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CORRECTION

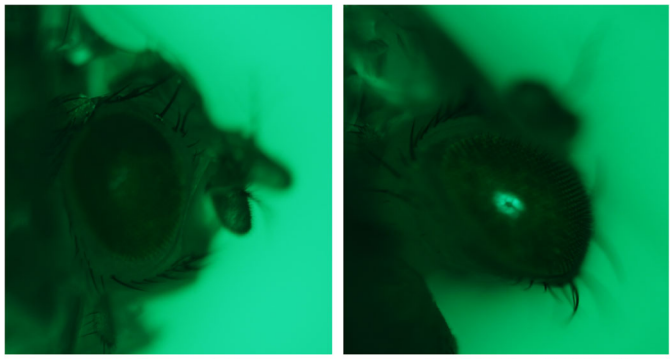
Correction: Functional INAD complexes are required to mediate degeneration in photoreceptors of the *Drosophila rdgA* mutant

Plamen Georgiev, Isaac Garcia-Murillas, Danny Ulahannan, Roger C. Hardie and Padinjat Raghu

There were errors in *J. Cell Sci.* (2005) **118**, 1373-1384 (doi:10.1242/jcs.01712).

Several image assembly errors were found in Figs 1, 3, 4, 6, 7, 9 and 10.

Specifically, the Fig. 7A *rdgA^1;su(100);+* image was erroneously duplicated in the following panels: Fig. 1 *w,rdgA^1, P[rh1-Rh1EGFP];trp^{343}*; Fig. 3A *rdgA^1;su(100)*; Fig. 7A *rdgA^1;su(100)/InaD^1*. The correct images have been retrieved and the corrected panels are shown below.



w,rdgA^1,P[rh1-Rh1EGFP] *w,rdgA^1,P[rh1-Rh1EGFP];trp^{343}*

Fig. 1 (corrected). Assessment of retinal degeneration in live flies using the FDPP. Images of *w,rdgA1, P[rh1-Rh1EGFP]* and *w,rdgA^1, P[rh1-Rh1EGFP];trp^{343}* viewed on a stereomicroscope and recorded digitally. Rescue of retinal degeneration by *trp343* results in a crisp pseudopupil in which the rhabdomeres of photoreceptors R1-R6 can be distinctly seen and counted. Images were viewed on an Olympus MVX10 stereomicroscope and captured using an Olympus DP71 digital camera.

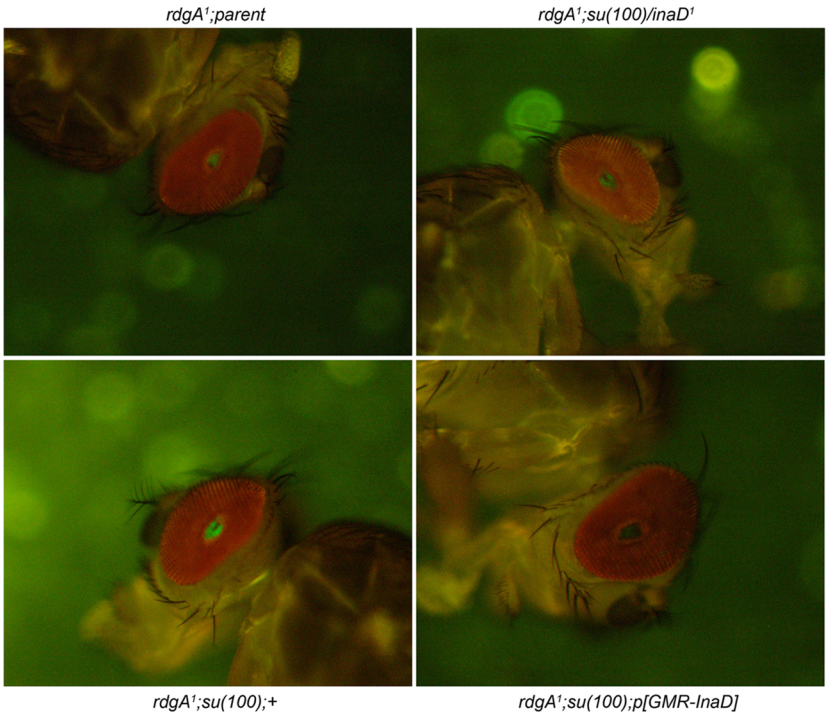


Fig. 7A (corrected panel). (A) The rescue of degeneration (assayed by FDPP) seen in *rdgA^1;su(100)/InaD^1* and *rdgA^1;su(100)* mutants can be reversed by introducing the transgene *P[GMR-InaD]* in *rdgA^1;su(100);P[GMR-InaD]*. The original *rdgA^1;parent* that shows degeneration is shown for comparison.

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In addition, there was duplication of electron microscopy images across panels in Figs 3B (*rdgA*^{1}*; parent*), 6A (*rdgA*^{1}), 9A (*w,rdgA*^{1}) and 10B (*rdgA*^{1}). The authors state that all of the electron microscopy presented in this paper was carried out as a single set in 2003. Samples were fixed, embedded and sectioned, following which imaging was carried out. While many samples were viewed, only the best images in terms of section quality were captured on film. The whole series of electron microscopy images documents rescue of the *rdgA*^{1} phenotype by various other mutants such as *su(1)*, *su(40)*, *su(100)* and *InaD*^{1}. Therefore, the entire set of genotypes (samples) was imaged as a single set and the *rdgA*^{1} sample/image used for comparison with all other genotypes is the same.

Finally, there was an erroneous duplication of images in Figs. 4C and 6A (*rdgA*^{1}*;InaD*^{1}). The correct images have been retrieved and the corrected panel is shown below.

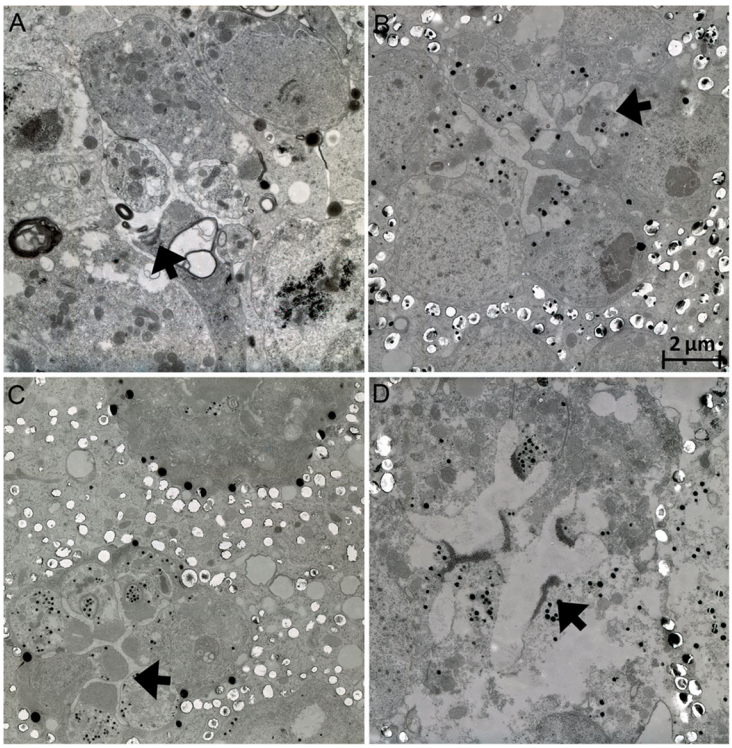

Fig. 4 (corrected). Rescue of rhabdomere degeneration in *w;Trp*^{365}. In comparison to wildtype flies, *w;Trp*^{365} homozygous flies show severe rhabdomere degeneration (A). This can be rescued by *su(40); Trp*^{365} (C) but not in *su(100);Trp*^{365} (B) double mutants. The effect of *su(1)* on *Trp*^{365} was equivalent to that of *su(100)* with minimal, if any, rescue of rhabdomere structure (D). Arrows indicate rhabdomeres.

The authors apologise to the journal and scientific community for any inconvenience caused by these errors, which do not impact the results or conclusions of this paper.

Functional INAD complexes are required to mediate degeneration in photoreceptors of the *Drosophila rdgA* mutant

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Accepted 7 January 2005
Journal of Cell Science 118, 1373-1384 Published by The Company of Biologists 2005
doi:10.1242/jcs.01712

Summary

The TRP family of ion channels mediates a wide range of calcium-influx phenomena in eukaryotic cells. Many members of this family are activated downstream of phosphoinositide hydrolysis but the subsequent steps that lead to TRP channel activation in vivo remain unclear. Recently, the lipid products of phosphoinositide hydrolysis (such as diacylglycerol and its metabolites) have been implicated in activating TRP channels in both *Drosophila* and mammals. In *Drosophila* photoreceptors, lack of diacylglycerol kinase (DGK) activity (encoded by *rdgA*) leads to both constitutive TRP-channel activity and retinal degeneration. In this study, using a novel forward-genetic screen, we identified InaD, a multivalent PDZ domain protein as a suppresser of retinal degeneration in *rdgA* mutants. We show that *InaD* suppresses *rdgA* and that the rescue is correlated with reduced levels of phospholipase Cβ (PLCβ), a key enzyme for TRP channel activation. Furthermore, we show that light, Gq and PLCβ all modulate retinal degeneration in *rdgA*. The results demonstrate a previously unknown requirement for a balance of PLCβ and DGK activity for retinal degeneration in *rdgA*. They also suggest a key role for the lipid products of phosphoinositide hydrolysis in the activation of TRP channels in vivo.

Key words: Phosphoinositides, Diacylglycerol kinase, TRP channels, Screen

Introduction

Transmembrane signalling cascades initiated by G-protein-coupled receptors are a widely used mechanism for signalling the detection of many sensory modalities. These cascades end with the activation of plasma-membrane ion channels whose activity alters membrane potential and initiates synaptic transmission of a signal to the central nervous system. Several different families of ion channels have been implicated in this process. Historically, the oldest and best characterized are cyclic-nucleotide-gated channels, whose role in vertebrate visual and olfactory transduction is well established (Matulef and Zagotta, 2003). More recently, members of the TRP family of ion channels have been implicated in the transduction of several sensory modalities in both vertebrate and invertebrate systems. These include light (*Drosophila* TRPC), pheromones (rodent TRPC2), taste (rodent TRPM), physical stimuli and temperature (*Drosophila* and mammal TRPV, TRPA and TRPN) (Montell et al., 2002). Currently, a crucial factor limiting our understanding of how TRP channels encode sensory modalities is the lack of information about how these channels are activated. In several cases, only a few transduction components have been identified and the inability to perform in vivo analysis of channel activation has been a major obstacle in revealing how TRP channels are activated.

The *Drosophila* phototransduction cascade is historically the oldest and to date the best understood model for the analysis of a TRP channel involved in sensory transduction (Hardie and Raghu, 2001). In the fly eye, rhodopsin, a seven-transmembrane-span G-protein-coupled receptor, activates phospholipase Cβ (PLCβ) (Bloomquist et al., 1988) via Gq (Scott et al., 1995). This initiates a biochemical cascade that ends with the opening of two classes of calcium- and cation-selective TRPC channels, TRP and TRPL (Niemeyer et al., 1996). Several key elements of the transduction cascade have been identified including Gq, PLCβ and protein-kinase C. Several of these components, along with the TRP channel, are clustered into a macromolecular signalling complex by the multivalent PDZ-domain protein INAD (Tsunoda et al., 1998). The INAD complex is thought to increase the speed and specificity of the light response (Montell, 1998; Tsunoda et al., 1998). However, despite this wealth of detail about the components of the transduction cascade, the mechanism of activation of TRP and TRPL remains poorly understood, and is one of the outstanding problems in both sensory neurobiology and intracellular calcium signalling.

Although the essential role of PLCβ in the activation of TRP and TRPL is well established (Bloomquist et al., 1988), the biochemical events initiated by this enzyme that lead to channel activation remain unclear. Inositol-1,4,5-trisphosphate (IP_3), the best-understood second messenger generated from phosphatidylinositol-4,5-bisphosphate [$PI(4,5)P_2$] hydrolysis by PLCβ (Berridge, 1997) was originally postulated to be the second messenger that leads to TRP and TRPL activation (Hardie and Minke, 1993). However, several recent lines of

evidence strongly indicate that IP_3-induced calcium (Ca^{2+}) release, or indeed a physical interaction between the IP_3 receptor (IP_3R) and the light-activated channels, is unlikely to underlie the mechanism of TRP and TRPL activation (Raghu et al., 2000a). More recently, lipid second messengers derived from $PI(4,5)P_2$ have been implicated in the activation of TRP and TRPL as well as their vertebrate homologues (Hardie, 2003). Polyunsaturated fatty acids, potential metabolites of diacylglycerol (DAG), the primary lipid generated by $PI(4,5)P_2$ hydrolysis, have been shown to activate TRP and TRPL in situ, as well as in inside-out patches of TRPL channels expressed in S2 cells (Chyb et al., 1999). In addition, both DAG and $PI(4,5)P_2$ have been shown to modulate TRPL channel activity in cell culture models (Estacion et al., 2001). Analysis of TRPC2 activation in the rodent vomeronasal organs shows considerable parallels to our current understanding of the mechanism of *Drosophila* TRP and TRPL activation (Lucas et al., 2003). However, despite these findings, the physiological relevance of $PI(4,5)P_2$-derived lipids as activators of *Drosophila* TRP channels in vivo remains to be established and the precise identity of the phospholipid species that is involved is unknown.

Recently, genetic evidence of a role for lipid messengers in the activation of TRPC channels in vivo has been obtained in *Drosophila* photoreceptors from an analysis of the *retinal degeneration A* (*rdgA*) mutant. The *rdgA* mutant was first isolated because it failed to respond to light in a behavioural assay (Hotta and Benzer, 1970). Analysis of retinal ultrastructure revealed that all alleles show varying degrees of photoreceptor degeneration (Harris and Stark, 1977). Biochemical analysis showed impaired diacylglycerol kinase (DGK) activity (Inoue et al., 1989) and reduced levels of light induced phosphatidic acid (PA) formation (Yoshioka et al., 1983) in head extracts of *rdgA* mutants. The gene that is defective in *rdgA* mutants has been cloned and found to encode an eye-enriched isoform of DGK (Masai et al., 1993), the principal enzyme that inactivates DAG by phosphorylation to PA. However, most significantly, under voltage-clamp conditions, several alleles including $rdgA^1$, $rdgA^3$, $rdgA^6$ and $rdgA^{KS60}$ (Raghu et al., 2000b) (Hardie, personal communication) all show a small constitutively active inward current, which, on the basis of its biophysical characteristics, genetics and pharmacology, has been shown to be composed largely of TRP channels. The retinal degeneration phenotype of *rdgA* can be rescued by genetically removing TRP channels (i.e. the double mutant *rdgA;trp*), whose photoreceptors now lack their principal plasma-membrane calcium-influx channels. These results suggested a model in which excessive calcium influx through constitutively active TRP channels results in retinal degeneration in *rdgA* (Raghu et al., 2000b). The light response of *rdgA;trp* photoreceptors shows defects in deactivation suggesting that DGK might play a role in terminating the light response (Raghu et al., 2000b) and recent evidence suggests that DGK plays a role in regulating signal amplification during the response to light (Hardie et al., 2002). Despite these recent observations that suggest a direct role for *rdgA* in phototransduction, previous studies have suggested a distinct mechanism underlying the retinal degeneration phenotype of *rdgA*. First, unlike most other phototransduction mutants, the retinal degeneration of *rdgA* is reported to be light independent (Harris and Stark, 1977). Second, *norpA* mutants, which lack the PLC activity essential for TRP channel activation, were reported not to suppress the retinal degeneration of *rdgA* (Masai et al., 1993). Third, several studies have suggested that a failure of rhabdomere biogenesis and protein trafficking underlies the *rdgA* phenotype (Masai et al., 1997; Suzuki et al., 1990).

To address these apparently conflicting results and to understand the mechanism of degeneration in *rdgA*, we are undertaking a genome-wide forward-genetic screen for mutants that suppress or enhance the retinal-degeneration phenotype of *rdgA*. Our goal is to identify molecules whose function might help us to understand the basis of the constitutive TRP-channel activity that is associated with the *rdgA* phenotype. Here, we describe the isolation and characterization of two mutants identified in our screen. We then describe experiments that address the requirement for the light response in the degeneration phenotype of *rdgA*.

Materials and Methods

Fly culture and stocks

Flies (*Drosophila melanogaster*) were reared on standard cornmeal, dextrose, yeast medium at 25°C and 50% relative humidity in a constant-temperature laboratory incubator. There was no internal illumination within the incubator and flies were subject to brief pulses of light only when the incubator doors were opened. When required, flies were grown in a cooled incubator with constant illumination from a white light source. For experiments involving dark rearing, flies were grown in vials kept in tightly closed black photographic bags inside a cooled incubator. They were separated and handled in red light illumination before analysis of retinal degeneration. For experiments involving trp^{CM} flies, all relevant genotypes (including controls) were grown throughout development at 29°C, which we found to be the restrictive temperature required to obtain the *trp* phenotype.

The wild-type strain was Oregon-R. Other stocks included: *w,*$rdgA^1$ (E. Suzuki, Tokyo, Japan); $norpA^{P24}$, $norpA^{P12}$, $norpA^{P16}$ (W. L. Pak, Purdue University, IN); dGq^1, $InaD^1$ and $InaD^2$ (C. Zuker via B. Minke, University of California, San Diego, CA and Hebrew University, Jerusalem, Israel, respectively); $rdgA^3$ (S. Benzer, Caltech, USA); *w, P[rh1-Rh1EGFP]* (C. Desplan, Rockefeller University, NY); *P[GMR-InaD]* (S. Tsunoda, Boston University, MA); trp^{CM},$P[TRP^{\Delta 1272}]$ (C. Montell, Johns Hopkins University, Baltimore, MD); $w^{[1118]}$;P{ry[+t7.2]=neoFRT}42D P{w[+mC]=piM}45F (called the parent chromosome or 2120); and $y^{[1]}$ $w^{[*]}$;P{ry[+t7.2]=neoFRT}42D P{y[+t7.7] ry[+t7.2]=Car20y}44B, P{w[+mC]=GMR-hid}SS2, l(2)CL-R[1]/CyO; P{w[+m*]=GAL4-ey.H}SS5, P{w[+mC]=UAS-FLP1.D}JD2 (called 5251) (Bloomington Stock Center). The P{w[+m*]=GAL4-ey.H}SS5, P{w[+mC]=UAS-FLP1.D}JD2 element in this stock is henceforth referred to as EGUF.

When required, combinations of the above strains were constructed using standard genetic crosses. The *norpA,rdgA* double mutants were generated by meiotic recombination. Recombinants were screened for both mutations by back crossing to the individual mutants and scoring for their phenotypes.

Mutagenesis and screening

Mutagenesis with methanesulfonic-acid ethyl ester (EMS) was carried out using established methods (Lewis, 1968). Briefly, 2-3-day-old males were starved for 16 hours and then allowed to feed on 25 mM EMS in 1% sucrose dispensed onto filter paper in quarter-pint milk bottles for 24 hours at room temperature. Following a 24 hour period of recovery, the males were mated with *w,*$rdgA^1$*,P[rh1-Rh1EGFP];5251* virgin females. Development occurred at 25°C.

Male progeny with whole-eye mosaics were screened for rescue of retinal degeneration as described below.

Fluorescent deep pseudopupil analysis

Pseudopupil analysis was carried out on flies 0-12 hours after eclosion. Flies were immobilized using a stream of carbon dioxide and fluorescent pseudopupil analysis was carried out using an Olympus SZX12 stereomicroscope equipped with a fluorescent light source and green fluorescent protein (GFP) optics. When required, images were recorded using an Olympus DP11 digital camera.

Optical neutralization

Flies were immobilized by cooling on ice. They were decapitated using a sharp razor blade and fixed on a glass slide using a drop of colourless nail varnish. The refractive index of the cornea was neutralized using a drop of immersion oil (n=1.516 at 23°C); images were observed using a 40× oil-immersion objective (Olympus, UPlanApo, 1.00 Iris) with antidromic illumination (Franceschini, 1971). Images were collected on an Olympus BX-41 upright microscope and recorded using an Olympus DP11 digital camera.

Scoring retinal degeneration

In order to obtain a semiquantitative index of retinal degeneration, at least five independent flies were scored for each time point. A total of 50 ommatidia were assessed to generate a degeneration index. Owing to the convexity of the eye, only a proportion of the ommatidia can be in focus at any one plane during optical neutralization. To choose these ommatidia for scoring, we exploited the observation that retinal degeneration in *rdgA* spares R7 photoreceptors; only those ommatidia in which R7 could be clearly visualized were scored.

To quantify degeneration, a score of 1 was assigned to each rhabdomere that appeared to be wild type. Thus, a wild-type ommatidium would have a score of 7; severe alleles such as $rdgA^1$ would score 1 because only the rhabdomere of R7 is visible. Intermediate degenerations would have a score between 1 and 7. Scores were expressed as mean ± s.d.

Electron microscopy

Eyes were prepared for histology by dissecting in ice-cold fixative (2% formaldehyde, 2.5% glutaraldehyde in 0.1 M sodium-cacodylate buffer, pH 7.3). After a 4 hour fixation at 4°C, eyes were buffer washed, postfixed in 1% OsO_4 (1 hour) and stained en bloc in uranyl acetate (1 hour). Eyes were dehydrated in an alcohol series and embedded in Spurr's resin. Ultrathin sections (70 nm) were stained with uranyl acetate and lead citrate, and viewed on a Phillips CM100 transmission electron microscope (TEM).

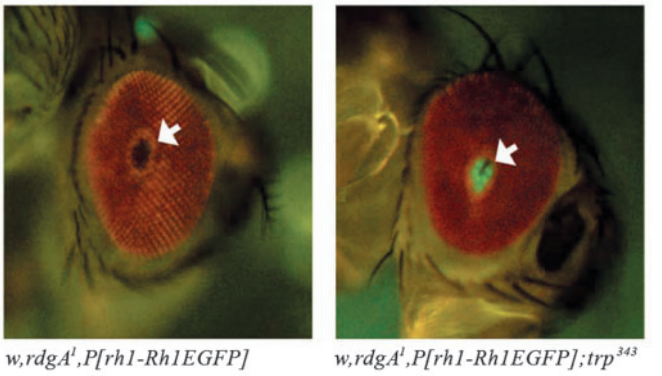

Fig. 1. Assessment of retinal degeneration in live flies using the FDPP. Images of *w,rdgA*^{1}*, P[rh1-Rh1EGFP]* and *w,rdgA*^{1}*, P[rh1-Rh1EGFP];trp*^{343} viewed on a stereomicroscope and recorded digitally. Rescue of retinal degeneration by *trp*^{343} results in a crisp pseudopupil in which the rhabdomeres of photoreceptors R1-R6 can be distinctly seen and counted.

Western-blot analysis

Protein analysis was carried out with flies aged 0-12 hours after eclosion. Heads were prepared by decapitating flies cooled on ice. They were homogenized in 2× SDS-PAGE sample buffer followed by boiling at 100°C for 5 minutes. Samples were separated using SDS-PAGE (Laemmli, 1970) (9% for INAD blots and 6% for NORPA blots) and electroblotted onto supported nitrocellulose membrane (Hybond™-C extra, Amersham) using semi-dry transfer. The uniformity of transfer onto membranes was checked by staining with Ponceau S. Following blocking in 5% non-fat milk (Marvel) blots were incubated for 1 hour at room temperature in appropriate dilutions of primary antibody. For INAD we used an anti-INAD antiserum provided by C. Montell (Johns Hopkins University, USA) at 1:5000. The anti-NORPA antiserum was provided by B. H. Shieh (Vanderbilt University, USA) and used at 1:1000 dilution. Because both of these polyclonal antisera showed multiple bands on western blots from fly heads, the presence and levels of the respective proteins in a sample were assessed by comparison to a sample from a known null allele for the appropriate gene run on the same gel. In the case of INAD, this allele was $InaD^1$ and, in the case of NORPA, this was $norpA^{P24}$. Immunoreactive protein was visualized following incubation in 1:10,000 dilution of donkey anti-rabbit IgG coupled to horseradish peroxidase (Jackson ImmunoResearch) for 1 hour at room temperature and the blots developed with Enhanced Chemiluminescence (ECL, Amersham).

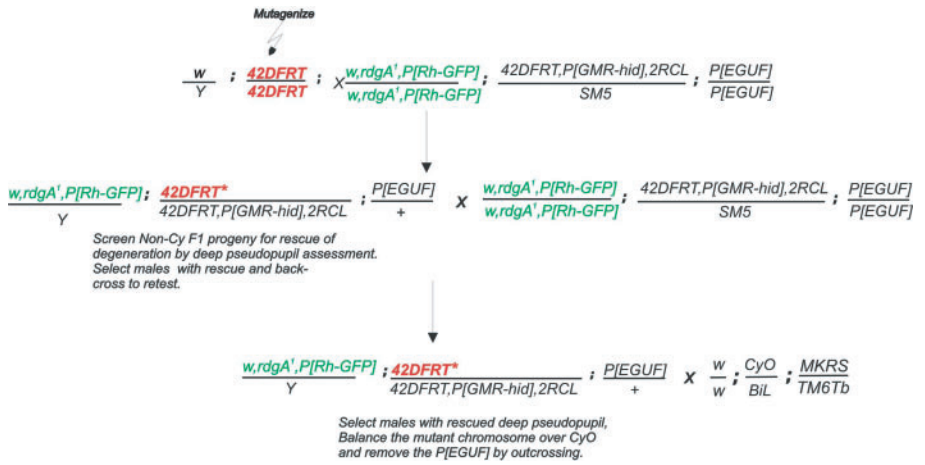

Fig. 2. Genetic scheme showing crosses that were used in the screen to isolate suppressers of *rdgA* using the whole-eye mosaic strategy. The stocks shown were generated from flies obtained.

Cloning and sequencing of *InaD*

Genomic DNA was prepared as previously described (Gloor et al., 1993). The *InaD* gene

was isolated by PCR amplification from genomic DNA using the following primers: INAD4, 5′-AGGAGGAGGAGAATGAGG-3′ and INAD8, 5′-GATCTCAAGCAGCGAGGATG-3′. These primers amplify a 3.3 kb fragment that includes the entire open reading frame for the *InaD* gene. PCR reactions were carried out at an annealing temperature of 55°C and extension time of 3 minutes using the high-fidelity, long-range EXL™ Polymerase (Stratagene). The PCR product was gel purified and cloned into pCR®2.1-TOPO® using the TOPO TA cloning kit (Invitrogen). Recombinant colonies were identified and three independent clones sequenced using a series of overlapping sequencing reactions. Sequencing was carried out using the dideoxy-chain-termination method by automatic DNA sequencing using ABIPRISM BigDye™ Terminator Cycle Sequencing Ready reaction kit (Perkin Elmer Life Sciences).

In order to distinguish between mutations and single-nucleotide polymorphisms, the *InaD* gene from the parent chromosome that was mutagenized was also sequenced. Three criteria were used to identify bona fide mutations: (1) base changes must not be present in all clones sequenced for the parent chromosome; (2) base changes must be present in all clones isolated from a single mutant strain [e.g. *su(1)*]; (3) base changes must be absent from all clones isolated from the other mutant strain [e.g. *su(100)*].

Results

Isolation of three suppressors of *rdgA* using a forward-genetic screen

The rationale for our screen is that TRP channels are constitutively active in *rdgA* mutants and that the genetic removal of these channels rescued both the constitutive activity and retinal degeneration (Raghu et al., 2000b). We reasoned that, if we performed a genetic screen looking for suppressors of *rdgA*, we were likely to discover at least two classes of mutants: (1) those in which the function of additional molecules required for TRP channel activation was affected; and (2) those that affected gene products involved in the degeneration process per se downstream of TRP channel activity. A crucial element of any genetic screen is the ease of phenotype scoring. To facilitate scoring retinal degeneration in live flies, we have modified the traditional method of assessing the deep pseudopupil (Franceschini, 1971), a measure of the intactness of photoreceptor rhabdomeres (the organelle involved in phototransduction). Because the traditional method of pseudopupil assessment is dependent on eye colour, it was unsuitable for use in our screen, in which the presence of several transgenes carrying the mini-white marker complicated the screening process. As an alternative, we used flies in which a fusion protein of rhodopsin1 with enhanced green fluorescent protein (RH1EGFP) had been expressed in photoreceptors R1-R6 under the control of the *rhodopsin1* promoter (Pichaud and Desplan, 2001). When viewed under a stereomicroscope equipped with GFP optics, these flies reveal a fluorescent deep pseudopupil (FDPP) in which the images formed by rhabdomeres R1-R6 can be clearly seen. When placed in the background of a retinal degeneration mutant, this FDPP becomes very indistinct or is lost. This is due to rhabdomere degeneration that destroys the optical system whose function is required for pseudopupil formation. To test whether the FDPP could be used to easily report suppression of degeneration, we generated the strain *w,rdgA*^{1}*,P[rh1-Rh1-EGFP];trp*^{343}. Unlike *w,rdgA*^{1}*, P[rh1-Rh1EGFP]*, these flies show a distinct FDPP in which the images of all six outer rhabdomeres can be clearly seen (Fig. 1).

Although several previous screens have been performed for mutants affected in adult photoreceptor function (Alloway and Dolph, 1999; Dolph et al., 1993; Hotta and Benzer, 1969; Pak

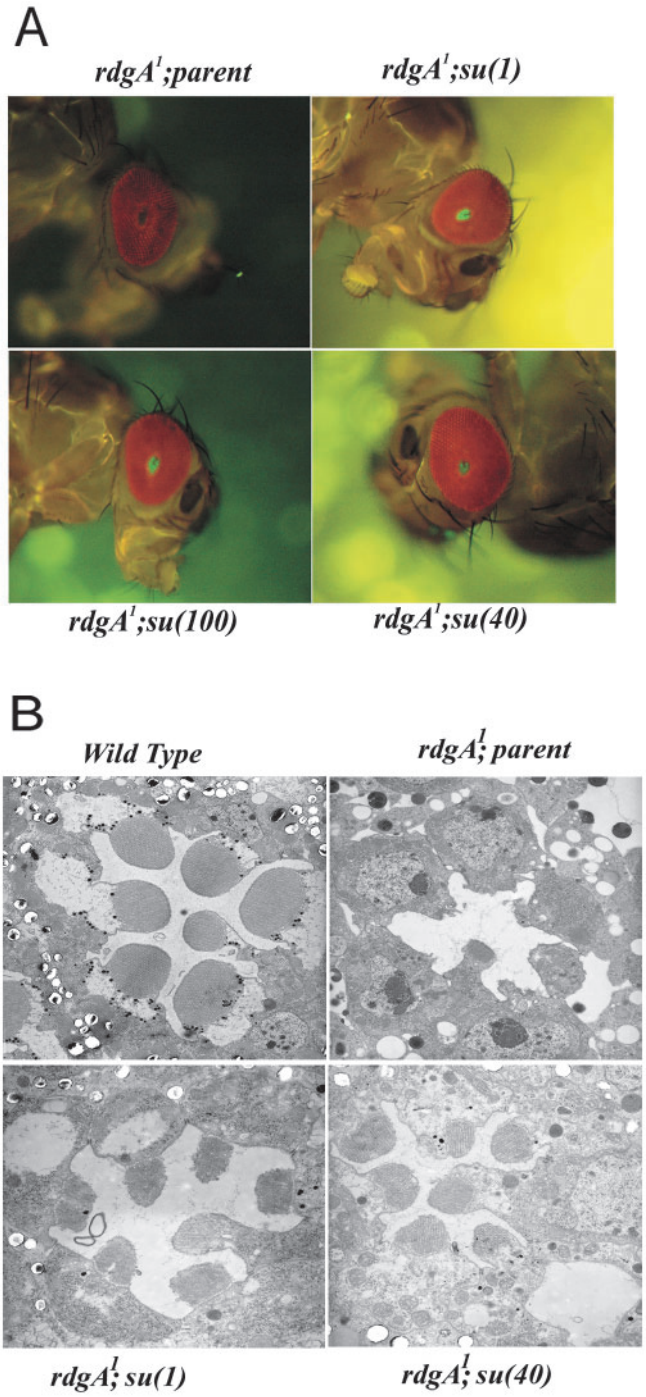

Fig. 3. (A) Visualization of the fluorescent deep pseudopupil (FDPP) in live flies. In the starting stock used for the screen (*w,rdgA*^{1}*, P[rh1-Rh1EGFP];parent*), no FDPP can be seen. A distinct FDPP can be seen in the three suppressers isolated [*rdgA*^{1}*;su(1)*, *rdgA*^{1}*;su(40)* and *rdgA*^{1}*;su(100)*]. (B) TEM image of a single ommatidium from wild-type and *rdgA*^{1} mutants compared with those from *rdgA*^{1}*;su(1)* and *rdgA*^{1}*;su(40)* confirming the rescue of retinal degeneration as assessed by the FDPP. Similar results were obtained with *su(100)* (data not shown).

et al., 1969), these have been designed to look for recessive, viable mutant alleles. Indeed, to date, most genes implicated in phototransduction are eye enriched, and mutants in these genes have proved to be homozygous viable. However it is possible that a subset of the gene products involved in phototransduction will also be involved in functions elsewhere in the fly, and mutants in such genes will be homozygous lethal. In particular, genes involved in the degeneration mechanisms per se might fall into this category. Taking this into account, we have performed a FLP/FRT screen in which mitotic recombination is exploited to generate homozygous mutant eyes in an otherwise heterozygous animal (Xu and Rubin, 1993). A feature of the classical FLP/FRT technique is that clones of homozygous ommatidia are interspersed with wild-type and heterozygous tissue. This represents a particular problem in a screen that involves assessing the integrity of the pseudopupil because both mutant and non-mutant ommatidia will contribute to the pseudopupil image. In order to overcome this problem, we have used a recently developed method (Stowers and Schwarz, 1999) that allows the generation of adult eyes composed entirely of homozygous mutant tissue.

Using the methods described above (Fig. 2), we performed a saturating screen for suppressors of $rdgA^1$ on the right arm of chromosome 2, screening ~6000 mutagenized chromosomes using the FDPP assay. Several potential suppressors were identified and, following retesting and clean-up of chromosomes 1 and 3, we isolated three mutants that clearly suppressed degeneration in $rdgA^1$ (Fig. 3A). We have named these *su(1)*, *su(40)* and *su(100)*. We examined retinal ultrastructure by transmission electron microscopy (TEM) to confirm that the rescue seen by green pseudopupil analysis was indeed present (Fig. 3B).

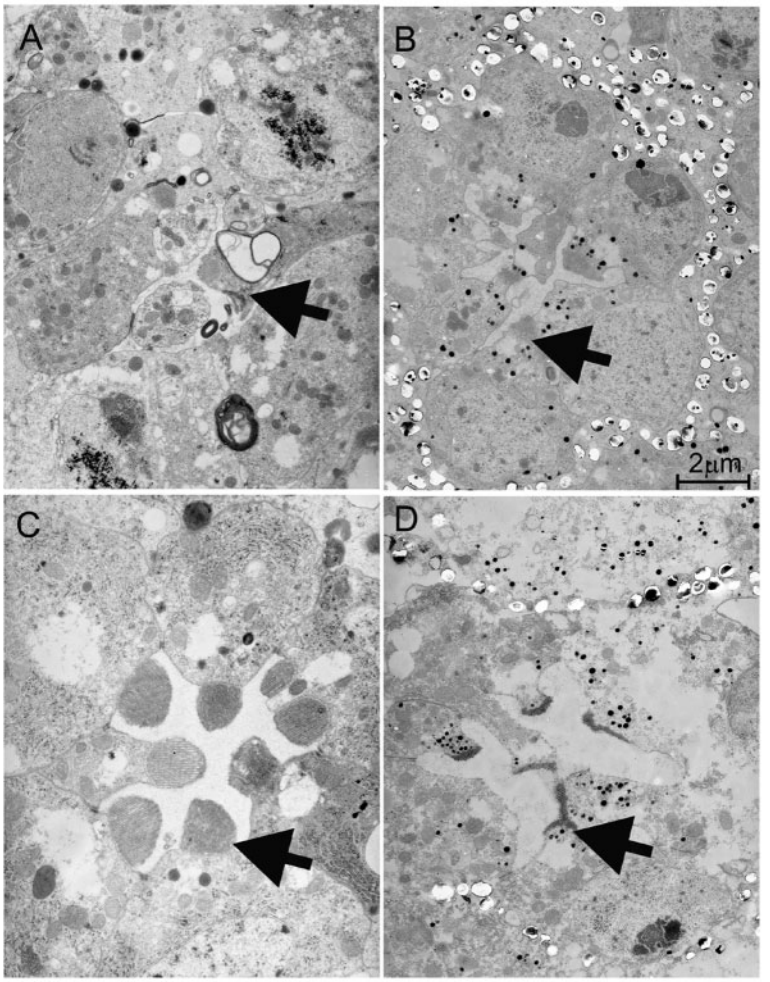

Fig. 4. Rescue of rhabdomere degeneration in $w;Trp^{365}$. In comparison to wild-type flies, $w;Trp^{365}$ homozygous flies show severe rhabdomere degeneration (A). This can be rescued by $su(40);Trp^{365}$ (C) but not in $su(100);Trp^{365}$ (B) double mutants. The effect of *su(1)* on Trp^{365} was equivalent to that of *su(100)* with minimal, if any, rescue of rhabdomere structure (D). Arrows indicate rhabdomeres.

su(1) and *su(100)* work at or above the level of the channel

In principle, suppressors of *rdgA* isolated in our screen could work by one of two mechanisms: (1) at or above the level of the channel, and therefore likely to encode members of the transduction cascade; (2) below the level of the channel, and therefore likely to be involved in the degeneration process. To understand the mechanism by which *su(1)* and *su(100)* modulate the degeneration of $rdgA^1$, we have exploited a recently described allele of *trp*, Trp^{365}, in which a mutation in the TRP channel is thought to render the channel constitutively active, resulting in retinal degeneration (Hong et al., 2002; Yoon et al., 2000). We hypothesized that, if the suppressors were to act at or above the level of the TRP channel in the transduction cascade, they were unlikely to suppress degeneration of Trp^{365}. Conversely, if the suppressors function by blocking the Ca^{2+}-dependent mechanisms of degeneration downstream of Ca^{2+} influx through TRP channels, they were also likely to suppress the phenotype of Trp^{365}. To test this, we generated double mutants of *su(1)*, *su(100)* and *su(40)* with Trp^{365}. We assayed retinal ultrastructure using TEM. The results clearly indicate that, whereas *su(1)* and *su(100)* show limited (if any) rescue of Trp^{365} photoreceptors, *su(40)* was able substantially to rescue degeneration. (Fig. 4). Because we are principally interested in the mechanism of TRP channel activation, we analysed *su(1)* and *su(100)* in detail in this study; the analysis of *su(40)* will be described elsewhere.

su(1) and *su(100)* map to a small region of 59B by deficiency mapping

In order to identify the affected genes in *su(1)* and *su(100)*, we carried out genetic complementation against the deficiency kit for chromosome 2R from the Bloomington *Drosophila* Stock Centre. This kit consists of 75 stocks that provide ~80% coverage of this arm. Non-complementing deficiencies were identified using the FDPP assay used in the initial isolation of the suppressors. We were able to identify a single deficiency, *Df(2)59AD* (Fig. 5B), that was unable to complement the phenotype of both *su(1)* and *su(100)* with respect to the rescue of degeneration in $w,rdgA^1,P[rh1\text{-}Rh1EGFP]$. Using the overlapping deficiencies *Df(2R)3_70*

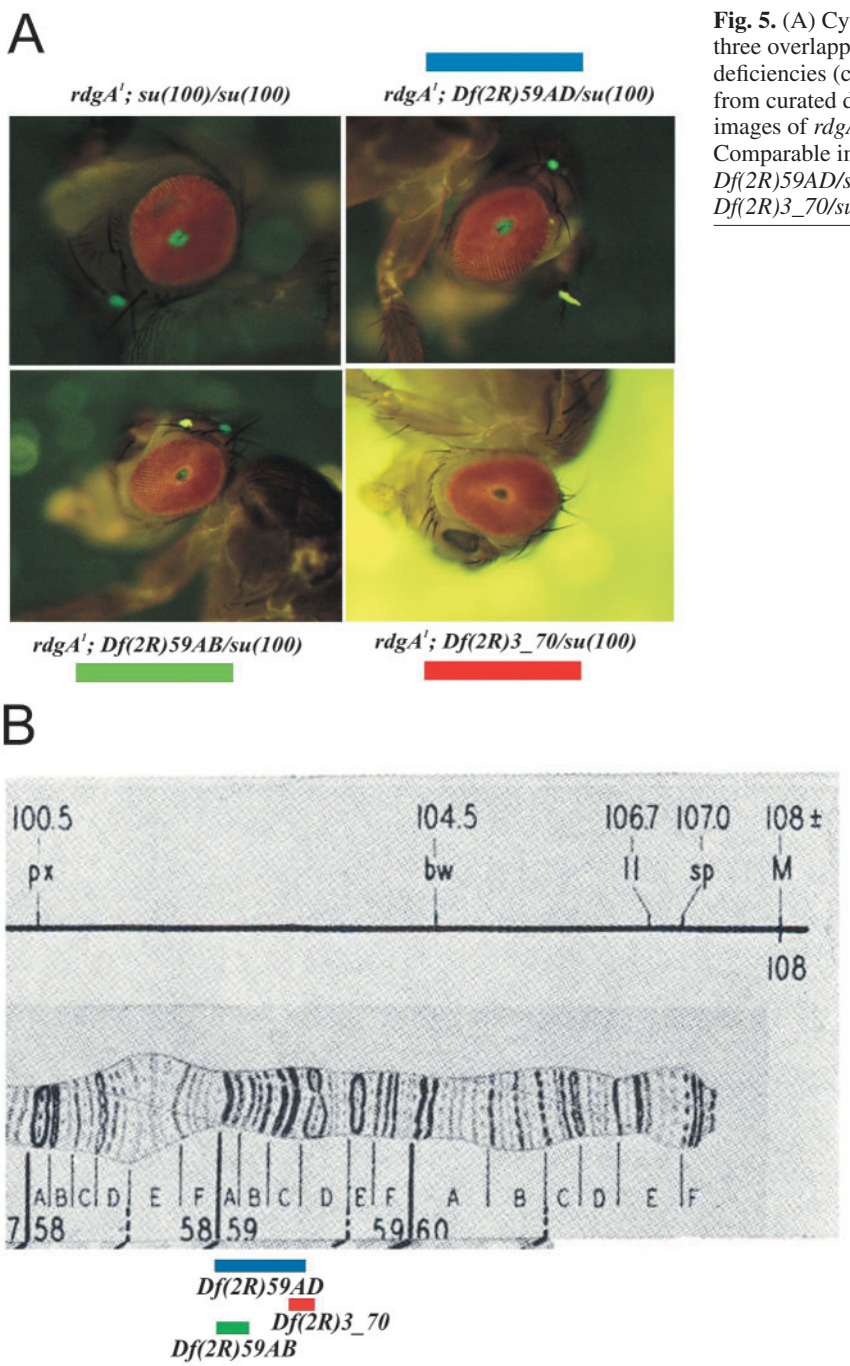

Fig. 5. (A) Cytogenetic representation of the 59B region showing the three overlapping deficiencies relevant to this study. The extent of the deficiencies (coloured boxes) and their breakpoints have been taken from curated data on the *Drosophila* database FlyBase. (B) FDPP images of *rdgA*^{1}*;su(100)* showing rescue of degeneration. Comparable images showing rescue of degeneration by *Df(2R)59AD/su(100)* but not *Df(2R)59AB/su(100)* and *Df(2R)3_70/su(100).*

and *Df(2R)59AB* (Fig. 5A), we were able to narrow down the location of *su(1)* and *su(100)* to a region that contain ~27 genes. Our analysis revealed that the *InaD*, an eye-enriched gene implicated in phototransduction, mapped to this region raising the question of whether *su(1)* and *su(100)* were novel alleles of *InaD*.

su(1) and *su(100)* are alleles of *InaD*

To test the possibility that *su(1)* and *su(100)* are alleles of *InaD*, we asked the following two questions. (1) Does *InaD*^{1} rescue *rdgA*^{1}? (2) Do *su(1)* and *su(100)* have altered levels of INAD protein?

To answer the first question, we generated the double mutant *rdgA*^{1}*;InaD*^{1} and analysed its retinal ultrastructure using TEM. *InaD*^{1} is a well-characterized null allele of *InaD* (Tsunoda et al., 1997). We found that *InaD*^{1} was able to rescue the degeneration of *rdgA*^{1} in much the same manner as *su(1)* and *su(100)* (Fig. 6A). We also tested whether *InaD*^{1}*/su(1)* and *InaD*^{1}*/su(100)* were able to rescue degeneration and found that this was indeed the case. These results suggest that *su(1)* and *su(100)* are likely to be either alleles of *InaD* or mutants in genes that interact strongly with it.

To answer the second question, we analysed levels of INAD protein in *su(1)* and *su(100)* retinas by western blots using an anti-INAD serum, comparing them to *InaD*^{1}. This analysis revealed that both *su(1)* and *su(100)* behave as protein-null alleles of INAD (Fig. 6B).

In principle, an alternate explanation for the results described above is that *Df(2R)59AD* contains another gene whose activity is required for the stable expression and function of INAD. To discriminate between these two possibilities, we tested the ability of *P[GMR-InaD]* (Tsunoda et al., 2001) to rescue the phenotypes of *su(1)* and *su(100)* using two assays: ability to restore the levels of INAD protein and ability to revert the suppression of degeneration in *rdgA*^{1}. We reasoned that, if *su(1)* and *su(100)* were alleles of *InaD*, *P[GMR-InaD]* would be able to restore INAD protein levels as well as to reverse the rescue of degeneration in *rdgA* by *su(100).* By contrast, if *su(1)* and *su(100)* were genes required for stable expression of INAD protein, it was unlikely that the *P[GMR-InaD]* transgene would reverse their phenotypes. In the event, we found that *P[GMR-InaD]* was able to revert both levels of INAD protein (Fig. 7A) as well as the ability of *su(100)* to suppress the degeneration of *rdgA*^{1}, strongly suggesting that they are new alleles of INAD (Fig. 7B).

Finally, to identify the molecular defect in the *InaD* gene, we cloned and sequenced a 3.3 kb fragment containing *InaD* from *su(1)*, *su(100)* and the parent chromosome used for the mutagenesis. This revealed that the *InaD* gene in *su(100)* contains a CAG→TAG change, introducing a stop codon just after the first PDZ domain. We could not detect this extremely small truncated protein on western blots. In *su(1)*, we identified an AAG→TAG change in the region between PDZ domains 3 and 4 (Fig. 8). More detailed analysis on western blots has revealed that this mutant produces a truncated INAD protein

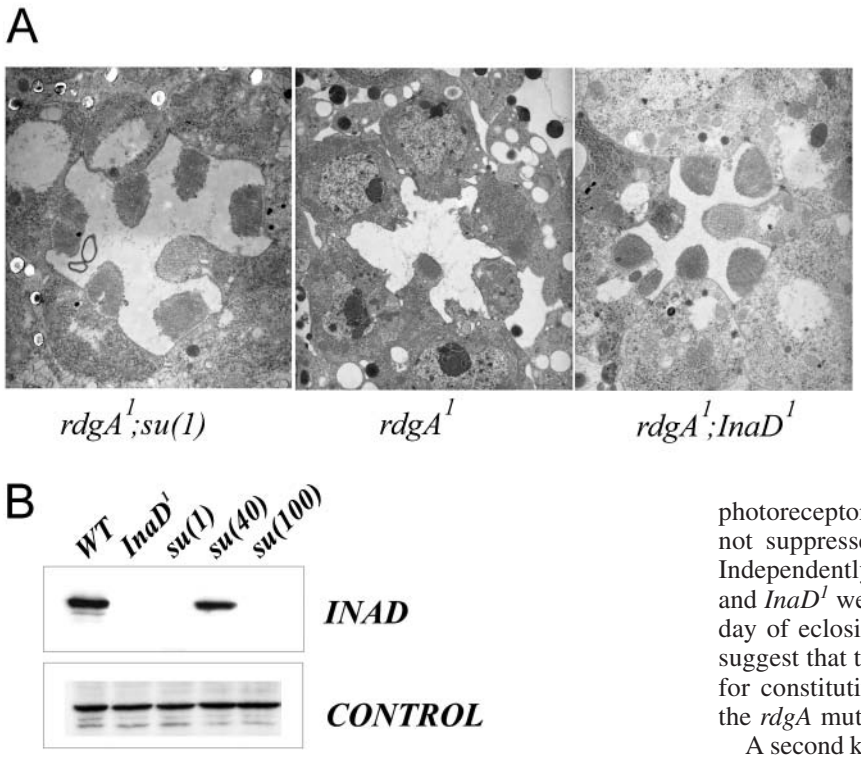

Fig. 6. (A) Rhabdomere structure in $rdgA^1$ is rescued to equivalent levels by *su(1)* and $InaD^1$. (B) Western blot using an antiserum against INAD showing wild-type INAD protein missing from head extracts of *su(1)* and *su(100)* but not *su(40)*. The established protein-null mutant $InaD^1$ is used as a negative control to identify the INAD band. Control panel shows the same blot detecting syntaxin as a loading control to show equivalent levels of protein in all samples.

encoding PDZ domains 1, 2 and 3 only (data not shown). The size of this truncated protein is consistent with the location of the stop codon that we have identified.

INAD is a multivalent PDZ-domain protein; some of its five PDZ domains interact with and assemble several known components of the phototransduction cascade into a macromolecular complex (Fig. 8). These include the TRP channel (Shieh and Zhu, 1996), PLCβ (Chevesich et al., 1997; Huber et al., 1996), protein kinase C (Huber et al., 1996; Tsunoda et al., 1997) and other INAD molecules (Xu et al., 1998). In addition, but more controversially, it has been suggested that rhodopsin, TRPL (Chevesich et al., 1997; Xu et al., 1998), NINAC (Wes et al., 1999) and calmodulin (Chevesich et al., 1997) might also bind to INAD. Because $InaD^1$ and *su(100)* are apparently both null mutants of INAD, we wondered which of the individual protein-protein interactions of INAD, when disrupted, contributes to the rescue of *rdgA*. Or, indeed, whether there are currently undiscovered protein partners of INAD that might be required to mediate constitutive channel activity and retinal degeneration in *rdgA*.

An obvious mechanism by which *InaD* could rescue $rdgA^1$ is by regulating the function or levels of TRP channels, because loss-of-function mutants in the *trp* gene are already known to rescue *rdgA* (Raghu et al., 2000b). To test this, we used two strategies. First, we tested whether $InaD^{P215}$ [an allele that carries a point mutation in PDZ3 and specifically abolishes the interaction of TRP with INAD while leaving other interactions intact (Fig. 8)] could suppress $rdgA^1$. We found that, in $rdgA^1;InaD^{P215}$, retinal degeneration was as severe as in $rdgA^1$ (Fig. 9A). Second, and conversely, we tested whether $TRP^{\Delta 1272}$ [a *trp* gene in which the last four amino acids shown to be crucial for interaction with INAD had been removed (Li and Montell, 2000)] could suppress degeneration in $rdgA^1$. To do this, we generated the double mutant $rdgA^1;trp^{CM},P[TRP^{\Delta 1272}]$. When grown at 29°C, the restrictive temperature for trp^{CM}, the only TRP channels expressed in these photoreceptors are $TRP^{\Delta 1272}$.We found that degeneration was not suppressed in $rdgA^1;trp^{CM},P[TRP^{\Delta 1272}]$ flies (Fig. 9B). Independently, we also found that TRP protein levels in *su(1)* and $InaD^1$ were at best minimally reduced or unaltered on the day of eclosion (data not shown). Taken together, these data suggest that the intact TRP-INAD interaction is not necessary for constitutive TRP-channel activation and degeneration in the *rdgA* mutant.

A second key member of the INAD complex is PLCβ, which in *Drosophila* photoreceptors is encoded by *norpA*. Although *norpA* has been reported not to suppress *rdgA* (Masai et al., 1993), given the apparent effects of the *rdgA* mutant on the light response (Hardie et al., 2002), we tested the contribution of intact levels of NORPA to the *rdgA* phenotype. Our analysis revealed that *su(1)* and *su(100)*, but not *su(40)*, showed reduced levels of NORPA, suggesting that reduced PLCβ activity might contribute to the rescue of *rdgA* by *InaD* (Fig. 10A). To test this hypothesis, we generated $rdgA^1;InaD^2$ double mutants. $InaD^2$ is an allele in which a point mutation in PDZ5 specifically disrupts the interaction between NORPA and INAD, resulting in delocalization of NORPA from the rhabdomere and reduced levels of this protein (Tsunoda et al., 1997). We found that $InaD^2$ produced almost equivalent rescue to the null allele $InaD^1$ (Fig. 10B). To test further the role of reduced NORPA levels in the rescue of *rdgA* by *su(100)*, we measured levels of NORPA in $rdgA^1;su(100)$ and $rdgA^1;InaD^1$ double mutants in which INAD levels had been restored using *P[GMR-InaD]*. We found NORPA levels restored to near-wild-type levels in both *su(100)* and $InaD^1$ (Fig. 10C), and that this correlated well with the reversion of suppression in $rdgA^1;su(100);P[GMR\text{-}InaD]$ (Fig. 7B). Taken together, these results suggest that the reduced levels of PLCβ contribute substantially to the rescue of degeneration in *rdgA* by *InaD*.

Role of phototransduction in the degeneration phenotype of *rdgA*

Because our findings on the mechanism by which *InaD* suppresses *rdgA* are apparently at odds with previous studies, which concluded that degeneration in *rdgA* was both light and PLCβ independent (Harris and Stark, 1977; Masai et al., 1993), we used several different approaches to re-examine the role of PLCβ and the phototransduction cascade on degeneration.

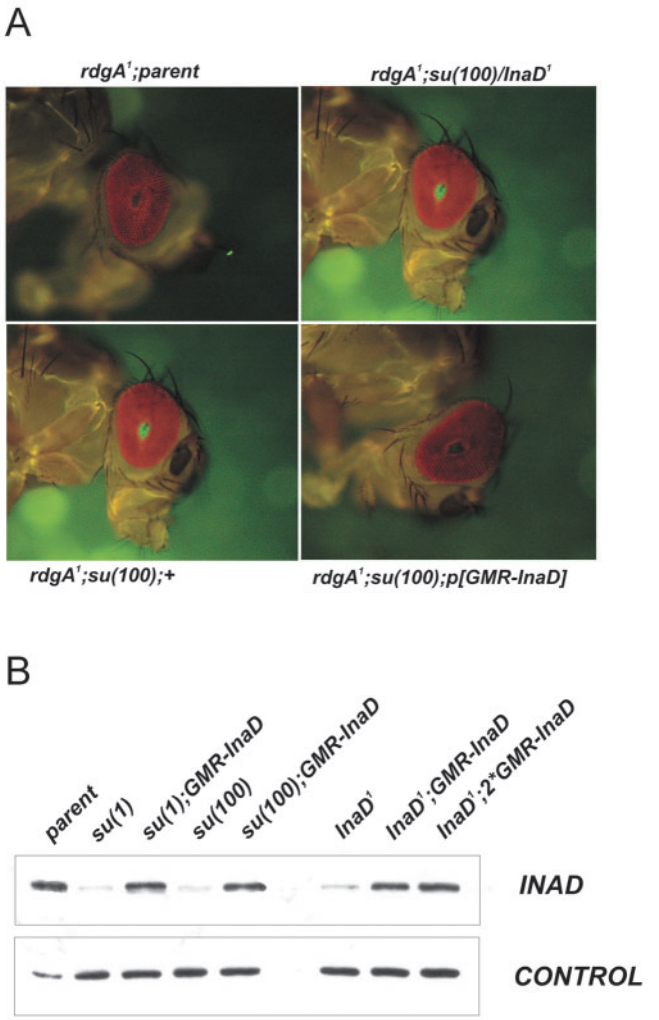

Fig. 7. (A) Rescue of INAD protein levels in *su(1)* and *su(100)*. Western-blot analysis of head extracts with an INAD antiserum. Levels of INAD protein in *su(1)*, *su(100)* and $InaD^1$ are compared to that in the same mutants also carrying the *P[GMR-InaD]* transgene. Levels of INAD in head extracts from the parent strain used for mutagenesis (2120) are also shown. A control blot shows equivalent levels of protein loading between the various samples. (B) The rescue of degeneration (assayed by FDPP) seen in $rdgA^1$*;su(100)/*$InaD^1$ and $rdgA^1$*;su(100)* mutants can be reversed by introducing the transgene *P[GMR-InaD]* in $rdgA^1$*;su(100);P[GMR-InaD]*. The original $rdgA^1$*;parent* that shows degeneration is shown for comparison.

Light dependence of degeneration

As previously reported (Harris and Stark, 1977), we found that there was no effect of dark rearing on the degeneration of $rdgA^1$. In addition, we were unable to suppress the degeneration of $rdgA^1$ using mutants that lacked Rh1, the principal receptor for light using the double mutant $rdgA^1$*;*$ninaE^{0117}$. Although $ninaE^{0117}$ flies (O'Tousa et al., 1985) also show photoreceptor degeneration, this phenotype is much less severe than that of $rdgA^1$, and $rdgA^1$*;*$ninaE^{0117}$ looked just as severe as the $rdgA^1$ single mutant (data not shown). We also analysed the degeneration of $rdgA^3$, a hypomorphic allele of *rdgA* (Harris and Stark, 1977). In $rdgA^3$, on eclosion, all seven rhabdomeres within an ommatidium can be distinctly counted and show near-wild-type morphology. Progressive degenerative changes can be seen in all six outer rhabdomeres over a period of time (at 25°C) so that, by 72 hours after eclosion, only the central R7 ommatidium can be seen (Fig. 11A). Once again, we were unable to suppress the degeneration of $rdgA^3$ by growing flies in complete darkness throughout development. Similar rates of rhabdomere degeneration were seen in flies grown in a normal laboratory incubator to that in $rdgA^3$ grown in constant darkness (Fig. 11B, NI v 24hD). However, the rate of degeneration was significantly higher in flies grown on a 12 hour light-dark cycle than in flies grown in complete darkness (Fig. 11B, 24h D v 12hL/12hD). These results suggest that activation of the phototransduction cascade enhances the rate of degeneration of *rdgA* photoreceptors.

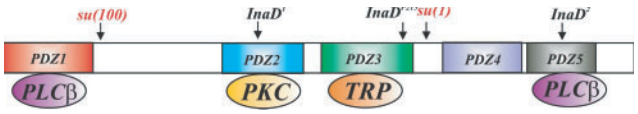

Fig. 8. Schematic representation of the multiple PDZ domains of INAD. The principal binding protein partners that are known to interact with INAD and the PDZ domains involved are shown; TRP-TRP channel; PKC eye-enriched protein kinase C and PLCβ-NORPA. Only key interactions about which there is general agreement among workers in the field are shown. The positions of point mutations in several INAD alleles including $InaD^1$, $InaD^2$, $InaD^{P215}$, *su(1)* and *su(100)* are shown with respect to the PDZ domains.

Effect of mutants that impair activation of the phototransduction cascade

We also tested the effects of mutants that remove two established, key elements of the activation cascade – dGq (Scott et al., 1995), the G-protein α subunit that is activated by rhodopsin, and phospholipase Cβ, the target of activated dGq. To do this, we generated double mutants of $rdgA^3$ separately with $G\alpha q^1$ and $norpA^{P24}$.

$G\alpha q^1$ is a severe hypomorph of dGq containing less than 1% of the wild-type protein levels and showing more than 1000-fold reduction in sensitivity to light (Scott et al., 1995). In $rdgA^3$*;*$G\alpha q^1$ mutants, the rate of degeneration monitored by optical neutralization was slower than that in $rdgA^3$ alone (Fig. 11C).

Mutants in phospholipase Cβ (*norpA*) lack the eye-enriched isoform of this enzyme (Bloomquist et al., 1988) and show dramatic reduction in sensitivity to light, with the most severe alleles ($norpA^{P24}$) being virtually unresponsive to light. We generated a $norpA^{P24}$*,*$rdgA^3$ double mutant and compared the rate of degeneration of these flies with that of $rdgA^3$ alone. We found that $norpA^{P24}$ was able completely to suppress the degeneration of $rdgA^3$ (Fig. 11D). Although Masai et al. reported that *norpA* does not suppress *rdgA* (Masai et al., 1993), no information was provided about which allele of either gene was used in this study. In order to exclude any allele-specific effects, we also generated a double mutant with the more severe $rdgA^1$ allele ($norpA^{P24}$*,*$rdgA^1$). $norpA^{P24}$*,*$rdgA^1$ double mutants showed almost wild-type photoreceptor ultrastructure when examined by TEM (data not shown). Furthermore, we generated double

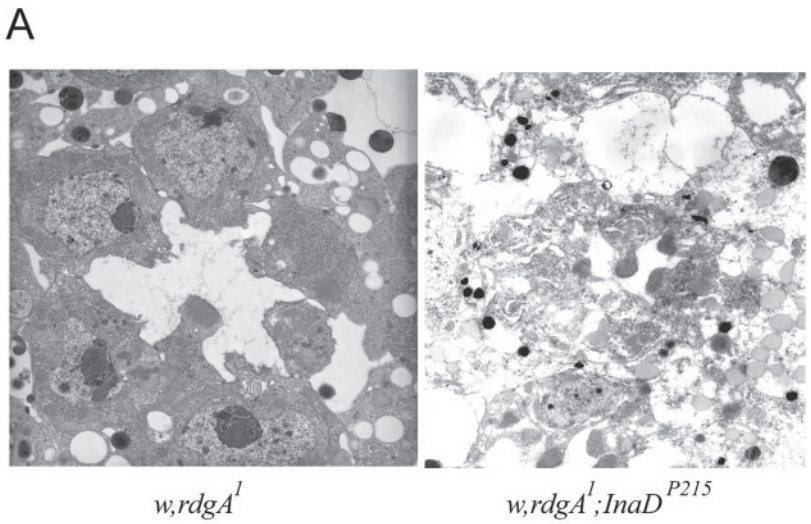

Fig. 9. Effect of specifically disrupting the TRP-INAD interaction on $rdgA^1$. (A) TEM of $rdgA^1;InaD^{P215}$ double mutants showing no rescue of degeneration. (B) Rescue of $rdgA^1$ by trp^{CM} assayed by optical neutralization. When grown at 29°C, the restrictive temperature for trp^{CM}, $rdgA^1;trp^{CM}$ show rescue of degeneration compared with that at the permissive temperature of 20°C. The images of rhabdomeres R1-R7 can be seen. Expression of $TRP^{\Delta 1272}$ channels in $rdgA^1;trp^{CM}$ mutants at 29°C results in severe retinal degeneration.

mutants of $rdgA^1$ with two additional severe hypomorphic alleles of *norpA*, $norpA^{P12}$ and $norpA^{P16}$ (Pearn et al., 1996). Again, both alleles were able to effect a robust and near-total rescue of degeneration in $rdgA^1$ (data not shown). These results clearly show that *norpA* does suppress *rdgA*.

Discussion

Despite their ability to regulate calcium influx during cell signalling, the mechanism underlying the activation of TRPC channels remains controversial and poorly understood. Owing to the lack of in vivo models systems (in which a well-defined signalling cascade would end with the activation of endogenous TRPC channels), most studies to date have been done on heterologously overexpressed TRPC channels using cell culture models. Although most studies agree that the hydrolysis of $PI(4,5)P_2$ by receptor-regulated PLCβ is required for activation, there is little agreement about the downstream biochemical mechanisms that result in TRPC channel opening. For some members, such as TRPC3, equally compelling studies have been published showing roles in activation for IP_3/IP_3R (Kiselyov et al., 1998; Vazquez et al., 2001) mediated store depletion and for the lipid products of $PI(4,5)P_2$ hydrolysis (Venkatachalam et al., 2001). Recently, it has become clear that many of these conflicting results arise from several experimental factors, including the level of overexpression of the channel (Vazquez et al., 2003), the presence of endogenous TRPC members in the cell lines used and the relative promiscuity of pharmacological agents used in manipulating their activation (Bootman et al., 2002). By contrast, in *Drosophila* photoreceptors, the detection of light by rhodopsin activates a signalling cascade that ends with endogenous TRPC (TRP and TRPL) activation. In this model, too, current debate centres around the identity of the products of $PI(4,5)P_2$ hydrolysis that are crucial for channel activation. Although there is substantial evidence to suggest that IP_3-mediated signalling is not essential, recent evidence suggests that the lipid products of $PI(4,5)P_2$ hydrolysis might be involved in activation (Chyb et al., 1999; Estacion et al., 2001). Analysis of photoreceptors lacking DGK activity (*rdgA*) has provided the first genetic evidence that suggests a role for lipid second messengers in activating TRP and TRPL in vivo (Raghu et al., 2000b; Hardie et al., 2002). However, questions remain about the biochemical basis of the *rdgA* phenotype and its relevance to the normal phototransduction cascade; for example, is the constitutive channel activity the cause or the consequence of the degeneration? In addition, how can one reconcile recent findings suggesting a role for *rdgA* in phototransduction with long-standing observations that imply a phototransduction-independent basis for the *rdgA* phenotype (Harris and Stark, 1977; Masai et al., 1993)?

To address this issue, we have carried out a forward-genetic screen to identify suppressors of the retinal degeneration phenotype of *rdgA* mutants. Such an approach is unbiased and makes no assumptions about the mechanisms underlying the degeneration process. Using a combination of deficiency mapping and bioinformatic analysis, we have identified *su(1)* and *su(100)* as new alleles of *InaD*, a PDZ-domain protein required for the assembly of signalling complexes in *Drosophila* photoreceptors and suggested to have a role in the regulation of signalling specificity and speed (Tsunoda et al., 1998). *InaD* has not previously been reported to interact with *rdgA*. Trp^{365} contains a point mutation at the cytoplasmic end of S5 in the TRP channel and shows constitutive channel

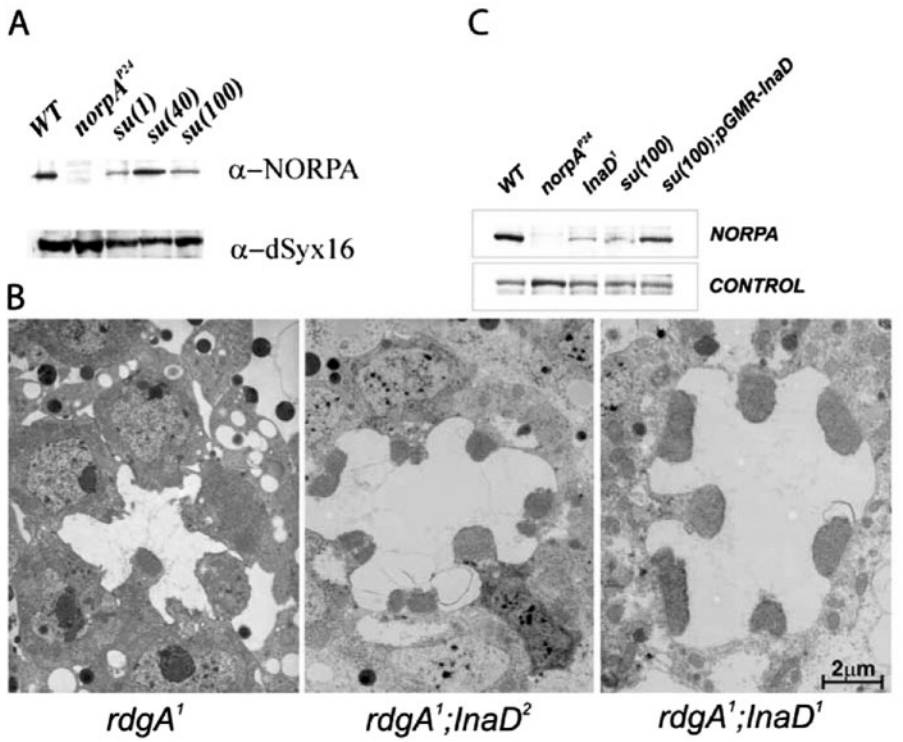

Fig. 10. (A) Western blot showing reduced levels of NORPA in head extracts from *su(1)* and *su(100)* but not *su(40)*. Levels of dSyx on the same blot were used as a loading control. (B) TEM of a single ommatidium showing equivalent levels of rhabdomere rescue by $InaD^1$ and $InaD^2$ in $rdgA^1$. (C) Western blot showing that the reduced levels of NORPA in $InaD^1$ and *su(100)* can be restored by complementation with *P[GMR-InaD]*. An irrelevant band from the same blot is used as a control. Extracts from $norpA^{P24}$, a null allele were used to identify the NORPA band.

activity and degeneration (Hong et al., 2002; Yoon et al., 2000). The finding that *InaD* was largely ineffective at suppressing the degeneration of Trp^{365} suggested to us that the mechanism of suppression was at or above the level of the channel in the transduction cascade rather than by blocking events downstream of excessive calcium influx through constitutively active TRP channels. *InaD* clusters several key molecules required for phototransduction, including the TRP channel; this strongly suggested that the constitutive channel activity and degeneration in *rdgA* are a consequence of altered phototransduction.

To identify the specific known (or perhaps undiscovered) protein-protein interactions of *InaD* that contribute to the rescue of *rdgA*, we manipulated the *InaD* complex in a manner that allowed its interaction with specific transduction components such as the TRP channel and NORPA to be individually disrupted. We found wild-type TRP protein levels in *InaD* on eclosion and by analysing the effect of $InaD^{P215}$ (Shieh and Zhu, 1996) and $TRP^{\Delta 1272}$ (Li and Montell, 2000) on *rdgA* found that TRP channels that could not be recruited to the INAD complex were able to mediate degeneration in *rdgA* just as well as wild-type channels. Thus, loss of the TRP-INAD interaction is unlikely to be a significant mechanism by which *InaD* rescues *rdgA* and the function of TRP channels within the INAD complex is not crucial to retinal degeneration.

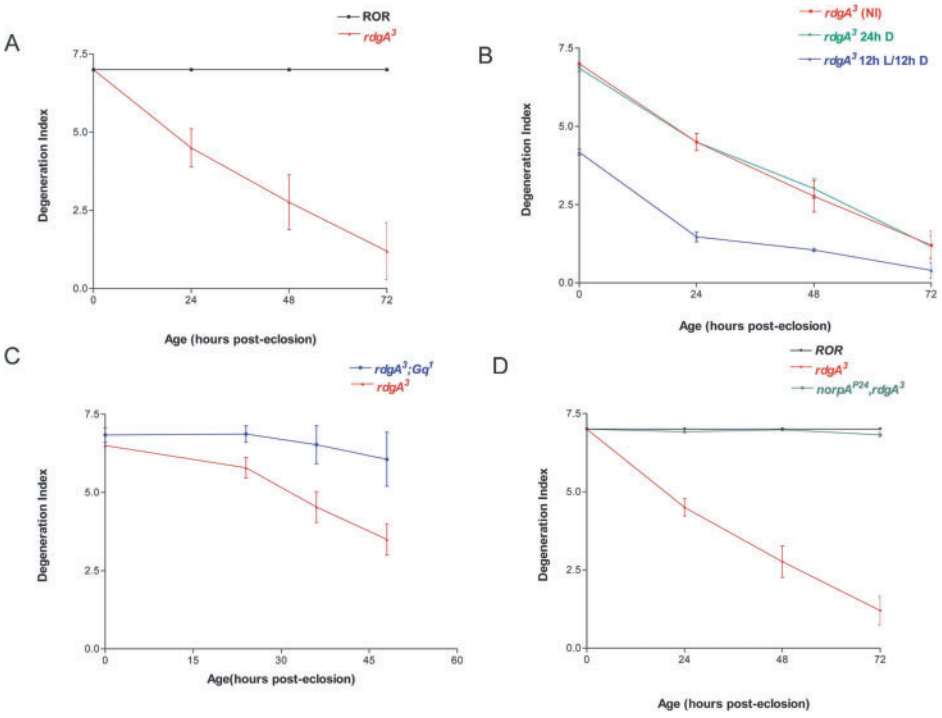

Fig. 11. Effects of modulating phototransduction on retinal degeneration in $rdgA^3$. (A) Time course of retinal degeneration in $rdgA^3$ compared with wild-type (Oregon-R ROR) flies. Flies were grown in a normal laboratory incubator and experienced brief pulses of light when the incubator doors were opened. ROR flies show no degeneration under these conditions. On eclosion, $rdgA^3$ flies show wild-type rhabdomere structure by optical neutralization but then progressively degenerate over 72 hours. (B) Rates of degeneration compared in $rdgA^3$ flies grown in normal incubator (NI), constant darkness in black photographic bags (24h D) and in a illuminated cooled incubator on light/dark cycles (12h L/12h D). (C) Rates of degeneration of $rdgA^3$ compared with $rdgA^3;Gq^1$. In this experiment, flies were grown in the normal incubator. (D) Rates of degeneration in ROR, $rdgA^3$ and $norpA^{P24},rdgA^3$. There is virtually complete suppression of degeneration in $norpA^{P24},rdgA^3$. In this experiment, flies were grown in a normal incubator.

By contrast, we found that NORPA levels in *su(1)* and *su(100)* mutants were reduced on eclosion, as has been reported for $InaD^1$ by other groups (Tsunoda et al., 1997), suggesting that a reduction in PLCβ activity might underlie the mechanism of suppression. We found that $InaD^1$, a protein-null allele, and $InaD^2$, an allele known to disrupt the INAD-NORPA interaction (Tsunoda et al., 1997), produce equivalent levels of rescue of $rdgA^1$, and that the levels of NORPA are inversely correlated to the extent of rescue. Thus, a major mechanism by which $InaD^1$, *su(1)* and *su(100)* suppress $rdgA^1$ is likely to involve the reduced levels of NORPA in these alleles. However, we cannot exclude the possibility that additional, unknown protein-protein interactions of PDZ5 in INAD that might also be disrupted in $InaD^2$ might also contribute to the rescue of *rdgA*. Given the essential role of PLCβ in the activation of TRP channels, this finding implies a key role for the balance of PLCβ and DGK activity in the degeneration of *rdgA*.

Although the degeneration phenotype of *rdgA* has been previously reported to be light independent (Harris and Stark, 1977) and not suppressed by *norpA* mutants (Masai et al., 1993), the finding that *InaD* suppresses *rdgA* and that it does so by reducing levels of NORPA suggested that defects in light-induced phosphoinositide turnover might underlie the degeneration phenotype of *rdgA*. In the light of our findings on the suppression of *rdgA* by *InaD*, we re-examined the effect of three key elements of the phototransduction cascade that are required for activation, namely light, Gαq and PLC. We found that, although the absence of light could not completely suppress the degeneration of $rdgA^3$, there was substantial suppression of degeneration in $rdgA^3$ flies grown in complete darkness compared with those grown on a 12 hour light/12 hour dark cycle. Degeneration could also be partially suppressed but not blocked by a strong hypomorph that reduced Gαq levels to <5% of the wild-type levels. However, most importantly, we found that *norpA* mutants that lack PLCβ could suppress the degeneration of *rdgA* in several allelic combinations for both genes. These results demonstrate a key role for activation of the phototransduction cascade in the degeneration phenotype of *rdgA*.

Although we show a requirement for light, Gαq and PLC activity in the degeneration phenotype of *rdgA*, as reported earlier (Harris and Stark, 1977), we were unable completely to suppress the degeneration of even the weakest allele, $rdgA^3$, by rearing flies in complete darkness. Indeed, reducing levels of Gαq using the strong hypomorph $G\alpha q^1$ (which has <5% of the wild-type Gαq levels) was able only to slow the rate of degeneration of $rdgA^3$. However mutants in $norpA^{P24}$ were able completely to suppress the degeneration of both $rdgA^3$ and $rdgA^1$. In a recent study that measured basal PLCβ activity in photoreceptors (Hardie et al., 2004), we found that, similar to wild-type photoreceptors, *rdgA* mutants showed reduced but still substantial basal PLCβ activity. This implies that, even in the dark, there is a basal turnover of $PI(4,5)P_2$ in *rdgA* photoreceptors. Thus, basal $PI(4,5)P_2$ hydrolysis could lead to the build up of a lipid metabolite of $PI(4,5)P_2$ that triggers constitutive TRP channel activity and retinal degeneration.

Implications for INAD function

Although several studies have demonstrated the importance of INAD in targeting and stabilizing members of the phototransduction cascade to the rhabdomere, there is little agreement about the requirement, if any, for intact INAD complexes once assembled and transported to the rhabdomere to activate TRP channels. Although some studies have suggested that an intact INAD complex is crucial for generating the channel activity that underlies a quantum bump, the response to a single photon of light (Scott and Zuker, 1998), others have suggested that this might not be the case (Li and Montell, 2000). In our analysis of the mechanism by which *InaD* suppresses *rdgA*, we found that that TRP channels not included within the INAD complex but still present in the rhabdomere were able to mediate retinal degeneration. These results support the idea that presence within the INAD macromolecular complex is not necessary for the constitutive activity of TRP channels seen in *rdgA*.

Although our data support the hypothesis that a principal mechanism by which *InaD* suppresses *rdgA* is via reduction in the levels of PLCβ, they do not exclude the possibility that the disruption of INAD interactions with currently undiscovered proteins that function downstream of NORPA might play a role in constitutive TRP channel activation and degeneration in *rdgA*. Testing this would require the generation of an *InaD* allele in which the INAD-NORPA interaction is intact while disrupting the function of the other protein-protein interactions of INAD. To our knowledge, no such allele exists, but the use of such an allele in conjunction with the *rdgA* mutant could be an useful approach to identifying currently undiscovered members of the INAD complex as well as the phototransduction cascade.

TRP channels appear to be key components of signalling cascades for the detection and coding of several sensory modalities. However, a limiting factor in advancing their role in sensory transduction is our poor understanding of their mechanism of activation. In the case of TRPC channels, this is limited by the lack of genetic model systems in which relevant components of the activation cascade can be identified. In the present study, we describe a novel modifier screen that should provide a powerful method for identifying the relevant transduction components in vivo. Starting with the *rdgA* mutant in which TRP channels are constitutively active and result in retinal degeneration, we have identified two new alleles of INAD, a known component of the phototransduction cascade, as suppressors of *rdgA*. This approach is likely to be a powerful tool to identify further components of the transduction cascade that are relevant in vivo.

This research was supported by funds from the Biotechnology and Biological Sciences Research Council, UK (PR, RCH) and the Medical Research Council (RCH). PR is a BBSRC David Phillips Research Fellow.

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