Genetic Analysis of the Maintenance of Neuronal Morphology in
Drosophila melanogaster

by

Jessica LaMae Whited

B. S., Biological Sciences
B. A., Philosophy
University of Missouri, 1998

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

June 2006

Signature of Author: _____
Department of Biology
May 3, 2006

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

Accepted by: _____
Stephen P. Bell
Professor of Biology
Chairman, Committee for Graduate Students

Genetic Analysis of the Maintenance of Neuronal Morphology in *Drosophila melanogaster*

By

Jessica LaMae Whited

Submitted to the Department of Biology on May 26, 2006, in partial fulfillment of the requirements for the Degree of Doctor of Philosophy in Biology

ABSTRACT

Precise control of cellular morphology is critical for both the development and maintenance of nervous systems. In the developing *Drosophila* eye, normal photoreceptor cells establish and maintain a highly polarized architecture, with cell bodies and nuclei located apically in the epithelium, and axons extending basally into the brain. Disruption of the Dynactin complex, which activates the minus-end-directed microtubule motor protein Dynein, causes mislocalization of photoreceptor nuclei basally, even into the optic stalk and brain. Photoreceptors in animals mutant for the Dynactin subunit *Glued* retain apical markers, but have a bipolar-like morphology with the cell body translocated toward the brain and an apical process extending to the surface of the eye disc. Dynactin is required post-mitotically to maintain proper nuclear positioning. Using a genetic screen, I identified loss-of-function alleles of *kinesin heavy chain*, encoding a subunit of the plus-end-directed microtubule motor Kinesin, as suppressors of the rough eye and nuclear mispositioning in *Glued* mutants. Thus, a balance of minus-end-directed and plus-end-directed microtubule motor forces may be required to maintain nuclear position within postmitotic neurons. Establishment and maintenance of complex axonal trajectories is also a key feature of neuronal mophology. I identified a requirement for a novel cytoplasmic tyrosine phosphatase, PTPMEG, in these processes. Normal mushroom bodies, structures critical for insect learning and memory, have dorsally-projecting alpha lobe and medially-projecting beta lobe axons. Alpha lobes develop normally in *ptpmeg* mutants, but their pattern is not maintained. Instead, alpha lobe axons retract during pupation, resulting in thin and/or shortened alpha lobes. Meanwhile, beta lobe axons overextend at the midline. Removing *ptpmeg* function in mushroom bodies does not cause mutant phenotypes. *ptpmeg* mutants are rescued by pan-neuronal expression of wild-type Ptpmeg, but not by versions with disrupted phosphatase activity. These data suggest that Ptpmeg activity is required in another type of neuron to prevent mushroom body axon retraction. Ellipsoid body axons normally form a ring structure in the central brain. In *ptpmeg* mutants, the ellipsoid body axons develop abnormally, with the ventral side of the ring being discontinuous; the defect can be rescued by expression of wild-type Ptpmeg pan-neuronally.

Thesis supervisor: Paul A. Garrity
Title: Assistant Professor of Biology

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

I thank my advisor, Dr. Paul A. Garrity, for his advice and support. He has always found time to discuss whatever was on my mind, without appointment, and I consider this to have been critical for my intellectual development as a scientist. I am lucky to have found an advisor with such a zest for science as his. I thank the members of the Garrity Laboratory, especially those directly involved in the projects described here and whose work is separately acknowledged at the beginning of chapters. I also thank members of my thesis committee for their time, suggestions, and advice, including Dr. Terry Orr-Weaver, Dr. Mary-Lou Pardue, Dr. Bob Horvitz, and Dr. Michael Welte. Dr. Frank Gertler and Dr. Andy Chess were instrumental in my early pursuit of the photoreceptor cell body positioning story, and for that I thank them. Thanks to Dr. Frank Solomon and Dr. Steve Bell for advice. I also acknowledge the many wonderful teachers I had in high school and as professors at the University of Missouri, especially Dr. Diann Jordan and Vicki Hubbard, who gave me my first job in science; Dr. Miriam Golomb, my undergraduate research advisor; and Dr. Alexander von Schonborn, my philosophy advisor who guided me through the decision to pursue graduate studies in biology. Many of my fellow students have provided me with an important outlet for scientific conversation, and I thank David MacPherson and Mark Rosenzweig in particular. I thank Hilda Harris-Ransom for her guidance regarding bureaucratic matters.

Finally, I thank my family for their love and for the closeness that we share despite our geographical separation. My mom and dad, Debbie and Tim, instilled in me a sense of awe for nature at a very young age and I am deeply indebted to them for having

done so. I am also grateful for the friendship of my sister, Kimmi, and her family, Danny and Finn.

INTRODUCTION

Biological form requires both change and maintenance. Change encompasses processes of cell division, cell growth, differentiation, cell migration, and others, which are generally thought of as development. Once changes have occurred, however, the result must either be maintained or serve as substrate for additional changes. Maintenance of particular states may either be an active process, for instance, requiring the activity of particular proteins, or a default-type process in which further change is not possible. The *Drosophila melanogaster* nervous system provides an ideal arena for exploration of both the developmental changes and maintenance systems required for biological form.

NERVOUS SYSTEM SPECIFICATION

Evolutionarily conserved developmental programs partition early embryos into areas fated to become endoderm, mesoderm, and ectoderm. The nervous system is specified from ectoderm (reviewed in Harland, 2000). Acquisition of neural fate can occur either via cell-cell interactions or the enactment of a lineage-dictated cell fate.

NEURONAL MIGRATION

Many newborn neurons must migrate from their birthplace to their eventual locations. These migrations can occur over relatively small distances, for example, just a few cell lengths, or they can occur over large distances (reviewed in Kriegstein and Noctor, 2004). Neurons in the developing human cortex migrate several hundred cell lengths. Neuronal migration is tightly regulated and must occur precisely to avoid

developmental defects that can impact function. The mechanisms used for neuronal migration share many features with those used to prevent aberrant movement of cellular components during maintenance stages and will thus be discussed more fully in later sections.

ESTABLISHING NEURONAL POLARITY

Once neurons have been created and have reached their migratory destinations, they need to grow and connect the processes that will allow them to function. A neuron must first acquire a polarity, which will allow it to develop separate parts for sending and receiving information. The axonal compartment will be enriched with factors specialized for presynaptic transmission of information, while the dendritic compartment will be enriched with factors specialized for postsynaptic receipt of information. These two compartments share the same membrane, so proper intracellular localization of compartment identity factors is essential. Neuronal cell polarity is particularly apparent in the organization of the microtubule cytoskeleton and the function of the motor proteins that travel along it, as summarized in Figure 1. Early studies showed that neurons have highly ordered microtubule arrays, and that the ends of the microtubules were distinguishable from one another, suggesting they were polarized (Chalfie and Thomson, 1979). Microtubules in frog axons were shown to have a single polarity, prompting speculation that bidirectional transport in axons must therefore depend on the transport machinery as opposed to the polarity of the microtubules used as tracks (Burton and Page, 1981). Cat axons were also shown to have polarized microtubules, and it was determined that the fast-growing microtubule end (the plus end) is oriented toward the

cell cortex in axons, away from the soma, while the slow-growing minus ends were toward the soma (Heidemann et al., 1981). Dendrites were later shown to have mixed orientations of microtubules, with some plus ends at the cortex as well as some minus ends (Baas et al., 1988).

Microtubule formation and localization in neurons

Microtubule nucleation is often initiated at a specific sites in cells referred to as a microtubule organizing centers (MTOC) (reviewed in Desai and Mitchison, 1997). In most animal cells, the MTOC is the centrosome, which is composed of two centrioles as well as a cloud of surrounding proteins, such as pericentrin and gamma-tubulin (γ-tubulin) (reviewed in Dammermann et al., 2003). The centrosome is often situated close to the nucleus in interphase and post-mitotic cells (reviewed in Gonczy, 2004). Microtubules are formed from polymerized dimers of α and β tubulin, with one α-tubulin joined to one β-tubulin to form a dimer (Weisenberg et al., 1968). Tubulin dimers link with other tubulin dimers to form long chains—or protofilaments—with alternating α and β subunits (Amos and Klug, 1974). In most cases, thirteen protofilaments are joined laterally into a tube to form one microtubule (Evans et al., 1985). Once formed, tubulin dimers can be directly added to or subtracted from either end of the microtuble in vitro. The fast-growing end is referred to as the plus end, and the slow-growing end is the minus end. In vivo, however, subunit addition has only been reported at the plus end (Dammermann et al., 2003). The nucleation steps in microtubule formation are facilitated by γ-tubulin located at the MTOC, which is thought to cap the minus end and perhaps inhibit subunit addition there; microtubules can also form outside of a MTOC, in

the cytoplasm (Dammermann et al, 2003). Polymerized microtubules are often highly dynamic, alternating between phases of growing and shrinking (Desai and Mitchison, 1997). In general, the plus end of the microtubule is directed away from the MTOC while the minus end is either still embedded in the MTOC or is interior to the cell cortex (Dammermann et al, 2003). Hence, in many cells, the minus-ends of microtubules can be used as a marker for the location of the MTOC. Both tubulin subunits bind GTP, but the β tubulin subunit can hydrolyze the GTP to GDP once it has become incorporated into a protofilament or microtubule (Weisenberg et al., 1976; Spiegelman et al., 1977). Stretches of tubulin polymers with GDP are less stable and more weakly connected to one another than those at the newly-added ends which still have GTP (Chretien and Wade, 1991; Hyman et al., 1995). When the microtuble has many fresh subunits at its tip which are in the tightly-connected, GTP-bound state, the growth of the microtubule is promoted (Mejillano et al., 1990). However, if growth stops and GTP hydrolysis catches up such that the end contains GDP, the microtubule will shrink.

The specialized structure and, often, incredible length of neurons and their processes require specific mechanisms for localizing microtubules (reviewed in Baas et al., 2005). In neurons, microtubule formation is initiated at the MTOC, but polymerized microtubules are later liberated from the MTOC by specific proteins so they can move down the lengths of axonal and dendritic processes to ensure adequate microtubule supply at distances far from the soma. Katanin is a protein complex that localizes to the centrosome and enzymatically cleaves microtubules there (Hartman et al., 1998). It is involved in cleaving microtubules during mitosis, but also works in post-mitotic neurons. Loss of Katanin function in cultured neurons causes microtubules to be too long and to

fail to be released from the MTOC, and it inhibits axon growth, suggesting it may be required in vivo to ensure that short pieces of microtubules are available for axon extension away from the cell body (Ahmad et al., 1999; Karabay et al., 2004). Shorter microtubules, formed by cleavage of longer ones, are thought to be more easily transported down the length of the axon by motor proteins (discussed later) (Wang and Brown, 2002). The arrangement of microtubules with respect to one another is controlled, in part, by microtubule-associated proteins (MAPs), which bind to microtubules and can crosslink them, and also increase microtubule stability. MAPs with shorter crosslinking spans, such as tau and MAP2C, are expressed in axons and those with longer crosslinking spans, such as MAP2 are expressed in dendrites; hence, the microtubules are packed closer together in axons and further apart in dendrites (Chen et al., 1992).

Microtubule-based transport

The inherent polarity of microtubules allows them to be used as tracks for machinery that translocates cellular cargos. Microtubule polarity allows for directional transport; translocation machinery, which binds particular sites in tubulin subunits, can travel distances simply by binding to and moving along one part of the microtubule polymer to an adjacent site. Microtubule polarity also allows for bidirectional transport if different machineries exist for travel in one direction versus the other, since the topography of the microtubule polymer is inherently different between the two directions.

Motor proteins are protein complexes that couple chemical energy to mechanical force. Those motor proteins that are used for cargo transport in the cell use the energy derived from nucleotide hydrolysis to cause a conformational change in the protein

allowing it to bind a new site on the molecular tracks it travels (reviewed in Mallik and Gross, 2004). Both microtubules and actin filaments have dedicated motor proteins. While microtubule-based transport is important for all cells, it is particularly essential for the function of neurons (reviewed in Hirokawa and Takemura, 2005). A single motor axon in an adult human body can be more than one meter in length. The specialized microtubule transport system is required for intracellular movement to take place quickly between the soma and the distal parts of the axon. Fast axonal transport rates in mammals, calculated by measuring the distance traveled by radiolabeled proteins within a cat sciatic nerve, are in excess of 4 cm per day (Ochs, 1972). Retrograde movement of membraneous vesicles occurs from the distal axon toward the cell body, while those vesicles traveling toward the distal axon tip are undergoing anterograde transport.

Cytoplasmic dynein, an ATPase, is responsible for most of the minus-end directed microtubule motor activity in eukaryotic cells (reviewed in Vallee et al., 2004). A large protein complex, cytoplasmic dynein's several polypeptide chains are named for their relative sizes, for example, dynein heavy chain, dynein intermediate chain, and dynein light chain. The processivity of a motor protein describes the average amount of time a single motor is engaged in one translocation session before it falls off or becomes disengaged from the microtubule. Dynein's processivity—and hence its activity as a cargo transporter—is greatly enhanced by a protein complex that binds both dynein and microtubules, dynactin (Gill et al., 1991; King and Schroer, 2000; Culver-Hanlon et al., 2006). Dynactin is also critical for the coupling of membranous cargo to the dynein motor (Holleran et al, 1996, 2001; Burkhardt et al., 1997; Kumar et al., 2001; Muresan et al, 2001). Dynactin's ability to tightly bind microtubules and skate along them in one

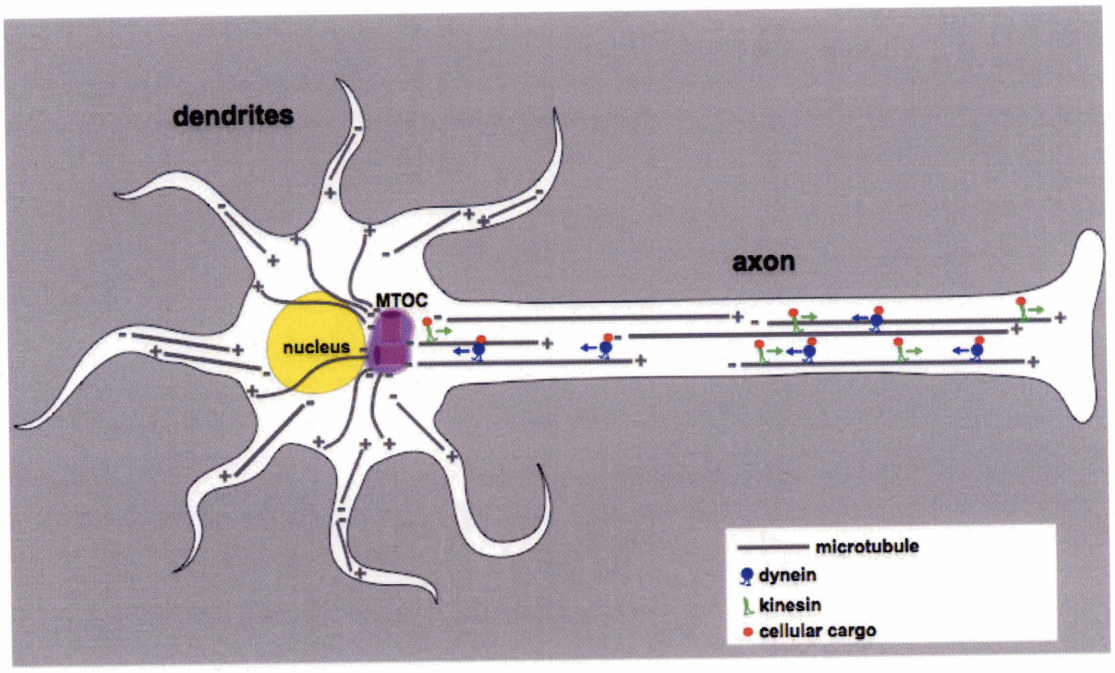
direction has been proposed to account for its function in increasing dynein's processivity (Culver-Hanlon et al., 2006).

Conventional kinesin, first purified from squid axons and chick brain extracts (Vale et al., 1985a; Brady, 1985), and also composed of several polypeptide chains (Bloom et al., 1988), uses ATP hydrolysis to move toward the plus-ends of microtubules. Conventional kinesin has been shown to be required for plus-end-directed, anterograde transport along microtubules in neurons (Vale et al., 1985b; Hall and Hedgecock, 1991). An entire superfamily of kinesin proteins (KIFs) has since been discovered, and many, such as KIF3A and KIF2, are also responsible for anterograde transport (Kondo et al., 1994; Noda et al., 1995). However, a few proteins with sequence similarity to conventional kinesins move cargo in the opposite direction. *Drosophila* Ncd, identified based on its requirement for chromosome segregation during meiosis (Endow et al., 1990; McDonald and Goldstein, 1990), was later shown to be a minus-end directed microtubule motor (Walker et al., 1990; McDonald et al., 1990b)

Microtubule motor proteins can carry cargos along microtubules, but they can also move the microtubules themselves. This activity is accomplished through the binding of the motor protein to a fixed position in a cell, for instance, the cell cortex, while the walking action of the motor pushes or pulls the microtubule. An example of microtubule positioning by motor proteins is the role of cortically-associated dynein in positioning the yeast mitotic spindle (Carminati and Stearns, 1997). Finally, other roles for the microtubule motor proteins have been identified. Dynactin is required for proper microtubule focusing and for coupling the minus ends of microtubules to the centrosome (Gaglio et al., 1997; Quintyne et al., 1999).

Figure 1. Neuronal polarization and microtubule-based transport. A simplified neuron is shown. Note that the axon is not drawn to scale and would actually be longer and thinner than depicted. Microtubules are initiated at the microtubule organizing center (MTOC). Those that remain associated with the MTOC have their slow-growing minus ends fixed near the MTOC and their fast-growing plus ends toward the periphery of the soma. Microtubules destined for neuronal processes are liberated from the MTOC by specific proteins. Dendrites contain mixed polarities of microtubules; some are oriented with the plus-end distal to the cell body, and some are oriented with the minus-end distal to the cell body. Microtubules in the axon have a singular polarity: plus-ends are directed distal to the cell body, while minus ends are proximal. In vivo, microtubule motor proteins move along microtubules in only in one direction. Depicted are cytoplasmic dynein, a minus-end directed microtubule motor, and conventional kinesin, a plus-end directed microtubule motor, shown with cargos.

Figure 1


dendrites
axon
MTOC
nucleus
microtubule
dynein
kinesin
cellular cargo

Development of Axons and Dendrites from Neurites

Early steps in acquisition of neurite polarity have been most extensively explored in cultured mammalian neurons. Cultured neurons extend many small processes, called neurites, of which one will become the axon (reviewed in Bradke and Dotti, 2000; Anderson and Bi, 2000). Neurite extension is dependent upon microtubules (Seeds et al, 1970). Among a population of cultured neuroblastoma cells induced to differentiate by removal of serum, coalescence of many microtubule initiation sites into a single site in some cells precedes the outgrowth of a neurite from any of the cells (Spiegelman et al., 1979). At any given time point, more cells have coalesced their initiation sites (as identified by anti-tubulin antibody) into one site than have extended a neurite, and a coalesced microtubule initiation site is always located directly on the path from the neurite to the nucleus (Spiegelman et al., 1979). These data suggest that neurite outgrowth is preceded by coalescence of microtubule initiation centers, but they do not prove that microtubule reorganization is required nor do they address the mechanism by which microtubule organization influences neurite polarity.

In fact, the specification of one neurite as the axon remained a biological mystery until recently. Many studies found a correlation between the neurite which will become the axon and the amount of cytoskeletal activity in this neurite's growth cone versus the cytoskeletal activity in the other neurites (reviewed in Andersen and Bi, 2000). Actin dynamics in the presumptive neurite's growth cone were investigated, and it was found that depolymerization of the actin cytoskeleton in a single neurite by local application of cytochalasin D causes that neurite to assume the polarity of the axon (Bradke and Dotti, 1999). Thus, a hypothesis was formed that local actin instability determined the polarity

of the neuron and specified the axon among many neurites (Bradke and Dotti, 1999). This actin instability was proposed to allow for more flexibility in the growing neurite's distal tip and, hence, a more permissive environment for microtubule advancement into the distal tip (Bradke and Dotti, 1999). Once a single neurite became more axon-like, positive molecular feedback loops were proposed to amplify the axon's specification and growth and negative feedback loops were proposed to work on the other neurites to keep them from adopting the axon fate (reviewed in Andersen and Bi, 2000).

However, new work has uncovered much earlier polarity-determining events in neurons. Centrosome location has recently been shown to be a necessary and sufficient factor for axon specification (de Anda et al., 2005). Following the last cell division, a neuron's centrosome is located opposite of the cleavage plane. The membrane area directly adjacent to the centrosome undergoes ruffling first, and this spot is the first to sprout a neurite. This first neurite, which is closest to the centrosome, becomes the axon. Blocking centrosome function, using a chromophore-assisted light inactivation of centrosomin in cultured *Drosophila* neurons, blocks axon formation, and ectopic centrosomes cause the development of ectopic axons (de Anda et al., 2005). More complete understanding of the connections between centrosome location and intracellular compartment identity and selective axon growth awaits future investigations. New molecular markers for axon specification, such as the constitutively active truncated kinesin-1 which preferentially accumulates in the tip of the future axon before it is morphologically distinct from other neurites (Jacobson et al., 2006), will be useful for further exploration.

CONNECTING THE NERVOUS SYSTEM

Axons and dendrites must grow, navigate, and branch to achieve their morphologies. Navigation of axons, or axon guidance, is achieved through the axon's reception of extracellular signals by the axon's growth cone (located at the distal tip) and the intracellular transduction of these signals to the cytoskeleton, which undergoes changes that result in directional movement (reviewed in Kalil and Dent, 2005). Much work over the last two decades has elucidated several of the molecular pathways whereby axons are attracted to or repelled by particular signal sources (reviewed in Huber et al., 2003). Signals are often diffusible proteins, so the concentration of the signal is dependent upon the distance to the source. Axons express different signal receptors, different combinations of receptors, different amounts of receptors, and/or different configurations of downstream effectors. Thus, they can respond differently to the same signals. As such, precise connectivity can be achieved even with vast numbers of neurons.

MAINTAINING NERVOUS SYSTEM FORM

Once a neuron reaches its final destination and has made the necessary cellular connections, its precise architecture must be maintained to allow normal functioning. Each part of a mature neuron must be maintained. This thesis focuses on the processes that are required to maintain neuronal morphology, the consequences of disrupting these processes, and the potential implications for development, maintenance, and diseases of the nervous system.

Nuclear Positioning in Neurons

An obvious morphological feature of a neuron is its cell body, or soma. The cell body contains the nucleus and closely-connected organelles such as the Golgi apparatus. In many cells, the nucleus itself constitutes a large proportion of the total cell volume, and the shape of the cell body is largely determined by the nucleus. As a neuron's branching pattern becomes more complex, the relative amount of space occupied by the nucleus becomes smaller, but the location of the nucleus is still important. Where the cell body is positioned relative to the rest of the parts of the cell—such as the axons and dendrites—is a defining feature of many specific types of neurons. For example, bipolar neurons have two main processes, each originating from opposite sides of the cell body. Current understanding of how the nucleus acquires and maintains its specific position in a neuron is limited. Of the identified components, several have an evolutionarily conserved role in nuclear positioning in other cell types and in simpler organisms.

Regulation of nuclear position in filamentous fungi

Key studies of the mechanisms responsible for proper nuclear positioning have made use of simple model organisms. Nuclear positioning has been most extensively studied in the filamentous fungus *Aspergillus nidulans*. *Aspergillus* extend a filament, or mycelium, that progressively becomes filled with more nuclei as the fungus grows. In wild-type *Aspergillus*, all nuclei are spaced evenly apart along the length of the filament. Nuclear migration into the filament was shown to be dependant upon microtubules (Oakley and Morris, 1980). To uncover genes and proteins involved in spacing the nuclei

evenly apart, screens were devised that recovered mutants that do not space nuclei apart properly (Osami et al., 1990; Xiang et al., 1994; Xiang et al., 1995). Instead, nuclei become clumped together in these "nuclear distribution," or "nud" mutants. Many of the mutants had mutations in genes that encoded key components of microtubule motor proteins or their regulators. Importantly, mutations in the minus-end directed microtubule motor protein Dynein caused a severe nuclear mispositioning phenotype in *Aspergillus* (Xiang et al., 1994).

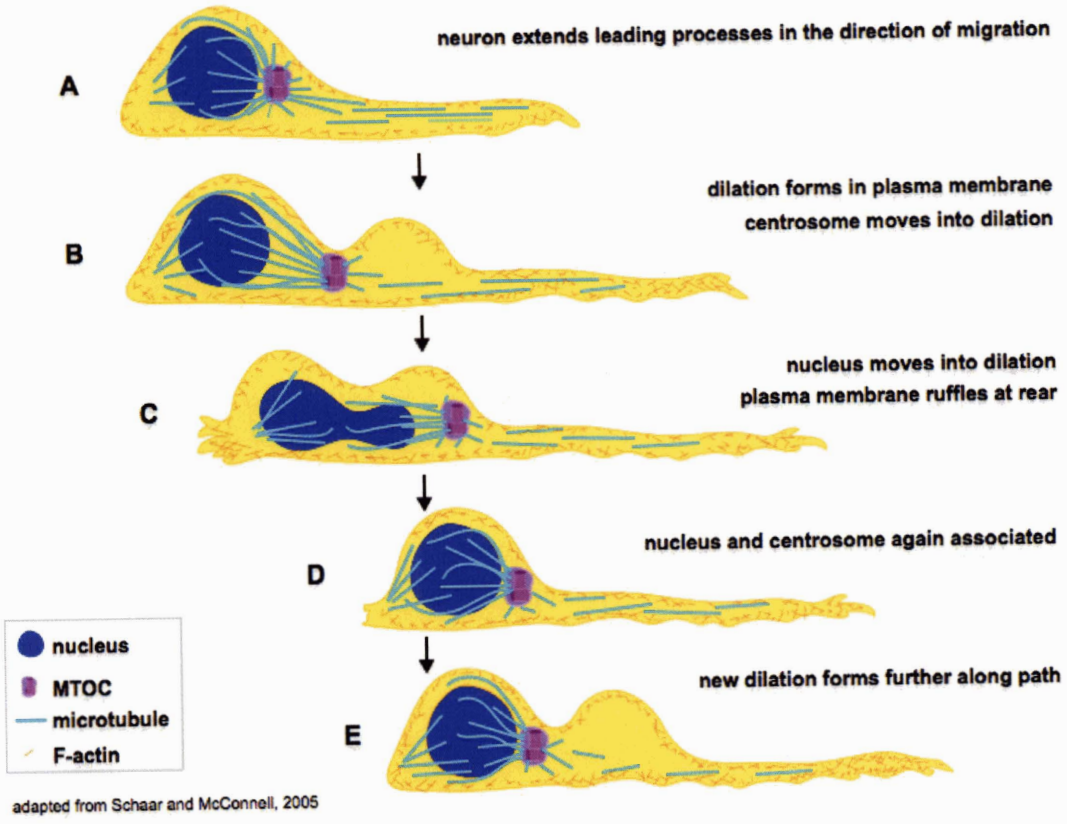
Nuclear Positioning and Neuronal Migration

Most cells, including young neurons, employ specific mechanisms for translocating the nucleus during cell migration. Neurons have a distinct mode of locomotion, as shown in Figure 2. Studies of live cells have revealed the behavior of individual neurons during migration (Edmondson and Hatten, 1987; Solecki et al., 2004; Schaar and McConnell, 2005). Neuronal migration involves the extension of a long, thin leading process in the direction of migration and subsequent translocation of the nucleus-containing soma toward the leading process (Edmondson and Hatten, 1987). The leading process is dynamic and appears to be sampling the environment, displaying both extensions and retractions, but having a net extension (Edmondson and Haten, 1987). The movement of the nucleus occurs in a saltatory fashion, with movement phases and stationary phases (Edmondson and Hatten, 1987). Microtubules are highly organized in migrating neurons, with a cage-like morphology around the nucleus which has been hypothesized to link the nucleus to the centrosome (Solecki et al., 2004). Recently, a dilation of the migrating neuron's cell membrane has been observed to occur after

leading process extension, but before nuclear translocation (Schaar and McConnell, 2005). Mechanisms involved in creating the dilation have not yet been identified. After the dilation is created, the centrosome becomes decoupled from the nucleus and moves into the dilation. The decoupling of the centrosome and nucleus is an unresolved issue, and little is known about how cells modulate centrosome-nucleus coupling during migration. Following centrosomal translocation, the dilation is subsequently occupied by the nucleus, which requires an intact microtubule cytoskeleton to translocate there (Schaar and McConnell, 2005). Movement of the nucleus into the dilation is accompanied by "blebbing," membrane ruffling at the trailing edge behind the nucleus. This blebbing is dependent upon the activity of non-muscle myosin II and, hence, it has been hypothesized that contractile actin-myosin forces may act to push the migrating neuron's nucleus into the dilation (Schaar and McConnell, 2005). These imaging studies have outlined the basic scheme of neuronal migration, and they highlight both the dynamic nature of a migrating neuron's morphology as well as the need for more understanding of the mechanisms that are used to position the nucleus in both migrating and stationary neurons.

Figure 2. Morphological changes during neuronal migration. Shown is a side view of a simplified migratory neuron, traveling toward the right. An environmental cue is assumed to guide the exploratory movements of the filapodia at the leading process so that a net movement toward the right occurs. Following process extension, a dilation in the cytoplasm is created between the nucleus and the leading process. The centrosome is first to move into the dilation. As the nucleus moves into the dilation, myosin-dependent membrane ruffling occurs at the rear of the cell. The nucleus remains closely associated with microtubules during this process. Once the nucleus has moved into the dilation, it is again associated with the centrosome and the process can begin anew. Adapted from Schaar and McConnell, 2005.

Figure 2


A
neuron extends leading processes in the direction of migration
B
dilation forms in plasma membrane
centrosome moves into dilation
C
nucleus moves into dilation
plasma membrane ruffles at rear
D
nucleus and centrosome again associated
E
new dilation forms further along path
nucleus
MTOC
microtubule
F-actin
adapted from Schaar and McConnell, 2005

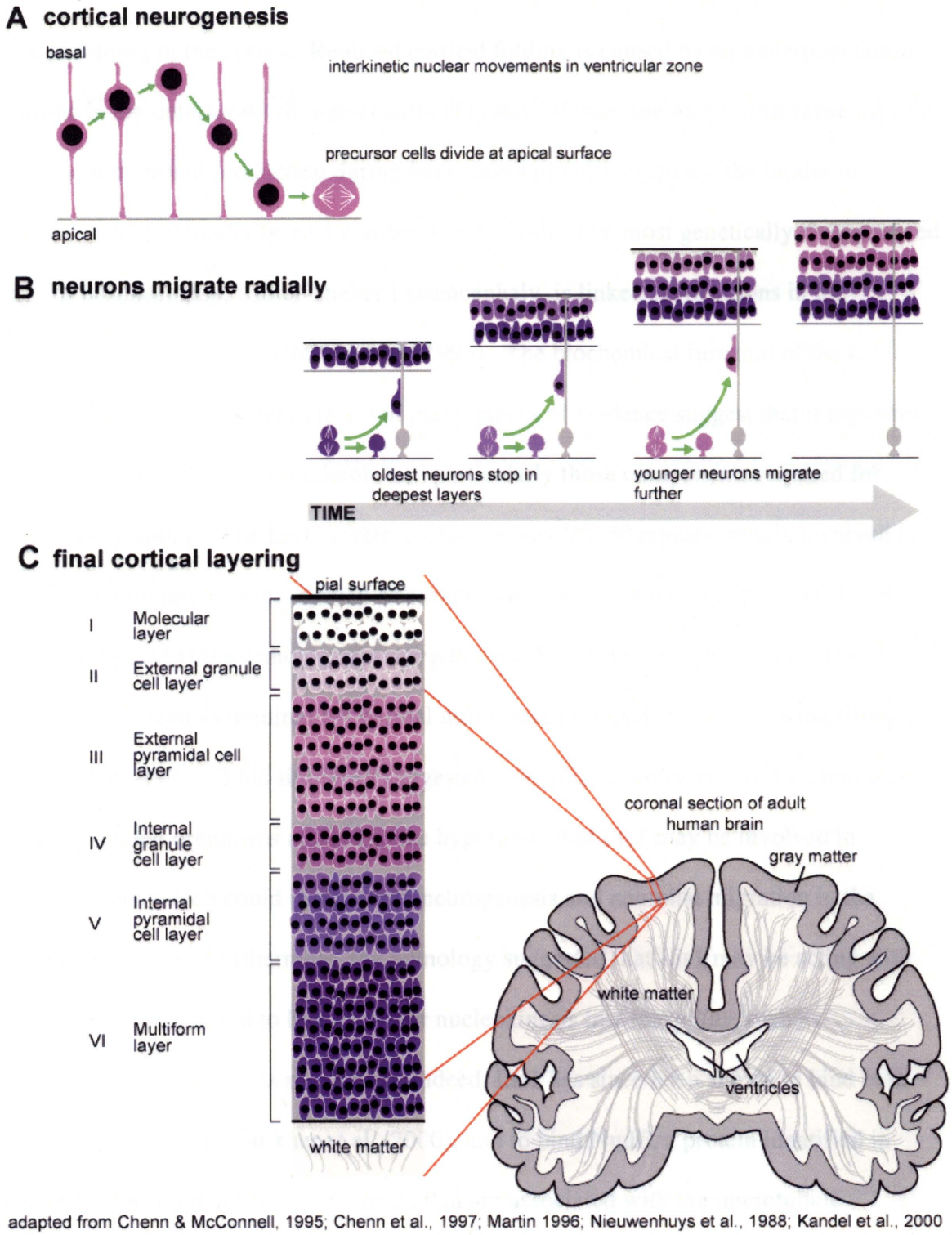
Nuclear Positioning and Developmental Disorders of the Nervous System

The neurons that compose a fully developed cortex are born in a specific region of the developing brain and many must travel long distances to reach their final positions (reviewed in Kriegstein and Noctor, 2004). They are dependent upon nuclear translocation processes not only during their migrations but also during their production. Current understanding of key aspects of cortical development is illustrated in Figure 3. Cortical neurons are born in the ventricular zone. Cell division, which occurs in the ventricular zone to produce the neurons, is tightly linked to changes in the position of the progenitor cell nucleus. Progenitors undergo "interkinetic nuclear migration," moving their nuclei apically and basally within the epithelial sheet. Cell division occurs only when the nucleus/cell body has reached the apical surface of the ventricular zone, which, in cortical development, is the most interior surface, next to the ventricle. Defects in interkinetic nuclear migration can result in defects in the number of neurons produced in the ventricular zone and, thus, can affect the overall number of neurons available to the developing cortex (for example, see the later discussion regarding the role of Lis1) (Tsai et al, 2005). Early-born pyramidal cortical neurons migrate outward radially, into the subventricular zone. There, they pause and either temporarily return to the ventricular zone, or continue to migrate radially. Some neurons also migrate tangentially. Migrating young neurons are guided radially by radial glia, whose cell bodies reside in the ventricular zone but have processes that extend radially, through the cortical plate. These same radial glia have been demonstrated to serve as the progenitors for many cortical neurons (Noctor et al., 2001). The neurons are directed to their destinations by extracellular signals, secreted in particular layers of the developing cortex. Normal

cortical development proceeds in an "inside-out" fashion whereby the younger neurons migrate further radially, past previously born neurons, to form distinct layers contingent upon their birthdates. Migrating neurons must receive these signals, via membrane receptors, and intracellular signaling pathways must work to direct the neuron. If migration signals are defective or neurons are defective in receiving or transducing these signals, the complex and precise architecture of a normal cortex does not develop. Defective cortical patterning can lead to embryonic death or to mental retardation.

Figure 3. Key elements of mammalian cortical development. (A) Cortical neurons are generated in the ventricular zone, the inner-most area in the developing cortex, adjacent to the fluid-filled ventricles. Neural progenitors undergo interkinetic movements during the cell cycle and only divide when they reach the apical surface of the epithelium (the surface closest to the ventricle). (B) Newly-born neurons migrate radially, to a position dependent upon their birthdates. Those neurons born first, the oldest neurons, stop their migrations in the deepest layers of the cortex. Neurons born later travel past the previously created layers to populate more superficial areas of the brain. (C) Final cortical layering in the adult human brain is illustrated. In histological stainings, cell bodies and nuclei stain darkly and thus the area enriched for them is referred to as "gray matter," while unstained axons that connect various regions of the brain appear as "white matter." Cortical neurons' cell bodies and nuclei are located in the highly folded superficial gray matter area. Beneath the pial surface lie six distinguishable types of cortical neurons, with the layer VI (multiform) neurons having been born earliest.

Figure 3



adapted from Chenn & McConnell, 1995; Chenn et al., 1997; Martin 1996; Nieuwenhuys et al., 1988; Kandel et al., 2000

Several disorders are linked to defects in the ability of cortical neurons to migrate. Lissencephaly, meaning "smooth brain," is a class of brain disorders characterized by a lack of folding in the cortex. Reduced cortical folding is caused by an underpopulation of neurons in the outermost (youngest) cortical layers. Hence, the underlying cause may be the lack of neuronal production during later stages of corticogenesis, the failure of neurons to migrate radially, or a combination of both. The most genetically characterized form of lissencephaly, Miller-Dieker Lissencephaly, is linked to mutations in the *lissencphaly-1* (*lis1*) gene (Reiner et al., 1993). The biochemical function of the Lis1 protein is not yet completely clear, but many pieces of evidence suggest that it regulates key components of the cytoskeleton, and particularly those components required for nuclear positioning. The Lis1 protein contains seven WD40 repeats, motifs involved in protein-protein interactions, as well as a microtubule binding activity (Sapir et al., 1997). Importantly, *lis1* is the ortholog of *Aspergillus nudF*, a gene identified in a nuclear distribution screen as required for normal movement of nuclei into the growing filament (Xiang et al., 1995). This similarity suggested a possible conservation of function across distantly related organisms and led to the hypothesis that Lis1 may be involved in nucleokinesis, which could impact both neurogenesis and neuronal migration in the developing cortex. Furthermore, the homology suggested that Lis1 may be acting with other components found to be critical for nucleokinesis in *Aspergillus*, such as cytoplasmic dynein and its regulators. Indeed, Lis1 has since been shown to bind both dynein and dynactin (Faulkner et al., 2000), and to bind NudE, a protein identified in *Aspergillus* with homologs in mammals that are associated with the microtubule organizing center (Efimov and Morris, 2000; Feng et al., 2000). Lis1 may also be

involved in regulating microtubule dynamics and its association with the plus-ends of microtubules may influence the interactions between microtubule plus-ends and the cell cortex or cargo loading at these sites (reviewed in Xiang, 2003).

Recent experiments have imaged cortical neurons with reduced *lis1* function, accomplished through an RNA-interference strategy, in developing embryonic brain slices (Tsai et al., 2005). These experiments revealed several distinct cell biological defects in the cells lacking *lis1*. For instance, the *lis1*-deficient animals showed a lack of interkinetic nuclear movements within the neuronal progenitor cells, reduced progenitor proliferation, and hence too few cortical neurons. Cortical neurons that were produced did not resolve their multiple neurites into two, a step where the neuron adopts a "bipolar" morphology which occurs before it can travel radially along radial glia. Those *lis1*-deficient neurons that do achieve a bipolar morphology do not successfully migrate radially, and they are specifically defective in somal translocation following leading process outgrowth (Tsai et al., 2005). Lis1 may specifically be involved in coupling the centrosome to nucleus during neuronal migration; cerebellar granule neurons derived from heterozygous Lis1 knockout mice have increased distance between the centrosome and nucleus and do not migrate as far in culture as wild-type neurons (Tanaka et al., 2004). Thus, the current data show that Lis1 is required for interkinetic nuclear movements required for neurogenesis, for neuronal polarization, and for the somal translocation required for neuronal migration.

Another type of cortical malformation, X-linked lissencephaly or "double cortex" syndrome, is also linked to defective cortical neuron migration. In this case, the normally six-layered cortex shows extra layers of neurons that failed to migrate far enough to reach

their proper locations. The causative gene was mapped to the X chromosome in humans and called *doublecortin* (*dcx*; des Portes et al., 1998; Gleeson et al., 1998). *Dcx* encodes a phosphorylatable microtubule-associated protein that is highly enriched in post-mitotic cortical neurons during stages of neuronal migration (Francis et al., 1999; Gleeson et al., 1999). DCX has been shown to increase the overall concentration of polymerized microtubules in vitro and may thereby influence the motile properties of migrating neurons (Francis et al., 1999; Gleeson et al., 1999; Horesh et al., 1999).

Extracellular signals guide migrating neurons and thus act upstream of Lis1 and DCX. The best understood extracellular signal required for proper cortical lamination is Reelin. Reeler mutant mice have severe ataxia (correlated with a failure of cerebellar neurons to migrate properly) and mispositioning of cortical neurons. Cortical layering is essentially inverted in reeler mutants, with the oldest neurons populating the outer-most cortical layers, and the youngest neurons found in the deepest layers (Caviness, 1982). The disrupted gene was mapped, allowing the identification of *reelin*, which encodes the secreted protein Reelin (D'Arcangelo et al., 1995). Reelin is produced by the Cajal-Retzius neurons, a group of cortical pioneer neurons that migrate early to the marginal zone and are thus in a prime location to produce signals that influence the radial migration of neurons born later (D'Arcangelo et al., 1995). Consistent with a role in guiding newborn cortical neurons to their destinations, Reelin is most highly expressed in very young mice (D'Arcangelo et al., 1995). The Reelin receptors expressed on migrating cortical neurons were identified as VLDL Receptor (very low-density lipoprotein receptor, or VLDLR) and ApoE Receptor 2 (apolipoprotein E receptor 2, or ApoER2), loss of either receptor causes mild cortical layering defects, and loss of both

receptors causes defective neuronal migration and cortical layering defects similar to those in *reeler* mutants (Trommsdorff et al., 1999). Humans with mutations in Reelin have cerebellar and cortical defects as well (Hong et al., 2000).

Disruption of the mouse *disabled* gene causes an almost identical phenotype to *reeler* mutants (Howell et al., 1997). Disabled encodes a scaffolding protein involved in signal transduction, and known to be a regulator of actin cytoskeletal dynamics (reviewed in Winder 2004). Phenotype similarity prompted the investigation of the relationship between mDab1 and Reelin. *mDab1* mutants have normal Cajal-Retzius cells, and these cells express the Reelin protein, suggesting that if Reelin and mDab1 do act in the same signaling pathway, mDab1 is likely downstream of Reelin (Howell et al., 1997). Reelin mutants were shown to have extremely reduced levels of mDab1 tyrosine phosphorylation, and treatment of cells derived from embryonic brains with Reelin-conditioned media caused the specific upregulation of tyrosine phosphorylation on mDab1 (Howell et al., 1999). Interestingly, mDab1 protein expression is upregulated in reeler mutants, a phenomenon proposed to occur due to lack of Reelin-induced downregulation of mDab1 expression, which occurs in wild-type neurons after they have migrated (Howell et al., 1999). Finally, the link between Reelin signal, VLDLR/ApoER2 reception, and mDab1-facilitated transduction during cortical neuron migration was shown to be dependant upon the specific binding of mDab1 to VLDLR and ApoER2 receptors via NPxY motifs on their cytoplasmic tails (Hiesberger et al., 1999). Thus, a direct binding pathway has been elucidated from a factor secreted from Cajal-Retzius neurons near the pial surface to a known cytoskeletal regulator of actin in the cortical neurons.

Because many of the same cytoskeletal proteins are involved in regulation of both cell division and cell motility, and both processes are essential for brain development, precise assignment of gene function in neural development requires the experimental separation of these processes. In the study of *lis1*, for example, this has led to prolonged debate as to which defects are responsible for which phenotype. One way to achieve such separation is to assay the consequence of loss of gene function after a cell's final division, to determine if there is a postmitotic requirement for the gene. For example, a postmitotic promoter element can be used to express either double-stranded RNA to induce RNA interference for a particular gene, or it can be used to express a dominant-negative version of a protein which will antagonize endogenous wild-type activity.

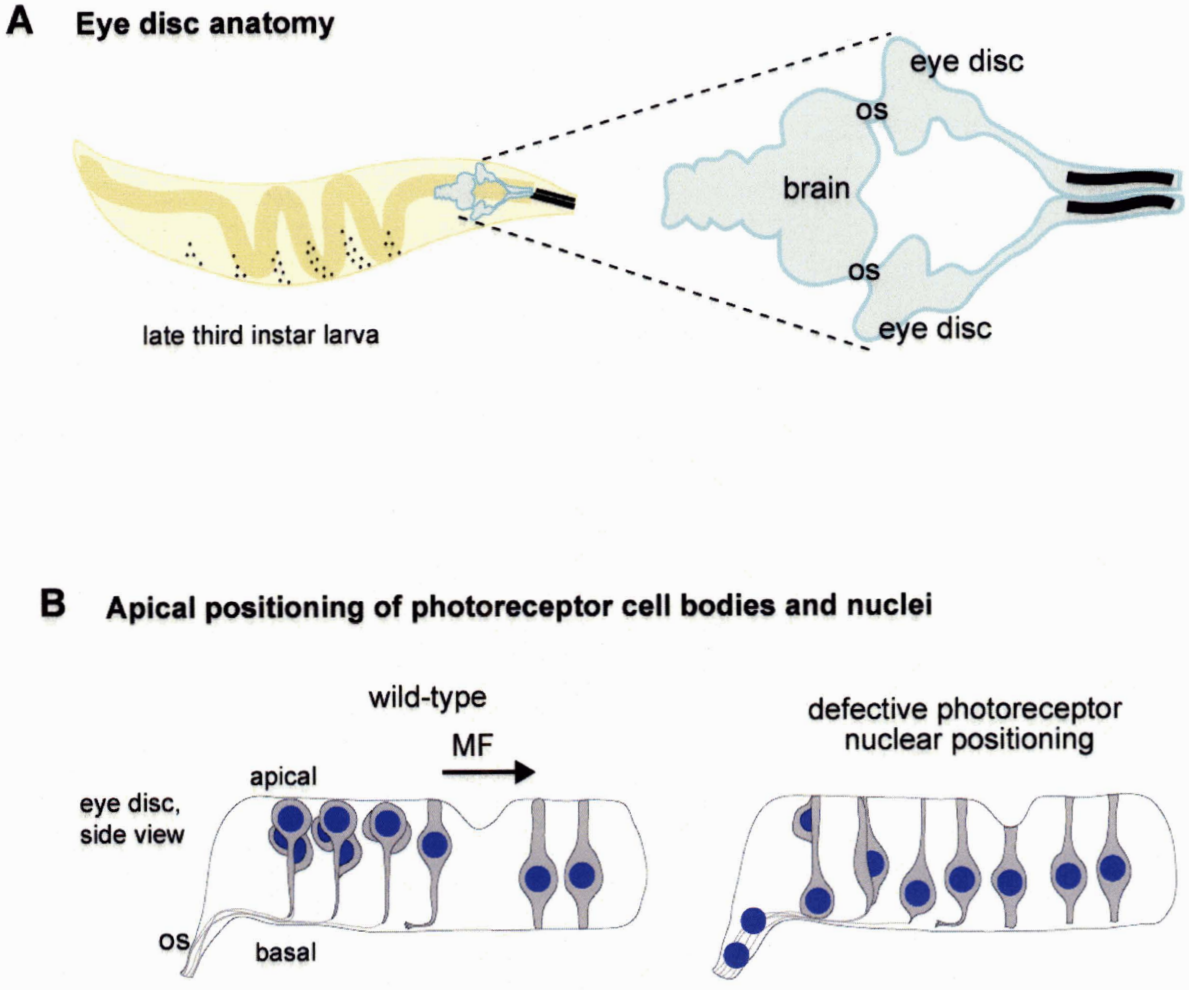
Maintenance of Nuclear Position in the Developing Drosophila Eye

The Drosophila compound eye develops during larval and pupal life as an epithelium within the eye imaginal disc (reviewed in Wolff and Ready, 1993; illustrated in Figure 4). Imaginal discs serve as sites of proliferation and patterning during the development of complex adult structures. The cells within the eye disc are patterned from the future posterior margin of the eye to the anterior margin, and morphogenetic events are coupled to the posterior to anterior progression of the morphogenetic furrow, a visible indentation in the eye disc caused by local changes in cell shape (Tomlinson, 1985) (Figure 5B). Similar to those that occur during neurogenesis in mammals, photoreceptor progenitors undergo rounds of bidirectional interkinetic nuclear movement spanning the depth of the eye disc epithelium (Tomlinson, 1985). Cell division occurs when progenitors "round up," or arrive at the apical surface. Hence, nuclear positioning

mechanisms may be instrumental in the production of normal amounts of photoreceptors. Following differentiation, postmitotic photoreceptors extend axons basally, which pierce the basement membrane, travel through the optic stalk, and innervate target regions in the brain. Importantly, the nuclei of postmitotic photoreceptors are positioned within the apical third of the eye disc and are never found basally or in the optic stalk.

Figure 4. Developing eye disc and photoreceptor nuclear positioning. (A) Compound eyes develop from eye imaginal discs. Third instar stage larvae are dissected to obtain the two eye discs, which are connected to the brain by the optic stalk (os). (B) When viewed from the side, the morphogenetic furrow (MF) is seen to progress from posterior to anterior, with the most differentiated cells posterior to the furrow. Photoreceptor cell bodies and nuclei are positioned toward the apical surface in normal animals, but in mutants with defective nuclear positioning, they can be situated basally or in the optic stalk.

Figure 4


A
Eye disc anatomy
eye disc
os
brain
os
eye disc
late third instar larva
B
Apical positioning of photoreceptor cell bodies and nuclei
wild-type
MF
apical
eye disc,
side view
os
basal
defective photoreceptor
nuclear positioning

The developing compound eye is especially conducive for studying the genes required for nuclear and cell body positioning in neurons. Each eye will eventually develop about 800 ommatidial facets, and each ommatidium contains eight photoreceptors. Thus, there are thousands of opportunities for detection of a mutant phenotype in any given eye disc and hence assays here are extremely sensitive. Additionally, perturbations of photoreceptor nuclear positioning are relatively easy to see along the apical/basal axis of the eye disc, particularly if the defect is strong enough to give the impression of holes in the apical pattern of photoreceptor nuclei. More severe defects, such as nuclei becoming displaced into the optic stalk or photoreceptor cell bodies becoming displaced into the brain, are quite obvious. Many genetic tools are available for the disruption of genes in specific cell types and at specific stages of development in the *Drosophila* eye. Finally, because mutants whose photoreceptor nuclei and cell bodies are displaced have a concomitant malformation of the adult eye, the adult eye is a useful, and readily accessible, organ for screening for genes that may be directly involved in regulating nuclear and cell body positioning in neurons.

Several genes have been identified as regulators of photoreceptor nuclear positioning in *Drosophila*. *Glued* encodes p150, a component of dynactin, which activates the minus-end directed microtubule motor dynein. Flies with a transposable element insertion in the *Glued* locus, which causes translation of a truncated Glued protein that acts as a dominant-negative by binding to dynein intermediate chain but not to other dynactin components, have severe eye defects, including photoreceptor nuclei being positioned at the basal surface (Fan and Ready, 1997). Loss-of-function mutations in *klarsicht* (previously known as "*marbles*") also cause photoreceptor nuclear

mispositioning (Fischer-Vize and Mosley, 1994; Welte et al., 1998). Klarsicht is proposed to function as a regulator of microtubule motors that works to coordinate motors that move in opposite directions (Welte et al., 1998). Other genes required for photoreceptor nuclear positioning include *nuclear lamin*, whose protein has been proposed to link the nuclear membrane to the microtubule organizing center through a possible interaction with Klarsicht (Patterson et al., 2004) and *misshapen*, which encodes a Ste20-like kinase (Houalla et al., 2005). Interestingly, the *Drosophila* ortholog of *LIS1*, *dlis1*, is required for proper photoreceptor nuclear positioning (Swan et al., 1999), and overexpressing mouse Disabled1 in *Drosophila* photoreceptors causes mispositioning of photoreceptor nuclei (Pramatarova et al., 2006). These results further suggest that nuclear positioning in neurons and neuronal cell migration are intimately linked processes, and they demonstrate that biological principles learned from studies in photoreceptor nuclear positioning in *Drosophila* may be relevant to human brain development.

Of the genes identified to be required for proper photoreceptor nuclear positioning along the apical-basal axis, only a few have been examined for a specific role in maintaining nuclear position. That is, it is unclear which of these genes are required to initially move the nucleus to the apical portion of the eye (a process linked to cell division), which of these genes are required to maintain the position of the nucleus once it has been established (either in the proper place or in an improper place, such as the basal part of the eye disc), and which genes may be required to do both. These are important distinctions because they address when in the development or life of a particular cell nuclear positioning mechanisms are engaged. If, for instance, a gene is required to

maintain nuclear positioning, loss of this gene's function in a cell that has already stopped dividing and has differentiated may lead to the ectopic movement of the nucleus in the cell. Alternatively, if the gene is required in a post-mitotic, differentiated cell to move the nucleus (for instance, during cell migration), a defect in nuclear movement, and hence cell migration, might be expected. It is also possible that nuclear mispositioning in a photoreceptor could be the indirect consequence of an earlier defect. For example, if, during the mitotic event that created the photoreceptor, the plane of cell division was disrupted and daughter cells received disrupted polarity determinants, the position of the nucleus might be normal with respect to the cell's polarity, but abnormal because the polarity is not correct.

Maintenance of Axons and Dendrites

The adult brain was once thought to be static, with its cells and their connections undergoing little or no turnover or change . For example, as late as 1959, W.R. Russell wrote, "They [neurons] are formed before birth and cannot be replaced if they are killed by injury or disease" (Russell, 1959). However, by 1978, C. Cotman wrote, "Only a few years ago it was a commonly held belief that the nervous system could be 'wired' in only one way during development and, once formed, the only changes in its circuitry were those due to neuronal loss. . . Recent findings, however, clearly show that neuronal circuitry is highly adaptable at a structural level even in the mature nervous system" (Cotman, 1978). For example, neurogenesis has been demonstrated to occur in several discrete locations in the adult mammalian brain (reviewed in Ming and Song, 2005). Adult neurogenesis has been proposed to play a role in homeostasis of brain circuits and

in repair of injured tissue. However, the mechanisms of functional integration of newly born neurons into an otherwise developed adult brain are not known. Recent work has shown that neurons in adult brains modulate the structure of their axons and dendrites over the course of normal life (reviewed in Callaway, 2006). This neurite remodeling involves both the elimination and addition of branches and synapses. Conversely, axon and dendrite maintenance defects, such as retraction or overextension, have been linked to several human diseases and have been shown to occur in mouse models of these diseases (reviewed in Luo and O'Leary, 2005). Thus, maintenance of axon and dendrite morphologies is an essential component of normal brain functioning.

Neurite Retraction

Neurite retraction is an important means for controlling the precise circuitry of the nervous system. During early stages of circuit development in mammals, many neurons' axons form temporary connections with extra targets or grow an exuberant amount of branches (reviewed in Luo and O'Leary, 2005). One of the earliest examples of developmental neurite retraction to be studied molecularly occurs in the motor axons that innervate muscles. Here, many axons can form temporary connections with a particular muscle cell, but only one axonal input will be stabilized and maintained throughout adulthood (reviewed in Hall and Sanes, 1993). In the mammalian visual system, axons originating from both eyes initially overlap their projection fields in the brain, only to undergo axon pruning and refinement to sort them into separate regions later. Once a neuronal connectivity pattern is established, neurons must inhibit retraction from occurring to maintain the precise connectivity pattern. While genetic programs and

energy must be devoted to inhibiting retraction, many researchers speculate that because some synaptic remodeling does occur in adulthood to facilitate learning and memory, the ability of many neurons to change their precise wiring is crucial for their function (reviewed in Sur and Rubenstein, 2005; Chklovskii et al., 2004). Retraction of neurites has been studied both *in vivo* and *in vitro*, and several key findings support the idea that retraction is an active cellular process that can be stimulated by environmental factors.

Neurite Retraction In Vitro

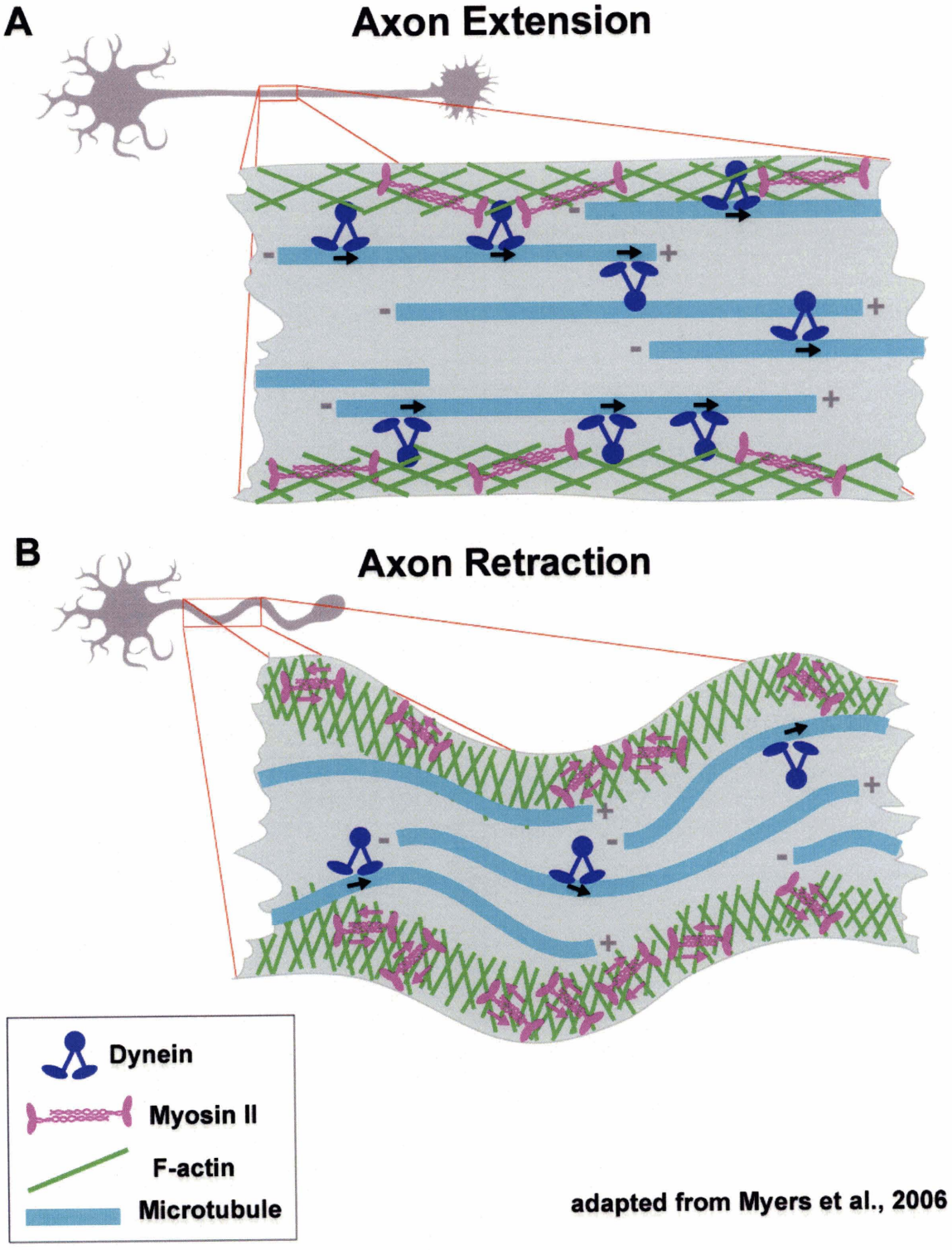
Early studies in cultured neurons demonstrated that axons can be induced to undergo retraction by manipulation of the microtubule cytoskeleton. Treatment of neuroblastoma cells with nocadazole, which depolymerizes microtubules, causes neurites to rapidly retract toward the cell body (Solomon and Magendantz, 1981). This retraction effect can be blocked by simultaneously exposing the cells to cytochalasin D, which inhibits actin polymerization by binding the fast-growing barbed end of filamentous actin (Solomon and Magendantz, 1981). These results suggest that retraction is an active cellular process that can be blocked by perturbing actin dynamics rather than simply a default state that occurs when microtubules are disassembled.

Later, a hypothesis was developed to explain how a neuron might integrate microtubule and actin dynamics to control neurite outgrowth and retraction (Ahmad et al., 2000). Rather than simply the strength of the microtubules themselves opposing the contractility of the actin cytoskeleton, this hypothesis predicted that molecular motor proteins are instrumental to neurite outgrowth, maintenance, or retraction. In this case, microtubule motors such as dynein (which can exert force on microtubules) might be

expected to counteract actin motors such as myosin (which are involved in exerting force along actin filaments to control cell shape) (Figure 5). If microtubules were depolymerized, microtubule motors would no longer function, and actin motors would no longer be counteracted and, thus, the neurite would retract. If actin filaments were also perturbed, the actin motors could not function, and thus retraction would not occur. The new hypothesis predicted that simply inhibiting the function of the microtubule motors would have the same effect—neurite retraction—as depolymerizing the microtubules. If true, inhibiting the actin motors simultaneously would block the retraction. By overexpressing dynamitin (a component of dynactin that when overexpressed disrupts dynactin and therefore dynein activity), the researchers showed that inhibiting dynein causes neurite retraction (Ahmad et al., 2000). Additionally, if myosin activity is also inhibited by injecting the cells with a dominant-negative version of myosin, retraction is blocked (Ahmad et al., 2000). These experiments prove that molecular motors that work on microtubules and actin are directly involved in controlling whether a neurite extends, retracts, or is maintained.

Figure 5. Model for action of motor proteins during axon extension and retraction. A possible model for how dynein and myosin might influence axon extension versus retraction. (A) A neuron undergoing axon extension is depicted, and a segment of the axon is magnified. The neuron's cell body and dendrites are to the left, and axon, tipped with growth cone, to the right. Cytoplasmic dynein motors acting on long microtubules exert forces that move the microtubules toward the growth cone (shown at right), hence facilitating axon elongation. (B) During retraction events, myosin II motors likely contract the filamentous actin meshwork that underlies the axon's cell membrane. Dynein motors may become dislodged from their positions within the cortical cytoskeleton. Microtubules buckle and coil, and less force can be generated by dynein to move them toward the axon's tip. Adapted from Myers et al., 2006.

Figure 5



adapted from Myers et al., 2006

Neurite Retraction In Vivo: During Initial Development, Organismal Maturation, and Adulthood

Neuronal activity and experience can shape precise synaptic connections. Many experiments have defined critical periods for the establishment of neuronal morphologies and circuitry. During the critical period, neuronal activity can influence the shape of a neuron's arbors. For instance, in kittens, if one eye is experimentally closed, the corresponding geniculocortical neurons' axons undergo rapid morphological remodeling (Antonini and Stryker, 1993). Lack of visual stimulation and neuronal activity during the critical period causes the retraction of many axon branches, creating a permanent condition whereby the kitten will not see properly from that eye later in life (Antonini and Stryker, 1993).

Precise synaptic connectivity is controlled, in part, by axon retraction processes. As mentioned earlier, the one-to-one ratio of motor axon to muscle cell in the mammalian neuromuscular junction (NMJ) is only achieved through a process of axon overgrowth followed by retraction of extra axons. In vertebrates, at birth, more than one motor neuron synapses with each muscle fiber. However, within two weeks, motor axon pruning occurs, resulting in the elimination of some synapses so that only one motor neuron innervates each muscle fiber (reviewed in Frank, 1997, and summarized in Figure 6). Elimination of the other motor neurons' synapses onto a muscle fiber is followed by the growth of the remaining motor axon and its acquisition of the vacated synaptic sites. Several pieces of evidence suggested that synaptic activity might control motor axon connectivity at the NMJ. For instance, blocking synaptic activity at the rat soleus muscle NMJ by implanting tetrodotoxin-infused beads into the sciatic nerve prolongs the time

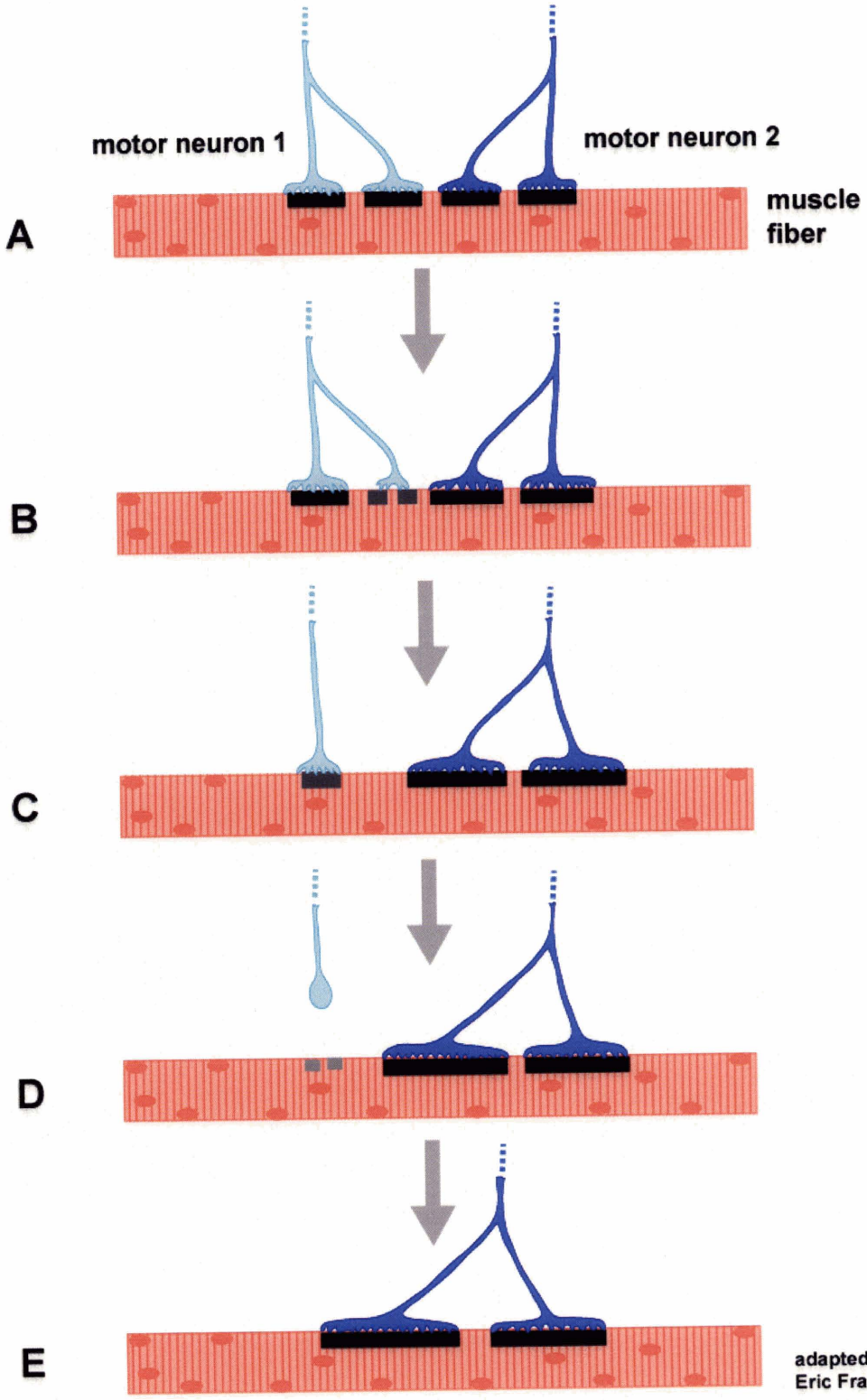
required for synapse elimination (Thompson et al., 1979) Increased stimulation, produced by implanting electrodes into the soleus muscle and providing a set stimulus at regular time intervals, results in several days less time required for synapse elimination in newborn rats (Thompson, 1983). In adult rats, when pruning has already resulted in the establishment of only one motor neuron per muscle fiber, the anatomy of the motor axons can be changed by experimentally modulating synaptic activity (Balice-Gordon and Lichtman, 1993). Blocking synaptic receptors in adult mouse muscle, by local application of α-bungarotoxin (which irreversibly binds acetylcholine receptors and prevents their activation by acetylcholine) puffed over a portion of an NMJ with a micropipette, leads to the retraction of motor axons immediately opposite of the blocked site (Balice-Gordon and Lichtman, 1994). However, if all of the receptors are blocked in a single NMJ, ectopic synapse elimination does not occur, suggesting that competition between the different motor axons must occur to elicit axon withdrawal of any one of them (Balice-Gordon and Lichtman, 1994).

Since these studies suggested that activity at the NMJ might control axon withdrawal during normal vertebrate development, more recent studies have confirmed this hypothesis. Immediately after birth, two motor neurons innervating the same muscle fiber release similar amounts of neurotransmitter (Colman et al., 1997). However, one week later, fewer muscle fibers have more than one motor neuron synapsed. Those fibers that still have input from two motor neurons show one of the neurons having more than four times the activity of the other (Colman et al., 1997). Overall, during this process, one motor axon's synapse becomes stronger, while the other's becomes weaker. Eventually, the one with reduced activity retreats, and the bulbous morphology of the

retreating axon suggests it is being retracted. The vacated synapse spot is taken over by the stronger motor axon. Synaptic efficiency stemming from variation in the amount of receptors expressed by the muscle can also affect which motor neuron retracts; for example, NMJ synapses with acetylcholine receptors on the muscle are more likely to undergo axon retraction (Colman et al., 1997). Therefore, changes in synaptic strength can lead to changes in the anatomy at the neuromuscular junction and the morphologies of neurons (Colman et al., 1997).

Figure 6. Retraction of motor neuron axons during normal postnatal mammalian neuromuscular development. (A) Immediately after birth, each muscle fiber is synapsed with axons from multiple motor neurons. (B) Soon thereafter, some motor neuron's synapses weaken and these axons lose synaptic connections with the muscle fiber. Adjacent axons from another motor neuron, with increased synaptic activity, begin to expand their synapses with the muscle fiber (C), and axonal branches from the weaker motor neuron are lost. Eventually, the weaker motor axon retracts from the muscle fiber, showing a bulbous morphology at its tip (D). Meanwhile, the remaining motor neuron's axon continues to add synapses in the area vacated by the retracted axon (D and E). This process is complete in rodents by two weeks after birth.

Figure 6


motor neuron 1
motor neuron 2
muscle fiber
A
B
C
D
E
adapted from Eric Frank, 1997

Interestingly, retraction of motor axons is also a key feature of developing *Drosophila* neuromuscular junctions. Young larvae display evidence of synaptic bouton retraction in many synapses, but these retraction events are decreased by third instar (Eaton et al., 2002). Animals with reduced activity (either by RNA interference or by genetic lesions) in either of two dynactin components, Arp1 or p150Glued, have high levels of axon retraction into third instar stage (Eaton et al., 2002). By expressing dominant-negative Glued only in nerves, a presynaptic requirement for dynein/dynactin activity in promoting synapse stability was revealed (Eaton et al., 2002). These experiments demonstrate that motor proteins are also important for synapse stabilization and maintenance of axon morphologies, and they suggest a hypothesis that loss of dynein/dynactin function might cause an activation of a retraction program within an axon.

Recent experiments have demonstrated a surprising degree of plasticity in neuronal morphologies during normal adult rodent life. Two-photon confocal microscopy of labeled neurons in live animal brains have revealed the structural changes of individual neurons over time. The fraction of persistent (i.e., not changing over a given observation time) dendritic spines in the mouse somatosensory cortex increases steadily after birth, and continues to increase at least well into adulthood (Holtmaat et al., 2005). For example, between postnatal days 16-25, only 35% of dendritic spines could be classified as persistent (unchanged over at least eight days), but by 175-225 days old, 73% of dendritic spines were persistent (Holtmaat et al., 2005). Persistent spines had thicker morphologies than transient spines (Holtmaat et al., 2005). While dendrites are exhibiting less morphological plasticity, some are nonetheless still changing their

morphologies. Similar changes occur on the presynaptic side, in axonal synaptic boutons. Elimination of presynaptic boutons and counterbalancing addition of other boutons was seen to occur in the both adult mouse (De Paola et al., 2006) and adult monkey cortex (Stettler et al, 2006). Additionally, axons from different types of neurons display different amounts of plasticity (De Paola et al., 2006). Synapses vary in morphology, with some types appearing as short spines on the axon and others as simply a thickening of the axon. A single neuron can also have varying amounts of plasticity among the different types of synapses that it forms (Stettler et al., 2006). For example, in the adult macaque visual cortex, changes in pyramidal neurons' en passant boutons (a synapse on the length of the axon, not a branch tip) were compared to changes in pyramidal neurons' terminaux boutouns (synapes at the axon branch tips) (Stettler et al., 2006). En passant boutons were found to have a lower turnover rate (about 6% lost and 7% gained per week) than terminaux boutons (about 14% lost and 14% gained per week) (Stettler et al., 2006). In all cases of axon subtraction or addition, the length of axon involved changed less than 11 μm per week (Stettler et al., 2006). New technologies for studying neuronal morphologies in living adults will increase understanding of the amount of morphological change neurons undergo, and it may help identify molecular regulators of adult neuronal morphologies.

Axonal degeneration and disease

Degeneration of axons has been observed in the progression of many different diseases that affect the nervous system, for example, Huntington's, Alzheimer's, and Parkinson's diseases (reviewed in Luo and O'Leary, 2005; Coleman, 2005). In most

cases, it is unclear whether degeneration of axons may be proceeding through the same or similar mechanisms to the retraction of neurites observed during normal development. These diseases are also accompanied by neuronal cell death, and the correlations between axon degeneration and neuronal death, as well as potential causal relationships, are only beginning to be defined. Thus, it is possible that increased understanding of normal and aberrant axon retraction might lead to better understanding of neurodegenerative diseases and potential therapies.

Axon degeneration that occurs with disease is morphologically similar to the degeneration that occurs following injury to an axon (reviewed in Coleman, 2005). As such, models for how axons degenerate following injury might be relevant to understanding degenerative diseases. A particularly fruitful experimental system has been the mouse. In vertebrates, one type of axon degeneration that occurs after nerve transection take place in a stereotypical series of steps is referred to as "Wallerian degeneration (Waller, 1850)." Portions of the axon distal to the lesion undergo morphological changes such as the growth of a swelling at the proximal edge of the severed axon piece, followed by breakdown of the cytoskeleton in the free axon piece and recruitment of glia to the scene (Griffin et al., 1995). Importantly, Wallerian degeneration events amount to the loss of part of the axon, but the neuron itself does not die. A strain of mouse has been discovered, "slow Wallerian degeneration" or *Wld*^{S}, in which severed axons do not degenerate as quickly as severed axons in wild-type animals (Lunn et al., 1989). The genetic aberration was identified in *Wld*^{S} mice and found to encode a novel fusion protein with portions from UFD2/EA, a ubiquitination pathway protein, and Nmnat, an enzyme needed for nicotinamide adenine dinucleotide (NAD)

biosynthesis (Conforti et al., 2000). This discovery provides important starting points for examining potential cellular pathways involved in axon degeneration. Expressing the Wld^s fusion protein confers delayed axon degeneration following injury (Mack et al., 2001). Recent work has shown the Nmnat portion likely confers the degeneration delay (Araki et al., 2004; Wang et al., 2005). Introducing Wld^s into a genetic background that causes neurodegeneration, such as progressive motor neuronopathy or inherited peripheral neuropathy, can significantly delay the onset of axon degeneration (Ferri et al, 2003; Samsam et al., 2003). However, it cannot prevent the degeneration of the axon or the death of the cell body.

Animal disease models for human neurodegenerative diseases may help elucidate the connection between axon degeneration and neuronal death, and one will briefly be discussed here. Huntington's disease has been linked to the aggregation of huntingtin proteins in the brain (Scherzinger et al., 1997). In human brain tissue, aggregates are more numerous in neurite-rich regions compared to cell bodies, and neuropil aggregates have been found in the brains of people not yet expressing Huntington's symptoms (Gutekunst et al, 1999). These aggregates are thought to interfere with the brain's ability to conduct normal cell biological business such as ubiquitin-dependent protein degradation (Bence et al., 2001) and vesicle transport along the length of the axon (Szebenyi et al., 2003), and thus interfere with the health of the neuron such that it eventually dies. In a mouse model, huntingtin aggregates in the neuropil have been shown to increase as the animal ages, and particular axons with the highest amounts of aggregated huntingtin are also the axons undergoing the most obvious signs of degeneration (Li et al., 2001). The onset of axonal degeneration, characterized by fewer

synapses and synaptic vesicles and dark, enlarged organelles in electron microscopy sections, occurs before any evidence of neuronal cell death in the nucleus (Li et al., 2001). These results suggest that axonal degeneration might be a necessary prerequisite for neuronal death, or may even cause it. Therefore, inhibiting axon degeneration may slow the progress of Huntington's disease and others with similar pathologies.

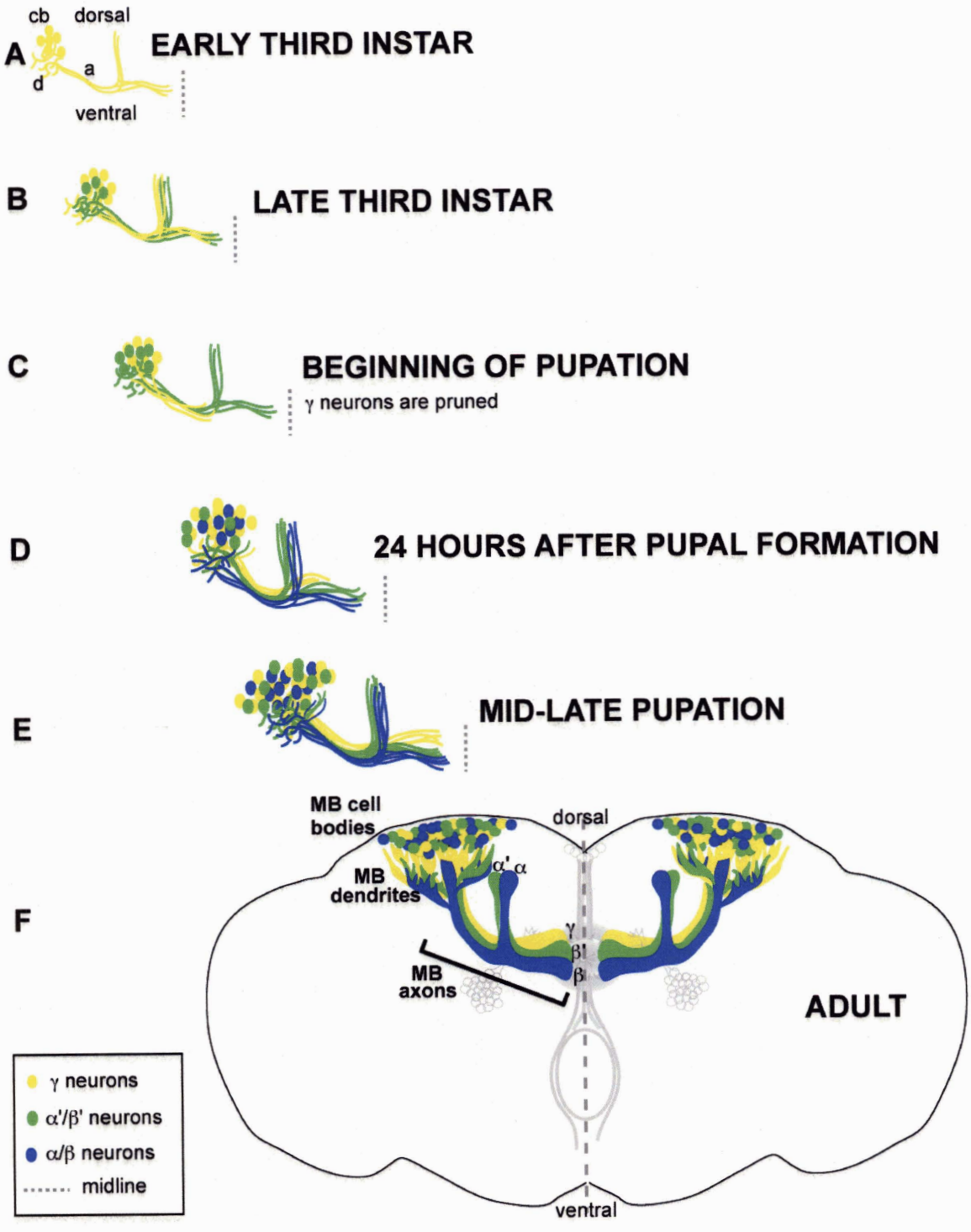
Drosophila Mushroom Bodies as a Model for Studying Mechanisms of Neuronal Morphology Maintenance

The mushroom bodies of the Drosophila central nervous system provide a genetically accessible context for probing the requirements of establishment and maintenance of neuronal morphologies. Mushroom bodies are large, easily identifiable, bilaterally symmetric clusters of neurons located in the brain and common to many—if not all—insects. Mushroom bodies have been implicated in learning and memory, particularly of olfactory stimuli, and in walking behaviors (reviewed in Zars, 2000). Each *Drosophila* mushroom body is comprised of approximately 2,500 neurons. There are three distinct classes of Drosophila mushroom body cells. Each mushroom body neuron develops a precise projection morphology dependent upon its type (Figure 7). The earliest-born mushroom body neurons are the γ neurons, which are born in the first larval stage (first instar), project a fiber ventrally from which a dendrite branches while the axon continues ventral-medially. The γ axon splits to form two branches: one projects dorsally, while the other projects medially. This branch pattern is maintained until early pupation, when the projection pattern is then remodeled in response to hormonal signals. During the first few hours of pupation, an ecdysone-triggered

signaling cascade instructs the programmed degeneration, or pruning, of the γ neuronal projections (Lee et al., 1999; Awasaki and Ito, 2004). Subsequently, γ neurons regrow projections, but the new projections do not branch to form a dorsal projection and, rather, simply extend medially (Lee et al., 1999). Pruning of γ lobe axons requires reception of the ecdysone signal by the γ neurons as well as infiltration of the γ axon bundles by glia and engulfment of γ axons by these glia (Lee et al., 2000; Awasaki and Ito, 2004). In contrast to the γ neurons, the α'/β' and α/β neurons, which are born later, do not undergo any axon pruning (Lee et al., 1999). Their axons simply form a dorsal and a medial branch that persist into adulthood (Lee et al., 1999). Notably, although the γ, β', and β lobes project toward the midline, they stop short of touching or crossing it in wild-type animals. Together, the branched axons from many mushroom body neurons are fasciculated to form thick lobed structures. α and β lobes express high amounts of the neuronal cell adhesion molecule Fasciclin II during late pupal stages and throughout adulthood, which provides a simple way to assay their structure. Hence, the *Drosophila* mushroom lobes are an ideal system for analyzing the cell biological and genetic requirements for axon pruning to occur (in the γ lobes) as well as the requirements for the maintenance of axon morphologies (in the α'/β' and α/β lobes).

Figure 7. Development of the *Drosophila* mushroom bodies. The left hemisphere is depicted in A-E, and the entire brain is depicted in F. (A) Earliest-born mushroom body neurons, the γ neurons (yellow), have already been generated and cell bodies (cb) have grown projections by early third instar stage. Each neuron grows a single process which branches to form a dendrite (d) and an axon (a). The axon continues to grow until it bifurcates. One branch grows dorsally, while the other continues medially but does not grow into the midline. (B) α/β neurogenesis (green) begins at late third instar, and their dendrites and axons branch similarly. At the beginning of pupation, γ neurons are pruned, and both dorsal and medial axon branches are removed. By 24 hours after pupal formation, the γ axons have begun their regrowth stage, but, notably, new γ axons do not form dorsal branches. Meanwhile, α'/β' neurons continue to be added and α/β neurons (blue) are also generated, and they form both dorsal and medial axon branches. Neurogenesis proceeds until late pupation. The final adult mushroom body pattern is shown in (F), along with other central brain features, which are shown in gray (see Chapter 3 for more detailed anatomy).

Figure 7


A
cb
dorsal
a
d
ventral
EARLY THIRD INSTAR
B
LATE THIRD INSTAR
C
BEGINNING OF PUPATION
γ neurons are pruned
D
24 HOURS AFTER PUPAL FORMATION
E
MID-LATE PUPATION
F
MB cell bodies
dorsal
α'α
MB dendrites
γ
β'
β
MB axons
ADULT
ventral
γ neurons
α'/β' neurons
α/β neurons
midline

Several genes have been shown to be required for proper Drosophila mushroom body axon patterning. Some genes act cell-autonomously, in the mushroom body cells themselves, while others are likely to act in other cells to control mushroom body patterning. Animals with loss-of-function *Dscam* (a neuronal cell adhesion molecule with many thousands of predicted isoforms) mushroom body clones have abnormal segregation of axon branches, with too few axon branches innervating the dorsal α and α' lobes and too many in the β and β' lobes (Wang et al., 2002). Overexpression of *dFMRP*, the Drosophila homolog of the gene encoding human Fragile-X Mental Retardation Protein, in mushroom bodies causes a similar α lobe reduction and β lobe fusion (Michel et al., 2004), while loss-of-function alleles of dFMRP show ectopic axon branching and mild β fusion (Pan et al., 2004). Loss-of-function alleles of *Src64*, a cytoplasmic tyrosine kinase and homolog of the human *Src* oncogene, have reduced α lobes in the adult and β fusion (Nicolai et al., 2003), although only the β fusion reflects a cell-autonomous mushroom body neuron requirement for *Src64*. Loss-of-function alleles of *derailed*, which encodes a protein with receptor tyrosine kinase homology, cause adult α lobes to be too short and β lobes to fuse across the midline (Simon et al., 1998). Interestingly, Derailed is not expressed in the mushroom body cells but is instead found during the critical pupal development stages in a novel interhemispheric ring in the vicinity of the β lobe termini, suggesting it may work there to prevent the β axons from crossing the midline (Simon et al., 1988). For both *derailed* and *Src64*, it is not known whether the mushroom body α axons develop abnormally or whether these axons develop normally but fail to be maintained.

In one case, a molecule has been identified that is required for maintenance of

mushroom body axon projections. Mushroom-body-specific depletion of p190RhoGAP, a GTP-ase activating protein which enhances RhoA's GTPase activity and therefore promotes the inactive GDP-bound state, causes the progressive retraction of both α and β lobe axons (Billuart et al., 2001). Regulation of the Rho signaling pathway has since been shown to be critical for consolidation of long term memories in rats (Lamprecht et al., 2002), which could be linked to its function in neurite stability. Overexpression of the protocadherin Flamingo in mushroom bodies causes the progressive retraction of α lobes alone during pupation, and may therefore be ectopically activating a retraction pathway (Reuter et al., 2003). Analysis of *Drosophila* mushroom body axon maintenance is a promising approach for discovery of novel factors which can modulate the maintenance of axon branches.

Potential roles for phosphatases in the maintenance of nervous system morphologies?

Many genes required for proper nervous system development may also be required later for maintenance of neuronal morphologies. Tight control of protein phosphorylation levels within neurons is important for many pathfinding decisions navigated by neurons during development (reviewed in Ensslen-Craig et al., 2004); for instance, phosophorylation is important for the cortical neuronal migrations discussed earlier. Several tyrosine phosphatases have been implicated in axon guidance; for example, the receptor tyrosine phosphatase Ptp69D is required for proper retinal axon target selection in the *Drosophila* brain (Garrity et al., 1999). The receptor tyrosine phosphatase LAR has recently been shown to be required in mice for the maintenance of

dendritic spines and excitatory synapses (Dunah et al., 2005). In the *Drosophila* visual system, LAR is required for synaptogeneis by preventing the retraction of a particular class of photoreceptor axons after they have reached their target layer in the brain, and it is also required for the pathfinding of other photoreceptor axons (Clandinin et al., 2001; Maurel-Zafran et al., 2001)

Because phosphatases are required in many contexts for nervous system development, we were interested in exploring the potential role of a novel cytoplasmic tyrosine phosphatase, Ptpmeg, in the establishment and maintenance of the nervous system. PTPMEG is an evolutionarily conserved protein that shares high homology with two human proteins, PTPN3 and PTPN4 (PTPMEG). All three proteins have a FERM domain, a domain often involved in linking cytoplasmic proteins to the membrane (reviewed in Cho and Stahelin, 2005), and a PDZ domain, a protein binding motif often found in protein scaffolds (Kim and Sheng, 2004). Mutations in the gene encoding PTPN3 were identified in a screen designed to uncover mutations in kinases and phosphatases common in colorectal cancer cell lines, and therefore PTPN3 is a putative tumor suppressor (Wang et al., 2004). The other homolog, PTPN4/PTPMEG, has been shown to inhibit cell growth in culture when overexpressed, suggesting that it could also be involved in regulating cell cycle or growth (Gu et al., 1996). Interestingly, PTPN4/PTPMEG is highly expressed in the mammalian nervous system (Gu and Majerus, 1996, Hironaka et al., 2000), where it has been shown to bind the Glutamate receptor subunits GluRδ2 (GRID2) and GluRε1 (NMDAR2B) (Hironaka et al., 2000). GluRd2/GRID2 is required for synapse formation in the mouse CNS, and it is also required during adulthood for synaptic maintenance (Kashiwabuchi et al., 1995; Kurihara

et al, 1997; Takeuchi et al., 2005). However, the role of PTPN4/PTPMEG in mammalian nervous system development and maintenance is unknown and elucidating this role awaits loss-of-function analysis there. Our analysis of PTPMEG in the *Drosophila* nervous system is the first in vivo study of the consequences of loss of *ptpmeg* function, and it demonstrates that PTPMEG's phosphatase activity is important for the development of proper neuronal connectivity in some neurons, but is required later for maintenance of neuronal connectivity in other neurons (see Chapter 3).

SUMMARY

Nervous system form and function requires both the development and maintenance of complex neuronal morphologies and connections. Patterning the nervous system involves the specification of neurons, neuronal migration, axon and dendrite outgrowth, guidance of these processes to their targets, axon and dendrite branching along the way, pruning of exuberant branches, and selective maintenance of wiring patterns. At present, relatively little is known about how complex neuronal morphologies are maintained throughout the life of an animal. The *Drosophila* nervous system offers several advantages for the study of maintenance of neuronal morphologies, including the accessibility of easily assayed structures, the possibility of genetic screening, and numerous genetic tools for assigning time and place of gene activity. These features have allowed for the identification in this thesis of factors required for the maintenance of nuclear and cell body position within postmitotic neurons and for the maintenance of axonal projections. Future experimentation in *Drosophila* may expand on understanding of both the maintenance of nuclear and cell body positioning and axonal trajectories as

well as the maintenance of other key attributes of neuronal morphology. Hopefully, these future experiments will provide important insights into nervous system diseases, learning and memory, and other topics related to the morphologies of neurons over time.

REFERENCES CITED

Ahmad, F., Hughey J, Wittmann T, Hyman A, Greaser M, Baas PW. (2000). Motor proteins regulate force interactions between microtubules and microfilaments in the axon. *Nature Cell Biology* **2**.

Ahmad, F., Yu, W, McNally, FJ, and Baas, PW. (1999). An essential role for katanin in severing microtubules in the neuron. *Journal of Cell Biology* **145**, 305-315.

Amos, L., and Klug, A. (1974). Arrangement of subunits in flagellar microtubules. *Journal of Cell Science* **14**, 523-549.

Andersen, S., and Bi, G-q. (2000). Axon formation: a molecular model for the generation of neuronal polarity. *BioEssays* **22**, 172-179.

Antonini, A., Stryker, MP. (1993). Rapid remodeling of axonal arbors in the visual cortex. *Science* **260**, 1819-1821.

Araki, T, Sasaki, Y, and Milbrandt, J. (2004) Increased nuclear NAD biosynthesis and SIRT1 activation prevent axonal degeneration. *Science* **305**, 1010-1013.

Awasaki, T., and Ito, K. (2004). Engulfing action of glial cells is required for programmed axon pruning during Drosophila metamorphosis. *Current Biology* **14**, 668-677.

Baas, P., Deitch, JS, Black, MM, and Banker, GA. (1988). Polarity orientation of microtubules in hippocampal neurons: uniformity in the axon and nonuniformity in the dendrite. *Proc Natl Acad Sci U S A* **85**, 8335-8339.

Baas, P., Karabay, A, and Qiang, L. (2005). Microtubules cut and run. *Trends in Cell Biology* **15**, 518-524.

Balice-Gordon, R., and Lichtman, JW. (1994). Long-term synapse loss induced by focal blockade of postsynaptic receptors. *Nature* **372**, 519-524.

Bence, N., Sampat, RM, and Kopito, RR. (2001). Impairment of the ubiquitin-proteasome system by protein aggregation. *Science* **292**, 1552-1555.

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

Bloom, G., Gagner, MC, Pfister, KK, and Brady, ST. (1988). Native structure and physical properties of bovine brain kinesin and identification of the ATP-binding subunit polypeptide. *Biochemistry* **27**, 3409-3416.

Bradke, F., and Dotti, CG. (1999). The role of local actin instability in axon formation. *Science* **283**, 1931-1934.

Bradke, F., and Dotti, CG. (2000). Establishment of neuronal polarity: lessons from cultured hippocampal neurons. *Current Opinion in Neurobiology* **10**, 574-581.

Brady, S. (1985). A novel brain ATPase with properties expected for the fast axonal transport motor. *Nature* **317**, 73-75.

Burkhardt, J., Echeverri, CJ, Nilsson, T, and Vallee, RB. (1997). Overexpression of the dynamitin (p50) subunit of the dynactin complex disrupts dynein-dependent maintenance of membrane organelle distribution. *Journal of Cell Biology* **139**, 469-484.

Burton, P., and Paige, JL. (1981). Polarity of axoplasmic microtubules in the olfactory nerve of the frog. *Proc Natl Acad Sci U S A* **78**, 3269-3273.

Callaway, E. (2006). Should I stay or should I go? Presynaptic boutons in the adult cortex still haven't made up their minds. *Neuron* **49**, 780-783.

Carminati, J., and Stearns, T. (1997). Microtubules orient the mitotic spindle in yeast through dynein-dependent interactions with the cell cortex. *Journal of Cell Biology* **138**, 629-641.

Caviness, V. J. (1982). Neocortical histogenesis in normal and reeler mice: a developmental study based upon [^{3}H]thymidine autoradiography. *Developmental Brain Research* **4**, 293-302.

Chalfie, M., and Thomson, JN. (1979). Organization of neuronal microtubules in the nematode *Caenorhabditis elegans. Journal of Cell Biology* **82**, 278-289.

Chen, J., Kanai, Y, Cowan, NJ, and Hirokawa, N. (1992). Projection domains of MAP2 and tau determine spacings between microtubules in dendrites and axons. *Nature* **360**, 674-677.

Chenn, A., and McConnell, SK. (1995). Cleavage orientation and the asymmetric inheritance of Notch1 immunoreactivity in mammalian neurogenesis. *Cell* **82**, 631-641.

Chenn, A., Braisted, JE, McConnell, SK, and O'Leary, DDM. (1997). Development of the cerebral cortex: mechanisms controlling cell fate, laminar and areal patterning, and axonal connectivity. In *Molecular and Cellular Approaches to Neural Development*, (ed. W. Cowan, Jessell, TM, and Zipursky, SL). New York: Oxford Univ. Press.

Chklovskii, D., Mel, BW, and Svoboda, K. (2004). Cortical rewiring and information storage. *Nature* **431**, 782-788.

Cho W, S. R. (2005). Membrane-protein interactions in cell signaling and membrane trafficking. *Annu Rev Biophys Biomol Struct* **34**, 119-151.

Chretien, D., and Wade, RH. (1991). New data on the microtubule surface lattice. *Biology of the Cell* **71**, 161-174.

Clandinin, T., Lee, CH, Herman, T, Lee, RC, Yang, AY, Ovasapyan, S, and Zipursky, SL. (2001). Drosophila LAR regulates R1-R6 and R7 target specificity in the visual system. *Neuron* **32**, 237-238.

Coleman, M. (2005). Axon degeneration mechanisms: comonality amid diversity. *Nature Reviews Neuroscience* **6**, 889-898.

Colman, H., Nabekura, J, and Lichtman, JW. (1997). Alterations in synaptic strength preceding axon withdrawl. *Science* **275**, 356-361.

Conforti, L., Tarlton, A, Mack, TG, Mi, W, Buckmaster, EA, Wagner, D, Perry, VH, and Coleman, MP. (2000). A Ufd2/D4Cole1e chimeric protein and overexpression of Rbp7 in the slow Wallerian degeneration (WldS) mouse. *Proc Natl Acad Sci U S A* **97**, 11377-11382.

Cotman, C. (1978). Neuronal Plasticity. New York: Raven Press.

Culver-Hanlon, T., Lex, SA, Stephens, AD, Quintyne, NJ, and King, SJ. (2006). A microtubule-binding domain in dynactin increases dynein processivity by skating along microtubules. *Nature Cell Biology* **8**, 264-270.

D'Arcangelo, G., Miao, GG, Chen, S-C, Soares, HD, Morgan JI, and Curran, T. (1995). A protein related to extracellular matrix proteins deleted in the mouse mutant reeler. *Nature* **374**, 719-723.

Dammermann, A., Desai, A, and Oegema, K. (2003). The minus end in sight. *Current Biology* **13**, R614-R624.

de Anda, F., Pollarolo, G, DaSilva, JS, Camoletto, PG, Feiguin, F, and Dotti, CG. (2005). Centrosome localization determines neuronal polarity. *Nature* **436**, 704-708.

De Paola V, H., A, Knott, G, Song, S, Wilbrecht, L, Caroni, P, and Svoboda, K. (2006). Cell type-specific structural plasticity of axonal branches and boutons in the adult neocortex. *Neuron* **49**, 861-875.

des Portes, V., Pinard, JM, Billuart, P, Vinet, MC, Koulakoff, A, Carrie, A, Gelot, A, Dupuis, E, Motte, J, Berwald-Netter, Y, Catala M, Kahn, A, Beldjord, C, and

Chelly, J. (1998). A novel CNS gene required for neuronal migration and involved in X-linked subcortical laminar heterotopia and lissencephaly syndrome. *Cell* **92**, 51-61.

Desai, A., and Mitchison, TJ. (1997). Microtubule polymerization dynamics. *Annual Review of Cell and Developmental Biology* **13**, 83-117.

Dunah, A., Hueske, E, Wyszynski, M, Hoogenraad, CC, Jaworski, J, Pak, DT, Simonetta, A, Liu, G, and Sheng, M. (2005). LAR receptor protein tyrosine phosphatases in the development and maintenance of excitatory synapses. *Nature Neuroscience* **8**, 458-467.

Eaton BA, F. R., Davis GW. (2002). Dynactin is necessary for synapse stabilization. *Neuron* **34**, 729-741.

Edmondson, J., and Hatten, ME. (1987). Glial-guided granule neuron migration in vitro: a high-resolution time-lapse video microscopic study. *The Journal of Neuroscience* **7**, 1928-1934.

Efimov, V., and Morris, NR. (2000). The LIS1-related NUDF protein of Aspergillus nidulans interacts with the coiled-coil domain of the NUDE/RO11 protein. *Journal of Cell Biology* **150**, 681-688.

Endow, S., Henikoff, S, and Soler-Niedziela, L. (1990). Mediation of meiotic and early mitotic chromosome segregation in Drosophila by a protein related to kinesin. *Nature* **435**, 81-83.

Ensslen-Craig SE, B.-K., SM. (2004). Receptor protein tyrosine phosphatases regulate neural development and axon guidance. *Developmental Biology* **275**, 12-22.

Evans, L., Mitchison, T, and Kirschner, M. (1985). Influence of the centrosome on the structure of nucleated microtubules. *Journal of Cell Biology* **100**, 1185-1191.

Fan, S.-S., and Ready, D. F. (1997). *Glued* participates in distinct microtubule-based activities in *Drosophila* eye development. *Development* **124**, 1497-1507.

Faulkner, N., Dujardin, DL, Tai, CY, Vaughan, KT, O'Connell, CB, Wang, Y, and Vallee, RB. (2000). A role for the lissencephaly gene LIS1 in mitosis and cytoplasmic dynein function. *Nature Cell Biology* **2**, 784-791.

Feng, Y., Olson, EC, Stukenberg, PT, Flanagan, LA, Kirschner, MW, and Walsh, CA. (2000). LIS1 regulates CNS lamination by interacting with mNudE, a central component of the centrosome. *Neuron* **28**, 665-679.

Ferri, A., Sanes, JR, Coleman, MP, Cunningham, JM, and Kato, AC. (2003). Inhibiting axon degeneration and synapse loss attenuates apoptosis and disease progression in a mouse model of motoneuron disease. *Current Biology* **13**, 669-673.

Fischer-Vize, J., and Mosley, KL. (1994). Marbles mutants: uncoupling cell determination and nuclear migration in the developing Drosophila eye. *Development* **120**, 2609-2618.

Francis, F., Koulakoff, A, Boucher, D, Chafey, P, Schaar, B, Vinet, MC, Friocourt, G, McDonnell, N, Reiner, O, Kahn, A, McConnell, SK, Berwald-Netter, Y, Denoulet, P, and Chellly, J. (1999). Doublecortin is a developmentally regulated, microtubule-associated protein expresed in migrating and differentiating neurons. *Neuron* **23**, 247-256.

Frank, E. (1997). Synapse elimination: for nerves it's all or nothing. *Science* **275**, 324-325.

Gaglio, T., Dionne, MA, and Compton, DA. (1997). Mitotic spindle poles are organized by structural and motor proteins in addition to centrosomes. *Journal of Cell Biology* **138**, 1055-1066.

Garrity PA, L. C., Salecker I, Robertson HC, Desai CJ, Zinn K, Zipursky SL. (1999). Retinal axon target selection in Drosophila is regulated by a receptor protein tyrosine phosphatase. *Neuron* **22**, 707-717.

Gill, S., Schroer, TA, Szilak, I, Steuer, ER, Sheetz, MP, and Cleveland, DW. (1991). Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. *Journal of Cell Biology* **115**, 1639-1650.

Gleeson, J., Allen, KM, Fox, JW, Lamperti, ED, Berkovic, S, Scheffer, I, Cooper, EC, Dobyns, WB, Minnerath, SR, Ross, ME, and Walsh, CA. (1998). Doublecortin, a brain-specific gene mutated in human X-linked lissencephaly and double cortex syndrome, encodes a putative signaling protein. *Cell* **92**, 63-72.

Gleeson, J., Lin, PT, Flanagan, LA, and Walsh, CA. (1999). Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron* **23**, 257-271.

Gonczy, P. (2004). Centrosomes: hooked on the nucleus. *Current Biology* **14**, R268-R270.

Griffin, J., George, EB, Hsieh, S-T, and Glass, JD. (1995). Axonal degeneration and disorders of the axonal cytoskeleton. In *The axon: structure, function and pathphysiology*, (ed. S. Waxman, Koesis, JD, and Sytys, PK), pp. 375-390. New York: Oxford University Press.

Gu, M., and Majerus, PW. (1996a). The properties of the protein tyrosine phosphatase PTPMEG. *J Biol Chem* **241**, 27751-27759.

Gu, M., Meng, K, and Majerus, PW. (1996b). 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**, 12980-12985.

Gutekunst, C., Li, SH, Yi, H, Mulroy, JS, Kuemmerle, S, Jones, R, Rye, D, Ferrante, RJ, Hersch, SM, and Li, XJ. (1999). Nuclear and neuropil aggregates in Huntington's disease: relationship to neuropathology. *Journal of Neuroscience* **19**, 2522-2534.

Hall, D., and Hedgecock, EM. (1991). Kinesin-related gene unc-104 is required for axonal transport of synaptic vesicles in C. elegans. *Cell* **65**, 837-847.

Hall, Z., and Sanes, JR. (1993). Synaptic structure and development: the neuromuscular junction. *Cell* **72 Supplement**, 99-121.

Harland, R. (2000). Neural induction. *Current Opinion in Genetics and Development* **10**, 357-362.

Hartmann, J., Mahr, J, McNally, K, Okawa, K, Iwamatsu, A, Thomas, S, Cheesman, S, Heuser, J, Vale, RD, and McNally, FJ. (1998). Katanin, a microtubule-severing protein, is a novel AAA ATPase that targets to the centrosome using a WD40-containing subunit. *Cell* **93**, 277-287.

Heidemann, S., Landers JM, and Hamborg, MA. (1981). Polarity orientation of axonal microtubules. *Journal of Cell Biology* **91**, 661-665.

Hiesberger, T., Trommsdorff, M, Howell, BW, Goffinet, A, Mumby, MC, Cooper, JA, and Herz, J. (1999). Direct binding of Reelin to VLDL receptor and ApoE receptor 2 induces tyrosine phosphorylation of disabled-1 and modulates tau phosphorylation. *Neuron* **24**, 481-489.

Hirokawa, N., and Takemura, R. (2005). Molecular motors and mechanisms of directional transport in neurons. *Nature Reviews Neuroscience* **6**, 201-214.

Hironaka, K., Umemori, H., Tezuka, T., Mishina, M., and Yamamoto, T. (2000). The protein-tyrosine phosphatase PTPMEG interacts with glutamate receptor δ2 and ε subunits. *J. Biol. Chem.* **275**, 16167-16173.

Holleran, E., Ligon, LA, Tokito, M, Stankewich, MC, Morrow, JS, and Holzbaur, EL. (2001). beta III spectrin binds to the Arp1 subunit of dynactin. *J Biol Chem* **276**, 36598-36605.

Holleran, E., Tokito, MK, Karki, S, and Holzbaur, EL. (1996). Centractin (ARP1) associates with spectrin revealing a potential mechanism to link dynactin to intracellular organelles. *Journal of Cell Biology* **135**, 1815-1829.

Holtmaat, A., Trachtenberg, JT, Wilbrecht, L, Shepherd, GM, Zhang, X, Knott, GW, and Svoboda, K. (2005). Transient and persistent dendritic spines in the neocortex in vivo. *Neuron* **45**, 279-291.

Hong, S., Shugart, YY, Huang, DT, Shahwan, SA, Grant, PE, Hourihane, JO, Martin, ND, and Walsh, CA. (2000). Autosomal recessive lissencephaly with cerebellar hypoplasia is associated with human RELN mutations. *Nature Genetics* **26**, 93-96.

Horesh, D., Sapir, T, Francis, F, Wolf, SG, Caspi, M, Elbaum, M, Chelly, J, and Reiner, O. (1999). Doublecortin, a stabilizer of microtubules. *Human Molecular Genetics* **8**, 1599-1610.

Houalla, T., Hien Vuong, D, Ruan, W, Suter, B, and Rao, Y. (2005). The Ste20-like kinase misshapen functions together with Bicaudal-D and dynein in driving nuclear migration in the developing Drosophila eye. *Mechanisms of Development* **122**, 97-108.

Howell, B., Hawkes, R, Soriano, P, and Cooper, JA. (1997). Neuronal position in the developing brain is regulated by mouse disabled-1. *Nature* **389**, 733-737.

Howell, B., Herrick, TM, and Cooper, JA. (1999). Reelin-induced tyrosine phosphorylation of disabled 1 during neuronal positioning. *Genes and Development* **13**, 643-648.

Huber, A., Kolodkin, AL, Ginty, DD, and Cloutier, J-F. (2003). Signaling at the growth cone: ligand-receptor complexes and the control of axon growth and guidance. *Annual Review of Neuroscience* **26**, 509-563.

Hyman, A., Chretien, D, Arnal, I, and Wade, RH. (1995). Structural changes accompanying GTP hydrolysis in microtubules: information from a slowly hydrolyzable analogue guanylyl-(alpha,beta)-methylene-diphosphate. *Journal of Cell Biology* **128**, 117-125.

Jacobson, C., Schnapp, B, and Banker, GA. (2006). A change in the selective translocation of the kinesin-1 motor domain marks the initial specification of the axon. *Neuron* **49**, 797-804.

Kalil, K., and Dent, EW. (2005). Touch and go: guidance cues signal to the growth cone cytoskeleton. *Current Opinion in Neurobiology* **15**, 521-526.

Kandel, E., Schwartz, JH, and Jessell, TM. (2000). Principles of Neural Science. New York: McGraw-Hill.

Karabay, A., Yu, W, Solowska, JM, Baird, DH, and Baas, PW. (2004). Axonal growth is sensitive to the levels of katanin, a protein that severs microtubules. *Journal of Neuroscience* **24**, 5778-5788.

Kashiwabuchi, N., Ikeda, K, Araki, K, Hirano, T, Shibuki, K, Takayama, C, Inoue, Y, Kutsuwada, T, Yagi, T, Kang, Y, Alzawa, S, and Mishina, M. (1995). Impairment of motor coordination, purkinje cell synapse formation, and cerebellar long-term depression in GluRδ2 mutant mice. *Cell* **81**, 245-252.

Kim, E., and Sheng, M. (2004). PDZ domain proteins of synapses. *Nature Reviews Neuroscience* **5**, 771-781.

King, S., and Schroer, TA. (2000). Dynactin increases the processivity of the cytoplasmic dynein motor. *Nature Cell Biology* **2**, 20-24.

Kondo, S., Sato-Yoshitake, R, Noda, Y, Aizawa, H, Nakata, T, Matsuura, Y, and Hirokawa, N. (1994). KIF3A is a new microtubule-based anterograde motor in the nerve axon. *Journal of Cell Biology* **125**, 1095-1107.

Kriegstein, A. R., and Noctor, S. C. (2004). Patterns of neuronal migration in the embryonic cortex. *Trends in Neuroscience* **27**, 392-399.

Kumar, S., Zhou, Y, and Plamann, M. (2001). Dynactin-membrane interaction is regulated by the C-terminal domains of p150(Glued). *EMBO Rep.* **2**, 939-944.

Kurihara, H., Hashimoto, K, Kano, M, Takayama, C, Sakimura, K, Mishina, M, Inoue, Y, and Watanabe, M. (1997). Impaired parallel fiber --> Purkinje cell synapse stabilization during cerebellar development of mutant mice lacking the glutamate receptor delta2 subunit. *Journal of Neuroscience* **17**, 9613-9623.

Lamprecht, R., Farb, CR, and LeDoux, JE. (2002). Fear memory formation involves p190 RhoGAP and ROCK proteins through a GRB2-mediated complex. *Neuron* **36**, 727-738.

Lee, T., Lee, A, and Luo, L. (1999b). Development of the *Drosophila* mushroom bodies: sequential generation of three distinct types of neurons from a neuroblast. *Development* **126**, 4065-4076.

Lee, T., Marticke, S, Sung, C, Robinow, S, and Luo, L. (2000). Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila. Neuron* **28**, 807-818.

Li, H., Li, SH, Yu, ZX, Shelbourne, P, and Li, XJ. (2001). Huntingtin aggregate-associated axonal degeneration is an early pathological event in Huntington's disease mice. *Journal of Neuroscience* **21**, 8473-8481.

Lunn, E., Perry, VH, Brown, MC, Rosen, H, and Gordon, S. (1989). Absence of Wallerian degeneration does not hinder regeneration in the peripheral nerve. *European Journal of Neuroscience* **1**, 27-33.

Luo, L., and O'Leary, DD. (2005). Axon retraction and degeneration in development and disease. *Annual Review of Neuroscience* **28**, 127-156.

Mack, T., Reiner, M, Beirowski, B, Mi, W, Emanuelli, M, Wagner, D, Thomson, D, Gillingwater, T, Court, F, Conforti, L, Fernando, FS, Tarlton, A, Andressen, C, Addicks, K, Magni, G, Ribchester, RR, Perry, VH, Coleman, MP. (2001). Wallerian degeneration of injured axons and synapses is delayed by a Ube4b/Nmnat chimeric gene. *Nature Neuroscience* **4**, 1199-1206.

Mallik, R., and Gross, SP. (2004). Molecular motors: strategies to get along. *Current Biology* **14**, R971-R982.

Martin, J. (1996). Neuroanatomy: Text and Atlas. Stamford, CT: Appleton and Lange.

Maurel-Zaffran, C., Suzuki, T, Gahmon, G, Treisman, JE, and Dickson, BJ. (2001). Cell-autonomous and -nonautonomous functions of LAR in R7 photoreceptor axon targeting. *Neuron* **32**, 225-235.

McDonald, H., and Goldstein, LS. (1990a). Identification and characterization of a gene encoding a kinesin-like protein in Drosophila. *Cell* **61**, 991-1000.

McDonald, H., Stewart, RJ, and Goldstein, LS. (1990b). The kinesin-like ncd protein of Drosophila is a minus end-directed microtubule motor. *Cell* **63**, 1159-1165.

Mejillano, M., Barton, JS, and Himes, RH. (1990). Stabilization of microtubules by GTP analogues. *Biochemical and Biophysical Research Communications* **166**, 653-660.

Michel, C., Kraft, R, Restifo, LL. (2004). Defective neuronal development in the mushroom bodies of *Drosophila fragile X mental retardation 1* mutants. *Journal of Neuroscience* **24**, 5798-5809.

Ming, G., and Song, H. (2005). Adult neurogenesis in the mammalian CNS. *Annual Review of Neuroscience* **28**, 223-250.

Muresan, V., Stankewich, MC, Steffen, W, Morrow, JS, Holzbaur, EL, and Schnapp, BJ. (2001). Dynactin-dependent, dynein-driven vesicle transport in the absence of membrane proteins: a role for spectrin and acidic phosophlipids. *Molecular Cell* **7**, 173-183.

Myers, K., He, Y, Hasaka, TP, and Baas, PW. (2006). Microtubule transport in the axon: re-thinking a potential role for the actin cytoskeleton. *Neuroscientist* **12**, 107-118.

Nicolai, M., Lasbleiz, C, and Dura, JM. (2003). Gain-of-function screen identifies a role of the Src64 oncogene in Drosophila mushroom body development. *Journal of Neurobiology* **57**, 291-302.

Nieuwenhuys, R., Voogd, J, and van Huijzen, C. (1988). The Human Central Nervous System: A Synopsis and Atlas. Berlin: Springer-Verlag.

Noctor, S., Flint, AC, Weissman, TA, Dammerman, RS, and Kriegstein, AR. (2001). Neurons derived from radial glial cells establish radial units in the neocortex. *Nature* **409**, 714-720.

Noda, Y., Sato-Yoshitake, R, Kondo, S., Nangaku, M, and Hirokawa, N. (1995). KIF2 is a new microtubule-based anterograde motor that transports membranous organelles distinct from those carried by kinesin heavy chain or KIF3A/B. *Journal of Cell Biology* **129**, 157-167.

Oakley, B., and Morris, NR. (1980). Nuclear movement is β-tubulin-dependent in Aspergillus nidulans. *Cell* **19**, 255-262.

Ochs, S. (1972). Fast transport of materials in mammalian nerve fibers. *Science* **176**, 252-260.

Osmani, A., Osmani, SA, and Morris, NR. (1990). The molecular cloning and identification of a gene product specifically required for nuclear movement in *Aspergillus nidulans. The Journal of Cell Biology* **111**, 543-551.

Pan, L., Zhang, YQ, Woodruff, E, and Broadie, K. (2004). The *Drosophila fragile X* gene negatively regulates neuronal elaboration and synaptic differentiation. *Current Biology* **14**, 1863-1870.

Patterson, K., Molofsky, AB, Robinson, C, Acosta, S, Cater, C, and Fischer, JA. (2004). The functions of Klarsicht and nuclear lamin in developmentally regulated nuclear migrations of photoreceptor cells in the Drosophila eye. *Molecular Biology of the Cell* **15**, 600-610.

Pramatarova, A., Ochalski, PG, Lee, C-H, and Howell, BW. (2006). Mouse Disabled 1 regulates the nuclear position of neurons in a Drosophila eye model. *Molecular and Cellular Biology* **26**, 1510-1517.

Quintyne, N., Gill SR, Eckley, DM, Crego, CL, Compoton, DA, and Schroer, TA. (1999). Dynactin is required for microtubule anchoring at centrosomes. *Journal of Cell Biology* **147**, 321-334.

Reiner, O., Carrozzo, R, Shen, Y, Wehnert, M, Faustinella, F, Dobyns, WB, Caskey, CT, and Ledbetter, DH. (1993). Isolation of a Miller-Dieker lissencephaly gene containing G protein β-subunit-like repeats. *Nature* **364**, 717-721.

Reuter, J., Nardine, TM, Penton, A, Billuart, P, Scott, EK, Usui, T, Uemura, T, and Luo, L. (2003). A mosaic genetic screen for genes necessary for Drosophila mushroom body neuronal morphogenesis. *Development* **130**, 1203-1213.

Russell, W. (1959). Brain, Memory, and Learning: A Neurologist's View. Oxford: Clarendon Press.

Samsam, M., Mi, W, Wessig, C, Zielasek, J, Toyka, KV, Coleman, MP, and Martini, R. (2003). The Wlds mutation delays robust loss of motor and sensory axons in a genetic model for myelin-related axonopathy. *Journal of Neuroscience* **23**, 2833-2839.

Sapir, T., Elbaum, M, and Reiner, O. (1997). Reduction of microtubule catastrophe events by LIS1, platelet-activating factor acetylhydrolase subunit. *EMBO J.* **16**, 6977-6984.

Schaar, B., and McConnell, SK. (2005). Cytoskeletal coordination during neuronal migration. *Proc Natl Acad Sci U S A* **102**, 13652-13657.

Scherzinger, E., Lurz, R, Turmaine, M, Mangiarini, L, Hollenbach, B, Hasenbank, R, Bates, GP, Davies, SW, Lehrach, H, and Wanker, EE. (1997). Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates in vitro and in vivo. *Cell* **90**, 549-558.

Seeds, N., Gilman, AG, Amano, T, and Nirenberg, MW. (1970). Regulation of axon formation by clonal lines of a neural tumor. *Proc Natl Acad Sci U S A* **66**, 160-167.

Simon, A., Bouquet, I, Synguelakis, M, Preat, T. (1998). The Drosophila putative kinase linotte (derailed) prevents central brain axons from converging on a newly described interhemispheric ring. *Mechanisms of Development* **76**, 45-55.

Singer, W. (1995). Development and plasticity of cortical processing architectures. *Science* **270**, 758-764.

Solecki, D., Model, L, Gaetz, J, Kapoor, TM, and Hatten, ME. (2004). Par6α signaling controls glial-guided neuronal migration. *Nature Neuroscience* **7**, 1195-1203.

Solomon, F., and Magendantz, M. (1981). Cytochalasin separates microtubule disassembly from loss of asymmetric morphology. *89* **1**.

Spiegelman, B., Lopata, MA, and Kirschner, MW. (1979). Aggregation of microtubule initiation sites preceding neurite outgrowth in mouse neuroblastoma cells. *Cell* **16**, 253-263.

Spiegelman, B., Penningroth, SM, and Krischner, MW. (1977). Turnover of tubulin and the N site GTP in Chinese hamster ovary cells. *Cell* **12**, 587-600.

Stettler, D., Yamahachi, H, Li, W, Denk, W, and Gilbert, CD. (2006). Axons and synaptic boutons are highly dynamic in adult visual cortex. *Neuron* **49**, 877-887.

Sur, M., and Rubenstein, JL. (2005). Patterning and plasticity of the cerebral cortex. *Science* **310**, 805-810.

Swan, A., Nguyen, T, and Suter, B. (1999). *Drosophila Lissencephaly-1* functions with *Bic-D* and dynein in oocyte determination and nuclear positioning. *Nature Cell Biology* **1**, 444-449.

Szebenyi, G., Morfini, GA, Babcock, A, Gould, M, Selkoe, K, Stenoien, DL, Young, M, Faber, PW, MacDonald, ME, McPhaul, MJ, and Brady, ST. (2003). Neuropathogenic forms of huntingtin and androgen receptor inhibit fast axonal transport. *Neuron* **40**, 41-52.

Takeuchi, T., Miyazaki, T, Watanabe, M, Mori, H, Sakimura, K, and Mishina, M. (2005). Control of synaptic connection by glutamate receptor delta2 in the adult cerebellum. *Journal of Neuroscience* **25**, 2146-2156.

Tanaka, T., Serneo, FF, Higgins, C, Gambello, MJ, Wynshaw-Boris, A, and Gleeson, JG. (2004). Lis1 and doublecortin function with dynein to mediate coupling of the nucleus to the centrosome in neuronal migration. *Journal of Cell Biology* **165**, 709-721.

Thompson, W. (1983). Synapse elimination in neonatal rat muscle is senstive to pattern of muscle use. *Nature* **302**, 614-616.

Thompson, W., Kuffler, DP, and Jansen, JK. (1979). The effect of prolonged, reversible block of nerve impulses on the elimination of polyneuronal innervation of new-born rat skeletal muscle fibers. *Neuroscience* **4**, 271-281.

Tomlinson, A. (1985). The cellular dynamics of pattern formation in the eye of *Drosophila. Journal of Embryology and Experimental Morphology* **89**, 313-331.

Trommsdorff, M., Gotthardt, M, Hiesberger, T, Shelton, J, Stockinger, W, Nimpf, J, Hammer, RE, Richardson, JA, and Herz, J. (1999). Reeler/Disabled-like disruption of neuronal migration in knockout mice lacking VLDL receptor and ApoE receptor 2. *Cell* **97**, 689-701.

Tsai, J. W., Chen, Y., Kriegstein, A. R., and Vallee, R. B. (2005). LIS1 RNA interference blocks neural stem cell division, morphogenesis, and motility at multiple stages. *Journal of Cell Biology* **170**, 935-945.

Vale, R., Reese, TS, Sheetz, MP. (1985a). Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. *Cell* **42**, 39-50.

Vale, R., Schnapp, BJ, Mitchison, T, Steuer, E, Reese, TS, and Sheetz, MP. (1985b). Different axoplasmic proteins generate movement in opposite directions along microtubules in vitro. *Cell* **43**, 623-632.

Vallee, R., Williams, JC, Varma, D, and Barnhart, LE. (2004). Dynein: An ancient motor protein involved in multiple modes of transport. *Journal of Neurobiology* **58**, 189-200.

Walker, R., Salmon, ED, and Endow, SA. (1990). The Drosophila claret segregation protein is a minus-end directed microtubule motor. *Nature* **347**, 780-782.

Waller, A. (1850). Experiments on the section of glossopharyngeal and hypoglossal nerves of the frog and observations of the alternatives produced by thereby in the structure of their primitive fibers. *Philosophical Transactions of the Royal Society of London* **140**, 423-429.

Wang, J., Zugates, CT, Liang, IH, Lee, CH, and Lee, T. (2002a). Drosophila Dscam is required for divergent segregation of sister branches and suppresses ectopic bifurcation of axons. *Neuron* **33**, 559-571.

Wang, J, Zhai, Q, Chen, Y, Lin, E, Gu, W, McBurney, MW, and He, Z. (2005). A local mechanism mediates NAD-dependent protection of axon degeneration. *J Cell Biol.* **170**, 349-355.

Wang, L., and Brown, A. (2002b). Rapid movement of microtubules in axons. *Current Biology* **12**, 1496-1501.

Wang, Z., Shen D, Parsons DW, Bardelli A, Sager J, Szabo S, Ptak J, Silliman N, Peters BA, van der Heijden MS, Parmigiani G, Yan H, Wang TL, Riggins G, Powell SM, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE. (2004). Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science* **304**, 1164-1166.

Weisenberg, R., Borisy, GG, and Taylor, EW. (1968). The colchicine-binding protein of mammalian brain and its relation to microtubules. *Biochemistry* **7**, 4466-4479.

Weisenberg, R., Deery, WJ, and Dickinson, PJ. (1976). Tubulin-nucleotide interactions during the polymerization and depolymerization of microtubules. *Biochemistry* **15**, 4248-4254.

Welte, M., Gross, SP, Postner, M, Block, SM, and Wieschaus, EF. (1998). Developmental regulation of vesicle transport in Drosophila embryos: forces and kinetics. *Cell* **92**, 547-557.

Winder, S. (2004). Filopodia formation and Disabled degradation downstream of Reelin. *Biochem J* **384**, e1-2.

Wolff, T., and Ready, DF. (1993). Pattern formation in the *Drosophila* retina. In *The Development of Drosophila melanogaster*, vol. II (ed. M. Bate, and Martinez Arias, A), pp. 1277-1325. Plainview, NY: Cold Spring Harbor Laboratory Press.

Xiang, X. (2003). LIS1 at the microtubule plus end and its role in dynein-mediated nuclear migration. *Journal of Cell Biology* **160**, 355-364.

Xiang, X., Beckwith, SM, and Morris, NR. (1994). Cytoplasmic dynein is involved in nuclear migration in *Aspergillus nidulans*. *Proc. Natl. Acad. Sci. USA* **91**, 2100-2104.

Xiang, X., Osmani, AH, Osmani, SA, Xin, M., and Morris, NR. (1995). *NudF*, a nuclear migration gene in *Aspergillus nidulans*, is similar to the human *LIS-1* gene required for neuronal migration. *Molecular Biology of the Cell* **6**, 297-310.

Zars, T. (2000). Behavioral functions of the insect mushroom bodies. *Current Opinion in Neurobiology* **10**, 790-795.

Chapter 2.

Dynactin is required to maintain nuclear position within postmitotic *Drosophila* photoreceptor neurons

Jessica L. Whited, Andre Cassell, Monique Brouillette, and Paul A. Garrity

As published in Development 2004 131, 4677-4686.

Screen to generate new *cpb* alleles, described in Materials and methods, was performed with the assistance of Andre Cassell and Monique Brouillette.

SUMMARY

How a nucleus is positioned within a highly polarized postmitotic animal cell is not well understood. In this work, we demonstrate that the Dynactin complex (a regulator of the microtubule motor protein Dynein) is required to maintain the position of the nucleus within post-mitotic Drosophila melanogaster photoreceptor neurons. We show that multiple independent disruptions of Dynactin function cause a relocation of the photoreceptor nucleus toward the brain, and that inhibiting Dynactin causes the photoreceptor to acquire a bipolar appearance with long leading and trailing processes. We find that while the minus-end directed motor Dynein cooperates with Dynactin in positioning the photoreceptor nucleus, the plus-end directed microtubule motor Kinesin acts antagonistically to Dynactin. These data suggest that the maintenance of photoreceptor nuclear position depends on a balance of plus-end and minus-end directed microtubule motor function.

INTRODUCTION

Neurons are highly polarized cells whose cell bodies extend processes specialized for receiving and transmitting information. The location of a neuron's cell body is defined in large part by the location of its nucleus, and the positioning of a neuron's cell body with respect to its processes (e.g. axons and dendrites) varies in a neuron-specific way, contributing to the great diversity of neuronal morphologies. Little is known about how the position of the nucleus is maintained as a neuron undergoes the morphological changes accompanying differentiation.

Nuclear positioning makes an important contribution to brain architecture. In insects, for example, neuronal nuclei and neuronal processes are spatially segregated within the brain, with nuclei populating cortical regions while neuritis extend into neuropil regions and establish connections (Cajal, 1990; Strausfeld and Meinertzhagen, 1998). While such extreme spatial segregation is not as widely observed in mammals, neuronal nuclear positions are highly stereotyped throughout the mammalian nervous system (Cajal, 1990; Strausfeld and Meinertzhagen, 1998). Genes mutated in several human neurological disorders (including isolated lissencephaly sequence, Miller-Dieker syndrome, and some forms of lissencephaly with cerebellar hypoplasia) have been implicated in nuclear positioning in other systems (Gupta et al., 2002; Olson and Walsh, 2002), although whether defects in the maintenance of nuclear positioning within neurons contribute to these disorders is unknown. Nonetheless, functional studies of these genes in model organisms suggest that the molecular mechanisms that control nuclear positioning may be relevant to human neuronal mispositioning disorders (Morris, 2003; Reinsch and Gonczy, 1998).

Their highly polarized nature and complex morphologies make neurons a favorable system for studying nuclear positioning, yet the mechanisms that maintain nuclear position in postmitotic neurons have not been extensively explored. Both the microtubule cytoskeleton and the actin cytoskeleton have been implicated in positioning nuclei within non-motile animal cells (Starr and Han, 2003). The nucleus is often associated with the focus of microtubule minus ends, and work in non-dividing cultured mammalian cells indicates that the cytoplasmic microtubule network and the minus-end directed microtubule motor Dynein are important for maintaining the focus of

microtubule minus ends and nuclear position (Quintyne et al., 1999). In multinucleate *Caenorhabditis elegans* muscle cells and in *Drosophila melanogaster* nurse cells, nuclei require anchorage to the actin cytoskeleton to maintain their appropriate positions (Starr and Han, 2003).

In the *Drosophila melanogaster* compound eye, the precise packing of photoreceptor neurons within each facet of the eye involves the highly stereotyped localization of photoreceptor nuclei. The photoreceptors are generated within a polarized monolayer epithelium (the eye imaginal disc), and the coordinated movements of differentiating photoreceptor nuclei have been described in detail (Tomlinson, 1985). As each photoreceptor differentiates, its nucleus rises toward the apical surface of the eye disc and remains apical while the photoreceptor axon extends toward the basal surface of the eye disc and into the brain. Several mutations that cause photoreceptor nuclei to be displaced toward the brain have been identified and include mutations in genes encoding the Dynactin subunit Glued (Fan and Ready, 1997), the Dynein-associated protein Lis1 (Swan et al., 1999) (the human homolog of which is disrupted in isolated lissencephaly sequence and Miller-Dieker syndrome; Olson and Walsh, 2002; Reiner et al., 1993), the putative microtubule motor regulator Klar (Mosley-Bishop et al., 1999; Welte et al., 1998), and the nuclear lamin Lam DM(0) (Patterson et al., 2004). These studies have demonstrated that the location of the photoreceptor nucleus depends on factors associated with the microtubule cytoskeleton. However, it is essential to determine whether such nuclear relocation reflects nuclear mispositioning within the cell or migration of the entire cell, and whether the defect is simply a secondary consequence of earlier disruptions in mitosis or alterations in the overall apical/basal polarity of the retinal

epithelium. The many molecular and genetic tools available in the Drosophila retina facilitate the critical examination of these issues.

The Dynactin complex is an assembly of 11 different subunits that functions as a activator of Dynein (Gill et al., 1991), serving as an adaptor for cargo (Holleran et al., 1996, 2001; Muresan et al., 2001) and enhancing motor processivity (King and Schroer, 2000). The Dynactin subunit Glued couples Dynactin to Dynein by binding to the Dynein intermediate chain (Dic) (Karki and Holzbaur, 1995; Vaughan and Vallee, 1995). Overexpression of a truncated form of Glued that binds to Dic but cannot associate with the rest of the Dynactin complex acts a s powerful inhibitor of Dynein and Dynactin function (Allen et al., 1999; Eaton et al., 2002; Fan and Ready, 1997). Overexpression of the Dynactin subunit Dynamitin disrupts Dynactin complex assembly and also inhibits Dynactin function (Echeverri et al., 1996; Eckley et al.,, 1999). Biochemical studies have shown that the Dynactin complex also contains Capping Protein (Schafer et al., 1994), a heterodimer composed have the Capping Protein alpha (Cpa) and Capping Protein beta (Cpb) subunits (Cooper et al., 1999). Although best known for capping the barbed ends of filaments of actin, Capping Protein also associates with filaments of the actin-related Arp1 protein, which is a central element of the Dynactin complex (Cooper et al., 1999; Schafer et a l., 1996).

In this work we demonstrate, using multiple independent strategies to disrupt Dynactin function, that the Dynactin complex is critical for photoreceptor nuclear positioning and that Dynactin inhibition causes photoreceptor nuclei to leave the retina and move toward the brain. We show that Dynactin acts in postmitotic photoreceptors and that the disruption in nuclear positioning observed reflects the movement of the

nucleus within the neuron rather than photoreceptor migration. We isolate loss-of-function mutations in *kinesin heavy chain* (*khc*) as strong suppressors of nuclear mispositioning in *Glued*^{1} mutants, and we demonstrate that Kinesin antagonizes Glued function in positioning the nuclei of postmitotic photoreceptors, both in the adult eye and in the larval photosensory organ. Our data demonstrate that the maintenance of photoreceptor nuclear position relies on Dynactin activity and suggest that the positioning of photoreceptor nuclei depends on the antagonistic activities of plus-end and minus-end directed microtubule motors.

MATERIALS AND METHODS

Genetics and molecular biology

Unless otherwise indicated, fly stocks were obtained from the Bloomington Stock Center. *Glued*^{1} and *UAS:Glued*^{DN} have been described (Boylan and Hays, 2002; Fan and Ready, 1997; Harte and Kankel, 1982). Glued^{DN} contains the N-terminal 922 amino acids of Glued and behaves similarly to the product of Glued^{1} (Allen et al., 1999; Eaten et al., 2002; Fan and Ready, 1997). *UAS-Dynamitin-GFP* flies have been described (Januschke et al, 2002).

cpb^{F44} was recovered from approximately 4400 lines of EMS-mutagenized *FRT40A* flies screened for failure to complement *cpb*^{M143} lethality. Both *cpb*^{M143} and *cpb*^{F44} were sequenced and contained a G to A transition introducing a stop codon after amino acid 147 of Cpb. *cpb*^{M143} and *cpb*^{F44} were independently induced as 14 sequence differences were detected between *cpb*^{M143} and *cpb*^{F44} within the Cpb transcription unit (flanking the truncation mutation); all *cpb*^{F44} polymorphisms were shared with the

FRT40A stock used for mutagenesis. *cpb*^{M143} was generated in the lab of E. Wieschaus. *Df(2L)E.2* was provided by M. Welte. DNA from homozygous *Df(2L)E.2* embryos was examined by PCR using multiple primer pairs covering the entire Cpb transcription unit; no Cpb DNA was detected in these animals, suggesting *Df(2L)E.2* deletes Cpb. *UAS-Gl*$^{\Delta 84}$ was provided by G. Davis, and *UAS-Nod:LacZ* by S. Thor. Homozygous mutant visual system clones were produced using the eyeless-FLP system (Newsome et al., 2000).

pUAS:Cpb contains a full-length Cpb cDNA (SD07714, Research Genetics) cloned into pUAST (Brand and Perrimon, 1993). pGlass38-1:Gal4 contains 38-1, a pentamer of a 38 bp glass-responsive fragment from the Rh1 enhancer upstream of an hsp70 minimal promoter (Ellis et al., 1993), cloned into pGATb (Brand and Perrimon, 1993). Transgenic flies were created as described (Spradling and Rubin, 1982). pGlass38-1:Gal4 drove expression in the anticipated pattern (Ellis et al., 1993), with expression initiating in photoreceptors seven to eight rows behind the onset of detectable Elav expression. As one row of ommatidia is added every 90 minutes (Wolff and Ready, 1993) and photoreceptor axons reach the brain four to five rows after the initiation of Elav expression, the onset of detectable transgene expression lags photoreceptor axon innervation of the target by $\geq$ 3 hours. Rescue was obtained by crossing *Df(2L)E.2, Bc/+;tubulin:GAL4,UAS:mCD8GFP/+* males to *p{w+,UAS:cpb}, cpbM143/SM6:TM6b,Tb* virgins. A total of 235 third-instar progeny were scored for UAS:Cpb transgene rescue of *cpb* lethality. All 37 *cpb/Df(2L)E.2* larvae, (*Bc*, non-*Tb* larvae) were GFP-positive and thus contained both the Gal4 driver and UAS:Cpb; no GFP-negative *cpb/Df(2L)E.2* larvae, which did not contain the Gal4 driver, were

recovered. Single-cell analysis in Fig. 2 was performed by crossing *hsFLP-Actin-FRT-FRT-GAL4,UAS:GFP/Y* males to *w; c-s* or Gl^{1}/*TM6b* virgins. Progeny were heat-shocked at 38°C for 1 hour each day.

Histology

Third-instar eye-brain complexes were stained as described (Garrity et al., 1999). Primary antibodies were used as indicated: mouse Mab 24B10 anti-Chaoptin (Fujita et al., 1982) (1:200), rat Mab 7E8A10 anti-Elav (O'Neill et al., 1994) (1:50), mouse Mab 40-1a anti-LacZ (1:200) and rabbit anti-PATJ (1:2000). 24B10, 7E8A10, and 40-1a were obtained from the Developmental Studies Hybridoma Bank at the University of Iowa. Secondary antibodies were obtained from Jackson Laboratories and used as described: goat anti-mouse HRP (1:200), goat anti-rat HRP (1:500), goat anti-mouse Cy3 (1:400), goat anti-rabbit FITC (1:200), goat anti-rat Cy5 (1:200), goat anti-mouse FITC (1:200). Confocal images were obtained using a Nikon PCM2000 microscope. SEM was performed as described (Wolff, 2000).

RESULTS

Dynactin is required for proper localization of photoreceptor cell bodies and nuclei

Patterning of the adult compound eye of Drosophila initiates during the third instar phase of larval life, and mutations in the Dynactin subunit Glued strongly disrupt eye development (Fan and Ready, 1997; Harte and Kanke, 1982). Normally the nuclei of differentiating photoreceptors occupy apical regions of the eye disc. In animals heterozygous for the dominant-negative *Glued* allele $Glued^{1}$, many photoreceptor nuclei

have been shown to accumulate within basal regions of the eye disc (Fan and Ready, 1997). We further characterized the effect of *Glued*^{1} on photoreceptor development using an antibody recognizing photoreceptor cell surfaces. In wild type, the region of the differentiating photoreceptor neuron containing the nucleus remained in the retina, while the photoreceptor axon extended through the optic stalk into the brain (Fig. 1A). However, in *Glued*^{1} animals, while photoreceptors still extended axons into the brain, the region of the photoreceptor containing the nucleus often appeared to leave the retina and travel through the optic stalk into the brain (Fig. 1B). Staining of photoreceptor nuclei directly demonstrated the movement of photoreceptor nuclei out of the eye disc and into the optic stalk in *Glued*^{1} mutants (see below).

To further establish that *Glued*^{1} defects reflected disruptions in Dynactin function, we used two other approaches to disrupt the Dynactin complex. As described below, we overexpressed Drosophila Dynamitin, which also inhibits Dynactin function in flies (Duncan and Warrior, 2002; Januschke et al., 2002), in photoreceptor neurons. We also examined loss-of-function mutations in the Dynactin subunit Cpb by generating animals whose visual systems contained homozygous mutant clones of the *cpb* strong loss-of-function mutation *cpb*^{M143}. In these *cpb*^{M143} mosaic animals, the nuclear regions of many photoreceptors were observed in the optic stalk and brain (Fig. 1C,D).

To confirm that the *cpb*^{M143} mutant photoreceptor defect was due to a loss of *cpb* function, we isolated an additional strong loss-of-function *cpb* allele, *cpb*^{F44}, from an EMS mutagenesis and obtained a chromosomal deficiency uncovering the *cpb* locus, *Df(2L)E.2* (see materials and methods for details). When animals contained homozygous mutant clones of *cpb*^{F44} or homozygous mutant clones of *Df(2L)E.2*, a similar movement

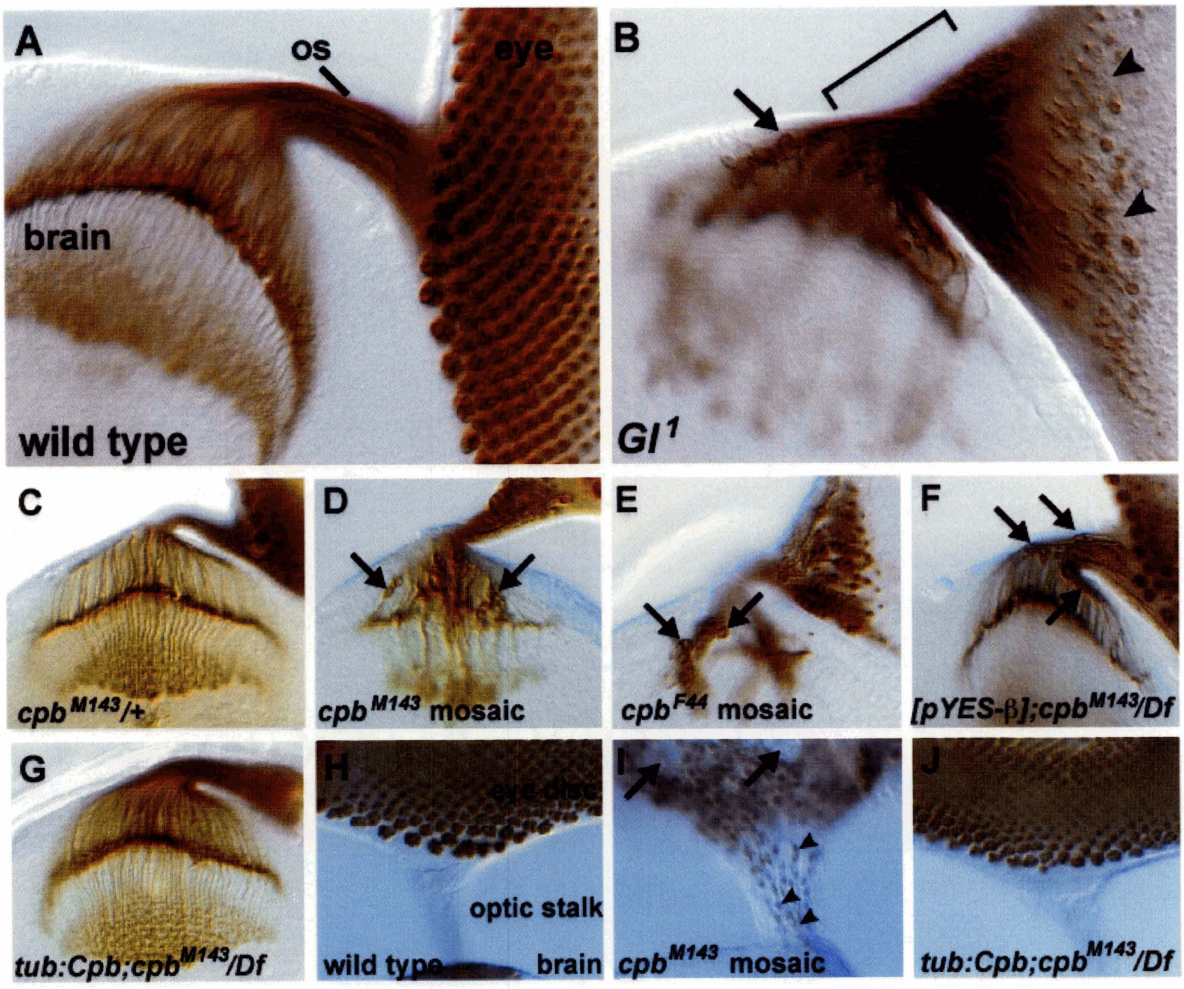
of photoreceptor nuclear regions toward the brain was observed (Fig. 1E and data not shown). *cpb/Df(2L)E.2* animals did not survive to third instar, preventing the classic genetic demonstration that these *cpb* alleles behaved as strong loss-of-function mutations. Fortunately, we found that the *[pYES-β]* genomic transgene, which contains the Cpb coding region (Hopmann et al., 1996), was able to rescue the lethality of *cpb/Df(2L)E.2* animals, but did not rescue the previously described *cpb* bristle defect (Hopmann et al., 1996). This suggested that *[pYES-β]* was a partially functional rescue construct that could be used to examine the visual systems of otherwise *cpb/Df(2L)E.2* animals. We found that *[pYES-β];cpb^{M143}/Df(2L)E.2* animals displayed a photoreceptor defect similar to that of other *cpb* mutants, consistent with nuclear mispositioning resulting from the loss of *cpb* function (Fig. 1F). We further confirmed that the defect was due to the loss of *cpb* function by successfully rescuing the *cpb^{M143}/Df(2L)E.2* photoreceptor defects (as well as the cpb bristle defects) by expression of a wild-type Cpb cDNA under the control of a heterologous promoter (Fig. 1G and data not shown). Staining of photoreceptor nuclei directly demonstrated the movement of photoreceptor nuclei out of the eye disc and into the optic stalk in *cpb* mutants (Fig. 1H-J).

The bifunctional nature of Cpb, which associates with filaments of actin as well as filaments of Arp1, means that loss of Cpb also increases filamentous actin levels (Hopmann and Miller, 2003). Nonetheless, previous studies have shown that increases in filamentous actin alone, such as those observed in hypomorphic *cpb* alleles or in *actup* mutants, do not cause photoreceptor nuclear mispositioning (Benlali et al., 2000; Hopmann and Miller, 2003). Together with the *Glued^{1}* and Dynamitin data, the *cpb* observations yield a consistent picture that alterations in Dynactin subunits cause

mispositioning of photoreceptor cell bodies and nuclei, and indicate that Dynactin, and not just the Glued subunit, has an important role in photoreceptor development.

Figure 1. Dynactin is required to position photoreceptor cell bodies and nuclei in the developing third instar eye disc. Photoreceptor cell membranes are stained with anti-chaoptin in A-G. (A) In wild type, photoreceptor cell bodies (as defined in this figure by the position of the nucleus) are precisely arranged in clusters in the apical region of the eye disc and project axons through the optic stalk (os) into the brain's optic lobe. (B) In *Glued*^{1} mutants, many photoreceptor neuron cell bodies leave the apical region of the eye disc (arrowheads) and travel into the optic stalk (bracket) and brain (arrow). (C) Heterozygous *cpb*^{M143} animals have a wild-type photoreceptor axon projection pattern, with photoreceptor cell bodies positioned in the eye disc. (D) In heterozygous *cpb*^{M143} animals with homozygous *cpb*^{M143}/*cpb*^{M143} patches in the visual system, many photoreceptor cell bodies leave the eye disc and enter the brain (arrows). (E) An independently generated *cpb* allele, *cpb*^{F44}, also causes photoreceptor cell bodies to enter the brain (arrows) in eye clones. (F) Photoreceptor mispositioning (arrows) is also observed in *cpb*^{M143}/*Df(2L)E.2* animals rescued from early lethality by expression of Cpb from a genomic transgene [pYES-b] (see text for details). (G) *cpb*^{M143}/*Df(2L)E.2* animals rescued by ubiquitous expression of a wild-type Cpb cDNA have normal photoreceptor positioning. Photoreceptor nuclei are stained with anti-Elav in H-J. (H) In wild type, photoreceptor nuclei remain in the eye disc and do not enter the optic stalk. (I) Photoreceptor nuclei are mispositioned in *cpb*^{M143} mosaic eye discs, with patches of eye tissue missing nuclei (arrows) and Elav-staining nuclei found in the optic stalk (arrowheads). (J) *cpb*^{M143}/*Df(2L)E.2* animals rescued by ubiquitous expression of a Cpb cDNA have normal photoreceptor nuclear positioning.

Figure 1

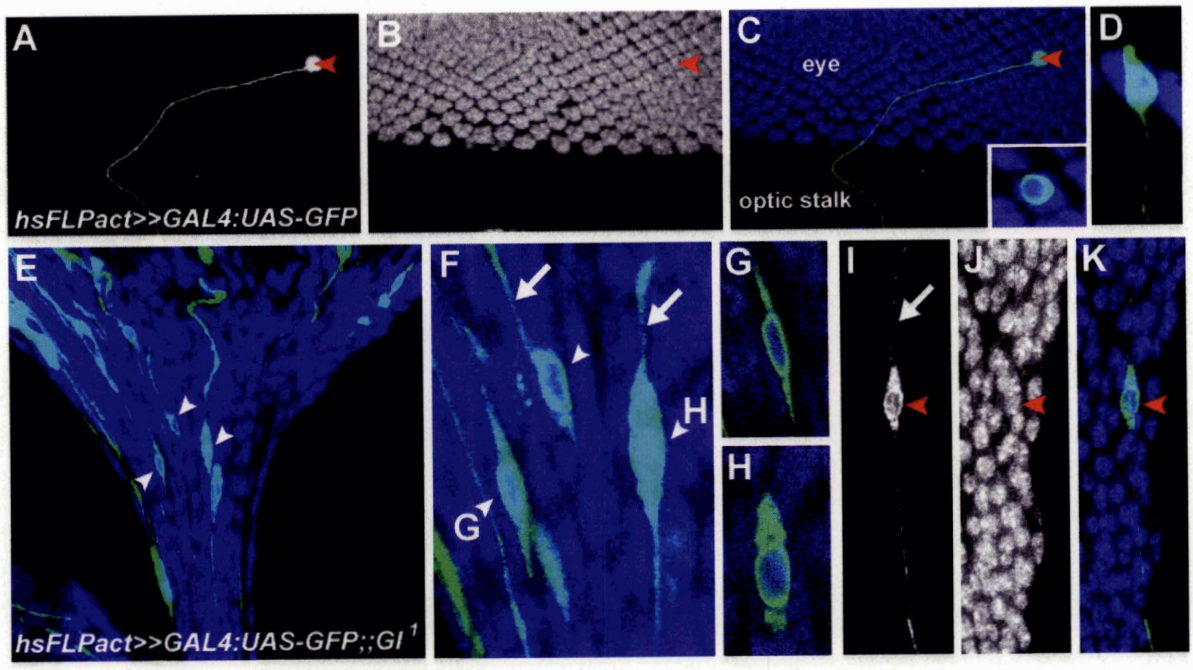

A
os
eye
brain
wild type
B
Gl 1
C
cpb M143 /+
D
cpb M143 mosaic
E
cpb F44 mosaic
F
[pYES-β];cpb M143 /Df
G
tub:Cpb;cpb M143 /Df
H
eye disc
optic stalk
wild type
brain
I
cpb M143 mosaic
J
tub:Cpb;cpb M143 /Df

Dynactin is required for maintenance of nuclear positioning within postmitotic photoreceptors

The mispositioning of photoreceptor nuclei in Dynactin mutants raised the question of whether these disruptions reflect altered positioning of the nucleus within the photoreceptor or simply migration of the entire photoreceptor. To address this question, single photoreceptors were labeled in wild type and *Glued*^{1} mutants. Wild-type photoreceptors exhibit a highly polarized morphology in which the region of the photoreceptor containing the nucleus lies in the apical region of the eye disc and an axon extends basally into the brain (Fig. 2A-D). *Glued*^{1} mutant photoreceptors whose nuclei have entered the optic stalk had highly altered morphologies, with both leading and trailing processes extending from the regions of the cell where the misplaced nucleus was located (Fig. 2E-K). We quantified leading and trailing processes of misplaced *Glued*^{1} photoreceptors, considering only those with no other labeled cells or processes nearby. Of these 13 neurons, 12 had clearly detectable leading and trailing processes. The leading process (axon) extended into the target region and the trailing process extended back into the eye disc. These data demonstrate that inhibition of Dynactin function dramatically alters the position of the nucleus within the photoreceptor.

Figure 2. Nuclei are mispositioned within *Glued* mutant photoreceptors. (A-K) All neuronal nuclei in a third instar eye-brain complex are labeled with anti-Elav (blue in C-H, K, white in B, J). Individual cells are labeled with GFP (green in C-H, K, white in A, I) using a *heat-shock FLP Act-FRT-FRT-Gal4:UAS-GFP* chromosome in a wild-type background (A-D) or a $Glued^{1}$ mutant animal (E-K). An individual wild-type photoreceptor is marked with GFP in A, and all neuronal nuclei labeled with anti-Elav in B. A and B are merged in C, with a higher magnification view of the marked photoreceptor containing a nucleus in the inset. (D) An individual photoreceptor in another sample is imaged from a side view, with apical at the top. In $Glued^{1}$ mutants, individual cells in the optic stalk in E (marked with arrowheads) are shown at higher magnification in F. Examples of trailing processes are marked with arrows in F. Cells in F marked with both an arrowhead and a letter are shown at right, with their corresponding letter, at higher magnification. An individual labeled cell in another animal is shown in I, with the trailing process indicated by the arrow. I and J are merged in K.

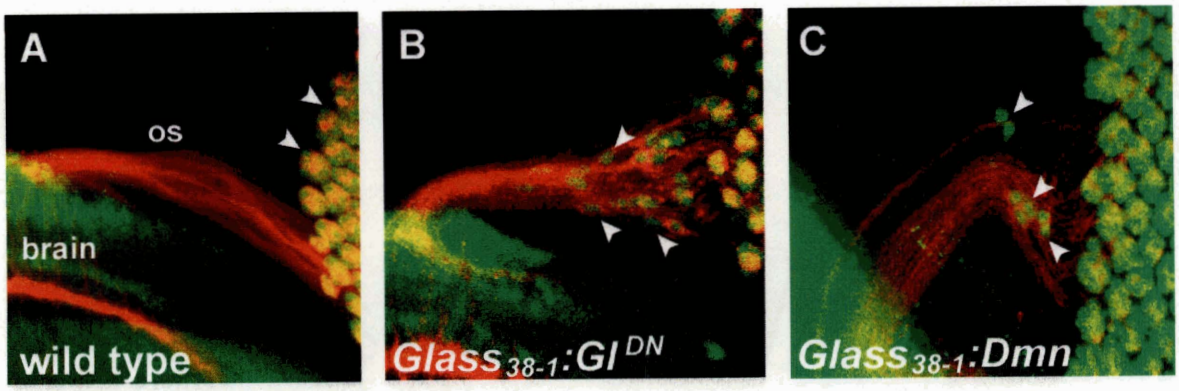
Figure 2


A
B
C
eye
optic stalk
D
hsFLPact>>GAL4:UAS-GFP
E
F
G
H
I
J
K
hsFLPact>>GAL4:UAS-GFP;;Gl 1

The Dynactin complex also controls the pattern of mitoses within the *Drosophila* retina (Fan and Ready, 1997). To determine whether nuclear mispositioning is a secondary consequence of the earlier mitotic requirement for Dynactin, we examined the effects of specifically inhibiting the Dynactin complex in postmitotic photoreceptors. Conditional inhibition of Dynactin function can be achieved through inducible expression of a truncated, dominant-negative form of Glued ($Glued^{DN}$) that resembles the protein product of $Glued^{1}$ (Allen et al., 1999; Fan and Ready, 1997). $Glued^{DN}$ was expressed under the control of the postmitotic photoreceptor-specific Glass 38-1 promoter, which initiates expression in the photoreceptors only after their axons have entered the brain (see Materials and methods). Expression of $Glued^{DN}$ under the control of Glass 38-1 caused photoreceptor nuclei to move into the optic stalk (Fig. 3A,B). Overexpression of Dynamitin under the control of Glass 38-1 caused similar photoreceptor nuclear positioning defects (Fig. 3C). These data demonstrate that Dynactin is required postmitotically in photoreceptors to maintain nuclear position and that the disruptions in nuclear positioning observed are not simply a secondary consequence of mitotic defects.

Figure 3. Dynactin is required postmitotically to maintain photoreceptor nuclear positioning. Third instar eye-brain complexes are stained with anti-Chaoptin (red) and anti-Elav (green). No photoreceptor nuclei (arrowheads) are seen in the optic stalk in wild type (A). Expression of dominant-negative Glued or overexpression of Dynamitin in differentiated photoreceptors under the control of Glass38-1 causes nuclei to leave the eye disc and enter the optic stalk (B, C, arrowheads).

Figure 3



Photoreceptor nuclear movement occurs without disruption of apical/basal polarity

The displacement of photoreceptor nuclei from apical regions of the eye disc toward more basal regions could reflect an overall disruption in apical/basal polarity of the eye disc. The apical/basal polarity of developing photoreceptors was assessed by examining the distribution of the Drosophila β-catenin Armadillo and the PDZ-domain-containing protein PATJ (Pielage et al., 2003). Armadillo localizes to the zonula adherens separating the apical and basolateral membrane domains of developing photoreceptors (Pellikka et al., 2002), while PATJ localizes to the apical membrane domain (Izaddoost et al., 2002). In wild-type eye discs, Armadillo is concentrated just beneath the apical tips of the developing photoreceptors (Fig. 4A). In *Glued*^{1} animals, Armadillo was still present near apical regions of the eye disc, even in areas completely devoid of apical photoreceptor nuclei (Fig. 4B). Thus, this marker of apical/basal polarity was retained even when photoreceptor nuclei moved basally. Similar results were obtained when *Glued*^{1} mutants were visualized in cross-section using both Armadillo and PATJ. Apical localization of PATJ and Armadillo were observed in *Glued*^{1} and the relative apical/basal ordering of these markers was maintained (Fig. 4C,D). These data suggest that the alterations in photoreceptor morphology are not caused by a loss of apical/basal polarity within the developing photoreceptors.

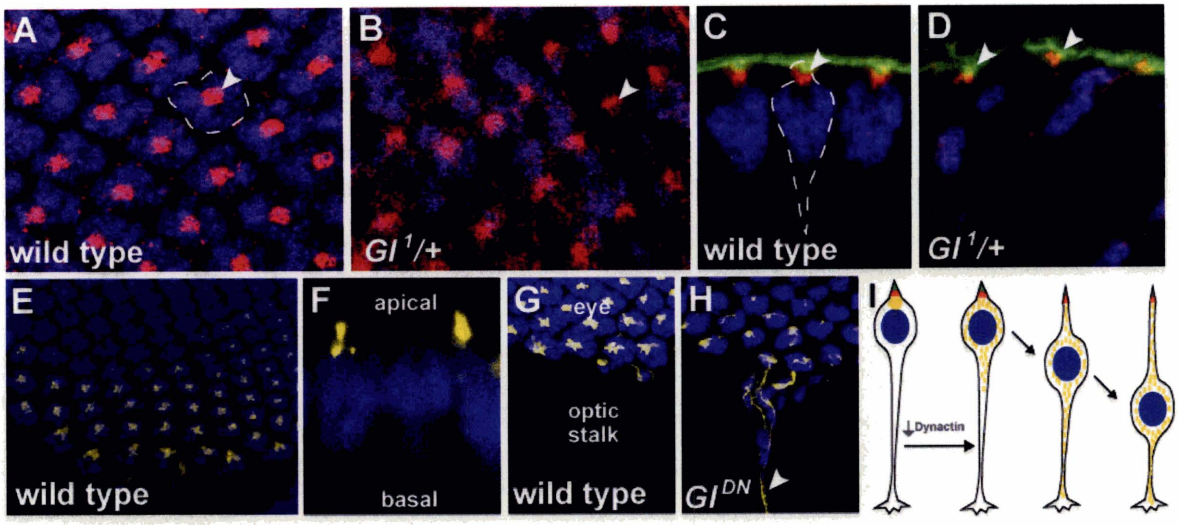
Glued maintains microtubule cytoskeleton organization

Dynactin has important functions in the organization of the microtubule cytoskeleton in many systems. The microtubule cytoskeleton of developing photoreceptors is highly polarized, with microtubule minus ends concentrated apical to

the nucleus as detected using antisera recognizing gamma-tubulin (Swan et al., 1999). A similar apical focus is observed when using the fusion protein Nod:LacZ (Fig. 4E,F), which often co-localizes with microtubule minus ends (Clark et al., 1997). The relatively ubiquitous expression of gamma-tubulin in the retina complicated the analysis of gamma-tubulin localization when retinal patterning was disrupted (J.L.W. and P.A.G., unpublished). Therefore, we examined the effect of Glued on factors associated with the microtubule cytoskeleton by expressing Nod:LacZ specifically in postmitotic photoreceptors. In animals expressing Glued^{DN} in postmitotic photoreceptors as well as in *Glued*^{1} mutants, Nod:LacZ was no longer exclusively concentrated in apical regions of photoreceptors, but rather spread into the photoreceptor axons (Fig. 4H, data not shown). Thus while the overall apical/basal polarity of the photoreceptors was not disrupted in *Glued* mutants, the spatial organization of the microtubule cytoskeleton-associated factor Nod:LacZ was affected.

Figure 4. Apical markers are not disrupted, but Nod:LacZ is mislocalized, in *Glued* mutants. Third instar eye discs are stained with anti-Elav (blue), anti-Armadillo (red), and anti-PATJ (green) (A-D). Views from the apical surface (A, B) show evenly spaced apical markers in wild type (A), with each ommatidial cluster (dashed outline) centered under a concentration of Armadillo (arrowhead). Armadillo staining is largely normal in *Glued*^{1} (B), despite the presence of ommatidia devoid of nuclei (arrowhead). Side views show that in wild type (C), each ommatidium (dashed line) has a distinct apical clustering of Armadillo and PATJ (arrowhead). *Glued* animals appear to retain apical markers (arrowheads) even when photoreceptor nuclei are mispositioned (D). Nod:LacZ (yellow) expressed in postmitotic photoreceptor neurons under the control of the $Glass_{38\text{-}1}$ promoter localizes apical to photoreceptor nuclei (anti-Elav, blue) in the most mature photoreceptor neurons (E, apical surface, and F, side view) and is not found in axons or in the optic stalk (G). When dominant-negative Glued is expressed in photoreceptor neurons using the $Glass_{38\text{-}1}$ promoter, Nod:LacZ staining is distributed throughout the axons of the most mature cells (H, arrowhead). In (I), consequences of Dynactin disruption are summarized, synthesizing the data obtained in Figures 2-4. Inhibition of Dynactin function in the postmitotic neuron causes the photoreceptor nucleus (blue) to be displaced toward the axon terminal. Despite nuclear movement, a trailing process remains and apical markers (PATJ in green, Armadillo in red) are retained. Nod:LacZ (yellow), however, becomes mislocalized from its wild-type apical position and enters the axon.

Figure 4

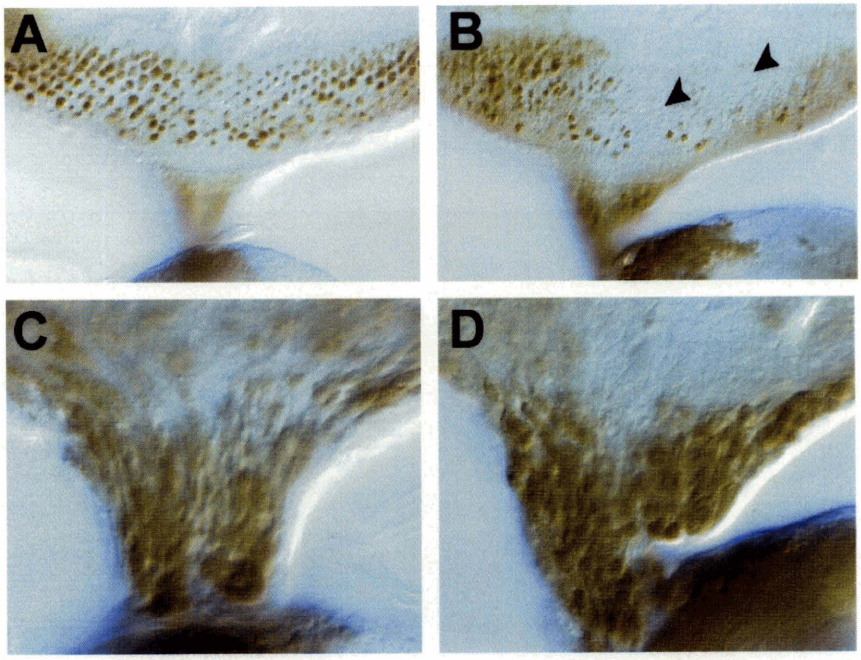

A
wild type
B
Gl 1/+
C
wild type
D
Gl 1/+
E
wild type
F
apical
basal
G
eye
optic
stalk
wild type
H
Gl DN
I
Dynactin

Glued cooperates with Dynein in photoreceptor nuclear positioning but is antagonized by Kinesin

Dynactin activates the microtubule motor Dynein, and strong loss-of-function mutations in *dynein intermediate chain* (*dic*) are dominant enhancers of the rough eye phenotype of *Glued*^{1} mutants (Boylan and Hays, 2002). As Dynein and Dynactin may play multiple roles together during eye development, we examined the effect of a reduction in *dic* gene dosage upon photoreceptor nuclear positioning in *Glued*^{1} animals. A twofold reduction in *dic* gene dosage caused a further decrease in the number of photoreceptor nuclei in apical regions of *Glued*^{1} mutant eye discs (Fig. 5A,B). This did not reflect a simple reduction in the number of photoreceptors generated, as large numbers of photoreceptor nuclei were crowded at the base of the eye disc and entered the optic stalk in both animals (Fig. 5C,D). Thus, a larger fraction of photoreceptor nuclei left apical positions when the level of *dic* gene activity was reduced, consistent with Dynein and Dynactin acting together in this process.

Figure 5. *Glued* nuclear mispositioning is enhanced by *dynein intermediate chain* reduction. Third instar eye discs were stained with anti-Elav. (A, B) Apical surface of the eye disc. *Glued*^{1} mutants show small areas devoid of apical photoreceptor nuclei (A), while *dic*^{2}/+ ; *Glued*^{1}/+ animals (B) have much larger areas devoid of nuclei (arrowheads). (C, D) Basal surface of eye disc and optic stalk. The greater absence of photoreceptor neuron nuclei in apical regions of the eye disc in *dic*^{2}/+ ; *Glued*^{1}/+ animals is not simply due to an absence of photoreceptor neurons, as large numbers of photoreceptor nuclei are clustered at the base of the optic stalk in both *Glued*^{1} (C) and *dic*^{2}/+ ; *Glued*^{1}/+ animals (D).

Figure 5



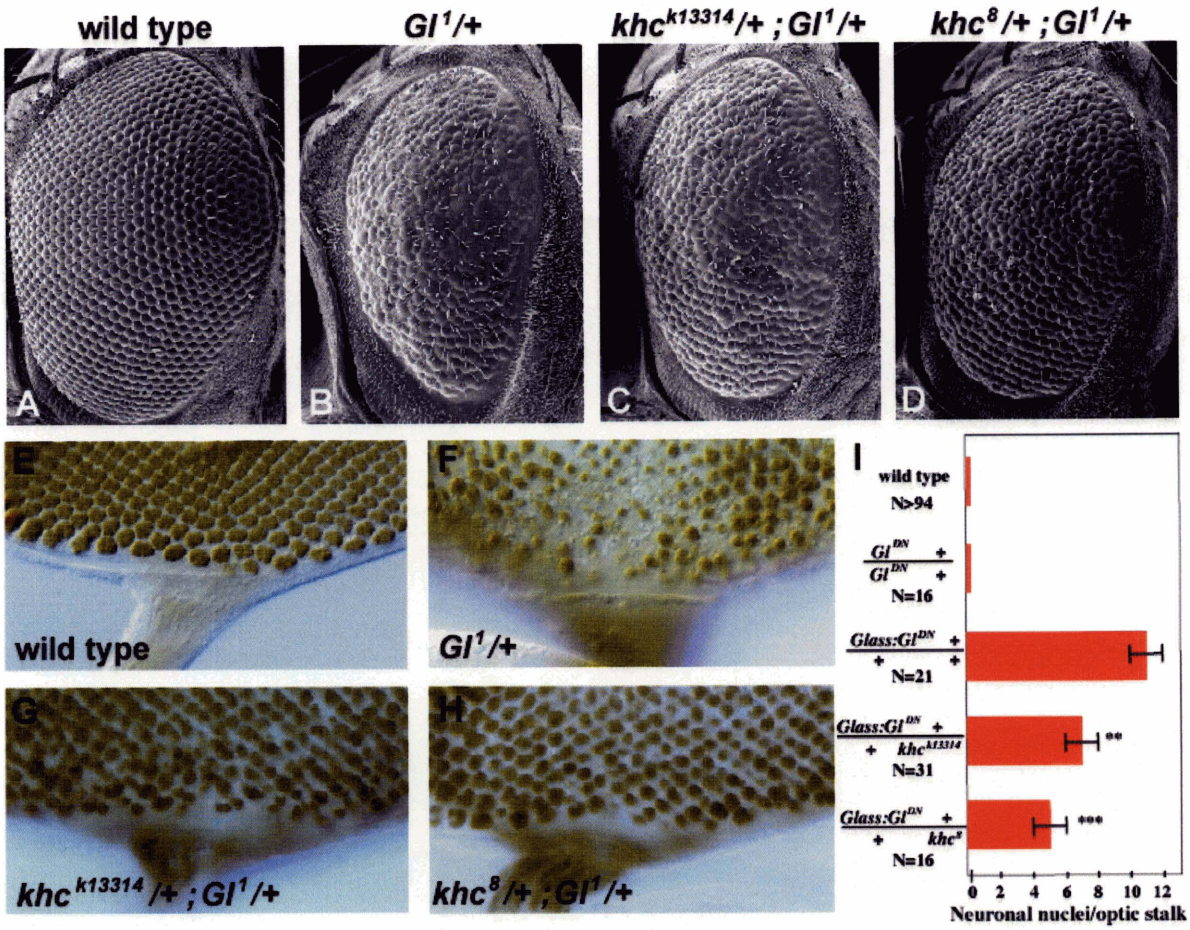
To identify additional factors that interact with Dynactin to control nuclear positioning, a genetic screen was performed to identify genes that dominantly enhanced or suppressed the *Glued*^{1} external eye phenotype. From a collection of approximately 1800 stocks containing transposon-induced lethal mutations, several stocks were identified that had no dominant effect on eye development in a wild-type background, but were dominant enhancers or suppressors of *Glued*^{1}. Two dominant suppressors of *Glued*^{1}, *khc*^{k13219} and *khc*^{k13314}, were alleles of *kinesin heavy chain* (*khc*), which encodes a subunit of the plus-end directed microtubule motor kinesin (Fig. 6A-C). The interaction with *Glued*^{1} was further confirmed using the null allele *khc*^{8} (Fig. 6C). Examination of developing eye discs demonstrated that a twofold reduction of *khc* gene dosage greatly increased the number of photoreceptor nuclei present in apical regions of *Glued*^{1} mutant eye discs (Fig. 6E-H). This suggested that *khc* acts antagonistically to *Glued* in photoreceptor nuclear positioning.

To determine whether *khc* mutations interacted with *Glued*^{1} in postmitotic photoreceptors, *khc* gene dosage was reduced in animals expressing dominant-negative Glued under the control of the postmitotic Glass38-1 promoter (Fig. 6I). Wild-type animals (n>50 hemispheres) or animals containing the dominant-negative Glued transgene without the Glass 38-1 promoter (n>20) never contained photoreceptor nuclei within their optic stalks. *Glass38-1:Glued*^{DN} animals contained an average of 11±1 photoreceptor nuclei within the optic stalk (± s.e.m., n=21). However, *Glass38-1:Glued*^{DN} animals heterozygous for either *khc*^{k13314} or *khc*^{8} showed a significant reduction in the number of photoreceptor nuclei in the optic stalk (7±1, n=31, unpaired t-test $P<0.01$ for *khc*^{k13314}/+ and 5±1, n=16, $P<0.001$ for *khc*^{8}/+). Thus, a twofold reduction in

khc gene dosage suppressed the effects of postmitotic expression of dominant-negative Glued, consistent with *Glued* and *khc* acting antagonistically within differentiated photoreceptors to regulate nuclear positioning.

Figure 6. *Glued* nuclear mispositioning is suppressed by *kinesin heavy chain* reduction. Scanning electron micrographs of adult eyes (A-D). (A) Wild type. (B) Gl^{1}/+ animals have smaller eyes with disorganized ommatidia. (C, D) Reduction of *kinesin heavy chain* gene dosage partially suppresses Gl^{1}/+ eye defect. Apical regions of third instar eye discs in which photoreceptor nuclei are stained with anti-Elav (E-H). (I) Suppression of the Glass-Glued^{DN} phenotype quantified by counting the number of Elav-positive nuclei in the optic stalks of animals with 15 to 22 rows of photoreceptor development. The average for each genotype was 19 rows of development. Error bars are s.e.m. and asterisks denote P-value of unpaired t-test (**P<0.01, ***P<0.001).

Figure 6


wild type
Gl1/+
khck13314/+ ;Gl1/+
khc8/+ ;Gl1/+
A
B
C
D
E
wild type
F
Gl1/+
G
khck13314/+ ;Gl1/+
H
khc8/+ ;Gl1/+
I
wild type
N>94
GlDN +
GlDN +
N=16
Glass:GlDN +
+ +
N=21
Glass:GlDN +
+ khck13314
N=31
Glass:GlDN +
+ khc8
N=16
**
***
0 2 4 6 8 10 12
Neuronal nuclei/optic stalk

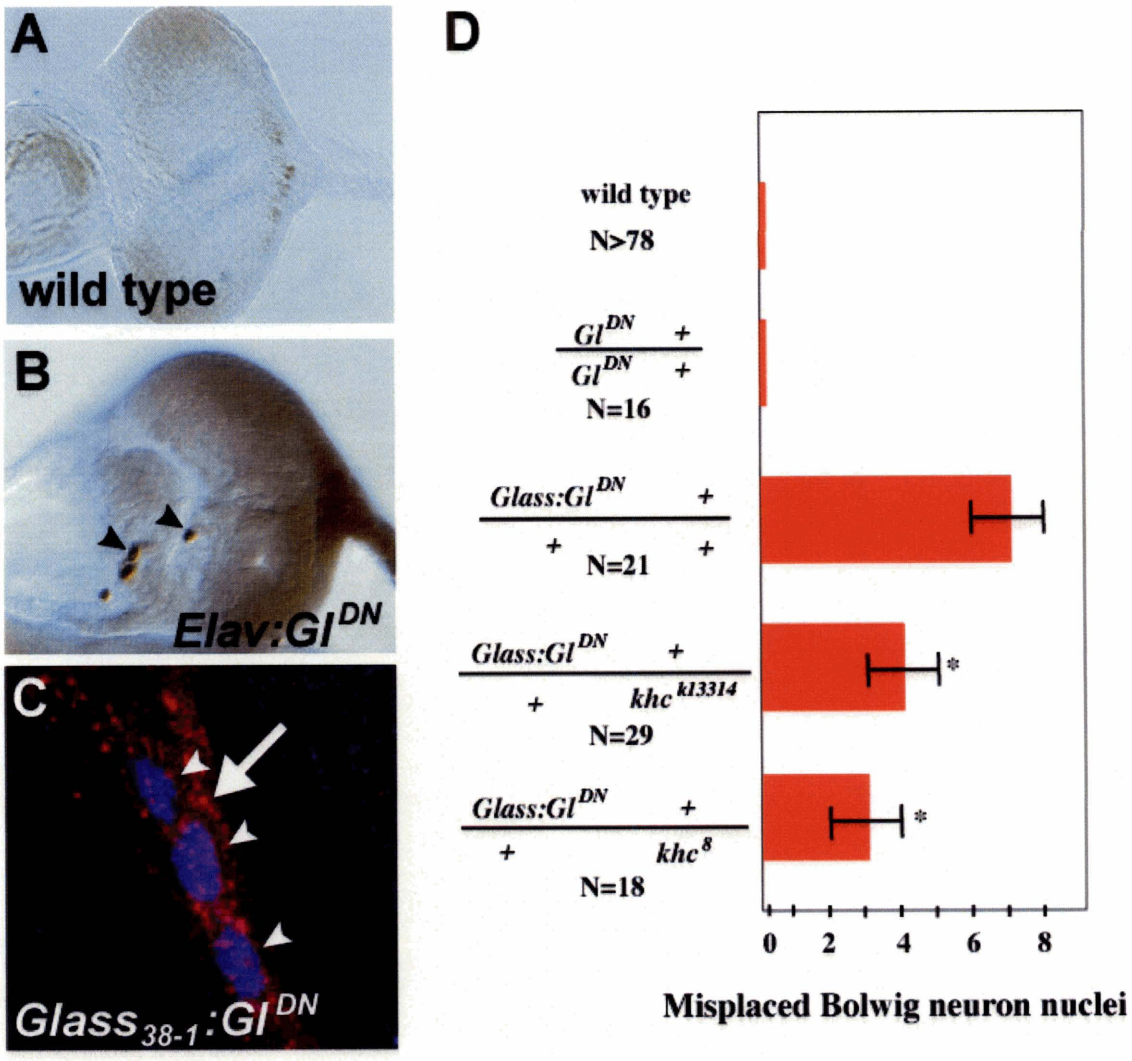
Glued and Kinesin Heavy Chain also act antagonistically in positioning Bolwig photoreceptor nuclei

We examined the interaction between *Glued* and *khc* in other photoreceptors by examining the Bolwig organ, a cluster of 12 photosensitive neurons that differentiate during embryonic development and extend axons into the brain (Schmucker et al., 1997). By second and third instar larval stages, Bolwig photoreceptor nuclei are located near the anterior tip of the larva and their axons extend over the eye/antennal disc into the brain, a distance of >200 um. In wild-type second instar animals, photoreceptor neuron differentiation has not yet begun in the eye disc and no neuronal nuclei are present there (Fig. 7A). However, when Glued^{DN} was expressed in postmitotic Bolwig photoreceptors, their nuclei appeared on the surface of the eye/antennal disc (Fig. 7B,C). Thus, as in the photoreceptors of the adult eye, expression of Glued^{DN} in Bolwig photoreceptors caused their nuclei to be positioned closer to their axon termini; in many cases, the Bolwig nuclei were over 150 um closer than normal to their axon terminals in the brain.

The interaction between *Glued* and *khc* in Bolwig photoreceptors was assessed by counting the number of Bolwig nuclei on the surface of the eye/antennal disc (Fig. 7D). While wild-type and *UAS:Glued^{DN}* animals had no neuronal nuclei in this region, *Glass38-1:Glued^{DN}* animals contained 7±1 (n=21). A reduction of *khc* gene dosage in *Glass38-1:Glued^{DN}; khc^{k13314}/+* and *Glass38-1:Glued^{DN}; khc^{8}/+* animals significantly reduced this to 4±1 (n=29, $P<0.05$) and 3±1 (n=16, $P<0.05$), respectively. These data further support the functional antagonism of *Glued* and *khc* in photoreceptor nuclear positioning.

Figure 7. *Glued* function is required to position Bolwig organ nuclei, where it is antagonized by *kinesin heavy chain*. Wild-type second instar eye-antennal discs stained with anti-Elav show no neuronal nuclei in the eye-antennal disc along the path of the Bolwig nerve (A), while animals expressing dominant-negative Glued in postmitotic Bolwig photoreceptor neurons, using either the Elav promoter (B) or $Glass_{38\text{-}1}$ (C), have neuronal nuclei (arrowheads in B, C) along the path of the Bolwig nerve (in C, anti-Chaoptin in red, anti-Elav in blue, arrow indicates Bolwig nerve). Misplaced Bolwig nuclei in animals that express dominant-negative Glued under $Glass_{38\text{-}1}$ control were quantified in D. The number of misplaced Bolwig neuron nuclei was reduced when animals were heterozygous for loss-of-function mutations in *khc*. $*P<0.05$.

Figure 7


A
wild type
B
Elav:Gl DN
C
Glass 38-1 :Gl DN
D
wild type
N>78
Gl DN +
Gl DN +
N=16
Glass:Gl DN +
+ +
N=21
Glass:Gl DN +
+ khc k13314
N=29
Glass:Gl DN +
+ khc 8
N=18
*
*
0 2 4 6 8
Misplaced Bolwig neuron nuclei

Discussion

Although the proper positioning of neuronal cell bodies and the nuclei they contain is a central feature of brain morphogenesis, relatively little is known about how the position of a nucleus is maintained within a postmitotic neuron. Here we have examined the function of Dynactin in maintenance of nuclear positioning in postmitotic photoreceptor neurons. We saw that nuclear positioning shows impressive plasticity, as disruption of Dynactin function after photoreceptor axons had extended into their target region in the brain caused the nucleus to move away from the neuron's apical tip and toward the growth cone, giving the photoreceptor a 'bipolar' morphology (compared with its normally 'unipolar' morphology). Interestingly, we found that reductions in Kinesin partially compensated for the effects of reduced Dynactin activity. Taken together, these data establish an essential role for Dynactin in the morphological organization of postmitotic photoreceptors and suggest that a balance of plus- and minus-end directed microtubule motor activity could influence the position of the nucleus.

Glued acts in postmitotic photoreceptors to control nuclear positioning

Establishing and maintaining appropriate nuclear position is a general challenge for eukaryotic cells and the mechanisms that control nuclear positioning vary with cell type and developmental stage (Morris, 2003; Starr and Han, 2003). In neurons, the position of the nucleus is initially established at the end of the precursor cell's mitosis and changes as the neuron migrates into position and acquires its differentiated morphology. Thus, nuclear positioning is a dynamic process integrated into the differentiation program of a neuron.

Several alternative models have been proposed for the role of the Dynactin subunit Glued in neuronal positioning in the fly eye (Fan and Ready, 1997). As Glued1 mutations affect both mitosis and nuclear positioning in the eye, it has been difficult to assess whether Glued activity is required specifically in postmitotic neurons. In fact, broad expression of the cell-cycle inhibitor p21 behind the morphogenetic furrow (the region in which photoreceptors differentiate) partially suppressed the *Glued*^{1} nuclear positioning defect, suggesting that disruptions in Dynactin might lead to a nuclear positioning defect by simply disrupting the coordination of cell-cycle progression and nuclear movement (Fan and Ready, 1997). Our results demonstrate that Dynactin activity is required within the postmitotic photoreceptor to regulate nuclear positioning. We also see nuclear mispositioning when Glued function is inhibited in postmitotic photoreceptors of the Bolwig organ, indicating that this function is not specific for photoreceptors generated in the eye disc.

Our analysis has focused on the positioning of the nucleus within the photoreceptor neuron. It is interesting to consider whether other constituents of the cell body are similarly mispositioned when Dynactin function is disrupted. Our analysis of single Glued1 photoreceptors indicates that mispositioned nuclei are surrounded by a concentration of other cellular material, as evidenced by the accumulation of CD8:GFP (a transmembrane protein associated with cell surfaces as well as secretory vesicles) around the nuclei in Fig. 2H,I. Thus it is possible that not only nuclei, but also other elements of the cell body, are mispositioned in these animals.

Kinesin exerts an antagonistic influence on photoreceptor nuclear positioning

Our finding that Glued collaborates with Dynein in photoreceptor neuron nuclear positioning raises the question of whether other motor proteins contribute to this process. From a screen for genes that promote or antagonize Glued function in the retina, we identified loss-of-function alleles of *kinesin heavy chain* (*khc*) and demonstrated that a reduction in *khc* dosage reduced the amount of photoreceptor nuclear mispositioning observed in $Glued^{1}$ animals. These data suggest that nuclear mispositioning does not result simply from the poisoning of axonal transport, as a decrease in *khc* function exacerbates the axonal transport defects of $Glued^{1}$ animals (Martin et al., 1999). Furthermore, the observation of a *Glued/khc* interaction in postmitotic photoreceptors of the adult eye and the larval Bolwig organ indicates that the interplay between Glued and Kinesin occurs within the differentiating photoreceptor. Taken together, our data suggest that the two may normally play antagonistic roles in positioning the photoreceptor nucleus. The fact that strong photoreceptor nuclear mispositioning is not observed in animals containing homozygous mutant clones of *khc* tissue in the retina (Brendza et al., 2000) (J.L.W. and P.A.G., unpublished) is perhaps not surprising, as the nucleus normally resides near the apical surface of the retina and adjacent to the focus of microtubule minus ends, leaving little room for further apical movement. While the microtubule motor proteins Dynein and Kinesin are important for nuclear positioning in many cell types (Cottingham and Hoyt, 1997; DeZwaan et al., 1997; Duncan and Warrior, 2002; Januschke et al., 2002; Morris, 2003; Reinsch and Gonczy, 1998; Requena et al., 2001), a role for microtubule motors in nuclear positioning in postmitotic neurons has not been previously established.

Roles of Dynein, Dynactin, and Kinesin in photoreceptor nuclear positioning

Dynein and Dynactin control a number of cellular processes through their effects on the structure of the microtubule cytoskeleton and through the transportation of cargo along microtubules. In particular, Dynein and Dynactin regulate nuclear positioning in many dividing and migrating eukaryotic cells (Morris, 2003; Reinsch and Gonczy, 1998). How do Dynein and Dynactin control photoreceptor nuclear position and how might kinesin exert an antagonistic influence? One possibility is that the photoreceptor nucleus may be a cargo moved directly by the Dynein/Dynactin complex. The proximity of the photoreceptor nucleus to the focus of microtubule minus ends in wild-type animals would be consistent with Dynein and Dynactin working to move the nucleus toward the focus of microtubule minus ends, while the antagonistic interaction between Dynactin and Kinesin could reflect the direct coupling of the nucleus to both minus-end and plus-end directed motors. Thus, the position of the photoreceptor nucleus would reflect the relative balance of opposing motor activities, with Dynein predominating under normal circumstances in the photoreceptors. Such coupling to opposite-polarity microtubule motors has been implicated in the movement of other organelles, such as mitochondria and lipid droplets (Gross, 2003). This scenario would be consistent with the movement of the photoreceptor nucleus away from the focus of microtubule minus ends in animals mutant for *klar*, a gene implicated in the coordination of plus- and minus-end directed motors attached to lipid droplets in the Drosophila embryo (Mosley-Bishop et al., 1999; Welte et al., 1998). Although the mechanism by which Klar may regulate microtubule motors is unknown, *klar* genetically interacts with the nuclear lamin LamDM(0) (Patterson et al.,

2004), raising the possibility that Klar could be involved in the coordination of Dynein and Kinesin motors associated with the photoreceptor nuclear envelope.

Alternatively, Dynein and Dynactin could also play more indirect roles in photoreceptor nuclear positioning. For example, in non-motile interphase mammalian tissue culture cells, Dynactin co-localizes with the focus of microtubule minus ends and Dynactin disruption defocuses these minus ends (Quintyne et al., 1999). Since the photoreceptor nucleus normally lies adjacent to the focus of microtubule minus ends, it is possible that nuclear movement could then be a secondary consequence of microtubule minus-end redistribution. Such redistribution could potentially be dependent upon Kinesin activity. In *C. elegans* embryos, *zyg-12* is required for close association of the nucleus with the focus of microtubule minus ends and the ZYG-12 protein may act as a physical link between Dynein and the nuclear envelope (Malone et al., 2003). However, no functional equivalent of ZYG-12 has been identified in Drosophila. While ZYG-12 has homology to the Hook family of proteins, analysis of Drosophila hook indicates that it is involved in regulating secretory and endocytic pathways rather than photoreceptor nuclear localization (Walenta et al., 2001). In a similar model, Dynein and Dynactin could also control the apical/basal positioning of the focus of microtubule minus ends. In *Saccharomyces cerevisiae*, Dynein associated with the cell cortex is postulated to control the movement of microtubules along the interior surface of the cell (Lee et al., 2003). In photoreceptors, association of Dynein with the apical cortex of the cell might act similarly to move microtubule minus ends toward the apical tip of the photoreceptor.

To begin to test the effect of Dynactin inhibition on factors associated with the microtubule cytoskeleton, we have examined the distribution of the fusion protein,

Nod:LacZ, which colocalizes with microtubule minus ends in wild-type animals. We see a strong delocalization of Nod:LacZ in *Glued* mutants (Fig. 4) and in *cpb* mutants (J.L.W. and P. A. G., unpublished). The movement of Nod:LacZ into the axon would be consistent with a defocusing of microtubule minus ends and even alterations in the overall polarity of the microtubule cytoskeleton. Such microtubule disorganization would cause Nod:LacZ to no longer travel to a single destination in $Glued^{1}$ mutants. However, an alternative explanation is that Dynactin is required for the movement of Nod:LacZ to microtubule minus ends. In this scenario, Nod:LacZ would not necessarily be localized at minus ends and thus no longer serve as an effective microtubule minus-end marker in $Glued^{1}$ mutants. It is interesting to note that despite the strong effects of reducing the gene dosage of *dic* and *khc* on nuclear mispositioning in $Glued^{DN}$ mutants, we did not see detectable effects of reducing *dic* or *khc* gene dosage on Nod:LacZ distribution in Glued DN animals (J.L.W. and P,A.G., unpublished). Thus the redistribution of Nod:LacZ may be unrelated to the mispositioning of the photoreceptor nucleus, although only a dramatic alteration in the distribution of Nod:LacZ would be detected in our assay. A more detailed analysis of microtubule organization in photoreceptors with disruption in Dynactin functions awaits the development of additional tools.

How the distinct regions of a neuron (the axons, dendrites, and nucleus-containing cell body) are properly positioned is a central question in neuronal cell biology, about which little is known. Here we have shown that Dynein and Dynactin play a major role in maintaining the position of the nucleus within a postmitotic photoreceptor neuron, and that Kinesin can antagonize this function. It will be of interest to determine whether

Dynactin may be directly involved in coupling the apical/basal polarity of the photoreceptor neuron to the polarity of the microtubule cytoskeleton, for example, through association with factors involved in apical/basal polarization of the photoreceptor. Another key issue for the future is to determine whether these effects of Dynein, Dynactin, and Kinesin on photoreceptor nuclear migration reflect their association with the photoreceptor nucleus and/or the effects of these complexes on the microtubule cytoskeleton more generally.

Acknowledgements

We thank Hugo Bellen, Graeme Davis, Tom Hays, Roberta Hopmann, Katherine Miller, William Saxton, Stefan Thor, Michael Welte, Kevin Cook and the Bloomington Stock Center, and the Developmental Studies Hybridoma Band for reagents, Nicki Watson for SEM, Tim Tayler and Frank Miskevich for microscopy assistance, and Garrity lab members and Frank Gertler for discussions. We thank Linda Huang, Mark Rosenzweig, Frank Solomon, and an anonymous reviewer 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. J.L.W. was supported by an NIH Predoctoral Training Grant and A.C. by an undergraduate research grant to MIT from Howard Hughes Medical Institute.

REFERENCES CITED

Allen, M. J., Shan, X., Caruccio, P., Froggett, S. J., Moffat, K. G. and Murphey, R. K. (1999). Targeted expression of truncated glued disrupts giant fiber synapse formation in Drosophila. *J. of Neuroscience*. **19**, 9374-9384.

Benlali, A., Draskovic, I., Hazelett, D. J., and Treisman, J. E. (2000). Act up controls actin polymerization to alter cell shape and restrict Hedgehog signaling in the *Drosophila* eye disc. *Cell* **101**, 271-281.

Boylan, K. L., and Hays, T. S. (2002). The gene for the intermediate chain subunit of cytoplasmic dynein is essential in Drosophila. *Genetics* **162**, 1211-1220.

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.

Brendza, R. P., Sheehan, K. G., Turner, F. R., and Saxton, W. M. (2000). Clonal tests of conventional kinesin function during cell proliferation and differentiation . *Mol. Biol. Cell* **11**, 1329-1343.

Cajal, S. R. (1990). *New Ideas on the Structure of the Nervous System in Man and Vertebrates*. Cambridge, MA: MIT Press.

Clark, I. E., Jan, L. Y., and Jan, Y. N.. (1997). Reciprocal localization of Nod and kinesin fusion proteins indicates microtubule polarity in the Drosophila oocyte, epithelium, neuron, and muscle *Development* **124**, 461-470.

Cooper, J. A., Hart, M. C., Karpova, T. S., and Schafer, D. A. (1999). Capping protein. In *Guidebook to the Cytoskeletal and Motor Proteins* (ed. T. Kreis and R. Vale), pp. 62-64. New York: Oxford University Press.

Cottingham, F. R. and Hoyt, M. A. (1997). Mitotic spindle positioning in Saccharomyces cerevisiae is accomplished by antagonistically acting microtubule motor proteins. *J. Cell Biol.* **138**, 1041-1053.

DeZwaan, T. M., Ellingson, E., Pellman, D., and Rof, D. M. (1997). Kinesin-related KIP3 of Saccharomyces cerevisiae is required for a distinct step in nuclear migration. *J. Cell Biol.* **138**, 1023-1040.

Duncan, J. E. and Warrior, R. (2002). The cytoplasmic dynein and kinesin motors have interdependent roles in patterning the *Drosophila* oocyte. *Curr. Biol.* **12**, 1982-1991.

Eaton, B. A., Fetter, R. D., and Davis, G. W. (2002). Dynactin is necessary for synapse stabilization. *Neuron* **34**, 729-741.

Echeverri, C. J., Paschal, B. M., Vaughan, K. T., and Vallee, R. B. (1996). Molecular characterization of the 50-kD subunit of dynactin reveals function for the complex in chromosome alignment and spindle organization during mitosis. *J. Cell Biol.* **132**, 617-633.

Eckley, D. M., Gill, S. R., Melkonian, K. A., Bingham, J. B., Goodson, H. V., Heuser, J. E., and Schroer, T. A. (1999). Analysis of dynactin subcomplexes reveals a novel actin-related protein associated with the arp1 minifilament pointed end. *J. Cell Biol.* **147**, 307-320.

Ellis, M. C., O'Neill, E. M., and Rubin, G. M. (1993). Expression of Drosophila glass protein and evidence for a negative regulation of its activity in non-neuronal cells by another DNA-binding protein. *Development* **119**, 855-865.

Fan, S. S. and Ready, D. F. (1997). Glued participates in distinct microtubule-based activities in Drosophila eye development. *Development* **124**, 1497-1507.

Fujita, S. C., Zipursky, S. L., Benzer, S., Ferrus, A., and Shotwell, S. L. (1982). Monoclonal antibodies against the *Drosophila* nervous system. *Proc. Natl. Acad. Sci. USA* **79**, 7929-7933.

Garrity, P. A., Lee, C. H., Salecker, I., Robertson, H. C., Desai, C. J., Zinn, K., and Zipursky, S. L. (1999). Retinal axon target selection in *Drosophila* is regulated by a receptor protein tyrosine phosphatase. *Neuron* **22**, 707-717.

Gill, S. R., Schroer, T. A., Szilak, I., Steuer, E. R., Sheetz, M. P., and Cleveland, D. W. (1991). Dynactin, a conserved, ubiquitously expressed component of an activator of vesicle motility mediated by cytoplasmic dynein. *J. Cell Biol.* **115**, 1639-1650.

Gross, S. P. (2003). Dynactin: coordinating motors with opposite inclinations. *Curr. Biol.* **13**, R320-R322.

Gupta, A., Tsai, L. H., and Wynshaw-Boris, A. (2002). Life is a journey: a genetic look at neocortical development. *Nat. Rev. Genet.* **3**, 342-355.

Harte, P. J. and Kankel, D. R. (1982). Genetic analysis of mutations at the Glued locus and interacting loci in *Drosophila melanogaster*. *Genetics* **101**, 477-501.

Holleran, E. A., Ligon, L. A., Tokito, M., Stankewich, M. C., Morrow, J. S., and Holzbaur, E. L. (2001). Beta III spectrin binds to the Arp1 subunit of dynactin. *J. Biol. Chem.* **276**, 36598-36605.

Holleran, E. A., Tokito, M. K., Karki, S., and Holzbaur, E. L. (1996). Centractin (ARP1) associates with spectrin revealing a potential mechanism to link dynactin to intracellular organelles. *J. Cell Biol.* **135**, 1815-1829.

Hopmann, R. and Miller, K. G. (2003). A balance of capping protein and profiling functions is required to regulate actin polymerization in *Drosophila* bristle. *Mol. Biol. Cell* **14**, 118-128.

Hopmann, R., Cooper, J. A., and Miller, K. G. (1996). Actin organization, bristle morphology, and viability are affected by actin capping protein mutations in *Drosophila*. *J. Cell Biol.* **133**, 1293-1305.

Izaddoost, S., Nam, S. C., Bhat, M. A., Bellen, H. J., and Choi, K. W. (2002). *Drosophila* Crumbs is a positional cue in photoreceptor adherens junctions and rhabdomeres. *Nature* **416**, 178-183.

Januschke, J., Gervais, L., Dass, S., Kaltschmidt, J. A., Lopez-Schier, H., St. Johnston, D., Brand, A. H., Roth, S., and Guichet, A. (2002). Polar transport in the *Drosophila* oocyte requires Dynein and Kinesin I cooperation. *Curr. Biol.* **12**, 1971-1981.

Karki, S. and Holzbaur, E. L. (1995). Affinity chromatography demonstrates a direct binding between cytoplasmic dynein and the dynactin complex. *J. Biol. Chem.* **270**, 28806-28811.

King, S. J. and Schroer, T. A. (2000). Dynactin increases the processivity of the cytoplasmic dynein motor. *Nat. Cell Biol.* **2**, 20-24.

Lee, W. L., Oberle, J. R., and Cooper, J. A. (2003). The role of the lissencephaly protein Pac1 during nuclear migration in budding yeast. *J. Cell Biol.* **160**, 355-364.

Malone, C. J., Misner, L., Le Bot, N., Tsai, M. C., Campbell, J. M., Ahringer, J., and White, J. G. (2003). The *C. elegans* hook protein, ZYG-12, mediates the essential attachment between the centrosome and nucleus. *Cell* **115**, 825-836.

Martin, M., Iyadurai, S. J., Gassman, A., Gindhart, J. G., Jr., Hays, T. S., and Saxton, W. M. (1999). Cytoplasmic dynein, the dynactin complex, and kinesin are interdependent and essential for fast axonal transport. *Mol. Biol. Cell* **10**, 3717-3728.

Morris, N. R. (2003). Nuclear positioning: the means is at the ends. *Curr. Opin. Cell Biol.* **15**, 54-59.

Mosley-Bishop, K. L., Li, Q., Patterson, L., and Fischer, J. A. (1999). Molecular analysis of the klarsicht gene and its role in nuclear migration within differentiating cells of the *Drosophila* eye. *Curr. Biol.* **9**, 1211-1220.

Muresan, V., Stankewich, M. C., Steffen, W., Morrow, J. S., Holzbaur, E. L., and Schnapp, B. J. (2001). Dynactin-dependent, dynein-driven vesicle transport in the

absence of membrane proteins: a role for spectrin and acidic phospholipids. *Mol. Cell* **78**, 173-183.

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

O'Neill, E. M., Rebay, I., Tjian, R., and Rubin, G. M. (1994). The activities of two Ets-related transcription factors required for *Drosophila* eye development are modulated by the Ras/MAPK pathway. *Cell* **78**, 137-147.

Olson, E. C. and Walsh, C. A. (2002). Smooth, rough and upside-down neocortical development. *Curr. Opin. Genet. Dev.* **12**, 320-327.

Patterson, K., Molofsky, A. B., Robinson, C., Acosta, S., Cater, C., and Fischer, J. A. (2004). The functions of Klarsicht and nuclear lamin in developmentally regulated nuclear migrations of photoreceptor cells in the *Drosophila* eye. *Mol. Biol. Cell* **15**, 600-610.

Pellikka, M., Tanentzapf, G., Pinto, M., Smith, C., McGlade, C. J., Ready, D. F., and Tepass, J. (2002). Crumbs, the Drosophila homologue of human CRB1/RP12, is essential for photoreceptor morphogenesis. *Nature* **416**, 143-149.

Pielage, J., Stork, T., Bunse, I., and Klambt, C. (2003). The *Drosophila* cell survival gene discs losts encodes a cytoplasmic Codanin-1-like protein, not a homolog of tight junction PDZ protein Patj. *Dev. Cell* **5**, 841-851.

Quintyne, N. J., Gill, S. R., Eckley, D. M., Crego, C. L., Compton, D. A., and Schroer, T. A. (1999). Dynactin is required for microtubule anchoring at centrosomes. *J. Cell Biol.* **147**, 321-334.

Reiner, O., Carrozzo, R., Shen, Y., Wehnert, M., Faustinella, F., Dobyns, W. B., Caskey, C. T., and Ledbetter, D. H. (1993). Isolation of a Miller-Dieker lissencephaly gene containing G protein beta-subunit-like repeats. *Nature* **364**, 717-721.

Reinsch, S. and Gonczy, P. (1998). Mechanisms of nuclear positioning. *J. Cell Sci.* **111**, 2283-2295.

Requena, N., Alberti-Segui, C., Wizengurg, E., Horn, C., Schliwa, M., Philippsen, P., Liese, R., and Fischer, R. (2001). Genetic evidence for a microtubule-destabilizing effect of conventional kinesin and analysis of its consequences for the control of nuclear distribution in *Aspergillus nidulans*. *Mol. Microbiol.* **42**, 121-132.

Schafer, D. A., Gill, S. R., Cooper, J. A., Heuser, J. E., and Schroer, T. A. (1994). Ultrastructural analysis of the dynactin complex: an actin-related protein is a component of a filament that resembles F-actin. *J. Cell Biol.* **126**, 403-412.

Schafer, D. A., Jennings, P. B., and Cooper, J. A. (1996). Dynamics of capping protein and actin assembly in vitro: uncapping barbed ends by polyphosphoinositides. *J. Cell Biol.* **135**, 169-179.

Schmucker, D., Jackle, H., and Gaul, U. (1997). Genetic analysis of the larval optic nerve projection in *Drosophila. Development* **124**, 937-948.

Spradling, A. C. and Rubin, G. M. (1982). Transposition of cloned P elements into *Drosophila* germline chromosomes. *Science* **218**, 341-347.

Starr, D. A. and Han, M. (2003). ANChors away: an actin based mechanism of nuclear positioning. *J. Cell Sci.* **16**, 211-215.

Strausfeld, N. and Meinertzhagen, I. A. (1998). The insect neuron: types, morphologies, fine structure, and relationship to the architectonics of the insect nervous system. In *Microscopic Anatomy of Invertebrates*. Vol. 11(B), (ed. F. W. Harrison and M. Locke), pp. 487-538.

Swan, A., Nguyen, T., and Suter, B. (1999). *Drosophila* Lissencephaly-1 functions with Bic-D and dynein in oocyte determination and nuclear positioning. *Nat. Cell Biol.* **1**, 444-449.

Tomlinson, A. (1985). The cellular dynamics of pattern formation in the eye of *Drosophila. J. Embryol. Exp. Morphol.* **89**, 313-331.

Vaughan, K. T. and Vallee, R. B. (1995). Cytoplasmic dynein binds dynactin through a direct interaction between the intermediate chains and p150Glued. *J. Cell Biol.* **131**, 1507-1516.

Walenta, J. H., Didier, A. J., Liu, X., and Kramer, H. (2001). The Golgi-associated hook3 protein is a member of a novel family of microtubule-binding proteins. *J. Cell Biol.* **152**, 923-934.

Welte, M. A., Gross, S. P., Postner, M., Block, S. M., and Wieschaus, E. F. (1998). Developmental regulation of vesicle transport in *Drosophila* embryos: forces and kinetics. *Cell* **92**, 547-557.

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.

Wolff, T. and Ready, D. F. (1993). Pattern formation in the Drosophila retina. In *The development of Drosophila melanogaster* (ed. M. Bate and A. Martinez-Arias), pp. 1277-1325. Cold Spring Harbor, NY: Cold Spring Harbor Press.

Chapter 3.

PTPMEG is a novel cytoplasmic tyrosine phosphatase required for development and maintenance of neuronal connectivity in the *Drosophila* brain

Jessica L. Whited, Joyce C. Yang, and Paul A. Garrity

Manuscript in preparation.

***ptpmeg*^{1} allele was created by Joyce C. Yang and Myles B. Robichaux. Anti-Ptpmeg antibody was generated by Caleb Kennedy. Mushroom body defects in *ptpmeg* mutant adults were discovered by Paul A. Garrity.**

SUMMARY

We have identified two novel functions for the cytoplasmic tyrosine phosphatase PTPMEG in neuronal connectivity in the *Drosophila* brain. PTPMEG is an evolutionarily conserved protein with predicted FERM, PDZ, and phosphatase domains. We generated a loss-of-function allele of *Drosophila ptpmeg*. *ptpmeg* is required for patterning mushroom body (MB) axons. Ptpmeg is not required for initial MB axon outgrowth and branching. Instead, ptpmeg is needed to prevent axon retraction following initial connectivity. We also show that *ptpmeg* is required for the development of normal ellipsoid body (EB) axonal projections. PTPMEG's phosphatase activity is required for its functions in patterning both the MB and EB axons. For both the MB and EB, *ptpmeg* function appears to be required non-cell-autonomously, in other neurons, for proper axon patterning. Expression of PTPMEG protein indicates that it is enriched in neuronal processes. These data show that *ptpmeg* is required for proper development and maintenance of the *Drosophila* nervous system.

INTRODUCTION

Neuronal wiring patterns are crucial determinants of brain function. During development, axons navigate to reach their appropriate targets in response to guidance information in their environment. Once established, axonal projection patterns must be appropriately refined and maintained as the nervous system matures and ages. The maintenance of axonal projections is an active process whose regulation contributes to normal development as the selective retention of axonal input helps sculpt patterns of

neuronal connectivity (Katz and Shatz, 1996; Lichtman and Colman, 2000). In addition, disruptions in the maintenance of axonal projections potentially contribute to several common neurological diseases, including Alzheimer's, Parkinson's, and Huntington's diseases, in which axonal atrophy is observed (Luo and O'Leary, 2005). Understanding the molecular mechanisms that control the maintenance of neuronal connectivity patterns is therefore critical for understanding how the brain's wiring pattern arises during development and is maintained in healthy adults.

The initial pathfinding of axons is modulated by extracellular guidance cues that bind guidance receptors on the axon surface and act to repel or attract the growth cone present at the axon's tip (Dickson, 2002). The maintenance of axonal projections is also influenced by cell-to-cell signaling. During the maturation of the nervous system, patterns of axon branch retention and pruning are strongly influenced by environmental signals provided by the surrounding tissue. These signals can be systemic, as in Drosophila where the hormone ecdysone modulates neuronal remodeling throughout the nervous system (Levine et al., 1995; Lee et al., 2000), as well as more local, as in the developmentally programmed pruning of forebrain axon branches under the control of the Semaphorin family of guidance cues (Bargi et al., 2003). Axon branching can also be modulated by axon-target interactions, as most extensively described in vertebrate systems where neuronal activity and target-derived signals have been shown to be critical for controlling axon branch retention and elimination (Katz and Shatz, 1996; Lichtman and Colman, 2000).

Like pruning, the long-term retention of axon branches can also be an active process and can involve the persistent inhibition of axon retraction pathways. Axon

branch maintenance in the mushroom bodies requires the inhibition of axon branch retraction by RhoGAP, a negative regulator of Rho (Billuart et al., 2001), and related pathways are proposed to act in mice, where focal adhesion kinase negatively regulates axon branch stabilization via Rho GTPases (Rico et al., 2004). While at present less is known about the molecular mechanisms involved in modulating axon branch maintenance than about the initial establishment of axonal projections, the mechanisms involved in axon branch maintenance are likely to be of great importance in understanding neuronal connectivity in the brain.

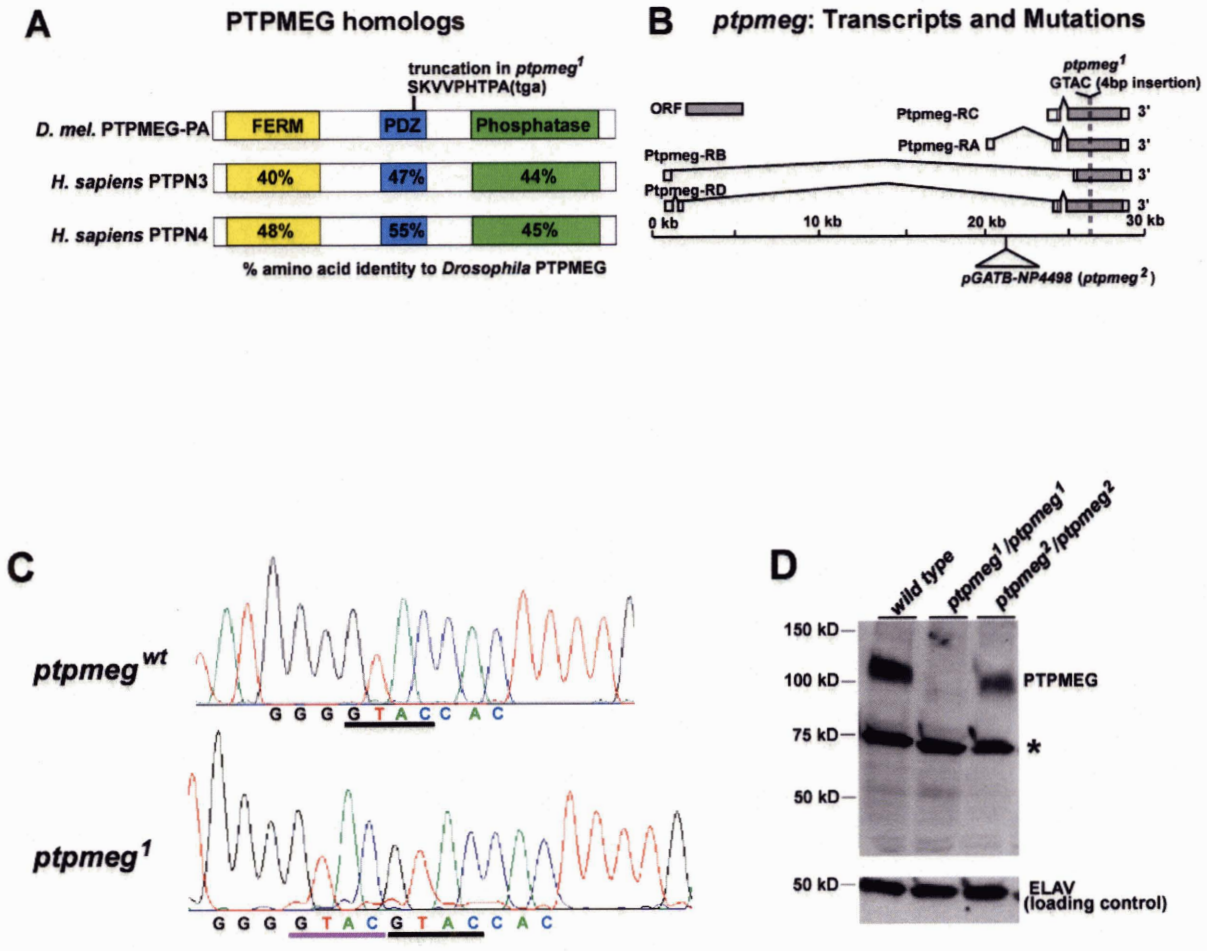
Tyrosine phosphatases have important roles in the establishment of neuronal connectivity. In Drosophila, the neuronally expressed receptor tyrosine phosphatases LAR, PTP10D, PTP52F, PTP69D, and PTP99A contribute to axon guidance decisions (Johnson and Van Vactor, 2003), and LAR has also been shown to regulate synaptogenesis at the neuromuscular junction (Kaufmann et al., 2002). In vertebrates, LAR also regulates both the formation and maintenance of excitatory synapses (Dunah et al., 2005). Drosophila PTPMEG encodes an evolutionarily conserved cytoplasmic protein tyrosine phosphatase that is characterized by the presence of an N-terminal FERM domain followed by a single PDZ domain (Figure 1A). FERM domains are multi-functional protein- and lipid-binding domains commonly found in membrane-associated signaling and cytoskeletal proteins (Bretscher et al., 2002). PDZ domains are protein-binding motifs often found in scaffolding proteins that contain additional protein-interaction motifs (Kim and Sheng, 2004). Orthologs of PTPMEG are found in animals from flies to humans (Bretscher et al., 2002), and mammals have two homologs, PTPN3 and PTPN4 (Figure 1A). Previous studies have shown that these PTPMEG relatives are

both neuronally expressed (Hironaka et al., 2000; Sahin et al., 1995; Takeuchi et al., 1994) and PTPN4 is present in post-synaptic density fractions in the mouse brain (Hironaka et al., 2000). While no neuronal function for PTPN3 has been established, PTPN3 is mutated in several human colon cancers leading to the suggestion that it is a colon cancer tumor suppressor gene (Wang et al., 2004). The developmental functions of PTPMEG, PTPN3, and PTPN4 have not been previously examined.

Here we characterize the function of *ptpmeg* in *Drosophila* by creating a mutation in *ptpmeg* using homologous recombination and analyzing the effects of loss of *ptpmeg* function on neuronal development. We find that *ptpmeg* acts in neurons and is required for the maintenance of neuronal connectivity patterns in the fly brain. In *ptpmeg* mutants, mushroom body axons initially form normal projection patterns, but one set of axon braches later retracts while another set overextends. Genetic mosaic analyses suggest that *ptpmeg* acts in neurons that communicate with MB axons and structure/function studies demonstrate that PTPMEG phosphatase activity is critical for *ptpmeg* function in the maintenance of connectivity patterns. The progressive loss of proper neuronal connectivity in the mushroom bodies of *ptpmeg* mutants indicates that PTPMEG-regulated signaling pathways are involved in the stabilization of neuronal circuits.

Figure 1. Ptpmeg homologs, locus structure, and mutations. (A) Alignment of *Drosophila* PTPMEG protein with human PTPN3 and PTPN4. The effect of the frameshift mutation in *ptpmeg*^{1} on PTPMEG translation is noted. (B) *Drosophila ptpmeg* transcripts, P-element insertion alleles, and *ptpmeg*^{1} allele (generated by homologous recombination). (C) DNA sequencing trace from genomic *ptpmeg* locus in wild-type animal compared to *ptpmeg*^{1} homozygous mutants. (D) Western blot using adult heads confirms that *ptpmeg*^{1} does not produce detectable levels of wild-type PTPMEG protein, and *ptpmeg*^{2} produces reduced amounts of PTPMEG protein. Asterisk indicates background band. The neuronally expressed Elav protein was used as a control for equal loading of lanes.

Figure 1


A
PTPMEG homologs
truncation in ptpmeg1
SKVVPHTPA(tga)
D. mel. PTPMEG-PA
FERM
PDZ
Phosphatase
H. sapiens PTPN3
40%
47%
44%
H. sapiens PTPN4
48%
55%
45%
% amino acid identity to Drosophila PTPMEG
B
ptpmeg: Transcripts and Mutations
ptpmeg1
GTAC (4bp insertion)
ORF
Ptpmeg-RC
Ptpmeg-RA
Ptpmeg-RB
Ptpmeg-RD
3'
0 kb
10 kb
20 kb
30 kb
pGATB-NP4498 (ptpmeg2)
C
ptpmeg wt
G G G G T A C C A C
ptpmeg1
G G G G T A C G T A C C A C
D
wild type
ptpmeg1/ptpmeg1
ptpmeg2/ptpmeg2
150 kD
100 kD
75 kD
50 kD
PTPMEG
*
50 kD
ELAV
(loading control)

MATERIALS AND METHODS

Genetics and Molecular Biology

ptpmeg^{1} allele was generated by homologous recombination as described (Sears et al., 2003). A donor fly strain was created that harbored a transgene carrying a portion of the *ptpmeg* gene modified to contain one I-Sce1 and one I-Cre1 restriction site (neither otherwise found in the *Drosophila* genome) and a four-bp insertion in the coding region. These elements were followed by sequence encoding the white eye marker, and the entire transgenic construct was flanked by FRT sites, allowing for FLP recombinase recognition. Donor flies, which contained the transgenic element inserted into a random genomic location, were crossed to flies carrying genetic elements encoding the FLP recombinase and the I-Sce1 enzyme, both under the control of an inducible promotor. FLP and I-Sce1 expression was induced in the germline, allowing for the excision of a circular donor element via the FRT sites, followed by cleavage into a linear fragment by I-Sce1. Subsequent homologous recombination occurred at the *ptpmeg* locus to produce a ptpmeg duplication. Flies with the *ptpmeg* duplication (and hence the 4-bp insertion, I-Cre1 site, white marker, and an FRT site) were induced to undergo another round of excision which resulted in the creation of the *ptpmeg*^{1} allele that simply contains the 4-bp insertion and therefore is predicted to encode only one version of the PTPMEG protein, with a wild-type FERM domain but truncated in the PDZ domain.

Western blot was performed as described (Sears et al., 2003). 10 adult heads were used for each lane. The blot was probed with guinea pig anti-PTPMEG (1:2500) followed by goat anti-guinea pig (1:5000) and detected using Amersham

chemiluminescence detection system. For loading control, blot was stripped and reprobed using rat anti-Elav (1:1000) followed by goat anti-rat (1:5000).

Wild-type rescue construct was made by cloning *ptpmeg* cDNA derived from BDGP clone LD22982 into pUASt. ΔFERM was created similarly, but with a truncated cDNA that does not encode any of the FERM domain, clone LD27491. Other mutant *ptpmeg* constructs were created by modifying the wild-type cDNA using the Invitrogen GeneTailor Site Directed Mutagenesis System.

MARCM analysis was performed by crossing *hsFLP UAS-GFP;;tubGAL80 tubGAL4 FRT80B* flies to *ptpmeg*^{1} *FRT80B/S:T* flies, allowing adults to lay eggs over several days, heat-shocking larval progeny once a day at 38°C for one hour, and picking adults without *S:T* for dissection.

Histology

Polyclonal guinea pig anti-Ptpmeg antibody, directed against the PDZ domain-containing region, was generated by Covance, and used 1:750 for histology, 1:5000 for Western blotting. The following other antibodies were used at the indicated dilutions: mouse anti-FasII (4D1), 1:50, rat anti-Elav, 1:2000, donkey anti-guinea pig (subtracted against mouse) Cy5, 1:200, donkey anti-mouse (subtracted against guinea pig) FITC 1:200, goat anti-mouse Cy3, 1:400. All fluorescent antibodies were obtained from Jackson Laboratories (Bar Harbor, ME). All other antibodies were obtained from DSHB (Iowa).

Third instar stainings were performed as described (Whited et al., 2004). Pupal brains were prepared by removing pupal case covering head, incubating for five minutes

in 2% PFA (8% stock diluted 1:4 in PBL) with 3uL/1mL PBT, followed by slicing head cuticle open using tungsten needle, squeezing contents through hole from back, recovering brain, and fixing 45 minutes in 2% PFA with PBT. Adult brains were dissected in PBS and fixed for 45 minutes in 2% PFA with PBT. All primary and secondary antibodies were incubated overnight, 4°C, washes were performed for 1 hr at RT or overnight at 4°, brains were cleared in Vectashield overnight, 4°C, and mounted in Vectashield. Imaging was performed on a Nikon PCM2000 confocal microscope.

RESULTS

ptpmeg mutant animals are viable, but uncoordinated

To study the function of *ptpmeg* in the animal, we disrupted the *ptpmeg* locus using homologous recombination to introduce a 4-bp insertion into the PTPMEG open reading frame, creating *ptpmeg*^{1} (see materials and methods for details). The insertion in *ptpmeg*^{1} introduces a frameshift into the PTPMEG open reading frame within the PDZ encoding region, truncating the protein after the introduction of nine novel amino acids (Figures 1B, 1C). Homozygous *ptpmeg*^{1} animals were viable and fertile, but appeared uncoordinated and often became trapped alive in their food when cultured under moist conditions, a phenotype rescued by neuronal expression of a PTPMEG cDNA (J.L.W. and P.A.G., unpublished observations). (When care was taken to transfer adults to dry food upon eclosion from the pupal case, *ptpmeg*^{1} flies could survive for >20 days). To monitor PTPMEG protein expression in these animals, polyclonal antisera were raised against PTPMEG. This antisera recognized a major protein species of the expected

mobility for PTPMEG in wild-type but not *ptpmeg*^{1} mutant animals (Figure 1D), indicating that *ptpmeg*^{1} disrupted normal PTPMEG expression and confirming the specificity of the anitsera. As the antisera were raised against a region of PTPMEG spanning the PDZ domain, some truncated PTPMEG species could escape detection by this antisera. Nonetheless, all genetic and molecular data presented below are consistent with *ptpmeg*^{1} acting as a strong loss-of-function allele.

In addition to *ptpmeg*^{1}, we obtained two additional disruptions of the *ptpmeg* locus from publicly available collections. *pGATB-NP4498* (*ptpmeg*^{2}) contains a transposable element insertion upstream of the PTPMEG open reading frame. *ptpmeg*^{2} expresses reduced levels of PTPMEG protein (Figure 1D) and genetic data presented below suggests that it is a relatively weak loss-of-function allele of *ptpmeg*. In addition, we obtained *Df(3L)ED201*, a deficiency chromosome from which the entire *ptpmeg* locus has been deleted.

ptpmeg is required for mushroom body axon patterning

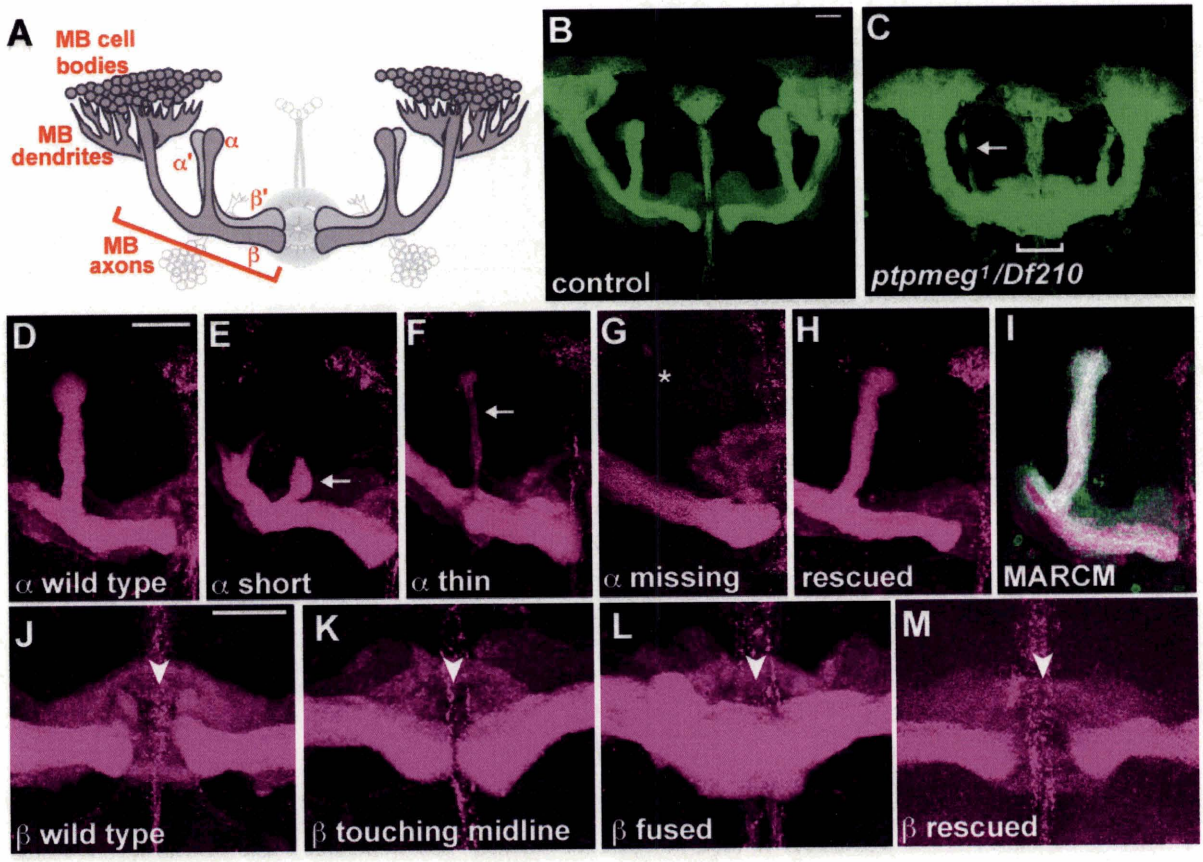
The external phenotype of *ptpmeg* mutant adults, uncoordinated, but largely normal morphologically, raised the possibility that *ptpmeg* mutants may have nervous system disruptions. We examined whether connectivity patterns in the adult brain were altered in *ptpmeg* mutants and observed significant disruptions of mushroom body (MB) axon projections in *ptpmeg* animals.

The MBs are involved in multiple behaviors including olfactory learning and memory and the modulation of walking (Zars, 2000). MBs are comprised of three subtypes of MB neurons: γ, α'/β', and α/β. Each MB neuron extends a single axon that

bifurcates to extend one axon branch into a dorsal lobe and one branch into a medial lobe that approaches the midline of the brain, but does not touch the midline (Lee et al., 1999b). During development, the γ subtype of mushroom body neurons loses its branches via developmentally programmed, ecdysone-triggered branch degeneration, and regrows only the lateral branch (Lee et al., 2000). In contrast, the α'/β' and α/β subtypes of mushroom body neurons normally retain both of their branches into adulthood (Figures 2A and 2B). The MB neurons provide a facile system for studying both the establishment and maintenance of neuronal connections involved in sophisticated behaviors such as associative olfactory memory.

Figure 2. *ptpmeg* is required for mushroom body (MB) axon patterning. (A) Mushroom body (MB) neurons are shown in bold in the central brain schematic. The location of α, α', β, and β' lobes, generated by the branching of α/β and α'/β' neuron axons, are indicated. (B, C) MB neurons are highlighted with mushroom-body-specific expression of mCD8-GFP. In normal adult brains (B), the dorsally-projecting α and α' lobes are of similar thickness as the medially-projecting β and β' lobes, and β/β' lobes project towards the midline but do not touch or cross it. In *ptpmeg* mutants (C), α/α' lobes are frequently reduced, appearing thin and/or short (arrow), and β/β' lobes are frequently overgrown, touching the midline or fusing with β/β' lobes from the contralateral MB (bracket). (D-I) MB axons from adult left brain hemispheres are labeled with anti-Fasciclin II which specifically highlights α and β lobes. A characteristic "L" shape is seen in normal brains (D), where the α lobe projects dorsally and terminates in a characteristic bulb and the β lobe projects medially and terminates prior to reaching the midline. Panels E-G highlight the types of α lobe defects in *ptpmeg* mutants, including short (E), thin (F), and missing (G) α lobes. (H) α lobe reduction in *ptpmeg* mutants is completely reduced by expression of wild-type Ptpmeg in neurons using *Elav-Gal4*. (I) *ptpmeg* is not required cell-autonomously in the MB cells, as animals with large clones of homozygous *ptpmeg*^{1} mutant MB tissue (marked in green) in otherwise heterozygous animals do not exhibit MB defects. (J) Normal midline (marked with an arrowhead) spacing of β lobe termini. (K, L) Examples of β lobes touching the midline (K) and fusing with the contralateral β lobe (L) in *ptpmeg* mutants. (M) Rescue of β lobe defects in *ptpmeg* mutant animal by expression of wild-type Ptpmeg in using *Elav-Gal4*.

Figure 2


A
MB cell bodies
MB dendrites
α'
α
β'
MB axons
β
B
control
C
ptpmeg1/Df210
D
α wild type
E
α short
F
α thin
G
α missing
H
rescued
I
MARCM
J
β wild type
K
β touching midline
L
β fused
M
β rescued

We found that *ptpmeg* was required for normal patterning of both distal and medial MB lobes. The cell bodies, dendrites, and axons of all MB neurons were visualized in *ptpmeg* mutants by expressing a membrane-targeted GFP (CD8:GFP) under the control of the MB-specific GAL4 source *OK107-GAL4*. While the cell body and dendritic regions of the MB neurons appeared normal in *ptpmeg* mutant adults, all five lobes containing MB axons appeared disrupted. In *ptpmeg* mutants, the dorsally projecting α and α' MB lobes were often reduced in thickness and/or length compared to wild-type controls. Meanwhile, the medially-projecting β, β', and γ MB lobes were often expanded with the medial lobes of one hemisphere reaching the midline and sometimes fusing with the medial lobes from the other brain hemisphere (Figure 2C).

We focused our analysis on the axons of the α/β neuron class of MB neurons. These neurons are the last set of MB neurons to be generated during development and are born during pupariation. In the adult, the α/β axons are the only MB processes that express the cell adhesion molecule Fasciclin II, permitting their selective labeling. In wild-type animals and in animals heterozygous for *ptpmeg* mutations, the α and β lobes had a highly regular morphology (Figure 2D, n>56 hemispheres). In contrast, *ptpmeg* mutant α lobes were frequently reduced, and were often short and/or thin (Figures 2E, 2F), and were sometimes completely missing (Figure 2G). In some instances, the knob-like appearance of the tip of the α lobe was whittled away in *ptpmeg* mutants, creating a "thin tip" appearance. Expression of a wild-type Ptpmeg cDNA in all neurons completely rescued a lobe reduction in $ptpmeg^{1}$ homozygous mutants and partially rescued the α lobe reductions observed in $ptpmeg^{1}/Df(3L)ED201$ animals (Figure 2H). MB axonal projections were also examined in $ptpmeg^{2}$ mutants. While $ptpmeg^{2}$

homozygotes did not exhibit MB defects, ~20% of MB hemispheres in *ptpmeg*^{2}/*Df(3L)ED201* animals had a lobe defects. This genetic evidence suggests that *ptpmeg*^{2} is a weaker allele than *ptpmeg*^{1}, a conclusion consistent with our protein expression data in Figure 1. The distribution and penetrance of the α lobe phenotypes in *ptpmeg* mutants is summarized in Figure 3A.

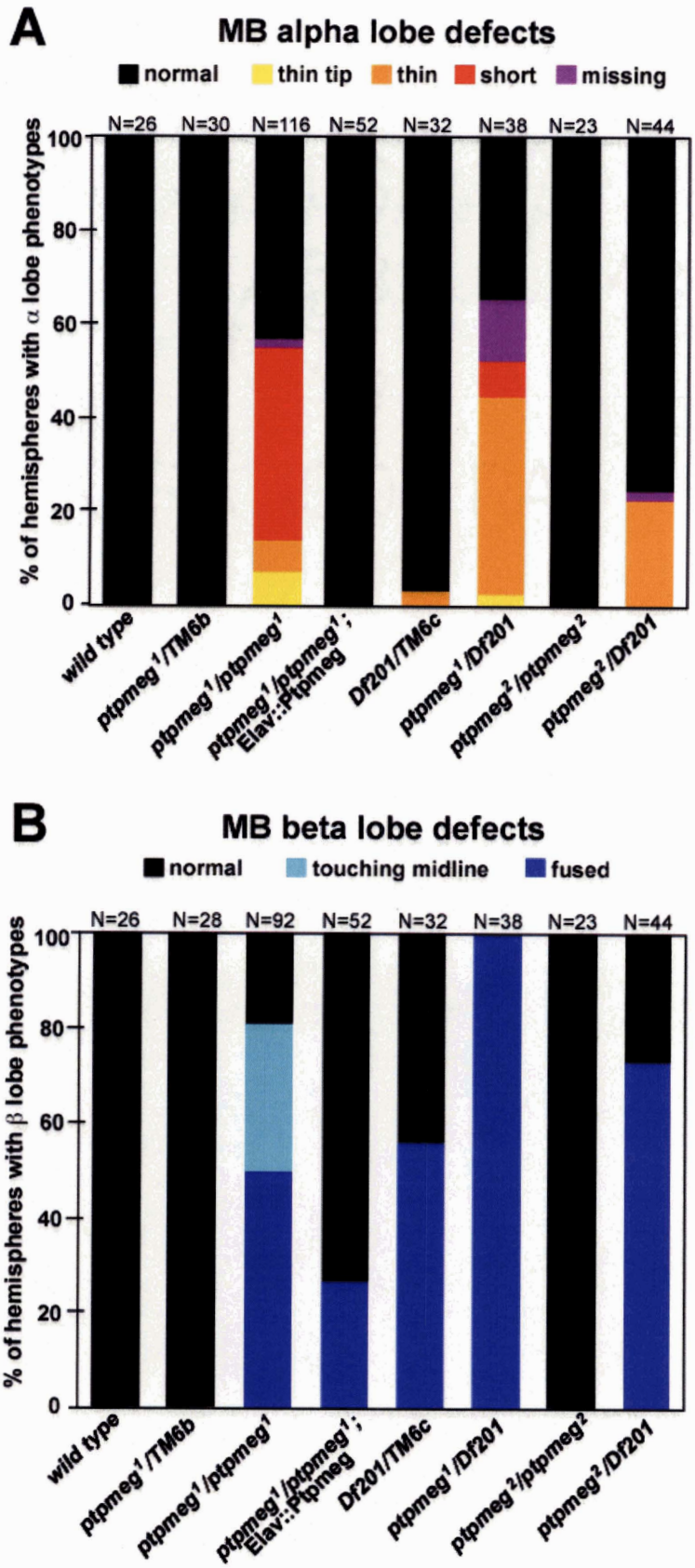
In addition to exhibiting α lobe defects, ptpmeg mutants also had disrupted β lobes. In wild-type animals and in *ptpmeg*^{1} heterozygotes, the β lobe terminated before reaching the brain's midline (marked by an arrowhead) at some distance from the β lobe of the contralateral hemisphere (Figure 2J). In *ptpmeg* mutants, β lobes often touched the midline (Figure 2K), in some cases completely fusing with the contralateral β lobe (Figure 2L). Similar to the α lobe, the β lobe defects of *ptpmeg* mutants were largely rescued by the expression of wild-type Ptpmeg protein in neurons, with the expression of the Ptpmeg transgene decreasing the penetrance of β lobe defects from ~80% to ~25% of hemispheres (Figure 2M). Note that *Df(3L)ED201* caused a dominant disruption of β lobe axons in ~50% of animals. While the penetrance of this β lobe defect increased to 100% in *ptpmeg*^{1}/*Df(3L)ED201* animals, consistent with a role for *ptpmeg* in β lobe development, the β lobe axon defects observed in *Df(3L)ED201* heterozygotes could not be rescued by Ptpmeg cDNA expression. This suggested that additional genetic lesions on *Df(3)ED201* were responsible for its dominant effects. The distribution and penetrance of β lobe phenotypes is summarized in Figure 3B.

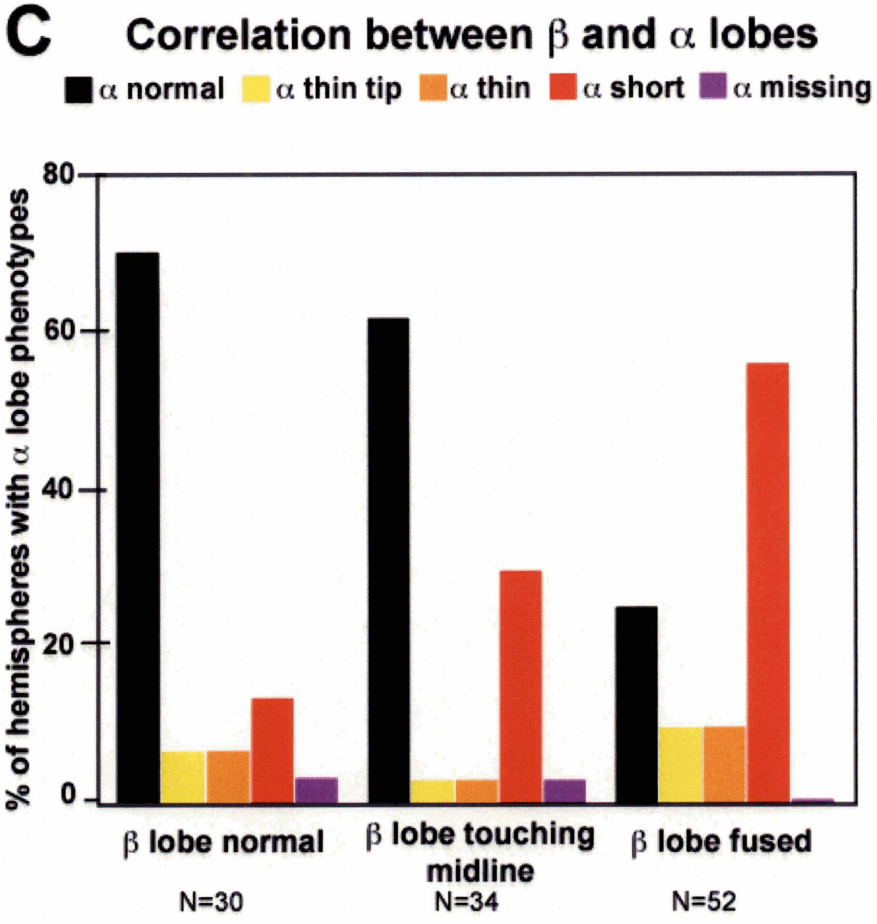
Taken together the previous data demonstrate that *ptpmeg* is required in neurons for patterning MB axon branches in the adult. To examine whether *ptpmeg* acts cell-autonomously in the MB neurons, marked clones of homozygous mutant *ptpmeg*^{1}

neurons were examined in otherwise heterozygous animals using the MARCM system (Lee et al., 1999a). Mutant clones of varying sizes were generated, including small clones containing 1 to 10 mutant α/β cells (n=14) and medium clones containing ~10-50 mutant α/β cells (n=11), but in no cases did we observe MB axon defects. Larger clones were also generated in which nearly all α/β neurons along with some α'/β' and γ neurons were mutant (n=16) (Figure 2I), but still no disruptions in MB axon branches were detected. Combined with the ability of Ptpmeg to rescue the MB axon defect when expressed in neurons under the control of Elav-GAL4, these data suggested that Ptpmeg may act in neurons other than the mushroom body neurons to control mushroom body axon patterning.

Figure 3. Quantification of MB axon defects in *ptpmeg* mutant animals. Panel C is shown on a separate page to facilitate legibility of genotypes. (A,B) Frequency of α lobe and β lobe defects in adults of the indicated genotypes. Df201 denotes *Df(3L)ED201* which is deleted for the entire *ptpmeg* locus. *ptpmeg*^{1}*/TM6b* and *Df201/TM6c* are heterozygous for *ptpmeg* as *TM6b* and *TM6c* are marked chromosomes wild-type for *ptpmeg*. (C) Correlation of α lobe defects with β lobe defects in *ptpmeg*^{1} mutants. Individual *ptpmeg*^{1} mutant brain hemispheres were sorted into classes based on their β lobe phenotypes and the frequency with which each class of hemisphere exhibited α lobe defects was calculated.

Figure 3


A
MB alpha lobe defects
normal
thin tip
thin
short
missing
N=26
N=30
N=116
N=52
N=32
N=38
N=23
N=44
% of hemispheres with α lobe phenotypes
100
80
60
40
20
0
wild type
ptpmeg1/TM6b
ptpmeg1/ptpmeg1
ptpmeg1/ptpmeg1; Elav::Ptpmeg
Df201/TM6c
ptpmeg1/Df201
ptpmeg2/ptpmeg2
ptpmeg2/Df201
B
MB beta lobe defects
normal
touching midline
fused
N=26
N=28
N=92
N=52
N=32
N=38
N=23
N=44
% of hemispheres with β lobe phenotypes
100
80
60
40
20
0
wild type
ptpmeg1/TM6b
ptpmeg1/ptpmeg1
ptpmeg1/ptpmeg1; Elav::Ptpmeg
Df201/TM6c
ptpmeg1/Df201
ptpmeg2/ptpmeg2
ptpmeg2/Df201


C
Correlation between β and α lobes
α normal
α thin tip
α thin
α short
α missing
% of hemispheres with α lobe phenotypes
80
60
40
20
0
β lobe normal
N=30
β lobe touching midline
N=34
β lobe fused
N=52

***ptpmeg* function stabilizes mushroom body dorsal and medial axon branches**

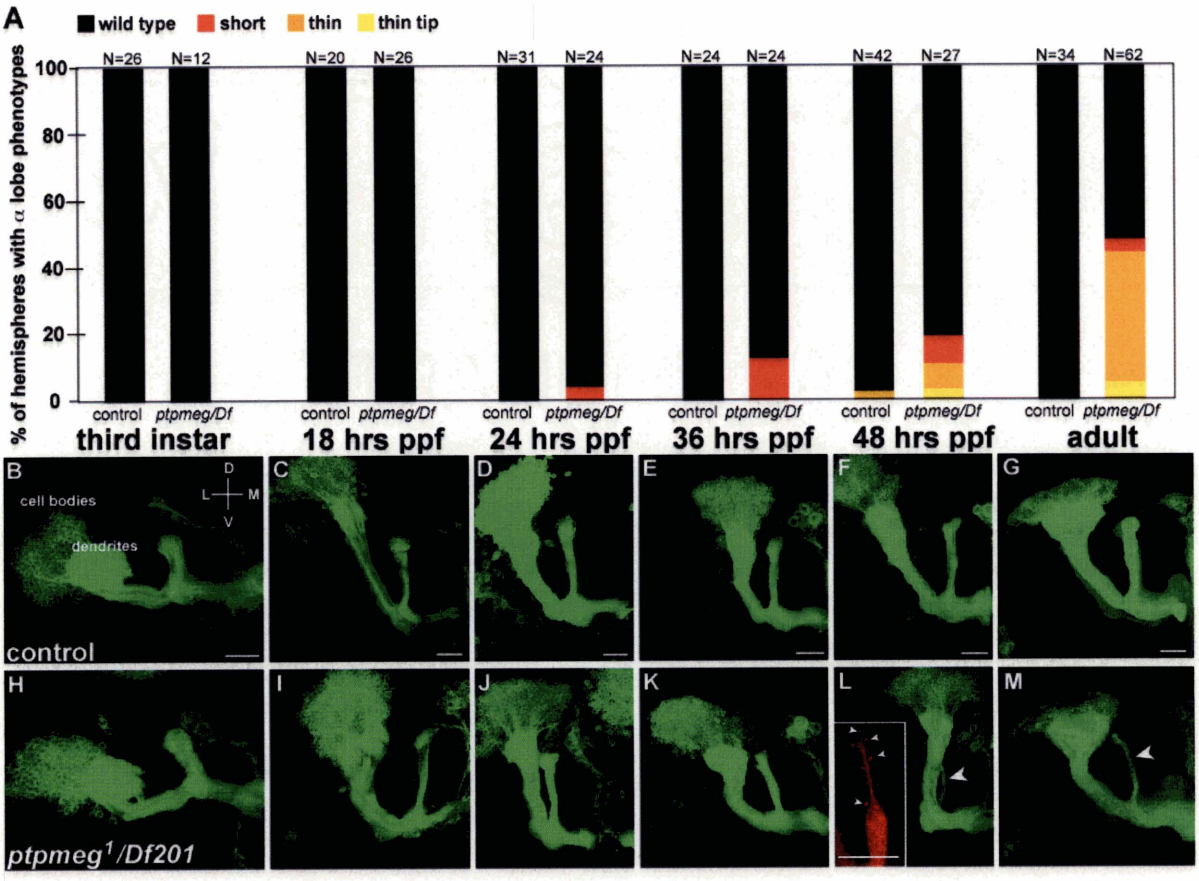
Two possible explanations could account for the defects in adult MB axon morphology in *ptpmeg* mutants. In the first scenario, *ptpmeg* mutants could be defective in the initial establishment of MB axon branches. Alternatively, *ptpmeg* MB axons might initially elaborate axon branches normally, but the branches might become progressively abnormal. To distinguish between these possibilities, we examined the morphology of MB axons in *ptpmeg* mutants at earlier developmental time points. The α/β neurons are born early during pupation (Lee et al., 1999b). By 18 hours of pupation, α/β dorsal and medial axon branches can be detected using Fasciclin II and by 48 hours their branching is well-established. In $ptpmeg^{1}$ animals, dorsal branches appeared normal at 18h (n=26) and 48h of pupation (n=36), and medial lobe branches were normal in all hemispheres at 18h (n=26) and in 33 of 36 hemispheres at 48h. These data suggested that *ptpmeg* was not essential for the initial branching of α/β axons, but rather their maintenance.

To observe the onset of MB axon projection defects more clearly, we monitored the development of MB axon branches at multiple time points in $ptpmeg^{1}/Df(3L)ED201$ animals using membrane-targeted GFP. Representative images from control animals are depicted in the top row of Figure 4 (B-G), while those from $ptpmeg^{1}/Df(3L)ED201$ animals are shown in the bottom row (H-M). In third instar animals, thick, bifurcated MB lobes are clearly visible in both wild-type and in $ptpmeg^{1}/Df(3L)ED201$ mutants (Figures 4B and 4H), indicating that the MB axon branches appear normal in *ptpmeg* mutants during larval life. By 18 hours post-pupal formation (PPF) in wild-type animals, the original dorsally-projecting mushroom body lobes have been lost and a thinner group has taken their place in a nervous system remodeling process that occurs between larval

and adult life during pupation (Figure 4C). *ptpmeg*^{1}*/Df(3L)ED201* mutants had normal dorsal lobe projections at 18 hours PPF (Figure 4I), indicating that dorsal axon branching during pupation is also normal. Dorsally-projecting mushroom body lobes continue to thicken with the addition of new branches as pupation proceeds. At 24 hours PPF, almost all *ptpmeg*^{1}*/Df(3L)ED201* brains analyzed still had dorsal lobe projection patterns indistinguishable from wild-type (Figures 4D and 4J), but by 36 hours PPF, a small proportion of the *ptpmeg*^{1}*/Df(3L)ED201* brains showed partial dorsal lobe reduction (Figures 4F and 4L). The percentage of mutant brains with reduced dorsal lobes continued to increase over later stages of pupal life, with approximately 20% of *ptpmeg*^{1}*/Df(3L)ED201* dorsal lobes exhibiting reductions at 48 hours PPF, increasing to approximately 50% by eclosion (the first day of adulthood). Phenotypic frequencies of dorsal lobe phenotypes in *ptpmeg*^{1}*/Df(3L)ED201* animals over the time-course of MB development are summarized in Figure 4A. Media lobe defects were not monitored in these *ptpmeg*^{1}*/Df(3L)ED201* animals. As noted above, the *Df(3L)ED201* caused a partially penetrant, dominant medial fusion phenotype that appears unlinked to lesions in *ptpmeg* (J.L.W. and P.A.G., unpublished data).

Figure 4. *ptpmeg* is required to prevent the retraction of MB α/α' axons. (A) α/α' lobe phenotypes over development. *ptpmeg*^{1}*/Df(3L)ED201* animals have completely normal α/α' lobes through 18 hours post pupal formation (PPF). Defects begin to manifest at 24 hours PPF, and the frequency with which reduced α/α' lobes are detected increases as pupal development proceeds. (B-M) Representative left hemispheres from animals in which MB neurons were highlighted through mushroom-body expression of mCD8-GFP. Both a control (top, B-G) and a *ptpmeg*^{1}*/Df201* mutant (bottom, H-M) are shown for each stage of development examined. Stages of development correspond to bar graph above each pair of images. Note that by 48 hours PPF, ~20% of α/α' lobes are reduced (L, arrowhead shows thin α/α') in *ptpmeg* mutants, while newly eclosed adult mutants have reduced α/α' lobes in >40% of cases. Inset in L is a high magnification view of an α lobe (visualized using FasII) that has partially withdrawn. Small arrowheads denote the concentrations of material that are occasionally observed near the dorsal region of α lobes that appear to be in the process of withdrawing.

Figure 4


A
wild type
short
thin
thin tip
% of hemispheres with α lobe phenotypes
100
80
60
40
20
0
N=26
N=12
N=20
N=26
N=31
N=24
N=24
N=24
N=42
N=27
N=34
N=62
control
ptpmeg/Df
third instar
18 hrs ppf
24 hrs ppf
36 hrs ppf
48 hrs ppf
adult
B
cell bodies
dendrites
D
L
M
V
control
H
ptpmeg1/Df201
C
D
E
F
G
I
J
K
L
M

ptpmeg can independently influence dorsal and medial branch maintenance

Taken together these experiments indicate that *ptpmeg* is not required for the initial outgrowth of MB axon branches, but rather for the maintenance of MB axon branches. However, the dorsal and medial axon branches of these MB neurons were differently affected by the loss of *ptpmeg*, as dorsal axon branches retracted while medial axon branches overextended. In one model, the two defects could be interdependent, with loss or overextension of one set of branches causing the defects in the other set of branches. Alternatively, the two defects could be largely independent. To begin to distinguish these models, we examined the correlation between distal lobe defects and medial lobe defects within a given MB. Of 58 *ptpmeg*^{1} mutant adults in which α/β axons were examined (116 hemispheres), the β (medial) lobes appeared normal in 30 hemispheres, contacted the midline but remained distinct from the contralateral β lobe in 34 hemispheres, and fused with the contralateral β lobe in 52 hemispheres. Among these animals, the frequency of disrupted α (medial) lobes positively correlated with disruption of the β (medial) lobes (Figure 3C). Only ~5% of hemispheres with normal β lobes had disrupted α lobes, while ~75% of hemispheres with fused β lobes had disrupted α lobes, an increase largely resulting from an increased frequency of α lobe shortening (Figure 3C). Thus, the severity of the defects in the two lobes were correlated. However, ~25% of α lobes remained normal in appearance in animals with fused β lobes, indicating that disruption of the β lobe did not require disruption of the α lobe. Similarly, ~15% of hemispheres with mutant α lobes exhibited normal β lobes. These data suggested that while the MB axons in some *ptpmeg* mutant animals were more disrupted than the MB axons of other animals, there was no obligate relationship between α and β lobe

phenotypes. Thus, overextension of the medial lobe did not depend on dorsal lobe retraction and the retraction of the dorsal lobe and the retraction of the dorsal lobe did not depend on medial lobe retraction, suggesting these defects are independent. These data support a model in which *ptpmeg* is required to stabilize both branches of MB axons, preventing the retraction of dorsal MB axon branches while preventing the overextension of medial MB axon branches.

PTPMEG protein is abundant on fiber tracts in the developing and adult brain

To obtain further insight into how *ptpmeg* might be involved in brain development, we examined the pattern of PTPMEG protein expression. We found that PTPMEG was enriched along multiple fiber tracts in the brain at all stages of development examined from third instar through adult. Strong PTPMEG immunostaining was detected during the periods at which MB axons begin to exhibit defects, from about 48 hours after pupal formation (PPF), and the staining persisted at slightly lower levels into the adult (Figures 5A, 5D, and 5F). *ptpmeg*^{1}*/Df(3L)ED201* mutants exhibited little or no anti-PTPMEG staining, confirming the specificity of the antisera (Figure 5B). Consistent with the rescue and genetic mosaic analyses presented above suggesting that PTPMEG did not act in MB axons, PTPMEG expression could not be detected on MB axons (Figures 5D, 5E, and 5E'). However, consistent with PTPMEG acting in neurons, PTPMEG immunoreactivity colocalized with membrane-targeted GFP expressed under the control of a neuron-specific promoter (*elav-Gal4*), indicating that PTPMEG protein is expressed in many nearby neurons (Figure 5C). Attempts to further define the subpopulations of neurons in which *ptpmeg* acts through the use of existing

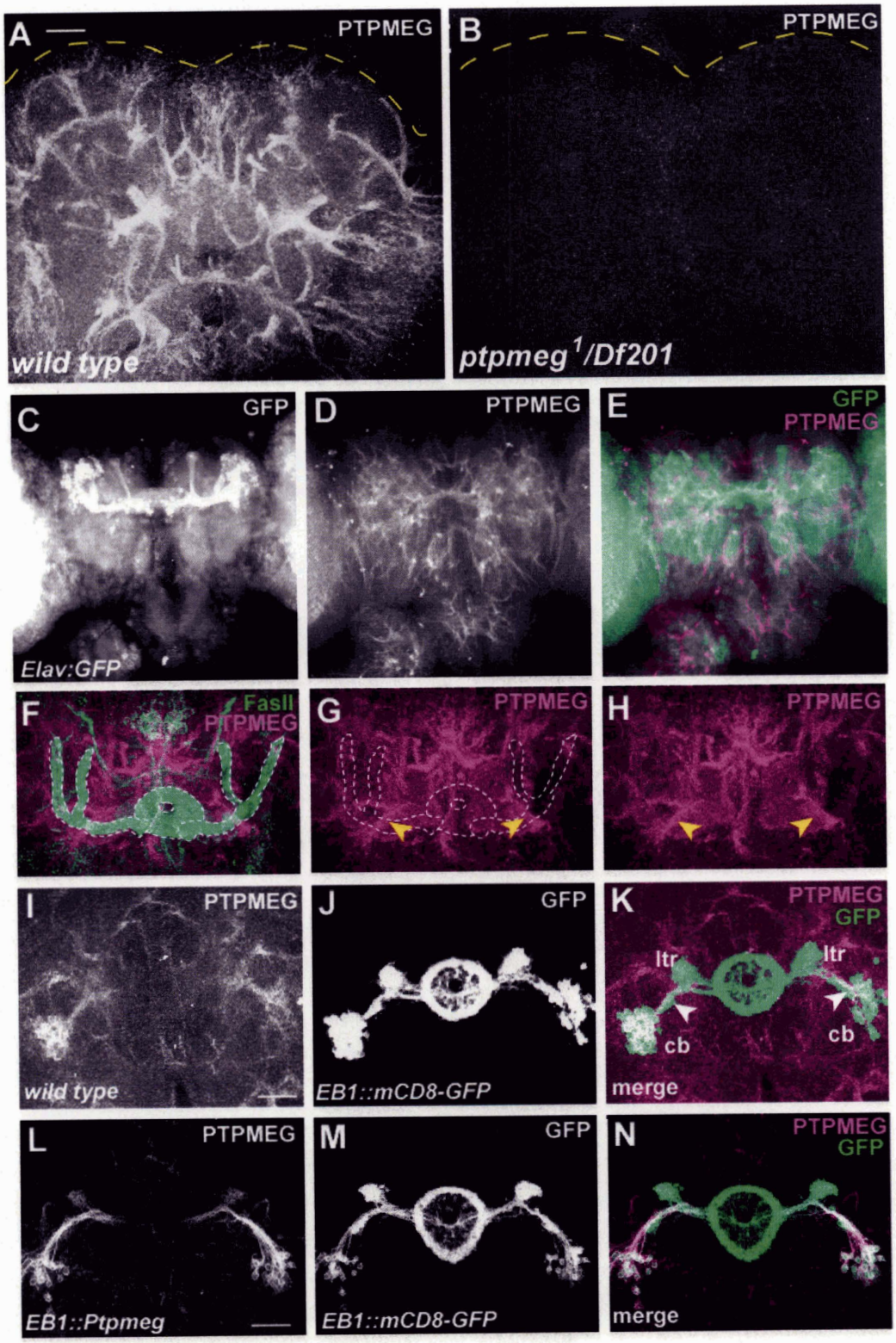
Gal4 sources more restricted in their expression than elav for transgenic rescue of *ptpmeg* and MARCM analysis were unsuccessful (J.L.W. and P.A.G., unpublished). Thus, the precise identity of the neurons in which PTPMEG acts remains an important issue to resolve in the future. As tools are not yet available to specifically manipulate many of the neurons that interact with MB axon branches (Ito et al. 1998), this is a general challenge presently faced in the studies of MB function.

Although PTPMEG was expressed on fiber tracts in the brain, it was not concentrated within the axon terminal-rich neuropil regions (Figure 5C). To examine the subcellular localization of PTPMEG in greater detail, PTPMEG expression was examined in a set of highly polarized neurons that strongly express PTPMEG protein, the ellipsoid body (EB) neurons. The EB neuron cell bodies are located in two clusters, one in each brain hemisphere, and send out a neurite toward the midline that elaborates dendritic tufts in the later triangle region (ltr) and, at the brain's midline, branches to form an elaborate circular axon terminal in the ellipsoid body ring (ebr). PTPMEG protein was concentrated on EB cell bodies and on the proximal region of the EB neurite. PTPMEG expression extended into the dendritic region, but was not detectable more distally in the axon terminals of the EB neurons (Figures 5F-5H). PTPMEG did not enter the EB axon terminal ring even when it was overexpressed in the EB neurons using an EB-specific promoter (*EB1-Gal4*) (Figures 5I-5K). Rather, it accumulated to increased levels in the EB cell bodies (particularly near the cell surface) and on the proximal neurite and the dendrites. This asymmetric distribution of PTPMEG suggested the presence of mechanisms for concentrating PTPMEG in EB cell bodies or excluding it

from EB axon terminals and was consistent with the observed absence of PTPMEG from neuropil regions in the brain where axon terminals are concentrated.

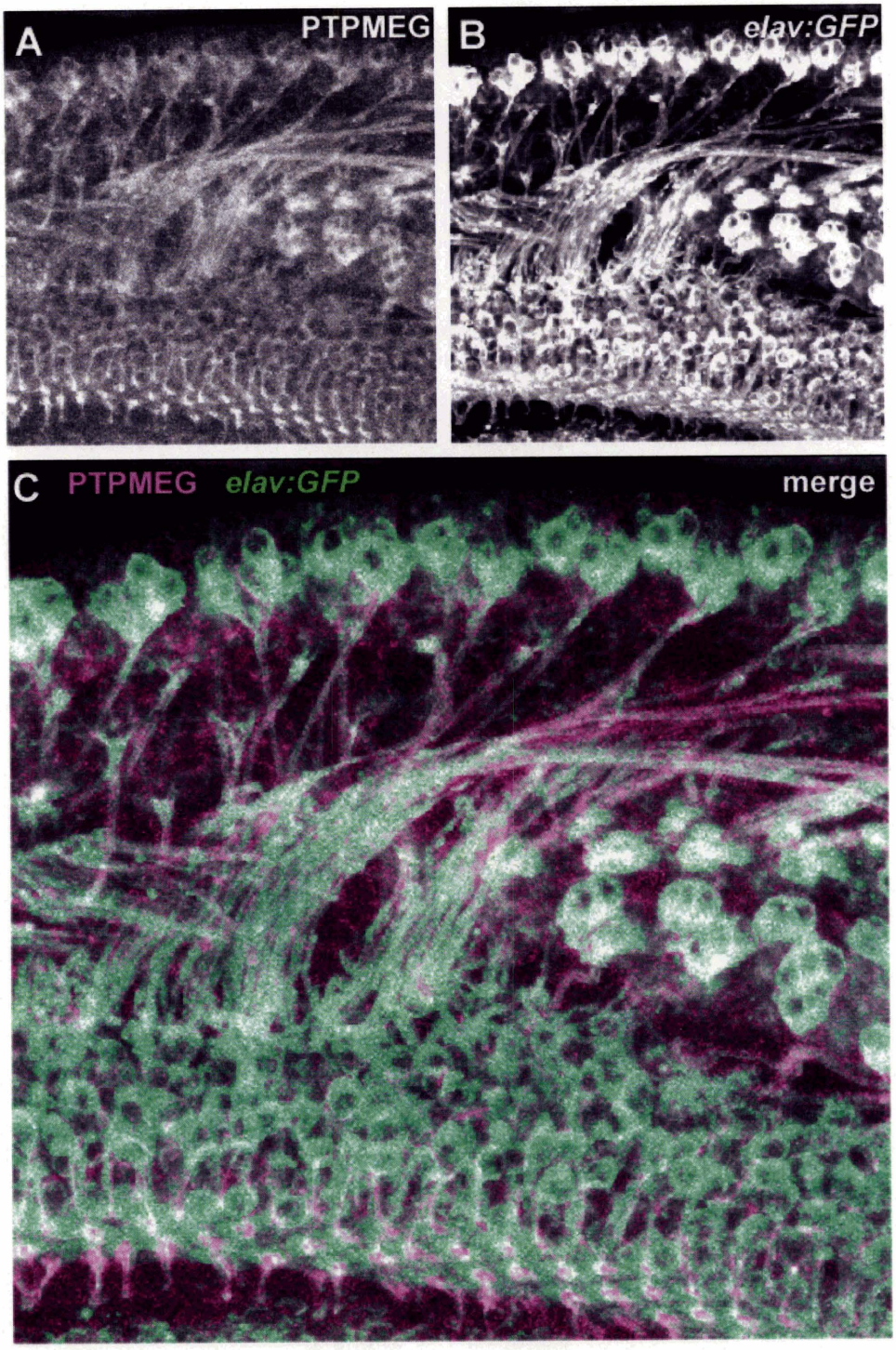
Figure 5. Pattern of PTPMEG protein expression in the developing and adult brain. (A) Expression of PTPMEG protein at 72 hours ppf in a wild-type brain. (B) Expression of PTPMEG is not detected in *ptpmeg*^{1}*/Df(3L)ED201* brains. (C) Approximately 42 hours PPF pupal brain, with Elav-GAL4 driving expression of mCD8-GFP in all neurons. (D) Same pupal brain, anti-PTPMEG channel. (E) Merge. PTPMEG (magenta) is expressed on neuronal fibers, as indicated by its overlap with mCD8-GFP expression driven from the pan-neuronal driver *Elav-GAL4* (green). Note that not all areas of PTPMEG expression appear to be neuronal. (F-H) PTPMEG is not detectably expressed on mushroom body axons. (F) PTPMEG expression (magenta) at 48 hours PPF. Mushroom body α and β lobe axons as well as ellipsoid body ring axons (ebr) are labeled with anti-FasII (green). (F) Merge. (G) PTPMEG channel only, with outline of α and β lobes and ebr axons superimposed. (H) PTPMEG channel only. PTPMEG is strongly expressed on neurites from ellipsoid body neurons (arrowheads). (I-K) PTPMEG is strongly expressed on ellipsoid body neuron cell bodies (cb) and neurites proximal to the cell bodies (arrowhead). PTPMEG expression extends to the dendritic region (ltr), but is not detected in EB axon termini in the ebr. (I) PTPMEG expression in adult. (J) EB neurons labeled with mCD8-GFP controlled by an EB-neuron-specific promoter (EB1). (K) Merge. (L-N) Ptpmeg protein still localizes to the cell bodies, initial projections, and dendrites and does not enter the axonal ring even when overexpressed in EB neurons (L-N). Note that overexpressed PTPMEG clearly outlines EB cell bodies, appearing to accumulate close to the surface of the cell.

Figure 5


A
PTPMEG
wild type
B
PTPMEG
ptpmeg 1/Df201
C
GFP
Elav:GFP
D
PTPMEG
E
GFP
PTPMEG
F
FasII
PTPMEG
G
PTPMEG
H
PTPMEG
I
PTPMEG
wild type
J
GFP
EB1::mCD8-GFP
K
PTPMEG
GFP
ltr
ltr
cb
cb
merge
L
PTPMEG
EB1::Ptpmeg
M
GFP
EB1::mCD8-GFP
N
PTPMEG
GFP
merge

Supplementary Figure 1. PTPMEG expression in the pupal visual system. A brain was dissected from an animal expressing mCD8-GFP under the control of the neuronal driver Elav at approximately 48 hours PPF. The brain was stained with anti-PTPMEG. Images of the photoreceptors and optic lobe were obtained. (A) PTPMEG expression. (B) Elav:GFP. (C) Merge. PTPMEG is clearly visible on the cell bodies and axons of photoreceptor cells (top of image). Its expression also overlaps with many other neurons (beneath the photoreceptors in this image), and we are working to positively identify these neurons.

Supplementary Figure 1



***ptpmeg* is required for the proper projection of ellipsoid body axons**

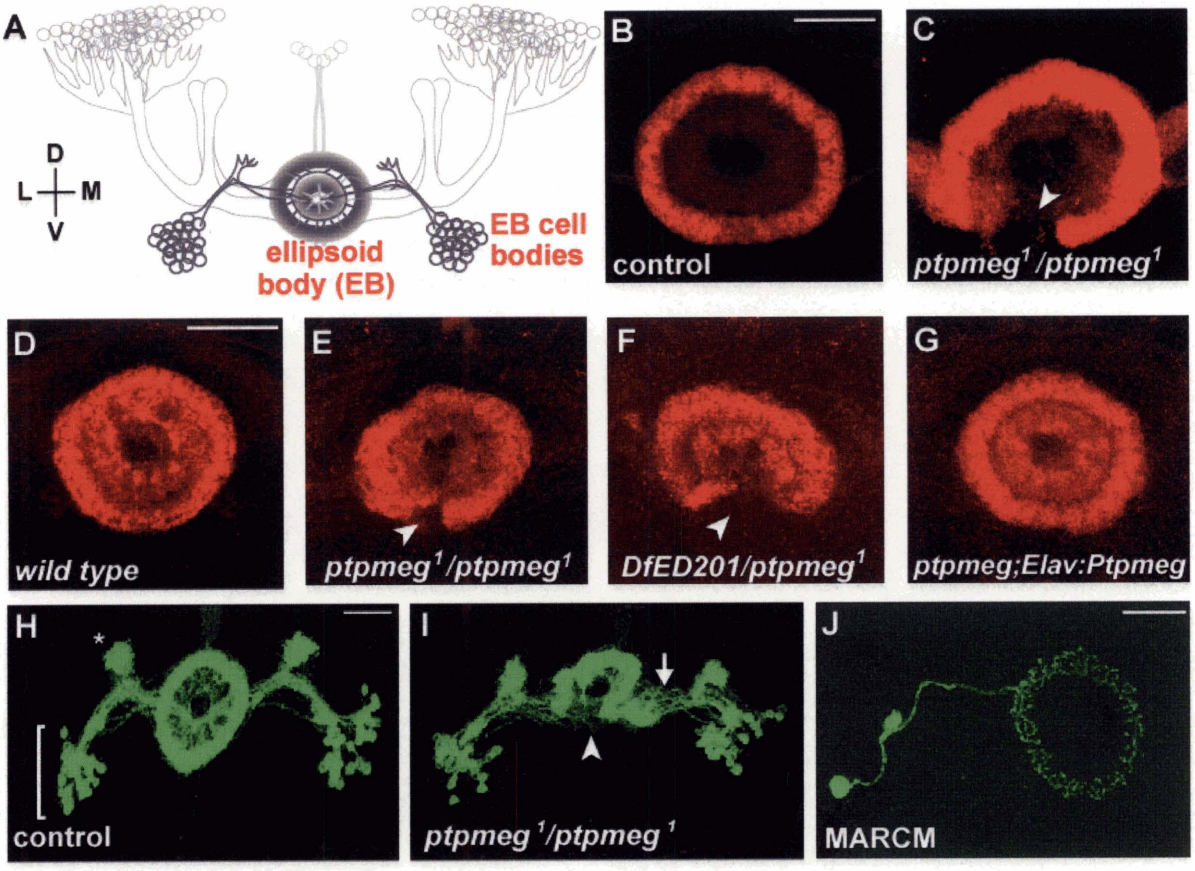
Given the strong PTPMEG expression in EB neurons, the effects of PTPMEG on the EB development were examined. The ellipsoid body (EB) develops late in pupal development, when axon termini from EB neurons from a ring at the midline of the brain (Zheng et al. 2006) (Figure 6A). The axon terminals of EB neurons strongly express the neuronal cell adhesion molecule Fasciclin II (FasII), facilitating their visualization (Figure 6A). In wild-type animals, the EB axons form a complete ring by 48 hours after pupal formation (Figure 6B, n=15). However, in *ptpmeg*^{1} mutants, EB axons formed an incomplete ring that was open along its ventral aspect (Figure 6C, n=19). EB axon projection defects persisted into the adult, as *ptpmeg*^{1} mutant adults frequently displayed a ventral notch or opening in the axonal ring (85%, n=46) (Figure 6E). Similar defects were observed in *ptpmeg*^{1}*/Df(3L)ED201* adults (100%, n=10) (Figure 6F), consistent with the defect in EB ring formation reflecting a strong loss of *ptpmeg* function. In contrast to the highly disrupted EB axon ring, the dendritic tufts and cell bodies of EB neurons appeared normal in *ptpmeg* mutants (Figures 6H and 6I), suggesting that EB projection defects in *ptpmeg* mutants were largely restricted to the distal regions of EB axons. Unlike the MB, where *ptpmeg* mutant defects increased in severity over time, the EB axon defects did not, suggesting that *ptpmeg* may be critical for the establishment of neuronal connectivity in the EB rather than for its maintenance.

To examine in which cells *ptpmeg* acts to control EB axon patterning, we performed tissue-specific rescue and genetic mosaic experiments. The EB patterning defect of *ptpmeg* mutants was completely rescued by expression of a wild-type Ptpmeg cDNA in all neurons using *Elav-GAL4* (Figure 6G, n=35/35 for *ptpmeg*^{1}*/ptpmeg*^{1};

n=23/23 for *ptpmeg*^{1}/*Df(3L)ED201*). These results indicated that *ptpmeg* function was required in neurons to correctly pattern the EB axonal ring. To determine if *ptpmeg* acts within the EB neurons, marked clones of homozygous mutant *ptpmeg*^{1} EB neurons were examined in otherwise hetereozygous animals using the MARCM system. Multiple animals containing *ptpmeg*^{1} mutant EB neurons were analyzed (n>13), including animals that contained large *ptpmeg* mutant clones encompassing essentially all EB neurons. In no case did we observe defects in EB axon projections (Figure 6H). Thus, while EB neurons express PTPMEG in their cell bodies and dendrites, the EB neurons do not require *ptpmeg* to control the trajectories of their axons. Combined with the ability of neuronally expressed Ptpmeg to rescue the EB axon branching defect, these mosaic data suggest that *ptpmeg* acts in other neurons that control EB axonal projections.

Figure 6. *ptpmeg* is required for normal ellipsoid body development. (A) Essential components of the adult *Drosophila* central brain are schematized, with the ellipsoid body highlighted in bold lines and the mushroom bodies shown in fainter outlines. Ellipsoid body axon termini in the EB ring are labeled with anti-Fasciclin II in B-G. By 48 hours post-pupal formation (ppf), control animals have complete EB rings (B); however, *ptpmeg* mutants have deformed EB rings, most often showing an incomplete area at the ventral side (C, arrowhead). Ellipsoid body axons appear as two concentric rings in control adults (D), but in *ptpmeg* mutants (E and F), adult EBs are open (arrowheads). Mutant EB phenotypes can be rescued by expression of wild-type Ptpmeg protein in neurons (G). The full outline of EB neurons are visualized in H and I by expressing mCD8-GFP with the EB-specific *EB1-Gal4*. In control (H), the EB cell bodies are present in groups (bracket) and elaborate a tight fascicle of neurites that branch into dendrites (asterisk) and axons, with the axon terminal elaborating a spoke-like pattern centrally. In *ptpmeg* mutants (I), axons appear slightly defasciculated (arrow), and fail to elaborate a normal ring (arrowhead). (J) *ptpmeg* is not required cell-autonomously in the MB cells. Example of an animal containing a homozygous $ptpmeg^{1}$ mutant EB neuron (marked in green) in an otherwise heterozygous animal. Axon ring formation appears normal.

Figure 6


A
D
L
M
V
ellipsoid body (EB)
EB cell bodies
B
control
C
ptpmeg1/ptpmeg1
D
wild type
E
ptpmeg1/ptpmeg1
F
DfED201/ptpmeg1
G
ptpmeg;Elav:Ptpmeg
H
control
I
ptpmeg1/ptpmeg1
J
MARCM

PTPMEG's phosphatase activity is required for proper neuronal connectivity in both the mushroom and ellipsoid bodies

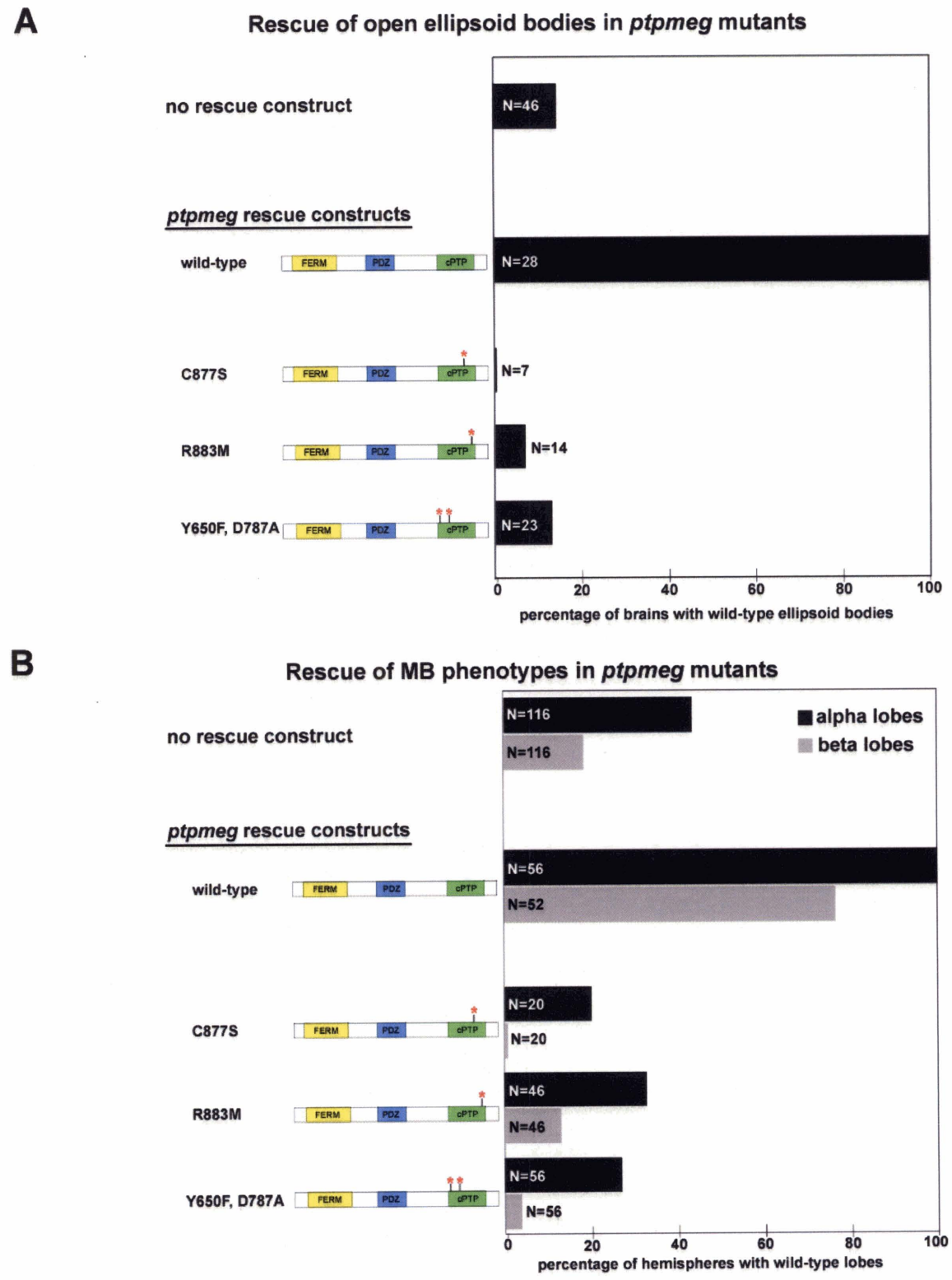
As ptpmeg encodes a putative protein tyrosine phosphatase, we tested the importance of phosphatase activity for *ptpmeg* function by examining whether mutant Ptpmeg transgenes with altered phosphatase domains could restore normal development in ptpmeg mutants. Three mutant forms of Ptpmeg were created. In Ptpmeg[C877S] the conserved cysteine essential for catalysis was replaced with serine (Flint et al., 1997). In Ptpmeg [Y650F,D787A], two mutations were introduced which drastically inhibit the ability of tyrosine phosphatases to dephosphorylate bound substrate, thereby "trapping" phosphorylated substrate in the catalytic site (Zhang et al. 1999). Finally, in Ptpmeg[R883M], a conserved arginine essential for the binding of tyrosine-phosphorylated substrates was replaced with methionine (Flint et al., 1997). Each mutant protein was introduced into *ptpmeg*^{1} animals and expressed in neurons under the control of Elav-GAL4. Western blot analysis demonstrated that each mutant protein was expressed at a level comparable to wild-type transgenic protein (data not shown). Each mutant was then tested for its ability to rescue the ptpmeg1 EB axon and MB axon projection defects.

While expression of a wild-type Ptpmeg cDNA in neurons completely rescued the EB axon defects of *ptpmeg* mutants, none of the three mutant versions of *ptpmeg* predicted to disrupt phosphatase activity significantly rescued EB defects (Figure 7A). Similarly, unlike a wild-type Ptpmeg cDNA, none of the phosphatase mutants rescued either the α lobe or β lobe defects in the MB (Figure 7B). In fact, defects may have been made even slightly more severe by the expression of the mutant protein. However, in no

case did expression of a mutant form of PTPMEG cause a dominant EB axon or MB axon phenotype in an otherwise normal animal. These results demonstrate that the phosphatase activity of PTPMEG is crucial for all three activities we find for *ptpmeg*: the phosphatase activity of PTPMEG is critical in promoting the normal establishment of the EB axonal ring, in preventing the retraction of MB dorsal lobes, and in preventing the delayed overextension of MB medial lobes.

Figure 7. Ptpmeg's phosphatase activity is required for both EB and MB axon branching. (A,B) EB neuron (A) and MB neuron (B) defects are rescued by expression of a wild-type Ptpmeg cDNA under the control of the neuronal promoter *Elav-Gal4*, but not by expression of Ptpmeg cDNA containing mutations in the phosphatase region. Quantification of phenotypes in adult *ptpmeg*^{1} animals containing indicated transgenes.

Figure 7


A
Rescue of open ellipsoid bodies in ptpmeg mutants
no rescue construct
N=46
ptpmeg rescue constructs
wild-type
FERM
PDZ
cPTP
N=28
C877S
N=7
R883M
N=14
Y650F, D787A
N=23
0
20
40
60
80
100
percentage of brains with wild-type ellipsoid bodies
B
Rescue of MB phenotypes in ptpmeg mutants
alpha lobes
beta lobes
no rescue construct
N=116
N=116
ptpmeg rescue constructs
wild-type
N=56
N=52
C877S
N=20
N=20
R883M
N=46
N=46
Y650F, D787A
N=56
N=56
0
20
40
60
80
100
percentage of hemispheres with wild-type lobes

DISCUSSION

We have identified a protein, PTPMEG, whose function is essential for the maintenance of proper patterns of neuronal connectivity in the fly brain. PTPMEG is a cytoplasmic protein tyrosine phosphatase and acts to promote the maintenance of mushroom body (MB) axon branch projections. While PTPMEG is dispensable for the initial establishment of MB axon branches, PTPMEG promotes the retention of dorsally-directed axon branches and inhibits the overgrowth of medially-directed branches. PTPMEG affects MB axons in a non-cell autonomous fashion, and PTPMEG expression and transgene rescue data suggest *ptpmeg* functions in surrounding neurons to modulate MB axon behavior. These studies identify PTPMEG as a critical factor required to stabilize neuronal connectivity patterns in the maturing and adult nervous system.

The maintenance of mushroom body axon branches is an active process

The regulated maintenance of axon branching patterns is critical for the generation of precise patterns of neuronal wiring. The selective retention or pruning of axon branches helps refine neuronal connectivity patterns as the nervous system matures (Katz and Shatz, 1996; Lichtman and Colman, 2000) and disruptions in axon branch maintenance are associated with the progression of several common neurological diseases (Luo and O'Leary, 2005).

What are the cell signaling events that control the long-term maintenance of axon branches not undergoing obvious remodeling, such as the α/β and α'/β' MB axon branches affected in *ptpmeg* mutants? Little is known about these issues, although recent evidence demonstrates that persistent inhibition of axon retraction-promoting pathways is

essential (Billuart et al., 2001). For both α/β and α'/β' MB neurons, maintenance of both their dorsal and medial axon branches requires RhoGAP, a negative regulator of the small GTPase Rho (Billuart et al., 2001). The activity of this Rho-dependent retraction pathway appears to be regulated by extracellular signaling pathways, as alterations in integrin signaling can modulate the effects of RhoGAP on axon retraction. Related pathways are proposed to act in mice, where focal adhesion kinase negatively regulates axon branch stabilization via Rho GTPases (Rico et al., 2004).

The effects of PTPMEG on axon branch stability

Here we have identified a novel signaling factor that can modulate axon branch stabilization. Like RhoGAP, PTPMEG promotes the retention of dorsal MB axon branches. In both RhoGAP and *ptpmeg* mutants, the withdrawal of the dorsal lobes does not resemble the branch degeneration observed when the dorsal lobe branches of γ MB neurons are pruned during metamorphosis. During γ neuron pruning, the full extent of the dorsal branch degenerates simultaneously (Watts et al., 2003), while in both RhoGAP (Billuart et al., 2001) and *ptpmeg* mutants, the distal tip of the dorsal lobe is preferentially lost and the proximal region is usually retained. In addition, the distal tip of the withdrawing branch often appears pointed in both RhoGAP and *ptpmeg* mutants and there are sometimes small dots of axonal material nearby. Such morphologies have been previously associated with branch retraction in other systems (Bernstein and Lichtman, 1999; Bishop et al., 2004).

In contrast to the similarities in RhoGAP and PTPMEG function observed in the dorsal lobe, RhoGAP and PTPMEG exert opposite effects on medial lobe axon branches.

While RhoGAP inhibition causes retraction of medial MB axon branches, the loss of *ptpmeg* causes medial lobe overextenstion. Interestingly, medial lobe overextension is observed in animals mutant for *drok*, a protein kinase negatively regulated by RhoGAP (Billuart et al., 2001). These phenotypic similarities raise the interesting possibility that PTPMEG and RhoGAP could affect related processes in the MB axons, with PTPMEG differentially affecting the activity of this pathway in the dorsal and medial branches. However, we have so far not detected genetic interactions between PTPMEG and the RhoGAP pathway (J.L.W. and P.A.G., unpublished data) and direct physical interactions between PTPMEG and RhoGAP or its downstream effectors are unlikely, as PTPMEG protein does not act in MB neurons. Nonetheless, these data are consistent with PTPMEG acting in cells that stabilize the pattern of MB axon branches, regulating signaling pathways that influence MB axon branch retention,

Proteins that interact with PTPMEG have not been identified in flies, but two proteins have been identified that associate with the PTPMEG homolog PTPN4 in the developing mouse brain. PTPN4 associates in vivo (as well as in yeast two-hybrid assays) with the NMDAR2B glutamate receptor subunit and with GRID2 (GluRd2), a glutamate-receptor related protein mutated in the ataxic mouse strains *lurcher* and *hotfoot* (Hironaka et al., 2000). Intriguingly, both of these PTPN4-interacting proteins can affect the behavior of innervating axons in a non-cell –autonomous fashion, acting in target cells. NMDA receptor activity in target cells can modulate the branching of innervating axons, as has been demonstrated both in the Xenopus visual system and the mouse somatosensory cortex (Cline and Constantine-Paton, 1989; Debski and Cline, 2002; Kutsuwada et al., 1996; Lee et al., 2005). GRID2 functions post-synaptically in mouse

cerebellar Purkinje cell to stabilize synaptic contact with incoming parallel fiber axon branches (Kurihara et al., 1997; Takeuchi et al., 2005), and elimination of GRID2 function after synapse formation results in a progressive loss of innervation by parallel fiber axons. Whether PTPN4 contributes to NMDAR- or GRID2-dependent axon stabilization in the mouse is unknown. While there is no clear fly ortholog of GRID2, the Drosophila ortholog of NMDAR2B, NMDAR2, contains a class I PDZ-binding motif at its C-termini, the site for the binding of the PDZ domain of PTPN4 to NMDAR2B (Hironaka et al., 2000). It will be of interest to examine the functional relationship between *ptpmeg* and *nmdar2* as genetic tools that permit the significant alteration of NMDAR function in flies become available.

Functional significance of PTPMEG-mediated regulation of axon branch stability

Our studies demonstrate an important role for the cytoplasmic tyrosine phosphatase PTPMEG in the stabilization of neuronal connectivity patterns in the fly mushroom body. As the mushroom bodies are critical for olfactory learning and memory in the fly, molecular pathways with the ability to elicit structural changes in the axons of mushroom body neurons, such as the one in which PTPMEG participates, are interesting candidates for mediating structural plasticity in this critical brain region. In addition, our studies show that PTPMEG activity is necessary to prevent a progressive loss of the animal's normal wiring pattern as it matures, inhibiting distal-to-proximal retraction of dorsal lobe MB axon branches and delayed overextension of medial lobe MB axon branches. Progressive distal-to-proximal disruptions in axonal branching are commonly observed in CNS neurodegenerative diseases like Alzheimer's and Parkinson's as well as

polyneuropathies associated with diabetes, alcoholism, and AIDS (Luo and O'Leary, 2005; Raff et al., 2002). Understanding the mechanisms that modulate axon branch maintenance, and the types of genetic lesions which give rise to axon branch destabilization, could therefore provide useful insights into the mechanisms that contribute to neurological disorders in humans.

ACKNOWLEDGEMENTS

We thank T. Schupbach, T. Preat, the Japanese stock center, and the Bloomington Stock Center for fly stocks. We thank J. Yang for the initial creation of a targeted insertion in the *ptpmeg* locus, M. Robichaux for converting the targeted insertion into a precise gene replacement, T. Tayler for Southern analysis of the replacement allele, and C. Kennedy for generating recombinant protein for antisera production. Thanks to M. Constantine-Paton and E. Nedivi for critical comments on the manuscript, and E. Hartwieg and H. R. Horvitz for microscope use.

REFERENCES CITED

Bargi, A., Cheng, HJ, Yaron, A, Pleasure, SJ, and Tessier-Lavigne, M. (2003). Stereotyped pruning of long hippocampal axon branches triggered by retraction inducers of the semaphorin family. *Cell* **113**, 285-299.

Bernstein, M., and Lichtman, JW. (1999). Axonal atrophy: the retraction reaction. *Current Opinion in Neurobiology* **9**, 364-370.

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

Bishop, D., Misgeld, T, Walsh, MK, Gan, WB, and Lichtman, JW. (2004). Axon branch removal at developing synapses by axosome shedding. *Neuron* **44**, 651-661.

Bretscher, A., Edwards, K, and Fehon, RG. (2002). ERM proteins and merlin: integrators at the cell cortex. *Nature Reveiws Molecular and Cellular Biology* **3**, 586-599.

Cline, H., and Constantine-Paton, M. (1989). NMDA receptor antagonists disrupt the retinotectal topographic map. *Neuron* **3**, 413-426.

Debski, E., and Cline, HT. (2002). Activity-dependent mapping in the retinotectal projection. *Current Opinion in Neurobiology* **12**, 93-99.

Dickson, B. (2002). Molecular mechanisms of axon guidance. *Science* **298**, 1959-1964.

Dunah, A., Hueske, E, Wyszynski, M, Hoogenraad, CC, Jaworski, J, Pak, DT, Simonetta, A, Liu, G, and Sheng, M. (2005). LAR receptor protein tyrosine phosphatases in the development and maintenance of excitatory synapses. *Nature Neuroscience* **8**, 458-467.

Flint, A., Tiganis, T, Barford, D, and Tonks, NK. (1997). Development of "substrate-trapping" mutants to identify physiological substrates of protein tyrosine phosphatases. *Proc Natl Acad Sci U S A* **94**, 1680-1685.

Hironaka, K., Umemori, H., Tezuka, T., Mishina, M., and Yamamoto, T. (2000). The protein-tyrosine phosphatase PTPMEG interacts with glutamate receptor δ2 and ε subunits. *J. Biol. Chem.* **275**, 16167-16173.

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

Johnson, K., and Van Vactor, D. (2003). Receptor tyrosine phosphatases in nervous system development. *Physiological Reviews* **83**, 1-24.

Katz, L., and Shatz, CJ. (1996). Synaptic activity and the construction of cortical circuits. *Science* **274**, 1133-1138.

Kaufmann, N., DeProto, J, Ranjan, R, Wan, H, and Van Vactor, D. (2002). Drosophila liprin-alpha and the receptor phosphatase Dlar control synapse morphogenesis. *Neuron* **34**, 27-38.

Kim, E., and Sheng, M. (2004). PDZ domain proteins of synapses. *Nature Reviews Neuroscience* **5**, 771-781.

Kurihara, H., Hashimoto, K, Kano, M, Takayama, C, Sakimura, K, Mishina, M, Inoue, Y, and Watanabe, M. (1997). Impaired parallel fiber --> Purkinje cell synapse stabilization during cerebellar development of mutant mice lacking the glutamate receptor delta2 subunit. *Journal of Neuroscience* **17**, 9613-9623.

Kutsuwada, T., Sakimura, K, Manabe, T, Takayama, C, Katakura, N, Kushiya, E, Natsume, R, Watanabe, M, Inoue, Y, Yagi, T, Aizawa, S, Arakawa, M, Takahashi, T, Nakamura, Y, Mori, H, and Mishina, M. (1996). Impairment of suckling response, trigeminal neuronal pattern formation, and hippocampal LTD in NMDA receptor epsilon 2 subunit mutant mice. *Neuron* **16**, 333-344.

Lee, L., Lo, FS, and Erzurumlu, RS. (2005). NMDA receptor-dependent regulation of axonal and dendritic branching. *Journal of Neuroscience* **25**, 2304-2311.

Lee, T., and Luo, L. (1999a). Mosaic analysis with a repressible cell marker for studies of gene function in neuronal morphogenesis. *Neuron* **22**, 451-461.

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**, 4065-4076.

Lee, T., Marticke, S, Sung, C, Robinow, S, and Luo, L. (2000). Cell-autonomous requirement of the USP/EcR-B ecdysone receptor for mushroom body neuronal remodeling in *Drosophila*. *Neuron* **28**, 807-818.

Levine, R., Morton, DB, and Restifo, LL. (1995). Remodeling of the insect nervous system. *Current Opinion in Neurobiology* **5**, 28-35.

Lichtman, J., and Colman, H. (2000). Synapse elimination and indelible memory. *Neuron* **25**, 269-278.

Luo, L., and O'Leary, DD. (2005). Axon retraction and degeneration in development and disease. *Annual Review of Neuroscience* **28**, 127-156.

Raff, M., Whitmore, AV, and Finn, JT. (2002). Axonal self-destruction and neurodegeneration. *Science* **296**, 868-871.

Rico, B., Beggs, HE, Schahin-Reed, D, Kimes, N, Schmidt, A, and Reichardt, LF. (2004). Control of axonal branching and synapse formation by focal adhesion kinase. *Nature Neuroscience* **7**, 1059-1069.

Sahin, M., Slaugenhaupt, SA, Gusella, JF, 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**, 7859-7863.

Sears, H., Kennedy CJ, Garrity PA. (2003). Macrophage-mediated corpse engulfment is required for normal Drosophila CNS morphogenesis. *Development* **130**, 3557-3565.

Takeuchi, K., Kawashima, A, Nagafuchi, A, and Tsukita, S. (1994). Structural diversity of band 4.1 superfamily members. *Journal of Cell Science* **107**, 1921-1928.

Takeuchi, T., Miyazaki, T, Watanabe, M, Mori, H, Sakimura, K, and Mishina, M. (2005). Control of synaptic connection by glutamate receptor delta2 in the adult cerebellum. *Journal of Neuroscience* **25**, 2146-2156.

Wang, Z., Shen D, Parsons DW, Bardelli A, Sager J, Szabo S, Ptak J, Silliman N, Peters BA, van der Heijden MS, Parmigiani G, Yan H, Wang TL, Riggins G, Powell SM, Willson JK, Markowitz S, Kinzler KW, Vogelstein B, Velculescu VE. (2004). Mutational analysis of the tyrosine phosphatome in colorectal cancers. *Science* **304**, 1164-1166.

Watts, R., Hoopfer, ED, and Luo, L. (2003). Axon pruning during Drosophila metamorphosis: evidence for local degeneration and requirement of the ubiquitin-proteasome system. *Neuron* **38**, 871-885.

Whited, J., Cassell A, Brouillette M, Garrity PA. (2004). Dynactin is required to maintain nuclear position within postmitotic Drosophila photoreceptor neurons. *Development* **131**, 4677-4686.

Zars, T. (2000). Behavioral functions of the insect mushroom bodies. *Current Opinion in Neurobiology* **10**, 790-795.

Zhang, S., Liu, J, Kobayashi, R, and Tonks, NK. (1999). Identification of the cell cycle regulator VCP (p97/CDC48) as a substrate of the band 4.1-related protein-tyrosine phosphatase PTPH1. *J Biol Chem* **274**, 17806-17812.

Zheng, X., Zugates, CT, Lu, Z, Shi, L, Bai, JM, and Lee, T. (2006). Baboon/dSmad2 TGF-beta signaling is required during late larval stage for the development of adult-specific neurons. *EMBO J.* **25**, 615-627.

CONCLUDING REMARKS

Neurons are highly polarized cells with distinctive morphologies dependant upon their functions. Complex molecular pathways are at work to ensure that neurons maintain their morphologies.

Here, the means by which the nucleus-containing cell body is positioned within a postmitotic neuron has been explored. Maintaining cell body position in *Drosophila* photoreceptor neurons requires the activity of Dynactin, the activator of the minus-end directed microtubule motor protein Dynein. The requirement for Dynactin in positioning postmitotic neuronal nuclei raises several fascinating issues. Does the maintenance of nuclear position require constant motor activity? Or, rather, is nuclear position normally fixed in a way that depends on some other molecular property of motor proteins, such as their acting as a cellular tether? If this is the case, it might be expected that further dissection of the requirement for Dynactin in photoreceptor nuclear positioning might reveal that motor proteins act to attach the nucleus to the apical cell cortex, perhaps through a membrane-bound protein complex.

Another exciting avenue for future exploration are the signals which control the positioning of Drosophila photoreceptor nuclei. Is there something in particular which guides the movement of the nucleus in the apical direction? If so, how is this signal produced and by which cells? Because loss-of-function alleles of the gene encoding one subunit of the Na^+/K^+ pump enhance the rough eye of *Glued* mutants, it is possible that the ion pumping function of the Na^+/K^+ pump is somehow involved in generating a polarity along the apical/basal aspect of photoreceptor cells that is used by molecular motors to guide nuclear positioning. Recently, calcium ion movement across gap

junctions has been linked to the interkinetic nuclear movements that occur in vertebrate retinal progenitor cells (Pearson et al., 2005), and perhaps ion movement may be involved in interkinetic nuclear movements in the *Drosophila* eye. Alternatively, because this Na^+/K^+ pump is also a component of the septate junctional complex in *Drosophila* (Genova and Fehon, 2003), which links cells together at their membranes near the apical surface, it is possible that it could be serving as a structural link as described above. A method for culturing developing *Drosophila* eye discs would allow for the testing of a potential functional requirement for ion translocation across the membrane in nuclear positioning. Elucidating the signals and the potential structural links will be exciting. A promising approach for identifying additional proteins involved in photoreceptor nuclear positioning is the enhancement/suppression screen in the adult eye. The enhancement/suppression screen here made use of the then available lethal transposable element insertions and thus only a fraction of the genes were considered.

While the movement of the nucleus during developmental events such as neuronal migration has been explored, the idea that the position of the nucleus might need to be actively maintained during later stages has received little attention. Here, I have identified a role for particular genes—*Glued*, *capping protein beta*, *dynein intermediate chain*, and *kinesin heavy chain*—in the maintenance of nuclear position within postmitotic neurons. Dynactin/Dynein have been previously characterized as being essential in various organisms for proper nuclear movement. Therefore, they represent examples of one class of nuclear positioning maintenance factors: those that are also used to move the nucleus. With additional studies, more factors required for both nuclear movement and nuclear maintenance might be identified. Additionally, some

maintenance-specific factors might also be uncovered. Perhaps some factors used to move the nucleus might need to be disengaged later to ensure that the nucleus does not move during maintenance stages. The role of any of these factors in nuclear positioning might be further defined by determining whether they act to control the space between the cell cortex and the microtubule organizing center, the space between the microtubule organizing center and the nucleus, or both.

In this thesis, the maintenance of axonal projections was also addressed, and a requirement for the predicted cytoplasmic tyrosine phosphatase PTPMEG was uncovered in the maintenance of a specific axonal branch in the *Drosophila* central brain. The role of PTPMEG in mushroom body axon maintenance could be further explored. For instance, which molecules act with PTPMEG to control maintenance? Is a specific molecular axon retraction pathway activated in the alpha lobes of *ptpmeg* mutants? How does the alpha lobe defect relate to the beta lobe overgrowth defect? Which neurons require *ptpmeg* function to maintain the alpha lobe axons trajectories?

There are several possible means of discovering genes and proteins that may work with *ptpmeg*. In the first, a genetic screen could be employed using the small eye associated with overexpression of Ptpmeg in the eye. A collection of mutants (deficiencies, P-element insertions, etc.) could be analyzed for their ability to enhance or suppress the small eye. Further experimentation would be required to determine if any such enhancers or suppressors worked to modify the *ptpmeg* loss-of-function phenotype in the nervous system. Another strategy for identifying *ptpmeg* interactors is to use biochemistry. The substrate trap mutants would be particularly beneficial for this approach. Head lysates could be made from transgenic flies carrying one of the substrate

trap insertions, and PTPMEG protein—wild-type and substrate trap version—could be pulled down using anti-PTPMEG. Bound substrates would also be expected to elute, and if the immunoprecipitate were applied loaded onto a protein gel, specific bands upregulated in flies with the substrate trap versus flies without the substrate trap might contain PTPMEG substrate. The substrate identity could be determined by mass spectrometry.

Both the maintenance of nuclear position and the maintenance of axonal trajectories will be interesting topics for further exploration in the *Drosophila* nervous system. The experiments presented in this thesis might serve as useful starting points for future experimentation regarding these topics.

References cited

Pearson, RA, Luneborg, NL, Becker, DL, and Mobbs, P. (2005). Gap junctions modulate interkinetic nuclear movement in retinal progenitor cells. *J. Neurosci.* **25**, 10803-10814.

Genova, JL, and Fehon, RG. (2003). Neuroglian, Gliotactin, and the Na+/K+ ATPase are essential for septate junction function in Drosophila. *J. Cell Biol.* **161**, 979-989.

APPENDIX A. Additional *cpb* alleles, phenotypes, and data.

Several aspects of the consequences of *cpb* loss of function were not included in Chapter 2, and will be discussed here.

A novel allele of *cpb*, cpb^{G141}, was recovered from the screen but not described in Chapter 2. Sequencing revealed that the cpb^{G141} allele has a G to A mutation that changes an aspartate to an asparagine at position 44 in the amino acid sequence (hence, D44N). cpb^{G141} acts as a hypomorphic allele; it is completel lethal (before third instar) in trans to the null cpb^{M143} allele, and it is semi-lethal in trans to the hypomorphic cpb^{F19} allele. Those cpb^{G141}/cpb^{F19} adults that do eclose have severely malformed bristles. Interestingly, however they do not display any obvious photoreceptor defects, and they do not have the cell body and nuclear mispositioning phenotype.

Animals which are heterozygous for the cpb^{M143} allele, but which have homozygous mutant eye tissue (as described in Chapter 2) grow into adults with extremely reduced eyes. Black necrotic tissue is commonly found on the eyes. Head bristles, which grow from tissue derived from the eye-antennal disc, are often missing; remaining head bristles have various abnormalities.

Apical/basal polarity was also performed on third instar eye discs from animals with cpb^{M143} homozygous mutant eye tissue. The apical marker Armadillo, which is found in adherens junctions, was missing in areas with large patches of mutant cpb^{M143} tissue. This result suggests that loss of *cpb* has different consequences than perturbation of Dynactin in $Glued^{1}$ mutants (where apical markers are retained despite nuclear mislocalization). Loss of apical markers in *cpb* mutant eye discs may reflect total migration of photoreceptors into the optic stalk and brain, without a trailing process seen

in *Glued*^{1}. Consistent with this possibility, anti-Chaoptin-labeled photoreceptors ectopically located in the brain have a more rounded shape than *Glued*^{1} photoreceptors, which are more elongated and oval-shaped. Further experiments could be devised to address the *cpb* phenotype in more detail. Two possibilities for the difference between *cpb* and *Glued*^{1} phenotypes can be proposed. In the first possibility, mutations in *cpb* affect both Dynein/Dynactin and filamentous actin function in photoreceptors. Proper filamentous actin capping activity may be required to establish or maintain apical contacts or to prevent retraction of the trailing process in photoreceptors with mispositioned nuclei. In the second possibility, *cpb*^{M143} produces a stronger Dynactin loss-of-function effect than the *Glued*^{1} mutation. In this case, *Glued*^{1} mutant photoreceptors might retain a trailing process because they still retain some Dynactin function. Further experimentation would be required to distinguish between these possibilities.

One experiment aimed at elucidating a potential role for F-actin capping in photoreceptor nuclear positioning was devised and performed. Since Capping protein caps barbed ends of F-actin, its activity is antagonistic to factors that promote subunit addition at the barbed end (such as Enabled) and to factors that sever F-actin, thereby creating more barbed ends (such as Profilin). In *Drosophila*, Profilin is encoded by the *chickadee* (*chic*) gene, and loss-of-function alleles of *chic* can partially suppress bristle defects caused by hypomorphic alleles of *cpb* (Hopmann and Miller, 2003). I tested whether the nuclear positioning defect and/or loss of apical markers in eye discs with *cpb*^{M143} homozygous mutant tissue could be suppressed by loss of *chic*. I created several lines of flies harboring recombinant chromosomes that were *cpb*^{M143} *FRT40A chic* and

analyzed eye disc mutant clones. I found that there was no obviously perceptible difference between eye discs that had clones of cpb^{M143} alone versus those that had cpb^{M143} *chic* clones with respect to either nuclear mispositioning or retention of apical markers. These chromosomes will be left with the Garrity lab should further experimentation become useful in the future.

I generated two additional types of transgenic flies harboring a cpb cDNA. The first cDNA contains a point mutation that results in an early stop codon at position 148 in the amino acid sequence (normally a tryptophan). These flies are designated *P[w+ UAS-cpbW148stop]*, and several lines were balanced and saved. This mutant was designed to express the truncated protein similar to the one produced by the two loss-of-function alleles described in chapter 2, cpb^{M143} and cpb^{F44}, which both encode a tryptophan to stop mutation at 148. The intention of making these flies was to determine if overexpressing this mutant version of the Cpb protein might cause a dominant-negative effect. I assayed consequences of overexpression with several drivers, including Elav-GAL4, GMR-GAL4, and Tubulin-GAL4. None produced any obvious external defects, and none caused photoreceptor cell body mispositioning. I also generated transgenic flies harboring a cpb cDNA with a leucine at amino acid 262 mutated to an arginine, designated *P[w+ UAS-cpbL262R]*. This point mutation was chosen because chicken capping protein beta subunits with the same mutation have severely impaired actin binding in vitro, but retain the ability to form heterodimers with capping protein alpha (Barron-Casella et al., 1995). When cpbL262R was overexpressed using Elav-GAL4, GMR-GAL4, or Tubulin-GAL4, there were no detectable defects (including photoreceptor morphology). I did not attempt to rescue the *cpb* photoreceptor

morphology defect with the cpbL262R construct. Several lines of each transgenic construct are balanced and have been left in the lab.

References cited

Barron-Casella, E. A., Torres, M. A., Scherer, S. W., Heng, H. H., Tsui, L. C., and Casella, J. F. (1995). Sequence analysis and chromosomal localization of human Cap Z. Conserved residues with the actin-binding domain may link Cap Z to gelsolin/severin and profilin protein families. *J. Biol. Chem.* **270**, 21472-21479.

Hopmann, R, and Miller, KG. (2003). A balance of capping protein and profilin functions is required to regulate actin polymerization in Drosophila bristle. *Molecular Biology of the Cell* **14**, 118-128.

APPENDIX B. Genetic screen for *Glued* interactors.

To gain mechanistic insight into other factors which may work with Dynactin to control nuclear positioning in photoreceptors, we devised a simple genetic screen to identify genes that interact with Glued. The screen was based on the dramatic, and easily scorable, adult rough eye in *Glued*^{1} mutants (described in chapter 2). *Glued*^{1} mutant eyes are narrower than wild-type, ommatidia are disorganized, distinct separations between adjacent ommatidia are often missing, and eye bristles are irregularly placed. The rough eye served as a sensitized background in which genetic disruptions in various other genes could be examined for enhancing or suppressing effects. To facilitate identification of causative genetic disruption, we chose to screen a collection of P-element insertions maintained at the Bloomington Stock Center. Only P-element insertions that were homozygous lethal were screened to increase the likelihood that each insertion screened represented a strong loss-of-function allele of their respective genes. Approximately 1800 lethal P-element insertion lines were obtained, in batches of 100, from Bloomington Stock Center over several months. Individual crosses were performed between Glued1/TM3 Sb Ser virgins and P-element males (balanced over various chromosomal balancers). Progeny which carried both *Glued*^{1} and P-element were scored by comparing their eyes to eyes of animals which were *Glued*^{1} without the P-element. Enhancers and suppressors were noted. Lines in which several animals were scored as enhancing or suppressing the *Glued*^{1} eye were investigated further.

To confirm a potential genetic interaction between *Glued*^{1} and a particular P-element insertion, other alleles of the gene closest to the insertion site were also tested. When available, published null alleles were obtained for the secondary screening.

Otherwise, alleles likely to cause loss-of-function were used. Approximately 15 P-element insertion lines were followed with this analysis. Data is summarized at beginning of "Jessica: Glued Enhancer/Suppressor" screen notebook. Briefly, the most promising insertion identified, other than the two insertions in *kinesin heavy chain* described in Chapter 2, was the insertion into *atpα,* which encodes a sodium/potassium pump subunit further described in Appendix C. Others showed either very mild changes in eye morphology, inconsistent results with multiple alleles, or did not show an interaction when re-tested.

An insertion into a novel gene, *CG10365*, was named "*sug1*" for "*suppressor of Glued-1*" because the only animal to survive to adulthood with both Gl^1 and sug^1 chromosomes had a very suppressed eye (total number of progeny analyzed was >129; only one of the correct genotype survived). *CG10365* is predicted to encode a novel protein of unknown function, with some homology to a bacterial protein, ChaC, of unknown function, that may associate with a cation transporter, ChaA (Flybase, InterPro). We (J. Whited and P. Phelps) made a "hop-out" of this P-element, in which the P-element was re-mobilized to determine if removing the insertion from this locus would revert the lethality/possible suppression phenotype. In brief, the various "hop-out" lines produced varied results, and the only commonality is that a couple (PHO4 and PHO5) seemed to enhance the Gl^1 eye, creating a shiny patch in the middle, although this phenotype was itself incompletely penetrant. Lethality with Gl^1 was rescued. These "hop-out" lines are balanced and will be left in the lab.

A similar screen was performed simultaneously by Andre Cassell, with assistance from J. Whited. In this screen, the same lethal P-element insertion collection was crossed

to flies expressing a myristoylated version of Pak (p21-activated kinase) under the control of *GMR-Gal4*. These flies also have rough adult eyes and mispositioned photoreceptor nuclei in eye discs (Hing et al., 1999). The most interesting P-element to be identified from this screen was an insertion in *capping protein alpha*, which enhanced the *GMR:Pak^{myr}* rough eye.

All of the data from the *Glued* and *GMR:Pak^{myr}* screens are contained in several notebooks left in the Garrity lab.

Reference cited

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

APPENDIX C. Interaction between *Glued* and *Atpα* and potential role of Na^+/K^+-ATPase in photoreceptor nuclear positioning.

A P-element insertion in *Atpα* was recovered in the screen as a dominant enhancer of *Glued*^{1} (Figure C1). The insertion was re-tested to verify that it was an enhancer. In all, >30 eyes were examined from *Glued*^{1}/*Atpα* flies, and all showed enhancement of the *Glued* rough eye. A deficiency uncovering the *Atpα* locus was obtained from Bloomington Stock Center. This deficiency also showed the same enhancement of the *Glued* phenotype, indicating that loss of *Atpα* function was likely to be responsible for the enhancement.

Atpα (*Na pump α subunit*) encodes the alpha subunit of the *Drosophila* sodium/potassium pump, Na^+/K^+-ATPase (Lebovitz et al, 1989). This is a multimeric protein which uses energy derived from ATP hydrolysis to move sodium ions out of the cell and potassium ions into the cell, both against their electrochemical gradients. The same pump also serves a structural role in *Drosophila* epithelia cells, as it is a part of the septate junctional complex (Genova and Fehon, 2003). To further explore a potential function for the Na^+/K^+-ATPase in photoreceptor nuclear positioning, I examined animals with mutations in the gene encoding the beta subunit, *nervana2* (*nrv2*). Homozygous null *nrv2* animals die before third instar, so I used a mosaic strategy (discussed in Chapter 2 for *cpb*) to produce homozygous mutant *nrv2* eye tissue in otherwise heterozygous animals. Third instar eye discs from *nrv2* mosaic animals were dissected and stained with anti-Elav to evaluate photoreceptor nuclear position. In all eyes examined (n>10), several photoreceptor nuclei were mispositioned along the apical-basal axis and had become positioned at the basal plane of the eye disc (Figure C2), suggesting *nrv2* is

required for normal apical/basal positioning of photoreceptor nuclei. However, in contrast to *Glued*^{1} or *cpb* mutants, there were no mispositioned *nrv2* photoreceptor nuclei within the optic stalk and, presumably, the brain. Adult animals with *Nrv2* homozygous eye tissue displayed a small eye similar in appearance, but less pronounced, to animals with *cpb* homozygous eye tissue. These experiments suggest that the Na^{+}/K^{+}-ATPase may be involved in photoreceptor nuclear positioning.

References cited

Lebovitz, RM, Takeyasu, K, and Fambrough, DM. (1989). Molecular characterization and expression of the (Na+/K+)-ATPase alpha-subunit in Drosophila melanogaster. *EMBO J.* **8**, 193-202.

Genova, JL, and Fehon, RG. (2003). Neuroglian, Gliotactin, and the Na+/K+ ATPase are essential for septate junction function in Drosophila. *J. Cell Biol.* **161**, 979-989.

Figure C1. Loss-of-function *Atpa* enhances *Glued* rough eye. A wild-type adult eye is shown at left. *Glued*^{1} eyes are significantly reduced in size and ommatidia are disorganized, giving a rough appearance. Loss of one copy of *Atpa* significantly worsens the roughness of the *Glued*^{1} eye, producing large, shiny ommatidia, and significantly reduces the eye's size.

Figure C1

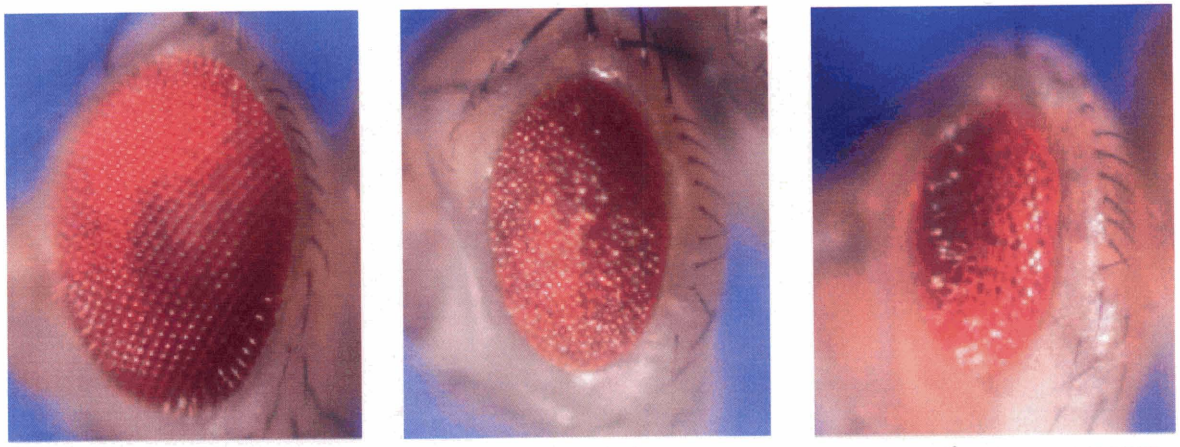
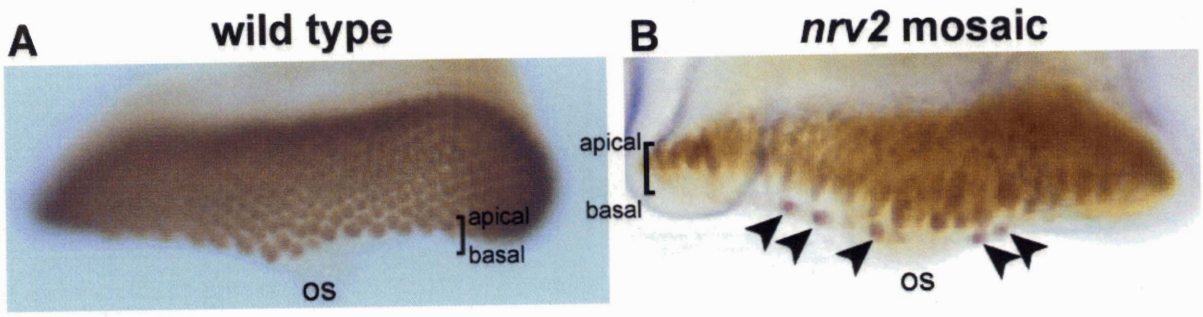


Figure C2. *nrv2* may be required for photoreceptor nuclear positioning. (A) An eye disc from a wild-type animal, as viewed from a top/lateral direction, unmounted, in glycerol. Anti-Elav stains photoreceptor nuclei, which are all arranged toward the apical surface. (B) An eye disc from a mosaic animal heterozygous loss-of-funciton *nrv2* with homozygous *nrv2* eye tissue. Note that several photoreceptor nuclei appear to be situated basally (arrowheads). (os) denotes optic stalk.

Figure C2



APPENDIX D. Effects of Ptpmeg overexpression on organ size.

Overexpression of wild-type Ptpmeg in several different tissues results in reduced tissue size, suggesting Ptpmeg may be capable of antagonizing growth-promoting pathways. Several transgenic fly lines carrying a wild-type Ptpmeg cDNA P-element insertion were created for use in rescue experiments and overexpression studies (Joyce Yang). When overexpressed in the eye using *eyeless-GAL4*, Ptpmeg caused a reduction in eye size (Figure D1). Reduced eyes appeared to be patterned normally. When overexpressed in a portion of the wing disc using *decapentaplegic-GAL4*, Ptpmeg caused a reduction in specifically in this portion (Figure D2, green versus purple areas). Additionally, the crossvein was missing in all cases (n>50). The ability of overexpressed Ptpmeg to cause the deletion of the crossvein appears to be reliant upon its phosphatase activity; when any of three transgenes predicted to disrupt phosphatase activity were overexpressed in the wing, the crossvein was present and appeared wild-type. The potential role of the phosphatase activity in mediating the tissue size effects could easily be assayed by measuring the size of the *dpp-GAL4* area in animals overexpressing these transgenes. I have already done the experiment and mounted the wings on coverslips, so if a future lab member is interested, she or he can simply take photographs on the microscope and calculate the relevant areas using Photoshop.

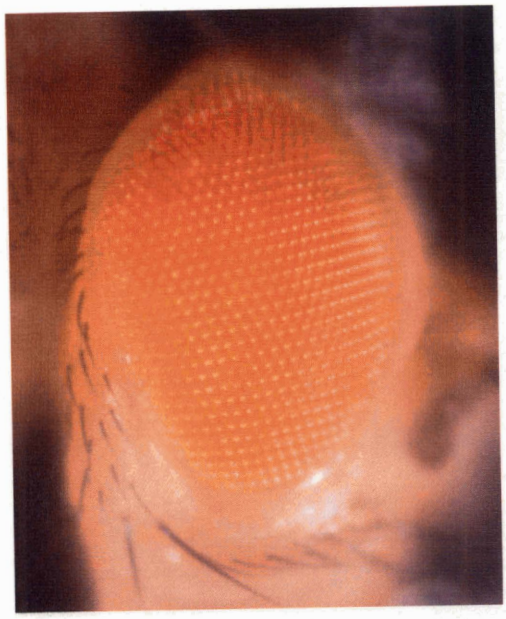
The potential role of endogenous PTPMEG in control of organ size remains unclear. Animals which are genetic nulls for *ptpmeg* are not statistically different in total body weight from control animals (J.L.W.). PTPMEG protein has been detected by immunohistochemistry in the eye (J.L.W.), however loss of *ptpmeg* function does not alter the eye's size or patterning in any perceptible manner (J.L.W.). Pseudopupils were

examined from *ptpmeg* null animals and found to be normal (P.A. G. and J.L.W.). Photoreceptor axon targeting was examined in *ptpmeg* mutants and was completely normal (J.L.W.).

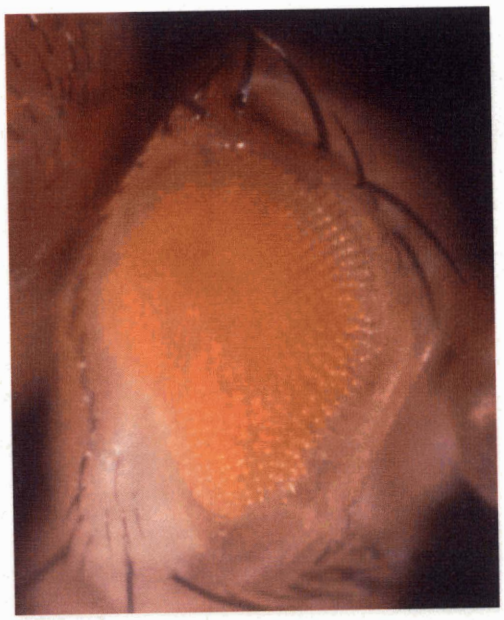
Overexpressed Ptpmeg was found to be capable of antagonizing the insulin signaling pathway. Overexpressing Ptpmeg in the eye strongly suppresses the effects of overexpression of activated insulin receptor (dInR) in the eye (Figure D3). Activated PI3-Kinase, which acts downstream of the insulin receptor, also causes eye and wing enlargement when overexpressed, and these effects are also suppressed by overexpression of Ptpmeg, as were the effects of insulin receptor overexpression in the wing (Figure D4).

Figure D1. Overexpression of Ptpmeg in the eye causes a reduction in eye size. At left is the adult eye from a control *eyeless-GAL4* animal expressing GAL4 in eye tissue. It has a wild-type appearance. At right is the adult eye from an animal in which *eyeless-GAL4* is used to overexpress Ptpmeg in the eye. Note that this eye has a reduced size, particularly in its ventral aspect. Ommatidial subunit organization appears relatively wild-type.

Figure D1



UAS-ptpmeg ; CyO•KrGFP



UAS-ptpmeg ; eyeless-GAL4

Figure D2. Overexpression of Ptpmeg causes a reduction in tissue size. Shown at left is the wing from an animal expressing *Dpp-GAL4*. This transgene drives expression of GAL4 specifically in the area of the developing wing which will give rise to the cells between longitudinal vein 3 (L3) and vein 4 (L4) in the adult wing, as shaded in green. A control area, between vein 4 (L4) and vein 5 (L5), which does not express *Dpp-GAL4* is shaded purple. The ratio of the area between L3-L4 and the area between L4-L5 [(L3-L4)/(L4-L5)] is about 1.0 in control animals. In animals in which *Dpp-GAL4* drives expression of the UAS-Ptpmeg transgene, thereby causing overexpression of Ptpmeg specifically in L3-L4 (green), the size of this area is significantly reduced compared to control areas in the same wing (purple). Note that the crossvein found in the *Dpp-GAL4* area is also deleted in animals overexpressing Ptpmeg there.

Figure D2

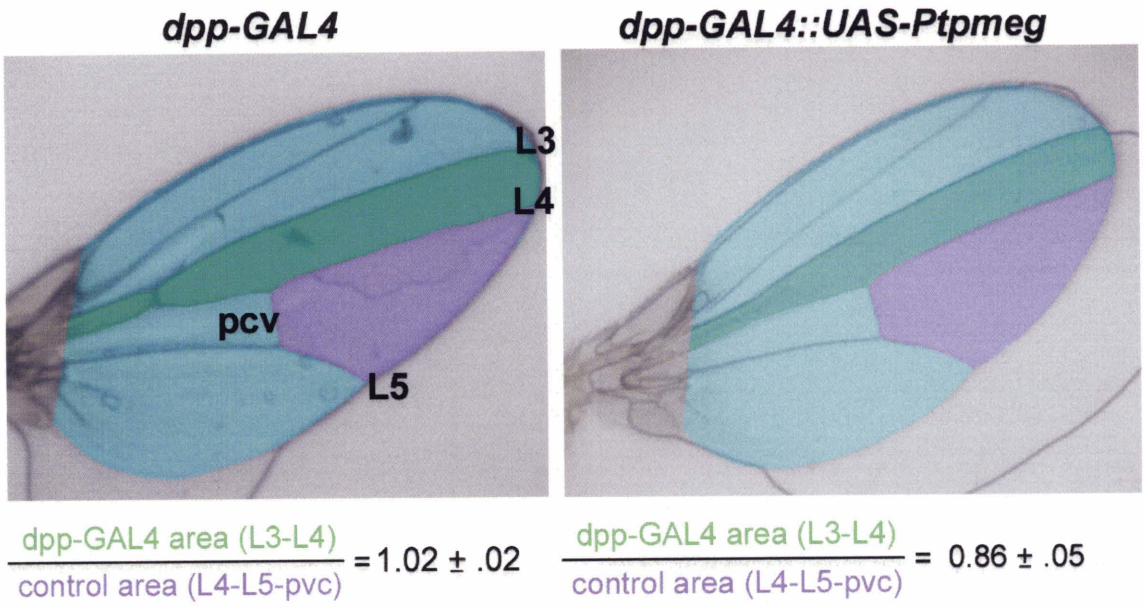
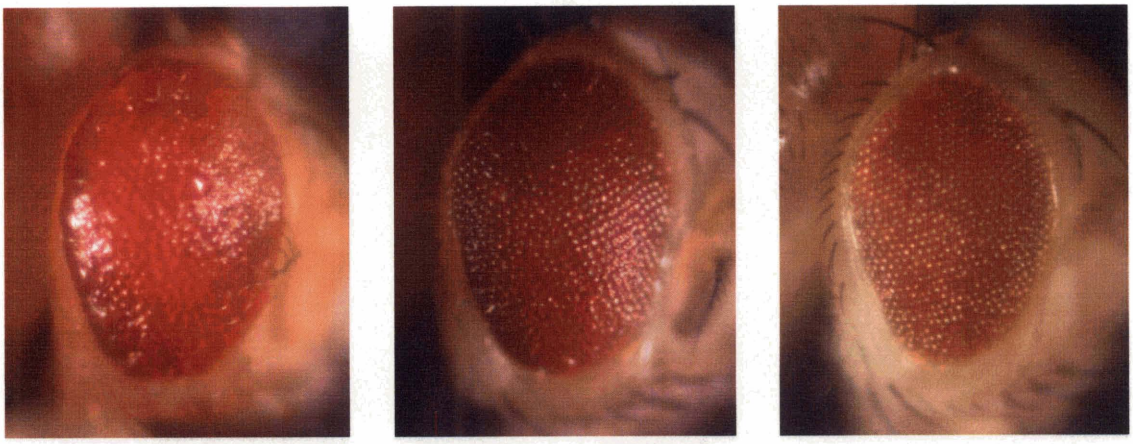

dpp-GAL4
dpp-GAL4::UAS-Ptpmeg
L3
L4
pcv
L5
dpp-GAL4 area (L3-L4) / control area (L4-L5-pvc) = 1.02 ± .02
dpp-GAL4 area (L3-L4) / control area (L4-L5-pvc) = 0.86 ± .05

Figure D3. Overexpression of Ptpmeg can antagonize the effects of Insulin signaling pathway activation in the eye. Pictured at left is a fly overexpressing activated insulin receptor in the eye, causing a bulging of the eye and a blistered appearance. Simultaneously overexpressing Ptpmeg (middle), restores a practically wild-type appearance to the eye. Overexpessing Ptpmeg alone in eye causes a reduction in eye size (right).

Figure D3

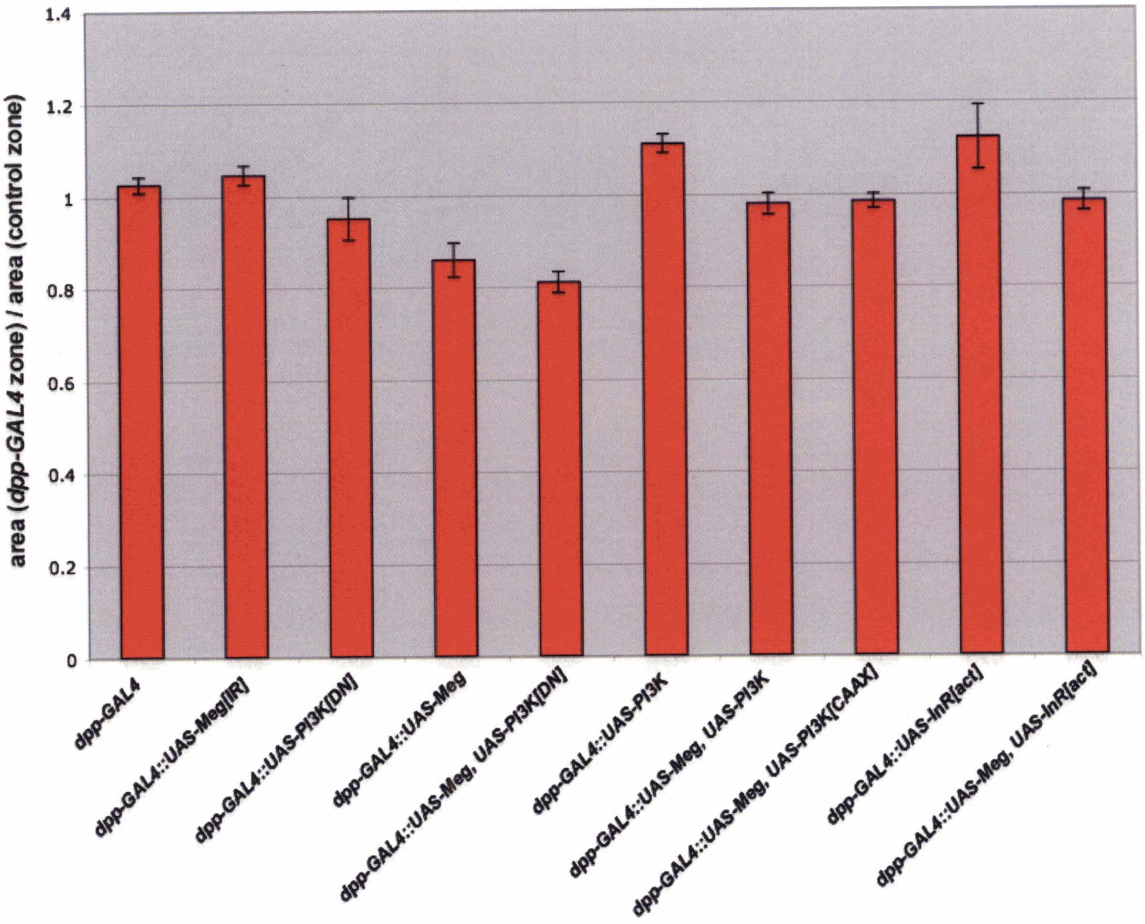


GMR-GAL4 ; UAS-InR^{A1325D}

UAS-ptpmeg ;GMR-GAL4 ; UAS-InR^{A1325D}

UAS-ptpmeg ;GMR-GAL4

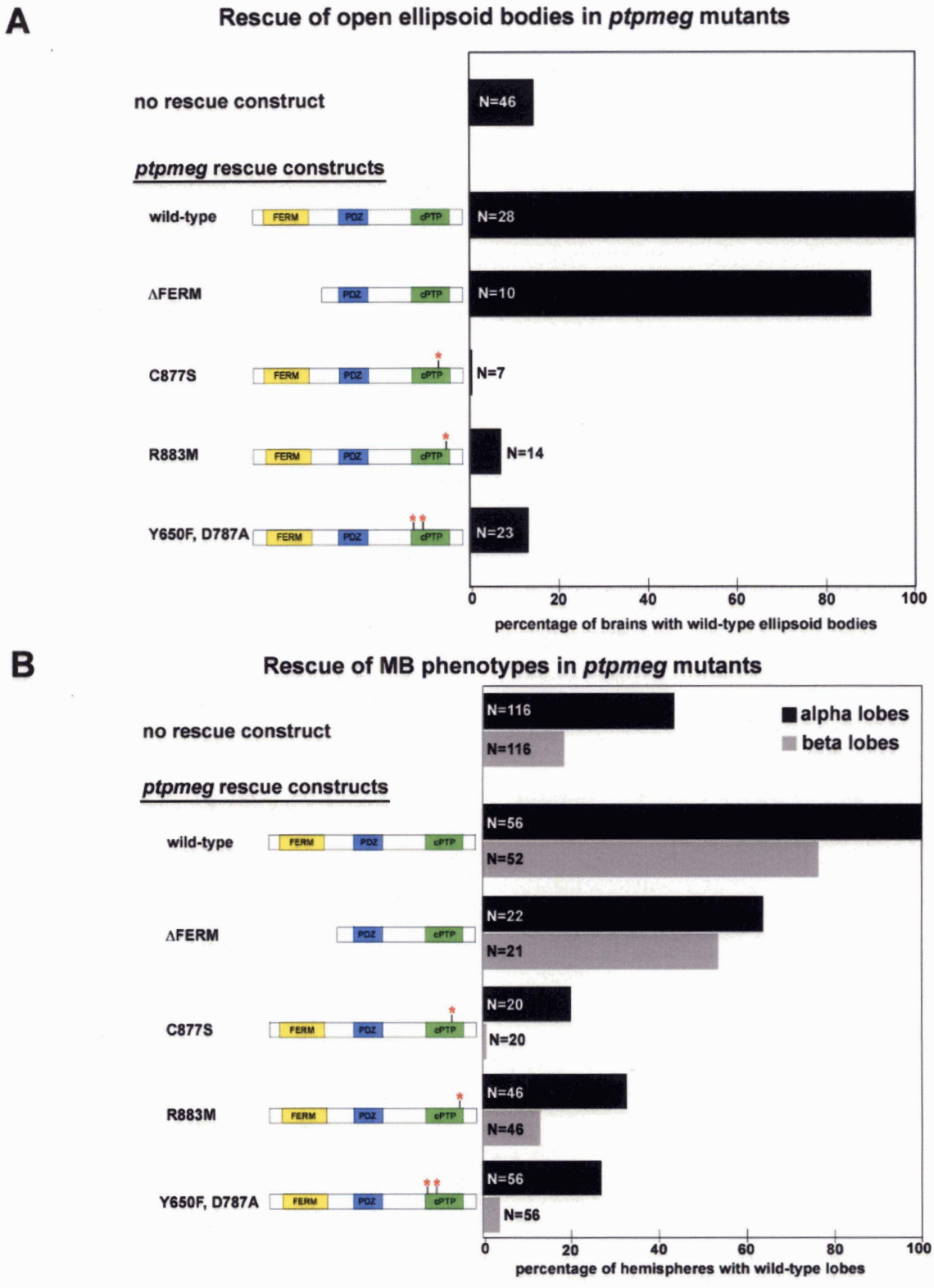
Figure D4. Ptpmeg overexpression can antagonize the effects of insulin signaling pathway activation in a compartment of the wing. Genotypes are shown on X-axis. *Dpp-GAL4* is a specific driver that is expressed only in cells which will form the area between longitudinal veins 3 and 4 in an adult wing. The area between longitudinal veins 4 and 5 is used as a control. In wild-type (not shown), and *dpp-GAL4* animals, the ratio of the *Dpp-GAL4* area to the control area (Y-axis) is close to 1.0. Overexpression of the *Meg[IR]* transgene, which induces reduction of Ptpmeg transcripts via RNA interference, in the *dpp-GAL4* area slightly increases the relative size of this compartment, but not significantly. Overexpression of dominant-negative [DN] PI3-Kinase (PI3K) significantly reduces tissue size. Overexpression of wild-type Ptpmeg reduces tissue size even more, and together, overexpression of dominant-negative PI3K and Ptpmeg has an additive effect in reducing wing area size. Conversely, activating the insulin signaling pathway by overexpressing wild-type PI3K (or constitutively active PI3K [CAAX], not shown) causes a relative increase in wing area size. This increase can be suppressed by simultaneously overexpressing wild-type Ptpmeg. Activating insulin signaling by expressing activated Insulin receptor (InR[act]) also causes an increase in wing area, an effect which is also suppressed when Ptpmeg is simultaneously overexpressed.


area (dpp-GAL4 zone) / area (control zone)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
dpp-GAL4
dpp-GAL4::UAS-Meg[IR]
dpp-GAL4::UAS-PI3K[DN]
dpp-GAL4::UAS-Meg
dpp-GAL4::UAS-Meg, UAS-PI3K[DN]
dpp-GAL4::UAS-PI3K
dpp-GAL4::UAS-Meg, UAS-PI3K
dpp-GAL4::UAS-Meg, UAS-PI3K[CAAX]
dpp-GAL4::UAS-InR[act]
dpp-GAL4::UAS-Meg, UAS-InR[act]

APPENDIX E. Role of the FERM domain in PTPMEG function in the ellipsoid body and mushroom bodies.

To characterize a potential requirement for PTPMEG's FERM domain in its nervous system function, we generated transgenic flies which harbor an incomplete Ptpmeg cDNA lacking the FERM domain under the control of UAS. We used these flies to test whether the FERM domain is required for the proper development of the ellipsoid body and for the proper maintenance of the mushroom bodies. Wild-type Ptpmeg expressed in all neurons using *Elav-Gal4* completely rescues EB defects in *ptpmeg*^{1} mutants (see Chapter 3), and the version missing the FERM domain (ΔFERM) also provides substantial rescue, although not complete, of the EB (Figure E1, A). ΔFERM's ability to rescue the EB defects was not significantly different from wild-type Ptpmeg's ability to rescue the EB defects (Fischer's test; P=0.2632). However, ΔFERM does not rescue the MB defects as well (Figure E1, B), suggesting there is a stricter requirement for PTPMEG's FERM domain in the maintenance of mushroom body axon architecture than for EB development. There was no statistically significant rescue of α lobe defects in *ptpmeg*^{1} mutants expressing ΔFERM versus those without a rescue construct (Fischer's test; P=0.1533). ΔFERM expression partially rescued the β lobe defects (Fischer's test, P=0.0016, comparing ΔFERM to no rescue construct). ΔFERM does rescue MB defects considerably more than PTPMEG versions with disrupted phosphatase activity.

Figure E1. Effects of loss of the FERM domain on the ability of neuronally-expressed Ptpmeg to rescue *ptpmeg*^{1} CNS phenotypes. (A) Substantial rescue of *ptpmeg*^{1} ellipsoid body defects is achieved with a transgene missing the FERM domain. (B) The transgene missing the FERM domain does not rescue mushroom body defects as well, although it is substantially more effective than ones which have disrupted phosphatase activity.


A
Rescue of open ellipsoid bodies in ptpmeg mutants
no rescue construct
ptpmeg rescue constructs
wild-type
ΔFERM
C877S
R883M
Y650F, D787A
FERM
PDZ
cPTP
N=46
N=28
N=10
N=7
N=14
N=23
0
20
40
60
80
100
percentage of brains with wild-type ellipsoid bodies
B
Rescue of MB phenotypes in ptpmeg mutants
no rescue construct
ptpmeg rescue constructs
wild-type
ΔFERM
C877S
R883M
Y650F, D787A
alpha lobes
beta lobes
N=116
N=116
N=56
N=52
N=22
N=21
N=20
N=20
N=46
N=46
N=56
N=56
0
20
40
60
80
100
percentage of hemispheres with wild-type lobes

BIOGRAPHICAL NOTE

Jessica LaMae Whited was born February 8, 1976, in Monroe, Michigan to Deborah Ann (Zinner) and Timothy Curtis Whited. She has one sister, Kimberly Susan (Whited) Tweedie, born in 1977. Ms. Whited spent the first eight years of her childhood in Monroe. The family moved to O'Fallon, Missouri, in 1984, and remained within a few miles of there. Ms. Whited graduated valedictorian from Fort Zumwalt South High School in 1994. She holds bachelors degrees in Biology and Philosophy from the University of Missouri in Columbia, where she graduated summa cum laude in 1998.