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Mutation of a TADR Protein Leads to Rhodopsin and G_q-Dependent Retinal Degeneration in *Drosophila*

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The *Drosophila* photoreceptor is a model system for genetic study of retinal degeneration. Many gene mutations cause fly photoreceptor degeneration, either because of excessive stimulation of the visual transduction (phototransduction) cascade, or through apoptotic pathways that in many cases involve a visual arrestin Arr2. Here we report a gene named *tadr* (for *t*orn *a*nd *d*iminished *r*habdomeres), which, when mutated, leads to photoreceptor degeneration through a different mechanism. Degeneration in the *tadr* mutant is characterized by shrunk and disrupted rhabdomeres, the light sensory organelles of photoreceptor. The TADR protein interacted *in vitro* with the major light receptor Rh1 rhodopsin, and genetic reduction of the Rh1 level suppressed the *tadr* mutation-caused degeneration, suggesting the degeneration is Rh1-dependent. Nonetheless, removal of phospholipase C (PLC), a key enzyme in phototransduction, and that of Arr2 failed to inhibit rhabdomeral degeneration in the *tadr* mutant background. Biochemical analyses revealed that, in the *tadr* mutant, the G_q protein of Rh1 is defective in dissociation from the membrane during light stimulation. Importantly, reduction of G_q level by introducing a hypomorphic allele of $G_{\alpha q}$ gene greatly inhibited the *tadr* degeneration phenotype. These results may suggest that loss of a potential TADR-Rh1 interaction leads to an abnormality in the G_q signaling, which in turn triggers rhabdomeral degeneration independent of the PLC phototransduction cascade. We propose that TADR-like proteins may also protect photoreceptors from degeneration in mammals including humans.

Key words: **retinal degeneration; rhodopsin; G-protein; photoreceptor; *Drosophila*; GPCR; cation amino acid transporter**

Introduction

Degeneration of rod and/or cone photoreceptors is a defining characteristic of retinitis pigmentosa (RP), a subset of human hereditary retinal diseases (Yamamoto et al., 1997) that cause night blindness followed by progressive loss of vision (Hartong et al., 2006). Many identified causal genes of RP encode key components of the visual transduction (phototransduction) cascade in photoreceptors (Hartong et al., 2006; Daiger et al., 2007). For instance, mutation in the light receptor rhodopsin is a prevalent cause of autosomal dominant RP (Kaushal and Khorana, 1994; Dryja, 2000; Wilson and Wensel, 2003), and loss of rhodopsin regulatory proteins, arrestins and a rhodopsin kinase, causes Oguchi disease, an autosomal recessive form of RP (Fuchs et al., 1995; Yamamoto et al., 1997; Dryja, 2000). In addition, several other RP genes are required for the trafficking and maturation of rhodopsin molecules (Hartong et al., 2006). Thus, abnormalities in rhodopsin signaling pathways are major causes of photoreceptor degeneration. Nonetheless, in many RP cases, it remains puzzling why the product of an affected gene is important for photoreceptor protection. More importantly, the mutant genes in ~40% of RP cases have yet to be identified (Hartong et al., 2006; Daiger et al., 2007).

The *Drosophila* photoreceptor is a genetic model system for the study of both phototransduction (Montell, 1999; Hardie and Raghu, 2001) and retinal degeneration (Ranganathan, 2003). The whole visual transduction cascade is localized in a packed microvillar structure rhabdomere (Hardie and Raghu, 2001), which is analogous to the outer segment of rod and cone photoreceptors. The fly rhodopsin is coupled to a G_q type G-protein (Lee et al., 1994; Scott et al., 1995). Instead of activating phosphodiesterase (PDE) to close cGMP-gated channels as in mammalian photoreceptors, this fly visual G-protein stimulates a *norpA* gene-encoded phospholipase C (PLC) to open TRP Ca^{2+}/cation channels (Bloomquist et al., 1988; Hardie and Minke, 1992; Montell, 1999). To rapidly terminate the light response, the stimulated rhodopsin molecule is deactivated promptly through a visual arrestin Arr2 (Dolph et al., 1993) and a dCAMTA/dFbxl4 pathway (Han et al., 2006).

Similar to those in humans, fly mutations in phototransduction molecules including rhodopsin (Leonard et al., 1992; Kurada and O'Tousa, 1995; Iakhine et al., 2004), PLC (Meyertholen et al., 1987; Zinkl et al., 1990; Alloway et al., 2000), TRP (Hong et al., 2002; Wang et al., 2005) and arrestins (Dolph et al., 1993; Satoh and Ready, 2005) all cause age-dependent photoreceptor degenerations, which are generally characterized by diminished rhabdomeres. Several other visual proteins such as a diacylglycerol kinase RDGA and a rhodopsin phosphatase RDGC are also essential for photoreceptor protection (Masai et al., 1993; Kiselev et

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al., 2000). Fly photoreceptors may degenerate in a necrotic, Ca^{2+}-dependent manner because of prolonged stimulation of the phototransduction cascade, or through apoptotic processes (Wang and Montell, 2007). In several mutants including *rdgC* and *norpA*, rhodopsin forms a stable complex with Arr2 to trigger photoreceptor apoptosis (Alloway et al., 2000; Kiselev et al., 2000). Here we report the isolation of a mutant fly *tadr* that undergoes rhabdomeral degeneration through a new pathway.

Materials and Methods

Fly genetics. The genotype of wild-type flies is *cn,bw* unless mentioned otherwise in the text. The *tadr* mutant was generated from *cn* progenitors using the chemical mutagen ethyl methanesulfonate (EMS), and recombined into a *cn,bw* background. Except for the dark-reared flies that were never exposed to light from the prepupal stage, all others were raised in an approximate 12 h light (~250 lux)/12 h dark cycle. The mutant alleles of other genes used in this work are $ninaE^5$, $arr2^5$, $norpA^{24}$, $G_{\alpha q}{}^1$, and $glass^2$.

A wild-type *CG9264* cDNA was obtained through RT-PCR, subcloned into a pCaSpeR-*hs* vector, and injected into w^{1118} flies to generate *p*[*hs-CG9264*] transgenic flies. The transgene was subsequently crossed into the *tadr* mutant background. To express the protein, flies were heat shocked for 1 h at 37°C in a water bath once a day from late pupal stage and examined at 7 d of age.

Optical neutralization analysis. This analysis was performed as described previously (Franceschini and Kirschfeld, 1971). In brief, fly heads were separated from the body and immersed in a layer of lens oil to optically neutralize the cornea. On the stage of a microscope, a spotlight was shone into the head from the neck side for antidromic illumination of the compound eye. The rhabdomeres that appeared as bright dots resulting from high transmission of light were counted for each upright ommatidium. The mean number of rhadomeres per ommatidium was calculated for each genotype and condition based on the results of 30 ommatidia from 5 flies. SEMs were presented as error bars in figures.

Toluidine blue staining and electron microscopy. Fly heads were hemisected, fixed with 2.5% glutaraldehyde in 50 mM sodium cacodylate buffer on ice for 4 h, washed with the buffer solution three times, and fixed again with 1% osmium tetroxide for 1 h. After ethanol dehydration, the head tissues were embedded in LR White resin. Eye cross-sections were cut either at 1 μm thickness and stained with 1% toluidine blue for light microscopy, or at 100 nm for electron microscopy.

Immunostaining of Rh1. Hemisected fly heads were fixed with 4% of paraformaldehyde in PBS, dehydrated in acetone, and embedded in LR White resin. Eye cross-sections of 1 μm were cut and stained with a monoclonal Rh1 antibody (DSHB) and a FITC-conjugated secondary antibody.

Electrophysiological recordings. Electroretinograms were examined as previously described (Li and Montell, 2000) with minor modifications. Flies were immobilized with thin stripes of tape. Two glass microelectrodes filled with Ringer's solution were put separately on the eye surface and the thorax (as reference). Five second light pulses (2500 lux) were used to stimulate the eye after adapting the fly in the dark for 1 min. The signal was amplified and recorded using a Warner IE210 Intracellular Electrometer. For the quantification of response amplitude, data from six flies were averaged and SEMs were calculated.

For whole-cell recordings, the ommatidia were isolated from flies enclosed within 2 h in Ca^{2+}-free Ringer solution, and individual peripheral photoreceptors were recorded as described previously (Han et al., 2006). The pipette and bath solutions were (in mM) 100 potassium gluconate, 40 KCl, 2 $MgCl_2$, 0.1 EGTA, 5 ATP, 0.5 GTP, 10 HEPES (pH 7.15) and 130 NaCl, 5 KCl, 1.8 $CaCl_2$, 5 proline, 25 sucrose, 10 HEPES (pH 7.15), respectively. The resistance of recording pipettes was 5–6 MΩ. Cells were clamped at −70 mV to examine light-induced currents.

Glutathione-Sepharose binding assay. cDNA fragments encoding the intracellular loop IV and the C-terminal tail of TADR were amplified through PCR and inserted into a pGEX-5X vector to express GST-fused proteins (GST-LOOP and GST-TAIL) in bacteria. The fusion proteins were purified with glutathione-Sepharose beads (Amersham). The proteins on beads were incubated with wild-type fly head extracts in PBS that contains 0.2% Triton X-100 and protease inhibitors (Roche). After three washes with the incubation solution, the bound proteins were eluted and subjected to SDS-PAGE and Western blot. The Arr2 antibody is as described (Han et al., 2006) and the sources of other antibodies were Montell lab (TRP), DSHB (Rh1), and Sigma-Aldrich (G_q).

Arr2 binding and release assays. Arr2 binding assays were performed as previously described (Satoh and Ready, 2005) with modifications. Five heads from dark-reared 1-d-old flies were added into a homogenization solution containing 250 mM sucrose, 120 mM KCl, 5 mM MgCl2, 1 mM DTT, 10 mM MOPS (pH7.0), and Complete protease inhibitors (Roche). For Arr2 binding, heads were exposed to bright blue light (700 lux) for 4 s, homogenized in the dark, and centrifuged at 13,000 × *g* for 5 min to precipitate the membrane fraction. For release of membrane-bound Arr2, the blue light-treated heads were exposed to orange light for 8 s before homogenization and centrifugation. The pellet and supernatant fractions were separated under very dim red light and subjected to SDS-PAGE and Western blot.

Assay of light-stimulated GTPγS binding. One-day-old, dark-reared flies were divided into two groups of 25 flies. Fly heads of the dark group were collected under dim red light, homogenized in 125 μl of the homogenization buffer, and centrifuged at 13,000 × *g* for 5 min to precipitate membrane. After one wash with the homogenization buffer, the membrane fraction was resuspended in 25 μl of ice-cold reaction buffer (2 mM 2-mercaptoethanol, 5 mM $MgCl_2$, 5 mM creatine phosphate, 50 U/ml creatine kinase, 0.25 mM ATP, 15 μM GDP, and 50 mM MOPS, pH 6.7). After 5 μl was saved for the determination of G_q protein level using Western blot, the membrane sample was added into 80 μl reaction buffer that contains 25 nM GTPγ^{35}S, and incubated in the dark for 10 min at room temperature. Membrane sample of the light group was prepared and incubated in the same way, except that the incubation and all other steps were conducted under blue light illumination (700 lux). The reactions were terminated by addition of 0.5 ml of ice-cold rinsing solution (2 mM 2-mercaptoethanol, 5 mM $MgCl_2$, and 50 mM Mops, pH6.7) followed by prompt filtration through glass-fiber filters (Whatman). The filters were rinsed with 2.5 ml of solution for 4 times, and air dried. The radioactivity on each filter was measured using a Beckman liquid scintillation counter. The level of GTPγS binding in each sample was normalized to the G_q protein level. The light-stimulated binding was calculated by subtracting the value of dark group from that of light group.

Assay of light-dependent G_q localization. The light-dependent G_q localization was examined following a previously described method (Kosloff et al., 2003). One group of 6 dark-reared flies less than one d old were exposed to bright blue light (700 lux) for one h, while another group were kept in the dark. The heads were removed under dim red light, homogenized in 30 μl of hypotonic homogenization solution [20 mM HEPES, pH 7.6, with protease inhibitors (Roche)], and centrifuged at 13,000 × *g* for 5 min to precipitate the membrane fraction. The pellet was washed, centrifuged again, and the supernatants were combined. Both the pellet and supernatant fractions were subjected to SDS-PAGE and Western blot.

Results

tadr flies undergo rhabdomeral degeneration

The *tadr* mutant were identified using an optical neutralization technique, which is for observation of light passing through each rhabdomere in the fly eye (Franceschini and Kirschfeld, 1971) (see method). In this method, all six peripheral (R1-R6) and the R7 central rhabdomere of each ommatidium are detected as individual light spots in wild-type fly eye (Fig. 1*A*). The light spot will be invisible if the rhabdomere diminishes or has dissembled microvilli. Based on this assay, we conducted a small-scale chemical mutagenesis screen for genes critical for rhabdomere integrity on chromosome 2. Of 273 homozygote-viable lines, we isolated two fly mutants with undetectable rhabdomeres at the age of 2 weeks (Fig. 1*A*, data not shown). One mutant is a new allele of the gene *Pph13*, which is required for rhabdomere morphogenesis (Zelhof et al., 2003), the other is *tadr*.

To confirm that the invisibility of rhabdomere in the optical neutralization assay (Fig. 1*A*) is caused by disruption of rhabdomere structure in the *tadr* mutant, we conducted electron microscopy (EM) to examine cross-eye sections of 2-week-old flies. In contrast to the tightly packed microvillar structure found in wild type, the mutant peripheral rhabdomeres had detached and broken microvilli (Fig. 1*B*), and the overall length of microvilli was much shorter than wild type. Based on this EM observation, we named the mutant *tadr*, for *t*orn *a*nd *d*iminished *r*habdomeres. The central R7 rhabdomeres in the *tadr* mutant contained normally attached microvilli, although many of these rhabdomeres were deformed.

The *tadr* phenotype could be caused either by rhabdomeral degeneration or by a defect in the development of rhabdomere. We examined eye sections of 1-d-old *tadr* mutant, and found that rhabdomeres were virtually normal in these young flies (Fig. 2*A*). In contrast, the microvilli of most peripheral rhabdomeres were severely shortened in 7-d-old flies, although they were not detached as in 2-week-old flies (Fig. 2*A*). Thus, the *tadr* phenotype may reflect a degeneration of rhabdomere. This is further supported by the following toluidine blue (TB) staining and optical neutralization assays.

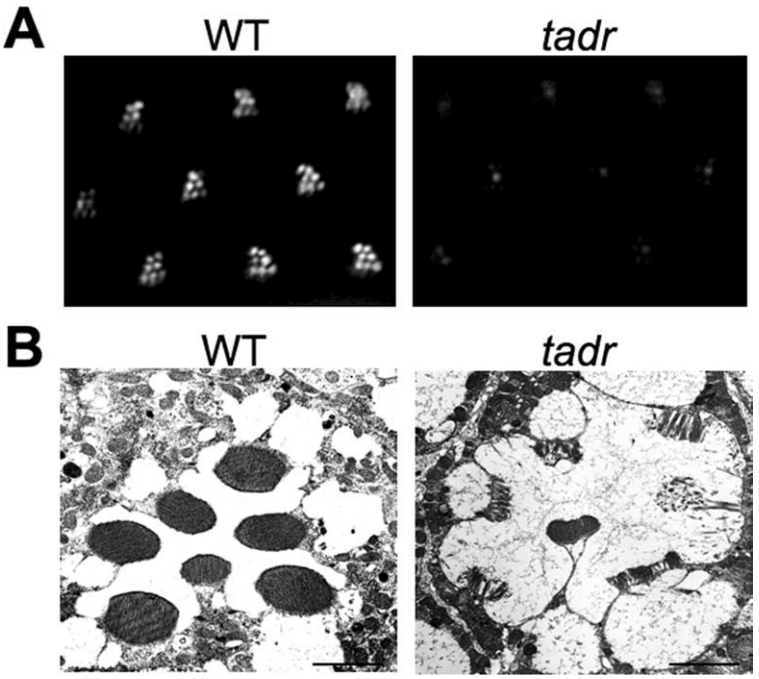

Figure 1. Impaired rhabdomere structure in *tadr* mutant flies. ***A***, A severe loss of peripheral rhabdomeres in 2-week-old *tadr* flies was observed in the optic neutralization assay. ***B***, EM analyses revealed that 2-week-old *tadr* flies contained detached and broken microvilli in peripheral rhabdomeres. Each picture shows a single ommatidium. Scale bars, 2 μm.

The TB staining of cross-eye sections revealed the rhabdomeres of wild-type fly as dark ovals, with peripheral ones much larger than the R7 (Fig. 2*B*). In *tadr* mutant flies, most rhabdomeres had normal morphology at 1 d old (Fig. 2*B*). When the fly became 7 d old, however, the average size of peripheral rhabdomeres severely decreased, with many of them even smaller than R7 (Fig. 2*B*,*C*). In optical neutralization analyses, most peripheral rhabdomeres were visible in 1-d-old flies, approximately half of them became invisible at 7 d old, and by the 13th d, almost no peripheral rhabdomeres were detected (Fig. 2*D*; supplemental Fig. 1, available at www.jneurosci.org as supplemental material). Those rhabdomeres showing abnormal shapes and reduced sizes in the TB staining were probably not detectable in the optical neutralization assay because of their low capabilities of light transmission.

In EM analyses, no significant abnormality was observed in the cell bodies of peripheral photoreceptors at early stages of rhabdomeral degeneration (Fig. 2*A*), suggesting that the degeneration originated from the rhabdomere. However, when the mutant fly grew to 2 weeks of age, many cell bodies also shrunk severely (Fig. 1*B*).

The *tadr* mutant has smaller light response

Despite the degeneration of rhabdomere, the peripheral photoreceptors in 7-d-old *tadr* mutant flies are responsive to light (supplemental Fig. 2, available at www.jneurosci.org as supplemental material). Nonetheless, in electroretinogram (ERG) recordings, the light responses of *tadr* fly had smaller amplitudes and slower termination when compared with wild type (Fig. 3*A*).

In 1-d-old *tadr* flies that only had a very subtle degeneration, the amplitude of ERG response was already smaller than wild type and was the same as that in the more degenerated, 7-d-old mutant flies (Fig. 3*A*). This temporal discrepancy between the ERG defect and the degeneration phenotype may suggest that the small ERG response is not simply caused by the morphological abnormality in rhabdomere. However, we found these two phenotypes are genetically linked to each other, and thus could be caused by mutation of the same gene.

CG9264 is the gene disrupted in the *tadr fly*

Because degeneration assays require long-time aging of flies, we instead mapped the *tadr* mutation based on the ERG phenotype. The ERG phenotype was uncovered by two deficiency chromosomes Df(2L)pr-A14 (missing a region from 37D2 to 39A4) and Df(2L)DS6 (missing 38F5 to 39E7), which located the mutation to the chromosome region 38F5–39A4. We further generated three small chromosomal deletions using FRT-containing piggyBac elements (Parks et al., 2004) and narrowed the mutant region to 39A1 (Fig. 3*B*). This region contains two predicted genes *CG9264* and *CG33511* and partially covers another two genes. By sequencing the genomic DNA, we identified a missense mutation in the gene *CG9264*, which changes the residue 532 Gly to Arg in the encoded product (Fig. 3*B*).

In quantitative RT-PCR analyses, the *CG9264* mRNA level was greatly reduced in fly heads of a *glass* mutant missing photoreceptor cells (Moses et al., 1989) (supplemental Fig. 3, available at www.jneurosci.org as supplemental material), suggesting a high expression level of *CG9264* in the photoreceptor. To confirm that the *CG9264* mutation is responsible for the visual phenotypes, we generated a transgenic fly (*tadr*;*P*[*hs-CG9264*]) that expresses a wild-type *CG9264* cDNA in the *tadr* mutant background through a heat-shock promoter. According to both EM and TB staining assays, overexpression of this cDNA by heat shocking the flies once a day from late pupal stage virtually eliminated the rhabdomeral degeneration observed in 7-d-old *tadr* flies (Fig. 3*C*). In addition, ERG responses after heat shock in these transgenic flies became almost identical to wild type (Fig.

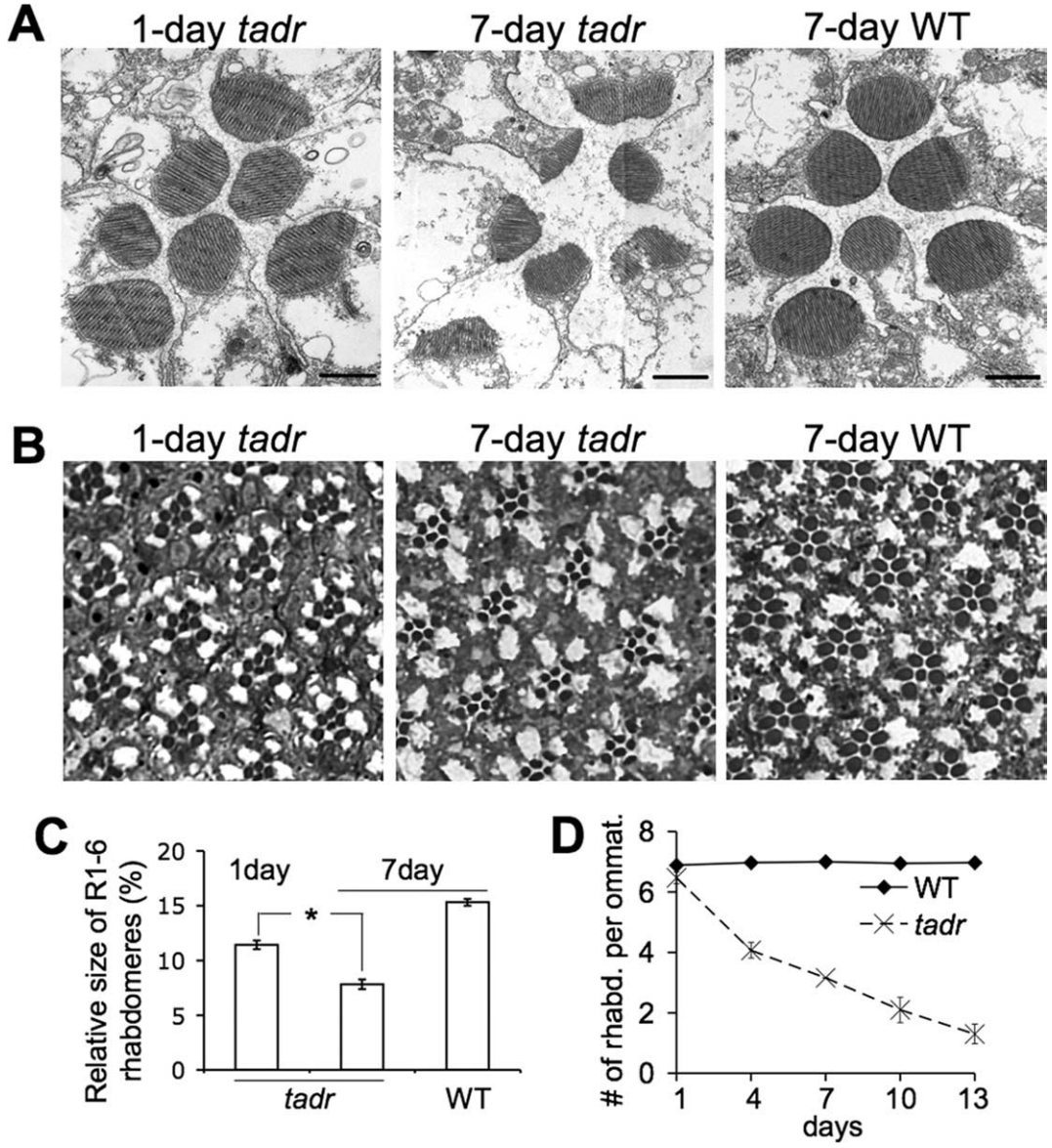

Figure 2. The rhabdomeral defect in the *tadr* mutant is caused by degeneration. ***A***, EM pictures for the comparison of rhabdomere structure between 1- and 7-d-old *tadr* flies. A wild-type picture is shown on the right. Scale bars, 1 μm. ***B***, TB staining of eye cross- sections revealed that 1-d-old *tadr* flies contained larger peripheral rhabdomeres than 7-d-old flies. Note the irregular shapes of some rhabdomeres in the 7-d mutant flies. ***C***, Quantification of the peripheral rhabdomere size based on the TB staining. The relative size represents the average ratio of the area occupied by all peripheral rhabdomeres to the total ommatidium area. Each ratio was calculated based on 6 ommatidia in a single eye section. Data from three experiments were averaged. SEMs are shown as error bars. The asterisk (*) indicates a significant difference. ***D***, In optical neutralization assays, the number of visible rhabdomeres decreased gradually during the aging of *tadr* flies. The mean number of rhabdomeres (rhabd.) per ommatidium (ommat.) was calculated based on 30 ommatidia of 5 flies for each genotype and age. Error bars represent SEMs.

3*D*). Thus, *CG9264* is indeed the mutant gene in the *tadr* fly, and is subsequently referred to as *tadr*.

Rhodopsin mediates rhabdomeral degeneration in the *tadr* mutant

The *tadr* gene encodes a 634 amino acid protein that has 12 putative transmembrane segments, with the eleventh disrupted by the Gly^{532} to Arg mutation in the mutant fly (Fig. 3*B*). The TADR protein does not contain any known protein domain or motif, except that the amino acid sequence is moderately homologous to several potential cation amino acid transporters (Verrey et al., 2004) including the human SLC7A4 (21% identical) and SLC7A1 (19% identical).

Because the protein structure of TADR did not provide enough clues to the understanding of the *tadr* mutation-dependent rhabdomeral degeneration, we attempted to explain the degeneration by looking for known rhabdomeral proteins that interact with TADR. We fused the two largest cytosolic fragments of TADR separately to a glutathione-S-transferase (GST) protein, immobilized them to glutathione-Sepharose beads, and used the beads to pull down proteins from fly head exacts. The result indicated that the intracellular loop IV of TADR but not its C-terminal tail specifically pulled down Rh1, the rhodopsin protein of peripheral rhabdomeres, from the head extracts (Fig. 4). Other examined visual proteins including G_q, PLC, TRP and Arr2 failed to interact with either TADR fragment. In control experiments, GST alone did not pull down any visual protein. These observations may suggest that the TADR protein, either directly or indirectly, associates with Rh1 in the photoreceptor.

Considering that loss of TADR-Rh1 interaction could cause

an abnormal rhodopsin signaling event disruptive to rhabdomere, we investigated whether rhabdomeral degeneration in the *tadr* mutant is stimulated by the activity of rhodopsin. We raised the mutant flies in a completely dark condition to prevent light activation of rhodopsin, and examined the rhabdomeres by TB staining at 7 d old. Although some rhabdomeres still showed irregular shape and/or reduced size (Fig. 5*A*), the average size of peripheral rhabdomeres was only slightly smaller than wild type and was much larger than in light-exposed mutant flies (Fig. 5*C*). In addition, the light deprivation greatly reduced the speed of rhabdomere loss in optical neutralization assays (Fig. 5*D*). Thus, degeneration of rhabdomere in the *tadr* mutant is largely stimulated by light.

Like other G-protein-coupled receptors, rhodopsin may have a low-level of spontaneous activity in the absence of light stimulation, as evident by quantum activation of the phototransduction cascade in the dark (Elia et al., 2005). We suspected that the mild degeneration of rhabdomere in dark-reared *tadr* flies might be stimulated by spontaneous rhodopsin activities, and that removal of the rhodopsin protein could have a greater effect on *tadr* phenotype rescue than light deprivation. To test this, we decreased the rhodopsin protein level by introducing a hypomorphic allele of *ninaE* (*ninaE*^{5}), the gene encoding the Rh1 opsin, into the *tadr* mutant background. Although the *ninaE* single mutant itself may undergo retinal degeneration at a much later stage (Leonard et al., 1992), the shape and integrity of rhabdomere remained intact in 7-d-old flies (Fig. 5*B*,*D*), except that the size of each peripheral rhabdomere appeared smaller than wild type (Fig. 5*B*,*C*). According to TB staining assays, the rhabdomeres in *tadr;ninaE* double mutant flies had the same size and shape as in the *ninaE* single mutant at 7 d old (Fig. 5*B*). The average sizes of peripheral rhabdomere in both flies are significantly larger than in the *tadr* single mutant. Moreover, the double mutant flies did not show any significant rhabdomeral loss in optical neutralization assays at least within 13 d after eclosion (Fig. 5*D*). Thus, decrease of Rh1 level suppresses the *tadr* mutation-caused rhabdomeral degeneration. Altogether, these observations suggest that the *tadr* degeneration is mediated by the activity of rhodopsin.

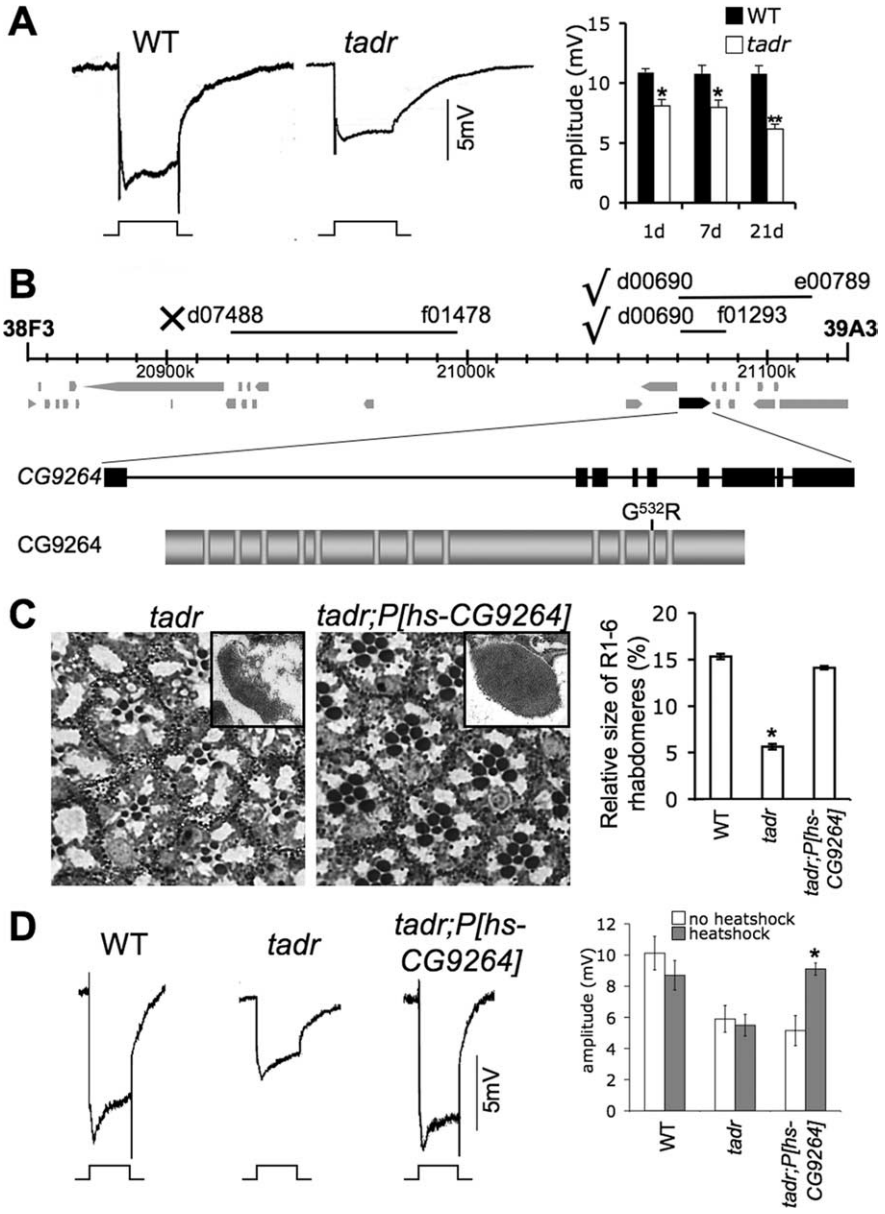

Figure 3. Identification of the *tadr* mutant gene. ***A***, ERG recordings revealed a small light response phenotype in the *tadr* mutant. Sample traces of ERG response in 7-d-old flies are shown on the left. The event markers underneath represent 5 s orange light pulses. The right panel shows the amplitudes of ERG response at different ages. ***B***, The *tadr* mutation was mapped to the gene *CG9264*. Three chromosomal regions (top) between the shown piggyBac insertion sites were deleted separately using a flipase. Deletion of the right two regions, not the left one, uncovered the *tadr* ERG phenotype in complementation tests. A missense mutation was identified in the gene *CG9264*, which encodes a 12-transmembrane-domain protein (bottom). ***C***, After being expressed through a heat-shock promoter, a wild-type *CG9264* cDNA prevented rhabdomeral degeneration in 7-d-old *tadr;p*[*hs-CG9264*] transgenic flies. TB staining and EM (inset) results were shown on the left. The relative sizes of peripheral rhabdomeres were calculated base on the TB staining and shown in the right panel. ***D***, The *tadr* ERG phenotype was also rescued by overexpression of the *CG9264* cDNA. The sample ERG traces on the left are from heat-shocked flies. All recorded flies had a *cn* background. The asterisk (*) indicates significant differences from the wild-type controls (***A***, ***C***) or from the *tadr* mutant (***D***).

Arr2 is not required for the rhabdomeral degeneration in *tadr* fly

Activated Rh1 rhodopsin forms a stable complex with a visual arrestin Arr2 to trigger apoptotic photoreceptor degeneration in severe mutant flies (Alloway et al., 2000; Kiselev et al., 2000). However, such an apoptotic mechanism may not underlie the rhabdomeral degeneration in the *tadr* mutant. First, the blue light-generated Rh1-Arr2 complex in the *tadr* fly dissembled on exposure to orange light as in wild type (Fig. 6*A*), indicating the lack of a stable complex in the mutant. Second, in both 1-d-old (Fig. 6*B*) and 10-d-old (supplemental Fig. 4, available at www.jneurosci.org as supplemental material) *tadr* flies, we failed to detect massive endocytosis of Rh1, which is required for the Rh1-Arr2 complex to trigger retinal degeneration (Orem and Dolph, 2002).

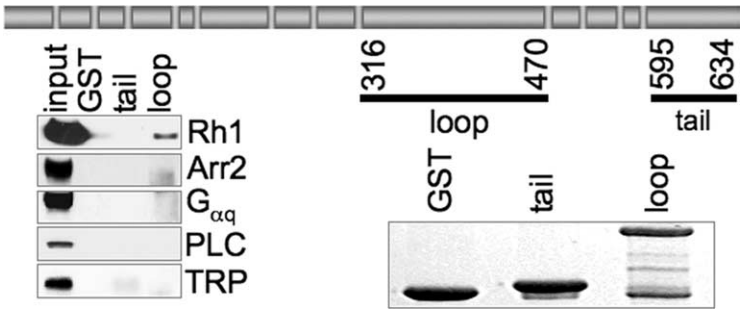

Figure 4. Rh1 interacts with TADR protein *in vitro*. A GST-fused intracellular loop IV of TADR specifically pulled down Rh1 rhodopsin from fly head extracts in a glutathione Sepharose binding assay. The tail of TADR did not bind to any visual protein. Lane one was loaded with 1/15 of extract input. The Coomassie-staining gel on the lower right shows the protein levels of GST and the GST-fusion proteins in the reaction mixtures.

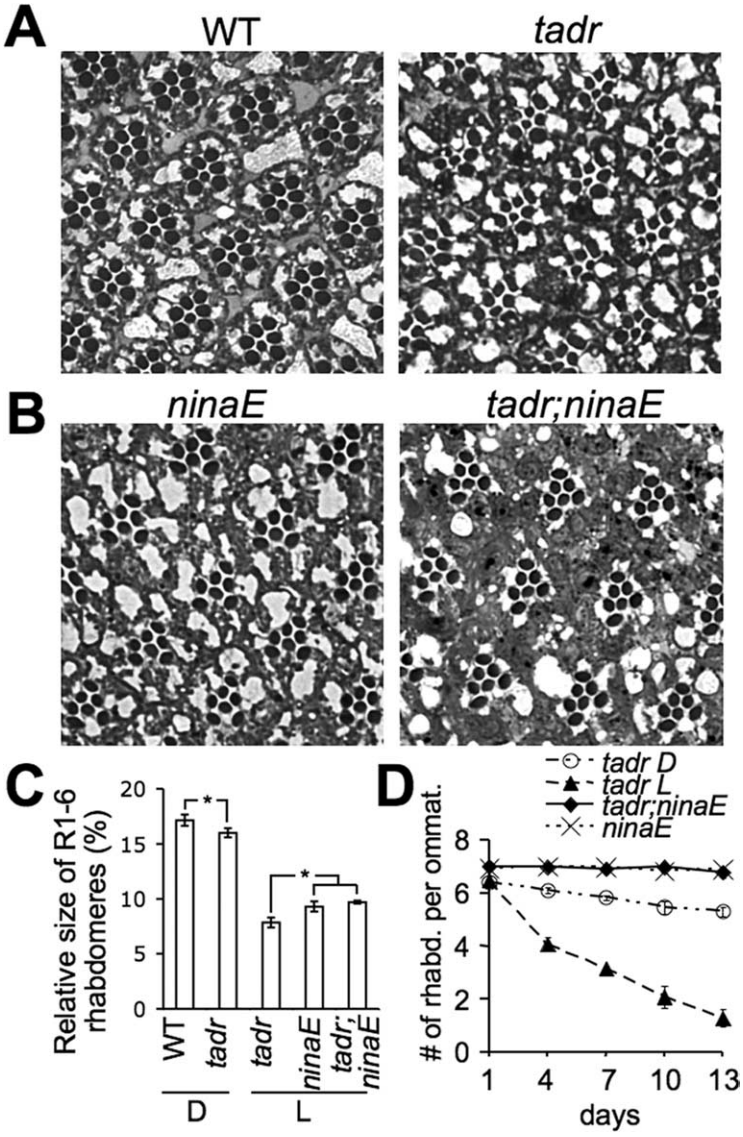

Figure 5. The *tadr* rhabdomeral degeneration depends on the rhodopsin activity. ***A***, Dark-reared *tadr* flies only showed a subtle rhabdomeral degeneration. Both the wild type and the mutant were dark-reared and 7 d of age. ***B***, Decreasing Rh1 rhodopsin level by a hypomorphic *ninaE* mutation prevented *tadr* mutation-caused rhabdomeral degeneration. ***C***, Relative sizes of peripheral rhabdomeres in different flies and light conditions. D, Dark reared; L, raised in a normal light/dark cycle. The asterisk (*) indicates significant differences between the paired samples. ***D***, Optical neutralization assays showed that both light deprivation and reduction of Rh1 level prevent the severe rhabdomere loss caused by *tadr* mutation.

To further investigate whether Arr2 is involved in the *tadr* rhabdomeral degeneration at all, we examined the effect of *tadr* mutation in an *arr2* null background. In regular, cyclic illuminating conditions, although *arr2* single mutant flies had reduced size of rhabdomere and large intracellular vacuoles in the photoreceptor (Fig. 6*C*,*D*) resulting from necrotic degeneration (Alloway et al., 2000), the rhabdomere shape was in general normal at 7 d old according to TB staining assays (Fig. 6*C*). In contrast, in *tadr;arr2* double mutant flies of the same age, many rhabdomeres either had irregular shape or completely disappeared (Fig. 6*C*). The average size of peripheral rhabdomeres was much smaller than that of both the *arr2* and the *tadr* single mutant (Fig. 6*D*). Moreover, in optical neutralization assays, the number of visible rhabdomeres in the double mutant decreased at a speed similar to that in the *tadr* single mutant, which is much faster than in the *arr2* mutant. (Fig. 6*E*). Thus, removal of Arr2 does not suppress the *tadr* rhabdomeral degeneration, suggesting that Rh1 mediates degeneration independent of Arr2 in the *tadr* mutant.

The tadr rhabdomeral degeneration is not caused by overstimulation of the phototransduction cascade

In the phototransduction cascade, rhodopsin stimulates Ca^{2+}-permeable TRP channels by the mediation of G_q and PLC. Because excessive Ca^{2+} influx through TRP may cause necrotic damage to the photoreceptor (Raghu et al., 2000), it is possible that the *tadr* rhabdomeral degeneration is caused by prolonged or excessive stimulation of TRP channels by rhodopsin. However, electrophysiological recordings of peripheral photoreceptors isolated from newly enclosed flies do not support this hypothesis. First, in the dark, *tadr* mutant photoreceptors had background inward currents as small as wild type (Fig. 7*A*) and a reversal potential similar to wild type (-54.4 ± 5.0 vs -51.8 ± 4.5 mV). These observations indicate a lack of TRP channel activity in the dark and thus cannot explain the moderate, light-independent degeneration. Second, consistent with the ERG phenotype, the light response of *tadr* photoreceptor had a smaller instead of a larger amplitude compared with wild type (Fig. 7*B*), indicating a lower level of TRP stimulation by light.

A PLC mutation *norpA* prevents rhodopsin from stimulating TRP channels (Bloomquist et al., 1988). To further test whether the *tadr* rhabdomeral degeneration depends on TRP activity or any other

PLC-mediated signaling event, we generated a *norpA;tadr* double mutant. When *norpA* single mutant flies were raised in cyclic illumination conditions to 7 d old, the peripheral rhabdomeres were only slightly different from wild type in shape, and were significantly larger than those in the *tadr* mutant (Fig. 7*C*,*D*). In contrast, the rhabdomeres in the *norpA;tadr* double mutant diminished more severely than in the *tadr* mutant (Fig. 7*C*,*D*). Additionally, the optical neutralization assay showed that the speed of rhabdomere loss in the double mutant was similar to that of *tadr* mutant fly, and was initially much faster than seen in the *norpA* single mutant (Fig. 7*E*). These observations suggest that rhodopsin mediates rhabdomeral degeneration independent of PLC and TRP activities in the *tadr* mutant.

Abnormal G_q signaling may trigger rhabdomeral degeneration in the *tadr* mutant

The small amplitude of light response suggests that a phototransduction step is impaired in *tadr* photoreceptors. If the abnormality at this step also leads to the rhabdomeral degeneration, it should occur at the level of rhodopsin or G_q, because the degeneration is independent of the downstream molecules PLC and TRP. To help understand the degeneration mechanism, we examined whether the step of G_q activation is impaired in the *tadr* photoreceptor. We prepared membrane samples from fly heads and measured blue light-stimulated GTPγS binding of membrane. Surprisingly, the level of stimulated GTPγS binding in the *tadr* membrane sample was even higher than in wild type (Fig. 8*A*).

To find out why light stimulated more GTPγS binding to the mutant membrane, we examined the effect of light stimulation on the level of membrane-associated $G_{\alpha q}$ molecule in the *tadr* mutant. As an important light adaptation mechanism of the fly photoreceptor, a large fraction of active $G_{\alpha q}$ molecules dissociate from membrane through depalmitoylation and diffuse out of the rhabdomere in bright light conditions (Kosloff et al., 2003; Cronin et al., 2004; Frechter et al., 2007). We found that, compared with wild type, a much lower amount of $G_{\alpha q}$ protein in the *tadr* mutant dissociated from membrane during light stimulation (Fig. 8*B*), which could partially explain the higher level of GTPγS binding to the mutant membrane. Because the higher density of active $G_{\alpha q}$ protein on the mutant membrane leads to a weaker but not a stronger activity of TRP channel, the above observations might suggest that

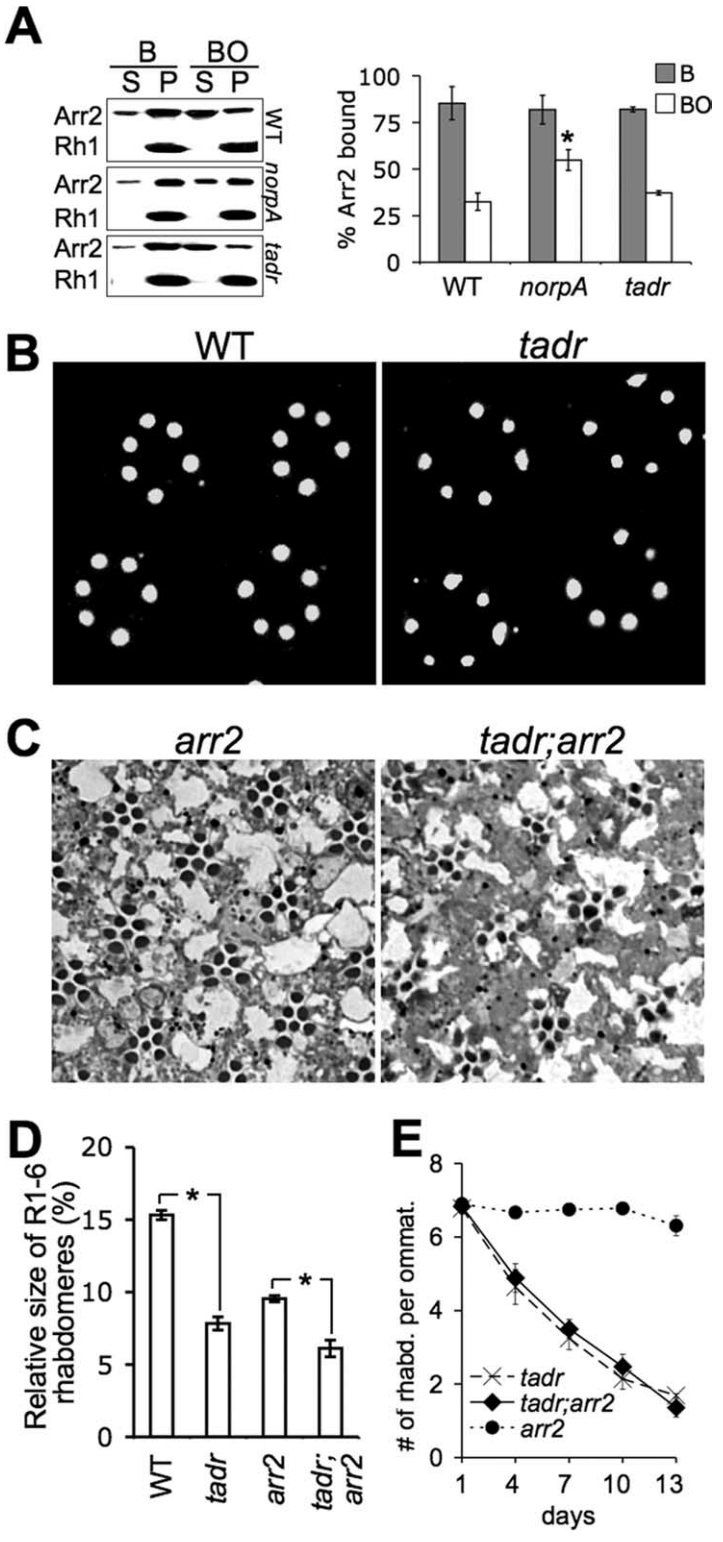

Figure 6. The Rh1-Arr2 complex is not involved in the *tadr* rhabdomeral degeneration. ***A***, Blue light-triggered binding between Arr2 and Rh1 was reversed by subsequent exposure to orange light in both wild-type and *tadr* mutant flies, but not in *norpA* mutant flies. All fly heads were collected in the dark. One group was exposed to blue light (B) and the other group was exposed to blue and then orange light (BO), before the homogenization. Supernatant (S) and membrane pellet (P) fractions were subjected to Western blot. The percentages of Arr2 bound to Rh1-containing membranes were quantified using NIH ImageJ software. The averaged data of four independent experiments is shown in the right panel. ***B***, Immunostainings showed that the majority of Rh1 protein in 1-d-old *tadr* flies was localized in the peripheral rhabdomeres (the 6 large spots of each ommatidium). The number of small Rh1 endocytic particles in the cell-body areas was as low as in wild type. ***C***, Micrographs of TB staining showing more severe rhabdomeral degeneration in the *tadr;arr2* fly than in the *arr2* single mutant at 7 d old. ***D***, Relative sizes of peripheral rhabdomeres measured in TB staining assays. ***E***, In optical neutralization assays, loss of visible rhabdomeres in the *tadr;arr2* fly was as rapid as in the *tadr* single mutant. The asterisk (*) indicates significant differences from the wild-type controls (***A***) or between the paired samples (***D***).

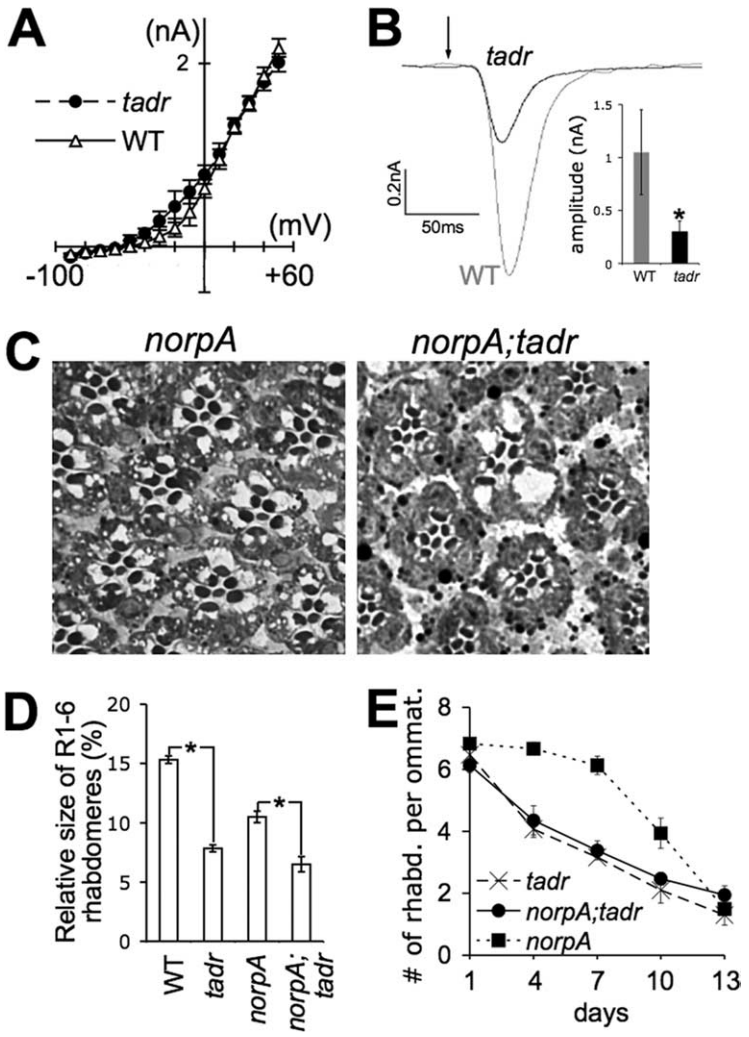

Figure 7. The *tadr* rhabdomeral degeneration is not caused by overstimulation of the phototransduction cascade. ***A***, In whole-cell current recordings, *tadr* mutant photoreceptors displayed normal background currents at different voltage levels in the dark. Data from three experiments were averaged. ***B***, A 10 ms light flash stimulated a smaller inward current in *tadr* mutant photoreceptors compared with that in wild type. The cells were clamped at −70 mV in whole-cell configuration. The averaged amplitudes are shown on the lower right. ***C***, According to TB staining, loss of the PLC NorpA did not inhibit rhabdomeral degeneration in 7-d-old *norpA:tadr* flies. ***D***, Relative sizes of peripheral rhabdomeres measured in TB staining assays. ***E***, In optical neutralization assays, the initial speed of rhabdomere loss in the *norpA;tadr* fly was the same as that in *tadr* mutant, and was much faster than in the *norpA* single mutant. The asterisk (*) indicates significant differences from the wild-type controls (***B***) or between the paired samples (***D***).

Discussion

We have identified TADR as a potential Rh1-interacting protein that is essential for the protection of fly photoreceptor from degeneration. In the *tadr* mutant fly, rhabdomeres undergo rhodopsin activity-dependent degeneration, which is mediated by G_q through a pathway different from the phototransduction cascade.

TADR is homologous to human membrane proteins SLC7A4 and SLC7A1, which belong to the family of cation amino acid transporters (Verrey et al., 2004). Nonetheless, not all members of this family function as amino acid transporters. For example, SLC7A4 could not transport any amino acid into the cell after being expressed on the membrane of *Xenopus* oocytes (Wolf et al., 2002). These TADR/SLC7A proteins may have functions in addition to amino acid transport. In the *tadr* mutant, the rhabdomeral degeneration is not likely the result of a shortage of amino acid supply in the photoreceptor. First, we did not detect a general problem in the synthesis of visual signaling proteins in Western blot assays (L. Ni and H.-S. Li, unpublished observations). Second, if the rhabdomeral degeneration were caused by the lack of particular amino acids, it should not be suppressed specifically by reducing the level of rhodopsin and G_q. It is more likely that loss of a different TADR function has caused the degeneration.

Our biochemical data indicates that TADR may interact, either directly or indirectly, with the Rh1 rhodopsin through the intracellular loop IV. In the *tadr* mutant, the $G^{532}R$ mutation disrupts the 11^{th} transmembrane domain, resulting in an extended loop V that could block the Rh1-interacting site in the neighboring loop IV. Because the TADR-Rh1 interaction may regulate Rh1-triggered signaling, loss of this interaction could lead to the Rh1-dependent rhabdomeral degeneration seen in the *tadr* mutant.

$G_{\alpha q}$ molecules in the *tadr* mutant are suffering from a problem, such as a low mobility on the membrane, which keeps them in the vicinities of rhodopsin molecules and restrains them both from depalmitoylation and from stimulating PLC.

Considering that prolonged possessing active $G_{\alpha q}$ molecules in a small membrane region could be harmful to the membrane structure, we examined the effect of decreasing $G_{\alpha q}$ level on the *tadr* rhabdomeral degeneration. A hypomorphic mutation ($G_{\alpha q}{}^{1}$) of the $G_{\alpha q}$ gene reduces $G_{\alpha q}$ to an undetectable level (Scott et al., 1995) without causing an obvious morphological change in rhabdomeres of 7-d-old flies (Fig. 8*C*). According to TB staining assays, the *tadr* mutation failed to induce significant rhabdomeral degeneration in this $G_{\alpha q}$ mutant background at the age of 7 d (Fig. 8*C*,*D*). Moreover, in optic neutralization assays, the *tadr*-dependent rhabdomere loss was greatly inhibited in the *tadr;*$G_{\alpha q}$ double mutant (Fig. 8*E*). Thus, an abnormal $G_{\alpha q}$ signaling event may mediate the rhodopsin-dependent rhabdomeral degeneration in the *tadr* mutant.

Abnormal signaling activities of rhodopsin may cause retinal degeneration through both apoptotic and necrotic pathways in the fly eye. In severe mutant flies including *rdgC*, *norpA* and *arr1*, activated Rh1 rhodopsin forms a stable complex with Arr2 to trigger apoptotic photoreceptor degeneration (Alloway et al., 2000; Kiselev et al., 2000). This apoptotic pathway does not underlie the rhabdomeral degeneration in the *tadr* mutant, because the degeneration depends on the G_q protein instead of Arr2. By the mediation of the PLC NorpA, G_q could stimulate excessive Ca^{2+} influx through TRP channels, which leads to necrotic degeneration of photoreceptor (Dolph et al., 1993). Nonetheless, this Ca^{2+}-dependent necrosis is not responsible for the *tadr* rhabdomeral degeneration: first, the degeneration is independent of NorpA; second, the TRP activity is even lower in the *tadr* mutant. A different mechanism is likely underlying this G_q-dependent degeneration.

G_q also mediates PLC/TRP-independent photoreceptor degeneration in a dominant *ninaE* mutant (Iakhine et al., 2004), although the mechanism remains unknown. After light stimulation, many more $G_{\alpha q}$ molecules in the *tadr* mutant are retained on the membrane compared with wild type. The lower level of phototransduction might suggest that most of the active $G_{\alpha q}$ molecules on membrane have failed to stimulate PLC in the mutant. We hypothesize that those extra $G_{\alpha q}$ molecules may instead have recruited an alternative effector to the membrane, which leads to the rhabdomeral degeneration. In addition to PLC, the G_q family proteins stimulate several other enzymes including an ADP-ribosylation factor ARF6 (Giguère et al., 2006) and a Rho guanine nucleotide exchange factor p63RhoGEF (Lutz et al., 2005, 2007; Rojas et al., 2007). Both ARF6 and Rho are monomeric GTPases that may change morphology of membrane structures by modulating the underneath actin cytoskeleton (D'Souza-Schorey and Chavrier, 2006; Linseman and Loucks, 2008). More importantly, Rho GTPases have been found to mediate both apoptotic and necrotic pathways of neuronal death (Linseman and Loucks, 2008). In the future it would be interesting to investigate whether any fly ARF or Rho GTPase signals downstream of G_q in the *tadr* rhabdomeral degeneration.

Another question remaining to be addressed in the future is exactly how the TADR protein helps to prevent the G_q signaling abnormalities observed in the mutant. We speculate that a potential association of TADR to rhodopsin could somehow promote the activated $G_{\alpha q}$ molecule to move away from the vicinity of rhodopsin, a step important for $G_{\alpha q}$ to dissociate from the membrane and to stimulate membrane-bound PLC molecules. This hypothesis explains why light stimulates a higher level of GTPγS binding to membrane but still causes a lower degree of photoreceptor depolarization in the *tadr* mutant.

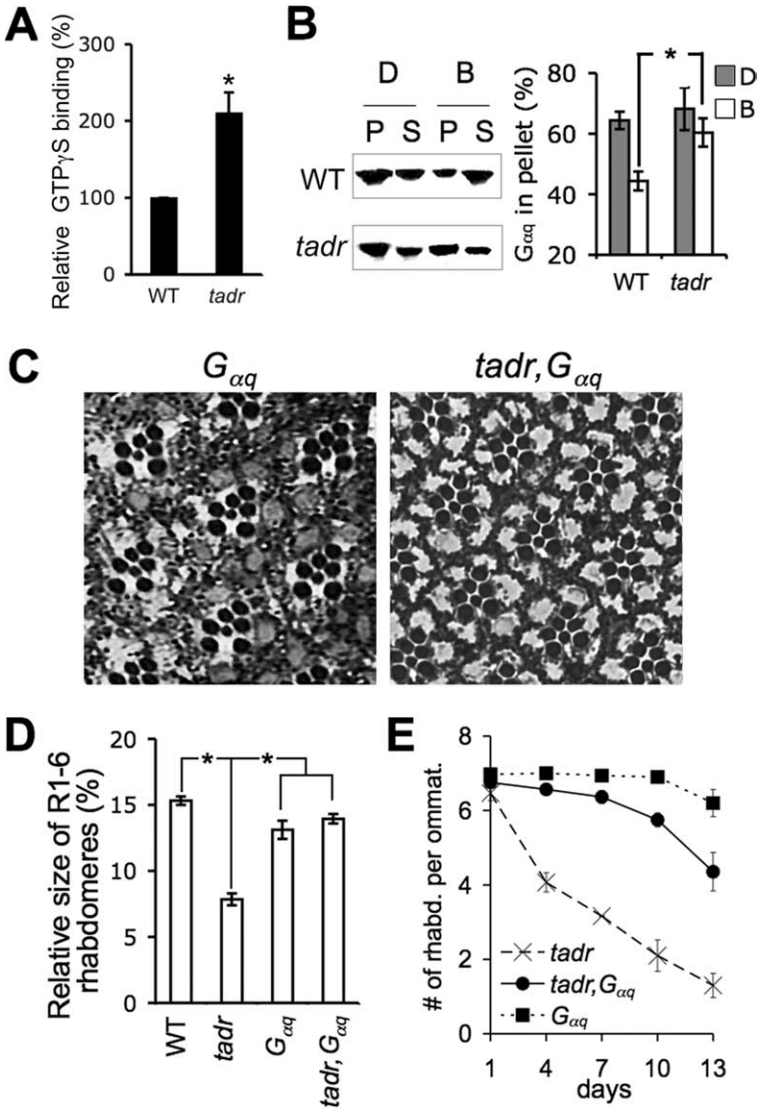

Figure 8. An abnormal G_q signaling mediates the *tadr* rhabdomeral degeneration. ***A***, The light-stimulated GTPγS binding (see Materials and Methods for the measurement) in the *tadr* mutant membrane sample was much higher than in wild type. Shown are the relative levels of GTPγS binding with the wild type set as 100%. Data from three experiments were averaged. ***B***, Blue-light treatment did not efficiently dissociate $G_{\alpha q}$ from membrane in the *tadr* mutant. Heads were collected from dark-reared flies either directly (D) or after 1 h exposure to blue light (B). After homogenization, supernatant (S) and membrane pellet (P) fractions were subjected to Western blot. The percentages of $G_{\alpha q}$ in the membrane pellet were quantified using NIH ImageJ software. The averaged data of four independent experiments is shown in the right panel. ***C***, Reduction of $G_{\alpha q}$ level suppressed rhabdomeral degeneration in a *tadr*,$G_{\alpha q}$ double mutant at 7 d old. The $G_{\alpha q}$ allele is $G_{\alpha q}{}^{1}$. ***D***, Relative sizes of peripheral rhabdomeres measured in TB staining assays. ***E***, In optical neutralization assays, the number of visible rhabdomeres in *tadr*,$G_{\alpha q}$ flies decreased much more slowly than in the *tadr* single mutant. The asterisk (*) indicates significant differences from the wild-type controls (***A***) or between the paired samples (***D***).

The mammalian visual G-protein transducin also mediates degeneration of the rod photoreceptor cell. In mouse mutants that have prolonged rhodopsin activities, rod photoreceptors undergo transducin-dependent apoptosis on exposure to low-intensity light (Hao et al., 2002). Thus, visual G-proteins may play a pivotal role in the degeneration of photoreceptor.

We propose that some mammalian TADR-like proteins could control the activities of rhodopsin/transducin and help to prevent degeneration of rod and cone photoreceptors. Although it has not been reported that a SLC7A protein exists in the mammalian eye, several transporter proteins that contain twelve transmembrane domains like TADR are highly expressed in the mouse retina (Blackshaw et al., 2001). One such protein, a taurine transporter, has been demonstrated to be essential for retinal protection using a knock-out mouse (Heller-Stilb et al., 2002; Rascher et al., 2004). It would be interesting to examine whether any of these mammalian retinal transporter proteins interact with rhodopsin and/or regulate the signaling of transducin. To identify additional affected genes in human RP, those encoding multiple-transmembrane-domain proteins in the super family of "amino acid transporter" should be evaluated as candidates of a high priority.

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