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A Glia-Mediated Feedback Mechanism for the Termination of Drosophila Visual Response: A Dissertation

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A GLIA-MEDIATED FEEDBACK MECHANISM FOR THE TERMINATION OF *DROSOPHILA* VISUAL RESPONSE

A Dissertation Presented

By

PEIYI GUO

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NEUROSCIENCE

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Program in Neuroscience
September 9, 2010

Dedicated to my parents

Yingtong Guo and Bohe Chen

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ABSTRACT

High temporal resolution of vision relies on the rapid kinetics of the photoresponse in the light-sensing photoreceptor neurons. It is well known that the rapid recovery of photoreceptor membrane potential at the end of light stimulation depends on timely deactivation of the visual transduction cascade within photoreceptors. Whether any extrinsic factor contributes to the termination speed of the photoresponse is unknown.

In this thesis, using *Drosophila* as a model system, I show that a feedback circuit mediated by both neurons and glia in the visual neuropile lamina is required for rapid repolarization of the photoreceptor at the end of the light response.

In the first part of my thesis work, I provide evidence that lamina epithelial glia, the major glia in the visual neuropile, is involved in a retrograde regulation that is critical for rapid repolarization of the photoreceptor at the end of light stimulation. I identified the gene affected in a *slrp* (*slow receptor potential*) mutant that is defective in photoreceptor response termination, and found it needs to be expressed in both neurons and epithelial glia to rescue the mutant phenotype. The gene product SLRP, an ADAM (*a disintegrin and metalloprotease*) protein, is localized in a special structure of epithelial glia, gnarl, and is required for gnarl formation. This glial function of SLRP is independent of the metalloprotease activity.

In the second part of my thesis work, I demonstrate that glutamatergic transmission

from lamina intrinsic interneurons, the amacrine cells, to the epithelial glia is required for the rapid repolarization of photoreceptors at the end of the light response. From an RNAi-based screen, I identified a vesicular glutamate transporter (vGluT) in amacrine cells as an indispensable factor for the rapid repolarization of the photoreceptor, suggesting a critical role of glutamatergic transmission from amacrine cells in this retrograde regulation. Further, I found that loss of a glutamate-gated chloride channel GluCl phenocopies vGluT downregulation. Cell specific knockdown indicates that GluCl functions in both neurons and glia. In the lamina, a FLAG-tagged GluCl colocalized with the SLRP protein in the gnarl-like structures, and this localization pattern of GluCl depends on SLRP, suggesting that lamina epithelial glia receive glutamatergic input from amacrine cells through GluCl at the site of gnarl. Since the amacrine cell itself is innervated by photoreceptors, these observations suggest that a photoreceptor — amacrine cell — epithelial glia — photoreceptor feedback loop facilitates rapid repolarization of photoreceptors at the end of the light response.

In summary, my thesis research has revealed a feedback regulation mechanism that helps to achieve rapid kinetics of photoreceptor response. This visual regulation contributes to the temporal resolution of the visual system, and may be important for vision during movement and for motion detection. In addition, this work may also advance our understanding of glial function, and change our concept about the effect of glutamatergic transmission.

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LIST OF ABBREVIATIONS

AC	amacrine cell
ADAM	a disintegrin and metalloprotease
ARR1	arrestin1
ARR2	arrestin 2
ATP	adenosine-5'-triphosphate
cGMP	cyclic guanosine monophosphate
CNS	central nervous system
DAG	diacylglycerol
DMSO	dimethyl sulfoxide
EGF	epidermal growth factor
EM	electron microscopy.
ERG	electroretinogram
GABA	γ-aminobutyric acid
GFP	green fluorescent protein
GluCl	inhibitory glutamate-gated chloride channel
GPCR	G-protein coupled receptor
GST	glutathione S-transferase
GTPase	guanosine triphosphatase
HisCl1	histamine-gated chloride channel 1,
HS	horizontal system
IgG	immunoglobulin G
INAC	inactivation no afterpotential C
INAD	inactivation no afterpotential D

IP3	inositol 1,4,5-triphosphate
IPL	inner plexiform layer
IVM	ivermectin
LCCHs	ligand-gated chloride channel homologs
LGICs	cys-loop ligand-gated ion channels
MBP	maltose binding protein
MMD	mind-meld
NA	nodulisporic acid
NINAC	neither inactivation nor afterpotential C
PIP2	phosphatidylinositol 4,5-bisphosphates
PLC	phospholipase C
PUFAs	polyunsaturated fatty acids
RACE	rapid amplification of cDNA ends
RDGC	retinal degeneration C
RH1	rhodopsin 1
RNAi	RNA interference
RT-PCR	reverse transcription-polymerase chain reaction
SEM	standard error of the mean
SLRP	slow receptor potential
TRP	transient receptor potential
TRPL	transient receptor potential-like
UTR	untranslated region
vGluT	vesicular glutamate transpoter
VS	vertical system
WT	wild type

CHAPTER I: INTRODUCTION

The importance and mechanisms of rapid termination of light response

Vision is the most important sensory system for human and most animals. It enables us to perceive the size, color and shape of objects, as well as their movements. For vision during movement and for the tracking of moving objects such as a predator, animal eyes need to sense light intensity changes with a high temporal resolution. This rapid kinetics depends on the responding speed of photoreceptors, light sensing neurons, which convert light signals into electrical signals that can be transferred into the brain (Squire et al., 2003).

In photoreceptor cells, the absorption of light photons causes the conformational change of the G-protein coupled light receptor rhodopsin, and activates the visual transduction cascade. In vertebrate photoreceptors, the light-stimulated rhodopsin catalyzes the activation a heterotrimeric G protein, transducin, which in turn activates the cyclic GMP hydrolyzing enzyme, phosphodiesterase, resulting in a rapid decrease in the intracellular cGMP concentration. This leads to the closure of cGMP-gated cation channels in the plasma membrane, causing hyperpolarization of the cell (Luo et al., 2008). When light stimulation is removed, the photoreceptor promptly returns to the original depolarization status. For the eye to achieve a high temporal resolution, both the activation and the termination of the photoreceptor light response need to be rapid enough. In mouse rod photoreceptors, the membrane potential recovers within 200ms

from the end of light stimulation (Lyubarsky and Pugh, 1996). So far most studies on the mechanism of photoreceptor light response termination have focused on the deactivation of the phototransduction cascade. It is now known that the rapid termination of photoreceptor light response depends on regulations at each step of the phototransduction, including the deactivation of rhodopsin, transducin, and phosphodiesterase, as well as the restoration of cGMP (Laitko and Hofmann, 1998; Burns and Baylor, 2001; Burns, 2010). However, it is unknown whether these intrinsic mechanisms of phototransduction deactivation are sufficient for the rapid termination of photoreceptor light response.

Besides the phototransduction cascade, retrograde regulation from retinal horizontal cells also contributes to the amplitude and voltage dependence of photoreceptors (Kamermans and Spekreijse, 1999; Cadetti and Thoreson, 2006). In addition, visual glial cells directly contact photoreceptors and may release important signaling molecules to act on the latter (Newman and Reichenbach, 1996; Bringmann et al., 2006). Theoretically, these extrinsic regulations may have effects on the membrane potential shift of photoreceptors at the end of light stimulation.

Feedback regulation of photoreceptor in visual systems

In both vertebrate and invertebrate visual systems, the output of photoreceptors is fine-tuned by feedback from interneurons, which is an important mechanism for shaping the transmission of light information (Gerschenfeld et al., 1980; Wu, 1991; Glantz et al.,

2000; Fahrenfort et al., 2005).

In vertebrate retina, the rod and cone photoreceptors synapse onto bipolar cells, which in turn synapse onto ganglion cells and conduct visual signals to the brain. Two types of laterally distributed interneurons, horizontal cells and amacrine cells, are thought to provide feedback information to photoreceptor and bipolar axon terminals, separately (Wässle, 2004; Cadetti and Thoreson, 2006). The negative feedback model from horizontal cells to cone photoreceptors is well established, which contributes to the formation of the center-surround receptive fields that enhance edge detection (Baylor et al., 1971). This feedback has also been proposed to contribute to light adaptation (Burkhardt, 1995), color opponent interactions (Twig et al., 2003), and function in retinal synchronization (Razjouyan et al., 2009). Similar negative feedback from horizontal cells to the rod photoreceptors was also discovered and proposed to influence vision under scotopic and mesopic conditions (Thoreson et al., 2008).

However, the mechanism of feedback from horizontal cells to cones and rods is still an issue of debate. The feedback was thought to be mediated by a GABAergic pathway (Murakami et al., 1982; Yazulla and Kleinschmidt, 1983; Schwartz, 1987), which has not been successfully reported in whole-retina preparations. Two different views on how horizontal cells communicate with photoreceptors have emerged: (1) horizontal cell hyperpolarization changes the extracellular potential through a hemichannel-mediated ephaptic mechanism, increases the calcium current in photoreceptors and promotes

transmitter release (Byzov and Shura-Bura, 1986; Kamermans et al., 2001; Fahrenfort et al., 2005; Fahrenfort et al., 2009). (2) hyperpolarization of the horizontal cells induces a pH increase within the synaptic cleft, enhances the calcium current in photoreceptors and reinforces transmitter release (Hirasawa and Kaneko, 2003).

Although electron-microscope studies have revealed small clusters of vesicles in human horizontal cell processes facing rod spherule or cone pedicle synapses, which may contribute to small punctate chemical synapses that mediate the feedback (Linberg and Fisher, 1988), current knowledge of the feedback from the horizontal cells to photoreceptors comes largely from simultaneous recordings of both cells (Baylor et al., 1971; Cadetti and Thoreson, 2006; Thoreson et al., 2008). It remains to be addressed, whether other cells affect this feedback, and what are the signaling molecules in this feedback. The limited understanding of the feedback is in part because of the lack of efficient *in vivo* approaches.

Functions of visual glia

In vertebrate retina, Müller glial cells are the major type of glia. They are radial astrocytes that span the entire length of the retina, contacting all retinal neuronal somata and processes (Bringmann et al., 2006). Müller cells are thought to function as optical fibers that transfer light to photoreceptors (Franze et al., 2007). They also play important roles in the development and maintenance of the retinal structure, and in survival of

retinal neurons from visual injuries (Newman and Reichenbach, 1996; Pekny and Pekna, 2004; Greenberg et al., 2007). In mature retina, Müller cells provide trophic factors to neurons and remove metabolic waste, regulate ion and water homeostasis, and maintain retinal blood flow. They may also regulate neuronal activities by rapidly uptaking and recycling neurotransmitters, and by releasing neurotransmitter precursors, gliotransmitters and other neuroactive substances (Bringmann et al., 2006; de Melo Reis et al., 2008).

Since Müller cells express various neurotransmitter receptors such as ionotropic and metabotropic glutamate receptors, GABA receptors, and glycine receptors (Malchow et al., 1989; Gerber, 2003; de Melo Reis et al., 2008; Roesch et al., 2008), they could also participate in the visual function through bidirectional communications between Müller cells and retinal neurons. A Ca^{2+} increase caused by light-evoked neuronal activity has been reported in Müller cells (Newman, 2004). Nonetheless, the detailed mechanisms and the significance of these communications remain unknown.

Accumulating evidence shows that astrocytes in the CNS respond to neurotransmitters from neurons and stimulate neurons by releasing gliotransmitters, thereby acting as an integral component of the synaptic network and contributing to the neuronal signal transmission. This leads to the emergence of the concept of “tripartite synapse” (Perea et al., 2009; Giaume et al., 2010). As the most important visual radial glia that interacts with almost all retinal neurons including photoreceptors, Müller cells

are likely to regulate the excitability and output of photoreceptors, and play important roles in the processing of visual signals.

The visual system of adult *Drosophila melanogaster*

We are using *Drosophila* as a genetic model system for the study of visual function. *Drosophila* and vertebrate visual systems share many common features in both structure and molecular mechanisms (Fig. 1-1). For example, both visual systems are characterized by layered and radial arrangement; photoreceptors in both systems respond to light through a G-protein-coupled signaling cascade; the light signals are transferred through graded potentials that depend on the release of neurotransmitters; signals are modulated by lateral neural integrators (Clandinin and Feldheim., 2009; Fain et al., 2010; Sanes and Zipursky, 2010). As a powerful genetic model organism, *Drosophila* has a huge advantage for *in vivo* characterization of visual signaling mechanisms.

In the adult *Drosophila* visual system, the light sensory organ, the compound eye, or retina, collects all visual information including light intensity, color and object movement, and transfers them to the brain through the optic lobe (Vogt and Desplan, 2007; Sanes and Zipursky, 2010). The optic lobe is divided into four neuropiles, i.e. lamina, medulla, lobula and lobula plate, the latter two comprise the lobula complex. The retina, lamina and medulla are comprised of an array of about 800 identical and well-organized units, called ommatidia, cartridges, and columns, respectively.

Compound eye and phototransduction

The structure of the *Drosophila* compound eye is well characterized. Each ommatidium contains 20 cells, 12 are supporting cells and pigment cells, and 8 are photoreceptors marked as R1-R8. The six outer photoreceptors, R1-R6, express the wide spectrum photopigment rhodopsin 1 (RH1) and are important for motion detection. The two inner photoreceptors, R7 and R8, express opsin sensitive to different wavelengths and are thought to be important in color vision (Montell, 1999).

In fly photoreceptor cells, light quanta are translated into electrical signals by a Gq protein-mediated signaling pathway. Exposure to light triggers the reversible conformational change of rhodopsin from an inactive state (R state) to metarhodopsin, the active state (M state). Metarhodopsin activates a heterotrimeric Gq protein. The α subunit of the activated Gq disassociates from the βγ subunits and stimulates a phospholipase C (PLC), which in turn catalyzes the hydrolysis of the membrane phospholipid phosphatidylinositol 4,5-bisphosphates (PIP2) into two secondary messengers, inostol 1,4,5-triphosphate (IP3) and diacylglycerol (DAG). Metabolites of DAG, polyunsaturated fatty acids (PUFAs), are thought to be involved in the opening of two classes of cation channels in the plasma membrane, the transient receptor potential (TRP) and TRP-like (TRPL) channels, and lead to the depolarization of photoreceptor cell membrane (Scott and Zuker, 1997; Hardie and Raghu, 2001; Squire et al., 2003; Katz and Minke, 2009). The underlying molecular mechanism of phototransduction is still not

entirely understood though.

As in vertebrate photoreceptors, fly photoreceptor light response terminates rapidly at the end of light stimulation for the achievement of high temporal resolution. This is largely dependent on the rapid inactivation of phototransduction cascade, involving the inactivation of rhodopsin by binding to arrestin, PLC-dependent deactivation of G protein through the GTPase-activating activity, and the closure of the channels (Smith et al., 1991; Scott and Zuker, 1997). In this thesis study, we attempt to identify more mechanisms that regulate the termination speed of photoreceptor light response.

Visual neuropiles and light signal transmission

Our current knowledge of *Drosophila* visual neuropiles is limited. The cellular diversity and synaptic organizations in the optic lobe have been mostly determined by Golgi impregnation and electron microscopy (Fischbach and Dittrich, 1989; Meinertzhagen and O'Neil, 1991; Takemura et al., 2008). To date, although accumulating projection mappings have been conducted through single-cell labeling and electron microscopy, very little is known about the function of individual cell types in the optic lobe (Morante and Desplan, 2004).

Outer photoreceptors project axons to the first neuropile, the lamina. Six axons from R1-R6 photoreceptors within six different ommatidia, innervate a single cartridge, and form synapses with lamina interneurons. Axons of R7 and R8 photoreceptors bypass the

lamina, terminate in the second and largest neuropile, the medulla, where they form synapses with processes of both lamina and medulla interneurons (Fig. 1-2).

The lamina neuropile contains five types of monopolar cells, L1-L5. In addition to R1-R8 axons and L1-L5 processes, each cartridge contains processes of two medullary centrifugal cells (C2 and C3), a third medullary cell (T1), a lamina intrinsic wide-field amacrine cell and two other wide-field neurons called tangential cells. Three epithelial glia cells surround each cartridge and extend processes into it (Fig. 1-2)

The medulla neuropile is subdivided into ten layers, M1-M10. R7, R8 and L1-L5 terminate and form synapses in either one or a few specific layers in the outer medulla M1-M6. Medullary cells including T1, tangential cells, C2 and C3 cells, multiple morphological species of medulla intrinsic neurons (M cells), medulla tangential neurons that project axons to lobula complex (Tm and TmY), also arborize in specific layers of the medulla. Synaptic connections among these cells in the medulla have been partially revealed by electron microscopy (Takemura et al., 2008), but the detailed visual pathways are still a mystery.

Different features of light information from the medulla are then transferred to the central brain through the lobula complex. The giant neurons, including the horizontal system (HS) cells and the vertical system (VS) cells, are responsible for motion detection. Their dendrites arborize broadly in the entire medulla neuropile, implying their importance in visual processing. It has been proposed that the VS cell dendrites receive

local, motion-sensitive elements that could be processed by L1 and L2 cells, the targets of outer photoreceptors (Joesch et al., 2008). However, details of this pathway remain unknown.

The *Drosophila* photoreceptors synthesize the inhibitory neurotransmitter histamine, which undergoes Ca^{2+}-dependent release upon photoreceptor depolarization (Hardie, 1987; Hardie, 1989). Histamine-gated chloride channels have been identified and are shown to be expressed in postsynaptic cells (Gengs et al., 2002; Zheng et al., 2002; Pantazis et al., 2008). Light-stimulated histamine release hyperpolarizes, and thus inhibits postsynaptic interneurons. Conversely, the removal of light abolishes histamine release, and excites the postsynaptic cells. Such a visual signaling pathway is called a sign-reversing pathway (Stuart et al., 2007).

While R7 and R8 photoreceptors mediate color vision and transfer the signal directly to the medulla, R1-R6 are responsible for high sensitivity vision and motion detection, and form tetrad synapses in the lamina. The postsynaptic cells of R1-R6 include L1, L2, one of the L3, amacrine cells, and epithelial glia. Amacrine cells are intrinsic to the lamina, while L1-L3 cells project to distinct layers in the medulla, where they synapse on unidentified medulla cells as well as terminals of other lamina neurons. The details of signal transmission in medulla and lobula complex are still unclear, but the large tangential neurons in the lobula complex provide motion sensitive pathways that project to the protocerebrum (Raghu et al., 2007; Raghu et al., 2009).

Feedback circuits in the visual system

Synaptic connections within the *Drosophila* lamina neuropiles were revealed by a serial electron-micrograph study in a single cartridge, in which the presence of synapse was characterized by a T-shaped presynaptic ribbon. It was shown that R1-R6 photoreceptors project synaptic terminals on monopolar cells and amacrine cells: 246 to L1, 269 to L2, 74 to L3 and 254 to amacrine cells, respectively. They also receive feedback inputs from the lamina interneurons: 6 from L2, 20 from L4, 48 from amacrine cells and 2 from C2 (Fig. 1-3A). This morphological evidence supports the idea that *Drosophila* photoreceptors undergo feedback regulation from lamina interneurons (Meinertzhagen and O'Neil, 1991; Meinertzhagen and Sorra, 2001). Since light triggers histamine release from photoreceptors，which causes hyperpolarization and inactivation of the postsynaptic interneurons, the feedback input from interneurons to photoreceptors should be triggered only in the dark when the histamine inhibition is absent. Because of the limited study, the function of the feedback input is unclear.

Immunocytochemistry and pharmacology studies have shown that amacrine cells are glutamatergic, while L2 may be a glutamatergic or cholinergic neuron (Yasuyama et al., 1996; Sinakevitch and Strausfeld, 2004; Kolodziejczyk et al., 2008). In combination with the histamine signals they receive from photoreceptors, it is reasonable to propose a simple model of feedback loop modulating photoreceptor response (Fig. 1-3B), in which L2 and amacrine cells receive inhibitory regulation from photoreceptor axons, and

activate depolarizing conductances on photoreceptor terminals by an excitatory neurotransmitter (glutamate or acetycholine). This model is supported by intracelluar recording from large monopolar cells (L1 and L2) and photoreceptors in flies with different genetic backgrounds (Zheng et al., 2006), and is proposed to contribute to retinal network adaptation (Nikolaev et al., 2009).

No studies on the feedback function of L4 cells have been reported. Although L4 cells do not receive direct input from photoreceptor terminals, they are innervated by L2 cells and send input directly to photoreceptors (Fig. 1-3A). Immunocytochemistry results show that they use acetycholine as a transmitter and express the $GABA_A$ receptor subunit RDL (Meinertzhagen and O'Neil, 1991; Kolodziejczyk et al., 2008), suggesting that they could also have an excitatory effect on photoreceptors and may be involved in a more complicated feedback circuit.

Both glutamate and acetycholine are excitatory neurotransmitters that may trigger depolarization of photoreceptors. However, to help to achieve the rapid repolarization at the end of a light response, interneurons need to apply an inhibitory regulation to photoreceptors when the light intensity decreases. It is unknown whether alternative feedback circuits exist for this purpose.

In addition to the identified synaptic contacts, nonsynaptic communication between photoreceptors and lamina cells such as glia may also play a role in regulating photoreceptor light response.

Glia cell functions in the adult visual system

Glia cells play important roles in neural circuit assembly, boundary formation, and axon guidance during the development of the fly visual system (Poeck et al., 2001; Parker and Auld, 2006; Chotard and Salecker, 2007). In adult fly lamina, there are six morphologically distinct classes of glia: two surface glia, fenestrated and pseudocartridge glia; two cortex glia, distal satellite and proximal satellite glia; and two neuropile glia, epithelial and marginal glia (Saint Marie and Carlson, 1983; Stuart et al., 2007). The epithelial glia is the major class, extending throughout the whole length of lamina neuropile, and surrounding the cartridge in a precise geometrical array. Unlike ensheathing glia that surround the neuropile to isolate neurons, lamina epithelial glia elaborate processes into the neuropile (Fig. 1-2B), and are comparable with astrocytes in the brain. This feature enables them to modulate synaptic connections (Murai and Van Meyel, 2007). Thus, most work on adult visual glia are focused on the lamina epithelial glia.

A major function of epithelial glia is to recycle neurotransmitters in the lamina (Stuart et al., 2007; Bringmann et al., 2009). To achieve a high temporal resolution of vision and avoid signal loss in the first neuropile, histamine released from the photoreceptor needs to be quickly removed from synaptic clefts. Lamina epithelial glial cells play critical roles in histamine clearance and recycling. They uptake histamine and convert it into an inactive conjugate, carcinine, through the β-alanyl biogenic amine synthase Ebony. After being

transferred back to the photoreceptor axon, carcinine is converted back to histamine by the cysteine peptidase Tan. The carcinine transport Inebriated Ine may mediate the carcinine shuttling from glia to photoreceptor (Soehnge et al., 1996; Gavin et al., 2007). This Ebony/Tan pathway is responsible for replenishing the histamine pool in the photoreceptor and ensuring signal transmission from photoreceptors to first-order interneurons (Stuart et al., 2007).

Epithelial glial cell processes are found postsynaptic to photoreceptor at tetrad synapses (Fig. 1-3A) (Meinertzhagen and O'Neil, 1991). One histamine-gated chloride channel, HisCl1, is expressed in epithelial glial cell, although its function remains unknown (Zheng et al., 2002; Pantazis et al., 2008).

In addition to forming potential synapses, epithelial glia may also communicate with neurons through two types of anatomically characteristic structures, capitate projections and gnarls (Fig. 1-2C) (Meinertzhagen and O'Neil, 1991).

Capitate projections are dynamic invaginations from epithelial glia to photoreceptor axons (Trujillo-Cenóz et al., 1965). Each capitate projection consists of a stalk and a spherical head that contains a widened extracellular space between membranes of glia and photoreceptor that contribute to a high electron density in EM. It has been shown that the number of capitate projections in a photoreceptor axon varies by the light condition (Rybak and Meinertzhagen, 1997), temperature (Brandstätter and Meinertzhagen, 1995), and by drug treatments that affect glial metabolism, or close gap juntions (Pyza and

Górska-Andrzejak, 2004). The dynamic features of capitate projections suggest that they may play a role in temporal regulation of visual signaling.

Endocytotic proteins, Clathrin and Endophilin, localize to capitate projections, and endocytotic vesicles are observed attached to and near the capitate projection stalk, indicating that they are sites of endocytosis in the photoreceptor axons. It has been proposed that capitate projections may mediate the transport of carcinine back to the photoreceptor terminal and thus are important for the histamine recycling (Fabian-Fine et al., 2003).

Gnarls are glial invaginations mostly observed between α-processes of amacrine cells and β-processes of T1 cells. They were first found in *Musca* (Campos-Ortega and Strausfeld, 1973). In *Drosophila* lamina, it displays a planar form of a slender glial process interposed between α and β elements (Meinertzhagen and O'Neil, 1991). Interestingly, superficially similar glial structures were also observed between β and β processes, as well as β processes and photoreceptor terminals (referred to as plaque junctions in *Drosophila*). The β process of T1 appears to be a common element of all gnarl structures. Although gnarl is thought to be a peculiar synaptic complex in which glia participate at postsynaptic sites (Burkhardt and Braitenberg, 1976; Saint Marie and Carlson, 1983), the function of gnarl is totally unknown.

In addition to contact neurons, epithelial glial cells are linked laterally to each other through gap junctions (Saint Marie and Carlson, 1983). The geometric array between

epithelial glial cells and cartridges suggest that epithelial glial cells may be able to transfer information between adjacent cartridges.

Findings in this study

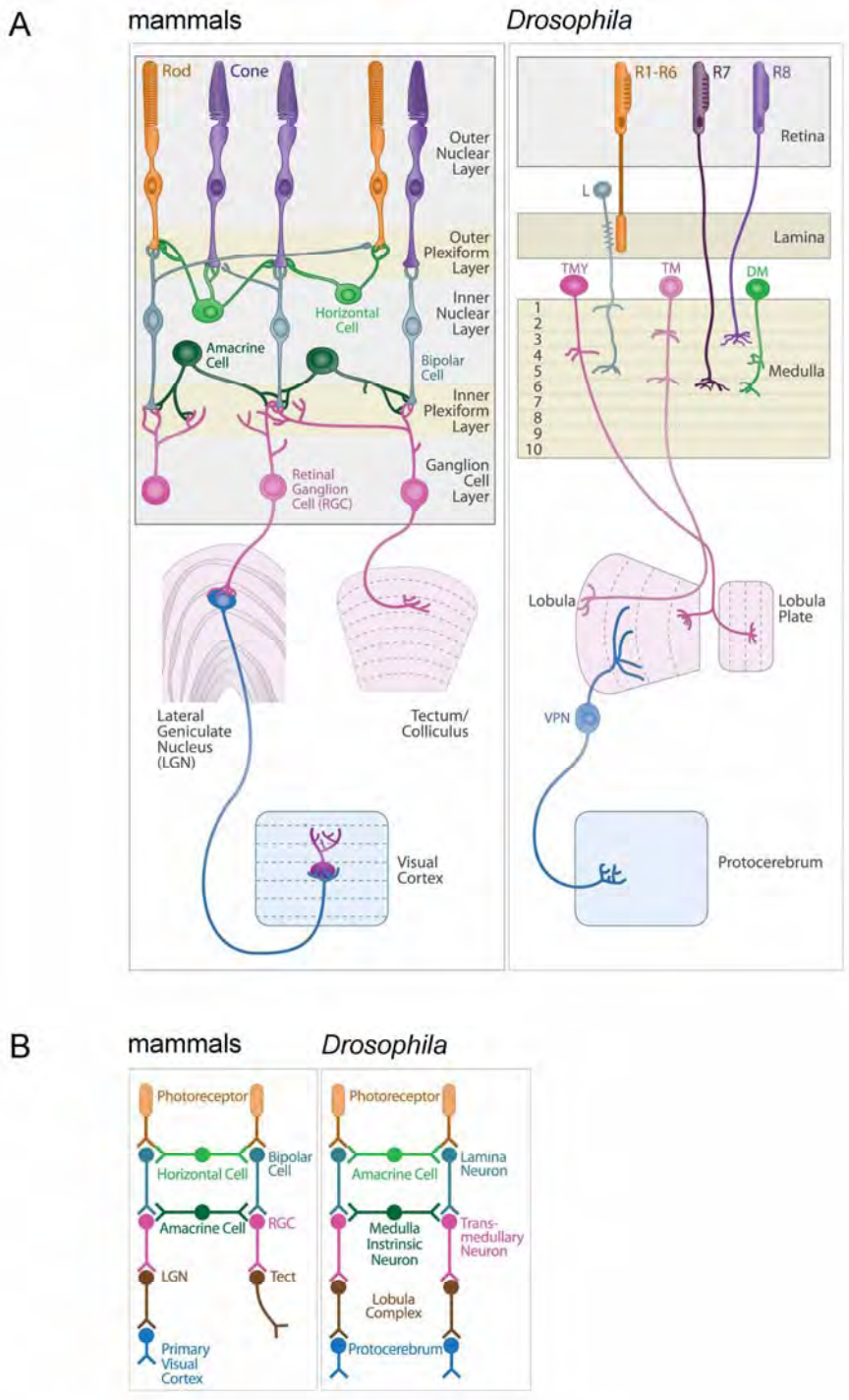
By using *Drosophila* as a model to study the termination of photoreceptor light response, we seek to answer the following questions: Is the deactivation of phototransduction enough for rapid repolarization of photoreceptor at the end of the light response? Is there any extrinsic factor involved? If yes, what is the underlying mechanism?

My research in this thesis has revealed a feedback mechanism that contributes to the termination of photoreceptor light response, in which the visual glia plays a critical role.

First, by characterizing the visual defect in a *slrp* (*slow receptor potential*) mutant fly that displays an abnormally slow termination of light response, I show that lamina epithelial glial cells facilitate the repolarization of photoreceptor at the end of the light response, and that gnarl, a SLRP-dependent special glial structure, may mediate this glial regulation.

Further, I have identified a photoreceptor — amacrine cell — epithelial glia — photoreceptor feedback loop that contributes to the rapid repolarization of photoreceptor. An inhibitory glutamate receptor GluCl is expressed in epithelial glia and mediates this feedback regulation.

Collectively, my research has revealed a novel feedback regulation mechanism that helps to achieve the rapid kinetics of photoreceptor light response termination. This work has advanced our knowledge about visual signaling modification, and emphasizes a important signaling role of glia in visual systems.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Amacrine Cell
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Retinal Ganglion Cell (RGC)
Lateral Geniculate Nucleus (LGN)
Tectum/ Colliculus
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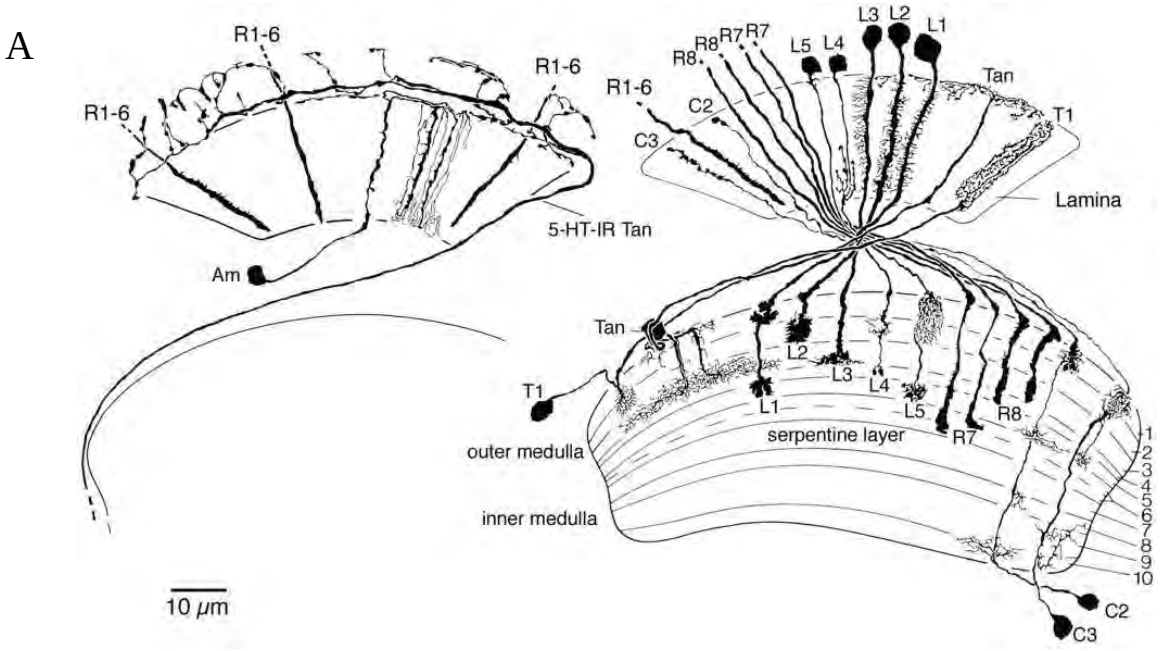
Amacrine Cell
Lamina Neuron
Medulla Instrinsic Neuron
Trans-medullary Neuron
Lobula Complex
Protocerebrum

Figure 1-1

Figure 1-1. Comparison between mammalian and *Drosophila* visual systems (modified from Sanes, 2010)

(A) Structures and light signaling transfer pathways from photoreceptors to brain in the visual systems of mammals (left) and *Drosophila* (right). Morphologies of major cell types and synaptic connections are shown.

(B) Major steps in light signal transfer in mammals (left) and *Drosophila* (right). In the mammalian visual system, as well as in other vertebrate visual systems, visual information is transferred to the brain through bipolar cells and retinal ganglion cells. Horizontal cells and amacrine cells form the lateral networks. In the *Drosophila* visual system, signals from photoreceptors are transferred to the brain through lamina, medulla, and lobula complex neuropiles. Amacrine cells and medulla intrinsic neurons distribute laterally in lamina and medulla, respectively.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R8
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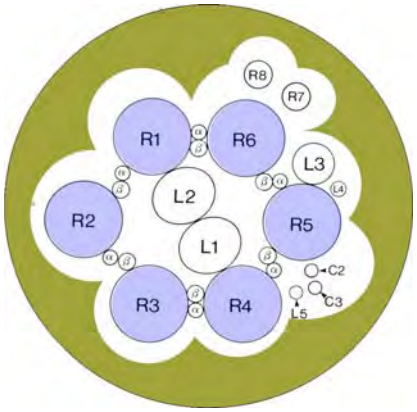
R7
L5
L4
L3
L2
L1
Tan
T1
C2
C3
Lamina
5-HT-IR Tan
Am
Tan
T1
L2
L3
L4
L1
L5
R8
R7
serpentine layer
outer medulla
inner medulla
1
2
3
4
5
6
7
8
9
10
10 μm
C2
C3


B
R8
R7
R1
R6
L3
L2
R2
R5
L1
C2
C3
R3
R4
L5

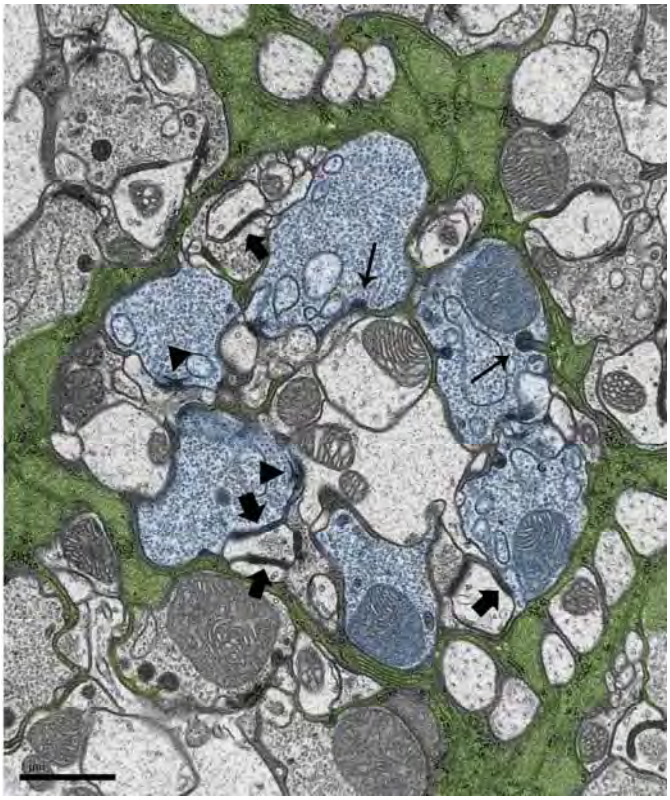

C

Figure 1-2

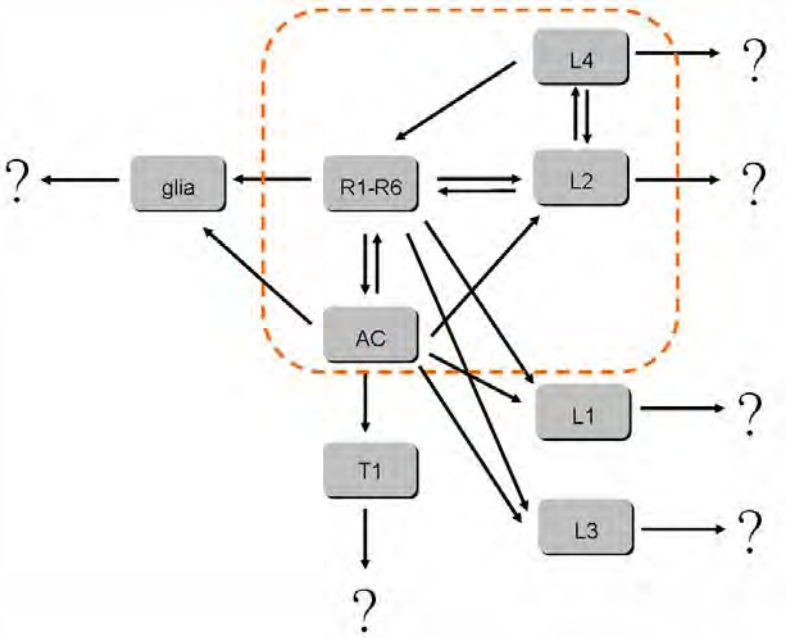
Figure 1-2. Cell types in *Drosophila* lamina

(A) Neuron types of the lamina revealed by Golgi impregnation (Kolodziejczyk et al., 2008, modified from Meinertzhagen and O'Neil, 1991), including photoreceptor R1-R6, R7, R8; lamina neurons L1-L5; lamina intrinsic wide-field amacrine cell; medullary cell T1; medullary centrifugal cells C2 and C3 and transmedullary wide-field neurons tangential cells Tan.

(B) Position of each component in the cross section of a *Drosophila* lamina cartridge. R1-R6 terminals (blue) form a ring surrounding the axons of L1 and L2. Coupled small processes of T1 and amacrine cells (α-processes of amacrine cells and β-processes of T1 cells) locate between R1-R6 terminals. Axons of other cells such as L3, L4, L5, C2 and C3 are shown. The cartridge is surrounded by epithelial glial cells (green).

(C) Electron Micrograph of the cross section of a *Drosophila* lamina cartridge. Synapses characterized by a T-shape presynaptic ribbon (arrowhead), glial invaginations in photoreceptors, capitate projections (narrow arrow), and glia-neuron structure gnarl (thick arrow) are shown. Each cartridge is surrounded by three epithelial glial cells (green).

A


L4
?
?
glia
R1-R6
L2
?
AC
L1
?
T1
L3
?
?

B

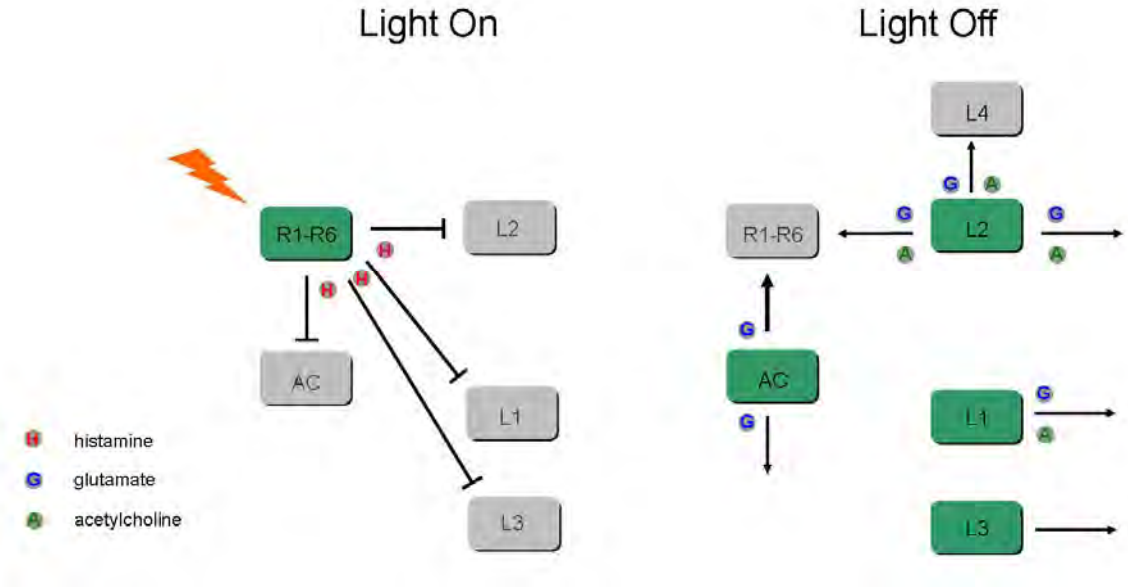

Light On
Light Off
R1-R6
L2
AC
L1
L3
L4
histamine
glutamate
acetylcholine

Figure 1-3

Figure 1-3. Signaling pathways between major cell types in *Drosophila* lamina

(A) Synaptic organizations between major cell types in lamina revealed by series EM studies. R1-R6 photoreceptors synapse to L1, L2, L3, amacrine cells, and epithelial glia, and receive feedback inputs from L2, amacrine cell and L4. L4 is invaginated by L2. Amacrine cells synapse to L1, L2, L3, T1 and glia cells. Outputs of other interneurons are not completely clear. R1-R6 photoreceptors and interneurons providing feedback synapses are shown inside the red box. AC: amacrine cells.

(B) Proposed negative feedback loop model. In light conditions, depolarization of R1-R6 triggers the release of histamine, which inhibits postsynaptic interneurons including L1, L2, L3 and amacrine cells. Removal of light abolishes the histamine release and actives these interneurons. Among them, L2 and amacrine cells release excitatory neurotransmitters (glutamate or acetycholine) and have feedback input to R1-R6 photoreceptors. Feedback from L4 is unclear. L1 may also use glutamate or acetycholine as transmitter.

CHAPTER II: A *DROSOPHILA* ADAM PROTEIN ASSEMBLES A GLIAL STRUCTURE AND IS CRITICAL FOR THE TERMINATION OF PHOTORECEPTOR LIGHT RESPONSE

This work was conducted under the direction of Dr. Hong-Sheng Li. My contribution to this work consists of electrophysiological recordings, gene identification and cloning, generating transgenic flies, fly genetic work, antibody generation and purification, western blot, immunostaining, electron microscopy and data analysis. Keith Reddig contributed by assisting in electron microscopy and cryosectioning for immunostaining. Ping Gong contributed by generating Arr1 and Arr2 antibodies. Dr. Hong-Sheng Li and I contributed to write the body of work.

Abstract

Timely deactivation of photoreceptor cells is one of the fundamental characteristics of visual systems. Although many key elements responsible for photoreceptor deactivation have been identified within photoreceptors, the role of factors outside photoreceptors remains unknown. Here we report a novel mechanism of photoreceptor deactivation, which depends on a retrograde regulation from visual glial cells. In *Drosophila* lamina, the first visual neuropile, the epithelial glia form slender sheets, called gnarl, between the processes of amacrine cells and those of T1 interneurons, and between T1 processes and photoreceptor terminals. We show that the gnarl structure depends on the ADAM (*a disintegrin and metalloprotease*) protein SLRP (*slow receptor potential*), and is critical for rapid termination of visual response in the photoreceptor. In the lamina, SLRP is expressed by glia, and is localized exclusively in gnarls. In a *slrp* mutant, the expression of SLRP in the lamina is completely eliminated, leading to disruption of gnarl. Intracellular recording of photoreceptor *in vivo* revealed a defect in the speed of light response termination, which was rescued by overexpression of a wild type SLRP protein. Since the light response in isolated cell bodies of mutant photoreceptors is normal, the slow termination of light response is most likely due to defective repolarization of photoreceptor axon at the end of light stimulation. This work reveals an important role of visual glia and SLRP protein in the regulation of the photoreceptor activity, which may contribute to the temporal resolution of *Drosophila* vision.

Introduction

To timely reflect the different aspects of visual information from the surrounding world such as shape, color and motion of a subject, the visual system of an organism needs to detect the light information with high temporal resolution, which depends on rapid response of photoreceptor, the primary light sensory neuron, not only to the increase of light intensity, but also to light dimming or light out.

Light activates a G-protein coupled receptor (GPCR) rhodopsin, which triggers the phototransduction cascade that regulates the activities of channels on photoreceptor membrane, and causes membrane potential change. This signal transduction process, referred to as phototransduction, enables light signals to be converted to electrical signals that can be transferred into the brain. Removal of light stimulation causes rapid recovery of the photoreceptor membrane potential and terminates the light response (Squire et al., 2003).

Previous research has greatly advanced the understanding of how rapid light response termination occurs in photoreceptor cells. Almost all research has focused on the deactivation of the GPCR phototransduction cascade (Sagoo and Lagnado, 1997; Burns and Baylor, 2001; Yau and Hardie, 2009; Burns, 2010). For example, rhodopsin can be rapidly shut off by phosphorylation and arrestin binding, which ensure the fast kinetics of termination response (Kuhn and Wilden, 1987). Nonetheless, it is unclear whether any

retrograde regulation contributes to the rapid termination of photoreceptor light response.

To address this question, we have been using *Drosophila* as a genetic model system. In *Drosophila*, many proteins are required for the rapid termination of photoreceptor light response, such as arrestin2 (ARR2) that binds and inactivates rhodopsin (Alloway and Dolph, 1999), through the facilitation of a myosin III, NINAC (Lee and Montell, 2004; Liu et al., 2008); a protein kinase C, INAC (Hardie et al., 1993), that could inactivate the light stimulated ion channels; and INAD, a scaffold protein that assembles the molecules together for rapid control (Shieh and Zhu, 1996; Chevesich et al., 1997). All these mechanisms function within the photoreceptor.

However, the rapid deactivation of phototransduction may not be sufficient for rapid termination of photoreceptor light response. It has been reported that the prompt restoration to the resting state in photoreceptors when light intensity dims undergoes a retrograde signaling regulation from the brain to the retina (Rajaram et al., 2005). Although no mechanism was provided, this work raised the possibility that the termination of the photoreceptor light response may be modified by retrograde modulation.

In the *Drosophila* visual system, the axons of the R1-R6 outer photoreceptor cells form synapses in the lamina, the first-order neuropile, where they send histaminergic signaling to interneurons including monopolar cells and amacrine cells. They also receive feedback input from the lamina interneurons, but the detailed feedback mechanisms are

still unclear (Meinertzhagen and O'Neil, 1991; Pantazis et al., 2008).

In this study, by identifying the *slrp* gene and its function, we report that the termination of light response in *Drosophila* photoreceptors undergoes a retrograde regulation mediated by lamina epithelial glial cells. A special glial process called gnarl, which depends on an ADAM protein SLRP for formation, may mediate this retrograde regulation.

Results

Photoreceptors in the *slrp* mutant display slow termination of light response

Induced by ethyl methanesulfonate mutagenesis, *slrp* mutants were isolated and reported in the 1970's (Pak, 1975; Homyk and Sheppard, 1977). All four alleles showed an abnormally slow termination of light response in ERG recording. In addition to the visual defect, they were generally hypoactive and displayed locomotion-deficient phenotypes. However, as the *slrp* gene had not yet been cloned, the underlying mechanism was unknown.

Electroretinogram (ERG) recording on the *slrp* mutant confirming previous reports, showed that when light stimulation is removed, the time required for the field potential to return to the baseline increased in the *slrp* mutant (Fig. 2-1A). ERG recording reveals the net extracellular potential change and represents the sum of the electrical activities of the entire fly eye in response to light stimulation. It not only reflects the electric change in photoreceptor cells, but is also affected by activities of pigment cells in the retina, as well as secondary neurons and visual glia in the lamina. To confirm that the slower termination of light response in the *slrp* mutant indeed occurs in the photoreceptor cells, we recorded the intracellular responses of photoreceptors in *slrp* flies. The results showed that photoreceptors in the *slrp* mutant could not repolarize as rapidly as the wild type at the end of light stimulation (Fig. 2-1B).

The visual defect in *slrp* photoreceptor is caused by loss of regulation on photoreceptor axons

The visual defect in *slrp* mutant photoreceptor may be due to abnormalities in eye development, defects in the regulation of the phototransduction cascade, or loss of extrinsic regulations from other cell types.

First, no morphological abnormality was observed in the compound eye of the *slrp* mutant using electron microscopy (Fig. 2-2A), suggesting that the visual defect is not caused by a developmental problem.

Next, we examined protein levels of key components of the phototransduction cascade, including TRP, TRPL, RDGC, INAD, INAC, RH1, ARR1, ARR2 and Gq (Scott and Zuker, 1997; Hardie and Raghu, 2001), and found that they all have normal levels (Fig. 2-2B). In addition, immunocytochemistry did not reveal any unusual distribution of these proteins, either. To find out whether the phototransduction cascade is regulated normally in the *slrp* mutant, we recorded whole-cell currents of photoreceptor cell bodies isolated from *slrp* mutant eyes. The light stimulated whole-cell responses in *slrp* mutant photoreceptors were terminated as rapidly as in wild type (Fig. 2-3). Thus, the slow repolarization of intact photoreceptors in the *slrp* mutant is not due to a defect in the regulation of the phototransduction cascade. The absence of a slow-termination defect in this recording paradigm suggests that the visual defect in the intact *slrp* photoreceptor is caused by loss of regulation on photoreceptor axons.

Still, there are two possibilities for the repolarization defect of photoreceptors in the *slrp* mutant: First, *slrp* photoreceptors may lack an intrinsic repolarization mechanism in the axon, for example, a potassium channel. Second, an extrinsic regulation is missing.

Mutation in the *mmd* gene is responsible for the *slrp* visual defect

To further understand the nature of the visual defect in the *slrp* mutant, we started out identifying the *slrp* mutant gene.

The *slrp* gene was previously mapped to the region 13F10-14B1 through complementation tests (Homyk and Pye, 1989). To narrow down the gene region, we generated five deficiencies that together delete most parts of the 13F17-14B3 region, using FRT-mediated, targeted deletion strategy (Parks et al., 2004). The *slrp* ERG phenotype was uncovered by Δe03798-f02910. Four additional deficiencies carrying smaller deletions in this region were then generated and tested for phenotype complementation. The result showed that the *slrp* gene was uncovered only by the deficiency Δe01671-e00903. Thus, four genes in this mapped region, including *cg8928*, *mind-meld (mmd)*, *or13a*, and *cg9170* are candidates for the *slrp* gene (Fig. 2-4A).

We conducted RT-PCR using mRNA from *slrp* mutant heads, but did not detect any change in the mRNA level of the four candidate genes. Two smaller genes, *cg8928* and *cg9170* were further excluded after genomic DNA sequencing, leaving *mmd* and *or13a* as the only two candidates. Since large genes may have multiple splicing forms, additional

RT-PCRs were conducted using primers targeting different cDNA fragments. The results showed that some *mmd* PCR products were smaller in the *slrp* mutant compared to wild type, although the mRNA level remained the same (Fig. 2-4B), indicating that the splicing pattern of *mmd* gene is altered in the *slrp* mutant.

Sequencing of the *mmd* cDNA from the *slrp* mutant showed that a 298bp sequence of intron18 was appended to exon18, which introduces a stop codon into the cDNA, and causes the truncation of the MMD protein. This abnormal splicing pattern could be due to an A to T point mutation located next to the splice donor site GT in intron18 (Fig. 2-4C).

To confirm that the *slrp* phenotype is caused by the loss of MMD, We attempted to rescue the phenotype by expressing wild type cDNAs of *mmd* in the mutant background. 5'UTR of *mmd* gene was identified based on a previously reported cDNA clone (GenBank DQ327771.1), and was confirmed by RT-PCR. Using 3'- RACE PCR and additional RT-PCRs, we identified and cloned four isoforms of *mmd* mRNA: *RB*, *RA1*, *RA2* and *RA3* (Fig. 2-4C). All these isoforms are affected by the splicing mutation in the *slrp* mutant. Next, we generated transgenic flies expressing each isoform through a heat shock promoter in the *slrp* mutant background. After heat shock-driven expression, the two longer forms, *RA2* and *RA3* rescued the *slrp* mutant phenotype (Fig. 2-4D). This result confirmed that mutation of *mmd* is responsible for the *slrp* defect. For consistency, we refer to the *mmd* gene as *slrp*.

SLRP does not function as a metalloprotease, and is not required during development

Sequence analysis has shown that SLRP belongs to the highly conserved ADAM protein family. Members of this family share a conserved multi-domain organization: an N-terminal signal sequence followed by a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich domain, an EGF-like domain, a transmembrane domain, and a cytoplasmic tail (Primakoff and Myles, 2000; Seals and Courtneidge, 2003).

A major function of ADAM family members is proteolysis depending on the metalloprotease domain that contains a conserved zinc-binding catalytic site sequence HExGHxxGxxHD (Gee and Knowlden, 2003; Higashiyama and Nanba, 2005). This sequence in SLRP protein, HMIGHNIGMGHD, is not totally conserved, suggesting that SLRP may not function as an active protease. To confirm this, a transgenic fly that expresses mutant variant SLRP with the metalloprotease domain disrupted was generated and examined for visual function. The results showed that SLRP with disrupted metalloproteinase domain still rescued the *slrp* phenotype, indicating that SLRP does not function as a protease in the visual system.

Another important function of ADAM proteins is cell-cell adhesion that is important for developmental events such as axon guidance and neuroblast migration, which is contributed by the disintegrin domain and other domains (Smith et al, 2002; White, 2003;

Huang et al., 2003; Tousseyn et al., 2006; Alfandari et al., 2009). In the rescue experiments, heat shocking *slrp;P[hs-SLRP-RA2]* flies at the adult stage rescued the visual phenotype (Fig. 2-4D; Fig. 2-5), indicating that SLRP is not required during the development of fly vision, but may mediate a physiological regulation in the eye.

SLRP does not function within photoreceptor

Since SLRP is required for rapid repolarization of photoreceptor at the end of light stimulation, we tested whether it functions inside the photoreceptor cell by expressing SLRP in photoreceptor cells. A *UAS-SLRP-RA2* transgenic fly was generated. We specifically expressed SLRP-RA2 in photoreceptors through the drive of a *RH1-GAL4* line in the *slrp* mutant background. The *slrp* ERG phenotype was not rescued (Fig. 2-6), indicating that SLRP expression outside of the photoreceptor cell is required for its visual function. This result suggested that the visual defect in the *slrp* mutant is due to loss of an extrinsic regulation that depends on SLRP.

SLRP is highly expressed in the lamina

To investigate SLRP protein localization, we generated a polyclonal antibody that recognizes all isoforms of SLRP. In western blot, the antibody revealed several major bands in wild type samples, which had the predicted sizes for each isoform. In *slrp* mutant samples, however, except for a trace amount of the longest form, almost no SLRP

signal was detected (Fig. 2-7A). Thus, this antibody is specific for SLRP proteins.

Western blot results also showed that the four forms of SLRP have different distribution. SLRP-RA1 and SLRP-RA2 are the only forms that can be detected in the lamina and retina extract (Fig. 2-7A), indicating that they are the major forms functioning in these tissues.

Immunostaining of fly head sections using the SLRP antibody showed that SLRP is not expressed in the retina at all, where the photoreceptor cell bodies and the phototransduction machinery are located. Instead, SLRP displayed a punctate distribution across almost all neuropiles in the brain, with the highest expression level observed in the first visual neuropile, lamina, where photoreceptor axon synapses with second-order interneurons. In control experiments, no SLRP protein was detected in the lamina region of *slrp* mutant flies, although a trace level of SLRP protein was observed in central areas of the brain (Fig. 2-7B).

Previous studies have shown that photoreceptor axons in the lamina cartridge receive input from interneurons L2, L4, and amacrine cells. If SLRP is required for the extrinsic regulation on photoreceptors, it is very likely to function in those interneurons. In co-localization tests, we labeled L2 and L4 cells with GFP proteins that were expressed through *L2-GAL4* line and *rdl-GAL4* line, respectively, and processes of amacrine cells with a vGluT antibody. To our surprise, SLRP was not localized in any of these neurons. However, the SLRP signal overlapped with processes of T1 cells, which were revealed by

T1-specific GFP signals (Fig. 2-8A).

Neuronal expression of SLRP is not sufficient for its visual function

To test whether SLRP functions in T1 cells, we overexpressed SLRP in T1 cells through a *T1-GAL4* line in *slrp* mutant flies. The *slrp* visual phenotype was not rescued (Fig. 2-8B). This may be caused by (1) SLRP is expressed in multiple cell types that are all required for the visual function. (2) Although SLRP signal is overlapped with T1 marker, considering the dense structure and the size of lamina dendrites, these signals may come from close membrane structure belonging to other cells.

To test the neuronal function of SLRP in visual system, we expressed SLRP-RA2 in all neurons through the drive of a pan-neuronal *elav-GAL4* line in the *slrp* mutant background. The *slrp* ERG phenotype was not rescued (Fig. 2-8B), indicating that neuronal expression of SLRP is not sufficient for its visual function**.**

Both neuronal and epithelial glial expressions of SLRP are required for its visual function

Since the visual defect in the *slrp* mutant was not rescued by neuronal expression of SLRP, next, we tested whether the SLRP visual function depends on its expression in glia. We expressed SLRP-RA2 in glia using a *repo-GAL4* line in the *slrp* mutant background. Although partial rescue was observed in a small fraction of *UAS-SLRP-RA2;repo-GAL4*

flies, the overall termination speed of light response in these flies was still much lower than that of wild type. Instead, driving SLRP expression by using a combination of *elav-GAL4* and *repo-GAL4* could rescue the *slrp* ERG phenotype, indicating that both neuronal and glial expression of SLRP are required for its visual function (Fig. 2-9A).

Immunostaining result shows that the SLRP signal is observed in the whole lamina neuropile, suggesting that it may express in the epithelial glia, which is the only glia that extends the full length of lamina neuropile and innervates between lamina neurons. To confirm this, we overexpressed SLRP using an epithelial glia specific driver, *HisCl1-GAL4*, in combination with an *elav-GAL4*. In ERG recordings this combined expression of SLRP rescued the *slrp* mutant phenotype (Fig. 2-9A), suggesting that SLRP functions in lamina epithelial glia.

To confirm that SLRP is expressed in lamina glia, we knocked down SLRP specifically in glial cells using a UAS-hairpin $slrp^{RNAi}$ line in combination with the *repo-GAL4* line. In the $slrp^{RNAi}$*;repo-GAL4* fly, the SLRP signal disappeared in the lamina, while no change was seen in other region of the brain. In contrast, in *elav-GAL4;*$slrp^{RNAi}$ flies, the SLRP level did not change much in the lamina, but decreased in the central brain and other parts of optic lobe (Fig. 2-9B). These results suggested that the laminar SLRP protein is mostly expressed by glia cells, while the majority of SLRP protein in other regions is in neurons.

Consistent with the expression pattern of SLRP, light responses of both

slrp^{RNAi}*;repo-GAL4* and *elav-GAL4;slrp*^{RNAi} flies terminated slowly in ERG assays (Fig. 2-9C), Thus, both epithelial glial and neuronal expressions are required for the visual regulation function of SLRP.

We then attempted to identify the neuron cell that expresses SLRP through phenotype rescue by expressing SLRP using the combination of *repo-GAL4* and a GAL4 line specifically expressed in a particular type of interneurons, including L1, L2, L4 and T1, but failed to observe significant rescue in any combination. This may suggest that either SLRP is expressed in other interneurons such as amacrine cells and centrifugal cells, which lack specific GAL4 lines; or SLRP is expressed in a medulla interneuron that regulates photoreceptor activity indirectly. In addition, SLRP visual function may need its expression in multiple neurons, so that overexpression of SLRP in glia and one type of neuron is not sufficient to rescue the *slrp* ERG phenotype.

Loss of SLRP leads to disruption of a glial structure, gnarl

The RNAi and phenotype rescue experiments indicate that SLRP is mostly expressed in epithelial glia. Nonetheless, immunostaining pattern of SLRP fits that of T1 cell terminals, and is not reminiscent of the glia distribution. To resolve this discrepancy, we localized SLRP in the lamina at the EM level using an immunogold labeling technique. The result showed that SLRP is located on a membrane structure of high electron density. The average length of these densities is over 200nm, and no accompanying synaptic

T-shape was observed. These SLRP positive densities were observed between interneurons (Fig. 2-10A), and between interneurons and photoreceptors (Fig. 2-10B). Based on the cartridge location of these density structures, and taking into consideration the overlapping of SLRP signal and T1 process in the immunofluorescence staining, these densities are very likely to be the glial structure gnarl that forms between T1 processes and photoreceptor, T1 or amacrine cell processes.

Localization of the SLRP protein on gnarl structure suggested that either SLRP functions for neuron-glia communication through this structure; or SLRP is required for the formation of this structure. To test whether loss of SLRP causes any visible defect in gnarl structure, we examined the ultrastructure of gnarl using electron microscopy. In *slrp* mutant lamina, almost no gnarl structures were detected. Overexpression of SLRP using a *tub-GAL4* not only rescued the *slrp* visual phenotype, but also recovered the gnarl numbers in the *slrp* mutant background (Fig. 2-11). Thus, SLRP is required for the formation of gnarl, which may mediate the visual function of SLRP.

Discussion

The rapid termination of photoreceptor light response depends on retrograde regulations

In *Drosophila* photoreceptors, photoresponse termination is thought to be achieved by fast deactivation of rhodopsin and calcium mediated intrinsic feedbacks (Dolph et al., 1993; Hardie et al., 2001; Gu et al., 2005). When light stimulation is removed, the deactivation of the GPCR signaling cascade leads to the repolarization of photoreceptor membrane potential to the baseline (Scott and Zuker, 1997).

A slow termination of photoreceptor light response has been reported in mutants with blocked photoreceptor transmission (Rajaram et al., 2005). Although the underlying mechanism is not provided, this finding suggests that a retrograde regulation is likely to contribute to the termination speed.

Previous studies have shown that the photoreceptor output signal undergoes feedback regulations (Meinertzhagen and O'Neil, 1991; Meinertzhagen and Sorra, 2001; Kolodziejczyk et al., 2008). It is thought that photoreceptor releases histamine to deactivate its postsynaptic interneurons in light, and receive excitatory feedback from interneurons in dark (Zheng et al., 2006). However, this model does not support the rapid repolarization of photoreceptor membrane potential at the end of light stimulation. Thus, a question has been raised: does another retrograde regulation exist facilitating the rapid

termination of photoreceptor light response?

In this work, we showed that the slow termination of light response in *slrp* photoreceptors is not caused by a defect in the regulation of phototransduction cascade, and that an extrinsic regulation modifies the repolarization speed of photoreceptor at the end of light stimulation. Our additional data shows that this extrinsic regulation is mediated by lamina epithelial glia. These results support the idea that the termination speed of photoreceptor light response undergoes a retrograde regulation, and shows that the lamina epithelial glia is involved in this retrograde mechanism, directly or indirectly. In neuroscience, retrograde signaling from postsynaptic cells such as neurons and muscles to presynaptic cells is present widely in different neuron systems (Fitzsimonds and Poo, 1998). Since lamina epithelial glia is also one of the postsynaptic partners of photoreceptor, here, we refer this epithelial glia-mediated regulation of photoreceptor as retrograde regulation, too.

In vertebrate visual system, photoresponse termination in cone or rod cells is thought to depend on the inactivation of phototransduction cascade (Burns and Baylor, 2001; Yau and Hardie, 2009). Known feedbacks from interneurons to photoreceptors play important roles in enhancing edge detection and regulating the sensitivity of photoreceptors in different light condition (Glantz et al., 2000; Fahrenfort et al., 2005). However, whether retrograde regulation contributes to the rapid kinetics of photoreceptor membrane potential shift is still unknown. Considering the similarities between vertebrate and

Drosophila visual system, our study suggests that the high speed of membrane potential shift upon acute decrease of light stimulation in photoreceptor may also undergo a retrograde regulation in the vertebrate visual system.

A function of glia in retrograde regulation of photoreceptors

In the adult fly visual system, lamina epithelial glial cells play importance roles (Meinertzhagen and O'Neil, 1991; Stuart et al., 2007; Bringmann et al., 2009; Edwards and Meinertzhagen, 2010). First, they ensheath lamina cartridge, and insulate it from surrounding units, which prevents the diffusion of solutes between cartridges and allows efficient electrical signal transmission. Second, they are involved in clearing and recycling of the extracelluar neurotransmitters, thus, modifying neurotransmitter levels. Third, lamina epithelial glial cells extend fine processes into the lamina neuropile and form specific neuron-glia structures such as capitate projections and gnarls. Although the functions of these structures are unclear, they are likely to mediate more direct regulations of neuronal activity.

Here we show that the gnarl structure of epithelial glia is involved in a retrograde regulation that modifies the termination speed of photoreceptor light response. The role of epithelial glia in this function could be:

(1) recycling the neurotransmitter that mediates the retrograde regulation through gnarl structure.

(2) mediating signal transmission from T1 to photoreceptor through gnarl. However, genetically activating (by expressing NaChBac, a bacterial depolarization-activated sodium channel with a lower threshold for activation and slower kinetics for inactivation), inactivating (by expressing Kir2.1, a mammalian inward-rectifier potassium channel that hyperpolarizes the plasma membrane), and ablating (by expressing DTI , diphtheria toxin A chain) T1 cells had no significance effects on the termination speed of photoreceptor light response (Guo et al. unpublished data), arguing against this possibility.

(3) receiving signal at gnarl from another interneuron and then modulating the membrane potential of photoreceptor through a separate unknown mechanism.

Lamina epithelial glia is morphologically and functionally comparable with CNS astrocyte (Reichenbach et al., 2010). Accumulating evidence is showing that astrocytes can release gliotransmitters such as glutamate, ATP, D-serine and GABA to modulate the neuronal excitability and output (Haydon and Carmignoto, 2006; Pfrieger, 2010). It is possible that similar gliotransmitters can also be released from epithelial glia, and apply regulation on photoreceptors.

In addition, epithelial glia invaginates into photoreceptor axons to form a large number of capitate projections, where a high level of endocytosis occurs (Fabian-Fine et al., 2003). A bsg^{d265} mutant that lacks the capitate projections displays a slow termination of light response phenotype (Curtin et al. 2007). Thus, it is very likely that capitate projections play a role in the rapid repolarization of photoreceptor.

Like epithelial glia in *Drosophila* visual system, Müller glial cells are the major glia in vertebrate retina. They contact almost all visual neurons and support their functions by maintaining extracelluar homeostasis, providing trophic factors and recycling neurotransmitters (Bringmann et al., 2006; de Melo Reis et al., 2008). It would be important to investigate whether Müller glia is involved in retrograde regulations of rod and cone cells.

Possible functions of other interneurons in retrograde regulation of photoreceptors

One question remains to address is, which neurons express SLRP and mediate the retrograde regulation? These neurons may either mediate a pathway of retrograde regulation separated from the glia-dependent one, or represent a different step in the same pathway.

We failed to identify these neuron types by phenotype rescue experiments, which may because that SLRP expression in multiple neurons is required for its visual function, or we do not have the proper GAL4 line for driving the neuronal expression in specific neuron type. Based on our study and current knowledge about lamina neurons, we can predict a few candidate neurons.

Amacrine cells: Amacrine cells are key element in feedback regulation. They directly receive input from photoreceptors and contribute majority of feedback synapses to photoreceptors, making them the most powerful source of retrograde signaling

(Meinertzhagen and O'Neil, 1991; Meinertzhagen and Sorra, 2001). Processes of amacrine cells are always next to T1 terminals, and either directly contact or are very close to gnarls, where SLRP is located.

L4 cells: When SLRP was expressed with the *repo-GAL4* in combination with a *rdl-GAL4* line, which drives expression in cells synthesizing $GABA_A$ receptor subunit RDL and labels L4 in the lamina, the visual defect of the *slrp* mutant was rescued (Guo et al., unpublished observation), suggesting that L4 may be the neuron that expresses SLRP and mediates the photoreceptor regulation. However, when *rdl-GAL4* line was replaced by other two Gal4 lines labeling L4, *Ln-GAL4* line and *AP-GAL4* line, no significant rescue was observed. Before making any conclusion on L4, we will need to address this discrepancy with additional experiments.

The function of SLRP in *Drosophila* eye

ADAM proteins are highly conserved proteins that exist in a wide variety of animal species including nematodes, flies, birds and mammals. They are involved in the regulation of many biological functions including cell signaling, cell adhesion and migration (Schlondorff and Blobel, 1999; Primakoff and Myles, 2000; Huovila et al., 2005; Duffy et al., 2009). Besides the metalloprotease activity that play important roles in shedding cell surface proteins and activating intracellular signaling pathways (Edwards et al., 2008), their non-proteolyic functions are involved in the axon guidance, neuroblast

migration and glial differentiation (Kurisaki et al., 2003; Liu et al., 2009). However, the physiological function of ADAM protein in visual systems has not been characterized.

Here we show that SLRP, a *Drosophila* ADAM protein, functions in both neurons and glias, and is required for the rapid kinetics of visual response. In lamina epithelial glia, SLRP localizes in the gnarl structure, which has a high electron density that usually represents a protein complex. Loss of SLRP leads to the disappearance of gnarl structure, suggesting that SLRP may function as an anchor protein on the cell membrane and recruit other components of the complex to the gnarl.

As a known active element in the synaptic function, mammalian astrocytes form highly dynamic processes that mediate neural-glia communications (Reichenbach et al., 2010; Zhang and Barres, 2010). Although some factors such as actin (Haber et al., 2006) and chloride channel (Mongin and Kimelberg, 2005) are thought to regulate the reshaping of astrocyte, the molecular mechanism underlying the formation of a functional process is unknown. Now we have found that the formation of a functional glial process, gnarl, depends on SLRP. It would be important to investigate a potential role of ADAM proteins in dynamic changes of vertebrate glia, including visual glia.

Besides the glial function, expression of SLRP in unidentified neurons is also required for the retrograde regulation of photoreceptors, probably through a different pathway. Given that mammalian ADAMs facilitate the formation of neuromuscular junction (Yumoto et al., 2008) and maintain the synapse function through serving as a

postsynaptic receptor (Futaka et al., 2006), we propose that SLRP may also contribute to synaptic functions in *Drosophila* visual neurons.

Materials and Methods

Fly genetics

All flies were reared at 22℃ in an approximate 12hr light (~250 lux)/12hr dark cycle. The genotypes of wild type flies are Canton-S for flies with red eye, and w^{1118} for flies with white eye. *slrp* mutant flies (previously named $slrp^{1}$) were from Dr. William L. Pak (Purdue University, Pak, 1975). *T1-GAL4*, *L1-GAL4*, *L2-GAL4*, and *L1L2A-GAL4* lines were from Dr. Martin Heisenberg (University of Würzburg, Rister et al., 2007). 21D-GAL4 line was from Dr. Elzbieta Pyza (Jagiellonian University, Górska-Andrzejak et al., 2005). *rdl-GAL4* line was from Julie Simpson (HHMI). *HisCl1-GAL4* line was from Dr. Chi-Hon Lee (NIH, Gao et al., 2008). Ln-*GAL4* line was from Dr. Mark Frye (UCLA; Zhu et al., 2009). The piggyBac insertion flies for deletion generation were obtained from Harvard Exelixis Collection. The $UAS\text{-}slrp^{RNAi}$ line (#1025) was from Vienna Drosophila RNAi Center (VDRC). All other flies were obtained from the Bloomington Drosophila stock center.

To generate deletion flies, piggyBac insertion flies carrying FRT sites were crossed with *hs-FLP* flies as described (Parks et al., 2004).

To generate SLRP transgenic flies, wild–type *slrp* cDNAs were obtained through RT–PCRs, subcloned into a pCaSpeR–hs vector or pUAST vector, and injected into w^{1118} flies. The transgenes were subsequently crossed into the *slrp* mutant background. Primers used in slrp gene identification are listed in Table 2-1S.

To generate the *p[hs-SLRP-RA2-MP]* transgenic flies, the point mutations (H407Q, G410A, H411Q and G414A) were introduced into *slrp-RA2* cDNA through PCR mutagenesis. The mutant cDNA was then subjected to cloning and injection.

To induce the protein expressions, flies carrying *p[hs–SLRP]* transgenes were heat shocked for 1 hr at 37℃ in a water bath in adult stage, flies carrying *UAS-SLRP* transgenes were crossed with GAL4 lines driving protein expression in specific cell types.

Electrophysiological recordings

Electroretinograms were examined as previously described (Li and Montell, 2000). Flies were immobilized with thin stripes of tape. Glass recording microelectrodes filled with Ringer solution were placed on the eye surface. A second extracellular recording electrode was maintained on the thorax (as reference). Five second orange light pulses (4000 lux) were used to stimulate the eye after adapting the fly in the dark for one minute. The signal was amplified and recorded using a Warner IE210 intracellular electrometer. To quantify the speed of response termination, the amplitude of the light-induced potential (LIP) was measured as the difference between the baseline voltage before lights-on and the voltage before lights-off. The time after light-off when the voltage shifted to LIP/2 was measured. For each genotype, data from 10 flies were averaged and SEM was calculated.

Intracellular recordings were performed as previously described (Wes et al., 1999) with modifications. Fly was immobilized with lab tapes, and a small opening was made

on the surface of the eye using fine tweezers. A thin glass microelectrode with resistance of about 100MΩ, filled with 2M KAc, was gradually inserted into the opening until light-induced depolarization of membrane potential was observed. The reference electrode, filled with Ringer's solution, was placed in the same eye opening. Five second orange light pulses (4000 lux) were used to stimulate the eye. The signal was amplified and recorded using a Warner IE210 intracellular electrometer. For each genotype, data from 10 photoreceptors in at least 3 flies were averaged and SEMs were calculated.

For whole cell recordings, the ommatidia were isolated from newly enclosed flies (<2 hr) in Ca^{2+}-free Ringer's solution, and photoresponses of individual photoreceptor were recorded as described previously (Hardie et al., 1991). The pipette and bath solutions were (in mM): 100 potassium gluconate, 40 KCl, 2 MgCl2, 0.1 EGTA, 5 ATP, 0.5 GTP, 10 HEPES (pH 7.15) and 130 NaCl, 5 KCl, 1.8 CaCl2, 5 proline, 25 sucrose, 10 HEPES (pH 7.15), respectively. The resistance of recording pipettes was 5.5-6.5 MΩ. The seal resistance was >3GΩ. For voltage-clamp recording, cells were clamped at -70 mV. The stimulating light pulses (1s) were delivered from a 100 W QTH light source (Oriel) to the cell after passing a high-speed shutter (76992, Oriel) and an orange filter. The signal was amplified using an Axopatch 200B, acquired at 1 kHz, and analyzed with pClamp 8 software (Axon Instruments, Inc.). For each genotype, data from at least five different flies were recorded.

Antibodies

The SLRP polyclonal antibody was raised in rabbit against a GST-fused extracelluar

fragment (A603 to G728). For purification, the same SLRP fragment was fused with MBP, purified with amylose resin (NEB), coupled to CNBr-activated Sepharose 4B (Amersham), and incubated with the anti-SLRP antiserum, the bound antibody was eluted.

For biotin-labeled SLRP antibody, 1mg (1mg/ml) purified SLRP antibody was dialyzed against carbonate buffer (100 mM carbonate, pH 8.4), and then incubated with 80 µg Biotin (N-Hydroxysuccinimidobiotin, H1759, Sigma, freshly prepared 1 mg/ml solution in DMSO) overnight at 4°C. Unbound Biotin was removed by dialyzing the antibody against PBS.

vGluT antibody was from Dr. Aaron Diantonio (WUSL, Daniels et al., 2004). Arr1 antibody was raised against a C–terminal fragment (aa226–319). Arr2 antibody was as described (Han et al., 2006). RH1 and DLG antibody were from Developmental Studies Hybridoma Bank (DSHB). GFP was from Invitrogen, Gq antibody was from Sigma, All others from Dr. Craig Montell.

Western blot

To examine total protein level in fly head, heads were removed and homogenized in 2×SDS-PAGE loading buffer, boiled for 3 min, spin at 13000rpm to remove any remaining tissue debris, and subjected to SDS-PAGE. Separated proteins were electrotransfered onto a PVDF membrane. After blocking with TBST (Tris-buffered saline with 0.01% Tween 20) containing 5% skimmed milk, the membranes were probed with appropriate primary antibodies diluted in blocking solution. Membrane-bound

primary antibodies were detected using secondary antibodies conjugated with horseradish peroxidase. Immunoblots were detected with LAS-3000 (FUJIFILM) using the enhanced chemiluminescence technique (Immobilon Western HRP Substrate; Millipore).

For retina-lamina preparation, fly heads were removed, dehydrated in 100% ethanol for at least 2 hours in room temperature. Retina and attached lamina were carefully separated from other part of the brain, air dried, homogenized in 2×SDS-PAGE loading buffer and subjected to western blot.

Immunofluorescence staining

For whole mount staining, the brains of adult flies were dissected and fixed in PLP fixation solution (2% paraformaldehyde, 0.01 M NaIO4, 75mM lysine, 37 mM sodium phosphate buffer, pH 7.4; McLean and Nakane, 1974) for 1.5 hr on ice. After 30 min incubation on ice with blocking buffer (5% fetal bovine serum in PBS containing 0.3% Triton X-100), the brains were then incubated overnight at 4°C in primary antibody diluted in blocking buffer. After three washes in PBS containing 0.3% Triton X-100, brains were incubated with FITC- or TRITC–conjugated secondary antibodies for 3 hr at room temperature, washed, and mounted in Vectashield medium (Vector Laboratories). Images were captured for confocal microscopy with either an LSM 510 instrument (Zeiss). The following primary antibodies were used: 1: 500 rabbit anti-SLRP; 1:200 mouse anti-DLG (4F3, DSHB); 1:200 mouse anti-RH1 (4C5, DSHB); 1:5000 anti-vGluT (Daniels et al., 2004); 1:500 mouse anti-GFP (A11120, Invitrogen).

For double staining with vGluT and SLRP rabbit polyclonal antibodies, after stained

with vGluT antibody and TRITC–conjugated secondary antibody, the brain was incubated with unrelated rabbit IgG such as Arr2 antibody, washed, and stained with biotin-labeled SLRP antibody and Streptavidin−FITC (S3762, Sigma).

For cryosection, fly heads were removed, incubated in 0.1M phosphate buffer with increasing concentrations of sucrose, infiltrate and embedded in a TFM tissue freezing medium (Ted Pella Inc.). 20 μm sections were cut at -20℃ and subjected to immunofluorescence staining

Electron Microscopy (EM)

Fly heads were removed and bisected and fixed in a solution of 2.5% glutaraldehyde in 0.05M sodium cacodylate buffer (pH7.4), and processed for EM as previously described (Meinertzhagen and O'Neil, 1991). After three times of wash, fly heads were post fixed with 2% osmium tetroxide for 5 hr, dehydrated in ethanol, infiltrated with propylene oxide, and embedded in polybed812 resin (08792-1; Polysciences). Embedded tissue was sectioned at 20 nm, stained in uranyl acetate and lead citrate. Sections were viewed at 80 kV in a Philips Tecnai 12 electron microscope

For immunogold EM, fly heads were fixed and embedded in LR White as described (Han et al., 2007). 100 nm sections of lamina were cut and immunostained with SLRP antibody and anti-rabbit IgGs conjugated with 10-nm gold particles. After staining with 1% aqueous uranyl acetate, the sections were examined under the transmission electron microscope.

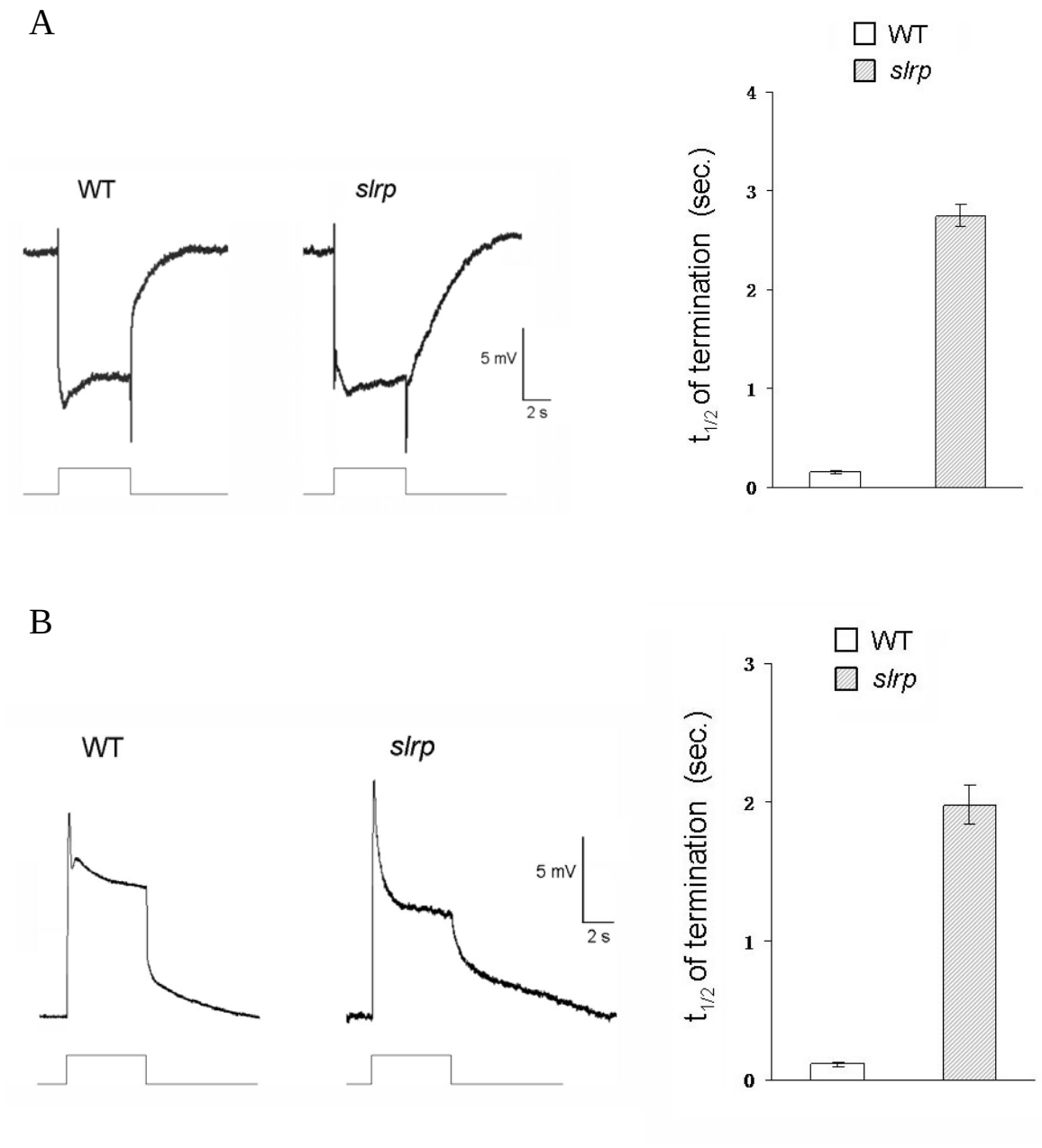

A
WT
slrp
5 mV
2 s
WT
slrp
$t_{1/2}$ of termination (sec.)
0
1
2
3
4
B
WT
slrp
5 mV
2 s
WT
slrp
$t_{1/2}$ of termination (sec.)
0
1
2
3

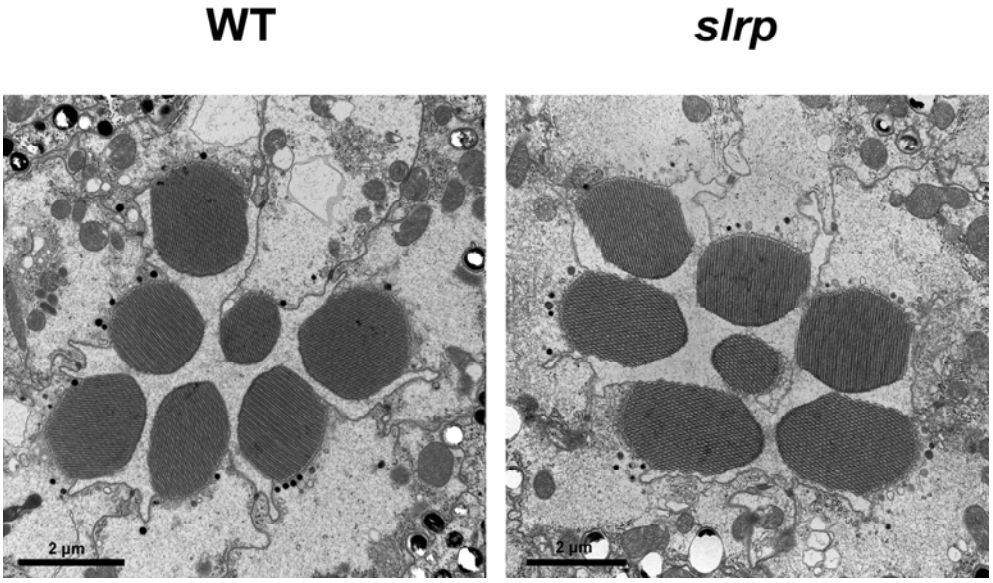
Figure 2-1

Figure 2-1. Photoreceptor of *slrp* flies displays slow termination of light response

(A) ERG response terminated slowly in *slrp* flies. Sample ERG traces from wild type and the *slrp* mutant are shown on the left. The error bars represent SEMs.

(B) Intracellular recordings of *in vivo* photoreceptors revealed a slower termination of light response in *slrp* flies. Sample traces are shown on the left. The error bars represent SEMs.

A


WT
slrp
2 µm
2 µm

B

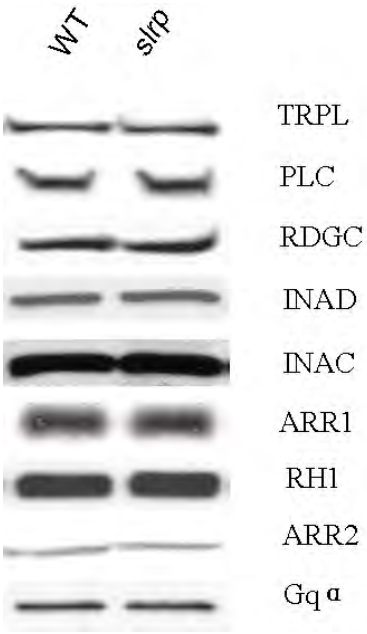

WT
slrp
TRPL
PLC
RDGC
INAD
INAC
ARR1
RH1
ARR2
Gq α

Figure 2-2

Figure 2-2. No abnormalities were detected in *slrp* mutant compound eye

(A) No morphological defects were observed in *slrp* mutant eye by EM analysis. One ommatidium is shown.

(B) Levels of most signaling proteins in the phototransduction cascade are normal in the *slrp* mutant.

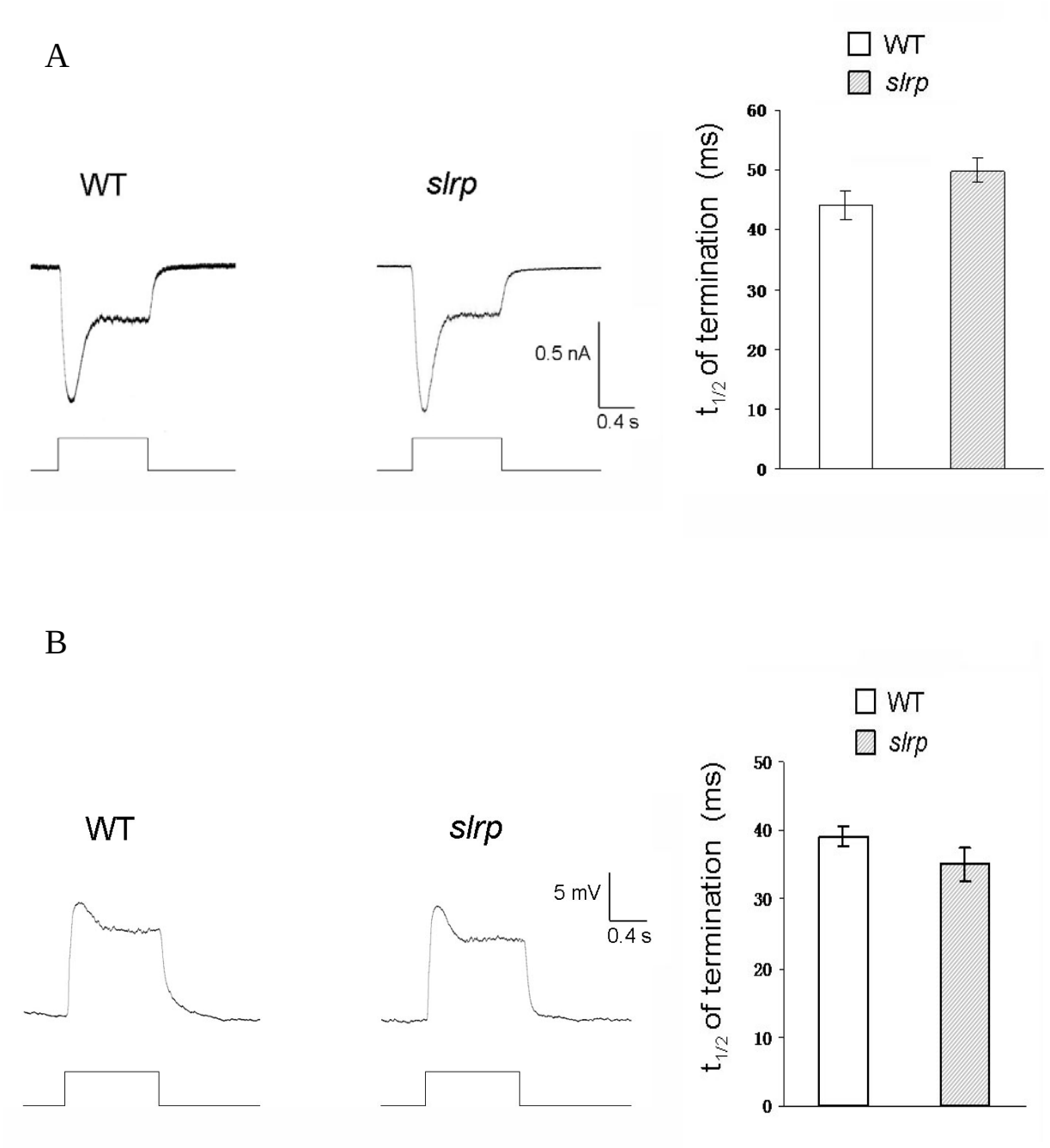
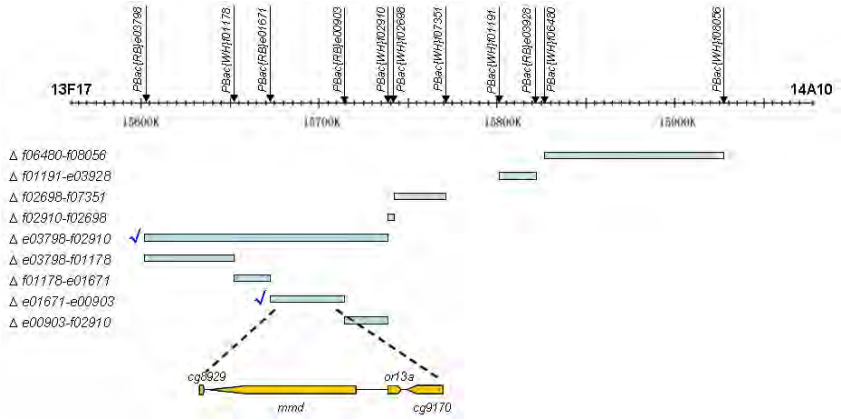

A
WT
slrp
0.5 nA
0.4 s
WT
slrp
$t_{1/2}$ of termination (ms)
60
50
40
30
20
10
0
B
WT
slrp
5 mV
0.4 s
WT
slrp
$t_{1/2}$ of termination (ms)
50
40
30
20
10
0

Figure 2-3

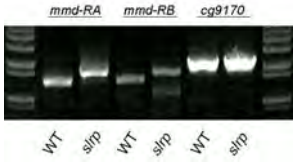
Figure 2-3. Phototransduction in *slrp* mutant photoreceptors is normal

(A) Voltage-clamp whole-cell recording and (B) Current-clamp whole-cell recording on isolated cell bodies of *slrp* photoreceptors did not reveal slow termination of light response. Sample traces are shown on the left. The error bars represent SEMs.

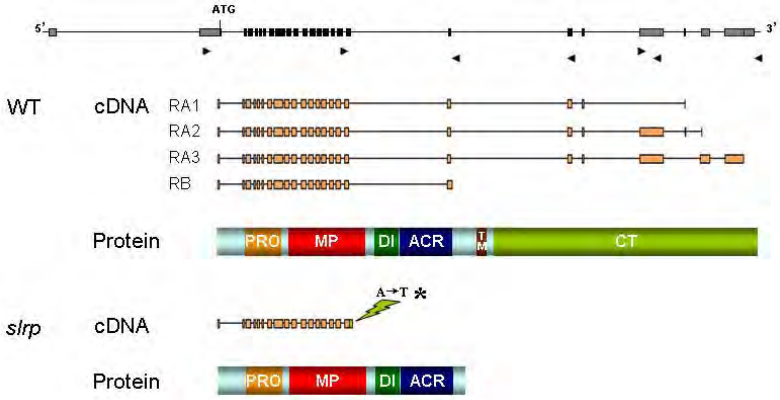
A



B



C



D

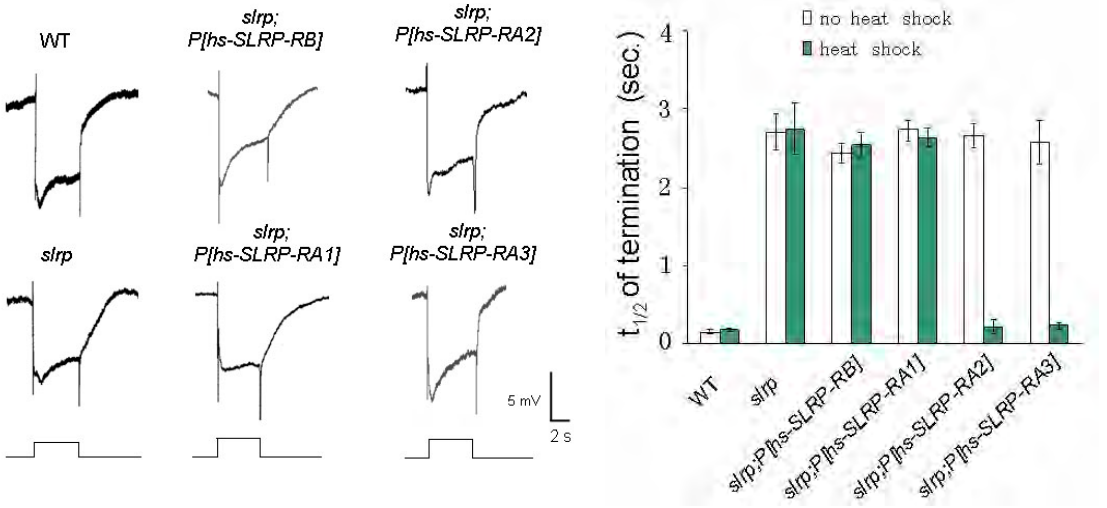


Figure 2-4

Figure 2-4. Identification of the *slrp* gene

(A) The *slrp* mutation was mapped to the region 13F10-14B1. Five chromosomal fragments in this region were deleted separately using a FRT-mediated targeted deletion strategy. The deficiency Δe01671-e00903 uncovered the *slrp* ERG phenotype. This region was divided into four smaller fragments that were deleted separately, only the deficiency Δe03798-f02910 uncovered the *slrp* ERG phenotype, leaving *cg8928*, *mind-meld (mmd)*, *or13a* and *cg9170* the candidates for the *slrp* gene.

(B) RT-PCR revealed *mmd* mRNA fragments with smaller size in the *slrp* mutant. The mRNA level remained the same as in wild type. Primers for *mmd-RA*: TTCACAGGGTCTAGAGTGCTCA/TACTTGAGCGTTGTGGTCTTTC; for *mmd-RB*: TTCACAGGGTCTAGAGTGCTCA/TTGCTTGTTCTTTTTGTGGTTG; for *cg9170*: ATGATCTGTCGCAACTGGACT/TCGCCCTTATAGCTGGTATCC.

(C) Structures of the *slrp* gene, the four identified cDNA isoforms, and SLRP protein. In the *slrp* mutant, a point mutation on the 18th intron changed the splicing pattern of the *slrp* gene, introduced a stop codon, and abolished expression of full length SLRP. Primers used in this study are shown (arrow heads). PRO: pro-domain, MP: metalloprotease domain, DI: disintegrin domain, ACR: ADAM cysteine-rich domain, TM: transmembrane domain, CT: cytosolic tail.

(D) The *slrp* ERG phenotype was rescued by overexpression of the cDNA of two *slrp* isoforms, RA2 and RA3, through a heat–shock promoter, but not the two shorter isoforms, RB and RA1. Sample traces are shown on the left. The error bars represent SEMs.

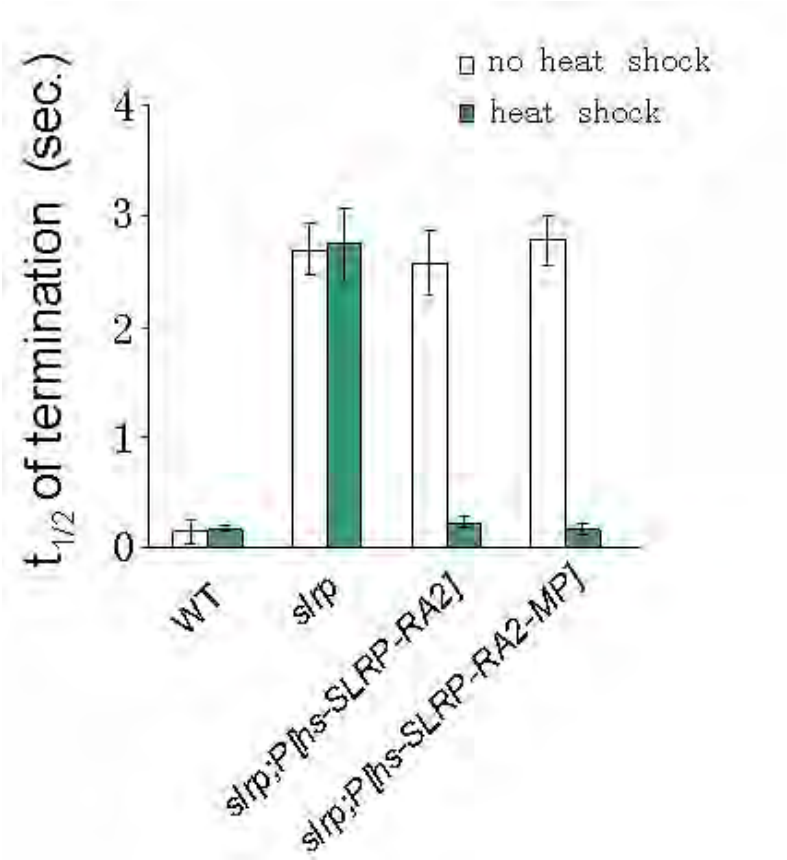

no heat shock
heat shock
$t_{1/2}$ of termination (sec.)
0
1
2
3
4
WT
slrp
slrp;P[hs-SLRP-RA2]
slrp;P[hs-SLRP-RA2-MP]

Figure 2-5

Figure 2-5. SLRP does not function as a metalloprotease in the visual system

Slow termination of light response in *slrp* flies was rescued by overexpression of a mutant SLRP with disrupted metalloprotease domain (s*lrp;P[hs-SLRP-RA2-MP]*). Error bars represent SEMs.

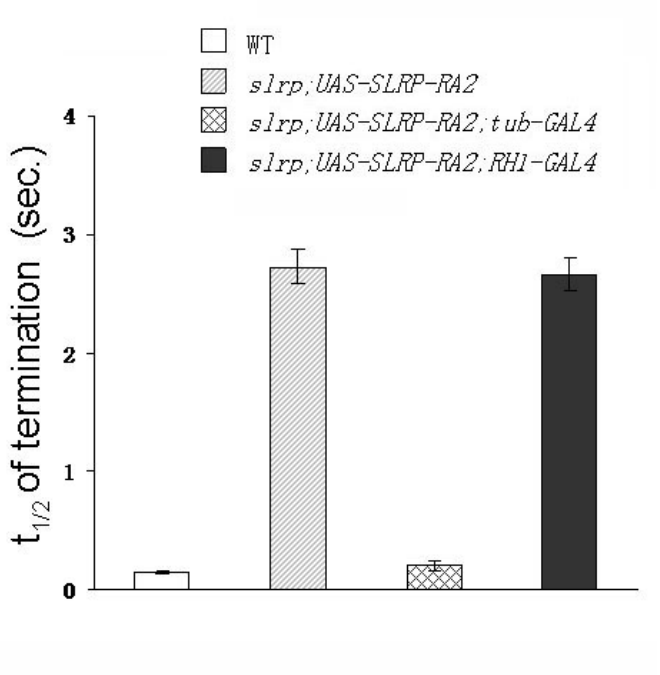
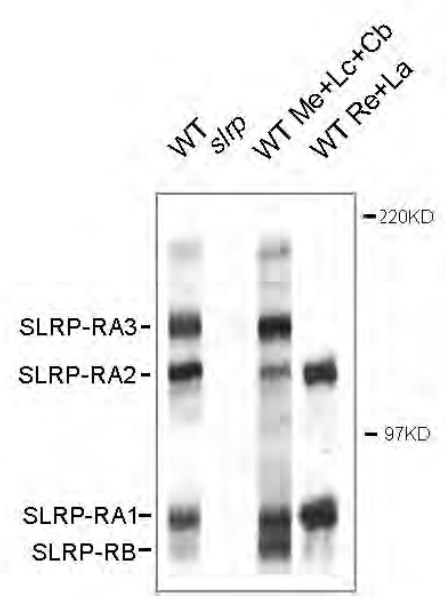

WT
slrp;UAS-SLRP-RA2
slrp;UAS-SLRP-RA2;tub-GAL4
slrp;UAS-SLRP-RA2;RH1-GAL4
t1/2 of termination (sec.)
0
1
2
3
4

Figure 2-6

Figure 2-6. SLRP does not function within photoreceptors

The *slrp* ERG phenotype was not rescued by overexpression of the *slrp* cDNA in photoreceptor cells through a *RH1-GAL4* promoter. In the control experiment, overexpression of the *slrp* cDNA using a pan GAL4, *tub-GAL4*, rescued the *slrp* ERG phenotype. The error bars represent SEMs.

A


WT
slrp
WT Me+Lc+Cb
WT Re+La
220KD
97KD
SLRP-RA3
SLRP-RA2
SLRP-RA1
SLRP-RB

B

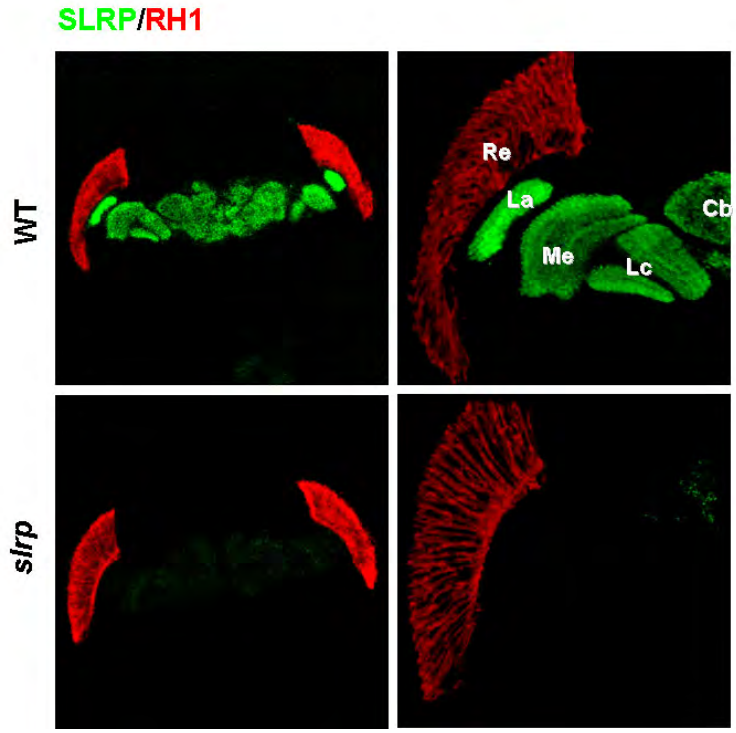

SLRP/RH1
WT
slrp
Re
La
Me
Lc
Cb

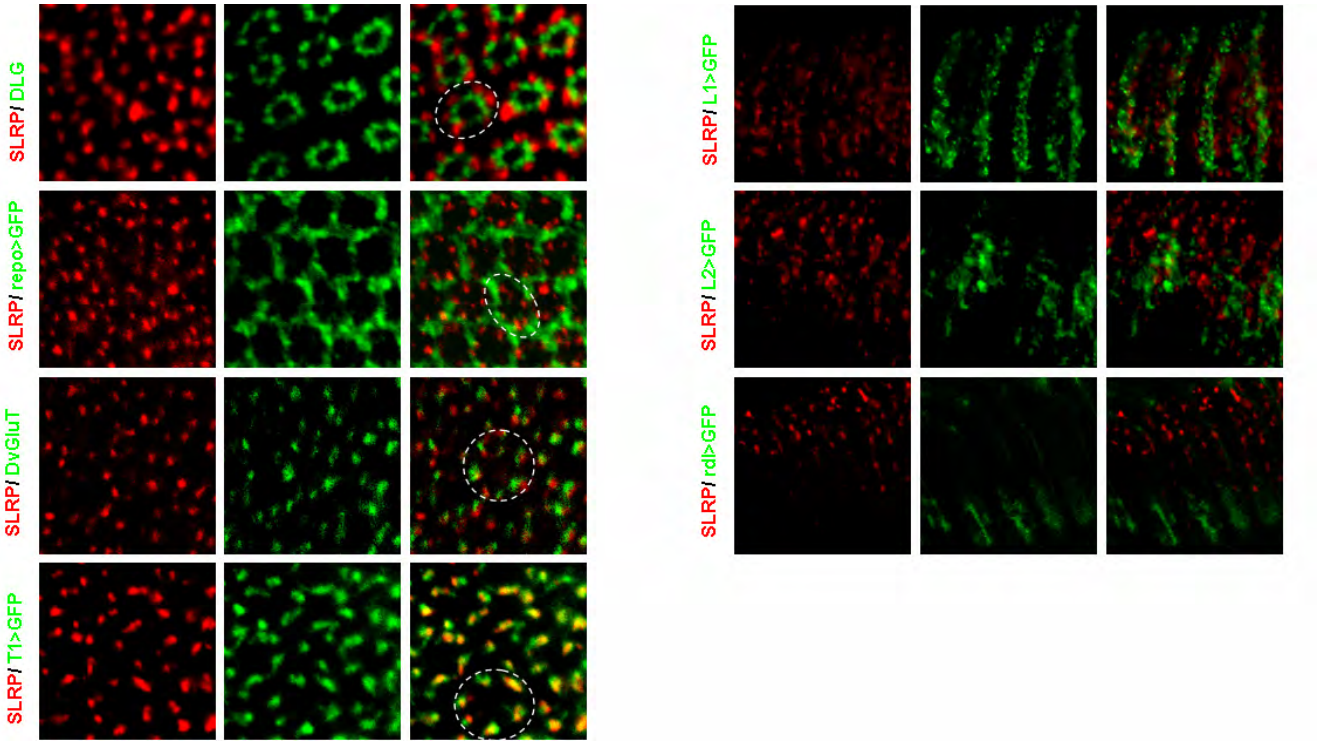
Figure 2-7

Figure 2-7. SLRP is highly expressed in the lamina

(A) Western blot with a SLRP antibody showed four bands corresponding to the predicted size of the four SLRP isoforms. The sizes of the background bands are consistent with precursor forms, in which the 30KD prodomain was not removed. Two forms of SLRP, SLRP-RA1 and SLRP-RA2, are expressed in the lamina. The loss of SLRP in the *slrp* mutant was shown. Re: retina, La: lamina, Me: medulla, Lo: lobula complex, Cb: central brain.

(B) Immunostaining showed SLRP is expressed in the whole brain, with the highest concentration in the lamina region, but not in the retina region. No SLRP protein was detected in the lamina region of *slrp* mutant flies, although a trace level of SLRP protein was observed in central areas of the brain.

A


SLRP/ DLG
SLRP/ repo>GFP
SLRP/ DvGluT
SLRP/ T1>GFP
SLRP/ L1>GFP
SLRP/ L2>GFP
SLRP/ rdl>GFP

B

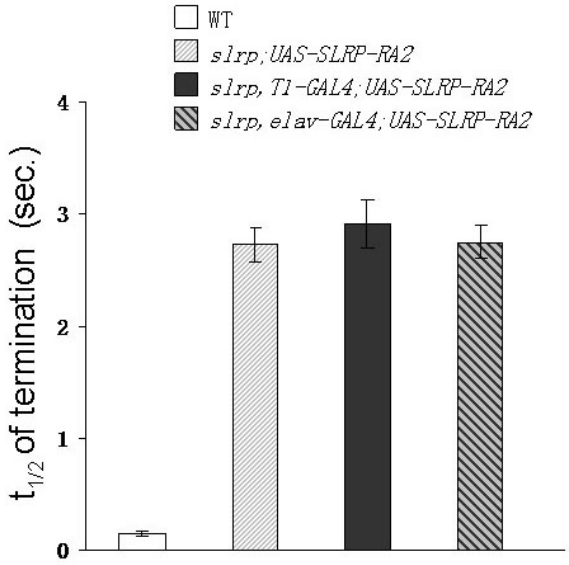

WT
slrp;UAS-SLRP-RA2
slrp, T1-GAL4;UAS-SLRP-RA2
slrp, elav-GAL4;UAS-SLRP-RA2
t1/2 of termination (sec.)
0
1
2
3
4

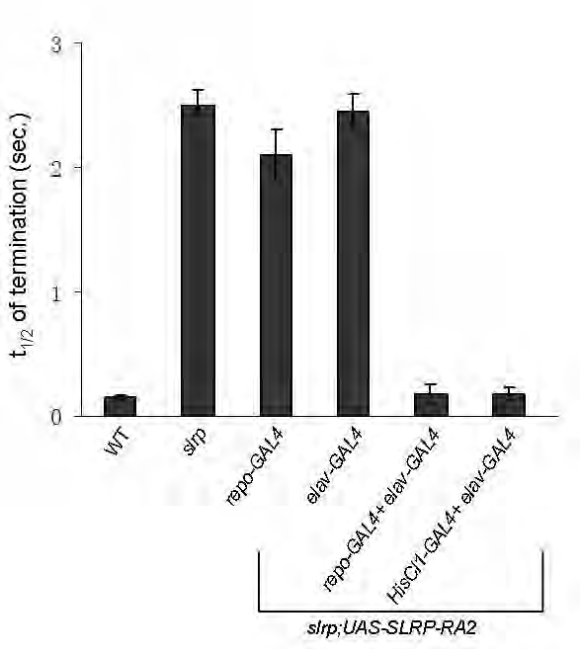
Figure 2-8

Figure 2-8. Neuronal expression of SLRP is not sufficient for its visual function

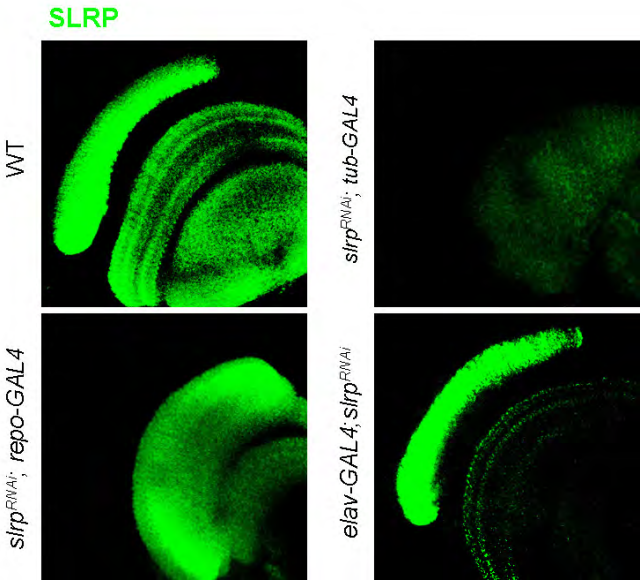
(A) SLRP signals overlap with T1 processes. Co-immunostaining revealed overlapping between SLRP signals and T1 processes (*T1-Gal4* driven GFP), and lack of co-localization between SLRP and photoreceptor (anti-DLG), amacrine cells (anti-dVGluT), glia, L1, L2, L4 cells (*repo-Gal4*, *L1-Gal4*, *L2-Gal4*, *rdl-Gal4* driven GFP) in the lamina. The cross-section of a single cartridge is labeled with white circle.

(B) The *slrp* ERG phenotype was not rescued by overexpression of the *slrp* cDNA in T1 cells. Pan neuronal expression of the *slrp* cDNA through an *elav-GAL4* promoter did not rescue the *slrp* ERG phenotype, either. The error bars represent SEMs.

A



B



C

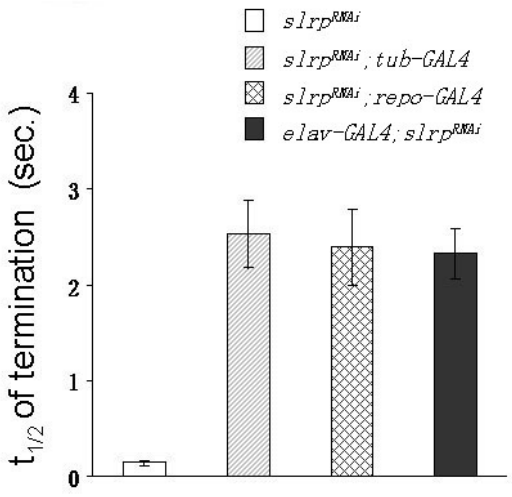


Figure 2-9

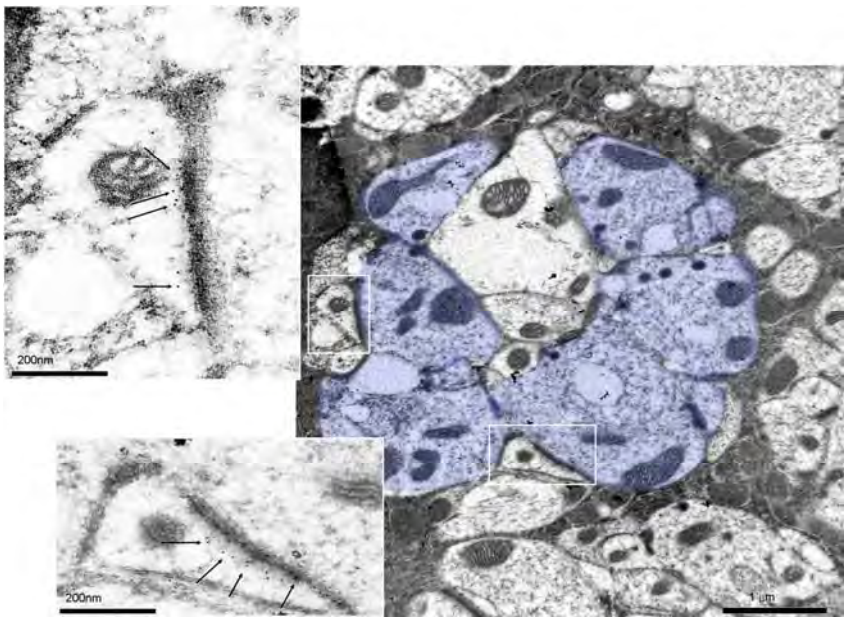
Figure 2-9. SLRP functions in both neuron and lamina epithelial glia

(A) The slow termination of light response phenotype of the *slrp* mutant was not rescued by expression of SLRP in neurons or glia alone, but rescued by expression of SLRP in both neurons and lamina epithelial glia. The error bars represent SEMs.

(B) Immunostainings showed knockdown of SLRP in glia abolished the SLRP expression in the lamina. Knockdown of SLRP in neurons decreased the SLRP level in other areas of the brain, but did not affect laminar SLRP.

(C) RNAi knockdown of the *slrp* gene caused a slower termination of light response in ERG (*slrp*^{RNAi}*; tub-GAL4*). The same ERG phenotypes were observed when the *slrp* gene was specifically knocked down in neurons *(elav-GAL4;slrp*^{RNAi}) or glial cells (*slrp*^{RNAi}*; repo-GAL4*). The error bars represent SEMs.

A



B

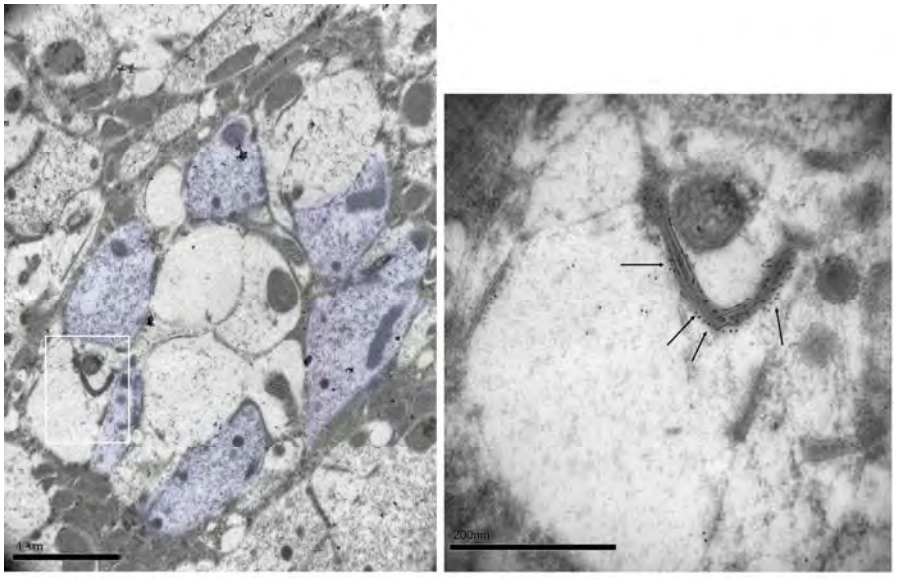
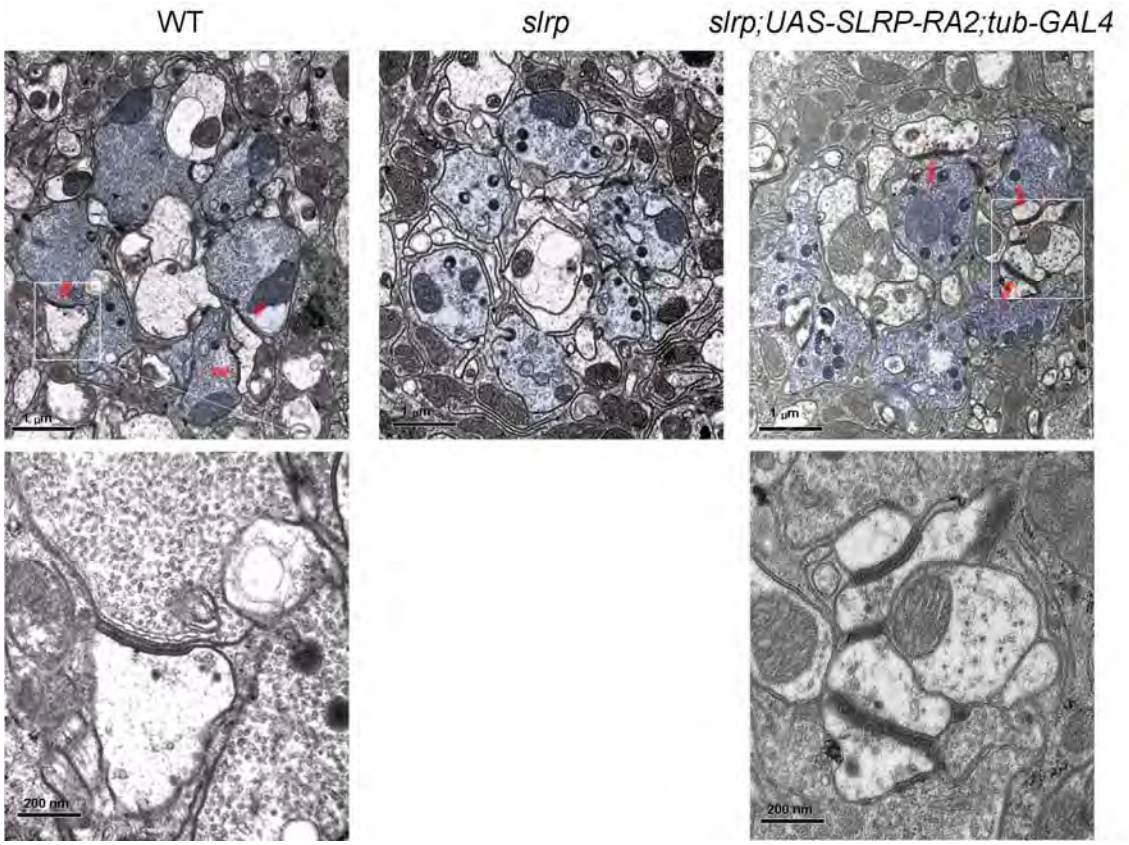


Figure 2-10

Figure 2-10. SLRP localizes in the gnarl structure of lamina epithelial glia

Immunogold-labeling of SLRP in lamina cross sections at the EM level revealed SLRP localization on the gnarl structures (arrows) between T1 cell processes and photoreceptor axons (blue) (A), and between T1 and amacrine cell processes, or T1 and T1 processes (B).

A



B

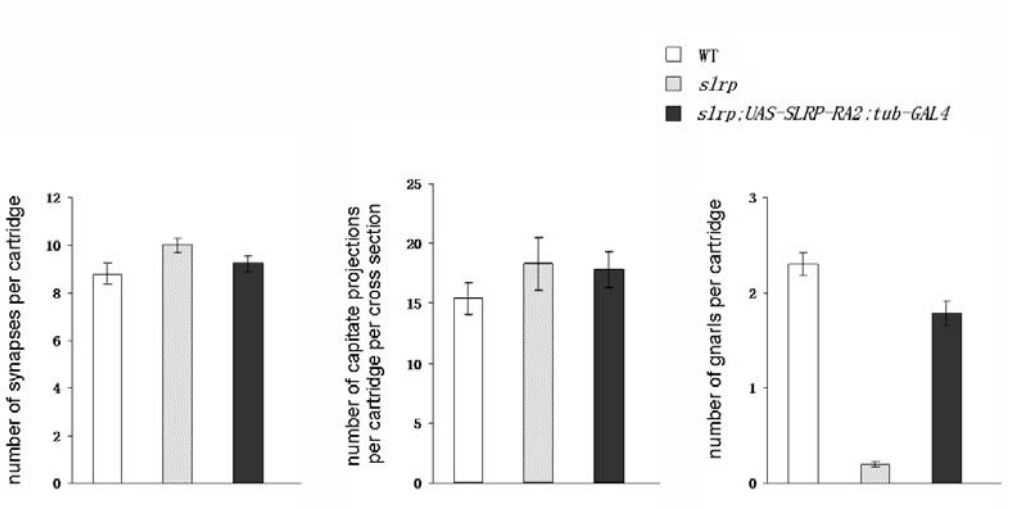


Figure 2-11

Figure 2-11. Loss of SLRP leads to disruption of gnarl

(A) Ultrastructure of lamina cross sections in wild type flies revealed gnarl structures (red arrows). Gnarl structures were missing in the *slrp* mutant, and were recovered by overexpression of SLRP in *slrp;UAS-SLRP-RA2;tub-GAL4* flies .

(B) The number of synapse and capitate projections remained the same in wild type, *slrp* mutant flies and *slrp;UAS-SLRP-RA2;tub-GAL4* flies. The number of gnarls decreased in *slrp* mutant flies and was recovered in *slrp;UAS-SLRP-RA2;tub-GAL4* flies. The mean number of these structures per cartridge was calculated based on 20 cartridges of at lease 3 flies for each genotype. Error bars represent SEMs.

Table 2-S1. Primers for *slrp* gene identification

gene	Sequence (5'-3')	5' position within the *Drosophila* genome
CG42354	Forward TGCTGTCTCACCTCTCGTTTT	15989480
RGP	Forward CCGCAGATTAATTTCCAACTTC	16092607
CG32577	Reverse CACGATGGATGTTTTGCTGA	15903378
CG32580	Forward GTTTCGGTTGCCGACAAATA	15904394
	Reverse CCATTCAGCATATGTGGTCC	15897747
CG9170	Forward CTAAATAAGCTGGCCTTGCTG	15875430
	Reverse TTGAACGACACTTCGAGTACG	15876651
	Reverse ATGATCTGTCGCAACTGGACT	15875559
	Forward TCGCCCTTATAGCTGGTATCC	15873435
CG8928	Forward ACAGCAGCATCGAACTCCT	15839195
	Reverse TTGGGAGTTTCTTGCTTGC	15839632
OR13a	Forward CCTAGTGCTGTGGCATTTATCA	15868429
	Reverse ACGAACCAGATTTAGGGCATTA	15870907
	Forward CATTGTTGTGTGCAAAAGAGC	15868933
SLRP/MMD	Forward TGAGCAAACACAGTCCGTG	15857521
	Reverse ATGGCTGTTCCCGAAACT	15857962
	Forward TACTTGAGCGTTGTGGTCTTTC	15848454
	Forward TTGCTTGTTCTTTTTGTGGTTG	15852410
	Reverse GAATTGGAGAATGTCTGGTTGA	15860926
	Forward CACGAGCAACGTATTGGTAACT	15842165
	Forward TTGCTTGTTCTTTTTGTGGTTG	15852410
	Reverse TTCACAGGGTCTAGAGTGCTCA	15856337
	Forward TGGCATAGACTAACTTGCTGGA	15848335
	Reverse ACGGTGAGCAGCTAAAAGACA	15845930
	Forward CCAATGTTACGCTTGTACTGCT	15845612
	Forward TTTTAAATGGCTTACCCTGTCG	15855578

CHAPTER III: AN INHIBITORY GLUTAMATERGIC FEEDBACK SIGNAL FACILITATES THE REPOLARIZATION OF DROSOPHILA PHOTORECEPTOR THROUGH LAMINA EPITHELIAL GLIA

This work was conducted under the direction of Dr. Hong-Sheng Li. My contribution to this work consists of fly genetic works, electrophysiological recordings, electron microscopy, behavior tests, generating transgenic flies, immunostaining, and data analysis. Keith Reddig contributed by assisting in electron microscopy, Dr. Zhuo Luan contributed by assisting in cloning work for generating transgenic flies. Dr. Hong-Sheng Li and I contributed to write the body of work.

Abstract.

Photoreceptors are responsible for receiving light signals, converting them into electrical signals and transmitting them to the brain through interneurons. In *Drosophila*, anatomic evidence suggests the existence of retrograde regulations on photoreceptor axons in the lamina, the first layer of visual neuropile. By manipulating levels of neurotransmitters synthesized in lamina interneurons, we found that the rapid termination of photoreceptor light response depends on glutamatergic signaling from amacrine cells, a type of wide-field lamina interneuron. An additional screen identified an inhibitory, glutamate-gated chloride channel GluCl that mediates the glutamatergic signaling. Intriguingly, cell-specific RNAi experiments show that GluCl functions in lamina epithelial glia, as well as in neurons other than photoreceptor. In transgenic flies, an FLAG-tagged GluCl colocalized with SLRP, a marker for a special epithelial glial structure, gnarl. Our results suggest the existence of a photoreceptor — amacrine cell — epithelial glia — photoreceptor feedback loop that facilitates the repolarization of photoreceptor at the end of the light response. More importantly, this work provides *in vivo* evidence for glutamate-mediated chloride channel activation, which may occur in both glia and neurons.

Introduction

In both vertebrate and invertebrate visual systems, photoreceptor cells are the primary light sensory neurons. They transfer light information to the brain through interneurons. In the meantime, they receive retrograde regulation from the interneurons (Glantz et al., 2000; Fahrenfort et al., 2005). For example, the horizontal cell in mammalian retina has negative regulation on cone and rod photoreceptors, which modulates the amplitude and voltage dependence of these cells (Wässle, 2004). Nonetheless, the effect of retrograde regulation on the kinetics of the photoreceptor response is largely unknown.

We have been using *Drosophila* as a genetic model system to study visual transmission and retrograde regulation of photoreceptors. An early electron microscopic study has provided a comprehensive description of the first visual neuropile, lamina, where peripheral photoreceptor axons synapse to and receive feedback from interneurons. Axons of the peripheral photoreceptors R1-R6 form tetrad synapses, whose postsynaptic components include L1-L3 monopolar cells, amacrine cells and epithelial glial cells. Conversely, they receive retrograde inputs from interneuron L2, L4 and amacrine cells (Meinertzhagen and O'Neil, 1991; Meinertzhagen and Sorra, 2001). Although no synaptic retrograde input from other interneurons has been observed, it is possible that other cells could modulate photoreceptors indirectly. In addition, nonsynaptic communication between photoreceptors and other types of cells may also contribute to

the regulation of photoreceptor activity.

It is reported that fly mutants with blocked photoreceptor transmission display a slow light response termination phenotype (Rajaram et al., 2005), suggesting that a retrograde regulation may contribute to the repolarization speed of photoreceptor at the end of light stimulation. This is supported by our recent finding that the rapid repolarization of photoreceptor requires an ADAM protein expressed in lamina epithelial glia (Guo et al., unpublished observation).

However, the current feedback model consisting of the inhibitory photoreceptor transmission to interneurons and an excitatory feedback from interneurons to photoreceptors (Zheng et al., 2006) is not in favor of the rapid repolarization of photoreceptors at the end of the light response. There must exist an additional, inhibitory feedback input to photoreceptor, which facilitates its repolarization.

To uncover such a feedback mechanism, we used an RNAi strategy to identify genes and cell types that are required for retrograde regulation of photoreceptors. In this work, we show that glutamatergic transmission from amacrine cells to lamina epithelial glia cells, which is mediated by a glutamate-gated chloride channel GluCl, is required for rapid repolarization of photoreceptors. We further propose that this retrograde regulation of photoreceptors occurs in a feedback manner through a photoreceptor — amacrine cell — epithelial glia — photoreceptor loop.

Results

Disruption of glutamatergic signaling causes slow repolarization of photoreceptor cells

Our previous study suggested that the repolarization speed of the photoreceptor at the end of light stimulation may be facilitated by a retrograde regulation from lamina interneurons. To understand the mechanism of this regulation, we attempted to identify the cell types that are involved in this retrograde regulation pathway.

Lamina contains more than 20 different types of interneurons. Except for clues from anatomical studies, their functions and underlying mechanisms of action are largely unknown. Due to the small cell size and the internal location, so far only few lamina large monopolar cells have been recorded from electrophysiological experiments (Zheng et al., 2006; Pantazis et al., 2008). The electrophysiological properties of lamina interneurons are mostly proposed based on recordings from larger fly species (Douglass and Strausfeld, 2005). However, some neurotransmitters used by lamina neurons have been revealed by immunocytochemistry studies. Amacrine neurons have been shown to be glutamatergic, L1 and L2 may be glutamatergic or cholinergic, acetylcholine may be the neurotransmitter in L4 and a type of Tan neuron, and GABA may be the transmitter of C2, C3 and another type of Tan neuron (Walther and Picaud, 2006; Kolodziejczyk et al., 2008).

By blocking these different transmission pathways, we can inhibit the function of different interneurons, and determine which interneuron types are involved in retrograde regulation of photoreceptors. Using an RNAi technique, we knocked down expression of key elements in different transmission systems, including enzymes involved in neurotransmitter metabolism, (vesicular) neurotransmitter transporters, and neurotransmitter receptors. A total of 32 UAS-hairpin RNAi lines, which target 25 genes were expressed through a *tub-GAL4* line in knockdown flies, which were subsequently screened for abnormal termination of light response in ERG assays (Table 3-1).

The screen showed that a decrease of vesicular glutamate transporter (vGluT) led to slow termination of light response (Fig. 3-1A). In contrast, no ERG abnormality was detected when elements in the GABAergic, cholinergic, and glycinergic transmissions were knocked down (Table 3-1).

No morphological abnormalities were detected in the $vglut^{RNAi}$*;tub-GAL4* fly eye under electron microscopy, suggesting this visual defect is not due to a developmental problem (Fig. 3-1B). To test whether the slow-termination defect of the $vglut^{RNAi}$*;tub-GAL4* fly derives from photoreceptors, intracellular recordings were conducted in photoreceptors of this fly. When light stimulation was removed, the membrane potential of photoreceptor recovered at a speed slower than that in wild type (Fig. 3-1C), indicating that vGluT is required for the rapid repolarization of photoreceptor at the end of light stimulation.

vGluT is the single vesicular glutamate transport in *Drosophila* genome (Daniels et al., 2004). In fly lamina, it is not expressed in photoreceptor axons, but is localized in processes of amacrine cells (Kolodziejczyk et al., 2008). This transporter is required to load glutamate into synaptic vesicles in axons and thus is important for the synaptic output of amacrine processes. Thus, the phenotype of the vGluT RNAi fly suggests that glutamatergic signaling from amacrine cells mediates a feedback regulation facilitating the recovery of photoreceptors from light response.

To test whether the vGluT function also attributes to other cells that might release glutamate, such as L1, L2, and glia, we examined *vglut^{RNAi};L1L2A-GAL4* flies and *vglut^{RNAi};repo-GAL4* flies, respectively. In ERG recordings, we did not detect any phenotype in these flies (Fig. 3-1A). Thus, vGluT may specifically function in laminar amacrine cells to facilitate the rapid repolarization of photoreceptor at the end of light stimulation.

The glutamate-gated chloride channel GluCl is the receptor mediating the retrograde regulation

During light stimulation, amacrine cells receive inhibitory input from R1-R6 photoreceptor axons through a histamine-gated chloride channel, Ort (Gengs et al., 2002; Zheng et al., 2002; Pantazis et al., 2008). At the end of light stimulation, amacrine cells become active and send synaptic feedback to photoreceptor axons. As glutamatergic

neurons, amacrine cells were thought to activate photoreceptors through direct synaptic transmission, which should slow down, but not accelerate, the repolarization of the photoreceptor. Thus, the amacrine-cell facilitation of photoreceptor repolarization may not occur through the direct synaptic input to photoreceptors.

To understand how glutamatergic amacrine cells facilitate the repolarization of photoreceptors, we attempted to identify the neurotransmitter receptors that are involved in this regulation of photoreceptors, including both the glutamate receptors and potential inhibitory receptors.

The *Drosophila* genome encodes a single metabotropic glutamate receptor, DmGluRA, and four ionotropic glutamate receptors that mediate excitatory neurotransmission: DGluRI, a kainate receptor expressed in the CNS; DNMDAR, an NMDA-like receptor in the brain, and DGluRIIA and DGluRIIB, two muscle-specific glutamate receptors (Ultsch et al., 1993, Schuster et al., 1991; Ultsch et al., 1992; Petersen et al., 1997). In addition, thirteen ligand-gated chloride channel homologs (LCCHs) have been found in *Drosophila*, which may mediate inhibitory synaptic transmission. These include receptors for histamine, GABA , and glycine (Knipple and Soderlund, 2010). Interestingly, one member of this family has been found to respond to glutamate *in vitro,* and is named glutamate-gated chloride channel GluCl (Cully et al., 1996). We investigated whether any of these receptors/channels are required for rapid termination of light response by knocking down their expression through RNAi (Table

3-2).

A slow termination of light response in ERG recordings was observed when GluCl level was decreased by RNAi in a *glucl*^{RNAi}*;tub-GAL4* fly (Fig. 3-2A). In contrast, no ERG defect was detected when other candidate receptors were knocked down (Table 3-2).

Since no morphological abnormalities were detected in the eye of the GluCl knockdown fly in electron microscopy (Fig. 3-2B), the visual defect in this fly is not due to a developmental problem, but may reflect an abnormality in visual physiology. We conducted intracellular recordings in photoreceptors of the *glucl*^{RNAi}*;tub-GAL4* fly, and found that the repolarization of photoreceptor was slow at the end of light stimulation (Fig. 3-2C), which indicates that GluCl is required for rapid termination of photoreceptor light responses.

To confirm that the visual defect was caused by a decrease in the GluCl level, we also examined a GluCl mutant, *glc*^{1}, using the ERG assay. The result showed that this mutant displayed the same ERG phenotype as in the *glucl*^{RNAi}*;tub-GAL4* fly (Fig. 3-2A). The similar visual defects observed in *vglut*^{RNAi}*;tub-GAL4*, *glucl*^{RNAi}*;tub-GAL4*, and *glc*^{1} flies suggest that glutamate released from amacrine cells acts on the inhibitory receptor GluCl for rapid repolarization of photoreceptor at the end of the light response.

GluCl functions in both neurons and visual glias

Does GluCl function in photoreceptors and mediate a direct inhibitory input from amacrine cell to photoreceptor? To test this, we knocked down GluCl specifically in photoreceptors using a *RH1-GAL4* line. In the knockdown fly *glucl*^{RNAi}*;RH1-GAL4*, we did not detect any ERG abnormality (Fig. 3-3), suggesting that GluCl does not function in photoreceptor axons to receive a direct input from amacrine cells, and should be expressed in a third cell type that links amacrine cells to photoreceptors. However, this result could be due to the low RNAi efficiency in photoreceptors. To further understand the localization of GluCl, we knocked down GluCl in other types of cells.

We knocked down GluCl in several interneurons, including L1, L2, L4 and T1 cells using cell-specific GAL4 lines. However, we did not detect any ERG phenotype in these knockdown flies either. To test whether GluCl functions in neurons at all, we knocked down the GluCl level using a pan-neuronal GAL4 line, *elav-GAL4*. In this knockdown fly we found light responses terminated slowly in ERG recordings (Fig. 3-3). Thus, GluCl probably functions in interneurons that lack cell-specific GAL4 drivers, such as C2, C3 and amacrine cells.

The slow-termination phenotype of GluCl knockdown flies is reminiscent of a *slrp* mutant. We recently found that the SLRP protein needs to be expressed in both neurons and lamina epithelial glias to make light responses terminate quickly. To investigate whether this is the same case for GluCl, we knocked down GluCl in glial cells using a *repo-GAL4* line, and found that the glia-specific knockdown caused a slow termination of

light response phenotype in ERG recordings (Fig. 3-3). Thus, both interneurons and glias receive glutamatergic signaling from amacrine cells, and may collaboratively facilitate the repolarization of photoreceptors at the end of light stimulation.

Similarities between the *slrp* and the *glc*^{1} mutants

slrp and *glc*^{1} mutants display the same ERG phenotype. According to RNAi experiments, both SLRP and GluCl proteins function in glias as well as in neurons. These similarities suggest that GluCl and SLRP are involved in the same pathway of neuron/glia regulation.

Besides the visual defect, the *slrp* mutant also displays behavioral defects such as hypo-activity, ether-induced leg shaking (Homyk, 1977; Homyk and Sheppard, 1977). Previous studies have also reported that the *glc*^{1} mutant has reduced locomotion and flight ability (Kane et al., 2000). We used a climbing test to compare the locomotion activities of *slrp* and *glc*^{1} mutants, and found that both mutants displayed locomotion defect (Fig. 3-4A). In addition, ether treatments caused leg shaking in both mutants.

GluCl was first discovered as the target of insecticides nodulisporic acid (NA) and ivermectin (IVM) (Cully et al., 1994; Cully et al., 1996). In the *glc*^{1} mutant, a P298S point mutation decreases the sensitivity of the channel to those insecticides and renders the fly resistant to them (Kane et al., 2000). To test whether the GluCl function is normal in the *slrp* mutant, we examined the survival rates of *slrp* mutant flies treated with IVM,

results showed a higher resistance in both *slrp* and *glc*^{1} flies, when compared to the wild type (Fig. 3-4B), suggesting that the GluCl function in the *slrp* mutant is disrupted.

GluCl is concentrated in gnarl, a special process of epithelial glia

To confirm the impairment of GluCl function in the *slrp* mutant, it would be important to look for changes in the protein level, distribution and function of GluCl. Since we do not have an antibody to GluCl, we instead generated a *UAS-GluCl-FLAG* transgene to express a FLAG-tagged GluCl, so that we could investigate the GluCl distribution using an FLAG antibody.

We introduced a *HS-GAL4* into the *UAS-GluCl-FLAG* fly and expressed the tagged GluCl protein through 37℃ heat shock for 30 minutes. Six hours after the heat shock, we removed the brain including lamina from the fly and stained the tissue with the FLAG antibody. We detected strong GluCl-FLAG signal both in the central brain and the optic lobe. In lamina area, no significant GluCl-FLAG expression was detected in photoreceptor axons or cell bodies of other lamina interneurons (Fig. 3-5A), suggesting that the GluCl protein is not stable in lamina neurons. However, strong GluCl-FLAG signal is observed in epithelial glia cell bodies and in unknown punctuate structures between photoreceptor axons (Fig. 3-5A, B). In flies without heat shock, the GluCl-FLAG signal from leaking expression was only observed in the punctuate structure (Fig. 3-5B). The punctuate GluCl-FLAG signal co-localized with SLRP in

double labeling experiments, suggesting GluCl is concentrated in gnarl, a special process of the epithelial glia (Fig. 3-5B).

In the *slrp* mutant background that has no gnarl structure, although heat shock still drove strong expression of GluCl-FLAG through the *HS-GAL4*, the signal was localized in the cell bodies of epithelial glia, with no punctate staining being observed in the inner region of the lamina cartridge (Fig. 3-5 B). This observation further supports that GluCl is concentrated in gnarl structures of epithelial glia.

Discussion

A role of amacrine cell glutamatergic transmission in feedback regulation of photoreceptors

Our previous studies on the *slrp* mutant have shown that the rapid termination of photoreceptor light response depends on a retrograde regulation. However, which neuron and neurotransmitter mediate this retrograde regulation was unknown. Here we identify lamina amacrine cells as an important factor in this regulation. Since amacrine cells themselves receive input from photoreceptors, they may facilitate the termination of photoreceptor light response in a feedback manner.

Similar to L1 and L2 monopolar projection neurons, amacrine cells receive inhibitory histaminergic input from photoreceptor axons at tetrad synapses upon light stimulation. As a feedback mechanism, they form ~48 synapses on photoreceptor axons in each cartridge (Meinertzhagen and Sorra, 2001). These synapses contribute over 60% input to photoreceptors. Unlike monopolar neurons, amacrine cells are wide field interneurons with lateral distributed branches. This morphological characteristic suggests that amacrine cells may mediate communication between neighboring cartridges, and are therefore important to feedback systems that enhances the synchronization in the entire eye and sharpens the visual field.

Because direct electrical recording from *Drosophila* lamina amacrine cells has been

unsuccessful, the functions and the mechanisms of amacrine cells in visual regulation circuits are proposed based on anatomic and immunochemical studies. Immunocytochemical studies have revealed glutamate in processes of amacrine as well as L1 and L2 cells. However, the single vesicular glutamate transporter in the *Drosophila* genome, vGluT, which is necessary for glutamate release from presynaptic sites, is only detected in amacrine cell processes, not in L1 or L2 cells (Kolodziejczyk et al., 2008). Therefore amacrine cells may be the only type of glutamatergic neuron in the lamina. Taking into consideration the large number of synapses from amacrine cells to photoreceptor terminals, it is reasonable to propose that amacrine cells provide a glutamatergic feedback to photoreceptors during the termination of light stimulation.

Although an electrophysiological study suggests that the amacrine cell-mediated feedback is excitatory to photoreceptors (Zheng et al., 2006), none of those excitatory glutamate receptors has been detected in the lamina so far. Our work, however, reveals a new feedback effect in which amacrine cells apply an inhibitory regulation on photoreceptors.

In this feedback regulation, glutamate released from amacrine cells does not activate photoreceptors through the direct amacrine cell to photoreceptor synapses, Instead, it stimulates the inhibitory glutamate receptor GluCl in the membrane of gnarl, an epithelial glia structure. Through an unknown mechanism (see discussion below), this inhibition of epithelial glia may facilitate the repolarization of photoreceptors (Fig. 3-6).

Processes of amacrine cells are always found next to those of T1 cells, in spaces between photoreceptor terminals. No synaptic output from T1 cells has been detected through electron microscopy in either the lamina or the medulla (Meinertzhagen and O'Neil, 1991; Takemura et al., 2008). However, our unpublished data shows that in the lamina, T1 cells are the only cell type labeled by a *dEAAT1-GAL4* line, which demonstrates the expression pattern of the gene encoding an excitatory amino acid transporter dEAAT1. This result is consistent with previous immunocytochemical studies with an anti-EAAT1 antibody (Rival et al., 2004). Since dEAAT1 uptakes glutamate into the cell and quickly clears extracellular glutamate, T1 cells may also play a role in the regulation of glutamatergic signaling.

Amacrine cells releasing glutamate, epithelial glia expressing GluCl, and T1 cells recycling glutamate, may together form an inhibitory glutamatergic transmission machinery that mediates rapid and transient neuron-glia feedback signaling.

The epithelial glia in the retrograde regulation of photoreceptor

The glial expression of the glutamate receptor, GluCl, is required for rapid termination of photoreceptor light response, suggesting that GluCl functions in the epithelial glia. This function is very likely dependent on the gnarl structure.

The effect of the chloride channel on the cell depends on the initial membrane potential, and the equilibrium potential of Cl^- ion (Williamson et al., 1998; Walz, 2002).

For most neurons, chloride channels mediate inhibitory signals and hyperpolarize cells. However, in glial cells and some immature neurons, the situation is different because of their very negative resting potential that leads to outward driving force of Cl^- (Walz, 2002). For example, the activation of ligand-gated chloride channel such as $GABA_A$ receptors or glycine receptors in astrocytes and oligodendrocytes glia produce depolarization of the cells (Belachew et al., 1998; Williamson et al., 1998). Recoding from isolated vertebrate visual glia, Müller cell, also showed a depolarization response to GABA through $GABA_A$ receptors (Malchow et al., 1989).

Drosophila lamina epithelial glia cells undergo slow hyperpolarization in response to light (Zheng et al., 2006), this could due to the closing of GluCl channel. Therefore, the activation of GluCl channel depolarizes epithelial glia. However, the hyperpolarization of epithelial glia may be caused by the opening of histamine-gated chloride channel during light stimulation, so it is also possible that GluCl introduces a repolarizating current, since it is opened when the epithelial glia is depolarized due to removal of the histaminergic inhibition at the end of light stimulation. To understand the GluCl-mediated regulation of epithelial glia, more direct evidences such as intracellular recording of epithelial glia in wild type and glc^1 mutant are required.

The next question is how epithelial glia modulates the membrane potential of photoreceptors? Although we don't have data addressing this, we propose two potential mechanisms.

First, the lamina epithelial glia is morphologically and functionally comparable to CNS astrocytes (Reichenbach et al., 2010), which release gliotransmitters such as glutamate, GABA, D-serine and ATP, to modulate neuronal and synaptic activity (Haydon and Carmignoto, 2005; Pfrieger, 2010). It is possible that epithelial glia release gliotransmitters to modulate the membrane potential of photoreceptors.

Second, epithelial glia invaginates into photoreceptor axons and forms a large number of capitate projections, where high levels of endocytosis occur (Fabian-Fine et al., 2003). A bsg^{d265} mutant that lacks capitate projections displays a slow termination of light response phenotype (Curtin et al. 2007). Thus, capitate projections could play a role in glia-photoreceptor transmission and facilitate the rapid repolarization of the photoreceptors.

Functional mechanism of SLRP in the visual system

We have shown that the SLRP protein is required for the formation of gnarl. However, why gnarl is required for rapid termination of photoreceptor light response? Here we found the GluCl is concentrated in gnarl, where it may receive glutamate input from amacrine cells. These observations suggest that gnarl is the structural base for an amacrine cell-epithelial glia communication that participates in the feedback regulation of photoreceptors. Interestingly, the gnarl structure is characterized by a high electron density, resembling that of a postsynaptic complex consisting of neurotransmitter

receptors, scaffolding proteins, cell adhesion molecules and other signaling proteins. Since GluCl colocalizes with SLRP in the gnarl, they are likely to be components of a large protein complex that includes additional signaling and regulating proteins. SLRP may be responsible for assembling this complex by recruiting other proteins to the gnarl.

In addition to their glial function, both SLRP and GluCl function in neurons as well, for the photoreceptor repolarization. This suggests a more direct interaction between these two proteins. It will be important to further investigate whether SLRP is required for the membrane targeting of GluCl in neurons.

The GluCl function in *Drosophila*

Glutamate-gated chloride channels were first identified as L-glutamate receptors that induce membrane potential hyperpolarization in locust muscle fibers (Lea and Usherwood, 1973a; Lea and Usherwood, 1973b; Cully-Candy and Usherwood, 1973; Horseman et al., 1988), and were later cloned from *C.elegans* (Cully et al., 1994). GluCls are pentameric transmembrane receptors belonging to the anionic channel of Cys-loop ligand-gated ion channels (LGICs) family. 6 genes that encode the GluClα and GluClβ subunits (Cully et al., 1994; Dent et al., 1997; Vassilatis et al., 1997; Laughton, 1997; Dent et al., 2000; Horoszok et al., 2001) have been identified in *C.elegans*, while only one GluCl gene has been identified in *Drosophila* genome, which is homologous to the *C.elegans* GluClα subunit (Cully et al., 1996).

Overexpressed GluCl channels in *Xenopus oocytes* display a glutamate-gated chloride permeability that is sensitive to IVM and NA. Therefore, GluCl has been used as a potential target for antiparasitic drug development (Raymond and Sattelle, 2002; Zhao et al., 2004). However, very few studies have focused on the biological function of this channel. The glc^{1} mutant has impaired locomotion and flight ability, suggesting a neurologic function of GluCl (Kane et al., 2000), but the site of function and underlying mechanism is unknown.

Here we reveal a function of GluCl in the *Drosophila* visual system. Both neuron and glia expressed GluCl proteins receive glutamatergic signals carrying light information from amacrine cells, which mediates a feedback regulation to ensure the rapid repolarization of photoreceptor membrane potential during termination of light response. Our study provides the first *in vivo* evidence for GluCl mediating glutamatergic transmission.

Although no vertebrate GluCl has been reported, gene structure comparisons and phylogenetic analysis indicate that GluCl is highly conserved with α subunits of vertebrate glycine-gated chloride channels (Vassilatis et al., 1997).

All glycine receptor α subunits have been immunocytochemically localized in the inner plexiform layer (IPL) of the mouse retina, with different characteristics of distribution. They are thought to be expressed in amacrine and bipolar cells, and mediate glycinergic inhibitory transmission (Balse et al., 2006; Heinze et al., 2007; Shen et al.,

2007; Majumdar et al., 2009). Interestingly, a couple of glycine receptor in bipolar cells are found postsynaptic to glutamatergic amacrine cells (Haverkamp et al., 2004), if those vertebrate glycine receptors also respond to glutamate, our current understanding of glutamate's role as a pure excitatory neurotransmitter in vertebrate will need to be changed.

Materials and Methods

Fly genetics

All flies were reared at 22℃ in an approximate 12 hr light (~250 lux)/12hr dark cycle. The genotypes of wild type flies are w^{1118}. *slrp* mutant flies (previously named $slrp^{1}$) were from Dr. William L. Pak (Purdue University, Pak, 1975). *L1L2A-GAL4* lines were from Dr. Martin Heisenberg (University of Würzburg; Rister et al., 2007). 21D-GAL4 line was from Dr. Elzbieta Pyza (Jagiellonian University; Górska-Andrzejak et al., 2005). All RNAi lines were from Vienna Drosophila RNAi Center (VDRC) (see Table 3-S1). All other flies were obtained from the Bloomington Drosophila stock center.

To generate *UAS-GluCl* transgenic fly, *GluCl* cDNA (*GluCl-RG*, GenBank accession number NM_001170185) was obtained from wild type total mRNA using RT–PCRs, the FLAG tag was added to the C terminal through PCR mutagenesis. The *GluCl-FLAG* sequence was subcloned into a pUAST vector, and injected into w^{1118} flies to generate the transgenic fly.

For RNAi screen, an UAS-hairpin RNAi fly was crossed with *tub-GAL4* fly, the offspring were then subjected to ERG assays. To ensure the effectiveness of the knockdown, when no ERG phenotype was detected, a *UAS-dicer2* transgene was added into the fly to enhance the knock down efficiency. Since most of the target genes are necessary for the survival of fly, lack of offspring indicated that the RNAi line was functional.

Electrophysiological recordings

Electroretinograms were examined as previously described (Li and Montell, 2000). Flies were immobilized with tape. Glass recording microelectrodes filled with Ringer's solution were placed on the eye surface. A reference electrode (filled with Ringer's solution) was placed on the thorax. After one minute of adaptation to the dark, the fly was stimulated by five second orange light pulses (4000 lux). The signal was amplified and recorded using a Warner IE210 intracellular electrometer. To quantify the speed of response termination, the amplitude of the light-induced potential (LIP) was measured as the difference between the baseline voltage before lights-on and the voltage before lights-off. The time after light-off when the voltage shifted to LIP/2 was measured. For each genotype, data from 10 flies were averaged and SEM was calculated.

Intracellular recordings were performed as previously described (Wes et al., 1999) with modifications. Flies were immobilized with lab tape. A small opening was made on the surface of the eye using fine tweezers. A thin glass microelectrode with resistance of about 100MΩ, filled with 2M KAc, was gradually inserted into the opening until light-induced depolarization of membrane potential was observed. A reference electrode filled with Ringer's solution was placed in the same eye opening. Five second orange light pulses (4000 lux) were used to stimulate the eye. The signal was amplified and recorded using a Warner IE210 intracellular electrometer. For each genotype, data from 10 photoreceptors in at least 3 flies were averaged and SEMs were calculated.

Immunofluorescence staining

The brains of adult flies were dissected and fixed in PLP fixation solution (2% paraformaldehyde, 0.01 M NaIO4, 75mM lysine, 37 mM sodium phosphate buffer, pH 7.4; McLean and Nakane, 1974) for 1.5 hr on ice. After 30 min incubation on ice with blocking buffer (5% fetal bovine serum in PBS containing 0.3% Triton X-100), the brains were incubated overnight at 4°C in primary antibody diluted in blocking buffer. After three washes in PBS containing 0.3% Triton X-100, brains were incubated with FITC- or TRITC–conjugated secondary antibodies for 3 hours at room temperature, washed, and mounted in Vectashield medium (Vector Laboratories). Images were captured for confocal microscopy with either an LSM 510 instrument (Zeiss). The following primary antibodies were used: 1: 500 rabbit anti-SLRP (raised against an A603 to G728 extracelluar fragment), 1:200 mouse anti-DLG (4F3, Developmental Studies Hybridoma Bank), 1:500 rabbit anti-FLAG (F7425, Sigma), and 1:500 mouse anti-FLAG (F1804, Sigma).

Electron Microscopy (EM)

Fly heads were bisected and fixed in 0.05M sodium cacodylate buffer with 2.5% glutaraldehyde (pH7.4), and processed for EM as previously described (Meinertzhagen and O'Neil, 1991). After washes, fly heads were post fixed with 2% osmium tetroxide for 2 hr, dehydrated in ethanol, infiltrated with propylene oxide, and embedded in polybed812 resin (08792-1; Polysciences). Embedded tissue was sectioned at 20 nm, stained in uranyl acetate and lead citrate. Sections were viewed at 80 kV in a Philips Tecnai 12 electron microscope.

Behavior assays

All behavioral assays were carried out under constant temperature (22℃) with 12:12 hr light/dark cycles. 3 to 7-day-old flies were used.

For the climbing test, 20 flies were placed in a 15 ml plastic tube with vertical distances marked. After a 30 min rest period, flies were tapped to the bottom of the tube, thereby eliciting an innate escape response during which the flies walk up the tube wall. The result was quantitated by recording the percentage of flies that are capable to climb the distance of 5 cm in 10 seconds. Data from 3 independent tests were averaged and SEM was calculated.

For the ether-induced leg shaking behavior test, flies were exposed to ether for 10 second. Wild type flies were immobilized except for occasional tarsal twitches. Both *slrp* mutant flies and glc^{1} mutant flies exhibit a rapid shaking of all six legs.

For the ivermectin treatment, a total of 20 flies were starved for 24 hr and placed in a empty vial, carrying glass-fibre soaked with ivermectin solution (5% (w/v) sucrose and 1% (v/v) DMSO containing ivermectin (I8898, sigma) at concentrations of 0, 0.025, 0.05, 0.075, 0.1, 0.2, 0.35 and 0.5 μM, freshly prepared before each test). Survival rates were scored 24 hr after treatment. Data from 3 independent tests were averaged and SEM was calculated.

gene		function	termination of light response
glutamate signaling			
cg2718	**GS1**	glutamine synthetase 1	normal
cg1743	**GS2**	glutamine synthetase 2	normal
cg9887	**vGluT**	vesicular glutamate transporter	slow
cg3747	**dEAAT1**	sodium-dependent excitatory amino acid transporter 1	—
cg3159	**dEAAT2**	sodium-dependent excitatory amino acid transporter 2	*
GABA signaling			
cg14994	**GAD1**	glutamic acid decarboxylase 1	normal
cg7811	**GAD2**	glutamic acid decarboxylase 2	normal
cg33310	**GB1**	metabotropic GABA-B receptor subtype 1	normal
cg6706	**GB2**	metabotropic GABA-B receptor subtype 2	normal
cg3022	**GB3**	metabotropic GABA-B receptor subtype 3	normal
cg17336	**LCCH3**	GABA receptor beta subunit	normal
cg7446	**GRD**	GABA-and-glycine-receptor-like-subunit; glycine receptor	normal
cg10537	**RDL**	GABA A receptor subunit	normal
acetylcholine signaling			
cg12345	**CHAT**	choline aetyl transferase metabotropic	normal
cg32848	**VAChT**	acetylcholine transmembrane transporter activity	—
cg17907	**ACE**	acetylcholine esterase	normal
cg5610	**ALS**	nicotinic acetylcholine receptor alpha 96Aa	normal
cg32538	**gfA**	nicotinic acetylcholine receptor alpha 18C	normal
cg11348	**ARD**	nicotinic acetylcholine receptor beta 64B	normal
cg4356	**mAchR-1**	muscarinic acetylcholine receptor 60C	normal
others			normal
cg5549	**CG5549**	glycine transporter	normal
cg8380	**DAT**	dopamine transporter	normal
cg8394	**VGAT**	amino acid transmembrane transporter	normal
cg1732	**GAT**	gamma-aminobutyric acid neurotransmitter transport	—
cg4545	**SERT**	cocaine-sensitive serotonin transporter	normal

Table 3-1. RNAi screen for genes involved in lamina signaling transmission

— RNAi driven by *tub-GAL4* line caused embryonic death.

* RNAi line showed abnormal termination speed of ERG light response, which may due to the insertion of the RNAi transgene.

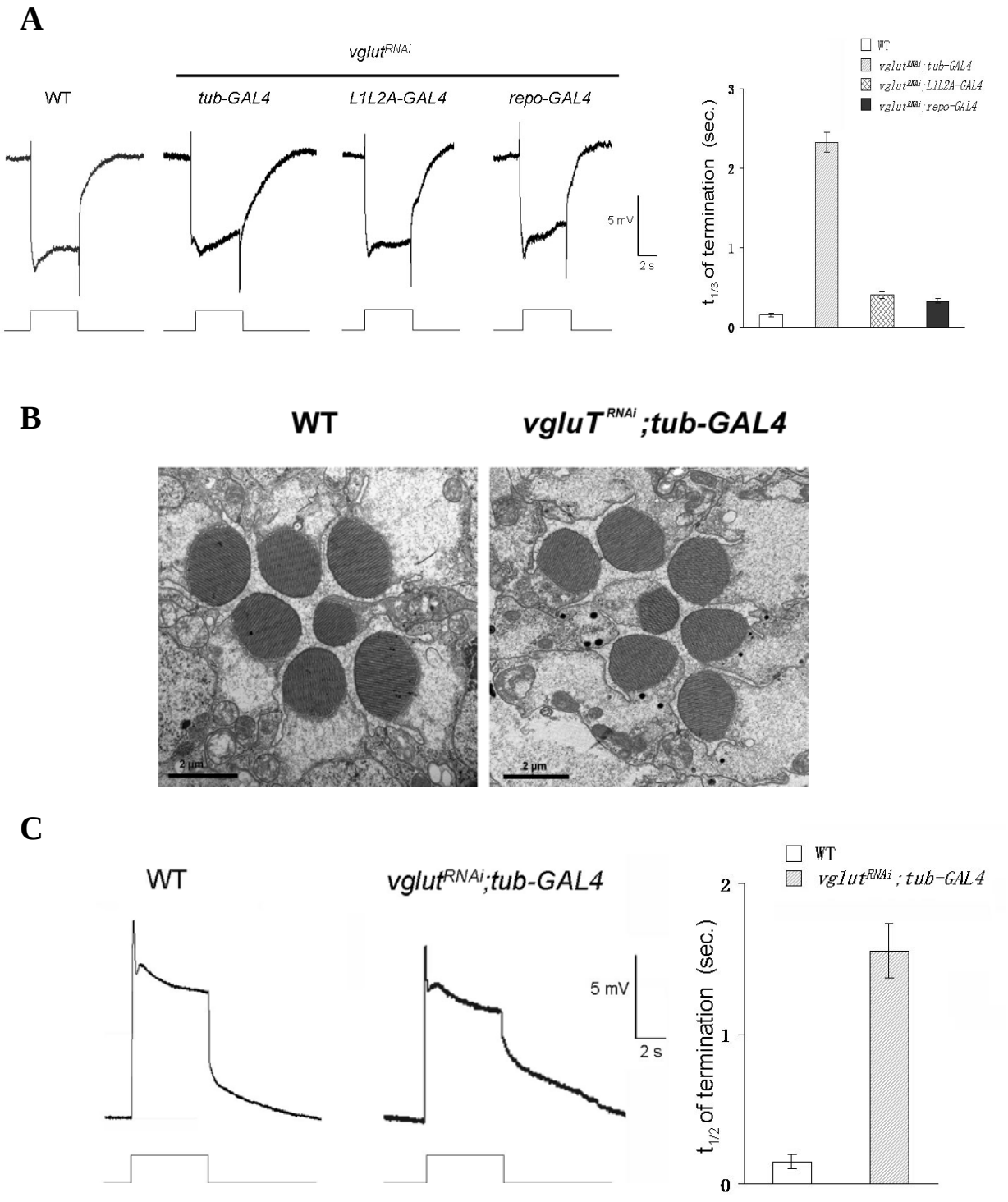

A
vglut^RNAi
WT
tub-GAL4
L1L2A-GAL4
repo-GAL4
5 mV
2 s
WT
vglut^RNAi;tub-GAL4
vglut^RNAi;L1L2A-GAL4
vglut^RNAi;repo-GAL4
t1/3 of termination (sec.)
B
WT
vgluT^RNAi;tub-GAL4
2 μm
2 μm
C
WT
vglut^RNAi;tub-GAL4
5 mV
2 s
WT
vglut^RNAi;tub-GAL4
t1/2 of termination (sec.)

Figure 3-1

Figure 3-1. Disruption of glutamatergic signaling pathway causes slow termination of light response in photoreceptor

(A) RNAi knockdown of the *vglut* gene caused a slower termination of light response in ERG (*vglut*^{RNAi}*;tub-GAL4*). This ERG phenotype was not observed when the *vglut* gene was specifically knocked down in L1, L2 interneurons (*vglut*^{RNAi}*; L1L2A-GAL4*) or glial cells (*vglut*^{RNAi}*; repo-GAL4*).

(B) No morphological defect was observed in *vglut*^{RNAi}*; tub-GAL4* fly eye by EM analysis compare to wild type. One ommatidium is shown.

(C) Intracellular recordings revealed slower termination of light response in photoreceptors *in vivo* when the *vglut* gene was knocked down. Sample traces are shown on the left. The error bars represent SEMs.

gene		function	termination speed of light response
glutamate receptor			
cg11144	**mGluRA**	metabotropic glutamate receptor	normal
cg8442	**DGluRI**	kinate-type ionotropic glutamate receptor	normal
cg2902	**DNMDAR**	NMDA-like ionotropic glutamate receptor 1	normal
cg6992	**GluRIIA**	muscle-specific glutamate receptor type A	normal
cg7234	**GluRIIB**	muscle-specific glutamate receptor type B	normal
LCCH family			
cg7535	**GluCLα**	inhibitory glutamate receptor	slow
cg14723	**HisCl1**	histamine receptor 1	normal
cg7411	**HisCl2**	histamine receptor 2	normal
cg17336	**LCCH3**	GABA receptor beta subunit	normal
cg7446	**GRD**	GABA-and-glycine-receptor-like-subunit; glycine receptor	normal
cg10537	**RDL**	GABA A receptor subunit	normal
cg33989	**pHCl**	pH sensitive chloride channel	normal
cg6927	**LCCH-4D**	unknown	normal
cg8916	**LCCH-14A**	unknown	normal
cg12344	**LCCH-47C**	unknown	normal
cg7589	**LCCH-74C**	unknown	—
cg11340	**LCCH-100C**	unknown	—

Table 3-2. RNAi screen for receptors mediating retrograde regulation of photoreceptor

— RNAi driven by *tub-GAL4* line caused embryonic death.

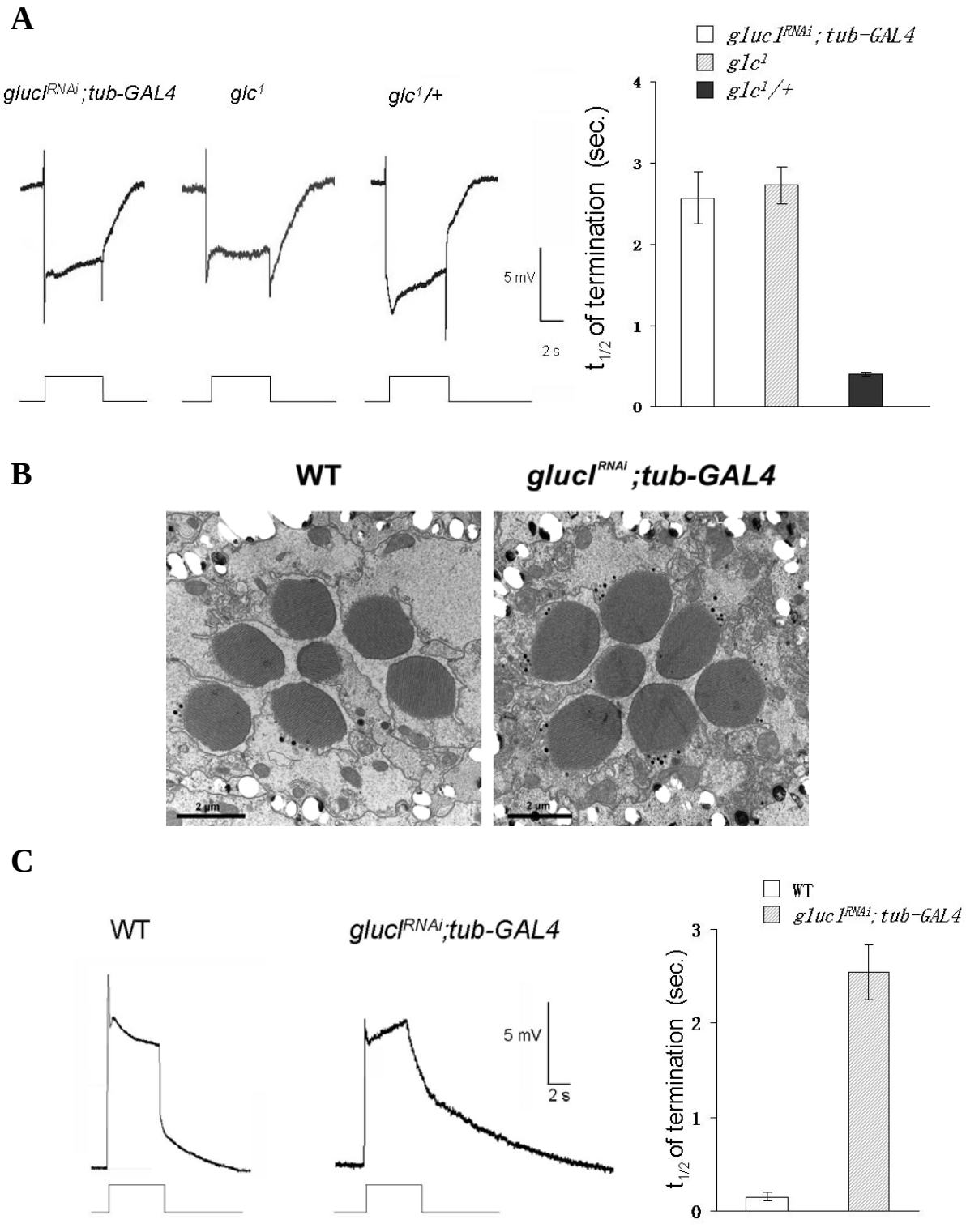

A
glucl^RNAi;tub-GAL4
glc^1
glc^1/+
5 mV
2 s
glucl^RNAi;tub-GAL4
glc^1
glc^1/+
t1/2 of termination (sec.)
0
1
2
3
4
B
WT
glucl^RNAi;tub-GAL4
2 μm
2 μm
C
WT
glucl^RNAi;tub-GAL4
5 mV
2 s
WT
glucl^RNAi;tub-GAL4
t1/2 of termination (sec.)
0
1
2
3

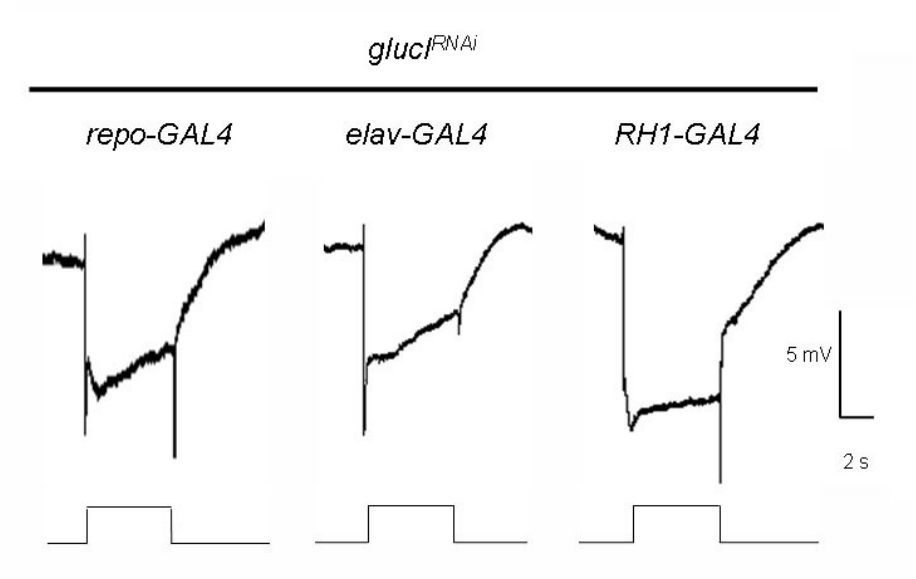
Figure 3-2

Figure 3-2. The glutamate-gated chloride channel GluCl is the receptor mediating the retrograde regulation of photoreceptors

(A) RNAi knockdown of the *glucl* gene (*glucl*^{RNAi}*; tub-GAL4*) caused a slower termination of light response in ERG recording, which was also observed in *glucl* mutant fliy *glc*^{1}, but not in *glc*^{1} /+ heterozygote fly. Sample traces are shown on the left. The error bars represent SEMs.

(B) No morphological defects were observed in *glucl*^{RNAi}*; tub-GAL4* fly eye by EM analysis. One ommatidium is shown.

(C) Intracellular recordings revealed slower termination of light response in photoreceptors *in vivo* when the *glucl* gene was knocked down. Sample traces are shown on the left. The error bars represent SEMs.


gluclRNAi
repo-GAL4
elav-GAL4
RH1-GAL4
5 mV
2 s

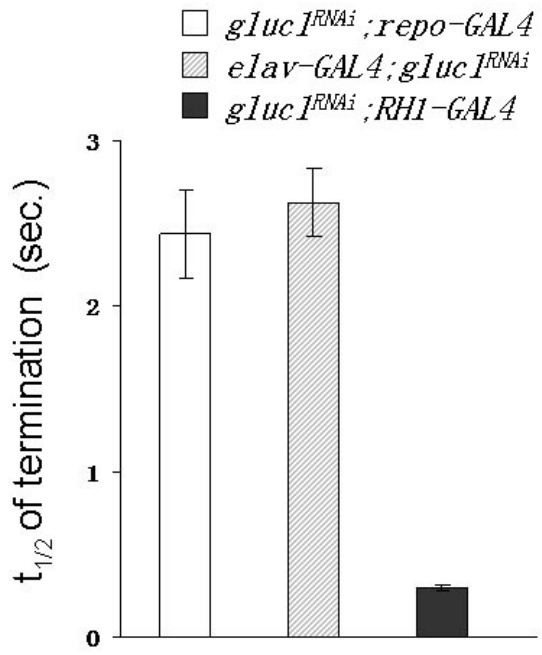
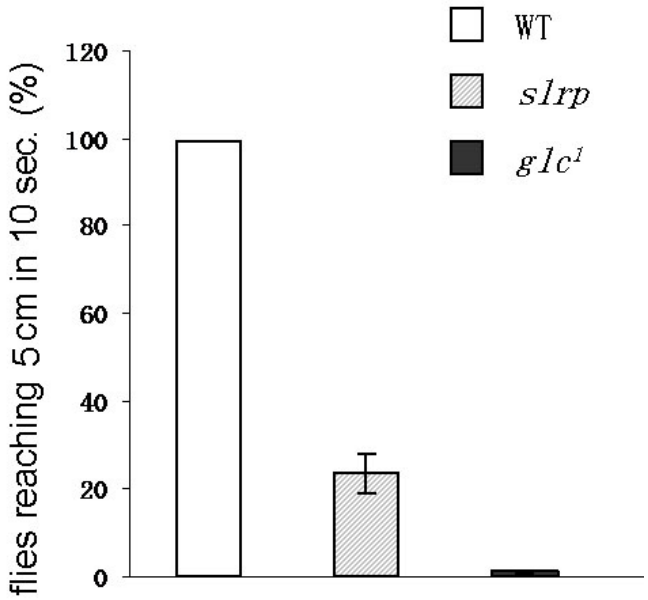

gluc1RNAi;repo-GAL4
elav-GAL4;gluc1RNAi
gluc1RNAi;RH1-GAL4
t1/2 of termination (sec.)
0
1
2
3

Figure 3-3

Figure 3-3. GluCl functions in both neuron and glia, but not in photoreceptors

ERG recording revealed a slower termination of light response when the *glucl* gene was specifically knocked down in glia (*glucl*^{RNAi}*;repo-GAL4*) or neurons (*elav-GAL4;glucl*^{RNAi}), but not in *glucl*^{RNAi}*;RH1-GAL4* flies. Sample traces are shown on the top. The error bars represent SEMs.

A


WT
slrp
glc1
flies reaching 5 cm in 10 sec. (%)
120
100
80
60
40
20
0

B

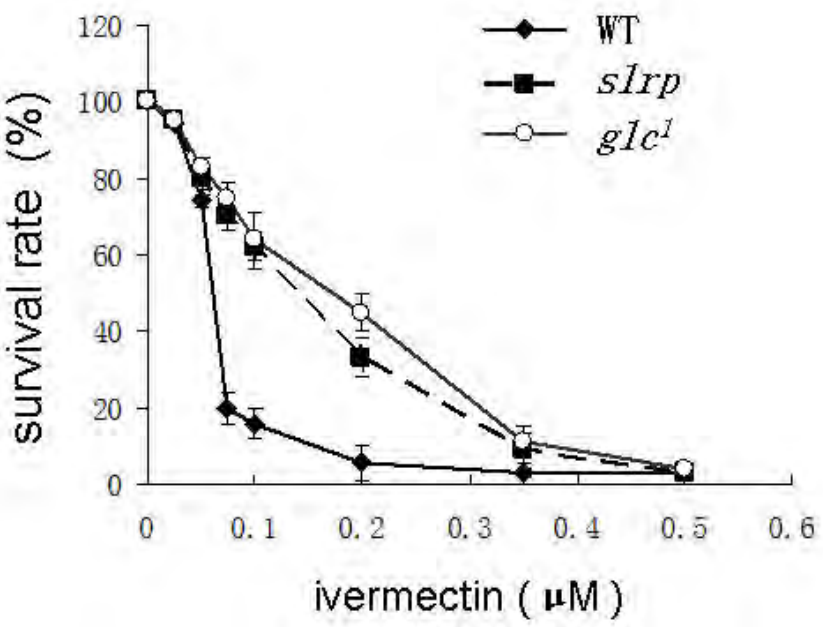

WT
slrp
glc1
survival rate (%)
120
100
80
60
40
20
0
0
0.1
0.2
0.3
0.4
0.5
0.6
ivermectin (μM)

Figure 3-4

Figure 3-4. The *slrp* and the *glc*^{1} mutants display similar behavior defects

(A) In climbing test, both the *slrp* and the *glc*^{1} mutants display locomotion defect. The error bars represent SEMs.

(B) Both the *slrp* and the *glc*^{1} mutants displayed resistance to ivermectin treatment. The error bars represent SEMs.

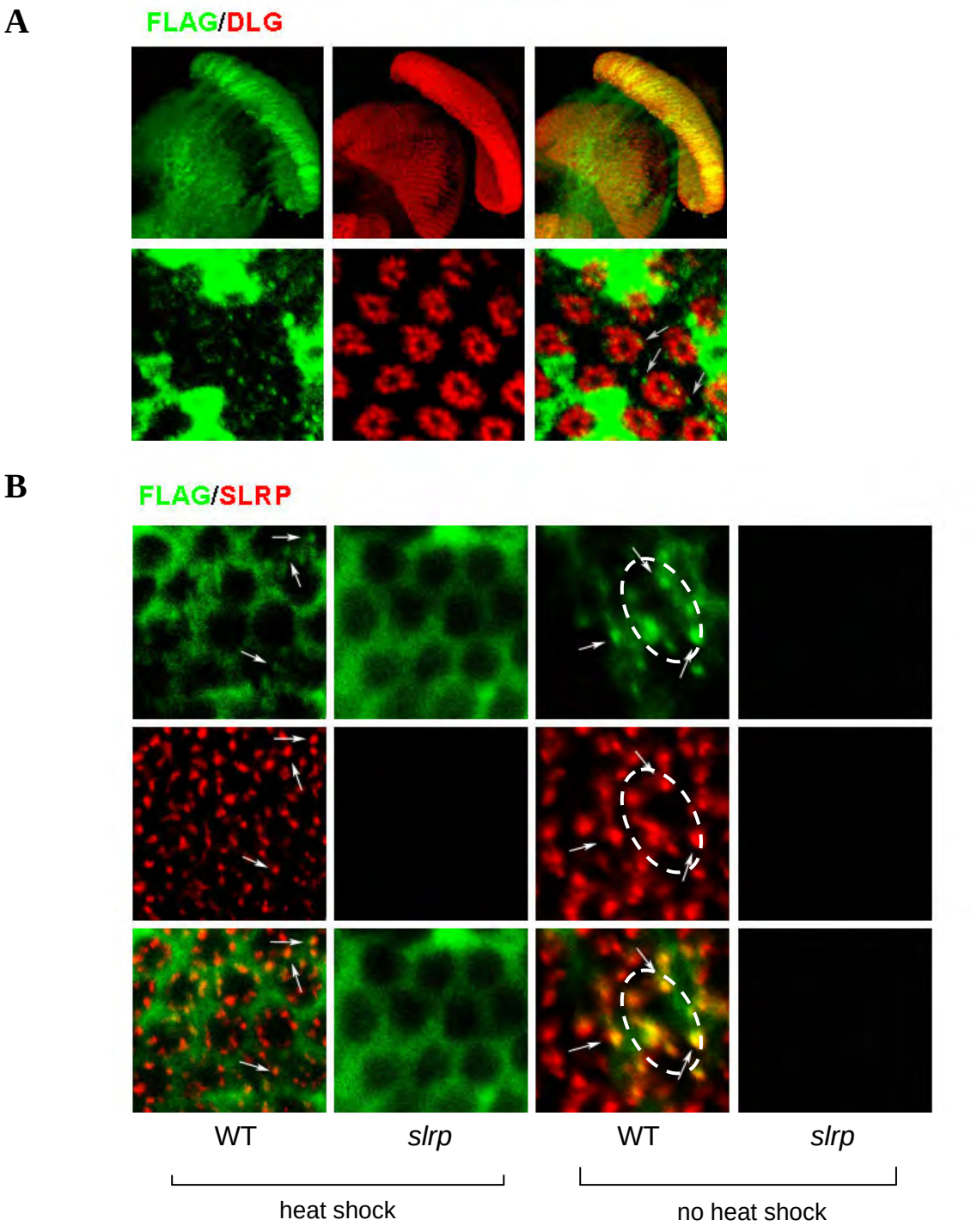


Figure 3-5

Figure 3-5. GluCl is concentrated in gnarl, a special process of epithelial glia

(A) GluCl-FLAG is expressed in *UAS-GluCl-FALG; HS-GAL4* flies after 30-minutes of heat shock at 37℃. Immunostainings showed that GluCl-FLAG signal (green) is observed in epithelial glia cell bodies and in punctuate structures (arrow) between photoreceptor axons (red, labeled with DLG antibody), but not in photoreceptor axons or cell bodies of other lamina interneurons.

(B) The punctuate GluCl-FLAG signal (green) co-localized with SLRP (red), a marker of gnarl structure. No punctate GluCl-FLAG signal is observed in the *slrp* mutant background that has no gnarl structure. Without heat shock, the GluCl-FLAG signal (green) from leaking expression was only observed in punctuate structures that co-localized with SLRP. No GluCl-FLAG signal was detected in the *slrp* mutant background. One cartridge is indicated by white circle.

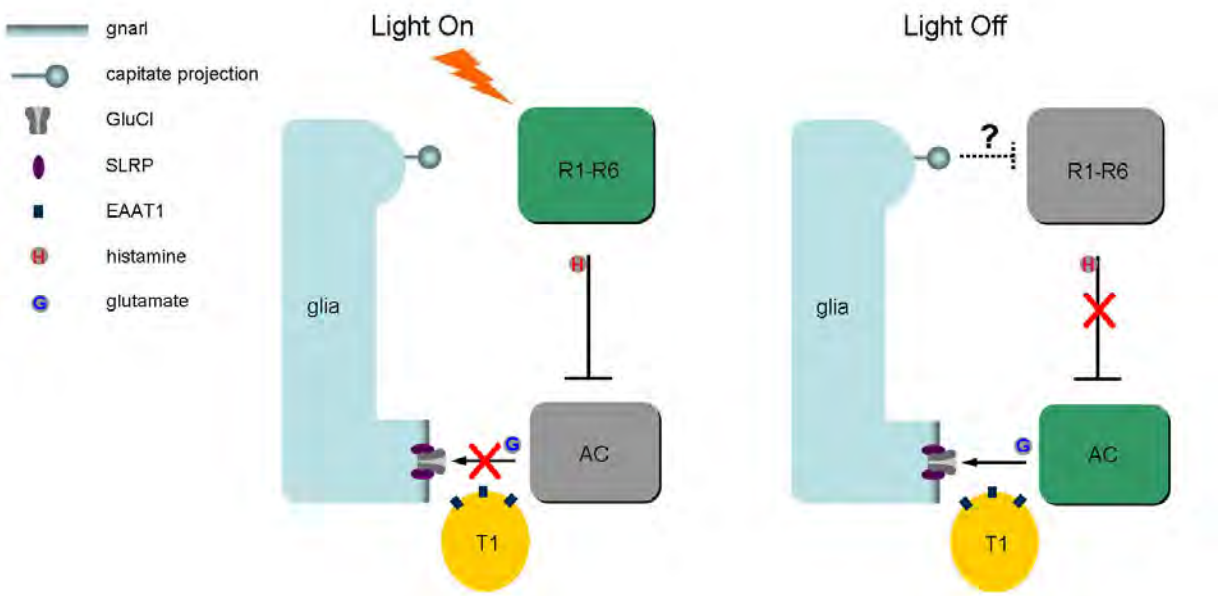

gnarl
capitate projection
GluCl
SLRP
EAAT1
histamine
glutamate
Light On
R1-R6
glia
AC
T1
Light Off
?
R1-R6
glia
AC
T1

Figure 3-6

Figure 3-6. The model of photoreceptor — amacrine cell — epithelial glia — photoreceptor feedback loop

In light conditions, depolarization of R1-R6 triggers the release of histamine, which inhibits postsynaptic cells including amacrine cell. When light stimulation is terminated, the removal of histamine activates amacrine cells, triggers the release of glutamate, which stimulates the inhibitory glutamate receptor GluCl in the gnarl membrane of epithelial glia. This inhibitory signal is then transferred from epithelial glia to photoreceptor through an unknown mechanism that probably involves capitate projections, the glial invaginations in the photoreceptor. This feedback loop accelerates the repolarization of photoreceptor membrane potential and facilitates the rapid termination of light response. The T1 cell expresses the glutamate transporter EAAT1, and thus may play a role, directly or indirectly in the glutamatergic signaling. The SLRP protein is required for the formation of gnarl structure, and the concentration of GluCl at the site of input.

Table 3-S1. RNAi lines examined in the screens (from Vienna Drosophila RNAi Center)

gene		transformant ID	gene		transformant ID
cg2718	GS1	40174	*cg5549*	CG5549	8222
cg1743	GS2	32929	*cg8380*	DAT	106961
cg9887	vGlut	104324 2574	*cg8394*	VGAT	45916
cg3747	dEAAT1	109401	*cg1732*	GAT	13359
cg3159	dEAAT2	104371	*cg4545*	SERT	11346
cg14994	GAD1	32344	*cg11144*	mGLuRA	1793
cg7811	GAD2	2890	*cg8442*	DGluRI	108019
cg33310	GB1	48300 101440	*cg2902*	DNMDAR	104773
cg6706	GB2	1785 110268	*cg6992*	GluRIIA	101686
cg3022	GB3	108036 50176	*cg7234*	GluRIIB	7878
cg17336	LCCH3	109606 37408	*cg7535*	GluCLα	105754
cg7446	GRD	5329	*cg14723*	HisCl1	104966
cg10537	RDL	100429 41101 41103	*cg7411*	ORT	107363
cg12345	CHAT	20183	*cg33989*	pHCl	11020 39283 103247
cg32848	VAChT	40918	*cg6927*	LCCH-4D	104345
cg17907	ACE	3968	*cg8916*	LCCH-14A	9138 101633
cg5610	ALS	48159	*cg12344*	LCCH-47C	37165 103271
cg32538	gfA	11329	*cg7589*	LCCH-74C	42582 102570
cg11348	ARD	39421	*cg11340*	LCCH-100C	22854 108337
cg4356	mAcHR-1	33123			

CHAPTER IV: DISCUSSION

A feedback pathway facilitating repolarization of photoreceptor

High temporal resolution of visual signaling depends on the rapid kinetics of photoresponse in photoreceptors, the light sensory neurons. For rapid recovery of photoreceptor membrane potential at the end of light stimulation, the phototransduction cascade is tightly controlled in photoreceptors (Scott and Zuker, 1997; Hardie et al., 2001; Gu et al., 2005). However, whether any extrinsic regulation is involved in the termination of photoreceptor light response is unknown.

In this dissertation, by using *Drosophila* as the model system, we identified a feedback regulation mechanism that is critical for the rapid termination of photoreceptor light response. The lamina epithelial glia is identified as one element of this feedback loop by the study on *slrp* mutant flies. Lamina amacrine cells act as another element in the feedback loop, which fills the gap from photoreceptors to glia. Based on current knowledge, we propose a complete photoreceptor — amacrine cell — epithelial glia — photoreceptor feedback loop for rapid repolarization of photoreceptor (Fig. 3.6). This feedback loop is different from a previously proposed negative feedback loop; instead, it transfers a positive feedback consisting of a sign-reversing signal from photoreceptors to amacrine cells and an inhibitory regulation from amacrine cells to photoreceptors through the epithelial glia. This feedback provides an important mechanism shaping photoreceptor output, especially during the light to dark shift.

Amacrine cells are wide-field neurons with lateral arborization within the lamina

(Fischbach and Dittrich, 1989), and epithelial glial cells are linked to each other through gap junctions (Saint Marie and Carlson, 1983), indicating that both cell types are capable of mediating the communication between adjacent cartridges. Their involvement in the feedback loop implies that this feedback may also work as a laterally regulating mechanism, and is critical for synchronization of photoreceptors in the eye.

Detail about the amacrine cell to glia signal transfer is revealed by RNAi screens and studies in the *slrp* mutant background. A glutamate-gated chloride channel GluCl locates to the gnarl, a glial structure that is probably equivalent to the postsynaptic complex. There, GluCl mediates glutamatergic input to the epithelial glia. In the future, we will need to characterize the last step of the feedback regulation: how glial cells modulate photoreceptor membrane potential.

Functions of glia in visual systems

Glial cells play important roles in development by providing diffusible and contact-mediated guidance cues that contribute to the neural connections and the formation of neural circuitry (Volterra and Meldolesi, 2005). In adult animals, they not only protect and maintain, but also modulate the nervous system. Accumulating evidence shows that glial cells participate in synaptic function and plasticity (Haydon and Carmignoto, 2004; de Melo Reis et al., 2008; Pfrieger, 2010; Wiedemann, 2010).

However, our understanding of glial cell function in visual systems is very limited.

Müller cell, the neuropile glia in the vertebrate retina that interacts with almost all retinal neurons, is thought to play roles in neurotransmitter metabolism and provide trophic factors (Bringmann et al., 2006; de Melo Reis et al., 2008). It is reported to function as optic fibers delivering light to photoreceptor (Franze et al., 2007). Although a light-evoked Ca^{2+} increase in Müller cells has been reported, suggesting they may participate in information processing in vertebrate retina (Newman, 2004), the specific effect of visual glia on the visual signal process was totally unknown.

Our study now shows that the *Drosophila* epithelial glia, a visual neuropile glia, is involved in a feedback loop that facilitates the termination of photoreceptor light response. Although we have not revealed the mechanism by which epithelial glias modulate the photoreceptor potential, we propose that the large amount of glial invaginations in photoreceptor terminals are underlying this modulation. Our work provides the first evidence that a visual glia directly transmit signals in a neural circuit, which demonstrates a neuron-like function of glia in the visual system.

Neuron-glia interaction mechanisms

Classic view states that glia act as cells that simply provide structural, metabolic and trophic support to neurons and synapses. However, to date, glial cells are thought to be active integrators of the brain circuitry as astrocytes, polarized glial cells in CNS, can exchange information with the neuronal elements of the associated synapses. This new

glial function in synaptic physiology is represented by the concept of “tripartite synapse”. Briefly, astrocytes can be activated by neuronal signals, exhibit Ca^{2+} excitability that triggers the release of gliotransmitters such as glutamate, D-serine, ATP, GABA, which in turn modulate neuronal activity and plasticity (Fields and Stevens-Graham, 2002; Perea and Araque, 2009; Giaume et al., 2010).

Astrocyte excitability depends on intracellular Ca^{2+} signaling, which can be selectively triggered by specific neurotransmitters (Perea and Araque, 2005). Almost all types of neurotransmitter receptors have been detected in different astrocytes *in situ* and *in vivo*, including glutamate receptors, GABA receptors, purinorecepotrs, glycine receptors, cholinoreceptors and adrenorecptors (Barres et al., 1990; Porter and McCarthy, 1997; Karadottir and Attwell, 2007). Immunostaining studies have shown that many of these glial receptors are concentrated in tripartite synapses (Aoki et al., 1994; Riquelme et al., 2002; Charles et al., 2003), suggesting they are involved in the signaling from neuron to glia. However, physiological functions of this neuron-glia signaling are unknown. In addition, the mechanism that concentrates glial receptors to the sites close to synapse has yet to be studied.

My dissertation studies show that, to facilitate rapid recovery of photoreceptors from light responses, the epithelial glia need to receive a glutamatergic signal from amacrine neurons through an inhibitory glutamate receptor, GluCl. This neuron to glia transmission occurs in a morphologically distinguishable glial structure, gnarl, which is adjacent to

glutamatergic synapses. An ADAM protein, SLRP, is required for the gnarl formation, while GluCl receives the glutamatergic signal in the gnarl membrane. Our findings have revealed a physiological function of neuron-glia signaling, and suggest that specifically assembled membrane domains are required for the concentration of neurotransmitter receptors on glia surface.

Future studies

(1) We have identified a new feedback loop that is required for rapid termination of light response. However, as discussed above, several gaps still need to be filled. For example, what is the exact mechanism by which the epithelial glia modulates photoreceptors? Which neuron(s) is involved in the glia-independent feedback regulation of photoreceptor? A wider RNAi-based screen directed to identifying additional genes in the retrograde regulation of photoreceptor would be helpful in addressing these questions.

(2) The major challenge in understanding visual circuits is to monitor activities of individual cells *in vivo*. Although we have identified a new feedback circuit by taking advantage of genetic tools, the strongest evidence would still come from direct recording of each cell involved in the feedback loop. The next step of our work would be conducting intracellular recording in genetically labeled cells such as epithelial glia. This is technically challenging but feasible. In addition, activities of individual neurons can be monitored through genetically expressed markers such as a Ca^{2+} indicator or Cl^{-} indicator

(Mank et al., 2008; Markova et al., 2008). These could be alternative ways of studying the visual circuits.

(3) Although no functional glutamate-gated chloride channel has been reported in vertebrates, gene structure comparisons and phylogenetic analysis demonstrate that the GluCl is orthologous to vertebrate glycine receptors (Vassilatis et al., 1997). All four glycine receptors are expressed in vertebrate retina, and are thought to mediate lateral inhibition of bipolar cells (Heinze et al., 2007; Majumdar et al., 2009). However, strong signals of glycine receptor GlyR2 and GlyR3 have been found in bipolar cells and postsynaptic to glutamatergic amacrine terminals in immunostaining assays (Haverkamp et al., 2004). It would be important to investigate whether any of those glycine receptors responds to glutamate.

The results of my dissertation work have led to a better understanding of how D*rosophila* eye responds rapidly to light intensity change, and provide important clues to the function of visual glia. This work may also advance our knowledge about how neuron and neuropile glia interact for achieving rapid signaling in the brain in general.

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