) are visualized using anti-Fasciclin II (FasII) (red). Dotted line indicates anterior edge of lobula cortex. os:optic stalk, lamc: lamina cortex, lamn: lamina neuropil; medn:medulla neuropil. (B) Lateral view (anterior at bottom) of Sca:GFP animal in which neuronal nuclei have been visualized using anti-Elav (blue) and glial nuclei using anti-Repo (red). Schematics of (C) horizontal view and (D) lateral view indicating cell populations and axons described in text. ep: epithelial glia, ma: marginal glia, mg: medulla glia.

Fig. 2. *Slit* is required for optic lobe development.

(A,B, E-H) Third instar visual systems, photoreceptor axons visualized with anti-Chaoptin. (A) In wild type, photoreceptor axons grow into the brain through the optic stalk. The R1-R6 subset of photoreceptor axons stop in the lamina neuropil while R7 and R8 continue into the medulla neuropil. (B) In $slit^{dui}$ mutants, there are gaps in the lamina neuropil (arrow) and increased numbers of axons enter the medulla (arrowhead). (C)Wild type and (D) $slit^{dui}$ visual systems in which R2-R5 photoreceptor axons are visualized using Ro:tau-LacZ (as in Garrity et al., 1999). (C) In wild type, all R2-R5 axons stop in the lamina neuropil. (D) In $slit^{dui}$ mutants, many R2-R5 axons pass through the lamina and enter the medulla (arrowheads). (E) $slit^{dui}/slit^{l(2)k05248}$, (F) $slit^{dui}/slit^{E158}$ and (G) $slit^{dui}/slit^{2}$ animals show photoreceptor axon targeting defects indistinguishable from $slit^{dui}$ homozygotes with gaps in the lamina (arrow) and increased numbers of axons entering the medulla (arrowhead). (H) *Omb-Gal4;UAS-Slit;* $slit^{dui}/slit^{2}$ visual system. Slit cDNA expression controlled by *Omb-Gal4* largely rescues *slit* targeting defects, restoring even layer of photoreceptor growth cones in the lamina (arrow). (I,J) Animals carrying *loco:LacZ* enhancer trap (which is strongly expressed in epithelial (ep) and marginal (ma)

glia) stained with anti-LacZ. (I) In wild type, continuous layers of epithelial and marginal glia are observed in the lamina. (J) In *slit* mutants, there are clumps of glia (arrowhead) and gaps (arrow) in the glial layers. (K,L) Photoreceptors axons are visualized with GMR:GFP (green) and glial nuclei with Repo:LacZ (magenta). (K) In wild type, R1-R6 axons stop in the lamina between layers of glia (open arrowheads). (L) In *slit* mutants, there are gaps in the photoreceptor innervation of the lamina, correlated with regions of the lamina devoid of glia (asterisk) and uneven innervation in regions containing clumps of glia (arrow). The clear separation between glia at the base of the lamina and glia surrounding the medulla observed in wild type is missing in *slit* mutants (arrowhead).

Fig. 3. Distal cell neurons invade the lamina in *slit* mutants.
(A-D) Third instar visual systems in which IPC neuroblasts and distal cell neurons are visualized using anti-Fasciclin III (FasIII, red), photoreceptor axons using GMR:GFP (green), and neuronal nuclei using anti-Elav (blue). (A-B) Horizontal view (anterior to left). (A) In wild type, IPC neuroblasts (which express FasIII) and their distal cell neuron progeny (dcn, which express FasIII and Elav) are adjacent to the posterior edge of the lamina (arrowhead). (B) In $slit^{dui}/slit^{2}$ mutants, distal cell neurons enter the base of the lamina (arrow) and reach the lamina's anterior edge (arrowhead). Distal cell neurons also enter the neuropil of the lamina (asterisk) and photoreceptor innervation is disrupted. (C-D) Lateral view (anterior at bottom). (C) In wild type, distal cell neurons are immediately adjacent to the posterior face of the lamina. (D) In $slit^{dui}/slit^{2}$ mutants, distal cell neurons enter the posterior face of the lamina (arrow) and reach its anterior edge (arrowhead).

Fig. 4. Slit is expressed in the developing optic lobe.
(A,B) Third instar nervous system stained with anti-Slit. (A) Slit is expressed in the medulla neuropil (medn) and the base of the lamina (arrow), as well as ventral ganglion midline (vg) and mushroom bodies (mb). (B) In $slit^{dui}$ mutants, Slit expression is greatly reduced in the optic lobe and ventral ganglion, though robust mushroom body staining is still observed. (C) Slit mRNA is expressed by cells surrounding the medulla. (D,E) Third instar visual systems stained with anti-Slit (magenta) and anti-Repo (green). (D)

Slit protein is found throughout the medulla neuropil (arrow), which is surrounded by medulla neuropil glia (mng). (E) Slit is present in the lamina neuropil and surrounds the epithelial (ep), marginal (ma) and medulla glia (mg). (F) Similar view as in E, stained with anti-Slit (magenta) and Sca:GFP (green). (G) Horizontal view stained with anti-Slit (red), anti-Elav (blue), and Sca:GFP (green). Slit protein localizes immediately adjacent to distal cell neurons at the base of the lamina and the optic chiasm. (H) Summary of Slit expression. (I) Third instar *eya*^{2} mutant visual system stained with anti-Slit. Slit protein is expressed in the medulla neuropil (arrow) in the absence of photoreceptor innervation. (I-J) Expression of the *slit*$^{l(2)k05248}$ (Slit:LacZ) enhancer trap. (J, K) Optic lobes stained with anti-LacZ (red) and anti-Repo (blue). (J) Slit:LacZ is expressed in medulla glia (arrowhead) and cells in the medulla cortex (arrow). (K) Slit:LacZ cells in medulla cortex (arrow) lie adjacent to medulla neuropil glia. (L) Optic lobe stained with anti-LacZ (upper panel), anti-Elav (middle panel) and a merged image (lower panel) with anti-LacZ in magenta and anti-Elav in green. Slit:LacZ cells in medulla cortex (arrows) co-express varying levels of neuronal marker Elav.

Fig. 5. Robo, Robo2 and Robo3 are expressed in overlapping patterns in the visual system.

Panels A, D, and G show third instar nervous systems stained with antisera against indicated Robo-family member. Panels B, E, and H show lateral view of optic lobe stained with antisera against indicated Robo-family member (magenta), neuronal nuclei stained with anti-Elav (blue), and photoreceptor axons visualized with GMR:GFP (green). Panels C, F and I show Robo-family staining alone. (A) Robo is expressed in the developing optic lobes (arrowheads). (B,C) Robo is expressed by IPC neuroblasts, by distal cell neurons, and in the medulla cortex. (D) Robo2 is expressed in the developing optic lobes (arrowheads). (E,F) Robo2 is expressed by IPC neuroblasts and distal cell neurons. (G) Robo3 is expressed in the developing optic lobes (arrowheads). (H,I) Robo3 expression is not detected on IPC neuroblasts, but is detected on distal cell neurons and in the medulla cortex as well as on photoreceptor axons.

Fig. 6. Knockdown of Robo-family proteins using transgenic RNAi.

(A) Western blot analysis of adult heads showing that animal homozygous for *robo3*^{1} express full-length Robo3 protein (carat) as well as a truncated Robo3 (asterisk). Anti-Elav used as loading control. (B) Western blot analysis of adult heads showing that expression of *UAS:RoboRNAi* in the nervous system controlled by *c155-Gal4* reduces Robo protein levels. (C) Ubiquitous expression of *UAS:Robo2RNAi* under the control of *tubulin-Gal4* reduces anti-Robo2 staining in the visual system. (D) Western blot analysis of adult heads showing that expression of *UAS-Robo3RNAi* controlled by *c155-Gal4* reduces Robo3 protein levels. (E-M) RNAi of an individual Robo-family member does not detectably reduce expression of other Robo-family proteins. (E-G) Anti-Robo staining in magenta. (H-J) Anti-Robo2 expression in magenta. (K-M) Anti-Robo3 expression in magenta. (E, H and K) Robo RNAi detectably reduces Robo expression (open arrowhead), but not Robo2 or Robo3 (closed arrowheads). (F, I and L) Robo2 RNAi detectably reduces Robo2 expression (open arrowhead), but not Robo or Robo3 (closed arrowheads). (G, J and M) Robo3 RNAi detectably reduces Robo3 expression (open arrowhead), but not Robo or Robo2 (closed arrowheads).

Fig. 7. Distal cell neurons intermingle with lamina glia in Robo-family knockdowns. (A,B) Lateral view (anterior at bottom). IPC neuroblasts and distal cell neurons are visualized with FasIII (red), c155:GFP is most strongly observed in IPC neuroblasts and photoreceptors (green), and neuronal nuclei are visualized with anti-Elav (blue). (A) *c155-Gal4;UAS-GFP*. (B) Distal cell neurons enter the lamina in *c155-Gal4;UAS-GFP;UAS-RoboRNAi;UAS-Robo2RNAi;UAS-Robo3RNAi* animals (arrow), reaching anterior edge of lamina (arrowhead). (C-F) Animals express GFP (green) under control of *Sca-Gal4*, labeling the IPC, distal cell neurons and medulla cortex (medc). Neuronal nuclei are visualized with anti-Elav (blue). (C,D) Photoreceptor axons are visualized using anti-Chaoptin (red). (C) *Sca-Gal4;UAS-GFP* animal. (D) Distal cell neurons enter the lamina in *Sca-Gal4;UAS-GFP;UAS-RoboRNAi;UAS-Robo2RNAi;UAS-Robo3RNAi* animals (arrow). (E,F) Lamina glia are visualized using anti-Repo (red). Distal cell neurons intermingle (arrows) with lamina glia in Robo-family knockdown animals. (E) *Sca-Gal4;UAS-GFP*; *UAS-RoboRNAi,UAS-Robo3RNAi;UAS-RoboRNAi,UAS-Robo3RNAi* animal. (While *Sca-Gal4;UAS-RoboRNAi;UAS-*

Robo3RNAi animals had no defects, animals containing two copies of both *UAS-RoboRNAi* and *UAS-Robo3RNAi* had modest defects, consistent with overlapping roles of Robo-family members.) (F) *Sca-Gal4;UAS-GFP;UAS-RoboRNAi;UAS-Robo2RNAi;UAS-Robo3RNAi* animal.

Fig. 8. Robo-family knockdown disrupts photoreceptor axon targeting. (A-C) Photoreceptor axons visualized using anti-Chaoptin. (A) *Tubulin-Gal4;UAS-GFP* control. (B) *Tubulin-Gal4;UAS-GFP;UAS-RoboRNAi;UAS-Robo2RNAi;UAS-Robo3RNAi* animal where many photoreceptor axons extend through the lamina (arrow) and too many photoreceptor axons enter the medulla (arrowhead). (C) *GMR-Gal4;UAS-GFP;UAS-RoboRNAi;UAS-Robo2RNAi;UAS-Robo3RNAi* animal. (D-F) Animals express GFP (green) under control of *Sca-Gal4* while photoreceptor axons are visualized using anti-Chaoptin (magenta). (D) *Sca-Gal4;UAS-GFP* animal. (E) *Sca-Gal4;UAS-GFP*; *UAS-RoboRNAi,UAS-Robo3RNAi;UAS-RoboRNAi,UAS-Robo3RNAi* animal in which GFP-expressing cells in the lamina correspond to regions of photoreceptor axon mistargeting (arrow). (F) *Sca-Gal4;UAS-GFP;UAS-RoboRNAi;UAS-Robo2RNAi;UAS-Robo3RNAi* animal. (G) Schematic of observed disruptions in visual system development. In wild type, distal cell neurons (blue) express Robos (orange outline) while Slit protein (red) surrounds glia (yellow with black outline) at the base of the lamina. Lamina glia serve as initial targets of incoming R1-R6 photoreceptor axons (green). When expression of all three Robo-family members is inhibited in distal cell neurons *(robo,robo2,robo3)*, distal cell neurons intermingle with the lamina glia and photoreceptor axon targeting is disrupted. Loss of Slit expression (*slit*) causes an indistinguishable defect.

Figure 1

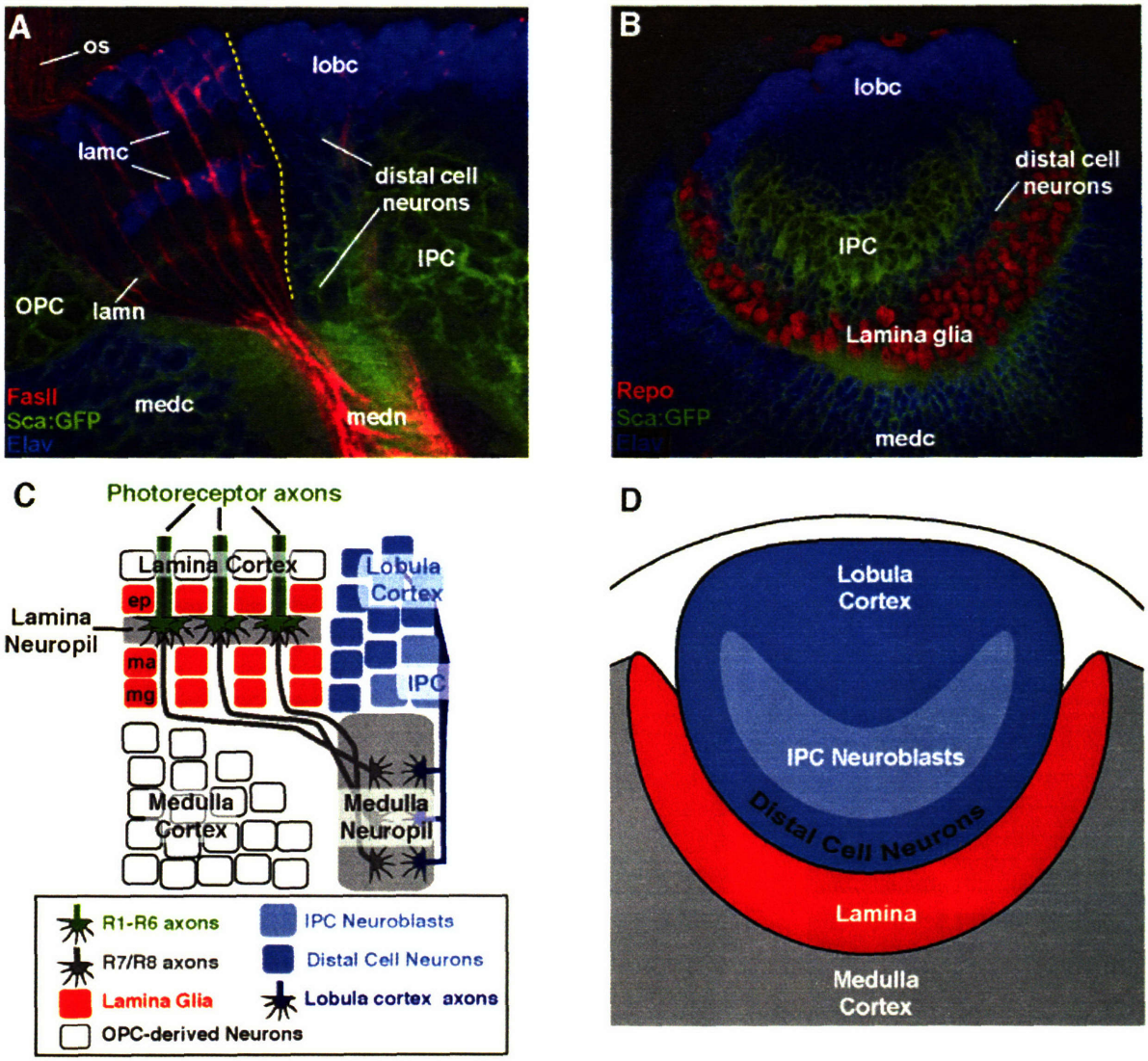
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Figure 2

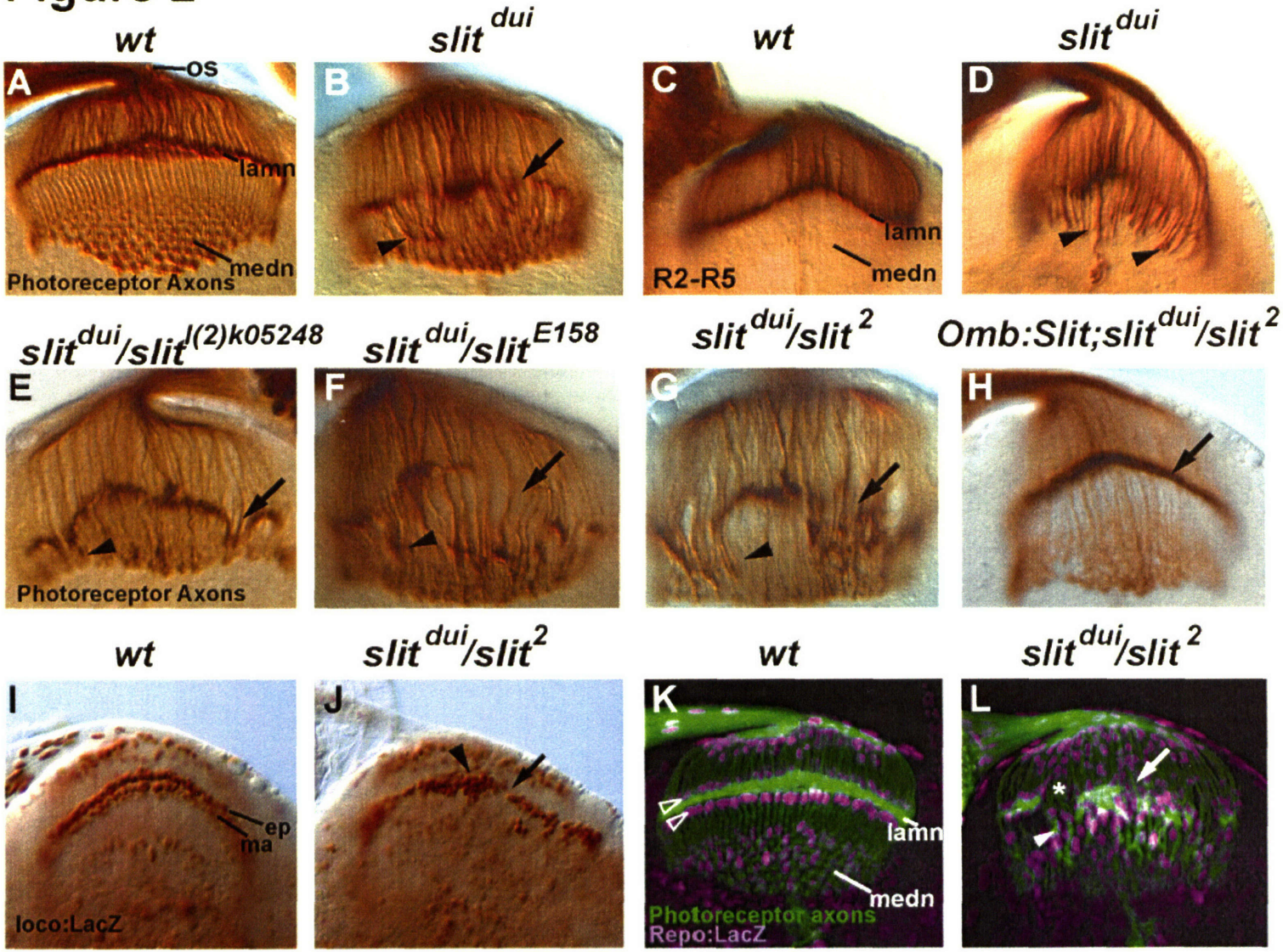
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Figure 3

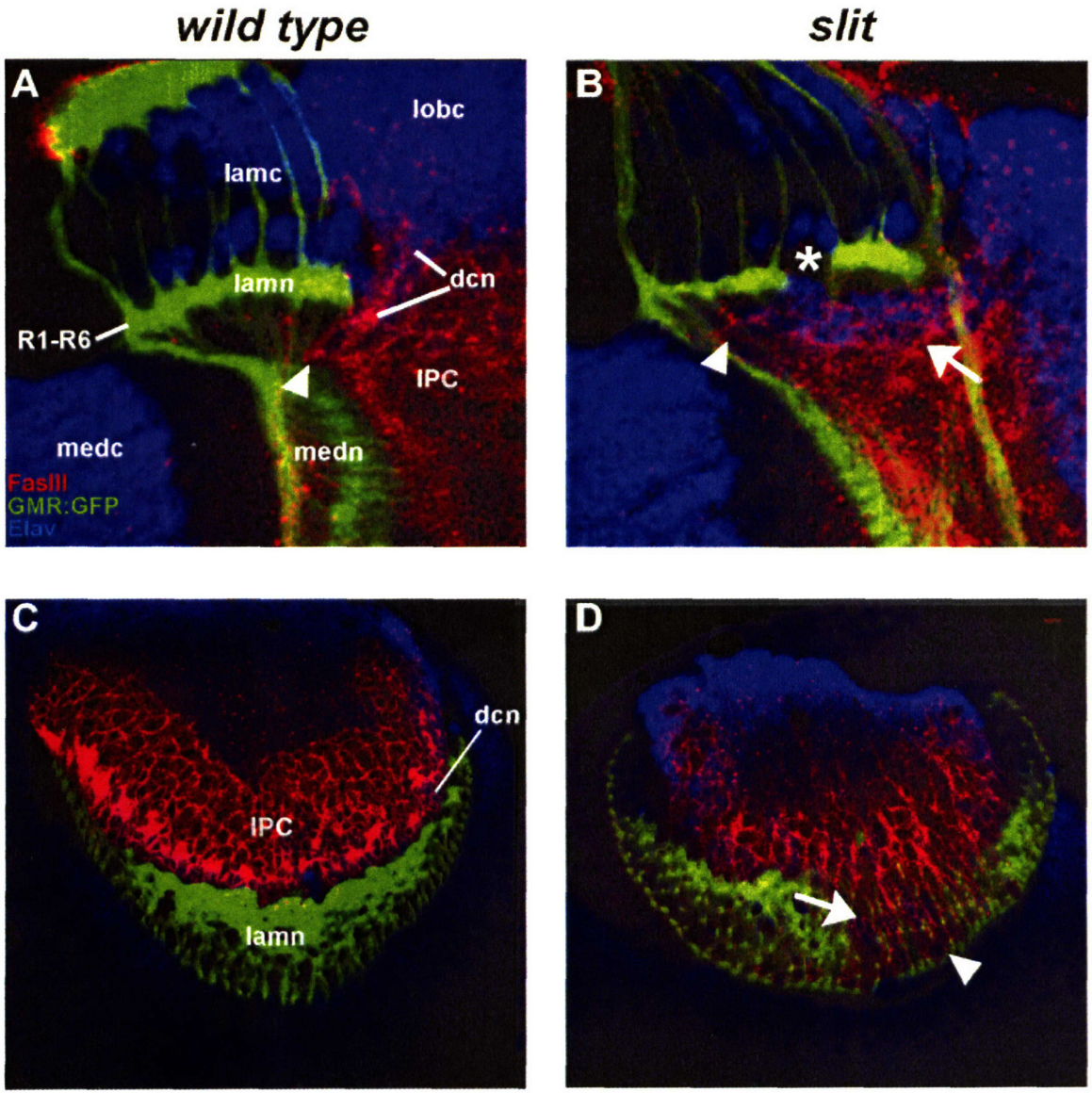
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Figure 4

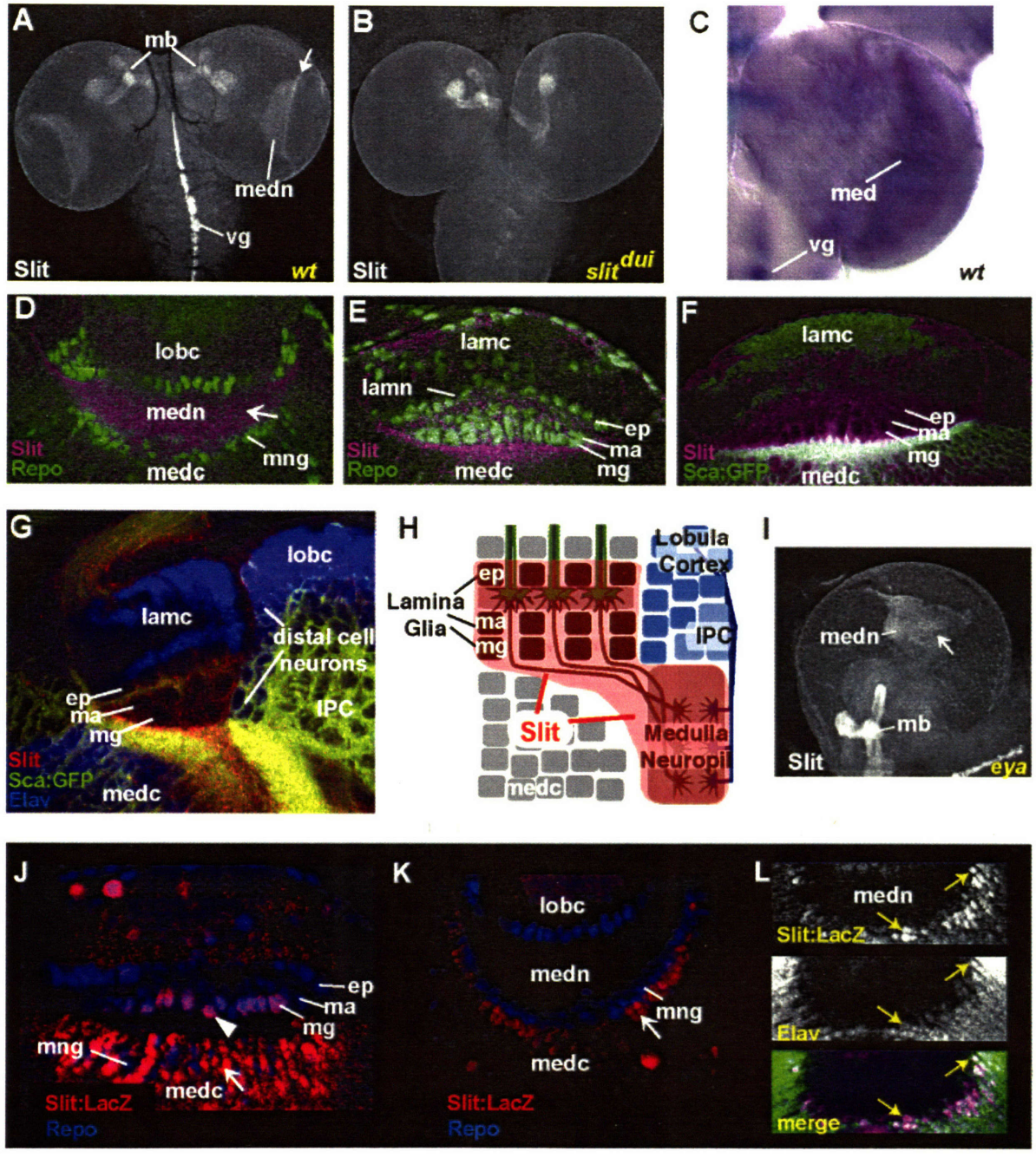
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Figure 5

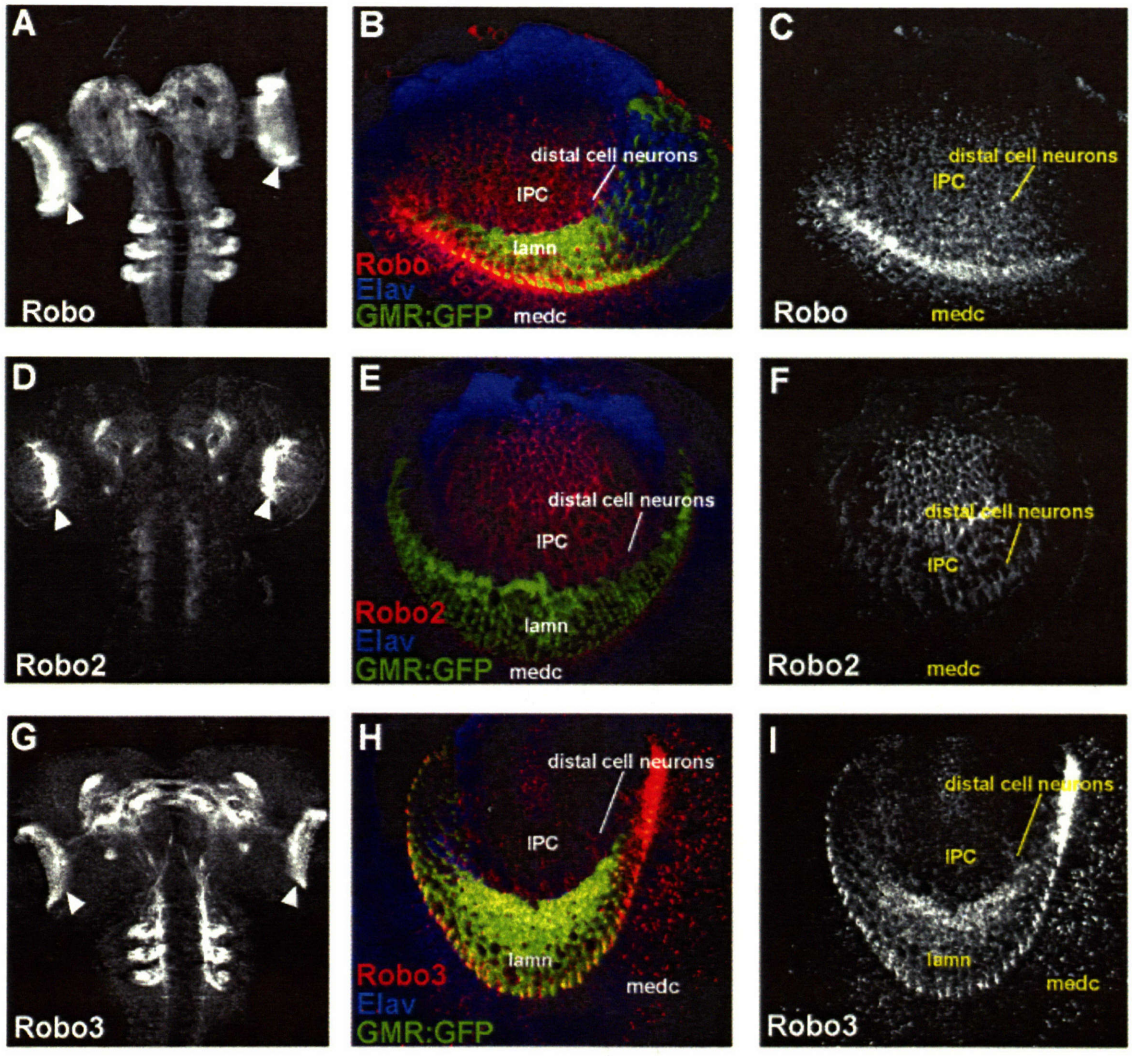
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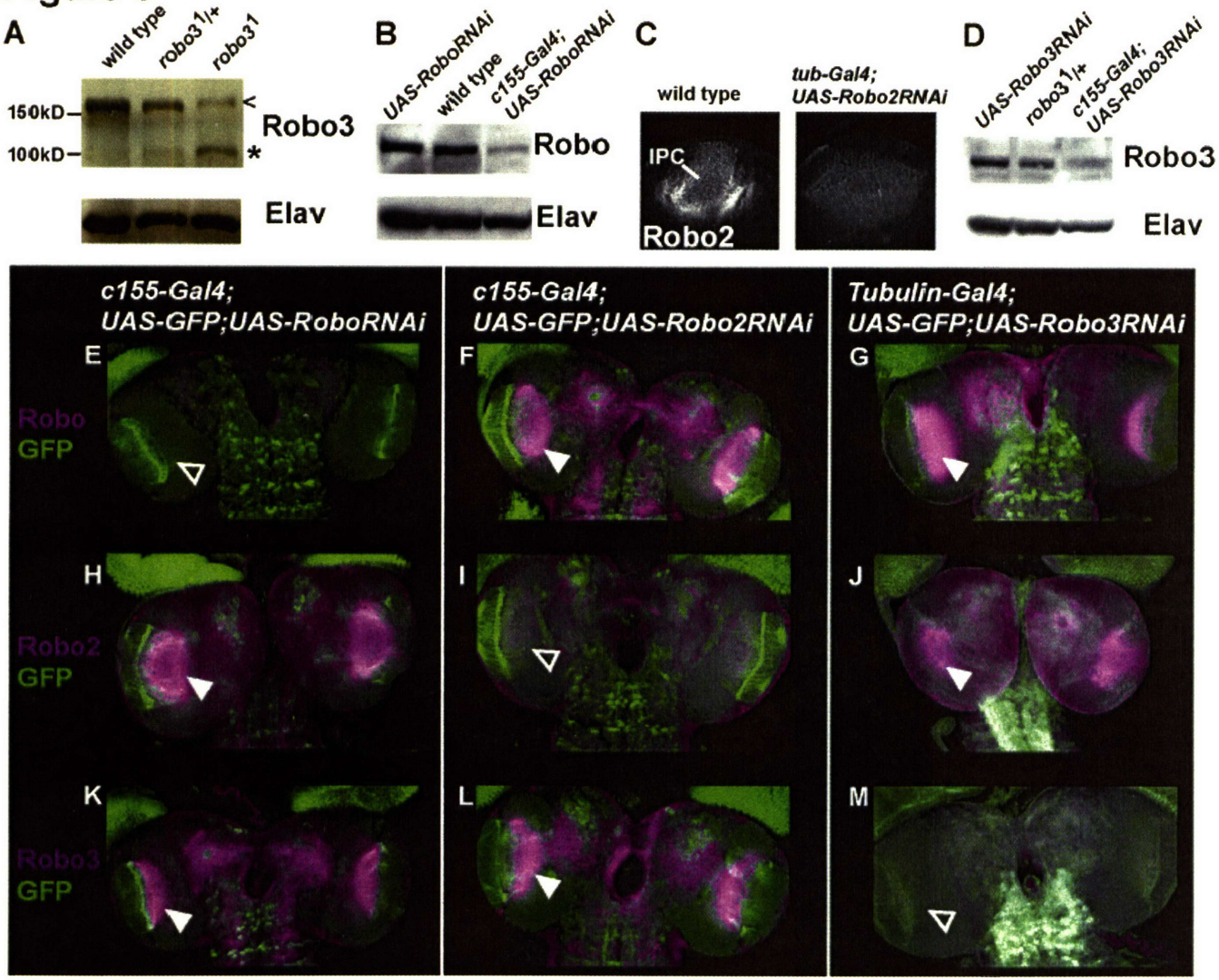
Figure 6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Robo
Elav
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Robo3
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Tubulin-Gal4; UAS-GFP;UAS-Robo3RNAi
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K
L
M
Robo3 GFP

Figure 7

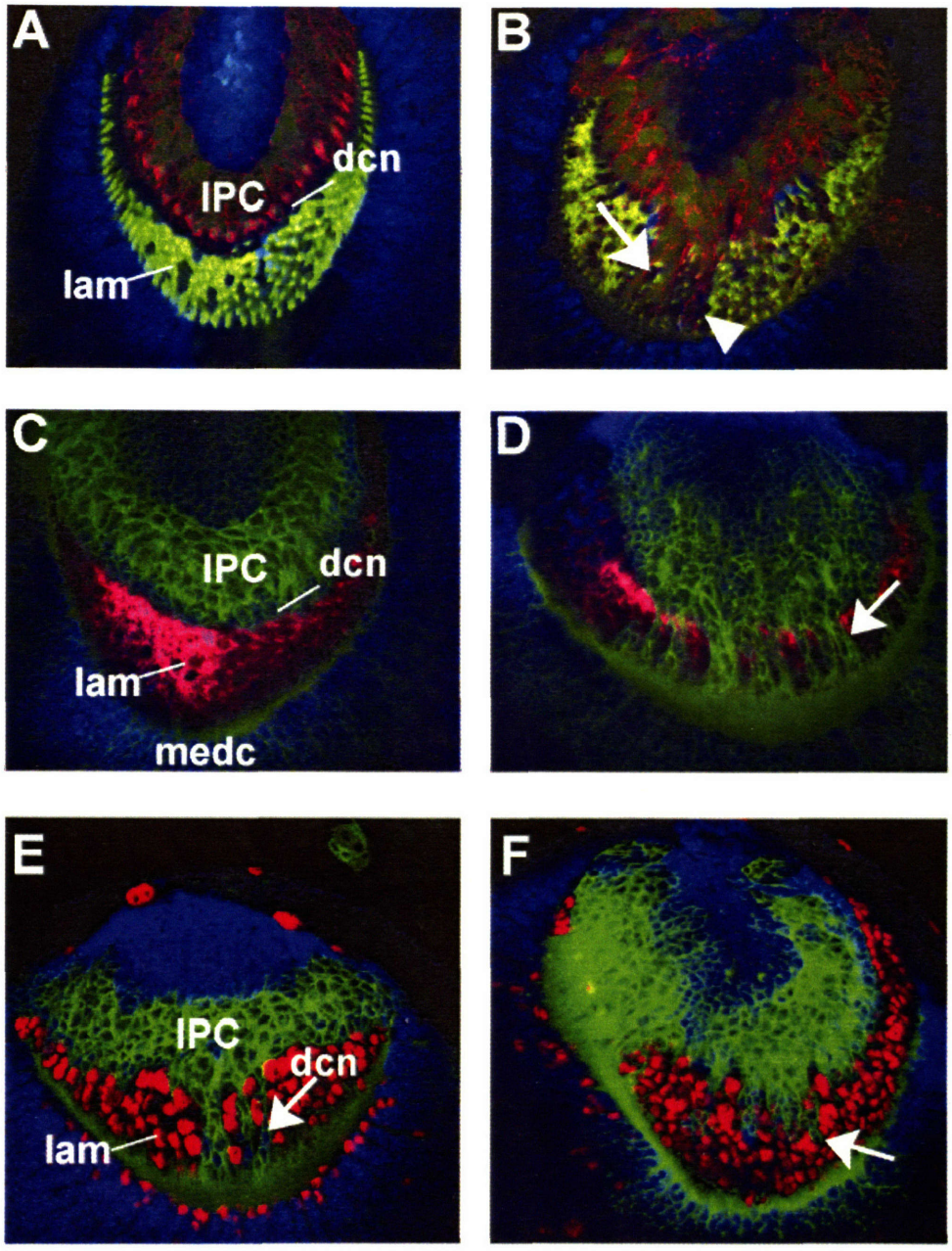
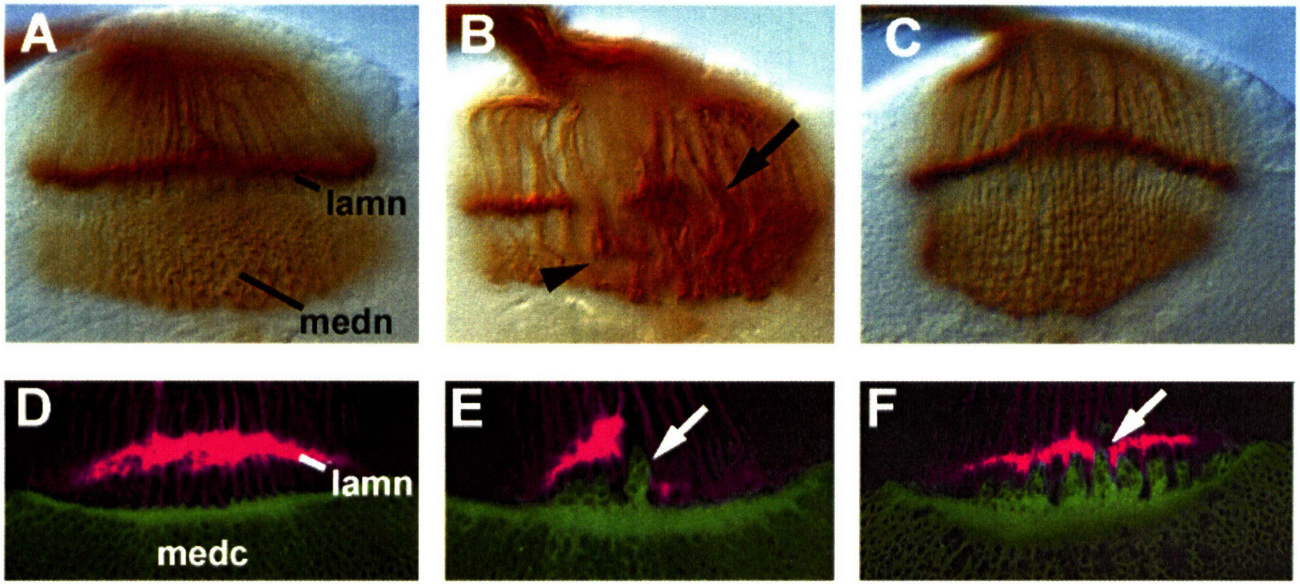
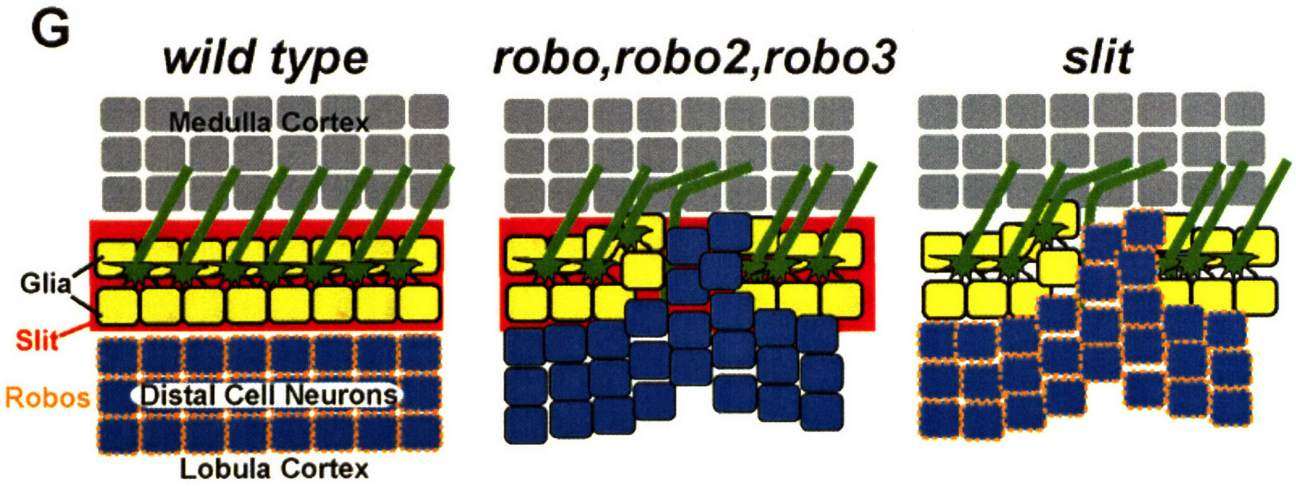


Figure 8


A
lamn
medn
B
C
D
lamn
medc
E
F

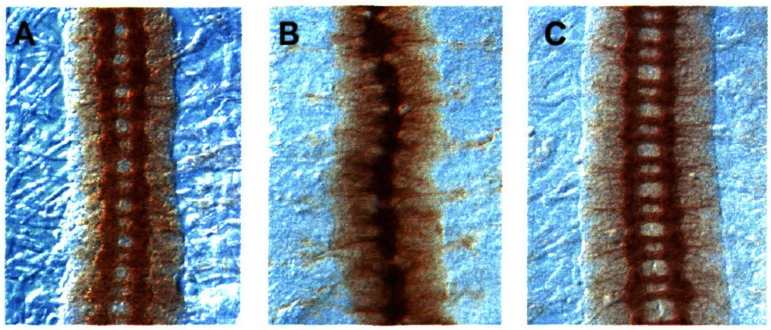

G
wild type
robo,robo2,robo3
slit
Medulla Cortex
Glia
Slit
Robos
Distal Cell Neurons
Lobula Cortex

Appendix to Chapter 2

The following section includes unpublished data and discussion pertaining to, but not included in Chapter 2.

Although the analysis of the visual system in the preceding chapter concentrates on the third larval instar stage of development, earlier and later stages of development were also examined. The developing CNS of wild type embryos has a characteristic appearance of a ladder-like scaffold when stained with BP102, a monoclonal antibody that labels all longitudinal and commissural axons (Fig. 1A). In *slit*^{2} null mutants, axons enter the midline and fail to exit. The result is a collapse of the CNS axon scaffold into a single longitudinal track within the midline (Fig. 1B). The CNS axon scaffold appears normal in *slit*^{dui} mutant embryos (Fig. 1C). Furthermore, whereas *slit*^{2} mutants die during late embryogenesis/early first larval instar, *slit*^{dui} mutants survive to adulthood. As mentioned in Chapter 2, the likely explanation for these differences is that the *slit*^{dui} allele functions as a tissue-specific allele of *slit*. This is most clearly seen in wild type third instar larvae, where Slit protein is prominently detected within the midline and optic lobe as well as on mushroom body axons. In *slit*^{dui} mutants, Slit protein is absent in the optic lobes, reduced

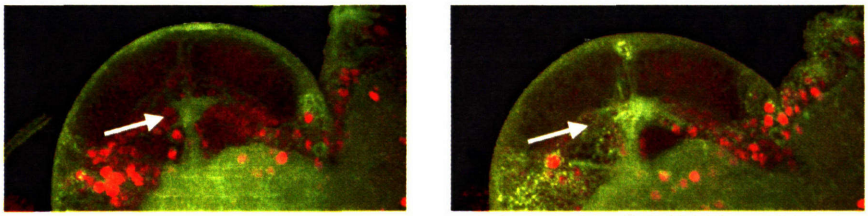
Figure1



in the midline, and present on mushroom body axons (See Chapter 2, Fig. 4A,B). Although significantly reduced, there is likely a sufficient level of Slit expression within the midline of *slit*^{dui} mutants to achieve normal embryonic CNS patterning.

Photoreceptor axons begin entering the brain at the beginning of the third larval instar. In order to examine the developing visual system of *slit* mutants before photoreceptor axon innervation, we used a number of markers, including anti-Robo2, anti-Fasciclin II, anti-HRP (a general neuropil marker) and the Slit:LacZ enhancer trap, to visualize the brains of second instar larvae. Figure 2 shows wild type and *slit* second instar larvae stained with anti-HRP (green) to visualize the medulla neuropil (arrow) and anti-LacZ (red) to visualize the Slit:LacZ enhancer trap pattern (at this stage of development the distal cell neurons and lamina neurons have not been generated). We were unable to detect any disruptions at this stage of development and thus focused our analysis on later stages.

Figure 2

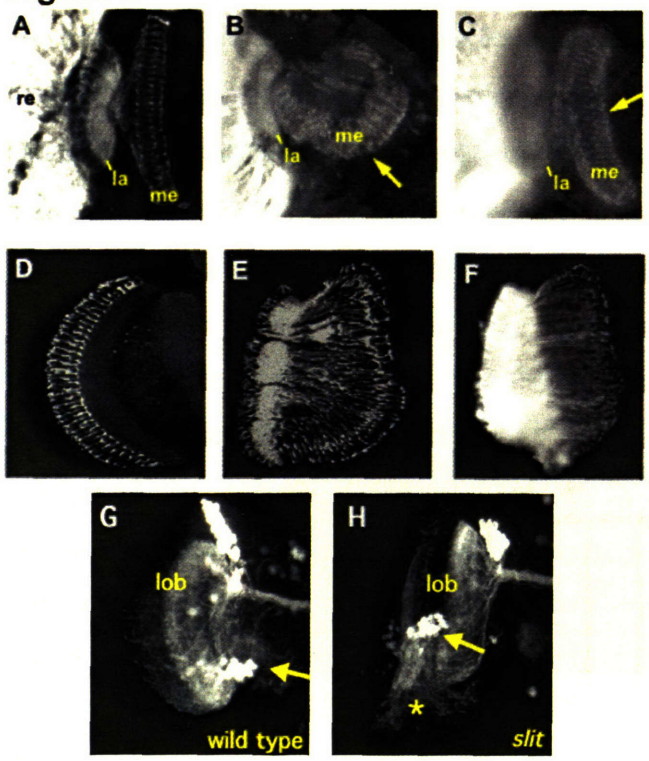


wild type ***slit***

The adult visual system in *slit* mutants and RoboRNAi animals has also been analyzed. Adult *slit* mutants exhibit striking defects in visual system morphogenesis, although it is not clear whether the adult defects are a consequence of the earlier third larval instar defects or represent another function for Slit and the Robo-family proteins. Cryosections of adult heads stained with anti-Chaoptin to visualize R-cells and their projections (Fig. 3A-C) reveal that the medulla (me) is misoriented with respect to the lamina (la) and retina (re) in *slit* mutants (Fig. 3B) as compared to wild type (Fig. 3A). This defect can be rescued by expressing a *slit* cDNA under the control of *omb-Gal4*, which drives expression broadly in the visual system (Fig 3C). Whole mount views of photoreceptor axon innervation of the medulla in wild type (Fig. 3D), *slit* mutants (Fig.

3E), and Robo-family RNAi knockdowns (Fig. 3F) show a similar disorganization of the medulla as seen in cryosections. The disruptions in adult *slit* mutants and adult Robo-family RNAi knockdowns are indistinguishable and provide further evidence that the Robo-family receptors and Slit are likely functioning to control the same developmental process.

Figure 3

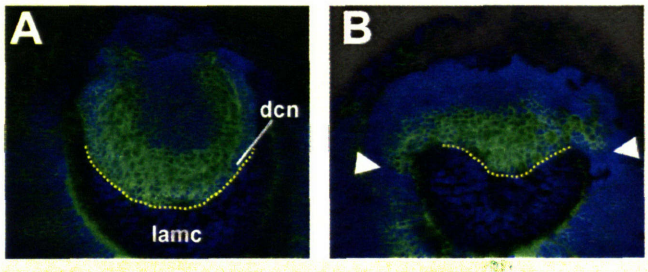


Examination of a subset of lobula (lob) neurons using *atonal-Gal4* (Hassan et al., 2000) to drive expression of a GFP reporter reveals defects in the positioning of ventral brain cluster (VBC) neurons (Fig. 3G,H, arrow). As compared to wild type (Fig. 3G), the VBC neurons in *slit* mutants (Fig. 3H) are located more centrally within the lobula. Additionally, the VBC axons project to regions outside the lobula in *slit* mutants (Fig. 3 H, asterisk). The primary cause of these defects is not presently known.

As noted in Chapter 2, overexpression of Robo2 in the lobula generates defects at the lamina/lobula interface. In wild type (Fig. 4A), the distal cell neurons (DCNs) normally lie at the posterior face (dashed yellow line) of the lamina cortex (lamc).

Overexpression of a *robo2* cDNA using *scabrous-Gal4* causes many DCNs to move away from the posterior face of the lamina toward the lamina edges (Fig. 4B, arrowheads). The cells in which *scabrous-Gal4* is expressed are labeled with UAS-mCD8-GFP and all neuronal nuclei are stained with anti-Elav (blue). One interpretation of this experiment is that increased levels of Robo2 in DCNs results in increased repulsion of DCNs from the posterior face of the lamina where they first encounter Slit. A caveat to this experiment is that *scabrous-Gal4* is also expressed in regions of the visual system where Robo2 protein is not detected. Therefore, it cannot be ruled out that misexpression of Robo2 in other regions of the visual system contributes to this phenotype.

Figure 4

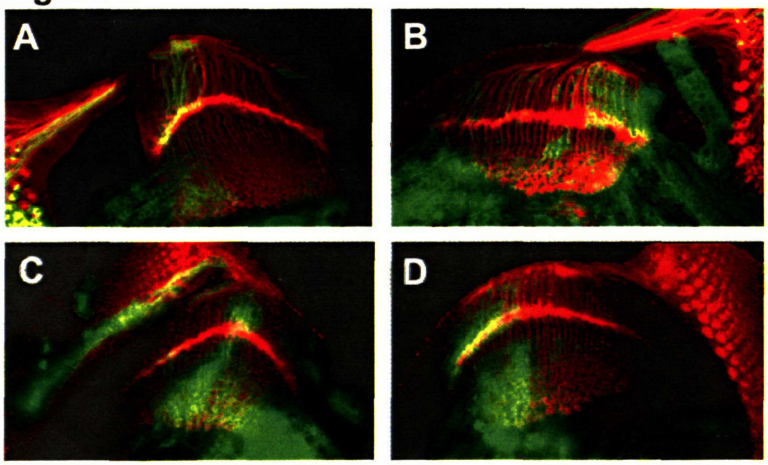


As mentioned in Chapter 2, to examine the effect of the reduction of Robo-family members in the visual system, I generated positively marked (GFP) *robo* mutant clones in a homozygous *robo3* mutant background (Fig. 5A,B). More than 40 third instar brain hemispheres were examined, however no photoreceptor axon disruptions were observed when examined with anti-Chaoptin (red). Interestingly, strong knockdown of Robo and Robo3 through RNAi does result in visual system disruptions (See Chapter 2, Fig. 7E). The discrepancy is likely due to the fact that *robo3* allele used in this study is hypomorphic (See Chapter 2, Figure 6A). The DCNs were not examined, as these experiments were performed before I had discovered that the DCNs were disrupted in $slit^{dui}$ mutants.

I also generated positively marked clones (GFP) of a null allele of *slit* ($slit^{2}$) (Fig. 5C,D). No photoreceptor axon disruptions were observed in the more than 50 third instar

brain hemispheres examined. A possible explanation for this result is that the mutant patches were not of sufficient size to generate phenotypes such as those seen in *slit*^{dui}. Slit is a secreted molecule and therefore diffusion of Slit from wild type tissue into regions where mutant patches were generated could compensate for loss of Slit in those regions. The MARCM system was used to generate both *robo* mutant clones (Fig. 5A,B) and *slit* mutant clones (Fig. 5C,D) (Lee and Luo, 1999).

Figure 5



The transposable element that is responsible for the *slit*^{dui} mutation is located approximately 30 kilobases away from the *slit* coding region. Although it is not uncommon for tissue-specific enhancers to be located long distances from the promoter of the regulated gene (Dorsett, 1999; Levine and Tjian, 2003; Merli et al., 1996), one concern is that the transposable element could be disrupting the expression of other genes in addition to *slit*. However, the ability to fully rescue the mutant phenotype with a *slit* cDNA (See Chapter 2, Figure 2H) argues that even if another gene is disrupted by the transposable element, it likely does not contribute to the mutant phenotype that we observe in the visual system.

The cDNA used to rescue the phenotype has been previously described (Kidd et al., 1999). Briefly, a *slit* cDNA was isolated from a *Drosophila* embryonic library and the entire open reading frame was sequenced, cloned into a UAS expression vector and subsequently transformed into flies. I expressed the *slit* cDNA under control of an eye-

specific promoter (*GMR-Gal4*) to verify that the expression construct generated Slit protein. As shown below in Figure 6 (arrows), high levels of Slit protein are detected in the eye disc and on R-cell axons where Slit is normally not expressed. Endogenous expression of Slit is also seen on mushroom bodies (MBs) and within the midline of the ventral nerve cord (VNC).

Figure 6

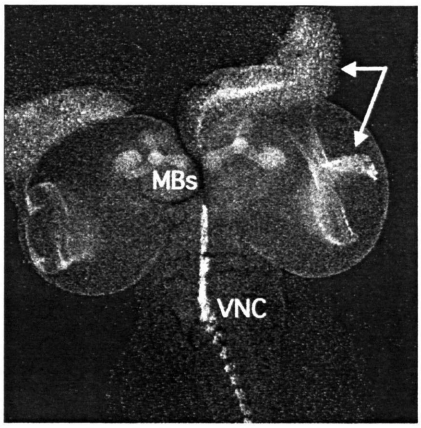
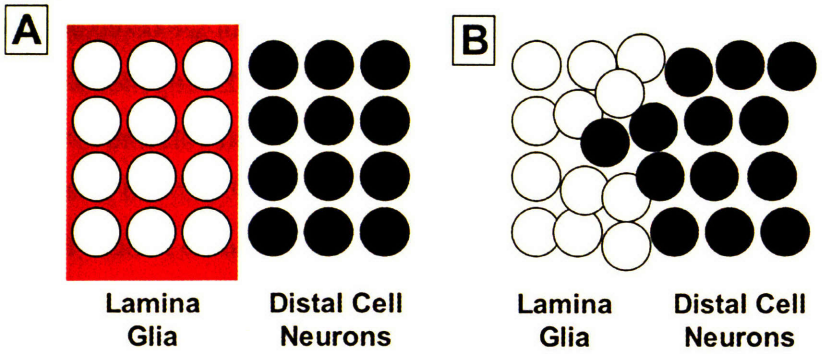


Figure 7 depicts a simplified model of the primary defect seen in the visual system of *slit*^{dui} mutants. In wild type (Fig. 7A), Slit protein is present within the lamina (red) and surrounds lamina glia (white circles). All three Robo-family proteins are expressed on the surface of distal cell neurons (DCNs) in the lobula (black circles). Normally, there is a distinct boundary between lamina glia and DCNs and these cells do not mix. In *slit*^{dui} mutants (Fig. 7B), Slit protein localization within the lamina is abolished, resulting in the invasion of DCNs into the lamina. The DCNs appear to ignore the boundary that is normally enforced by Slit and enter the lamina, displacing lamina glia. The displacement of lamina glia leads to a disruption in photoreceptor axon targeting (see Chapter 2, Figure 8G). The identical phenotype is also seen in animals in which the expression of Robo-family proteins is inhibited in DCNs.

Figure 7



References

Dorsett, D. (1999). Distant liaisons: long-range enhancer-promoter interactions in Drosophila. *Curr Opin Genet Dev* **9**, 505-14.

Hassan, B. A., Bermingham, N. A., He, Y., Sun, Y., Jan, Y. N., Zoghbi, H. Y. and Bellen, H. J. (2000). atonal regulates neurite arborization but does not act as a proneural gene in the Drosophila brain. *Neuron* **25**, 549-61.

Kidd, T., Bland, K. S. and Goodman, C. S. (1999). Slit is the midline repellent for the robo receptor in Drosophila. *Cell* **96**, 785-94.

Lee, T. and Luo, L. (1999). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-61.

Levine, M. and Tjian, R. (2003). Transcription regulation and animal diversity. *Nature* **424**, 147-51.

Merli, C., Bergstrom, D. E., Cygan, J. A. and Blackman, R. K. (1996). Promoter specificity mediates the independent regulation of neighboring genes. *Genes Dev* **10**, 1260-70.

CHAPTER THREE

Ptpmeg is required for Mushroom Body Morphogenesis in *Drosophila*

A number of individuals have contributed significantly to the initial characterization of *Ptpmeg*. Joyce Yang, a former graduate student in the lab, created the targeted disruption through homologous recombination. Three former lab technicians also contributed to the work. Myles Robichaux isolated the replacement allele and constructed the *UAS-PtpmegRNAi* transgene. Monique Brouillette analyzed the replacement allele by PCR and sequence analysis. Caleb Kennedy generated the peptide that was used to generate the polyclonal antibody. Paul Garrity originally identified the adult mushroom body phenotype. Timothy Tayler completed all other experiments. Jessica Whited, a current graduate student, is continuing the project.

SUMMARY

Drosophila Ptpmeg belongs to a family of highly conserved protein tyrosine phosphatases (PTPs) that contain an N-terminus FERM domain and a single PDZ domain. Although Ptpmeg homologs are expressed in the nervous system the physiological function of this class of proteins in the nervous system remains largely unknown. To study the role of Ptpmeg in *Drosophila*, we employed a targeted gene knockout strategy. Through homologous recombination, we introduced a disruption in the *Ptpmeg* locus that is predicted to generate a truncated form of the protein. The *Ptpmeg* mutant alleles are homozygous viable and express no visible external phenotypes. Although the homozygous mutants live to adulthood, they often fall into the food and die shortly after hatching. Examination of the adult brain revealed a mutant phenotype in the mushroom bodies, an important center for olfactory learning in *Drosophila*. In mutant animals, the mushroom body axonal structures are disorganized and fail to elaborate their characteristic shape. Ptpmeg is expressed on mushroom body axons and reducing levels of Ptpmeg protein from the mushroom bodies through tissue-specific RNAi generates the mutant phenotype. We propose that Ptpmeg is required in mushroom body neurons for proper mushroom body axon guidance and branching.

INTRODUCTION

Protein tyrosine phosphatases (PTPs) are signal-transducing enzymes that dephosphorylate proteins at tyrosine residues. In a balance with protein tyrosine kinases (PTKs), which catalyze the opposite reaction, PTPs regulate the level of protein tyrosine phosphorylation in cells. Regulation of tyrosine phosphorylation is important in a wide variety of cellular processes, including differentiation, proliferation, and cell growth, cell adhesion and cell-cell contact formation, and cellular metabolism (Neel and Tonks, 1997). Further, disruptions in tyrosine phosphorylation signaling can lead to a number of disease states (Hunter, 1997; Zhang, 2001). Depending on their cellular location, PTPs are classified as cytosolic or receptor-like (Tonks and Neel, 2001). The cytosolic PTPs contain a variety of non-catalytic protein domains, some of which are thought to regulate localization to the correct subcellular compartment (Fischer, 1999; Mauro and Dixon, 1994).

FERM domains (named for prominent family members 4.1 protein/Ezrin/Radixin/ Moesin) are found in a variety of cytoplasmic proteins that are involved in the control of cell adhesion, cell motility, cell shape and signal transduction (Bretscher et al., 2002). FERM domains participate in localizing proteins to the plasma membrane (Chishti et al., 1998). FERM domains have been shown to localize to the cytoplasmic surface of the plasma membrane and bind to PIP_2 (phosphatidylinositol 4,5-bisphosphate) and phosphatidylserine (Cohen et al., 1988; Hirao et al., 1996). A number of FERM proteins have been shown to function as membrane-cytoskeleton linkers (Chishti et al., 1998).

FERM-domain containing PTPs (FERM-PTPs) appear to fall into three classes based on their phylogeny and are generally designated as MEG, PEZ and BAS (Bretscher et al., 2002; Edwards et al., 2001). The MEG class has two members in vertebrates (Gu et al., 1991; Yang and Tonks, 1991), and a single representative in both *C. elegans* (Uchida et al., 2002) and *Drosophila* (Edwards et al., 2001). MEG proteins also contain a single PDZ domain which are commonly known to mediate protein-protein interactions(Kim and Sheng, 2004).

The MEG family member in *C. elegans*, PTP-FERM, is expressed in the nervous system and requires the FERM domain for its subcellular localization to the plasma membrane (Uchida et al., 2002). However, no loss-of-function mutants have been analyzed and the physiological role of this protein in the nervous system is not understood. In vertebrates, MEG family members have been shown to be expressed prominently in the brain and T-cells (Gjorloff-Wingren et al., 2000; Hironaka et al., 2000; Sahin et al., 1995). Both PTPN3 and PTPN4 have been shown to be located in membrane and cytoskeletal fractions and expressed in numerous cancer cell lines (Gjorloff-Wingren et al., 2000; Gu et al., 1991; Ikuta et al., 1994; Wang et al., 2004b; Warabi et al., 2000). PTPN4 was originally isolated as a cytosolic PTP from human megakaryoblast cell lines (Gu et al., 1991). PTPN4 is expressed in the mouse brain and physically associates with the glutamate receptor subunits delta-2 and epsilon (Hironaka et al., 2000). Overexpression of PTPN4 in cultured COS-7 cells, slowed proliferation and cell growth (Gu et al., 1996). PTPN3 was isolated from a HeLa cell library and has been shown to disrupt T-cell signaling when overexpressed in cultured cells (Han et al., 2000; Yang and Tonks, 1991). Although they have been predicted to have a role in regulating signaling at the plasma membrane, the physiological function of this class of proteins in the nervous system remains largely unknown.

In the present work, we identify *Ptpmeg* as a novel regulator of *Drosophila* mushroom body neurogenesis. The pattern of Ptpmeg protein expression and tissue-specific reduction of Ptpmeg suggest a role for this protein in mushroom body axon guidance and branching. The *Drosophila* mushroom bodies are a bilaterally symmetric structure in the central brain and play a key role in olfactory learning and memory (Heisenberg, 2003). Each mushroom body is derived from four neuroblasts, and by divisions through the embryonic, larval and pupal stages, each neuroblast generates ~500 neurons (Ito et al., 1997; Ito and Hotta, 1992), which can be classified into three types based on adult axonal projection patterns and the temporal order in which they are born. Axons extend from the mushroom body neuron and form a tight bundle known as the peduncle. At the base of the peduncle axons branch into dorsal and medial lobes. Neurons that form the γ lobe are born beginning in the late embryonic stages, neurons

that form the α' and β' lobes are born during larval development and lastly the neurons that form the α and β lobe are born after puparian formation (Ito et al., 1998; Lee et al., 1999). Mushroom body morphogenesis has been well described and more recently the mushroom bodies have become a useful system in which to study various aspects of neuronal development, including the growth, guidance and branching of axons (Ng et al., 2002). Further, a number of molecules have been identified that directly regulate mushroom body connectivity, including the cell adhesion molecules Dscam (Wang et al., 2004a; Wang et al., 2002; Zhan et al., 2004) and Flamingo (Reuter et al., 2003), members of the Rho family of small GTPases (Lee et al., 2000; Ng et al., 2002) as well as the Rho GTPase activating proteins (RhoGAPs) (Billuart et al., 2001).

RESULTS

Targeted disruption of the *Ptpmeg* locus

To initiate our investigation of the function of *Ptpmeg*, we disrupted the genetic locus by homologous recombination (Rong and Golic, 2000; Rong and Golic, 2001). The homologous recombination event resulted in a partial duplication of the *Ptpmeg* locus (Fig. 1A). To generate the *Ptpmeg* replacement allele (Gong and Golic, 2003), we genetically introduced a DNA double-stranded break with an inducible I-*Cre*I transgene. The homologous recombination vector had been engineered to include an I-*Cre*I restriction site and initiation of the DNA double-stranded break repair mechanism resulted in more than five putative *Ptpmeg* replacement alleles (Fig. 1A). In addition to the I-*Cre*I restriction site, the homologous recombination vector contained a four base pair insertion to create a frameshift mutation within the *Ptpmeg* coding region. The frameshift mutation is predicted to generate a truncated form of the Ptpmeg protein (see Fig. 2A).

Several replacement events were examined by PCR, sequence analysis and Southern blotting (Fig. 1B,C,D). The *Ptpmeg* replacement allele contained a new *Bsa*I restriction site and abolished an *Acc65*I restriction site. We first used a PCR-based assay to determine the presence or absence of these sites. As predicted, restriction digests with *Acc*65I generated two fragments in wild type, three fragments in *Ptpmeg* heterozygotes, and a single fragment in *Ptpmeg* homozygotes, while restriction digests with *Bsa*I generated one, three and two fragments in wild type, *Ptpmeg* heterozygotes and *Ptpmeg* homozygotes, respectively (Fig. 1B). Southern blot analysis also revealed the destruction of the Acc65I site and showed that no major genomic rearrangements had occurred within an ~4Kb region that included the site of homologous recombination and gene replacement (Fig. 1D). Thus, these analyses demonstrated that a targeted *Ptpmeg* replacement allele had been generated and the genomic region surrounding the locus was largely undisturbed.

Ptpmeg structure

The *Ptpmeg* gene, also known as *CG1228*, is predicted to have at least four transcripts, encoding proteins of 952 or 791 amino acids. Ptpmeg transcripts are present in cDNA

libraries from S2 cell, embryos, adult heads and testis (Berkeley *Drosophila* Genome Project). Ptpmeg is a highly conserved protein and the two closest human homologs, PTPN3 and PTPN4, share 32% and 38% overall amino acid identity respectively (Fig. 2B). Ptpmeg is composed of an N-terminal FERM domain, a PDZ-binding motif, and a C-terminal protein tyrosine phosphatase domain (PTP) (Fig. 2A). We generated antibodies to the central region of Ptpmeg, and found by immunohistochemistry that Ptpmeg protein is not detected in *Ptpmeg* mutants and is reduced in *Ptpmeg* heterozygotes (data not shown). Western blots probed with anti-Ptpmeg antiserum detect a single major protein species in wild type flies, which was absent in mutant flies (Fig. 2C)

Ptpmeg is required for Mushroom Body development

Homozygous *Ptpmeg* mutants are viable and fertile. Adults display no visible external defects, although many exhibit uncoordinated locomotion (J.C.Y., T.D.T. and P.A.G., unpublished). This observation prompted a histological investigation of the *Ptpmeg* mutant nervous system. Using the axonal marker, anti-Fasciclin 2 (1D4), we identified a defect in the mushroom bodies of the adult brain (Fig 3). In wild type adults the mushroom bodies form prominent bilateral structures in the protocerebrum. Each mushroom body is composed of ~2500 Kenyon cells, divided into three classes based on their axonal projection patterns into the mushroom body lobes and their time of birth. Tightly bundled axons form 5 distinct lobes in each hemisphere of the brain, γ, α, α', β, and β'. In the adult, anti-Fasciclin 2 strongly labels the axons of α/β neurons, the latest born Kenyon cells. These cells arise during puparian formation and project their bifurcated axons into dorsally-extending α lobes and medially-extending β lobes. In addition, anti-Fasciclin 2 weakly stains γ lobe axons. γ neurons are born during embryonic development and after remodeling events during development send a single axon into the medially-extending γ lobe.

We identified several common mushroom body defects in *Ptpmeg* mutants, most notably, the reduction or loss of α lobe axons (Fig 3A,B arrows) and the fusion of β lobe axons at the midline (Fig 3B arrowhead). α lobe reduction or loss was seen in 32.5% of mutants, β lobe fusion was seen in 27.5% of mutants, both α lobe reduction and β lobe

fusion was seen in 12.5% of mutants, while the remaining 12.5% of mutants appeared wild type (n=40). An increase in β lobe size was also commonly observed (Fig 3B arrowhead) but was not quantified. Although γ lobe defects were detected, the weak and variable staining of γ lobe axons by anti-Fasciclin 2 prevented an accurate assessment of the phenotype (T.D.T. and P.A.G., unpublished).

Using our antibody to Ptpmeg, we examined the localization of Ptpmeg within the adult brain. While we observed Ptpmeg expression in other parts of the brain, strong expression was detected on cells that appeared to associate with the mushroom bodies and on the mushroom body lobes (Fig. 6A,B). Mushroom body expression of Ptpmeg appears to be limited to the axonal lobes (Fig. 6B arrow and arrowhead). There is no detectable staining on the mushroom body cell bodies or calyx (Fig. 6B asterisk). Thus, it appears that Ptpmeg is expressed in mushroom bodies and preferentially localizes to axons.

Ptpmeg is required in neurons for α and β lobe targeting

To further address the role of Ptpmeg in mushroom body morphogenesis, we used transgenic RNAi to inhibit expression of Ptpmeg. Double-stranded RNA interference (RNAi) has been used effectively to reduce protein expression *in vitro* and *in vivo* (Caplen et al., 2000; Fire et al., 1998). The generation of a transgenic *UAS-PtpmegRNAi* construct allowed us to knockdown expression of Ptpmeg in a tissue-specific fashion. First we used tissue-specific RNAi to inhibit the expression of Ptpmeg in neurons. The nervous-system specific *c155-Gal4* was used to drive expression of the *UAS-PtpmegRNAi* construct. Expression of *UAS-PtpmegRNAi* under the control of *c155-Gal4* resulted in mushroom body defects similar to those seen in *Ptpmeg* mutant animals (Fig. 4). As in *Ptpmeg* mutants, β lobe defects included midline fusion and crossing (Fig. 4B,C arrowheads) while α lobe defects included reduction or absence (Fig. 4C). In several instances, the γ lobes appeared to fuse with the ellipsoid body structure (Fig. 4B, asterisk) or be misplace (Fig. 4C, asterisk). Of the 12 animals scored, 8 had detectable phenotypes (γ lobe phenotypes were not scored).

Although, the phenotype of *c155-Gal4*; *UAS-PtpmegRNAi* animals strongly resembles that of the *Ptpmeg* mutants, other cell types, such as glia or trachea, could not

be formally excluded from contributing to the mutant phenotype. We therefore expressed a *Ptpmeg* cDNA only in neurons under the control of *elav-Gal4*. The expression of this transgene rescued the mutant phenotype (data not shown). Expression of the *Ptpmeg* cDNA in the neurons of *Ptpmeg* mutants rescued the alpha lobe defects in 12 of 16 animals. The β lobe defects could not be scored due to a dominant mushroom body phenotype associated with the deficiency line used in the genetic cross. No mutant phenotypes have been observed in *Ptpmeg* heterozygous animals (n=8, unpublished data). Based on the phenotypes of the targeted knockout and RNAi knockdown experiments and based on our rescue experiments, we conclude that Ptpmeg is required within neurons for the proper establishment of the axonal lobe structures of the mushroom bodies.

Ptpmeg knockdown in mushroom body neurons disrupts α and β lobes

The identification of a neuronal function for *Ptpmeg* prompted further investigation of the neuronal class that required *Ptpmeg* activity. *OK107-Gal4* drives high levels of expression in all mushroom body neurons (Connolly et al., 1996). *OK107-Gal4* driven expression of *UAS-PtpmegRNAi* in *Ptpmeg* heterozygous animals results in strong α and β lobe phenotypes (Fig. 5). UAS-mCD8-GFP was used to visualize the entire mushroom body structure and anti-Fasciclin 2 was used to mark only the α, β, and γ lobes. We observed either an absence of both the α and α' lobe with a corresponding increase in the size of the β and β' lobes (Fig. 5A,B), or an absence of the β/β' lobes with a concomitant increase in size of the α/α' lobes (Figs. 5C and 5D). We did not observe similar defects when *UAS-PtpmegRNAi* was driven by *OK107-Gal4* in a wild type background. It is apparently necessary to reduce levels of Ptpmeg protein further than what can be achieved using *UAS-PtpmegRNAi* and *OK107-Gal4* alone. Finally, in OK107-Gal4, UAS-Ptpmeg-RNAi animals that are heterozygous for *Ptpmeg*, we observed a strong reduction in Ptpmeg staining (Fig 6C,D). In wild type adults, mushroom body axons bifurcate and send projections into two lobes. (The exception is γ neurons, which send a single projection into the γ lobe.) These data suggest that in *Ptpmeg* mutants, α and β axons often fail to branch. For instance, a missing or reduced α lobe often results in a corresponding size increase in the β lobes (See Figs. 3B,4B,5A).

DISCUSSION

In the present study, we have identified Ptpmeg, a FERM-containing protein tyrosine phosphatase, as a novel regulator of mushroom body development in *Drosophila.* Disruptions in mushroom body connectivity have been shown to affect the process of olfactory associative learning and memory (de Belle and Heisenberg, 1994; Heisenberg et al., 1985; Pascual and Preat, 2001). For instance, long-term memory requires the presence of the α/α' lobes but not the β/β' lobes (Pascual and Preat, 2001). Ptpmeg protein is expressed on mushroom body axons and our data suggest that it may directly regulate mushroom body axon targeting decisions, such as axon branching. Although it appears that Ptpmeg influences axon branching, the exact mechanism is not yet clear. Abnormalities in axon outgrowth, branch formation, branch maintenance, or branch retraction could potentially cause the mutant phenotypes that we have described. Experiments to further refine Ptpmeg's role in the mushroom bodies are ongoing.

The intracellular signaling pathways that regulate axon branching are not well understood. A number of extracellular cues have been shown to promote and restrict axon branch formation (Bures and Kazil, 1975; Dent et al., 2004; Wang et al., 1999; Yates et al., 2001), although the intracellular signaling pathways that are used in the process are not well described. In the mushroom bodies known cytoskeletal effectors, the Rac GTPases, control a number of processes including axon guidance and branching. Additionally, mutations in Dscam, a cell adhesion molecule, and Fmr1, a mRNA binding protein, also affect mushroom body axon branching, although the signaling mechanisms involved are unknown (Michel et al., 2004; Pan et al., 2004; Wang et al., 2004a; Wang et al., 2002; Zhan et al., 2004). In order to fully understand the process of axon branching, it will be important to identify the cytoplasmic signaling molecules that link transmembrane proteins to cytoskeleton, either directly or through effectors, such as the Rac GTPases. Ptpmeg may serve as one of these proteins.

The observed mushroom body phenotypes in *Ptpmeg* mutants suggest a number of possibilities. *Ptpmeg* function could be required within the mushroom bodies to regulate some aspect of Kenyon cell differentiation or axon guidance. For instance, missing or misguided axons could easily disrupt the overall neuropil structure of the α and β lobes. Alternatively, *Ptpmeg* function could be required in some other cell type,

thereby disrupting mushroom body development indirectly. For instance, glia that are closely associated with the mushroom bodies and their axons have been shown to be critical for their development (Awasaki and Ito, 2004; Watts et al., 2004). The mistargeting of other neurons, such as the mushroom body targets or intermediate targets, could also indirectly affect the pathfinding ability of α and β lobe axons. However, our experiments support a model in which Ptpmeg is functioning within the mushroom bodies themselves.

The spatial requirement of Ptpmeg appears to be in neurons and more specifically, in the mushroom body neurons. Expression of UAS-Ptpmeg-RNAi in neurons resulted in similar phenotypes as were seen in the targeted replacement allele mutants. The neuronal requirement for Ptpmeg was further substantiated by rescuing an aspect of the mutant phenotype (α lobe reduction) by expressing a *Ptpmeg* cDNA only in neurons of mutant animals. Using OK107-Gal4, which is strongly expressed in mushroom body neurons, we showed that knockdown of Ptpmeg protein in mushroom bodies results in strong mutant phenotypes. OK107-Gal4 is weakly expressed in other regions of the brain, however we did not observe significant reduction in Ptpmeg protein in these cells (data not shown). Taken together, these data suggest that the function of *Ptpmeg* is required in the mushroom bodies of *Drosophila.*

The mushroom body neurons develop in a sequential pattern. γ neurons develop first followed by α'/β' neurons and then α/β neurons. The temporal requirement of Ptpmeg appears to be after larval development. At this stage in development the γ and α'/β' neurons have already developed and the α/β neurons have begun to differentiate and project axons to the α and β lobes. Since we do not observe mutant phenotypes in *Ptpmeg* mutant larvae (data not shown) we believe it is likely that the axon branching defects seen in the adult are a result of developmental events that occur during puparian formation when the α/β neurons are developing. Therefore Ptpmeg could be required only in α/β neurons for normal mushroom body development. Alternatively, Ptpmeg could be required in the earlier born γ or α'/β' neurons. For example, initially α/β axons migrate along the axons of the earlier born α'/β' neurons. If Ptpmeg were required for the localization or regulation of molecules that are expressed on the surface of α'/β' axons

this may disrupt the substrate upon which α/β axons travel. Mosaic studies involving the different classes of mushroom body neurons will be required to distinguish these models.

We also examined the effects of ectopic expression and overexpression of Ptpmeg in the nervous system. Neither ectopic expression of Ptpmeg in neurons, using *c155-Gal4*, or overexpression in the mushroom bodies, using *OK107-Gal4*, generated detectable phenotypes. This could be due to compensatory effects of other proteins or simply reflect that neurons are not sensitive to excess levels of Ptpmeg.

MATERIALS AND METHODS

Genetics and Molecular Biology

Fly stocks and plasmids for creating the *Ptpmeg* replacement alleles were obtained from K. Golic. To create the targeting construct, bases 32244-35221 from AE003468 (corresponding to 3L 332,882-335,771) were cloned into pTV2. To create a four base insertion in the *Ptpmeg* coding region, the Acc65I site at base 34096 (corresponding to 3L 334,646) was filled in with Klenow polymerase. An I-SceI recognition site was inserted at the BstEII site at base 35698 (corresponding to 3L 334,247). Two-step allelic replacement was done as described [Rong, 2002 #1065]. Genomic DNA was prepared for PCR as described [Sears, 2003 #1066]. Primers used to amplify the genomic DNA surrounding the site of allelic replacement were: 5'-GAATTAATACGACTCACTATAGGGAGAGACGTCGGTTTTATTGAACAGTGC-3' (Primer A) and 5'-GAATTCTCATCCGATCTCATCGCTCTCCGGGAC-3' (Primer B). PCR products were sequenced by the MGH DNA Sequencing Core Facility.

To create the UAS-Ptpmeg-RNAi construct, genomic DNA sequences from bases 36733-37282 of AE003468 were fused via an SpeI site to an inverted Ptpmeg cDNA sequence spanning bases 2344-1869 of LD27491 and inserted into pUASt [Brand, 1993 #253][Kalidas, 2002 #1067]. Ptpmeg cDNA's LD16634 and LD27491 were inserted into pUASt to generate UAS-Ptpmeg full-length and UAS-PtpmegΔFERM (missing the N-terminal 387 amino acids), respectively.

Western Blot

Dissected larval nervous systems were homogenized in lysis buffer consisting of PBS (130mM NaCl, 175 mM Na2HPO4, 60mM NaH2PO4) and protease inhibitors (Roche). Lysates were run on a 7.5% poyacrylamide gel and transferred to Hybond-P membrane (Amersham Biosciences). Membranes were blocked overnight in 5% nonfat milk and then probed with anti-MEG (1:5000) or control anti-Elav (1:1000). HRP-conjugated secondary antibodies were used at a concentration of 1:5000. Each lane contained the nervous systems of approximately 5 larvae.

Southern Blot

Genomic DNA was isolated from 30 flies and digested with EcoRI/Acc65I or XhoI/Acc65I. Digested DNA was electrophoresed on an 0.8% agarose gel and transferred overnight onto a Hybond-XL nylon membrane (Amersham Biosciences). ^{32}P probes were labeled with the Rediprime DNA labeling system (Amersham Biosciences).

Immunohistochemistry

Polyclonal antiserum against Ptpmeg was produced in guinea pigs (Covance) against a 31 kD peptide containing 255 amino acids of Ptpmeg (aa 359-614) fused to a 6XHis tag. Anti-Ptpmeg antiserum was used at 1:750. Anti-Fasciclin 2 was used at 1:200. Secondary antibodies were obtained from Jackson Laboratories and used at 1:500. Fluorescent images were obtained using a Nikon PCM2000 confocal microscope.

REFERENCES

Awasaki, T. and Ito, K. (2004). Engulfing action of glial cells is required for programmed axon pruning during Drosophila metamorphosis. *Curr Biol* **14**, 668-77.

Billuart, P., Winter, C. G., Maresh, A., Zhao, X. and Luo, L. (2001). Regulating axon branch stability: the role of p190 RhoGAP in repressing a retraction signaling pathway. *Cell* **107**, 195-207.

Bretscher, A., Edwards, K. and Fehon, R. G. (2002). ERM proteins and merlin: integrators at the cell cortex. *Nat Rev Mol Cell Biol* **3**, 586-99.

Bures, Z. and Kazil, P. (1975). [Is it possible to influence the safety of deep colorectal anastomoses?]. *Rozhl Chir* **54**, 331-7.

Caplen, N. J., Fleenor, J., Fire, A. and Morgan, R. A. (2000). dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference. *Gene* **252**, 95-105.

Chishti, A. H., Kim, A. C., Marfatia, S. M., Lutchman, M., Hanspal, M., Jindal, H., Liu, S. C., Low, P. S., Rouleau, G. A., Mohandas, N. et al. (1998). The FERM domain: a unique module involved in the linkage of cytoplasmic proteins to the membrane. *Trends Biochem Sci* **23**, 281-2.

Cohen, A. M., Liu, S. C., Lawler, J., Derick, L. and Palek, J. (1988). Identification of the protein 4.1 binding site to phosphatidylserine vesicles. *Biochemistry* **27**, 614-9.

Connolly, J. B., Roberts, I. J., Armstrong, J. D., Kaiser, K., Forte, M., Tully, T. and O'Kane, C. J. (1996). Associative learning disrupted by impaired Gs signaling in Drosophila mushroom bodies. *Science* **274**, 2104-7.

de Belle, J. S. and Heisenberg, M. (1994). Associative odor learning in Drosophila abolished by chemical ablation of mushroom bodies. *Science* **263**, 692-5.

Dent, E. W., Barnes, A. M., Tang, F. and Kalil, K. (2004). Netrin-1 and semaphorin 3A promote or inhibit cortical axon branching, respectively, by reorganization of the cytoskeleton. *J Neurosci* **24**, 3002-12.

Edwards, K., Davis, T., Marcey, D., Kurihara, J. and Yamamoto, D. (2001). Comparative analysis of the Band 4.1/ezrin-related protein tyrosine phosphatase Pez from two Drosophila species: implications for structure and function. *Gene* **275**, 195-205.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. *Nature* **391**, 806-11.

Fischer, E. H. (1999). Cell signaling by protein tyrosine phosphorylation. *Adv Enzyme Regul* **39**, 359-69.

Gjorloff-Wingren, A., Saxena, M., Han, S., Wang, X., Alonso, A., Renedo, M., Oh, P., Williams, S., Schnitzer, J. and Mustelin, T. (2000). Subcellular localization of intracellular protein tyrosine phosphatases in T cells. *Eur J Immunol* **30**, 2412-21.

Gong, W. J. and Golic, K. G. (2003). Ends-out, or replacement, gene targeting in Drosophila. *Proc Natl Acad Sci U S A* **100**, 2556-61.

Gu, M., Meng, K. and Majerus, P. W. (1996). The effect of overexpression of the protein tyrosine phosphatase PTPMEG on cell growth and on colony formation in soft agar in COS-7 cells. *Proc Natl Acad Sci U S A* **93**, 12980-5.

Gu, M. X., York, J. D., Warshawsky, I. and Majerus, P. W. (1991). Identification, cloning, and expression of a cytosolic megakaryocyte protein-tyrosine-phosphatase with sequence homology to cytoskeletal protein 4.1. *Proc Natl Acad Sci U S A* **88**, 5867-71.

Han, S., Williams, S. and Mustelin, T. (2000). Cytoskeletal protein tyrosine phosphatase PTPH1 reduces T cell antigen receptor signaling. *Eur J Immunol* **30**, 1318-25.

Heisenberg, M. (2003). Mushroom body memoir: from maps to models. *Nat Rev Neurosci* **4**, 266-75.

Heisenberg, M., Borst, A., Wagner, S. and Byers, D. (1985). Drosophila mushroom body mutants are deficient in olfactory learning. *J Neurogenet* **2**, 1-30.

Hirao, M., Sato, N., Kondo, T., Yonemura, S., Monden, M., Sasaki, T., Takai, Y. and Tsukita, S. (1996). Regulation mechanism of ERM (ezrin/radixin/moesin) protein/plasma membrane association: possible involvement of phosphatidylinositol turnover and Rho-dependent signaling pathway. *J Cell Biol* **135**, 37-51.

Hironaka, K., Umemori, H., Tezuka, T., Mishina, M. and Yamamoto, T. (2000). The protein-tyrosine phosphatase PTPMEG interacts with glutamate receptor delta 2 and epsilon subunits. *J Biol Chem* **275**, 16167-73.

Hunter, T. (1997). Oncoprotein networks. *Cell* **88**, 333-46.

Ikuta, S., Itoh, F., Hinoda, Y., Toyota, M., Makiguchi, Y., Imai, K. and Yachi, A. (1994). Expression of cytoskeletal-associated protein tyrosine phosphatase PTPH1 mRNA in human hepatocellular carcinoma. *J Gastroenterol* **29**, 727-32.

Ito, K., Awano, W., Suzuki, K., Hiromi, Y. and Yamamoto, D. (1997). The Drosophila mushroom body is a quadruple structure of clonal units each of which contains a virtually identical set of neurones and glial cells. *Development* **124**, 761-71.

Ito, K. and Hotta, Y. (1992). Proliferation pattern of postembryonic neuroblasts in the brain of Drosophila melanogaster. *Dev Biol* **149**, 134-48.

Ito, K., Suzuki, K., Estes, P., Ramaswami, M., Yamamoto, D. and Strausfeld, N. J. (1998). The organization of extrinsic neurons and their implications in the functional roles of the mushroom bodies in Drosophila melanogaster Meigen. *Learn Mem* **5**, 52-77.

Kim, E. and Sheng, M. (2004). PDZ domain proteins of synapses. *Nat Rev Neurosci* **5**, 771-81.

Lee, T., Lee, A. and Luo, L. (1999). Development of the Drosophila mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development* **126**, 4065-76.

Lee, T., Winter, C., Marticke, S. S., Lee, A. and Luo, L. (2000). Essential roles of Drosophila RhoA in the regulation of neuroblast proliferation and dendritic but not axonal morphogenesis. *Neuron* **25**, 307-16.

Mauro, L. J. and Dixon, J. E. (1994). 'Zip codes' direct intracellular protein tyrosine phosphatases to the correct cellular 'address'. *Trends Biochem Sci* **19**, 151-5.

Michel, C. I., Kraft, R. and Restifo, L. L. (2004). Defective neuronal development in the mushroom bodies of Drosophila fragile X mental retardation 1 mutants. *J Neurosci* **24**, 5798-809.

Neel, B. G. and Tonks, N. K. (1997). Protein tyrosine phosphatases in signal transduction. *Curr Opin Cell Biol* **9**, 193-204.

Ng, J., Nardine, T., Harms, M., Tzu, J., Goldstein, A., Sun, Y., Dietzl, G., Dickson, B. J. and Luo, L. (2002). Rac GTPases control axon growth, guidance and branching. *Nature* **416**, 442-7.

Pan, L., Zhang, Y. Q., Woodruff, E. and Broadie, K. (2004). The Drosophila fragile X gene negatively regulates neuronal elaboration and synaptic differentiation. *Curr Biol* **14**, 1863-70.

Pascual, A. and Preat, T. (2001). Localization of long-term memory within the Drosophila mushroom body. *Science* **294**, 1115-7.

Reuter, J. E., Nardine, T. M., Penton, A., Billuart, P., Scott, E. K., Usui, T., Uemura, T. and Luo, L. (2003). A mosaic genetic screen for genes necessary for Drosophila mushroom body neuronal morphogenesis. *Development* **130**, 1203-13.

Rong, Y. S. and Golic, K. G. (2000). Gene targeting by homologous recombination in Drosophila. *Science* **288**, 2013-8.

Rong, Y. S. and Golic, K. G. (2001). A targeted gene knockout in Drosophila. *Genetics* **157**, 1307-12.

Sahin, M., Slaugenhaupt, S. A., Gusella, J. F. and Hockfield, S. (1995). Expression of PTPH1, a rat protein tyrosine phosphatase, is restricted to the derivatives of a specific diencephalic segment. *Proc Natl Acad Sci U S A* **92**, 7859-63.

Tonks, N. K. and Neel, B. G. (2001). Combinatorial control of the specificity of protein tyrosine phosphatases. *Curr Opin Cell Biol* **13**, 182-95.

Uchida, Y., Ogata, M., Mori, Y., Oh-hora, M., Hatano, N. and Hamaoka, T. (2002). Localization of PTP-FERM in nerve processes through its FERM domain. *Biochem Biophys Res Commun* **292**, 13-9.

Wang, J., Ma, X., Yang, J. S., Zheng, X., Zugates, C. T., Lee, C. H. and Lee, T. (2004a). Transmembrane/juxtamembrane domain-dependent Dscam distribution and function during mushroom body neuronal morphogenesis. *Neuron* **43**, 663-72.

Wang, J., Zugates, C. T., Liang, I. H., Lee, C. H. and Lee, T. (2002). Drosophila Dscam is required for divergent segregation of sister branches and suppresses ectopic bifurcation of axons. *Neuron* **33**, 559-71.

Wang, K. H., Brose, K., Arnott, D., Kidd, T., Goodman, C. S., Henzel, W. and Tessier-Lavigne, M. (1999). Biochemical purification of a mammalian slit protein as a positive regulator of sensory axon elongation and branching. *Cell* **96**, 771-84.

Wang, Z., Shen, D., Parsons, D. W., Bardelli, A., Sager, J., Szabo, S., Ptak, J., Silliman, N., Peters, B. A., van der Heijden, M. S. et al. (2004b). Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science* **304**, 1164-6.

Warabi, M., Nemoto, T., Ohashi, K., Kitagawa, M. and Hirokawa, K. (2000). Expression of protein tyrosine phosphatases and its significance in esophageal cancer. *Exp Mol Pathol* **68**, 187-95.

Watts, R. J., Schuldiner, O., Perrino, J., Larsen, C. and Luo, L. (2004). Glia engulf degenerating axons during developmental axon pruning. *Curr Biol* **14**, 678-84.

Yang, Q. and Tonks, N. K. (1991). Isolation of a cDNA clone encoding a human protein-tyrosine phosphatase with homology to the cytoskeletal-associated proteins band 4.1, ezrin, and talin. *Proc Natl Acad Sci U S A* **88**, 5949-53.

Yates, P. A., Roskies, A. L., McLaughlin, T. and O'Leary, D. D. (2001). Topographic-specific axon branching controlled by ephrin-As is the critical event in retinotectal map development. *J Neurosci* **21**, 8548-63.

Zhan, X. L., Clemens, J. C., Neves, G., Hattori, D., Flanagan, J. J., Hummel, T., Vasconcelos, M. L., Chess, A. and Zipursky, S. L. (2004). Analysis of Dscam diversity in regulating axon guidance in Drosophila mushroom bodies. *Neuron* **43**, 673-86.

Zhang, Z. Y. (2001). Protein tyrosine phosphatases: prospects for therapeutics. *Curr Opin Chem Biol* **5**, 416-23.

FIGURE LEGENDS

Figure 1: Disruption of the *Ptpmeg* locus through homologous recombination-mediated gene targeting. (A) A P-element containing a modified fragment of the *Ptpmeg* sequence, FRT sequences and a mini-white gene marker was randomly inserted into the *Drosophila* genome. Expression of FLP recombinase circularized the DNA. The plasmid was linearized through expression of the restriction enzyme I-*Sce*I and the linearized fragment recombined with the endogenous *Ptpmeg* locus. Expression of I-*Cre*I in fly lines carrying the gene targeting construct induces a double-stranded DNA break. Break repair mechanisms resulted in several *Ptpmeg* replacement alleles that carried only the GTAC repeat as shown by restriction digest analysis (B) and sequence analysis (C). (D) Southern Blot analysis of *Ptpmeg* replacement alleles. DNA was obtained from adult wild type or *Ptpmeg* replacement allele heterozygotes and digested with several restriction enzymes. The replacement allele destroys a previously existing *Acc65*I restriction site.

Figure 2: (A) Schematic of the Ptpmeg protein noting the prominent domains and the location of the stop codon generated in the replacement allele. Polyclonal antibodies were generated using a peptide containing amino acids 337-592, a region corresponding to the PDZ domain. (B) Homology between *Drosophila* Ptpmeg and the human homologs, PTPN3 and PTPN4. (C) Western blots containing adult head protein from wild type, $ptpmeg^{R4}$, $ptpmeg^{R5}$ or $ptpmeg^{R4}$ heterozygotes probed with anti-Ptpmeg antiserum. Blots were reprobed with anti-Elav antiserum to confirm similar levels of protein were present in each lane.

Figure 3: *Ptpmeg* is required for proper mushroom body morphogenesis. Adult brains of wild type (A) and *Ptpmeg* mutants (B, C) stained with anti-Fasciclin 2 to reveal mushroom body morphology. In wild type, α and β axons bifurcate forming the dorsal α lobe and the medial β lobe. The earlier born γ lobe axons are located behind the β lobe and stain weakly with anti-Fasciclin 2. In *ptpmeg* mutants, the α lobes are often reduced or missing (arrows) while the β lobes display defects such as thickening or fusion with

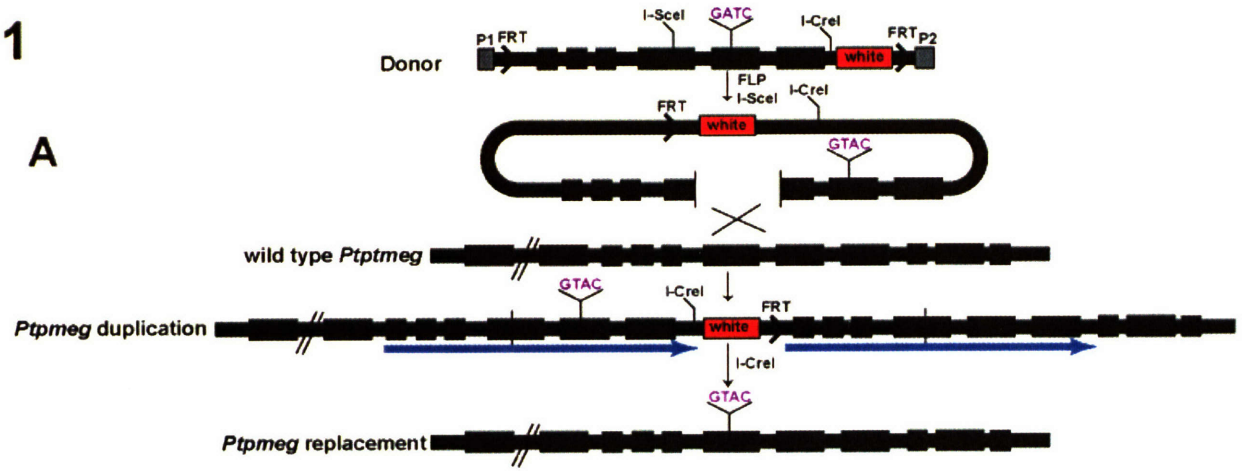
the other β lobe (arrowhead). β lobe absence is sometimes seen in other genetic backgrounds (see text).

Figure 4: Knockdown of Ptpmeg protein using transgenic RNAi. (A) Wild type. (B,C) Neuronal knockdown of Ptpmeg protein expression. c155-Gal4 driver flies were crossed to UAS-Ptpmeg-RNAi flies. The mushroom bodies of adult progeny were examined using anti-Fasciclin 2. Mushroom body lobe defects similar to those of *Ptpmeg* mutants were identified. Knockdown of Ptpmeg protein resulted in α lobe reduction (arrow in C) as well as β lobe fusion or mistargeting (arrowhead in B, C). γ lobe disruptions were also detected (asterisk in B,C). The ellipsoid body (eb) can be seen in (B) and (C).

Figure 5: Knockdown of Ptpmeg protein with the OK107-Gal4 Mushroom body driver. (A,C) Entire mushroom body structure in UAS-GFP; UAS-Ptpmeg-RNAi / $Ptpmeg^{R4}$; OK107-Gal4 animals. (B,D) Same animals with anti-Fasciclin 2 alone. GFP expression under OK107-Gal4 control shows the full mushroom body anatomy, including the Kenyon cells (Kc) and the dendritic arborizations that make up the calyx (ca). Knockdown of Ptpmeg protein in the mushroom bodies results in α lobe reduction (arrow in B) and a corresponding increase in the size of the β lobe (arrowhead in B). Absence of the β' lobe is also detected (arrow in D) with an apparent increase in the α lobe as compared to the α lobe in the other hemisphere.

Figure 6: Ptpmeg protein is detected on the α and β lobes of the mushroom bodies. (A,B) Wild type and (C,D) Ptpmeg mushroom body knockdown stained with anti-Ptpmeg (blue in A and C; white in B and D) and anti-Fasciclin 2 (red in A and C, not present in B and D). Ptpmeg protein is expressed throughout the brain, including on the axons that form the α and β lobe. (B) In wild type, Ptpmeg expression is detected on the α lobe (arrow) and β lobe (arrowhead), although strong staining is not detected on the Kenyon cells or calyx (asterisk). Anti-Ptpmeg staining is reduced in Ptpmeg mushroom body knockdown animals (Note: this is the same sample as in 5A,B.) Some anti-Ptpmeg staining persists on wild type-appearing lobes (asterisk in 6D).

Figure 1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Donor
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I-SceI
GATC
I-CreI
FRT P2
white
FLP
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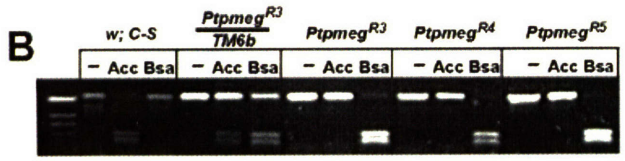
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w; C-S
Ptpmeg R3 / TM6b
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Ptpmeg R4
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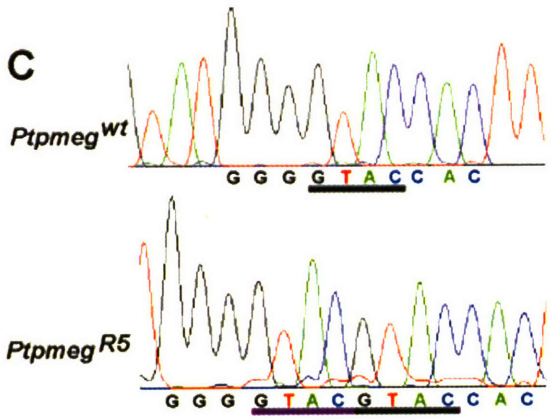
– Acc Bsa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Ptpmeg R5
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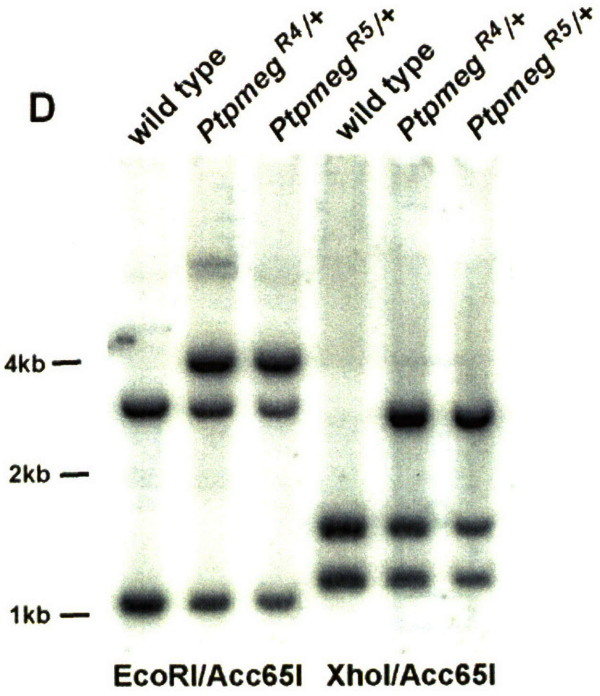
D
wild type
Ptpmeg R4/+
Ptpmeg R5/+
wild type
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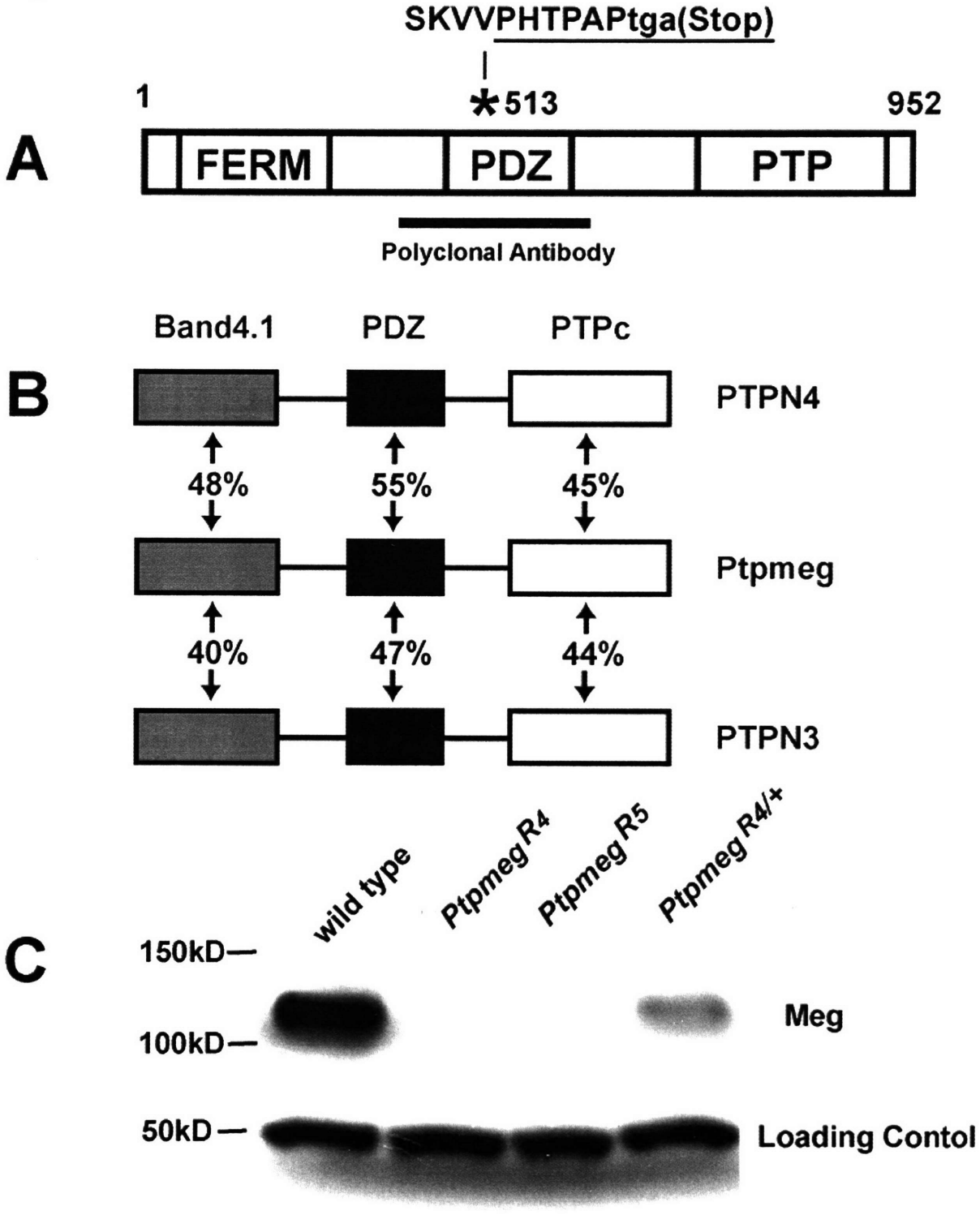
2kb
1kb
EcoRI/Acc65I
XhoI/Acc65I

Figure 2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952
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FERM
PDZ
PTP
Polyclonal Antibody
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PTPc
B
PTPN4
48%
55%
45%
Ptpmeg
40%
47%
44%
PTPN3
wild type
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Ptpmeg R5
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100kD
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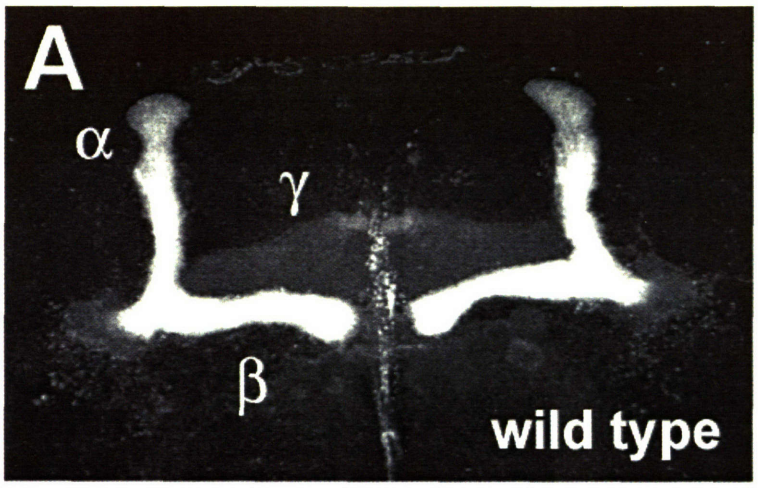
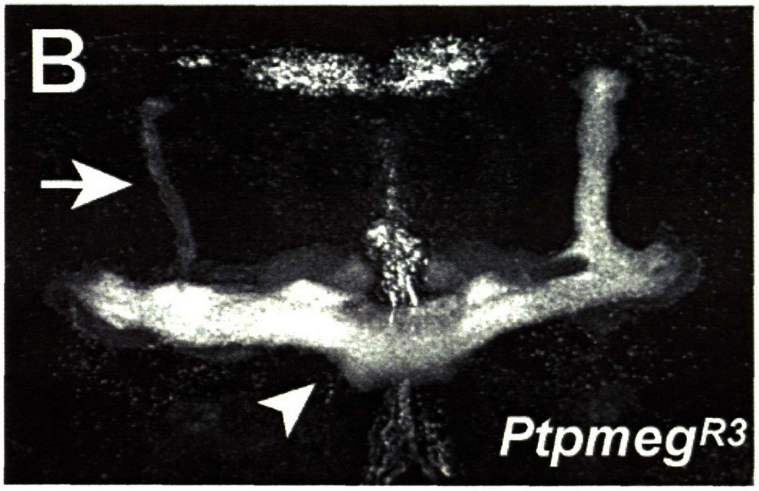
Meg
Loading Contol

Figure 3





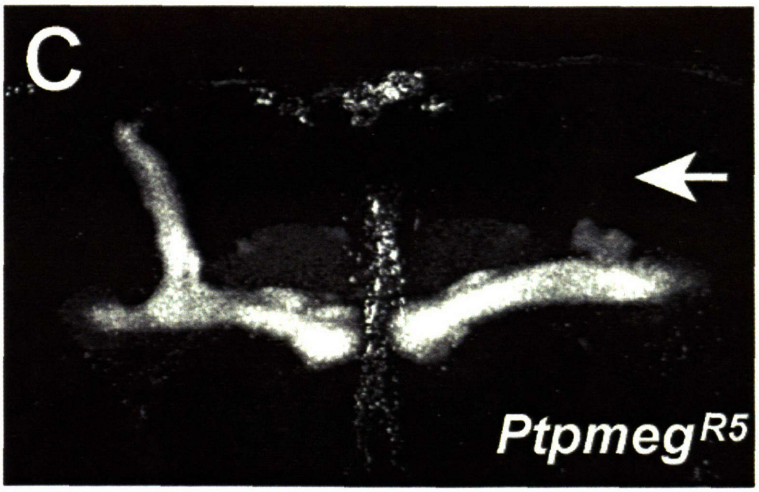


Figure 4

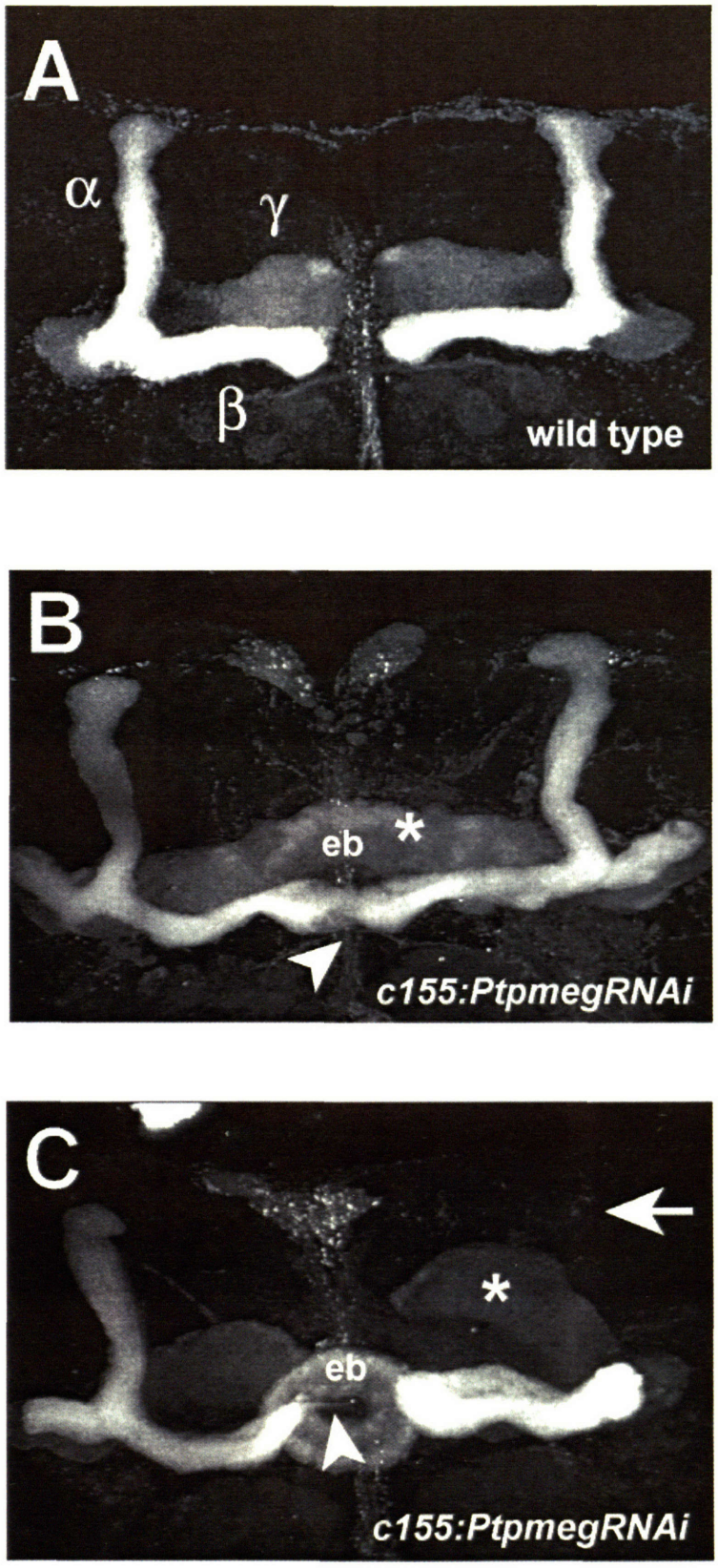
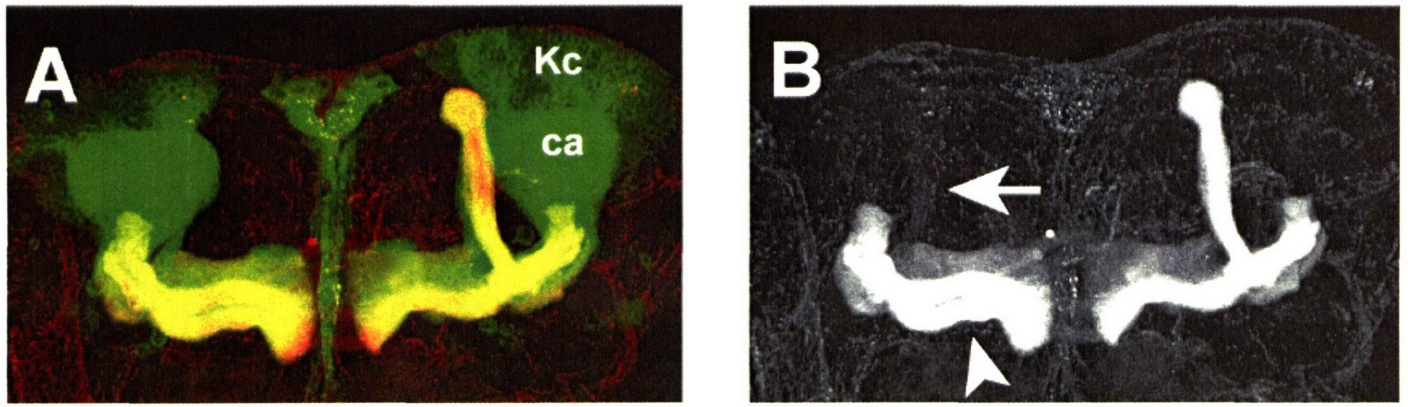
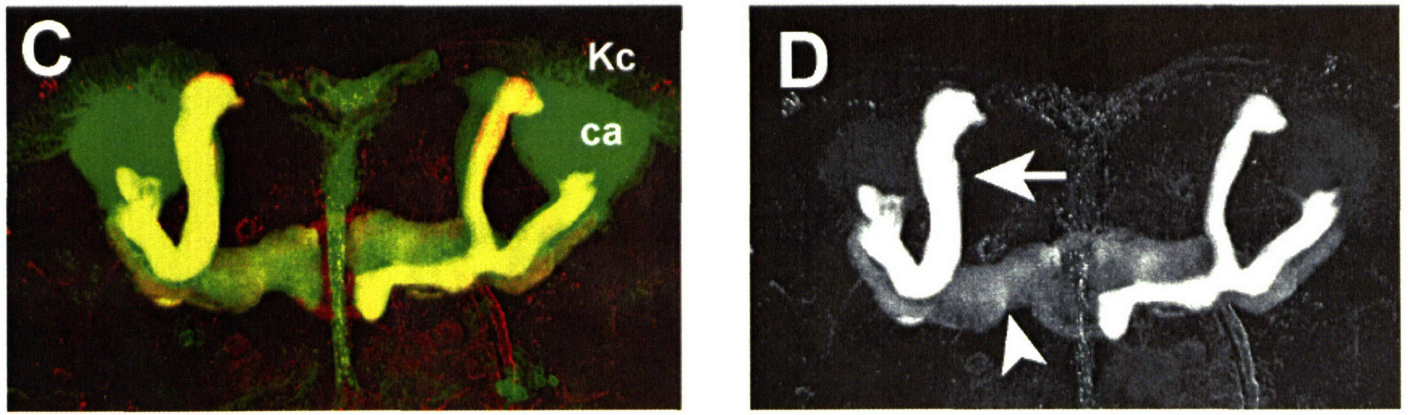


Figure 5

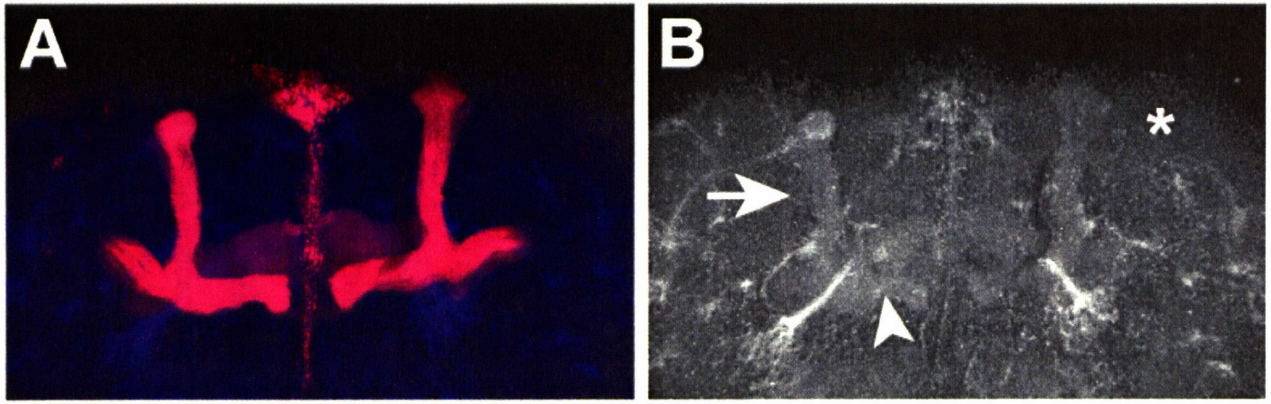


OK107:PtpmegRNAi ; Ptpmeg^{R4} / +

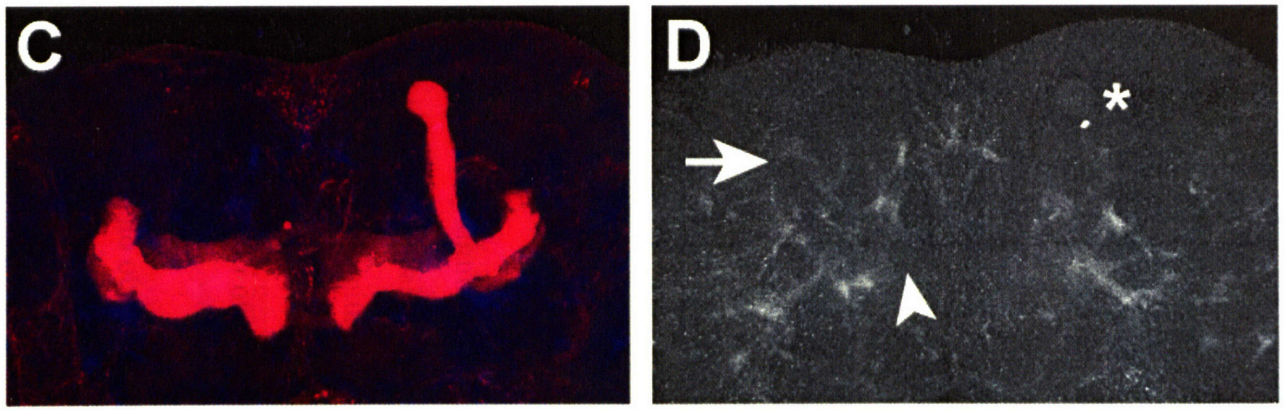


OK107:PtpmegRNAi ; Ptpmeg^{R4} / +

Figure 6



Wild Type



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CHAPTER FOUR

Discussion and Future Directions

In my thesis, I have shown that Slit and Robo-family proteins play a pivotal role in the compartmentalization of visual centers in the *Drosophila* brain by restricting the movement of cells across a boundary. My work defines a previously uncharacterized boundary between cells of the lamina and lobula in the developing visual system and provides an example of how sharp boundaries are maintained during development. I have also shown that a previously uncharacterized phosphatase, Ptpmeg, is essential for the development of the mushroom bodies, the center of olfactory learning and memory in the *Drosophila* brain. Ptpmeg is expressed in mushroom bodies and appears to regulate axon guidance and branching in these structures. Here, I will discuss the implications of my work as well as future experimental directions that could provide new insight into the processes of compartmentalization and axon guidance and branching.

Temporal requirement of Slit

My description of the *slit* mutant phenotype in Chapter 2 was mostly limited to the third larval instar stage of development, and my data support the idea that visual system development is largely normal up to that stage. However, due to some limitations in my analysis, I was unable to conclusively rule out that earlier developmental defects do not contribute to the mutant phenotype. Here, I will briefly discuss a number of experiments that could resolve this concern.

My analysis of the embryonic and early larval brain revealed no obvious defects in early visual system development. In addition, I was able to rescue the mutant phenotype by expressing a *slit* cDNA with a Gal4 line that drives expression in the photoreceptor neurons (GMR-Gal4). Because the photoreceptors develop during the third instar stage, this suggested that Slit, in the visual system, was also required at this stage and not earlier. However, GMR-Gal4 is also expressed in the larval optic nerve (Bolwig nerve), which arises during embryogenesis and sends a single bundle of axons into the brain (near the presumptive lamina and medulla regions) (Green et al., 1993).

Could expression of Slit from the Bolwig nerve account for the rescue of the mutant phenotype?

There are three experiments that could aid in resolving this concern. The first, and perhaps most simple approach, is to repeat the Slit rescue experiments using a heat-shock inducible Gal4 (hs-Gal4) line or a third instar-specific Gal4 line to restrict expression of Slit to the beginning of the third instar. The second approach is to repeat the Slit rescue experiments with GMR-Gal4 in an animal that is missing the Bolwig nerve. Elimination of the Bolwig nerve could be accomplished through genetic ablation or laser ablation (Kunes and Steller, 1991; Lohs-Schardin et al., 1979). If the mutant phenotype can be rescued using either of these approaches, I could conclude that Slit is required no earlier than the third instar stage, and that the phenotypes I reported in Chapter 2 were not due to some unidentified defect from earlier stages of development. A third complementary approach relies on the restriction of the Robo-RNAi transgenes (described in Chapter 2) to the early third instar stage. Again, using hs-Gal4 or a third instar-specific Gal4 line, the temporal requirement of the Robo proteins could be assessed. The goal of these experiments is to demonstrate that the Slit and Robo proteins exert their effects after compartment identities have been specified, further supporting our conclusion that Slit and the Robos are required for later aspects of compartmentalization (i.e. the establishment or maintenance of a compartment boundary).

Are the lobula neurons restricted by a repulsive signaling mechanism?

As mentioned in Chapter 1 and Chapter 2, a number of studies have convincingly shown that Slit can mediate repulsion through Robo receptors. In my work, the restricted movement of lobula neurons and the expression patterns of both Slit and the Robo proteins implied that a repulsive interaction was occurring in the visual system. However, I was not able to formally demonstrate that Slit was functioning through Robo receptors to repel lobula neurons from the lamina. Alternative explanations could be that Slit regulates the adhesive properties of lobula neurons (perhaps through Robo or Robo-independent mechanisms) or that Slit and the Robo proteins are engaged in different, independent processes that happen to result in the same phenotype. Here, I will suggest experiments to confirm whether or not a Slit-Robo interaction is triggering a repulsive

signaling mechanism in the process of compartmentalization in the *Drosophila* visual system.

Although, my initial experiments designed to overexpress or ectopically express Slit and Robo were largely unsuccessful (as discussed in Chapter 2), additional ectopic expression studies could be performed. Chimeric receptors have been used successfully in the *Drosophila* embryo to switch repulsion to attraction and attraction to repulsion (Bashaw and Goodman, 1999). As discussed in the introduction, Slit and Robo interactions often mediate repulsion, while Netrin/Frazzled interactions often mediate attraction (Guan and Rao, 2003). Robo/Frazzled chimeras, carrying the extracellular domain of one receptor and the intracellular domain of the other receptor, have been expressed in the embryonic CNS (Bashaw and Goodman, 1999). In the presence of Netrin the Fra-Robo chimera behaves as a repulsive receptor and in the presence of Slit the Robo-Fra chimera behaves as an attractive receptor. If the visual system is using Slit and Robo to repel lobula neurons from the lamina then one would predict that the expression of the Robo-Fra chimeric receptor in lobula neurons should lead to the opposite response, resulting in lobula neurons being drawn into the lamina. In such ectopic experiments, wild type Robo receptors would still be present in the lobula neurons, and therefore the normal signaling mechanism would still be in place. However, it is expected that high levels of Robo-Fra expression would "drown out" wild type signaling. Of course, expression of Robo-Fra in otherwise wild type cells could also lead to unforeseen consequences, such as dominant negative phenotypes. The experiment above, therefore, would be improved by expressing the chimera in a Robo knockdown (Robo-RNAi) animal. This would be the equivalent of a rescue experiment, but rather than using a wild type cDNA for the rescue, the Robo-Fra cDNA would be used. Finally, the creation of antibodies that recognize the "off" or "on" signaling state of Robo could be used and I will discuss this idea in the next section.

Robo Receptor Signaling and the Role of Phosphorylation

The dynamic regulation of Robo signaling is important for the migration of axons, neurons and other non-neuronal cell types (Fernandis and Ganju, 2001; Piper and Little, 2003). For example, as axons navigate across the CNS midline of the developing

Drosophila embryo, Robo signaling is turned "off and on" to allow crossing and prevent recrossing . Slit-expressing cells in the midline repel Robo-expressing axons by activating Robo repulsive signaling. This causes axons that express high levels of Robo on their growth cones to be prevented from crossing the midline, while axons that express low levels of Robo are able to cross the midline (Kidd et al., 1998). The Commissureless protein (Comm) negatively regulates Robo by preventing it from reaching the cell surface, allowing certain axons to cross the midline (Georgiou and Tear, 2003; Keleman et al., 2002). Once an axon crosses the midline, Robo levels are increased, due to a reduction in Comm levels, and this prevents recrossing. Comm is not conserved in vertebrates, and so vertebrates must use another mechanism to negatively regulate the Robo receptors. Considering the high degree of conservation of the Robo proteins across species, this mechanism of regulation may be used in *Drosophila* as well.

As with many receptors, phosphorylation appears to play a prominent role in Robo signaling. In mammalian cell culture, Abelson (Abl) kinase phosphorylates an evolutionarily conserved tyrosine residue (Y1040) on Robo. This phosphorylation event appears to be a critical component of Robo signaling, although there is conflicting genetic data as to whether Abl phosphorylation antagonizes or promotes Robo signaling (Bashaw et al., 2000; Wills et al., 2002). Y1040 is the major phosphorylation site (when expressed in cultured mammalian cells) and appears to be an important modulator of Robo activity. Mutating this tyrosine to a phenylalanine (Y1040F) in flies produces a "hyperactive" Robo protein (Bashaw et al., 2000).

How phosphorylation modulates Robo function remains an open question. For instance, is the phosphorylated or dephosphorylated form of Robo active? What is the phosphorylation state of Robo when Slit is present or absent? These and other important questions could be answered with an antibody that recognizes the phosphorylated form of *Drosophila* Robo. A straightforward experiment would be to examine the Robo phosphorylation state in the embryonic CNS of *slit* mutants. If the presence of Slit causes dephosphorylation of Robo, then *slit* loss-of-function mutants should show increased levels of phospho-Robo protein and Slit overexpression should decrease levels of phospho-Robo protein. On the other hand, if Slit binding causes phosphorylation of Robo, then the *slit* loss-of-function mutant should show decreased levels of phospho-

Robo protein and *slit* overexpression should increase levels of phospho-Robo protein.In the same manner, immunohistochemical examination of *Abl* mutants with the phospho-Robo antibody could determine whether Abl is the primary *in vivo* kinase of Robo. If Abl is the primary kinase for Robo, this would predict that phosphorylated Robo protein would be reduced or absent in *Abl* mutants. If phosphorylation positively or negatively regulates Robo signaling, dephosphorylation likely plays the opposite role. Genetic interaction studies have implicated the receptor protein tyrosine phosphatases (RPTPs) in Robo signaling and these same experiments could be used to help unravel their role in the process (Bashaw et al., 2000).

How do photoreceptors reach their targets?

Signals that control target layer selection often come from a population of "intermediate" target cells. For example, in the developing hippocampus, entorhinal axons target to their correct layer before the arrival of their eventual targets, the pyramidal neurons (Ceranik et al., 2000). A transient population of neurons, the Cajal-Retzius (CR) neurons, also occupy this layer and provide targeting signals to the entorhinal axons (Ceranik et al., 2000). Entorhinal axons fail to enter the hippocampus in the absence of the CR neurons. This suggests that an attractive signal from the CR neurons guides the entorhinal axons to the correct layer. Intermediate targets also play a central role in visual system axon targeting in *Drosophila.*

At the time my thesis work began, Slit had recently been identified as the ligand for the Robo receptor (Kidd et al., 1999). In *Drosophila*, Robo had been shown to be an axon guidance receptor that mediated midline crossing in the embryonic CNS (Kidd et al., 1998). Although we initially identified mutations in *slit* as disrupting the process of photoreceptor axon guidance, we later discovered that Slit/Robo interactions regulated compartmentalization in the visual system, and thereby indirectly affected axon guidance and target selection of the photoreceptors. What then are the signals directly required for the targeting of photoreceptors axons in the *Drosophila* visual system? Although the question of how photoreceptor (R-cell) axon targeting is accomplished has received a considerable amount of attention, the signals that are expressed and function in the optic lobe target region to directly control R-cell axon targeting remain a mystery.

R1-R6 photoreceptor axons project to the lamina and stop between precisely arranged rows of glial cells. The best evidence that lamina glial cells are the intermediate targets of R1-R6 axons is that in their absence R1-R6 axons extend past the lamina and project into the underlying medulla region, the target area of R7 and R8 axons (Poeck et al., 2001; Suh et al., 2002) (see Appendix for more details). These observations suggest that the lamina glia provide a "stop signal" for R1-R6 axons. Alternatively, the lamina glia could serve as a mechanical barrier that prevents R1-R6 axons from entering the medulla. However, this seems unlikely, as it would be predicted to prevent R7 and R8 from entering medulla as well. Therefore, it seems probable that there are molecules expressed on or secreted by the lamina glia that signal the R1-R6 axons to stop. However, none of these molecules has been identified. This should emphasize the need for directed screens that attempt to identify genes that are required in the visual system glia for proper photoreceptor axon targeting. The identification of these genes would be of considerable interest to the field.

Does differential cell adhesion contribute to visual system compartmentalization?

A commonly proposed mechanism for keeping cells of adjacent compartments separate is differential cell adhesion (Garcia-Bellido, 1975; Irvine and Rauskolb, 2001). If differential cell adhesion is playing a role in visual system compartmentalization, I would expect to find cell adhesion molecules expressed in distinct regions of the visual system. My analysis of the lamina/lobula boundary, in Chapter 2, identified Fasciclin III as one such candidate. Fasciclin III is an integral membrane protein that mediates homophilic cell adhesion and is often found as a component of septate junctions (Patel et al., 1987; Snow et al., 1989). In the *Drosophila* visual system, Fasciclin III is preferentially expressed on lobula neurons but not on cells of the lamina. It would therefore be interesting to examine *fasciclin III* mutants to see if there are defects in compartmentalization similar to *slit* mutants.

Integrins are a large family of transmembrane proteins that attach cells to the extracellular matrix and are therefore important in cell adhesion and cell movement. In *Drosophila*, *integrin* mutants were shown to genetically interact with *slit* mutants {Stevens, 2002 #302}. Although the nature of the interaction is unknown it suggests that

Slit and Integrins can work together to modulate cell motility. The cell adhesion protein, N-cadherin, is expressed in the *Drosophila* visual system and is known to be important for aspects of photoreceptor targeting (Lee et al., 2001). In vertebrate cells, Slit activation of Robo results in the formation of a receptor complex between N-cadherin and Robo. The interaction between Robo and N-cadherin appears to disrupt the connection of N-cadherin to the cytoskeleton (Rhee et al., 2002). The result of this interaction is to inhibit N-cad mediated cell adhesion. Whether these molecules disrupt the lamina/lobula boundary in the *Drosophila* visual system remains to be determined.

Finally, the extracellular portion of the Robo receptor contains domains that are commonly found in cell adhesion molecules, Ig (Immunoglobulin) domains and FNIII (fibronectin type III) domains. This raises the possibility that the Robo proteins can function both as cell adhesion molecules and as signaling transmembrane receptors. Human Robo and Robo2 proteins have been shown to participate in homophilic and heterophilic adhesion in vitro (Hivert et al., 2002).

Ptpmeg substrates and binding partners

Although the molecular mechanisms by which Ptpmeg regulates mushroom body morphogenesis are unknown, the protein domains contained within Ptpmeg have well characterized functions. Primarily, PDZ domains participate in protein-protein interaction, FERM domains associate with transmembrane proteins and the plasma membrane and PTP domains catalyze the removal of a phosphate group attached to a tyrosine residue. Taken together, these domains suggest that at least one role of Ptpmeg is to regulate phosphotyrosine signaling events at the cell surface.

Further characterization of Ptpmeg binding partners will be required to understand precisely how and where Ptpmeg is required within mushroom body neurons. The necessity of identifying the molecules that associate with Ptpmeg is clear and there are a number of common strategies that could be employed to identify physical interactors. Yeast two-hybrid screens have been used to identify PDZ-domain and FERM-domain interacting proteins (Kussel-Andermann et al., 2000; Schneider et al., 1999) and could be used to identify physical interaction with the Ptpmeg PDZ or FERM domain. Ptpmeg has been successfully immunoprecipitated from S2 cell extracts after transfection with a

Ptpmeg expression vector (L. Zipursky, personal communication). Hence immunoprecipitation experiments could prove to be an effective tool for identifying Ptpmeg partners.

"Substrate-trapping" has also been used successfully to identify the physiological substrates of protein tyrosine phosphatases (Flint et al., 1997). Mutating an invariant amino acid residue in the catalytic site creates a PTP domain that is able to bind but not cleave phosphotyrosine. Interacting proteins can then be isolated and sequenced to reveal their identity.

Mosaic Analysis

Although the initial characterization of *Ptpmeg* mutants revealed a requirement for its function in mushroom body formation, a more rigorous phenotypic analysis is necessary to understand the process that is being affected. The data presented in Chapter 3 are consistent with a role for Ptpmeg in axon guidance and branching. However, we have previously examined the phenotypes only at the level of the entire mushroom body. The pathfinding ability of individual axons or small populations of axons has not been determined. Furthermore, the branching and development of mushroom body dendrites, which lie just beneath the cell bodies, could not be analyzed with the markers that we had available to us. Examination of small mutant patches within an otherwise wild type or heterozygous mushroom body will help resolve a number of questions that arose from our work.

The MARCM (mosaic analysis with a repressible cell marker) system has been used effectively to identify the birth order and branching pattern of neurons within the mushroom body (Lee and Luo, 2001; Reuter et al., 2003). This technique can be used to label single neurons or small groups of neurons in the brain. Using this method, we will be able to selectively mark γ, α'/β', and α/β neurons that are lacking Ptpmeg protein. This will permit us to examine the behavior of individual axons that are homozygous mutant for *Ptpmeg* and more thoroughly classify any observed defects in processes such as axon growth, guidance or branching. This technique will also allow us to determine precisely where and when *Ptpmeg* function is required within the mushroom body neurons.

REFERENCES

Bashaw, G. J. and Goodman, C. S. (1999). Chimeric axon guidance receptors: the cytoplasmic domains of slit and netrin receptors specify attraction versus repulsion. *Cell* **97**, 917-26.

Bashaw, G. J., Kidd, T., Murray, D., Pawson, T. and Goodman, C. S. (2000). Repulsive axon guidance: Abelson and Enabled play opposing roles downstream of the roundabout receptor. *Cell* **101**, 703-15.

Ceranik, K., Zhao, S. and Frotscher, M. (2000). Development of the entorhino-hippocampal projection: guidance by Cajal-Retzius cell axons. *Ann N Y Acad Sci* **911**, 43-54.

Fernandis, A. Z. and Ganju, R. K. (2001). Slit: a roadblock for chemotaxis. *Sci STKE* **2001**, PE1.

Flint, A. J., Tiganis, T., Barford, D. and Tonks, N. K. (1997). Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc Natl Acad Sci U S A* **94**, 1680-5.

Garcia-Bellido, A. (1975). Genetic control of wing disc development in Drosophila. *Ciba Found Symp* **0**, 161-82.

Georgiou, M. and Tear, G. (2003). The N-terminal and transmembrane domains of Commissureless are necessary for its function and trafficking within neurons. *Mech Dev* **120**, 1009-19.

Green, P., Hartenstein, A. Y. and Hartenstein, V. (1993). The embryonic development of the Drosophila visual system. *Cell Tissue Res* **273**, 583-98.

Guan, K. L. and Rao, Y. (2003). Signalling mechanisms mediating neuronal responses to guidance cues. *Nat Rev Neurosci* **4**, 941-56.

Hivert, B., Liu, Z., Chuang, C. Y., Doherty, P. and Sundaresan, V. (2002). Robo1 and Robo2 are homophilic binding molecules that promote axonal growth. *Mol Cell Neurosci* **21**, 534-45.

Irvine, K. D. and Rauskolb, C. (2001). Boundaries in development: formation and function. *Annu Rev Cell Dev Biol* **17**, 189-214.

Keleman, K., Rajagopalan, S., Cleppien, D., Teis, D., Paiha, K., Huber, L. A., Technau, G. M. and Dickson, B. J. (2002). Comm sorts robo to control axon guidance at the Drosophila midline. *Cell* **110**, 415-27.

Kidd, T., Bland, K. S. and Goodman, C. S. (1999). Slit is the midline repellent for the robo receptor in Drosophila. *Cell* **96**, 785-94.

Kidd, T., Brose, K., Mitchell, K. J., Fetter, R. D., Tessier-Lavigne, M., Goodman, C. S. and Tear, G. (1998). Roundabout controls axon crossing of the CNS midline and defines a novel subfamily of evolutionarily conserved guidance receptors. *Cell* **92**, 205-15.

Kunes, S. and Steller, H. (1991). Ablation of Drosophila photoreceptor cells by conditional expression of a toxin gene. *Genes Dev* **5**, 970-83.

Kussel-Andermann, P., El-Amraoui, A., Safieddine, S., Nouaille, S., Perfettini, I., Lecuit, M., Cossart, P., Wolfrum, U. and Petit, C. (2000). Vezatin, a novel transmembrane protein, bridges myosin VIIA to the cadherin-catenins complex. *Embo J* **19**, 6020-9.

Lee, C. H., Herman, T., Clandinin, T. R., Lee, R. and Zipursky, S. L. (2001). N-cadherin regulates target specificity in the Drosophila visual system. *Neuron* **30**, 437-50.

Lee, T. and Luo, L. (2001). Mosaic analysis with a repressible cell marker (MARCM) for Drosophila neural development. *Trends Neurosci* **24**, 251-4.

Lohs-Schardin, M., Sander, K., Cremer, C., Cremer, T. and Zorn, C. (1979). Localized ultraviolet laser microbeam irradiation of early Drosophila embryos: fate maps based on location and frequency of adult defects. *Dev Biol* **68**, 533-45.

Patel, N. H., Snow, P. M. and Goodman, C. S. (1987). Characterization and cloning of fasciclin III: a glycoprotein expressed on a subset of neurons and axon pathways in Drosophila. *Cell* **48**, 975-88.

Piper, M. and Little, M. (2003). Movement through Slits: cellular migration via the Slit family. *Bioessays* **25**, 32-8.

Poeck, B., Fischer, S., Gunning, D., Zipursky, S. L. and Salecker, I. (2001). Glial cells mediate target layer selection of retinal axons in the developing visual system of Drosophila. *Neuron* **29**, 99-113.

Reuter, J. E., Nardine, T. M., Penton, A., Billuart, P., Scott, E. K., Usui, T., Uemura, T. and Luo, L. (2003). A mosaic genetic screen for genes necessary for Drosophila mushroom body neuronal morphogenesis. *Development* **130**, 1203-13.

Rhee, J., Mahfooz, N. S., Arregui, C., Lilien, J., Balsamo, J. and VanBerkum, M. F. (2002). Activation of the repulsive receptor Roundabout inhibits N-cadherin-mediated cell adhesion. *Nat Cell Biol* **4**, 798-805.

Schneider, S., Buchert, M., Georgiev, O., Catimel, B., Halford, M., Stacker, S. A., Baechi, T., Moelling, K. and Hovens, C. M. (1999). Mutagenesis and selection of PDZ domains that bind new protein targets. *Nat Biotechnol* **17**, 170-5.

Snow, P. M., Bieber, A. J. and Goodman, C. S. (1989). Fasciclin III: a novel homophilic adhesion molecule in Drosophila. *Cell* **59**, 313-23.

Suh, G. S., Poeck, B., Chouard, T., Oron, E., Segal, D., Chamovitz, D. A. and Zipursky, S. L. (2002). Drosophila JAB1/CSN5 acts in photoreceptor cells to induce glial cells. *Neuron* **33**, 35-46.

Wills, Z., Emerson, M., Rusch, J., Bikoff, J., Baum, B., Perrimon, N. and Van Vactor, D. (2002). A Drosophila homolog of cyclase-associated proteins collaborates with the Abl tyrosine kinase to control midline axon pathfinding. *Neuron* **36**, 611-22.

APPENDIX

Axon targeting in the *Drosophila* visual system

The following review entitled, "Axon targeting in the *Drosophila* visual system" by Timothy Tayler and Paul Garrity was published in the journal Current Opinion in Neurobiology (2003 Feb:13(1):90-95).

Abstract

The neuronal wiring of the *Drosophila melanogaster* visual system is constructed through an intricate series of cell–cell interactions. Recent studies have identified some of the gene regulatory and cytoskeletal signaling pathways responsible for the layer-specific targeting of *Drosophila* photoreceptor axons. Target selection decisions of the R1–R6 subset of photoreceptor axons have been found to be influenced by the nuclear factors Brakeless and Runt, and target selection decisions of the R7 subset of axons have been found to require the cell-surface proteins Ptp69d, Lar and N-cadherin. A role for the visual system glia in orienting photoreceptor axon outgrowth and target selection has also been uncovered.

Introduction

The fruitfly *Drosophila melanogaster* has a compound eye comprising roughly 750 ommatidia, each of which contains eight uniquely identifiable photoreceptor neurons, or 'R-cells', numbered R1 to R8 [1]. The R-cells provide a favorable system for examining axon targeting because the projections of R-cell axons are relatively simple and can be easily visualized and genetically manipulated [2, 3, 4 and 5]. R-cells fall into three basic classes: R1–R6, R7 and R8. R1–R6 extend axons to targets in the outermost optic ganglion of the brain, the lamina, whereas R7 and R8 extend axons through the lamina to targets in two distinct layers of the underlying optic ganglion, the medulla (Figure 1; [6]) precise layer-specific targeting provides a simple system for studying how axons choose appropriate targets.

In this review, we discuss recent progress towards understanding the molecular mechanisms that control the projection of photoreceptor axons to their appropriate target layers in the optic lobe.

R-cell axon extension into the optic stalk depends on retinal basal glia

The first stage of the journey of an R-cell axon involves its projection from the eye disc, where the R-cells differentiate, into the brain. R-cell axons reach the brain by projecting towards the posterior edge of the eye disc and through the optic stalk — a thin tube that connects the eye disc to the brain. To enter the optic stalk, R-cell axons rely on glial cells, known as retinal basal glia (RBG), which originate in the optic stalk and migrate into the eye disc (Figure2a; [7 and 8]). If RBG entry into the eye disc is inhibited, R-cell axons fail to enter the optic stalk (Figure 2b; [8]). Recent work suggests that the timing of RBG migration into the eye disc is crucial for R-cell axon guidance.

RBG enter the eye disc as retinal patterning commences at the early third instar larval phase with a wave of morphogenesis proceeding from posterior-to-anterior regions of the eye disc (Figure 2a). At the leading edge of this retinal patterning wave is a characteristic zone of cell shortening known as the 'morphogenetic furrow' [1]. The incoming RBG also travel from posterior to anterior to fill the basal layer of the eye disc, with the leading edge of the RBG trailing the advancing morphogenetic furrow (Figure 2a). The RBG are thus well positioned to provide a path for the R-cell axons to follow into the optic stalk.

Hummel et al. [9] have shown that the RBG may provide such a path in experiments that examined the effect of loss-of-function mutations in several genes, including *gish* (encoding casein kinase I), that cause the RBG to enter the eye disc prematurely before retinal differentiation. In these mutants, many RBG end up anterior to the morphogenetic furrow and, when R-cell differentiation commences, R-cell axons follow these ectopic RBG and project away from, rather than towards, the optic stalk (Figure 2c). Thus, a trail of RBG leading toward the optic stalk is important for directing R-cell axon growth into the optic stalk. The signals that RBG send the R-cell axons to regulate their extension are not known. The signals that come from the eye disc to control

RBG entry are also unknown, although the secreted proteins Hedgehog [9] and Dpp [10] have been identified as potential candidates.

Targeting of R1–R6 axons to the lamina

Once R-cell axons enter the brain, they are faced with a choice between two target regions: the lamina and the medulla (Figure 3a). What mechanisms cause the growth cones of R1–R6 axons to stop in the lamina, but let the growth cones of R7 and R8 axons pass through the lamina into the medulla? An important step towards answering this question has come from genetic analysis of the nuclear protein Brakeless.

In the absence of Brakeless function, nearly all R1–R6 axons proceeded into the medulla (Figure 3b; [11 and 12]). Brakeless controls the targeting of R1–R6 axons by acting in the retina but, somewhat surprisingly, Brakeless protein is present in the nuclei of all R-cell types. In addition, overexpression of Brakeless in all R-cells does not retarget R7 or R8 axons to the lamina. Thus, Brakeless function in the eye is necessary, but not sufficient, to target R-cell axons to the lamina.

The nuclear localization of Brakeless protein suggests that it has a role in gene regulation. Consistent with this hypothesis, Kaminker et al. [13] find that expression of the transcription regulator Runt is misregulated in brakeless mutants. Runt is normally expressed only in R7 and R8, but in brakeless mutants Runt is also expressed in R2 and R5. When Runt was ectopically expressed in R2 and R5 in otherwise wild-type animals, a brakeless-like phenotype is observed, with R1–R6 axons projecting into the medulla. Thus, Brakeless probably acts in concert with additional regulatory factors to restrict Runt expression and to regulate R1–R6 axon target selection. But although Runt misexpression may suffice to explain mistargeting in brakeless mutants, normally Runt may act redundantly with other molecules to enforce target specificity because runt loss-of-function mutants show no targeting defects. Although undoubtedly there are additional factors to be identified, it is encouraging that the gene regulatory pathways that are responsible for conferring R-cell subtype identity on their axons may be emerging.

Brakeless and Runt probably influence axon targeting by altering the composition of the guidance machinery at the axon tip. Although no direct links have been made among Brakeless, Runt and growth cone signaling molecules, two cell-surface proteins

have been implicated in the decision of R1–R6 axons to stop at the lamina: the receptor-like tyrosine phosphatases Ptp69d and Lar. In contrast to brakeless mutants, in which the majority of R1–R6 axons are mistargeted into the medulla, in Ptp69d and Lar mutants a minority of R1–R6 axons are mistargeted [4, 14 and 15]. This is consistent with these receptor phosphatases having auxiliary or overlapping roles in R-cell axon targeting, similar to their overlapping roles in motor axon targeting [16]. Like Brakeless, Ptp69d and Lar have permissive rather than instructive roles in targeting, because the expression of either protein in all types of R-cell does not retarget their axons to the lamina. The molecular mechanism by which these tyrosine phosphatases influence R1–R6 axon target selection is not known, and roles in controlling interactions among R-cell axons or interactions between R-cell axons and their targets are both plausible.

Cytoplasmic signaling pathways that participate in stopping the R1–R6 growth cones in the lamina have been identified but have not been linked to particular receptors as yet. A subset of R1–R6 axons extends past the lamina in animals carrying loss-of-function mutations in the Dock Src homology domain 2/Src homology domain 3 (SH2/SH3) adaptor protein [17] and the Misshapen serine/threonine kinase, a member of the Ste20 family that associates with Dock [18 and 19]. Interestingly, overexpression of Misshapen in R-cell axons causes R1–R6 axons to stop before reaching the lamina, suggesting that activation of Misshapen at the lamina may be sufficient to stop R1–R6 axons at the lamina [18]. Mutations affecting other cytoplasmic signaling molecules required for R-cell axon navigation have been identified [20, 21 and 22] but for at least two genes, Pak and Trio, loss of function does not detectably alter selection of the R1–R6 target layer [20 and 21], which is consistent with the differential use of distinct cytoplasmic signaling pathways in different R-cell axon guidance decisions.

Visual system glia regulate R-cell axon targeting

The targeting of R1–R6, R7 and R8 axons to different layers in the visual system suggests that there are molecular labels or signals in the target that allow these layers to be distinguished. The identity of guidance cues sent by the target are unknown, however, it had been suggested that glial cells in the lamina might be an important source of targeting information [23]. Although the R1–R6 axons eventually form synapses with

lamina neurons, R1–R6 growth cones initially stop between two layers of lamina glial cells: the epithelial glia and marginal glia (Figure 3a).

In fact, lamina neurons have been found to be dispensable for the initial stages of R1–R6 targeting. In hh1 mutant animals, no lamina neurons form but R1–R6 axons are targeted normally [24 and 25]. By contrast, mutations that disrupt the positioning of lamina glia severely disrupt R1–R6 targeting: both Poeck et al. [25] and Suh et al. [26] found that large numbers of R1–R6 axons projected through the lamina into the medulla when lamina glia were missing or reduced in number in nonstop and jab1/csn5 mutants (Figure 3c). A key goal for the future is to understand how these glia allow the R1–R6 axons to stop selectively in the lamina.

Targeting of R7 and R8 axons to distinct layers of the medulla

Both R7 and R8 axons project through the lamina into the medulla, however, each class of axon is targeted to a distinct layer within the medulla: R7 axons synapse with targets in the M6 layer, whereas R8 axons recognize targets in the more superficial M3 layer (Figure 4a; [27]). Recent work has identified three cell-surface proteins necessary for proper R7 axon targeting, the receptor tyrosine phosphatases Ptp69d and Lar (the same receptors required for R1–R6 layer-specific targeting) and N-Cadherin [4, 5, 15 and 28]. In animals with mutations in any of these genes, a large percentage of R7 axons stop short of their normal targets and project to M3 — the layer to which R8 axons are targeted.

Analysis of the effects of Lar mutations on R7 axon targeting has provided a detailed view of the R7 targeting process (Figure 4b; [15]). In Lar mutants, R7 axons initially project beyond the tip of the R8 axons; however, the Lar mutant R7 growth cones eventually retract to rejoin the R8 growth cones in the M3 layer. Thus, Lar mutant R7 growth cones may transiently contact the target but fail to sustain the interaction.

The similarity of the R7 axon targeting defects in animals with mutations in Lar, Ptp69d and N-Cadherin suggests that these genes may function together. Clandinin et al. [15] have proposed that N-Cadherin mediates R7 axon adhesion to the target and that Lar positively regulates N-Cadherin signaling, possibly by dephosphorylating catenin — a downstream effector of N-Cadherin and a target of receptor phosphatase regulation in

vertebrates [29 and 30]. Maurel-Zaffran et al. [28], who independently identified the R7 targeting defect of Lar mutants, showed that Trio, a Rho family guanine nucleotide exchange factor, and Enabled, a regulator of actin dynamics, show genetic interactions with Lar. This work implicates additional pathways through which Lar may regulate the targeting of R7 axons to the medulla.

Conclusions

A promising start toward the dissection of R-cell axon target selection has been made. A major challenge for the future is to explain the high degree of target-layer discrimination that R-cell growth cones show and, in particular, to characterize the target recognition receptors carried by the R-cell axons and the molecules produced by the targets that they recognize. The molecules now known to regulate target-layer selection by R-cell axons — Ptp69d, Lar and N-Cadherin — are necessary but not sufficient to target an R-cell axon to a particular layer. Whether these molecules are part of a combinatorial code that targets an axon to a particular layer or whether they are general mediators of growth cone navigation that permit as yet unidentified specificity receptors to do their jobs is an important issue for the future.

Another challenge for the future is the elucidation of the mechanisms that mediate R-cell synapse formation [6 and 31]. The initial layer-specific targeting decisions discussed in this review are essential for proper connectivity but are just the first step in assembling the circuitry of the visual system. In the lamina, for example, the R1–R6 axons undergo complex, highly stereotyped rearrangements later in development to contact their appropriate lamina neuron targets [32, 33 and 34]. The establishment of these connections requires some of the molecules discussed above, such as Lar and N-Cadherin [5 and 15], but the molecular dissection of this stage of visual system development is only just beginning [35].

Acknowledgements

We thank Linda Huang for her helpful comments. Work in the authors' laboratory is supported by the National Eye Institute, the Raymond and Beverly Sackler Foundation, and the McKnight Foundation.

References

1. Wolff T, Ready DF: Pattern formation in the *Drosophila* retina. In The Development of *Drosophila melanogaster*. Edited by Bate M, Martinez-Arias A. Cold Spring Harbor Press; 1993:1277-1325.

2. Pignoni F, Hu B, Zavitz KH, Xiao J, Garrity PA, Zipursky SL: The eye-specification proteins So and Eya form a complex and regulate multiple steps in *Drosophila* eye development. [published erratum appears in Cell 1998 Feb 20;92(4):following 585]. Cell 1997, 91:881-891.

3. R.S. Stowers and T.L. Schwarz, A genetic method for generating *Drosophila* eyes composed exclusively of mitotic clones of a single genotype. Genetics 152 (1999), pp. 1631–1639.

4. T.P. Newsome, B. Asling and B.J. Dickson, Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. Development 127 (2000), pp. 851–860.

5. C.H. Lee, T. Herman, T.R. Clandinin, R. Lee and S.L. Zipursky, N-Cadherin regulates target specificity in the *Drosophila* visual system. Neuron 30 (2001), pp. 437–450.

6. Meinertzhagen IA, Hanson TE: The development of the optic lobe. In The Development of *Drosophila melanogaster*. Edited by Bate M, Martinez-Arias A. Cold Spring Harbor Press; 1993:1363-1491.

7. K.W. Choi and S. Benzer, Migration of glia along photoreceptor axons in the developing *Drosophila* eye. Neuron 12 (1994), pp. 423–431.

8. R. Rangarajan, Q. Gong and U. Gaul, Migration and function of glia in the developing *Drosophila* eye. Development 126 (1999), pp. 3285–3292.

9. T. Hummel, S. Attix, D. Gunning and S.L. Zipursky, Temporal control of glial cell migration in the *Drosophila* eye requires gilgamesh, hedgehog, and eye specification genes. Neuron 33 (2002), pp. 193–203.

10. R. Rangarajan, H. Courvoisier and U. Gaul, Dpp and Hedgehog mediate neuron-glia interactions in *Drosophila* eye development by promoting the proliferation and motility of subretinal glia. Mech. Dev. 108 (2001), pp. 93–103.

11. Y. Rao, P. Pang, W. Ruan, D. Gunning and S.L. Zipursky, brakeless is required for photoreceptor growth-cone targeting in *Drosophila*. Proc. Natl. Acad. Sci. USA 97 (2000), pp. 5966–5971.

12. K. Senti, K. Keleman, F. Eisenhaber and B.J. Dickson, brakeless is required for lamina targeting of R1–R6 axons in the *Drosophila* visual system. Development 127 (2000), pp. 2291–2301.

13. J.S. Kaminker, J. Canon, I. Salecker and U. Banerjee, Control of photoreceptor axon target choice by transcriptional repression of Runt. Nat. Neurosci. 5 (2002), pp. 746–750.

14. P.A. Garrity, C.H. Lee, I. Salecker, H.C. Robertson, C.J. Desai, K. Zinn and S.L. Zipursky, Retinal axon target selection in *Drosophila* is regulated by a receptor protein tyrosine phosphatase. Neuron 22 (1999), pp. 707–717. SummaryPlus | Full Text + Links | PDF (546 K)

15. T.R. Clandinin, C.H. Lee, T. Herman, R.C. Lee, A.Y. Yang, S. Ovasapyan and S.L. Zipursky, *Drosophila* LAR regulates R1–R6 and R7 target specificity in the visual system. Neuron 32 (2001), pp. 237–248.

16. C.J. Desai, N.X. Krueger, H. Saito and K. Zinn, Competition and cooperation among receptor tyrosine phosphatases control motoneuron growth cone guidance in *Drosophila.* Development 124 (1997), pp. 1941–1952.

17. P.A. Garrity, Y. Rao, I. Salecker, J. McGlade, T. Pawson and S.L. Zipursky, *Drosophila* photoreceptor axon guidance and targeting requires the Dreadlocks SH2/SH3 adapter protein. Cell 85 (1996), pp. 639–650.

18. W. Ruan, P. Pang and Y. Rao, The SH2/SH3 adaptor protein dock interacts with the Ste20-like kinase misshapen in controlling growth cone motility. Neuron 24 (1999), pp. 595–605.

19. Y.C. Su, C. Maurel-Zaffran, J.E. Treisman and E.Y. Skolnik, The Ste20 kinase misshapen regulates both photoreceptor axon targeting and dorsal closure, acting downstream of distinct signals. Mol. Cell. Biol. 20 (2000), pp. 4736–4744.

20. H. Hing, J. Xiao, N. Harden, L. Lim and S.L. Zipursky, Pak functions downstream of Dock to regulate photoreceptor axon guidance in *Drosophila.* Cell 97 (1999), pp. 853–863.

21. T.P. Newsome, S. Schmidt, G. Dietzl, K. Keleman, B. Asling, A. Debant and B.J. Dickson, Trio combines with dock to regulate Pak activity during photoreceptor axon pathfinding in *Drosophila.* Cell 101 (2000), pp. 283–294.

22. T. Hummel, K. Leifker and C. Klambt, The *Drosophila* HEM-2/NAP1 homolog KETTE controls axonal pathfinding and cytoskeletal organization. Genes Dev. 14 (2000), pp. 863–873.

23. S.E. Perez and H. Steller, Migration of glial cells into retinal axon target field in *Drosophila* melanogaster. J. Neurobiol. 30 (1996), pp. 359–373.

24. Z. Huang and S. Kunes, Hedgehog, transmitted along retinal axons, triggers neurogenesis in the developing visual centers of the *Drosophila* brain. Cell 86 (1996), pp. 411–422.

25. B. Poeck, S. Fischer, D. Gunning, S.L. Zipursky and I. Salecker, Glial cells mediate target layer selection of retinal axons in the developing visual system of *Drosophila*. Neuron 29 (2001), pp. 99–113.

26. G.S. Suh, B. Poeck, T. Chouard, E. Oron, D. Segal, D.A. Chamovitz and S.L. Zipursky, *Drosophila* JAB1/CSN5 acts in photoreceptor cells to induce glial cells. Neuron 33 (2002), pp. 35–46.

27. K.F. Fischbach and A.P.M. Dittrich, The optic lobe of *Drosophila* melanogaster. I. A Golgi analysis of wild-type structure. Cell Tissue Res. 258 (1989), pp. 441–475.

28. C. Maurel-Zaffran, T. Suzuki, G. Gahmon, J.E. Treisman and B.J. Dickson, Cell-autonomous and -nonautonomous functions of LAR in R7 photoreceptor axon targeting. Neuron 32 (2001), pp. 225–235.

29. R.M. Kypta, H. Su and L.F. Reichardt, Association between a transmembrane protein tyrosine phosphatase and the cadherin–catenin complex. J. Cell. Biol. 134 (1996), pp. 1519–1529.

30. S.M. Brady-Kalnay, T. Mourton, J.P. Nixon, G.E. Pietz, M. Kinch, H. Chen, R. Brackenbury, D.L. Rimm, R.L. Del Vecchio and N.K. Tonks, Dynamic interaction of PTP with multiple cadherins in vivo. J. Cell. Biol. 141 (1998), pp. 287–296.

31. I.A. Meinertzhagen and K.E. Sorra, Synaptic organization in the fly's optic lamina: few cells, many synapses and divergent microcircuits. Prog. Brain Res. 131 (2001), pp. 53–69.

32. O. Trujillo-Cenoz and J. Melamed, Compound eye of dipterans: anatomical basis for integration — an electron microscope study. J. Ultrastruct. Res. 16 (1966), pp. 395–398.

33. V. Braitenberg, Patterns of projection in the visual system of the fly. I. Retina–lamina projections. Exp. Brain Res. 3 (1967), pp. 271–298.

34. I.A. Meinertzhagen, Wiring the fly's eye. Neuron 28 (2000), pp. 310–313.

35. T.R. Clandinin and S.L. Zipursky, Afferent growth cone interactions control synaptic specificity in the *Drosophila* visual system. Neuron 28 (2000), pp. 427–436.

FIGURES

Figure 1. Projection of R-cell axons to targets in the optic lobe. A single ommatidium containing eight R-cell neurons is shown. The *Drosophila* adult eye contains about 750 ommatidia. R-cell axons project through the optic stalk into the optic lobe, where they contact targets in two ganglia: the lamina and the medulla. The R1–R6 axons (green) stop at their target layer in the lamina, whereas the R7 axon (red) and the R8 axon (blue) continue into the underlying medulla, where they stop in two distinct layers.

Figure 2. R-cell axons (green) rely on glial cells (orange) to project into the optic stalk and enter the brain. These glial cells, known as the retinal basal glia (RBG), originate in the optic stalk and migrate into the eye disc. **(a)** In wild type, glia enter the eye disc as R-cell development begins, forming a trail extending from the eye disc to the optic lobe. The morphogenetic furrow marks the leading edge of R-cell differentiation as R-cell development proceeds from posterior to anterior across the eye disc (from bottom to top in this figure). **(b)** When glia are unable to enter the eye disc, due to expression of a dominant-negative form of the small GTPase Ras, R-cell axons fail to enter the optic stalk. **(c)** In *gish* mutants, glia enter the eye disc before the initiation of R-cell differentiation and migrate to ectopic locations anterior of the morphogenetic furrow. Many R-cell axons follow this ectopic glial trail and project away from the optic stalk and the brain, suggesting that glial cells are important for orienting early stages of R-cell axon outgrowth.

Figure 3. R1–R6 axon targeting to the lamina. **(a)** In wild type, the R1–R6 axons (green) stop between layers of glial cells (orange), whereas the R7 axons (not shown) and the R8 axons (blue) project through these glia into the medulla. These layers of glia are referred to as the epithelial glia (eg), the marginal glia (mg) and the medulla glia (me). **(b)** In *brakeless* mutants, the glia assume their normal positions, but the R1–R6 axons project through them to enter the medulla. **(c)** In *nonstop* or *jab1/csn5* mutants, the number of

glia at the lamina/medulla interface is reduced and the glia are often disorganized. In the absence of a properly patterned target, the R1–R6 axons project into the medulla.

Figure 4. R7 and R8 axons are targeted to distinct layers of the medulla. **(a)** In wild type, the R8 axon (blue) from each ommatidium enters the target first. The R7 axon (red) projects along the R8 axon and initially stops just beneath the R8 axon. The distance between the R7 and R8 growth cones increases during pupal development as additional fibers enter the medulla. In the adult, the R8 axon contacts targets in the M3 layer of the medulla, whereas the R7 axon contacts targets in the M6 layer. **(b)** In *Lar* mutants, the R7 axon initially extends beyond the R8 axon; however, the R7 growth cone is morphologically abnormal. The R7 axon subsequently withdraws to the position of the R8 axon tip as development proceeds, eventually terminating in the M3 layer.

Figure 1

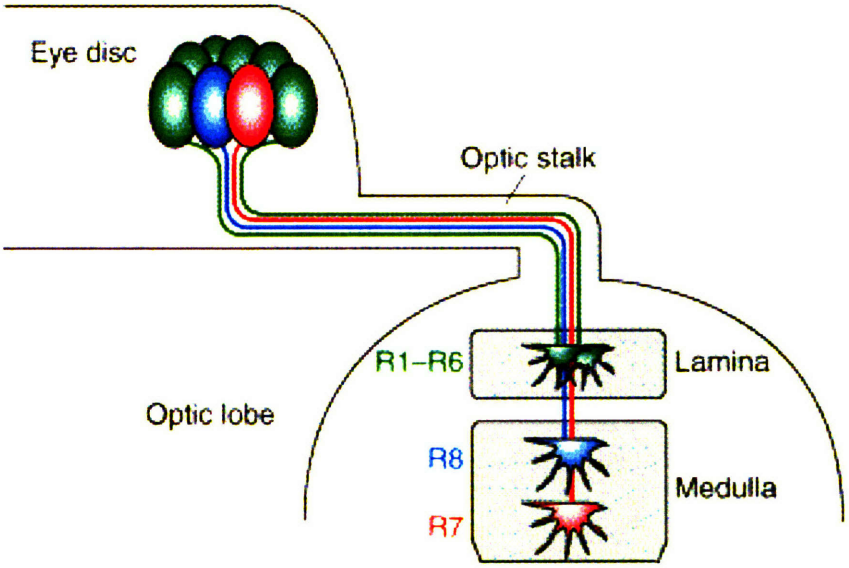


Figure 2

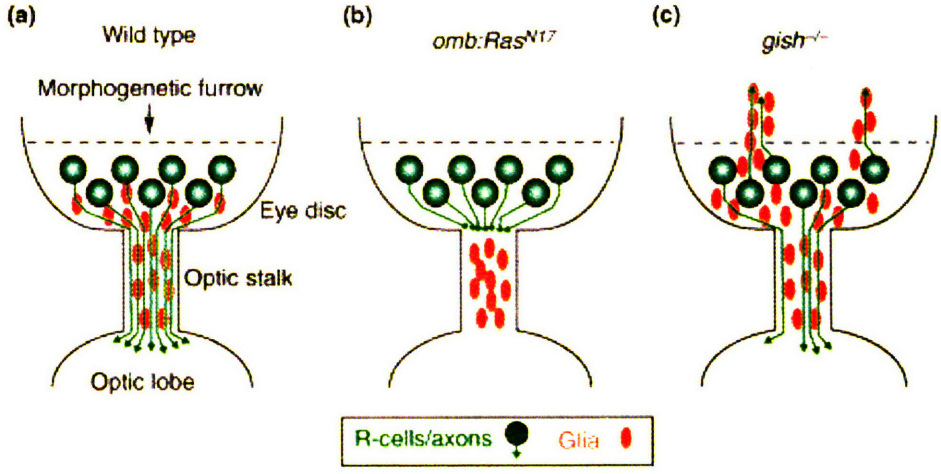


Figure 3

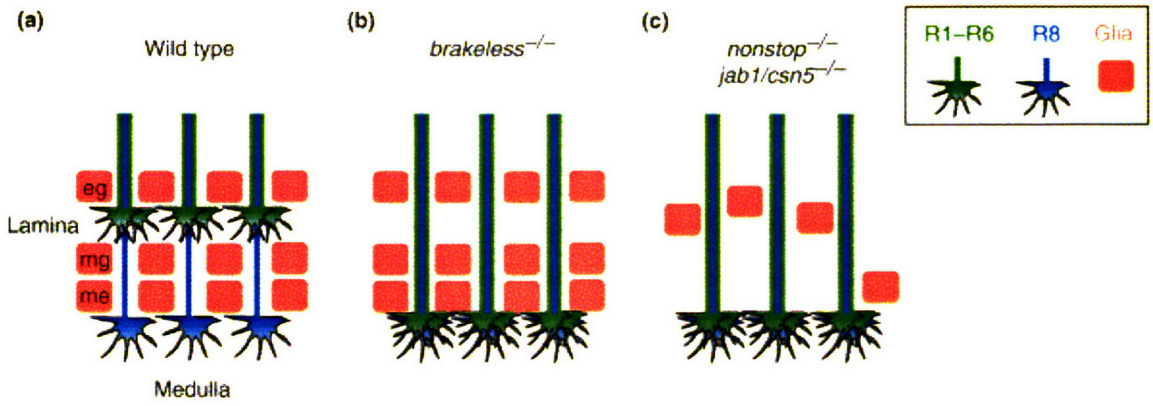
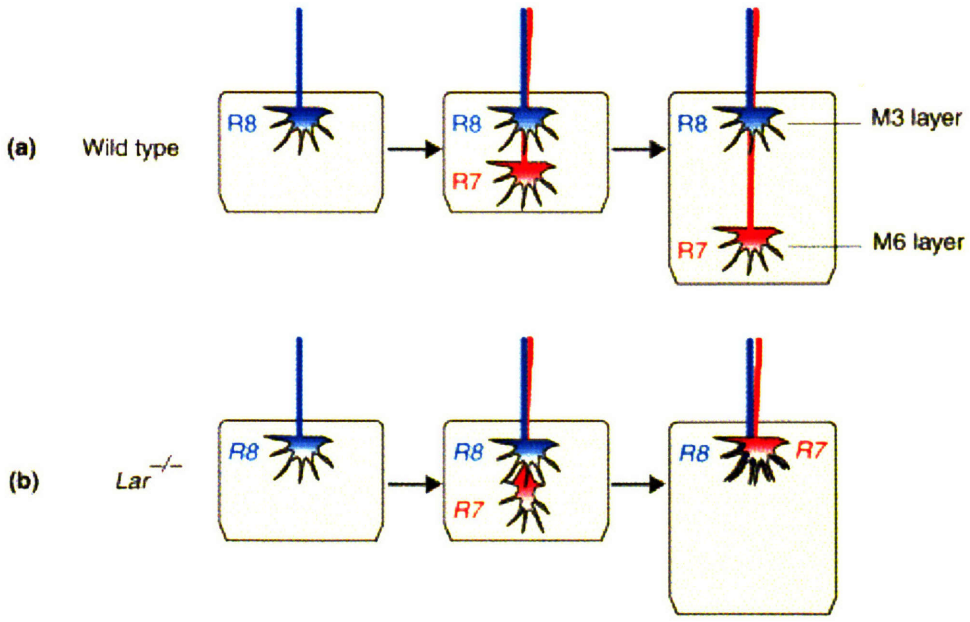


Figure 4



Acknowledgements

There are many people who have contributed to my graduate career and this thesis work would not have been possible without them. I would like to thank my advisor, Paul Garrity, who patiently introduced me to the tools and techniques of *Drosophila* neurobiology. His enthusiasm for science has been an inspiration. I was also fortunate to work with many wonderful scientists in the Garrity Lab. I thank them all for their curiosity, intelligence, and friendship. I would also like to thank the members of my thesis committee, Bob Horvitz, Harvey Lodish, Mary-Lou Pardue, and Morgan Sheng. Their constructive criticism and advice contributed significantly to my work. I am deeply grateful to Mala Murthy for commenting on and editing this thesis. Without her support, this thesis would not have been possible. Finally, I would like to acknowledge my parents, Barbara and Charles Tayler. Their love and support continues to guide me through life's challenges.