R7 PHOTORECEPTOR AXON TARGETING AND PRESYNAPTIC ASSEMBLY IN *DROSOPHILA*

by

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The development of a functional nervous system is paramount for the ability of animals to interact with their environments. Minor defects in nervous system function compromise the effectiveness of sensing and responding to stimuli. Severe defects in nervous system function often lead to extreme sensory, cognitive and motor skill impairment. The nervous system is a complex network of connections, with each neuron making functional contacts with several other neurons. Any single animal species generally exhibits a stereotyped pattern of neuronal connectivity, but the specific intrinsic and extrinsic signals that impart to a neuron its unique connective properties have only recently begun to be identified.

In this study, we use the *Drosophila* visual system to examine neuronal connectivity. Our screen for genes involved in R7 photoreceptor connectivity led to the identification of the RhoGAP domain-containing protein *dsyd-1* and the transcriptional

repressor *tramtrack*. Flies harboring homozygous mutant *dsyd-1* R7s fail to phototax towards UV light, an innate behavior mediated by the R7s. Subsequent analysis of axons of *dsyd-1* R7s showed abnormal morphology in the region of presynaptic sites, suggesting that similar to its role in *C. elegans*, *dsyd-1* is involved in presynaptic assembly. Further analysis demonstrated a requirement for *dsyd-1* function in docking presynaptic components to terminal sites of contact.

R7 axons are restricted to non-overlapping columns in the optic neuropil, thereby preserving spatial visual information in the retintopic map. The axon terminals of *tramtrack* mutant R7s exhibit overgrowth, similar to that observed in R7s that have loss of function mutations in genes involved in the activin signaling pathway. Previous studies have shown that activin signaling is involved in restricting R7 axons to their appropriate columns, and our results demonstrate that tramtrack may be functioning in the same pathway. One of two *tramtrack* isoforms, *ttk69*, is expressed in photoreceptors after they have differentiated, and expression of *ttk69* is specifically required for R7 axon targeting.

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I dedicate this document to my parents, who encouraged me to pursue an advanced degree in biology.

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CHAPTER I

INTRODUCTION TO R7 PHOTORECEPTOR AXON TILING AND PRESYNAPTIC ASSEMBLY

Nervous System Development and the Drosophila Visual System

The development of a fully functional nervous system is crucial for the ability of an animal to sense and respond to stimuli in the environment. Our cognitive abilities and motor skills rely on proper development and maintenance of our central nervous system. Failure to properly develop neural connections or the normal number and identity of neurons can leads to impairment of thought and motor processes, (Morfini et al., 2009). The central nervous system, (CNS), is a complex network of connections and how the network forms is a central question in neurobiology.

Development of the nervous system begins with the specification of neural precursor cells followed by the birth of neurons and glia from those progenitors, (Doe et al., 1998). After each neuron is born, it must migrate to its appropriate layer in the brain, extend neurites, and make contacts with proper synaptic partner cells. The tips of axons and dendrites are the sites of active growth, and they are specialized for sensing chemical attractants and repellents in the environment, (O'Donnell et al., 2009). Axons often extend long distances through multiple tissue layers. The unique identities of neurons impart to them the ability to distinguish different layers and also different potential

synaptic partner cells. Upon arrival in the proper layer in the brain, neurons of the CNS recognize their appropriate partner cell and form a functional connection, (Martin et al., 1995). In this study, I use the genetic model organism Drosophila to identify genes involved in axon tiling and presynaptic assembly.

The *Drosophila* visual system is an ideal model for studying axon target selection. The retina is composed of about 800 repeated units called ommatidia, and each ommatidium contains 8 photoreceptors and multiple support cells, (Fischbach and Hiesenger, 2008). The 8 photoreceptors can be divided into three classes based on their sensitivity to the light spectrum. The R1 through R6 photoreceptors, functionally orthologous to vertebrate rod cells, detect blue light and are largely involved in motion detection, (Hardie 2001). The R8 photoreceptor detects green light, and the R7 photoreceptor detects *uv* light. Fruit flies phototax towards *UV* light, an innate behavior of fruit flies that is exploited in this study.

The three classes of photoreceptors extend axons and form connections with partner cells in spatially distinct layers in the brain and also in a temporal progression, (Clandinin and Zipursky, 2000). The first photoreceptors to elaborate axons, the R1-R6s, project their axons to the first optic neuropil, the lamina. The R8 axon then extends through the lamina and into the second optic neuropil, the medulla, where it will eventually connect with its partner cell. Finally, the R7 sends its axon along the R8's axon into the medulla where it extends slightly past the R8 axon terminal. Since the R7 axon fasciculates with the previously elaborated R8 axon, the R7 growth cone likely makes few axon guidance decisions until it passes the R8 terminal.

Upon entry into the medulla early in pupal development, the R7 growth cone is dense with filopodia. The idle growth cone remains relatively stationary while other layers in the medulla are being elaborated. By early-mid pupal stages the filopodia retract and the R7 axon terminal begins to appear morphologically mature and indistinguishable from the adult R7 axon terminal, (Ting et al., 2005). It is not known whether the changes in R7 terminal structure during this stage in pupal development precede or are concomitant with presynaptic assembly. Electron microscopy experiments have shown that the locations of presynaptic sites in R7s are at the axon terminals, (Gao et al., 2008). I demonstrate in this study that presynaptic components are transported to the terminals of early pupal R7s, and thus a correlation exists between morphological changes and enrichment of presynaptic components in axon terminals.

Tiling of R7 axons

Retinotopy is a method employed by organisms to preserve the spatial features of visual stimuli detected by photoreceptors as they get transmitted to higher order neurons in the brain, (Roskies et al., 1995). In flies, the photoreceptors of neighboring ommatidia perceive a slightly different point in space. R7s from neighboring ommatidia project axons that terminate in neighboring columns in the medulla. The tiling of R7 and R8 axons is readily apparent when the medullas of brains are imaged by microscopy, but the mechanisms that prevent adjacent photoreceptors from invading neighboring columns are only just now starting to become known, (Ting et al., 2007).

A recent screen for genes involved in axon targeting identified the *activin* signaling pathway as key mechanism in restricting R7 axons to their individual columns. R7s lacking any component of the *activin* signaling pathway display invasive axon terminals at a low frequency. Genetic removal of a neighboring R7 increases the frequency of invasiveness of *activin* pathway mutant R7s, suggesting that R7 terminals receive instructive signals from neighboring R7s. The extracellular Activin is believed to be an autocrine signal, originating from an R7 that receives its own signal. Baboon, the Activin receptor localized to the R7 growth cone receives and transmits the signal to the nucleus via phosphorylation of Dsmad. Phosphorylated Dsmad is transported to the nucleus by Importin-α through a retrograde microtubule transport mechanism that uses Dynactin as a motor. Activin signaling is likely occurring during mid-pupal stages to restrict R7 growth cones to their individual columns. A paracrine signal also restricts R7 axons, as removal of neighboring axons in *baboon*, *importin*, or *dsmad* mutant R7s results in an increase in the frequency of invasiveness, (.

Screen for genes involved in R7 axon connectivity

Drosophila is highly amenable to genetic manipulations and as such we are able to efficiently perform forward genetic screens to identify genes involved in axon targeting. We exploit the flies' natural behavior to phototax towards *uv* light to identify genes involved in R7 photoreceptor axon targeting. Our screen involves first mutagenizing flies then balancing chromosome 3R to stably maintain flies with mutations in genes that are critical for early stages of development. The phototaxis towards uv light

behavior requires only a fraction of functional R7s in the fly's retina, and through genetic manipulation we make flies with a large fraction of non-functioning or dead R7s and a small fraction of homozygous mutant R7s. The flies are then subjected to a behavioral assay in a T-shaped tube that gives them the choice to phototax towards either *uv* light or bright light. Flies with homozygous wild type R7s prefer to move towards the *uv* light at a high frequency. The flies with homozygous mutant R7s that fail to move towards the *uv* light are set aside and eventually analyzed for R7 connectivity.

To analyze mutant flies that were isolated in the *uv* choice behavioral assay, I employed the MARCM (mosaic analysis with a repressible cell marker) system. Heterozygous flies containing homozygous mutant labeled R7s were dissected and analyzed for R7 axon connectivity defects. A variety of mutant phenotypes and complementation groups were isolated by this method of initial screening through the behavioral assay then histological analysis of R7 axon terminals. Two complementation groups are the focus of this study. In Chapter 3, I present data from the complementation group that harbors mutation in *tramtrack*, a gene that encodes a protein involved in heterochromatin formation. In Chapter 2, I will discuss my analysis of the single allelic complementation group W46. W46 mutant flies harbor a mutation in the *dsyd-1* gene, which encodes a protein involved in presynaptic assembly.

Assembly of Presynaptic Specializations

A fundamental question in developmental neurobiology is how presynaptic sites are assembled. Presynaptic sites, specialized for the chemical or electrical transmission of

information to a post-synaptic cell, are specified soon after an axon reaches its target. In *c. elegans*, the transmembrane molecule Syg-1 functions in the initial specification of presynaptic sites. Once the locations are specified, components of the presynaptic specialization are targeted to the specified sites. Molecular motors carry presynaptic cargo along microtubules in an anterograde direction. Syd-2/Liprin-α and Syd-1 function in HSN axons in *c. elegans* to localize cargo to presynaptic sites. Upon arrival at the sites of specification within axons, presynaptic components can begin to be assembled into structures specialized for the release of neurotransmitter. Genetic dissection of the presynaptic assembly pathway in c. elegans has demonstrated a hierarchy of proteins involved in the assembly program, and whether a similar mechanism exists in Drosophila will be addressed in this study.

CHAPTER II

DROSOPHILA DSYD-1 IS REQUIRED FOR PRESYNAPTIC ASSEMBLY

INTRODUCTION

Neurons are organized into circuits by synapses, the sites at which electrical signals from one neuron are conveyed to another. Chemical synapses translate these electrical signals into unidirectional neurotransmitter release and reception, requiring the assembly of distinct machineries at pre- and postsynaptic sites (reviewe in Colon-Ramos, 2009). Within the presynaptic cell, synaptic vesicles and active zone proteins must be transported down the axon and directed to sites of contact with appropriate postsynaptic targets (reviewed in Margeta and Shen, 2008; Owald and Sigrist, 2009). The R7 photoreceptor neurons in the Drosophila eye provide a genetically tractable system in which to study presynaptic development at terminal synapses (Ting and Lee, 2007). R7 axons select their synaptic targets in two stages (Ting et al., 2005). They first extend to a temporary target layer within the medulla, in which their growth cones remain expanded but paused; this temporary target corresponds approximately to the adult M3 layer. By 50 hours after puparium formation (hr APF) the R7 axons extend beyond this layer and by 60 hr APF have reached their final targets in M6; the R7 growth cones appear to mature directly into the ellipsoid bouton-like terminals seen in adult (Ting et al., 2005; data not shown). Recent electron microscopic reconstruction has shown that most presynaptic

sites in each R7 are located within these ellipsoid terminals or in the adjacent thickened "neck" region, which together span layers M5 and M6 (Takemura et al., 2008).

Previous genetic screens have identified three genes that are required for the second step of R7 synaptic target selection: N-cadherin (Ncad), LAR receptor phosphatase, and Liprin-a/SYD-2 (Lee et al., 2001; Clandinin et al., 2001; Maurel-Zaffran et al., 2001; Nern et al., 2005; Hofmeyer et al., 2006; Choe et al, 2006). Of these, Liprin-a/SYD-2 has emerged as a key regulator of presynaptic development in both worm and fly (Zhen and Jin, 1999; Kaufmann et al., 2002). Because Liprin-a/SYD-2 is found at nascent presynaptic sites and binds multiple active zone proteins, a simple model is that it acts as a scaffold for presynaptic components, which are diffusely localized in its absence. However, there is also evidence that Liprin-a/SYD-2 is required for efficient axonal transport of synaptic vesicles (Miller et al., 2005; Wagner et al., 2009). The atypical RhoGAP protein SYD-1 has recently been identified in C. elegans as an upstream regulator of Liprin-a/SYD-2 (Hallam et al., 2002; Dai et al., 2006; Patel et al., 2006; Patel and Shen, 2009). While homologs of SYD-1 exist in both fly and vertebrates, whether they perform similar functions is unknown. In addition, SYD-1's role in presynaptic assembly has previously been analyzed at synapses that occur en passant - that is, at one or more specific locations along the length of the axon.

Here we present our identification of null mutations in the fly homolog, dsyd-1, and present an analysis of their effects on presynaptic development within R7 terminals.

RESULTS

dsyd-1 is required for normal R7 function and axon terminal morphology

To identify genes required for R7 axon targeting and synaptogenesis, we have been screening for mutations that disrupt an R7-dependent behavior, phototaxis toward UV light (Lee et al., 2001; Ting et al., 2007). We analyze mosaic animals in which R7s but few other cells are homozygous mutant, and we subsequently determine histologically whether those with behavioral defects have R7s with abnormal connectivity. By this approach, we identified a new mutation, W46, that decreases UV preference (data not shown) and affects R7 axon morphology (Fig. 1A-B'). While wild-type R7 axons thicken in the region just beyond the M3 layer and terminate in an approximately ellipsoid bouton in the M6 layer of the medulla (Fig. 1A,A'), most W46 mutant R7 axons terminate in considerably reduced boutons in the M6 layer, many have thickenings prior to the M5 layer, and some terminate in the M3 layer (Fig. 1B,B'), phenotypes resembling those caused by loss of Ncad, LAR, or Liprin-a (Lee et al., 2001; Maurel-Zaffran et al., 2001; Clandinin et al., 2001; Hofmeyer et al., 2006; Choe et al., 2006). In addition, W46 mutant R7 terminals often have thin extensions that project beyond the M6 layer and terminate in small bouton-like structures (Fig. 1B,B'). We have previously found that mutant R7 axons can terminate at M3, the R8 recipient layer, as a consequence of adopting the R8 fate (Morey et al., 2008). To determine

whether the axon defects of W46 mutant R7s might be caused by such a cell fate transformation, we examined their expression of rhodopsin. We found that, as in wild type (Fig. 1C), W46 mutant R7 rhabdomeres (Fig. 1D) express the R7-specific rhodopsins Rh3 or Rh4. We conclude that, like mutations in Ncad, LAR, and Liprin-a, W46 specifically disrupts R7 axon morphology.

We mapped W46 to a small region that included the gene CG1976, the predicted fly homolog of C. elegans syd-1 (Hallam et al., 2002), which we have therefore named dsyd-1. We sequenced the dsyd-1 genomic DNA from a W46-containing chromosome and found a single basepair change within a conserved 3' splice site sequence adjacent to the seventh exon of dsyd-1 (Fig. 1E); this exon is predicted to encode part of the RhoGAP homology domain.

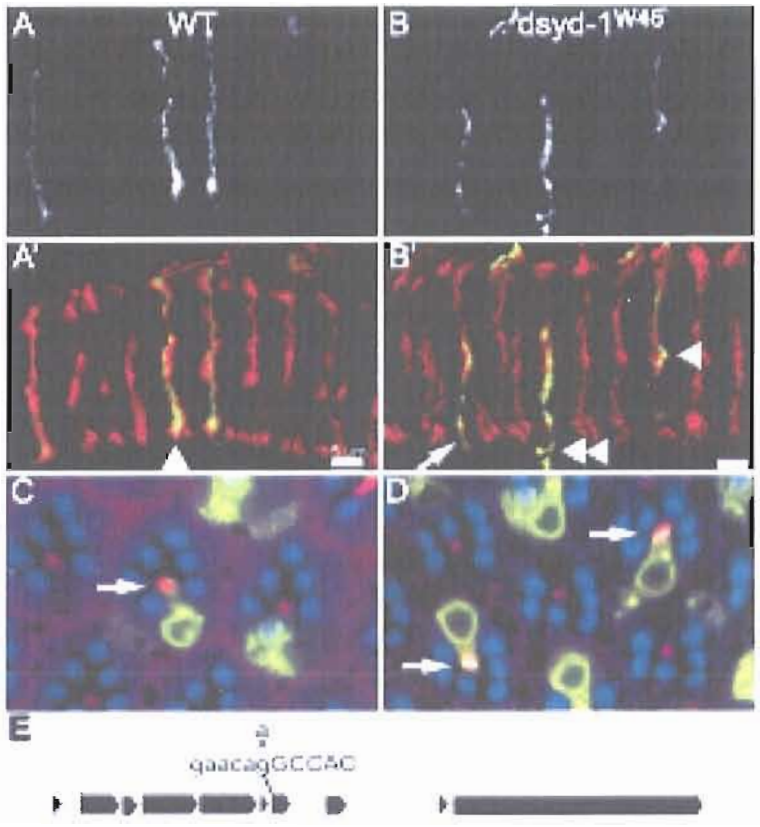

Figure 1. *dsyd-1* mutant R7 axon terminals are morphologically abnormal.
A-D, Mosaic animals in which ~10% of R7s are homozygous for a specific chromosome arm and express mCD8-GFP (green; see Experimental Procedures).
A-B', Young adult medullas. All R7 and R8 axons are labeled with mAb24B10 (red). A,A', Each wild-type R7 axon terminates at the M6 layer in an ellipsoidal bouton (arrows; note that mCD8-GFP does not label the full outline of each R7 terminal, as assessed by mAb24B10 staining). B,B', Most dsyd-1^{W46} mutant R7 axon terminals are reduced in diameter (arrow); some have minor extensions beyond the M6 layer (double arrowhead); and others are absent from the M6 layer and terminate instead at the M3 layer or between the M3 and M6 layers (arrowhead). C,D, Adult retinas in which R1-R6 rhabdomeres are labeled with anti-Rh1 antibody (blue). Wild-type (C) and dsyd-1^{W46} mutant (D) R7s (arrows) express R7-specific rhodopsins (red) and are morphologically indistinguishable. E, The W46 mutation is a G to A change that disrupts a conserved 3' splice site in the predicted sixth intron of dsyd-1.

Loss of dsyd-1 causes a weaker R7 axon targeting defect than does loss of Ncad, LAR, or Liprin-a

While loss of syd-1 or liprin-a from C. elegans HSN neurons causes identical defects in presynaptic assembly (Dai et al., 2006; Patel et al., 2006), R7s homozygous for the dsyd-1^{W46} allele have a less severe axon morphology defect than Liprin-a mutant R7s, indicating either that dsyd-1^{W46} does not completely eliminate dsyd-1 function or that the relationship between syd-1 and liprin-a may not be the same in C. elegans HSNs as in Drosophila R7s. To determine the null phenotype of dsyd-1, we generated a deletion of the predicted dsyd-1 coding sequence, which we refer to as "dsyd-1^{CD}", by inducing recombination between FRT-containing piggyBac transposons inserted at molecularly-defined locations (Fig. 2A); this deletion also removes most of a second gene, ferrochelatase, predicted to encode a mitochondrial enzyme. Homozygous dsyd-1^{CD} mutant R7 axons had defects indistinguishable from those of dsyd-1^{W46} mutant R7s, including reduced terminal boutons (Fig. 2B,B'), termination in the M3-M5 layers (Fig. 2B,B'), and extensions beyond the M6 layer that terminate in small boutons (Fig. 3). We quantified the first two of these phenotypes and found that dsyd-1^{W46} and dsyd-1^{CD} mutant R7 axons are indistinguishable (Fig. 2C). In confirmation that all three phenotypes are caused by loss of the dsyd-1 gene, we found that all are rescued by forced expression of FLAG-tagged Dsyd-1 (Fig. 2C; data not shown). We conclude that (1) W46 behaves like a null allele of dsyd-1 and (2) loss of dsyd-1 causes less frequent mistargeting of R7 axons to the M3 layer than loss of Ncad, LAR or Liprin-a, indicating that it does not act in a strictly linear pathway with any of these genes.

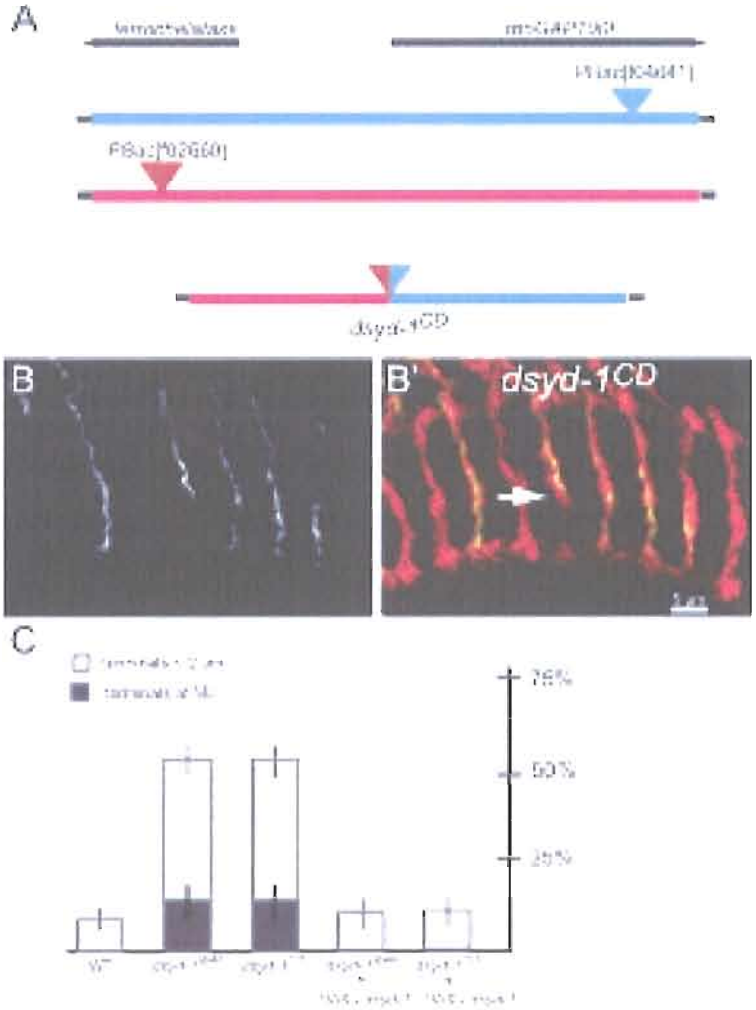

Figure 2. Loss of dsyd-1 causes fewer R7 axons to retract from their normal target layer than does loss of Ncad, LAR, or Liprin-α. A, Schematic showing the locations of the two FRT-containing PiggyBac transposons used to generate a deletion of dsyd-1 by FLP-induced recombination. The resulting dsyd-1^{CD} chromosome is depicted at the bottom and deletes most of dsyd-1, as well as the first half of the gene ferrochelatase. B,B', A young adult medulla containing dsyd-1^{CD} mutant R7 axons expressing mCD8-GFP (green); all R7 and R8 axons are labeled with mAb24B10 (red). Like dsyd-1^{W46} mutant R7 axons, most R7 axons entirely lacking dsyd-1 terminate in reduced boutons and some retract to the M3 layer (arrow). See Supp. Fig. 1 for dsyd-1^{CD} mutant R7 terminals with thin extensions. C, Quantification of the dsyd-1^{W46} and dsyd-1^{CD} R7 phenotypes and their rescue by expression of a FLAG-tagged UAS-dsyd-1 transgene. All were scored in mosaic animals in which ~10% of R7s were homozygous for either a wild-type (FRT82) or dsyd-1 mutant chromosome arm and expressed Synaptotagmin-GFP (Syt-GFP). dsyd-1^{W46} and dsyd-1^{CD} mutant R7 terminals are indistinguishable and retract less frequently than Ncad, LAR, or liprin-alpha R7s are reported to do.

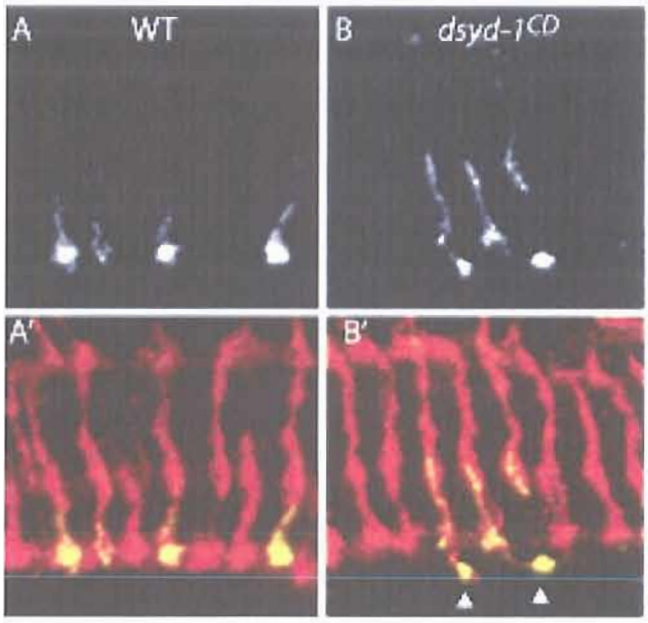

Figure 3. Like *dsyd-1*^{W46} mutant R7s, *dsyd-1*^{CD} mutant R7 terminals often project thin extensions beyond the M6 layer that terminate in bouton-like structures.
A-B', Young adult medullas containing homozygous R7 axons expressing the synaptic vesicle marker Syt-GFP (green). All R7 and R8 axons are labeled with mAb24B10 (red). A,A', Wild-type R7s. B,B', dsyd-1^{CD} homozygous mutant R7s. Note that the bouton-like structures at the ends of the thin extensions contain Syt-GFP (arrowheads).

dsyd-1 is required for a late step of R7 terminal development

R7 axon target selection can be divided into two stages (Ting et al., 2005). During late larval and early pupal development, R7 axons project to a temporary target layer, in which their terminal growth cones remain expanded. At 50 hours after puparium formation (hr APF) the R7 growth cones begin to project deeper into the medulla, ultimately stopping at their final target layer, M6. The ellipsoid shape of mature R7 terminals appears to originate directly from the shape of the R7 growth cones: we have observed no stage at which R7 terminals are not expanded, although their filopodia gradually disappear (data not shown; Ting et al., 2005). To distinguish whether, like Ncad, dsyd-1 is required for normal growth cone morphology during the first stage of R7 axon targeting, or, like LAR and Liprin-a, is required only later, we assessed R7 axon terminal morphology at 24 hr APF, 40 hr APF, and 60 hr APF. At both 24 hr APF (data not shown) and 40 hr APF (Fig. 4A-B'), wild-type and dsyd-1 mutant R7 axon terminals were indistinguishable. However, by 60 hr APF (Fig. 4C-D'), dsyd-1 mutant R7 terminals were reduced in diameter (as assessed by mAb24B10 staining; see also Fig. 5 and Fig. 6), and occasionally located in the M3-M5 layers. We conclude that dsyd-1 is not required for R7 growth cones to project to or interact with their temporary target layer and instead is required for the maintenance of normal terminal morphology and interaction with their final target layer. Because dsyd-1 mutant R7 terminals did not have thin, blobby extensions at 24 hr APF, 40 hr APF, or 60 hr APF, we conclude that these extensions are not simply remnants left after the failure to maintain an expanded terminal morphology but instead are the result of an active process that occurs at a later timepoint.

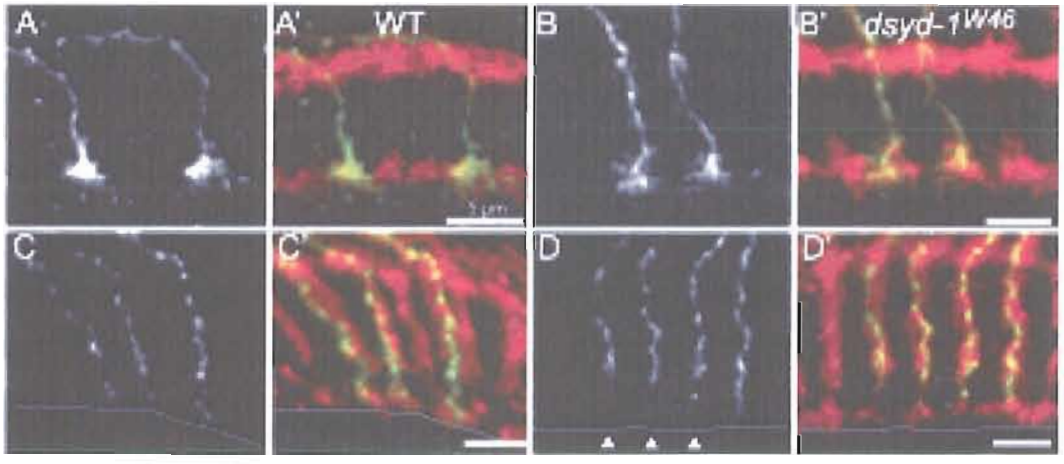

Figure 4. *dsyd-1* is required for a late step of R7 terminal development.
A-D, Pupal medullas containing homozygous R7 axons expressing CD8-GFP (green). All R7 and R8 axons are labeled with mAb24B10 (red). At 40 hours after puparium formation (hr APF), wild-type (A) and dsyd-1^{W46} mutant (B) R7 axons terminate in the correct target layer and are indistinguishable. However, by 60 hr APF many dsyd-1^{W46} mutant R7 terminals (D) are reduced in diameter compared with wild-type R7 terminals or have retracted (C; arrowheads). Note again that mCD8-GFP labels less of the R7 terminal than mAb24B10; see Supp. Fig. 2 for mAb24B10 alone. See also Fig. 4C-D'.

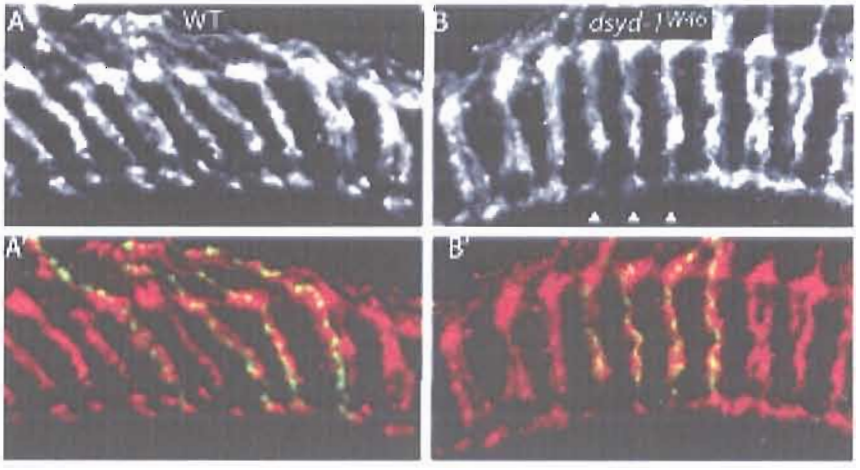

Figure 5. *dsyd-1* mutant R7 terminals are wild-type at 40 hr APF but reduced in diameter by 60 hr APF. A-B', Expanded views of Figure 3C' and D'. While mCD8-GFP does not always fill R7 terminals, a comparison of mAb24B10 staining alone (A,B) clearly demonstrates that many dsyd-1 mutant terminals (B,B') are reduced in diameter or have retracted by 60 hr APF (arrowheads).

dsyd-1 is required for the retention of synaptic vesicles at R7 axon terminals

In C. elegans, syd-1 is required either to recruit or to maintain presynaptic components at the appropriate subcellular locations. We therefore next tested whether the morphological defect of dsyd-1 mutant R7 terminals was correlated with a defect in localizing presynaptic components. A recent electron microscopic reconstruction of the medulla indicates that most R7 synapses and synaptic vesicles are located in layers M4-M6 (Takemura et al., 2008). Indeed, when we used our GMR-FLP/MARCM method to express the synaptic vesicle marker Synaptotagmin-GFP (Syt-GFP) in individual wild-type adult R7 axons, we found it to be predominantly localized to this region (Fig.

6A,A'). By contrast, Syt-GFP is broadly distributed within dsyd-1 mutant axons, in which it often accumulates in discrete puncta (Fig. 6B,B').

To distinguish whether dsyd-1 is required for the transport of Syt-GFP to R7 terminals or instead for its retention there, we examined the timecourse of Syt-GFP localization. In wild-type, Syt-GFP is enriched within R7 terminals at 24 hr APF (Fig. 6C,C'), even before they have projected to their final target layer at 60 hr APF (Fig. 6E,E'). We found that Syt-GFP is similarly enriched in dsyd-1 mutant R7 terminals at 24 hr APF (Fig. 6D,D'), indicating that loss of dsyd-1 does not prevent Syt-GFP from reaching R7 terminals. By 36 hr later, however, Syt-GFP is no longer predominantly at dsyd-1 mutant R7 terminals and instead accumulates in puncta at varying distances away (Fig. 6F,F'). We therefore conclude that dsyd-1 is required for the preferential retention of Syt-GFP at R7 terminals during their second step of target layer selection. Consistent with this model, we find that FLAG-tagged Dsyd-1 is localized to R7 terminals by early to mid-pupal development (Fig. 7; data not shown).

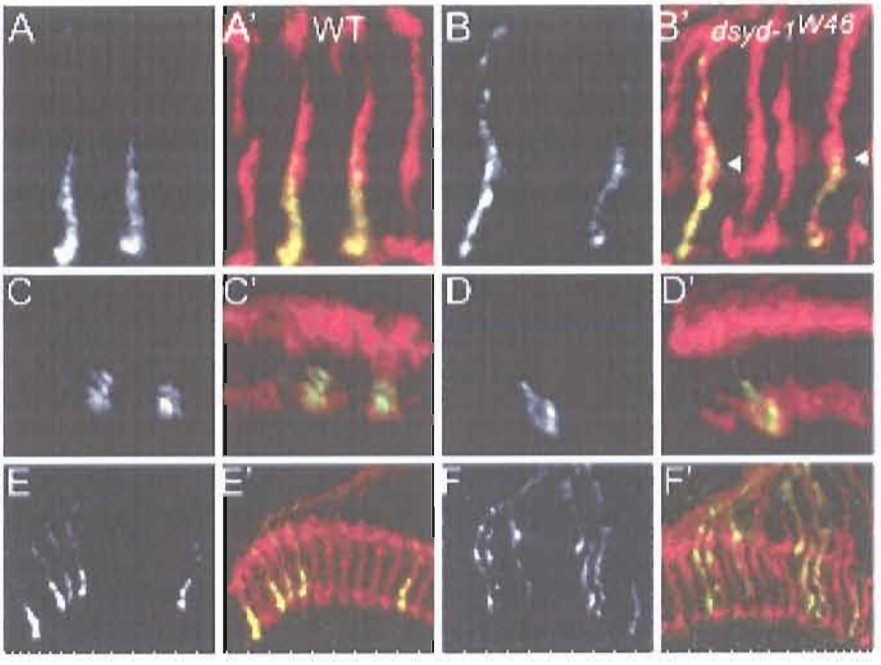

Figure 6. *dsyd-1* is required for the retention of synaptic vesicles at R7 terminals.
A-F', Medullas containing homozygous R7 axons expressing the synaptic vesicle marker Syt-GFP (green). All R7 and R8 axons are labeled with mAb24B10 (red).
Syt-GFP is enriched in wild-type R7 terminals in young adult (A,A') as well as in pupae at 24 hr APF (C,C') and 60 hr APF (E,E'). While Syt-GFP is enriched in dsyd-1^{W46} mutant R7 terminals at 24 hr APF (D,D'), it become more broadly distributed by 60 hr APF (E,E') and remains so in adult (B,B').

dsyd-1 is also required for the phototransduction-independent enrichment of mitochondria at R7 axon terminals

In addition to synaptic vesicles and other presynaptic components specialized for the transmission of information from pre- to post-synaptic cells, mitochondria are often enriched at synapses. However, it remains unclear what proportion of mitochondria, if any, might be physically associated with presynaptic sites rather than transported there in response to acute energy needs. To assess whether mitochondria are normally enriched at R7 terminals, we used the GMR-FLP/MARCM method to express the mitochondrial marker Mito-GFP in individual wild-type R7 axons. We found that, like Syt-GFP, Mito-GFP is predominantly localized to R7 terminals between the M4 and M6 layers (Fig. 8A,A'). By contrast, in dsyd-1 mutant R7 axons Mito-GFP is found in broadly distributed puncta (Fig. 8B,B'), a defect that is rescued by forced expression of FLAG-tagged Dsyd-1 (Fig. 8C,C'). To test whether this effect on Mito-GFP localization might be specific to dsyd-1, we examined Mito-GFP within those Liprin-a mutant R7 axons whose terminals remained at the M6 layer. We found a similar broad distribution of Mito-GFP (Fig. 8D,D'), indicating that this effect is instead shared by at least one other gene that regulates R7 connectivity. To test whether this Mito-GFP mislocalization might be an indirect consequence of the presumed disruption of evoked synaptic activity at R7 terminals caused by loss of dsyd-1 or Liprin-a, we examined the localization of MIto-GFP in animals hemizygous for a null mutation in norpA, a gene required for phototransduction (Bloomquist et al., 1988). We found that loss of norpA does not alter the enrichment of Mito-GFP in R7 axon terminals (Fig. 8E-F'), and therefore conclude

that loss of dsyd-1 or Liprin-a disrupts the localization of mitochondria that are enriched at R7 terminals independent of light-evoked activity. In turn, the morphological defects of dsyd-1 and Liprin-a mutant R7s are unlikely to be an indirect consequence of mitochondrial mislocalization, since R7s homozygous for a null mutation in dmiro, a gene required for normal axonal transport of mitochondria (Guo et al., 2005), have wild-type axon morphology (data not shown).

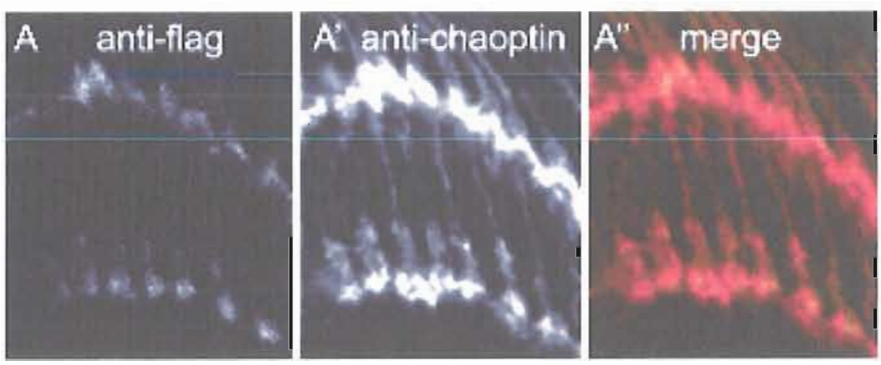

Figure 7. FLAG-tagged Dsyd-1 is enriched at R7 axon terminals at 50 hr APF. A-A'', Medullas of wild-type 50 hr APF pupae in which expression UAS-FLAG-dsyd-1 is driven in all R neurons by GMR-Gal4. Anti-FLAG staining is in green (A) and mAb24B10 in red (A').

dsyd-1 mutant animals can reach adulthood but have abnormal synapses

To determine whether, like LAR and Liprin-a, dsyd-1 might be required for normal neuron-muscle as well as neuron-neuron connectivity, we set out to examine the NMJs of dsyd-1^{W46}/dsyd-1^{CD} transheterozygous larvae. In generating these animals, we noticed that some survived to adulthood: in confirmation that their dsyd-1 function is

impaired, we found that, like individual dsyd-1 mutant R7 terminals, most R7 terminals in dsyd-1^{W46}/dsyd-1^{CD} animals were reduced in size, and some were absent from the M6 target layer (Fig. 9A,B). We therefore next examined the synaptic boutons at identified NMJs in dsyd-1^{W46}/dsyd-1^{CD} third instar larvae and found a slight but significant reduction in bouton number compared with wild type (Fig. 9C-E). The mildness of this phenotype compared with that caused by loss of LAR or Liprin-a is consistent with our finding that dsyd-1 mutant R7 axons have weaker defects than those of LAR or Liprin-a muant R7s. However, the functional consequences are considerable: both dsyd-1^{W46}/dsyd-1^{CD} larvae (Fig. 9F) and adults (data not shown) have strong locomotory defects, although we did not distinguish the degree to which the latter were caused by NMJ defects per se.

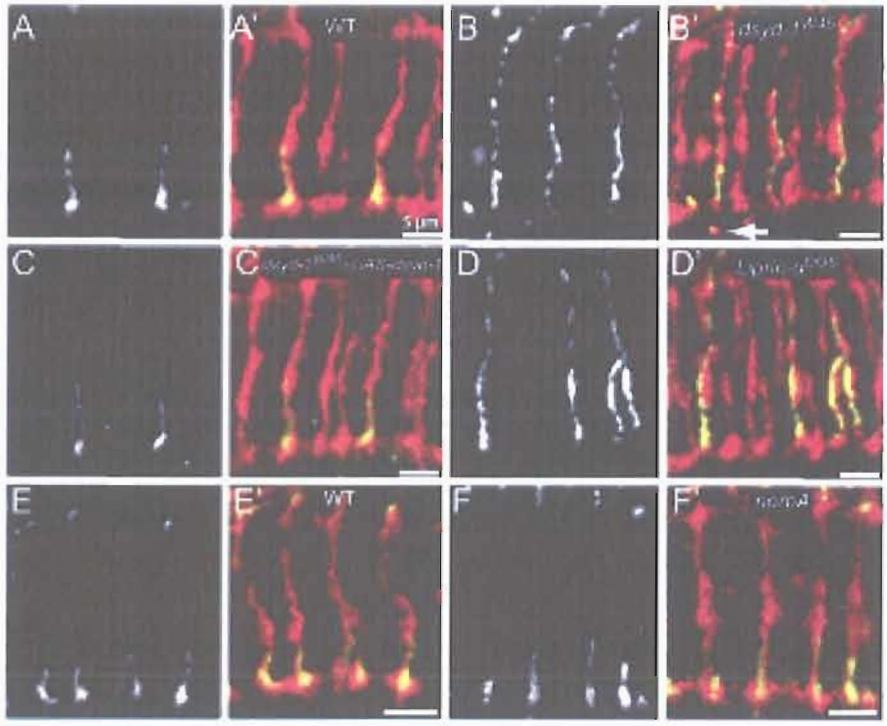

Figure 8. *dsyd-1* is required for the enrichment of mitochondria at R7 terminals. A-D', Young adult medullas containing homozygous R7 axons expressing the mitochondrial marker Mito-GFP (green). All R7 and R8 axons are labeled with mAb24B10 (red). Like Syt-GFP, Mito-GFP is enriched at wild-type R7 terminals (A,A') but is more broadly distributed in dsyd-1^{W46} mutant R7 axons (B,B'). Expression of FLAG-tagged UAS-dsyd-1 restores enrichment of Mito-GFP at dsyd-1^{W46} mutant R7 terminals (C,C'). Mito-GFP is also more broadly distributed in liprin-alpha mutant R7 axons (D,D'). E-F', Young adult medullas in which PANR7-Gal4 drives expression of UAS-mito-GFP in all R7s. As in wild type (E,E'), Mito-GFP remains enriched in the R7 terminals of hemizygous norpA mutants (F,F'), which lack phototransduction and therefore evoked activity.

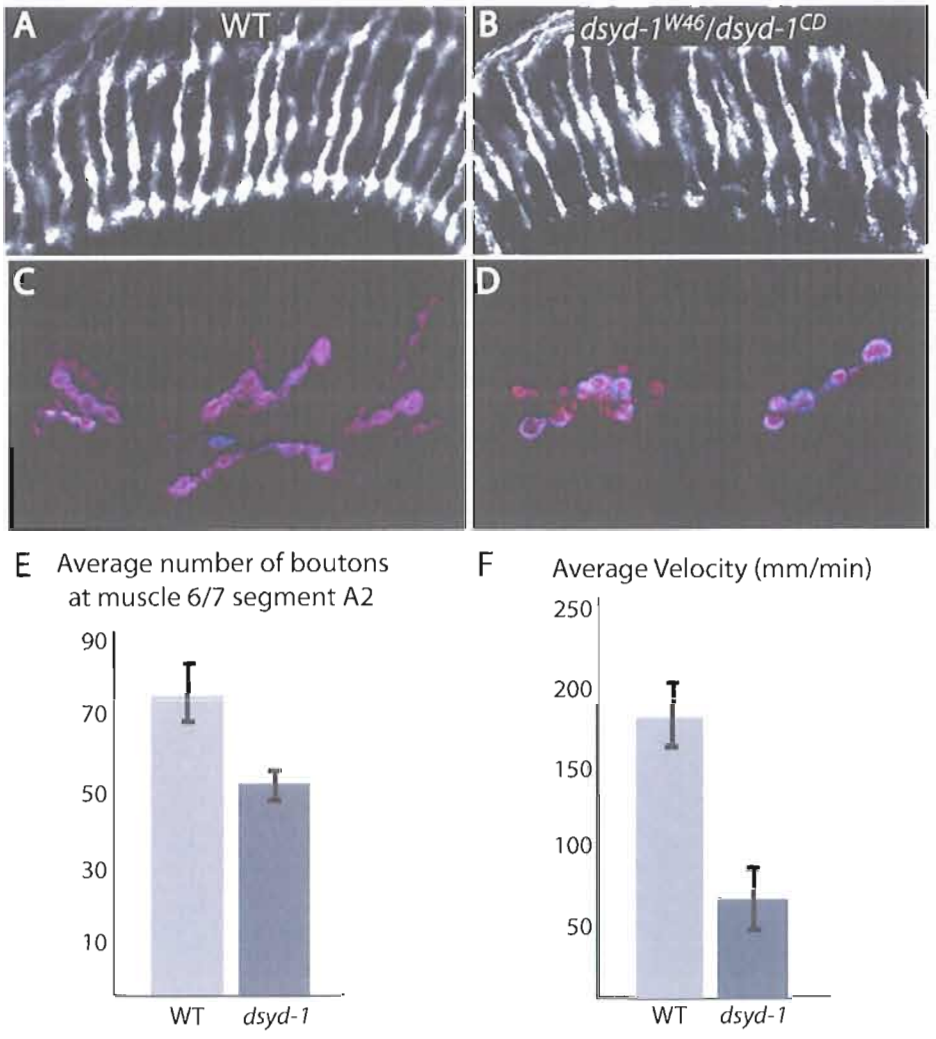

Figure 9. Animals lacking *dsyd-1* are viable but have abnormal synapses.
A,B, Young adult medullas in which all R7 and R8 axons are labeled with mAb24B10. Unlike R7s in wild type (A), R7s in dsyd-1^{W46}/dsyd-1^{CD} transheterozygotes have reduced or retracted terminals (B). C-D', Third instar larval muscle 6/7 segment A2 labeled with antibodies against the presynaptic marker Fasciclin II (red; C,D) and the postsynaptic marker Discs large (blue, C',D'). dsyd-1^{W46}/dsyd-1^{CD} transheterozygotes (D,D') have fewer synaptic boutons than wild type (C,C').E, Quantification of the phenotype sampled in C-D'. F, Comparison of the distances traveled by wild-type and dsyd-1^{W46}/dsyd-1^{CD} transheterozygous third instar larvae in a given time: loss of dsyd-1 impairs locomotion.

DISCUSSION

The fly R7 neurons provide a simple system in which to study the development of terminal synapses. We have found that most dsyd-1 mutant R7 axons extend to the correct final target layer but ultimately terminate in abnormally small boutons. We therefore conclude that dsyd-1 is required for the maintenance or subsequent remodeling of the growth cone's expanded shape. Some dsyd-1 mutant R7 terminals are absent from the M6 layer, indicating either that they failed to extend into this layer or that they initially extended but subsequently retracted. While loss of Ncad, LAR, or Liprin-a can also reduce R7 terminal bouton size, we have not previously observed the concomitant formation of thin extensions seen in some dsyd-1 mutant R7 terminals. These extensions project beyond the M6 layer and can branch (Fig. 1) and invade adjacent columns (Supp. Fig. 1). Intriguingly they generally terminate in small bouton-like structures that contain synaptic vesicles and mitochondria, indicating that they are not simply fliopodial extensions and may instead represent ectopic presynaptic sites.

We have found that, like worm syd-1, dsyd-1 is required for the enrichment of synaptic vesicles at presynaptic sites. In the C. elegans HSN neuron, syd-1 localizes presynaptic components by positively regulating Liprin-a/SYD-2: SYD-1 colocalizes with Liprin-a/SYD-2 at nascent synapses; SYD-2 is mislocalized in syd-1 mutants; SYD-1 directly enhances the ability of SYD-2 to bind the active zone protein ELKS-1; and overexpression of Liprin-a/SYD-2 suppresses the mislocalization of active zone proteins

and synaptic vesicles in syd-1 mutants (Dai et al, 2006; Patel et al, 2006; Patel and Shen, 2009). While we have found that dsyd-1 null mutant R7 axons have defects resembling those caused by loss of Liprin-a, they are weaker, as assessed by the frequency with which mutant R7 axons are absent from the M6 layer; we have similarly found that NMJ bouton number is less reduced in dsyd-1 than in Liprin-a mutants. If Liprin-a does act downstream of dsyd-1 in R7s, this difference in strength suggests that Liprin-a must also have dsyd-1-independent effects on presynaptic development. One possibility is that Liprin-a might both promote anterograde axon transport of presynaptic components and retain them at R7 terminals. Because we have found that a synaptic vesicle marker initially accumulates to wild-type levels in dsyd-1 mutant R7 terminals and only later accumulates in more distal puncta, we suggest that dsyd-1 may only be required for the retention and not transport of synaptic vesicles; to test this, one would need to directly compare synaptic vesicle movements in dsyd-1 and Liprin-a mutant axons. By the same token, dsyd-1 likely has Liprin-a-independent effects on R7 presynaptic development, since we were unable to rescue any of the dsyd-1 mutant R7 terminal defects by overexpressing HA-tagged fly Liprin-a (we used GMR-FLP/MARCM and confirmed expression by staining R7 axons with anti-HA antibodies; data not shown).

Mitochondria are frequently found in association with presynaptic sites, where they are required for the mobilization of synaptic vesicles from the reserve pool during periods of intense stimulation (Verstreken et al., 2005). Like synaptic vesicles, mitochondria are transported bidirectionally along microtubules in the axon (Hollenbeck and Saxton,

2005). While a proportion of mitochondria are highly mobile and therefore able to respond to acute energy needs, axons also contain clusters of stationary mitochondria, but often at non-synaptic, functionally undefined sites (Louie et al., 2008). Presumably because of this heterogeneous distribution within axons, mitochondrial markers are not typically included in studies of presynaptic development. However, when we examined a mitochondrial marker in R7s, we found it to be preferentially localized to R7 terminals, suggesting the presence there of stationary mitochondria. Because arthropod photoreceptor neurons continuously release neurotransmitter in response to light (Stuart et al., 2007), an obvious possibility was that the mitochondria at R7 terminals were kept there by extreme energy needs. However, we found that mitochondria remained enriched at R7 terminals even in the absence of light-evoked activity, indicating either that spontaneous release is sufficient for their recruitment or that an activity-independent mechanism is responsible. Loss of dsyd-1 or Liprin-a causes not only synaptic vesicles but also stationary mitochondria to accumulate in irregular puncta along the length of R7 axons. We speculate that the permanently high energy demands at photoreceptor synapses may have selected for the activity-independent docking of mitochondria at R7 synapses.

MATERIAL AND METHODS

Genetics

Single homozygous mutant R7 labeled clones were made using the MARCM system. Expression of FLP in R7s was under control of the GMR promoter and targeted FRT 82 sites on chromosomes in trans bearing either Gal80 or mutations in *dsyd-1*. Homozygous mutant R7s were labeled with CD8GFP, SynaptotagminGFP, or mitoGFP under control of UAS and driven by Actin Gal4. Rescue experiments were performed by generating single mutant labeled R7s that also express *UAS-dsyd-1-flag*. Analysis of SynaptotagminGFP localization in R7s revealed that SytGFP is enriched in R7s below the M3 in flies that are less than one day old. In flies older than 1 day, SytGFP is diffuse throughout the length of R7 axons. Analysis of SynaptotagminGFP localization in *dsyd-1* mutant R7 axons was done in flies that were less than 1 day old and compared to age-matched wild type control R7s. *Liprin*^{oos} (gift from J. Treisman) was used for mitochondrial distribution assays. I don't know which NorpA allele we used.

Mapping

W46 was mapped by using first low resolution visible markers,(*ru cu ca* from Bloomington *Drosophila* stock center), then high resolution PLP and RFLP markers, (Berger et al., 2001). Once W46 was narrowed to a region of roughly 500 b.p.'s near the distal tip of chromosome 3R, small custom genomic deletions were generated, (Parks et al., 2004), and used in complementation crosses with the W46 chromosome. Sequencing of *dsyd-1* in W46 revealed a guanine to adenosine transition in the 3' splice site of the sixth intron.

Cloning

CG1976, the DGC cDNA for *rhoGAP100F/dsyd-1*, is incomplete and only contains from nucleotide position 2736 to 5535 of the complete predicted cDNA sequence in flybase. The incomplete CG1976 fragment was subcloned into pGEM, then the complete cDNA was constructed by designing primers to the predicted *rhoGAP100F* open reading frame and performing reverse transcription PCR using RNA isolated from larval tissue. The actual boundary between exon 7 and intron 7 differs from the predicted boundary in flybase with the actual boundary being 5' -GGTGTTGCTTCAGgtaacgtactccc – 3'. An earlier version of *rhoGAP100F/dsyd-1* in flybase predicted the start of the first exon to be 27 nucleotides in length and to start 520 nucleotides upstream of the start of the second exon. RT PCR methods using primers within the first predicted exon sequence failed to amplify any fragments. Consequently, we completed assembly of CG1976 by performing PCR using a primer that contained the first predicted exon sequence, 5' –

atgacggtgcaaccggctgaaatggcg – 3'. Current versions of *rhoGAP100F/dsyd-1* in flybase predict the first exon to occur much further upstream from the previously predicted first exon which we used to construct our rescue and epitope-tagged constructs. The fully assembled sequence was then subcloned from pGEM into pUAST and also pUAST containing a C-terminal flag tag. Standard injection procedures were followed to generate *UAS-dsyd-1* and *UAS-dsyd-1-flag* transgene lines of flies.

Bridge to Chapter III

Another class of mutants to come out of out screen for genes involved in R7 connectivity is R7 axon terminals that are overgrown. Two different signaling mechanisms have been identified to restrict R7 axons to their individual columns. One mechanism is repulsion between two neighboring R7s. When two R7s exist in the same column, one of them experiences repulsion and grows into a neighboring column, thereby preventing the inappropriate doubling of two R7s in one single column. Another mechanism to ensure the R7 axons are tiled properly is intrinsic to R7s and involves the activin signaling pathway. Activin is secreted by R7s and the activin signal is similarly detected by R7s, thus regulating their own growth through a pathway that prevents R7 overgrowth into neighboring columns.

CHAPTER III

EXPRESSION OF TTK69 IS REQUIRED FOR R7 AXON TILING

INTRODUCTION

The trillions of neuronal connections in the brain are organized into topographical regions based on the type of sensory input being received. Within those regions another level of organization is achieved by layering the different neuronal subtypes that function in the region, (Montagnini and Treves, 2003). In vertebrates, input from adjacent photoreceptors is mapped relative to each other in the visual cortex, a method to provide the faithful reproduction of the outside environment that is being perceived. The molecular cues that are required to direct the neuronal connections in visual fields early in development are not well understood.

In Drosophila, a retinotopic map similar to that of vertebrates is observed. The Drosophila optic lobe is organized in layers, with each layer receiving visual input from different sets of photoreceptors, (Gao et al, 2008). R7 photoreceptors of neighboring ommatidia project their axons into adjacent but non-overlapping columns to a specific layer in the second optic neuropil, the medulla. The tiling of R7s as well as the other photoreceptor axons has recently been shown to require the functions of specific receptors, cell adhesion proteins, and transcription factors.

The axons of neurons in culture display an intrinsic promiscuity; they make connections often and with multiple partners, (Lotto et al., 1997). The innate connective tendency of the axons of neurons in culture suggests that in vivo, those tendencies are negatively regulated. Experiments in Drosophila have shown that the cell adhesion molecule Dscam localizes to axons and functions as a repulsive cue, signaling neighboring axons to remain in their own column, (Millard et al., 2009). The axons of R7s lacking specific isoforms of Dscam are disorganized and fail to stay restricted to their columns. A recent screen for genes required to restrict R7 axons to their columns identified the activin signaling pathway. The axons of R7s lacking the activin receptor baboon, activin, or the downstream transcriptional effector dsmad invade neighboring columns at a low frequency.

Through a similar screen for genes involved in R7 connectivity we isolated new alleles of the chromatin affecter *tramtrack*. Tramtrack is the functional homolog of vertebrate REST. Early experiments with REST demonstrated that it functions to silence expression of neuronal genes in non-neuronal cells through binding upstream regulatory regions of neuronal genes and promoting heterochromatin formation, (Belyaev et al., 2004). In addition to functioning in higher order chromatin remodeling to repress transcription, REST also has been implicated in gene activation. A truncated isoform of REST, REST4, interacts with REST in a dominant-negative manner to competitively inhibit REST from binding and repressing transcription, (Spencer et al., 2006). Experiments in stem cells have shown that REST expression levels are initially high in embryonic stem cells but progressively diminish as those cells differentiate into neural stem cells then neurons,

(Ballas et al., 2005). These experiments demonstrate that REST expression doesn't inhibit the ability of a cell to become a neuron; it prevents the premature progression of the neuronal differentiation program.

Drosophila ttk exists as two isoforms, ttk88 and ttk69, both of which function in multiple aspects of development and stasis. Ttk88 and ttk69 share the same N-terminal BTB-POZ domains but differ in their C-terminal zinc finger domains, suggesting that they also differ in their DNA binding specificities. In embryonic fly nervous system development, Ttk69 is required in post-mitotic glia to maintain their differentiated state, (Abrajano et al., 2009). Also, overexpression of Ttk69 has been shown to repress a subset of neuroblast markers and subsequent neuronal differentiation, (Badenhorst 2001). Misexpression of Ttk69 in post-mitotic neurons results in defects in axon pathfinding and axon tract morphology. Both Ttk isoforms have been shown to be regulated post-translationally. Phylopod and SINA ubiquitinate and consequently target both Ttk isoforms for degradation. Unlike Ttk88, Ttk69 is sumoylated and can be ubiquitinated by SINA in a phyllopod-independent manner, (Cooper et al., 2008). The deubiquitinating enzyme UBP64 antagonizes the function of SINA and stabilizes Ttk. UBP64 is expressed in cone cells where Ttk is required to repress neuronal fate, and SINA is expressed in photoreceptors where progression of R cell fate requires that Ttk be degraded, (Bajpe et al., 2003). The RNA binding protein, *musashi*, has been shown to downregulate Ttk in photoreceptors too (Hirota, 1999).

Although repression of Ttk is required for early photoreceptor differentiation, previous genetic experiments demonstrated a requirement for Ttk69 expression in photoreceptors

later in development. Overexpression of Ttk69 early in eye disc morphogenesis prevents photoreceptor differentiation, but interestingly Ttk69 protein is expressed at a high level in all photoreceptors in pupal development, (Wen et al., 2000). Unlike ttk88 mutant eye clones that contain no cone cells and have ectopic photoreceptors, ttk69 mutant clones are missing photoreceptors, suggesting that ttk69 plays a positive role in photoreceptor development, (Lai et al., 1996). We identified ttk in our screen for genes required during R7 axon connectivity. The overgrowth of the axons of ttk mutant R7s suggests that ttk may have a tiling function, one that restricts R7 axons to their individual columns after they reach their target layer.

RESULTS

The axons of adult photoreceptors lacking Ttk69 exhibit tiling defects.

Our method for generating homozygous mutant cells uses an eye specific promoter that is expressed in cone cells, R1, R6, and R7 photoreceptors. Hence, mutations that cause a fate change from cone cell to R7 identity should be discovered in our screen through the appearance of ectopic R7 axons. One phenotype of our new *tramtrack* alleles is extra R7 axons, which is consistent with results from previous experiments demonstrating cone cell to R7 fate transformation in *ttk88* mutant eyes. Also, the axons are overgrown and have a rough appearance compared to wild type axons, (figure 1B, 1A). Since all our ttk alleles are phenotypically the same, we chose the *ttk1E* allele for analysis in this study.

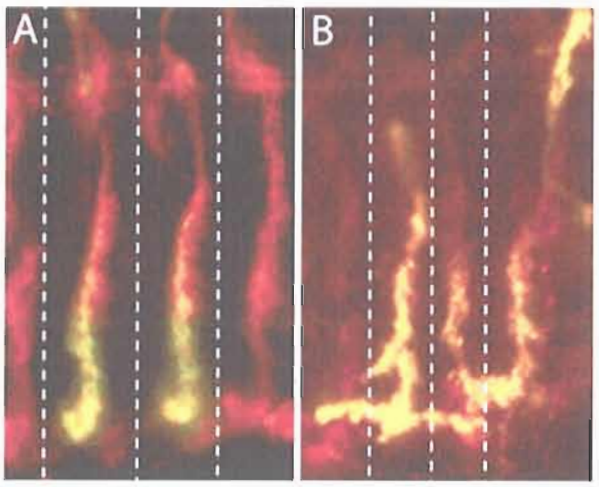

Figure 1. The axons of Tramtrack mutant R7s are rough, overgrown, and often invade neighboring columns of R7s. A Wild type adult R7 axons labeled with a pan photoreceptor marker 24B10 (red) and SynaptotagminGFP (green). Dashed lines indicate the borders of the columns. The terminals of wild type axons are restricted to their individual columns. B The axons of adult tramtrack mutant R7s exhibit invasive growth. Their axons extend into neighboring columns.

To determine whether *ttk1E* is specifically an allele of one or the other isoform of ttk, or whether it removes the function of both isoforms, we performed rescue experiments. Expression of a *UAS-ttk69*, (figure 2B), but not a *UAS-ttk88* transgene, (figure 2A) in *ttk1E* mutants rescues the axon defects but not the ectopic R7 axons. These results suggest that cone cells are still transformed to R7s, and Ttk69 function alone is sufficient to rescue the axon tiling defects. To determine whether loss of Ttk69 itself is responsible for the axon defects, we generated *ttk1e11* homozygous mutant R7s. The *ttk1e11* allele is a loss of function for the Ttk69 isoform, and Ttk88 expression and function is otherwise normal. The axons of *ttk1e11* mutant R7s exhibit the same overgrowth phenotypes as ttk1E mutant R7s, but whether invasive axons are from a single mutant R7 or a cone cell

that has been transformed into an R7 is difficult to distinguish. It's possible that the invasive R7 axons are the result of transformed cone cells that project an axon with the already present R7 axon, resulting in two R7 axons per single column. The invasive phenotype could then be partially the result of the crowding of two R7 axons in a column. To determine whether the invasive axon phenotype is the result of more than one R7 axon per column, we generated single mutant R7s using an R7 specific driver for making homozygous mutant cells. By this method, only one mutant cell will be generated in any given ommatidia, and that cell will be an R7 lacking functional Ttk69. The axons of homozygous mutant *ttk1e11* R7s exhibit tiling defects, (figure 2C), specifically indicating a function for Ttk69 in tiling, rather than tiling defects resulting from crowding of a single column with more than one R7 axon.

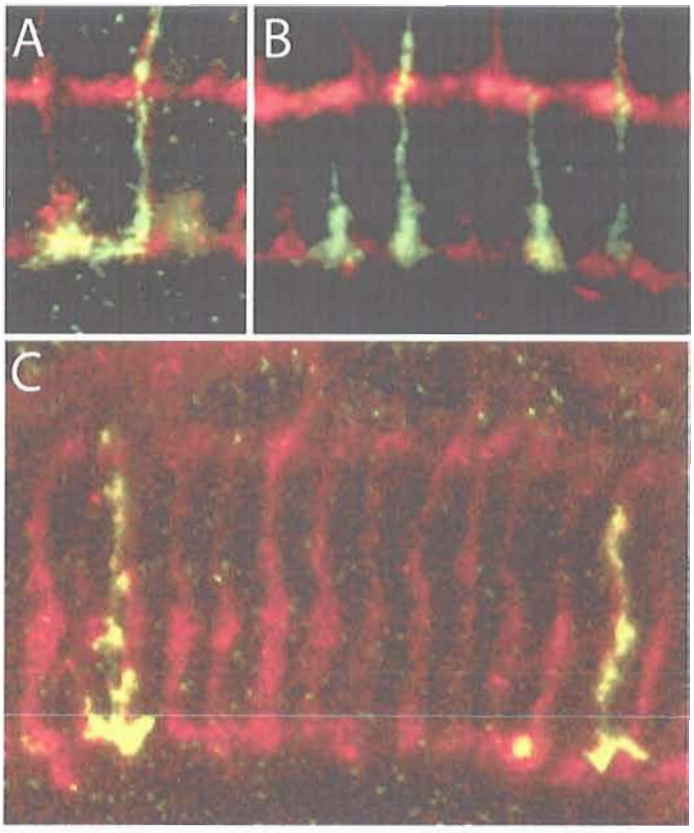

Figure 2. The Ttk69 isoform functions in tiling R7 axon terminals. A ttk1E mutant cell expressing *UAS-ttk88* and *UAS-SytGFP* (green). Expression of *UAS-ttk88* is not sufficient to rescue the axon tiling defects. B Expression of *UAS-ttk69* can rescue the R7 terminal axon tiling defects of *ttk1E* mutant cells. C Generation of single *ttk1e11* mutant R7s labeled with *UAS-SybGFP* (green) using an R7 specific driver to ensure only one mutant R7 per column still results in R7 axon tiling defects.

Previous experiments demonstrated a reduction in photoreceptor number per ommatidia in eyes with ttk69 mutant clones. Our method for generating *ttk69* homozygous mutant single cells results in R7 tiling defects, but whether the photoreceptor cell body in the retina is also affected by loss of Ttk69 is similarly of interest. To determine the fate of the cell bodies of ttk69 mutant photoreceptors, we used CD8-GFP to label the membranes of

homozygous mutant ommatidial cells. In retinas containing ttk69 homozygous mutant cells, only remnants of GFP labeling are evident, suggesting that the corresponding cell bodies have either degraded or aberrantly delaminated to an underlying layer, (figure 3).

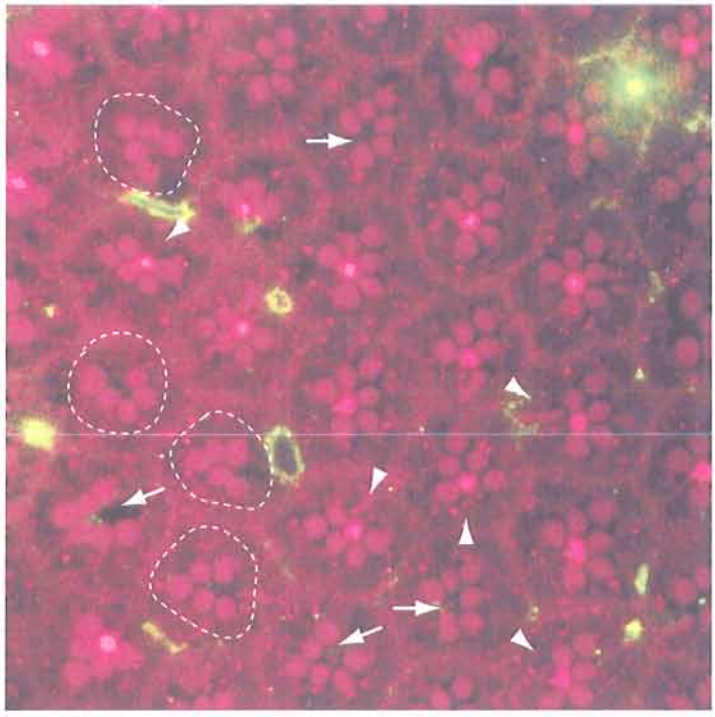

Figure 3. *ttk69* mutant cells are lost from the photoreceptor cell body layer in the adult retina. Phalloidin, (red) labels rhabdomeres. Homozygous mutant cells may delaminate, or migrate to a proximal layer in the retina. Alternatively, they may undergo apoptosis or be degraded. CD8 GFP (green) labels homozygous mutant cells and most of the membrane bound label has shifted to another layer or been degraded. Arrows highlight where R7 rhabdomeres are lost from ommatidia. Arrowheads illustrate that R1 and R6 homozygous mutant cells have also migrated or been degraded. Some ommatidia have two or more mutant cells. The ommatidia outlined with a dashed line have only 5 visible rhabdomeres compared to the normal complement of 7. These ommatidia likely have a homozygous mutant R7 and either a homozygous mutant R1 or R6.

Ttk69 is expressed in photoreceptors from early-mid through late pupal stages.

The cell bodies of adult photoreceptors lacking Ttk69 are lost from the proper layer in the retina, and the axons of mutant photoreceptors have severe terminal abnormal morphology. Ttk69 is likely functioning before adulthood to specify various aspects of photoreceptor development. To determine when Ttk69 is functioning, we performed a timecourse analysis of Ttk69 protein expression in fixed pupal eye disc tissue. The eye discs of pupae 24 hours after puparium formation, (24 APF), have no detectable Ttk69 in photoreceptors, (data not shown). Ttk69 expression comes on at about 30 APF, (figure 4A), and continues through mid stages of pupal development, (figures 4B, 4C, and 4D).

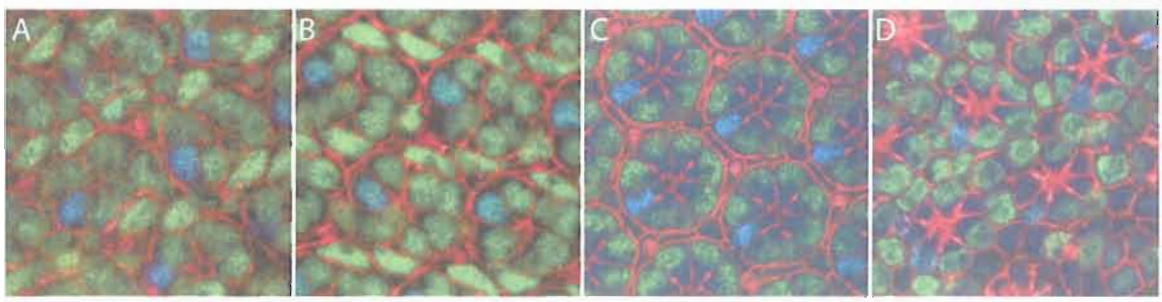

Figure 4. Ttk69 is expressed throughout mid-pupal development. Phalloidin, (red), labels all ommatidia and the membranes of individual photoreceptor cell bodies. The nuclear cell fate marker Prospero, (blue), is expressed only in R7s throughout pupal development. Ttk69 is barely detectable at 24% pupal development, (data not shown). A At 30% pupal development, P30, Ttk69 (green) is readily detectable in all cells of the ommatidia including photoreceptors. Ttk69 expression is maintained in photoreceptors through P60 at which point it's expression is downregulated in a subset of photoreceptors.

The time course of expression of Ttk69 in photoreceptors indicates that it functions at stages of R7 axon differentiation after R7 growth cones have entered their target layer in

the medulla. During late pupal stages, R7s as well as other photoreceptors are turning on expression of specific rhodopsin genes. Future experiments will address whether late pupal stages of photoreceptor differentiation are affected in *ttk1e11* mutant R7s.

Cell fate and polarity are not affected in photoreceptors lacking Ttk69.

To determine whether the axon overgrowth phenotype of R7s lacking Ttk69 could be the result of a change in cell fate, we assayed expression of Prospero and Sal proteins in *ttk1e11* mutant R7s. Sal is normally expressed in both R7 and R8. The axons of R7s lacking Prospero have mild axon targeting defects. Single *ttk1e11* mutant R7s have normal expression of the cell fate markers Prospero and Sal, (figure 5A, 5B), indicating that the axon growth defects are not the result of general misregulation of R7-specific genes. Interestingly, there are in some ommatidia two homozygous mutant *ttk1e11* cells and both of them express Prospero. Presumably, one of the cells is an R7 and the other is either an R1, R6, or cone cell that has undergone a fate switch to R7. A partial loss of cone cells has been reported in eye *ttk1e11* clonal analysis, so it's possible that cone cells lacking Ttk69 are misexpressing Prospero. In that case, the cone cell must have migrated to a position in the ommatida in close proximity to the originally specified R7. It's also possible that the extra R7 resulted from an R1 or R6 change of fate. It will be of interest to look at expression of Prospero in ttk1e11 whole eye mutant clones to learn how many cells per ommatidia can undergo transformation to R7 cell fate.

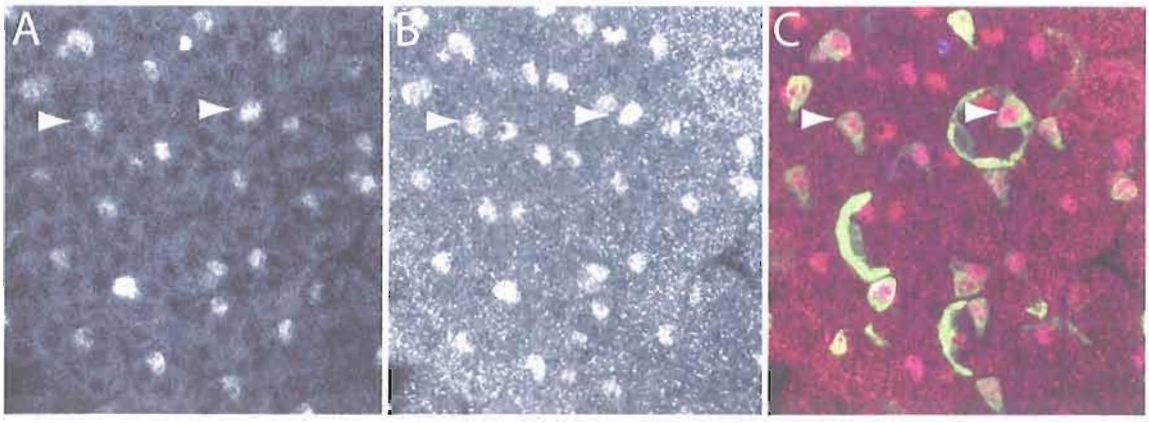

Figure 5. Cell fate is not affected in *ttk69* mutant R7s. A The nuclear transcription factor Prospero is expressed in all R7s. B The cell fate marker Sal is expressed in all R7s and R8s. C CD8-GFP (green) labels ttk69 homozygous mutant cells. Arrowheads point to ttk69 homozygous mutant R7s that are normally expressing Prospero and Sal.

The disappearance of *ttk1e11* mutant R7 cell bodies from the adult retina prompted us to examine the expression of cell polarity markers. Epithelial cells with defects in polarity are regularly lost from their corresponding tissue layer. Phalloidin labels the actin-dense inner-ommatidial rhabdomere structure in the center of ommatidia, and as such is used to assay loss of cell polarity. Bazooka specifies apical basal polarity early in photoreceptor development and its localization is restricted to the Zonula Adherens (ZA). Ommatidia lacking functional Bazooka have severe cell shape deformities, a phenotype detectable by staining with the actin marker Phalloidin. We tested *ttk1e11* mutant R7s for defects in Bazooka expression and localization. Aberrant cell polarity could possibly account for the loss of *ttk1e11* mutant photoreceptor cell bodies from the normal retinal layer. At 50% pupal development, *ttk1e11* mutant cells have normal expression and localization of Bazooka, and the overall morphology of ommatidia based on staining with Phalloidin appears normal, (figure 6A, 6B, and 6C). At this stage, Ttk69 does not seem to be

controlling photoreceptor polarity, hence loss of polarity cannot account for the loss of *ttk1e11* photoreceptor cell bodies from the retinal layer.

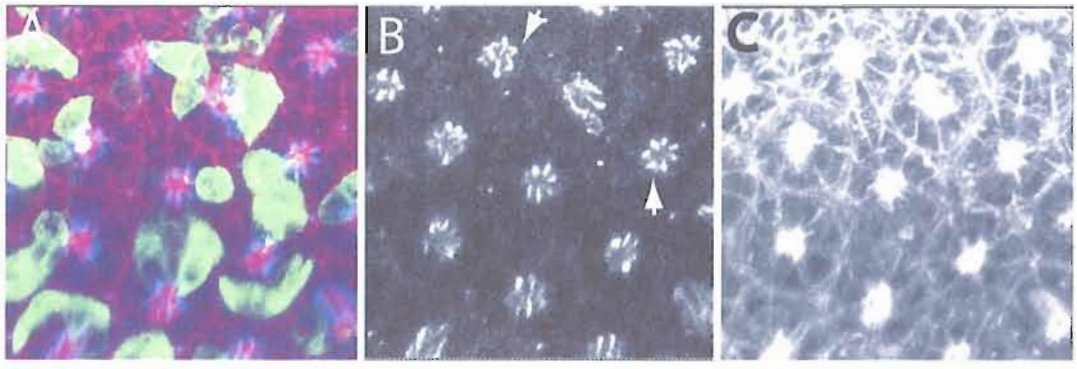

Figure 6. Cell polarity is normal in photoreceptors lacking Ttk69. A stage P50 eye disc expressing CD8-GFP, (green), which labels all *ttk1e11* homozygous mutant cells. Bazooka, (blue and panel B), is localized normally to the Zonula Adherens (arrows) in *ttk1e11* mutant cells. Phalloidin, (red and panel C), stains strongly in the center of all ommatidia. The morphology of the center actin-rich rhabdomere and stalk membrane are normal in ommatidia containing *ttk1e11* mutant cells.

Ttk69 functions in pupal photoreceptors to restrict axon growth.

Since Ttk69 expression comes on at early-mid pupal stages, we hypothesized that it functions in axon differentiation during those stages. In our R7 connectivity screen, we identified the *ttk* overgrowth phenotype in adult photoreceptor axons. It's possible that the axon overgrowth is a side effect of an unidentified phenotype in *ttk* mutant photoreceptors. An axon overgrowth phenotype that is manifest in pupal development when Ttk69 expression is normally on would indicate a direct role for Ttk69 in restricting axon growth. By P36, Ttk69 is expressed at a high level in photoreceptors, and at the same stage in *ttk1e11* mutants, the axon overgrowth phenotype is just starting to

manifest, (figure 7B). By mid-pupal development, the axon overgrowth phenotype of *ttk1e11* mutant R7s is revealed at a frequency similar to that observed in adult stages. These results demonstrate that the axon overgrowth phenotype corresponds to the temporal expression of Ttk69, which supports our hypothesis that Ttk69 functions in restricting R7 axon growth.

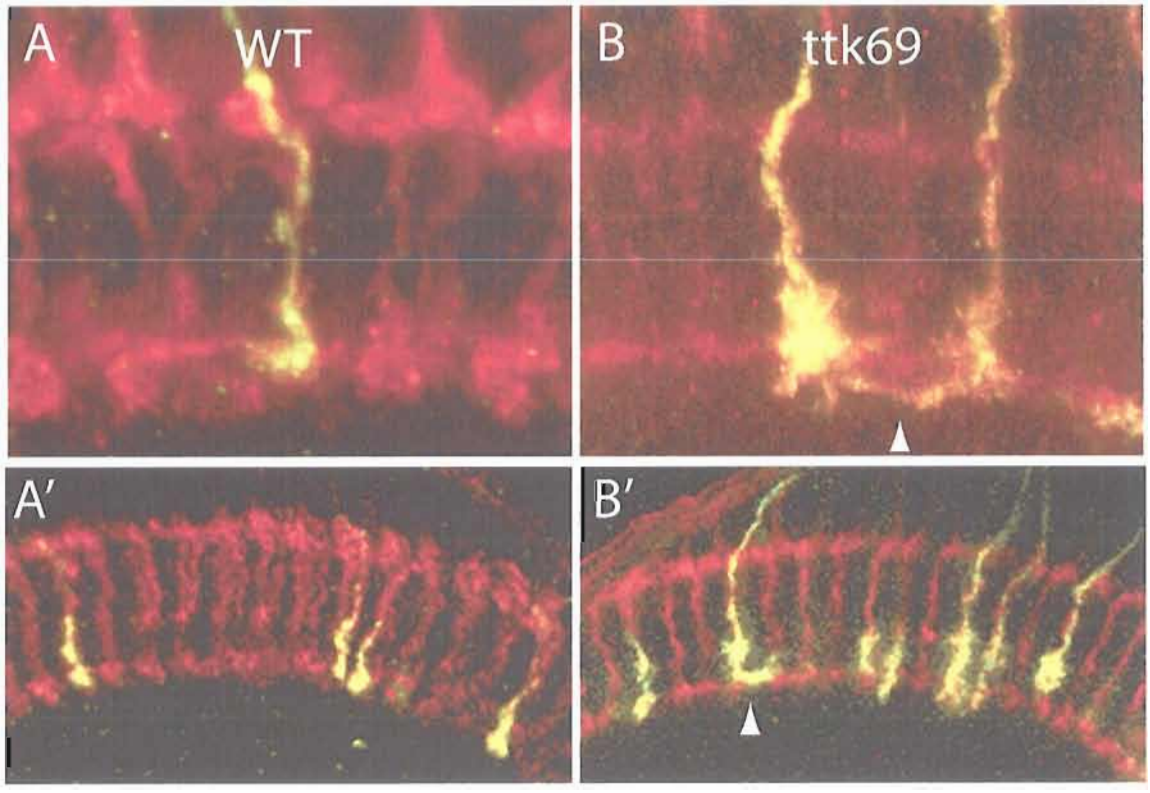

Figure 7. The axons of *ttk1e11* mutant R7s begin to exhibit overgrowth in early-mid pupal development. A Stage P36 single wild type R7 labeled with CD8-GFP (green) stays within its own column. A' Wild type axons at P50 remain within their individual columns. B At P36, a low frequency of *ttk1e11* mutant R7s grows into neighboring columns (arrowhead). B' By P50, the invasive axon phenotype of *ttk1e11* mutant R7s is readily apparent (arrowhead).

Dsmad levels are reduced in ttk69 mutant photoreceptors.

Previous reports demonstrate the requirement for Activin signaling in restricting pupal R7 axons to their columns. To determine whether Ttk69 regulates Activin signaling, we analyzed the levels of the transcription factor Dsmad in *ttk1e11* mutant R7s. Phosphorylated Dsmad is transported to the nucleus in response to extracellular Activin, and mutations in Dsmad or any of the components in the signaling pathway result in overgrowth of R7s. Loss of components in the Activin signaling pathway results in reduced levels of nuclear Dsmad, hence Dsmad levels can be used as a readout for Activin signaling in *ttk1e11* mutant cells. We do indeed find reduced levels of nuclear Dsmad in *ttk1e11* mutant R7s, indicating a role for Ttk69 in Activin signaling. Epistasis experiments are currently underway to determine whether Ttk69 is upstream or downstream of the Activin signaling pathway.

MATERIALS AND METHODS

Fly stocks and clonal analysis

To generate mosaic analysis with repressible cell marker (MARCM) clones we crossed flies bearing the GMR promoter driving expression of Flipase and also FRT 82 chromosomes bearing mutations in one of several alleles of *tramtrack* with a stock designed for performing histology. The histology stock is Actin Gal4 and UAS CD8-GFP on chromosome II and FRT 82 Gal80 over TM6B balancer on chromosome III. We

received the *ttk1e11* fly line from Y. Li at Pennsylvania State University. We recombined the *ttk1e11* allele onto a chromosome bearing a FRT82 site. A line harboring a *UAS-ttk69* transgene was also received from Y. Li. *UAS-dsmad-flag* was received from Chi-Hon Lee and was used to determine the relative levels of nuclear Dsmad in *ttk1e11* mutants compared to wild type and also mutants in the Activin signaling pathway. An primary antibody generated against Flag from Sigma was used at a concentration of 1:200 on pupal eye discs that had been dissected in Schneider's media and fixed in 4% paraformaldehyde. Dominant negative Activin, *UAS-ActDN*, and an activated Baboon, *UAS-bab*, transgenic lines were received from Chi-Hon Lee and used as controls when analyzing levels of nuclear Dsmad.

DISCUSSION

Recently, genetic screens for genes involved in R7 tiling are identifying and leading to the classification of a group of genes responsible for restricting R7 axons to their appropriate columns. The transmembrane proteins Capricious, Dscam2 and Turtle function in R7 tiling as well as the transcription factor Dsmad. Our results demonstrate a role for the chromatin modifying protein Tramtrack69 in tiling R7 axons. The overgrowth of pupal R7s lacking functional Ttk69 corresponds to its temporal expression in photoreceptors.

What happens to the cell bodies of Tramtrack69 mutant R7s? Although the polarity markers we analyzed in *ttk1e11* mutant R7s looked relatively normal, CD8-GFP used to mark the individual homozygous mutant cells had an unusual distribution

compared to homozygous wild type photoreceptors. CD8-GFP distribution was broadened compared to wild type, appearing as if it expanded into the rhabdomere. It is possible that normal membrane junctions that prevent the flow of components of the cell body membrane into the rhabdomere membrane are disrupted in R7s lacking Ttk69. It is possible that the cell bodies and rhabdomeres of *ttk1e11* mutant R7s fall below the basement membrane in the retina. Such a phenotype is manifest in photoreceptors that are missing components of the adherens junction. In addition to regulating R7 axon tiling, Ttk69 plays either a direct or indirect role in maintaining photoreceptor soma integrity, as the cell bodies and rhabdomeres are *ttk1e11* mutant R7s are either degraded, migrate to a different tissue layer, or fail to be properly maintained in their layer due to failure to express the appropriate integral membrane proteins. We hypothesize that it is unlikely that R7s are degraded, because their axons, although overgrown and exhibiting gross morphological defects, are maintained into adulthood.

Loss of Ttk88 from cone cells results in fate transformation to R7, and loss of Ttk69 results in some but not all cone cells transformed to R7s. Ttk69 may have a redundant function in specifying cone cell fate with Ttk88, and Ttk69 appears to be only partially required for this function. Still, the transformation of even some cone cells to R7s confounds the interpretation of our results, because we can not undoubtedly report whether the mutant axon we analyzed was that of an R7 lacking Ttk69 or either a cone, R1, or R6 cell with a fate transformation to R7. If we indeed analyzed the tiling defect of the latter scenario, the overgrowth could be partially or fully the result of crowding of two R7 axons in a single column rather than failure to express *ttk69*.

Future efforts to understand Ttk69's role in R7 axon tiling should extend to performing genomic studies to identify the downstream targets of Ttk69. In vertebrates, many of the targets of REST have been identified, and several of those targets are microRNA genes that regulate expression of neuronal genes. Clearly, in vertebrates there are multiple levels of regulation that ensure the appropriate temporal expression of neuronal genes. In this report, we demonstrate a new function for Ttk69 in photoreceptor development. Ttk69 expression comes on in mid stages of photoreceptor differentiation, and its expression is required to restrict the growth of axon terminals. Future experiments will aim to determine whether Ttk69 is expressed in other neuronal subtypes in the developing nervous system and if so whether it functions during differentiation to restrict axon growth similar to its function in photoreceptors.

CHAPTER IV

CONCLUSIONS

Throughout the experiments in this study, various aspects of the phenotypes of *dsyd-1* and *ttk69* mutant R7s were analyzed at early-mid pupal and adult stages. Interestingly, phenotypes of both mutants actually changed between mid-pupal and adult stages. In *dsyd-1* mutant R7s, axon terminals were reduced in size, retracted, or simply failed to make the proper extension to the final target layer in mid-pupal development. The extensions past the R7 target layer was a phenotype readily apparent by adulthood, but not ever observed at earlier, pupal stages. Pupal stages after about P50 were not analyzed in this study, the time point when a subset of *dsyd-1* mutant R7s presumably are growing past the target layer. R7 axon terminals are likely not just static throughout late pupal development. Various aspects of synaptogenesis or morphological alterations may very well still be occurring. Lack of a molecular toolkit, various presynaptic components fused to fluourophores, hinders our progress in understanding the aspects of photoreceptor differentiation that are occurring in late pupal stages. Also, performing live imaging of photoreceptor axon terminal growth in the medulla would significantly contribute to our understanding of pupal axon differentiation. The real time imaging of the transport of presynaptic components to axon terminals could provide more information to us about the mechanisms of presynaptic assembly. For instance, in the

dsyd-1 mutant R7s analyzed in Chapter II, I documented a mislocalization of the synaptic vesicle marker, SytGFP. At P24, SytGFP localization in dsyd-1 mutant R7 axons terminals is comparable to that of wild type. However at P48, SytGFP is localized in diffuse puncta throughout the axon. Does SytGFP initially reach the axon terminal and then it's docking and maintenance in the terminal fails in R7s lacking Dsyd-1? Or, do transport vesicles never reach the axon terminal, a defect in targeting presynaptic components to active zone sites. In the future, live imaging should resolve such questions.

In Chapter III, I showed that photoreceptors lacking Ttk69 are lost from the adult retinal layer. I also demonstrated that in mid-pupal stages, the morphology of the cell bodies of photoreceptors lacking Ttk69 appear similar to wild type. There is a loss of the photoreceptor soma between mid-pupal development and adult stages. The soma of photoreceptors must be stably maintained in the proper layer to ensure the integrity of photoreceptor morphology and the morphology of the retina as a whole. The disappearance of photoreceptor soma lacking Ttk69 suggests that Tramtrack has a direct or indirect role in stabilizing soma in the proper tissue layer. Future experiments will address what happens to the soma of *ttk1e11* mutant photoreceptors. Fixed preparations of eye discs at late pupal stages should provide information about whether *ttk1e11* mutant soma undergoes apoptosis; a fate we predict is unlikely due to the maintenance of the axons. Alternatively, we predict that *ttk1e11* mutant photoreceptors are lost from the proper layer in the retina and become localized to a distal layer.

Previously unrealized aspects of photoreceptor axon and cell body development in late pupal stages have become revealed indirectly from the experiments in this study. At P50, the terminals of R7 axons appear morphologically similar to adult axons. The extensions of R7 axons past the target layers that presumably are occurring at late pupal stages suggest that different aspects of axon development and maintenance are normally occurring then. Also, the loss of cell bodies from the retina in photoreceptors lacking Ttk69 indicates that regulation of soma morphology through expression of the proper transmembrane molecules and proper regulation of the underlying actin and microtubule cytoskeleton are required to maintain soma in the proper layer.

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