Common mechanisms regulating dark noise and quantum bump amplification in *Drosophila* photoreceptors.

Brian Chu¹, Che-Hsiung Liu¹, Sukanya Sengupta¹, Amit Gupta², Padinjat Raghu² and Roger C Hardie¹

Abbreviated title  Dark noise in *Drosophila* photoreceptors

1. University of Cambridge, Department of Physiology, Development and Neuroscience, Downing St, Cambridge, UK

2. National Centre for Biological Sciences, GKVK Campus, Bellary Road, Bangalore 560065 INDIA

Correspondence: Roger C Hardie rch14@cam.ac.uk  FAX 00441223 333840

University of Cambridge, Department of Physiology, Development and Neuroscience, Downing St, Cambridge, CB2 3EG U.K.

Number of pages 31

Number of Figures: 9 Figures,

Abstract 239 words

Key words: TRP channels, phototransduction, retinophilin, INAD, NINAC

Author contributions: BC performed most electrophysiological experiments; C-H L Western blot analysis and genetics; SS genetics, designing and performing crosses; AG & PR Generation of RDGA antibody and Western blot analysis; RCH, electrophysiology, experimental design, wrote paper with BC.
Abstract

Absolute visual thresholds are limited by “dark noise”, which in Drosophila photoreceptors is dominated by brief (~10 ms), small (~2 pA) inward current events, occurring at ~2 s\(^{-1}\), believed to reflect spontaneous G-protein activations. These dark events were increased in rate and amplitude by a point mutation in myosin III (NINAC), which disrupts its interaction with the scaffolding protein, INAD. This phenotype mimics that previously described in null mutants of \textit{ninaC} (no inactivation no after potential – encoding myosin III) and an associated protein, retinophilin (\textit{rtp}). Dark noise was similarly increased in heterozygote mutants of diacylglycerol kinase (\textit{rdgA/+}). Dark noise in \textit{ninaC}, \textit{rtp} and \textit{rdgA/+} mutants was greatly suppressed by mutations of the Gq \(\alpha\)-subunit (\textit{G}\(\alpha\)q), and the major light-sensitive channel (\textit{trp}), but not rhodopsin. \textit{ninaC}, \textit{rtp} and \textit{rdgA/+} mutations also all facilitated residual light responses in \textit{G}\(\alpha\)q and phospholipase C (PLC) hypomorphs. Raising cytosolic Ca\(^{2+}\) in the sub-micromolar range increased dark noise, facilitated activation of TRP channels by exogenous agonist, and again facilitated light responses in \textit{G}\(\alpha\)q hypomorphs. Our results indicate that RTP, NINAC, INAD and DGK, together with a Ca\(^{2+}\)-dependent threshold, share common roles in suppressing dark noise and regulating quantum bump generation; consequently, most spontaneous G-protein activations fail to generate dark events under normal conditions. By contrast, quantum bump generation is reliable, but delayed until sufficient G-proteins and PLC are activated to overcome threshold, thereby ensuring generation of full size bumps with high quantum efficiency.
Phototransduction in *Drosophila* is mediated by a phospholipase C (PLC) cascade, culminating in activation of two distinct Ca$^{2+}$ permeable channels encoded by the *transient receptor potential* (*trp*) and *trp-like* (*trpl*) genes (reviews: Hardie 2012; Katz and Minke 2009; Montell 2012). The light response is characterised by high sensitivity, rapid kinetics and wide dynamic range, in part achieved by the ultra-compartmentalisation inherent in the photoreceptors’ microvillar design (Fain et al. 2010; Hardie and Postma 2008; Yau and Hardie 2009). Photoisomerization of one rhodopsin results in generation of a quantum bump ~10 pA in amplitude representing the opening of ~15 TRP channels in a single microvillus of the light-absorbing rhabdomere (Henderson et al. 2000). Ca$^{2+}$ influx via TRP channels is essential for both amplification and rapid kinetics (Hardie 1991; Henderson et al. 2000; Ranganathan et al. 1991). In hypomorphic G-protein or PLC mutants, bump amplitudes are reduced several-fold, indicating amplification also requires activation of multiple G-proteins and PLC. Bump amplitudes in such mutants can be restored to wild-type levels by an additional mutation in the *rdgA* gene, which encodes diacylglycerol kinase (DGK), suggesting a role for this enzyme in regulating the supply of excitatory second messenger (Hardie et al. 2002).

To maximise sensitivity, photoreceptors must minimise noise caused by channel activity in the dark. Such “dark noise” can result from spontaneous activation of molecules at any phototransduction stage and sets a fundamental limit on absolute sensitivity (e.g. Aho et al. 1988; Rieke and Baylor 1996). In *Drosophila*, spontaneous isomerizations of rhodopsin are rare (< 1 min$^{-1}$) and dark noise is dominated by small (~2 pA) bump-like events at rates of 2-3 s$^{-1}$. These are eliminated in mutants of the Gq-protein α subunit (*Gαq*) suggesting they originate from spontaneous G-protein activation (Elia et al. 2005; Hardie et al. 2002).

An increase in spontaneous dark noise was first reported in mutants of *ninaC* (neither inactivation nor afterpotential C) (Hofstee et al. 1996), which encodes rhabdomeric and cytosolic isoforms of myosin III (Montell and Rubin 1988; Porter et al. 1992). Recently, a similar noise phenotype was found in *rtp* mutants lacking retinophilin (Mecklenburg et al. 2010), a novel rhabdomeric protein with homologies to junctophilins (Mecklenburg et al. 2010).
RTP protein, which associates physically with NINAC (Venkatachalam et al. 2010), was undetectable in ninaC null mutants, suggesting the increased dark noise in ninaC was due to lack of RTP (Mecklenburg et al. 2010).

The present study presents a detailed investigation of the determinants of dark noise, which both confirms and significantly extends some of these earlier findings. We show that dark noise is increased by a point mutation in NINAC that disrupts its interaction with the INAD scaffolding protein, and also in rdgA/+ heterozygotes with reduced DGK function. In addition, we show that dark noise is critically dependent upon cytosolic Ca\(^{2+}\) in the sub-micromolar range. We confirm that dark noise in wild-type photoreceptors as well as the enhanced dark noise in various mutant backgrounds is Gq protein dependent, mediated primarily by TRP channels and unaffected by genetic elimination of rhodopsin.

The results allow an in vivo estimate of the rate of spontaneous G-protein activations, and suggest that under normal conditions only a small fraction of these lead to channel activation. Importantly, we also show that the various mutations that increase dark noise (ninaC, rtp, rdgA) all increase the amplitude of the small quantum bumps in G\(\alpha_q\) and norpA hypomorphic backgrounds, suggesting that dark noise and quantum bumps are regulated by common mechanisms involving RTP, NINAC and DGK.

**Materials and Methods**

**Fly Strains**

Flies were reared on standard cornmeal-agar diet at 25°C in the dark. The wild-type strains included both white-eye (\(w^{1118}\)) and red-eye Oregon, with no difference being observed between them. Mutants used included:

- \(Gq^1\), a hypomorph of the Gq \(\alpha\) subunit expressing ca 1% of wild-type protein levels (Scott et al. 1995).
- \(norpA^{P16}\), PLC hypomorph (Pearn et al. 1996);
- \(rdgA^1/+,\) heterozygote for severe allele of \(rdgA\) encoding DAG kinase a.k.a \(rdgA^{BS12}\) (Masai et al. 1993b).
rdgA^K560 near protein-null allele of rdgA used for validating RDGA antibody (Masai et al. 1993a)

ninaE^II7, null mutant for the rhodopsin of R1-6 (Rh1).

rtp, null mutant of retinophilin, generated by combining the Df(3R)rtp^I deletion chromosome with the same chromosome rescued by transgenes for two other genes affected by the deficiency as previously described (Mecklenburg et al. 2010).

trpl^302 and trp^343, null mutants of the TRPL and TRP channel respectively (Niemeyer et al. 1996; Scott et al. 1997).

calx (= calx^A), severe loss of function mutant of CalX, the NCX Na^+/Ca^{2+} exchanger (Wang et al. 2005).

pCalX, flies overexpressing wild-type calx gene under the ninaE promoter enhancing NCX activity in the photoreceptors ~5-8-fold (Wang et al. 2005).

ninaC^P235, a null mutant of the NINAC myosin III, and ninaC^{J1501E} - a transgene with a point mutation in the C-terminal on ninaC^P235 null background (Montell and Rubin 1988; Wes et al. 1999). Seven further transgenic mutants of ninaC (mutagenised transgenes on null ninaC^P235 background) were tested, but none showed a dark noise phenotype. These included: ninaC^KD (deletion in kinase domain); ninaC^{45KR} (point mutation in kinase domain), ninaC^{M132} (lacking P132 cytosolic isoform); ninaC^{dC1} and ninaC^{dC2} (lacking calmodulin binding domains); ninaC^{1015.4} (4 amino acid deletion in myosin domain) and ninaC^{174PS} (mutations of two putative PKC phosphorylation sites (Li et al. 1998; Porter et al. 1995; Porter and Montell 1993).

Double mutant combinations were generated as required by standard genetic strategies.

Electrophysiology

Dissociated ommatidia were prepared as previously described (Hardie et al. 2002) from newly eclosed adult flies and transferred to the bottom of a recording chamber on an inverted Nikon Diaphot microscope. Standard bath contained (in mM): 120 NaCl, 5 KCl, 10 N-Tris-(hydroxymethyl)-methyl-2-amino-ethanesulphonic acid (TES), 4 MgCl_2, 1.5 CaCl_2, 25 proline and 5 alanine, pH 7.15. The intracellular pipette solution was (in mM):
140 K gluconate, 10 TES, 4 Mg-ATP, 2 MgCl₂, 1 NAD and 0.4 Na-GTP, pH 7.15. For
141 manipulation of Na⁺/Ca²⁺ exchange equilibrium the intracellular pipette was adjusted to
142 contain either 10 or 20 mM Na gluconate (and 130 or 120 mM K gluconate respectively).
143 Extracellular NaCl was substituted with LiCl (see individual figures) and solutions
144 applied from a closely positioned puffer pipette. Linolenic acid was kept as a 20 mM
145 DMSO stock at -20°C and diluted 1:1000 in appropriate bath solution. All chemicals
146 were obtained from Sigma-Aldrich. Whole-cell voltage clamp recordings were made at
147 room temperature (20 ± 1° C) at −70 mV (including correction for −10 mV junction
148 potential) using electrodes of resistance ~10-15 MΩ. Series resistance values were
149 generally below 30 MΩ. Series compensation of >80% was applied for macroscopic
150 responses, but not for sampling quantum bumps and dark noise (when series resistance
151 errors are negligible). Data were collected and analyzed using Axopatch 200 or 2D
152 amplifiers and pCLAMP8, 9 or 10 software (Molecular Devices, Union City CA).
153 Quantum bumps and spontaneous dark events were analysed using the Minianalysis
154 program (Jaejin Software Leonia, NJ), using a threshold criterion of 0.5 pA. Event rates
155 were analysed automatically, but for event amplitudes and waveforms, all events were
156 individually scrutinised before acceptance. Photoreceptors were stimulated via green
157 Light-Emitting-Diode; intensities were calibrated in terms of wild-type effectively
158 absorbed photons by counting quantum bumps at low intensities in wild-type cells.

Western immunoblotting
160 Heads from flies aged 0–24 h posteclosion were prepared by decapitating flies cooled on
161 ice. Samples were homogenized in 2x SDS-PAGE sample buffer followed by boiling at
162 90°C for 1 min. Samples were separated using SDS-PAGE and electroblotted onto
163 supported nitrocellulose membrane (Hybond-P or Hybond-C extra; GE Healthcare).
164 The uniformity of transfer onto membranes was checked by staining with Ponceau S.
165 Following blocking in 5% non-fat milk (Santa Cruz), blots were incubated for overnight
166 at 4°C in appropriate dilutions of primary antibodies (rabbit anti-α-tubulin (1:1000
167 dilution, ab15246, Abcam), rabbit anti-Gaq (1:3000 dilution), rabbit anti-RTP (1:500
168 dilution) and rabbit anti-RDGA (1:500 dilution). In each case antibody specificity was
169 confirmed by lack of staining in respective mutants. Immunoreactive protein was
visualized after incubation in appropriate dilution of secondary antibodies. The antirabbit IgG ECL HRP linked secondary antibodies (GE Healthcare) were used 1:5000 dilution for RTP and α-tubulin blot, 1:10000 for Gaq. The RDGA blot was probed with 1:10000 dilution of antirabbit IgG coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories). For Gaq, RTP and α-tubulin, bands were visualized by the Amersham ECL-Plus detection regents (GE Healthcare) and were quantitated by G:Box iChemi imaging system (SYNGENE). For RDGA, the blots were developed with ECL (GE Healthcare) using LAS4000 instrument (GE Healthcare) and the immunoblots were quantified using Quantity One 1-D analysis software (Bio-Rad).

**Results**

**Spontaneous dark noise in rdgA/+ heterozygotes**

The light-sensitive TRP and TRPL channels are constitutively active in rdgA mutants lacking diacylglycerol kinase (DGK), indicating that DGK, which converts DAG to phosphatidic acid (PA), is required to prevent spontaneous channel activation (Raghu et al. 2000). However, the uncontrolled Ca\(^{2+}\) influx through constitutively active channels in rdgA mutants results in severe retinal degeneration and loss of light response, leaving it unclear whether DGK plays any role in controlling dark noise under physiological conditions. To address this we investigated the effect of reducing DGK gene dosage in photoreceptors from rdgA/+ heterozygotes (Figure 1). These had normal morphology and sensitivity to light (see Figure 2C); however, immediately after establishing the whole-cell configuration in rdgA/+ photoreceptors, we detected a conspicuously high rate of spontaneous events in complete darkness, consisting of small quantum bump-like inward currents (Figure 1A). For quantitative analysis, we used event detection software to calculate the rates and amplitudes of dark events over the first 100 seconds after establishing the whole-cell configuration. Despite cell-to-cell variation (Figure 1F), event rates in rdgA/+ were on average four times faster than in wild-type (9.5 ± 0.8 events s\(^{-1}\), mean ± SEM n =20) and dark event amplitudes approximately twice as large (4.3 ± 0.2 pA). The differences were statistically highly significant (p < 10\(^{-5}\) 2-tailed unpaired *t*-test) and a scatter plot of event rates *vs* amplitudes for all cells showed no
overlap in distributions (Figure 1F). As in wild-type, the dark event rate (but not amplitude) gradually subsided over a recording period of 10-15 minutes (Figure 1B,C).

DGK has previously been proposed as a critical ATP-dependent enzyme in regulating bump amplification, because rdgA mutations leads to amplification of the small quantum bumps in norpA and Gaq hypomorphic mutants – a phenotype that can be mimicked by omission of nucleotide additives (ATP, NAD and GTP) from the recording electrode (Hardie et al. 2002). It is also known that under ATP-deprived conditions, TRP and TRPL channels become spontaneously activated, leading within minutes to an inward rundown current (RDC) associated with high frequency channel noise (Agam et al. 2000; Hardie et al. 2003; Hardie and Minke 1994). We re-examined the development of the RDC by recording from wild-type photoreceptors without ATP or other nucleotide additives in the recording pipette, concentrating on the initial phase of the RDC. Immediately after establishing the whole-cell configuration, small spontaneous dark events, similar to those using normal (ATP containing) electrode solution were observed. These then rapidly increased in rate and amplitude reaching rates of up to 10-20 events s$^{-1}$ within ~2 mins, after which the events fused to form a noisy inward current, heralding the onset of the full RDC (Figure 1D, lower). This pronounced time-dependent rise in spontaneous dark event rates might be explained by progressively reduced function of DGK as endogenous ATP is depleted. In support of this, in rdgA$^{+/}$ heterozygotes recorded under the same ATP-depleted conditions, there was now no obvious increase in dark event rate over time, but a stable high rate of ~20 event s$^{-1}$ was observed from the moment of establishing the whole-cell configuration until the onset of the full RDC (Figure 1D). We suggest that this apparently saturated event rate is likely to approximate the true rate of spontaneous G-protein activations, and that most of these fail to generate a dark event under normal conditions in wild-type photoreceptors (see Discussion).

### Spontaneous dark noise in alleles of ninaC

An increased spontaneous dark noise phenotype was first reported in ninaC null mutants (ninaC$^{P235}$) and ninaC$^{4174}$ mutants lacking the rhabdomeric p174 isoform of the NINAC protein (Hofstee et al. 1996). NINAC is a multifunctional non-conventional myosin III
with a kinase domain, two calmodulin binding domains and a myosin domain (review: Montell 1999). To determine which part of the protein was important for regulating dark noise we recorded from nine transgenic ninaC lines, expressing constructs with targeted deletions or mutations in different domains (see methods). Apart from ninaC<sup>P235</sup> and ninaC<sup>Δ174</sup>, the only other line reproducing the dark noise phenotype was ninaC<sup>Δ1501E</sup> – a point mutation in the C-terminal PDZ-domain binding motif that has been reported to anchor NINAC p174 to the INAD scaffolding protein (Wes et al. 1999). ninaC<sup>Δ1501E</sup> photoreceptors displayed high levels of spontaneous dark events (Figure 2) that were indistinguishable from those in ninaC<sup>P235</sup> null mutants in terms of both amplitude (mean, 3.8 ± 0.2 pA; n = 13; two-tailed unpaired t test, p = 0.1) and event rates (mean, 6.9 ± 0.7 events s<sup>-1</sup>; t test, p = 0.7). None of the other physiological phenotypes of the ninaC null or ninaC<sup>Δ174</sup> mutants, which include reduced sensitivity and a prolonged response decay (Hofstee et al. 1996; Porter et al. 1995), were reproduced in ninaC<sup>Δ1501E</sup> (Figure 2C).

We recently reported that dark noise was also substantially increased in mutants (rtp) of a novel rhabdomeric protein, retinophilin (Mecklenburg et al. 2010), which co-immunoprecipitates with NINAC (Venkatachalam et al. 2010). Comparison of dark noise in rtp mutants (Figure 2D-F, rtp data replotted from Mecklenburg et al., 2010) indicated that it was very similar to that in ninaC<sup>P235</sup>, ninaC<sup>Δ1501E</sup> and rdgA<sup>1/+</sup>. Because RTP protein was undetectable in Western blots of ninaC<sup>P235</sup> null mutants (Mecklenburg et al. 2010; Venkatachalam et al. 2010), we concluded that the increase in noise in ninaC mutants was most likely attributable to loss of RTP protein (Mecklenburg et al. 2010). Interestingly however, RTP is expressed at near wild-type levels in the ninaC<sup>Δ1501E</sup> point mutant (Figure 3 and Venkatachalam et al. 2010), leading us to propose that it is the association of RTP and NINAC with the INAD complex that is essential for suppressing dark noise.

**G<sub>αq</sub>, RTP ands DGK protein levels**

To control for the possibility that some of the dark noise phenotypes reported in this study might be due to be compensatory regulation of one or more of the genes implicated in controlling spontaneous noise, we ran Western blots to estimate the level
of expression of DGK, Gqα subunit and RTP in heads from various mutant
backgrounds. As shown in Figure 3, DGK was normally expressed in ninaC\(^{P^{235}}\),
ninaC\(^{I1501E}\) and rtp mutants; Gq\( \alpha \) was expressed normally in rdgA/+; ninaC\(^{P^{235}}\),
ninaC\(^{I1501E}\) and rtp mutants. RTP was expressed normally in rdgA\(^{I/+}\) and essentially
eliminated in ninaC\(^{P^{235}}\) as previously reported (Mecklenburg et al. 2010). RTP levels in
ninaC\(^{I1501E}\) were somewhat reduced (~45%), most likely because NINAC protein was
similarly reduced in this transgenic line (in which only one copy of the NINAC\(^{I1501E}\) was
expressed). To test whether the reduced levels of NINAC and/or RTP might have
contributed to the ninaC\(^{I1501E}\) phenotype, we recorded from photoreceptors from
ninaC\(^{P^{235}}/+\) heterozygotes (which have similarly reduced RTP protein levels). We
found no significant increase in dark event rate (3.1 ±0.4 events s\(^{-1}\) n=12) or amplitude
(2.3 ± 0.1 pA) above wild-type levels (p ≥ 0.15 for rate, and p ≥ 0.25 for amplitude).

**Dark noise is Gq\( \alpha \) dependent but independent of rhodopsin**

Dark noise events in wild-type photoreceptors are proposed to reflect spontaneous G-
protein activation and are effectively eliminated in Gq\( \alpha \) hypomorphic mutants (Elia et al.
2005; Hardie et al. 2002). To test whether the same is true for the enhanced noise in
rdgA\(^{I/+}\), ninaC and rtp photoreceptors, we introduced each genotype into a Gq\( \alpha \)
hypomorphic background. Indeed, dark noise was almost completely eliminated in
rdgA\(^{I/+}; \text{Gq}^{I} \text{ ninaC}^{P^{235}} \text{, Gq}^{I} \text{ and Gq}^{I}; \text{rtp} \) double mutants. In all double mutants, the
mean amplitude of any rare residual dark events was approximately halved to ~2 pA
compared to respective single mutants (Figure 4A,B).

In vertebrate rods, the G-protein (transducin) can be activated with greatly reduced
efficiency by bleached opsin (review Fain 2001). To ask whether Gq-protein activation
in the dark in *Drosophila* was truly spontaneous, or whether it might result from rare
activation by rhodopsin molecules in a non-activated state, we investigated null mutants
of the rhodopsin (*Rh1*) gene, ninaE (O’Tousa et al. 1985). Recordings from ninaE\(^{417}\)
mutants also revealed small spontaneous miniature events in complete darkness that
were indistinguishable from those seen in wild-type photoreceptors (Figure 4D-F). To test whether rhodopsin is also dispensable for the enhanced dark noise production in rdgA1/+ we generated rdgA1/+;ninaE117 double mutants and found that the phenotype (high rate of spontaneous dark events of larger amplitude) was similar to rdgA1/+ controls (Figure 4D-F). These results are consistent with a previous study reporting that enhanced dark noise in Gβ+/ heterozygotes could still be observed in flies reared on a vitamin-A deprived diet (Elia et al. 2005) and confirm that Goq dependent dark noise is generated independently of rhodopsin.

Dark noise is predominantly mediated by TRP channels

The wild-type light-induced conductance is mediated by two distinct Ca²⁺ permeable cation channels, TRP and TRPL (Hardie and Minke 1992; Niemeyer et al. 1996; Reuss et al. 1997), both activated downstream of Goq and PLC. To test which are responsible for dark noise we recorded from trp and trpl null mutants to isolate the respective channels (Figure 5A). Miniature dark events similar to those in wild-type photoreceptors were observed in trpl mutants lacking TRPL channels, showing no statistically significant difference in event rate or amplitude. In marked contrast, spontaneous dark events were largely absent in null trp343 mutants lacking the highly Ca²⁺-permeable TRP channels, with at most ~0.1 events s⁻¹ (n = 6). We also generated rdgA1/+;trp343 and ninaC335;trp343 double mutants; both again resulted in greatly reduced frequency of dark events (Figure 5B,D), while their mean amplitude was reduced to ~2 pA. We also perfused wild-type and rdgA1/+ heterozygote photoreceptors with La³⁺, which completely blocks all TRP channel activity (Hardie and Minke 1992; Niemeyer et al. 1996; Reuss et al. 1997). La³⁺ (80 µM) effectively eliminated most dark events within seconds, leaving only occasional small, residual events presumably mediated by TRPL channels (Figure 5C).

These results indicate that dark noise is predominantly mediated by TRP channels under the conditions of our experiments. Assuming a single channel conductance of ~8 pS
(Henderson et al. 2000) corresponding to single-channel current of 0.65 pA at resting potential, each miniature dark event of ~2 pA in amplitude in WT and trpl mutants can be interpreted as the summed opening of 2 to 3 TRP channels, while in trp mutants, the residual dark bumps probably represent the opening of single TRPL channels that have an estimated single-channel current of 2.5 pA, based on an effective single channel conductance of 35 pS (Henderson et al. 2000).

**Ca^{2+} dependence of spontaneous dark noise**

Ca^{2+} is critical in determining the shape and size of quantum bumps (Henderson et al. 2000), and recently it was reported that dark events were essentially eliminated in the absence of external Ca^{2+} in the bath (Katz and Minke 2012). To investigate the Ca^{2+} dependence of dark noise more quantitatively and in the physiological range, we exploited the Na^{+}/Ca^{2+} exchanger encoded by the calx gene (Schwarz and Benzer 1997). This is the major mechanism for Ca^{2+} extrusion in the photoreceptors, and dark resting cytosolic Ca^{2+} levels in calx mutant photoreceptors are elevated to ~400 nM (Wang et al. 2005). As shown in Figure 6, calx mutants invariably displayed a high frequency of spontaneous miniature bumps, with event rates at least twice as fast as in wild-type (mean, 6.0 ± 1.4 events s^{-1}; n = 6; p = 0.002 cf wild-type). The events again appeared to represent spontaneous G-protein activation as they were essentially eliminated in a \(\text{Ga}^q;\text{calx}\) double mutant. Conversely, overexpression of CalX in \(p\text{CalX}\) transgenic flies, which can be expected to lower the resting microvillar Ca^{2+} to levels below wild-type levels (normally ~ 150 nM), resulted in the near elimination of dark noise. In contrast to the effects of ninaC, rtp and rdgA/+ mutations, the mean amplitude of dark events in calx mutants was slightly, but significantly smaller (p = 0.0002) than in wild-type (Figure 6B). This is consistent with the known inhibition of the light-sensitive channels by Ca^{2+} (Gu et al. 2005); (see also Figure 6E) and also implies that the effects of the ninaC, rtp or rdgA/+ mutations (all of which increase dark event amplitude) are not mediated indirectly by an effect on Ca^{2+} levels.

To quantify the Ca^{2+} dependence of miniature dark events, we used ion substitution to control the transmembrane Na^{+} gradient (\(\text{Na}^i/\text{Na}^o\)) and thereby manipulate intracellular
Ca\textsuperscript{2+} (Ca\textsubscript{i}) according to the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger equilibrium (Gu et al. 2005; Wang et al. 2005).

\[
[Ca_i] = [Ca_o] \left[\frac{[Na_i]}{[Na_o]}\right]^3 e^{\frac{EF}{RT}}
\]

Eq. 1

In wild-type photoreceptors recorded with 10 mM Na\textsubscript{i} and 120 mM Na\textsubscript{o} (i.e. control bath, predicted Ca\textsubscript{i} at -70 mV, 55 nM), the dark event rate of 3.1 ± 0.2 events s\textsuperscript{-1} (n = 16; Figure 6C, E) was slightly above controls (t test, p = 0.03) without added internal Na\textsuperscript{+} in the electrode. As Na\textsubscript{o} was lowered (substituted for Li\textsuperscript{+}), the rate of dark events increased two-three fold as Ca\textsubscript{i} was raised (nominally) from 55 nM to 1.5 \textmu{}M (Figure 6C-E). Dark event amplitude, was significantly decreased (e.g. 2.23 ± 0.05 pA, n = 16 at 55 nM, cf 1.86 ± 0.14 pA at 180 nM, n = 8; t test, p = 0.00005), although not as much as the light-induced current, which was inhibited with an IC\textsubscript{50} of ~1 \textmu{}M as previously reported (Gu et al. 2005). Nominal Ca\textsuperscript{2+} concentrations beyond 1 \textmu{}M were not tested due to deteriorating signal-to-noise ratio that prevented discrete events from being reliably resolved.

Ca\textsuperscript{2+} sensitizes TRP channels

In principle there are several mechanisms by which Ca\textsuperscript{2+} could increase the rate of dark events. For example, we previously speculated that Ca\textsuperscript{2+} influx may sensitize the light-sensitive channels, by shifting their effective dose response function for excitatory second messenger(s) (Hardie et al. 2002). The identity of the second messenger is still debated (e.g. Delgado and Bacigalupo 2009; Hardie and Franze 2012; Huang et al. 2010; Lev et al. 2011); however, exogenously applied poly-unsaturated fatty acids (PUFAs) such as linolenic acid (LNA) are effective agonists, which act downstream of PLC probably acting directly on the channel or their lipid environment (Chyb et al. 1999; Hardie et al. 2003 ; Parnas et al. 2009). We therefore investigated the sensitivity of the light-sensitive channels to LNA as a function of cytosolic Ca\textsuperscript{2+} manipulated, as above, by exploiting the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange equilibrium (Figure 7). To assess sensitivity we
applied a standard concentration (20 μM) of LNA by a puffer pipette and measured the
time taken for a criterion current of 10 pA to develop. Sensitivity of TRP and TRPL
channels were investigated separately using \textit{trp} and \textit{trpl} mutants. In \textit{trp} mutants, where
only TRPL channels are present, the time taken to activate a criterion current was ~50 s
and independent of Cai in the range tested. By contrast, the time required to activate TRP
channels (in \textit{trpl} mutants) was very sensitive to imposed cytosolic Cai, accelerating from
~80 s at (nominally) 100 nM to ~20 s at concentrations above 1 μM with an EC50 of
~300 nM. A similar sensitisation to LNA by raised Ca2+ was also observed in a
\textit{norpA}^{P24};\textit{trpl} double mutant indicating that the effect was mediated independently of
PLC, and most likely at the level of the channel (Figure 7).

The ability to facilitate dark events and LNA-induced channel activity by raising Ca2+ in
this manner contrasts with the effect of Ca2+ on the wild-type light response, where
raising Cai via the Na+/Ca2+ exchange equilibrium only inhibited the response (Gu et al.
2005). We suspected that this might be because quantum bumps in wild-type
photoreceptors already represent an effectively near-saturating response at the level of
individual microvilli: i.e. quantum efficiency (Q.E.) is near 100% and the majority of
TRP channels in a microvillus are activated following successful absorption of a photon.
We therefore also investigated the effect of raising Ca2+ in \textit{Gaq}\textsuperscript{4} hypomorphs, where
Q.E. is reduced, with many photon absorptions failing to reach threshold for bump
generation (Hardie et al. 2002) – see also discussion. Indeed in \textit{Gaq}\textsuperscript{4} the response to
light was facilitated 2-3 fold by Cai in a narrow, submicromolar (~100-400 nM) range,
similar to the Ca2+ dependence of dark event rate (Figure 7D-F). The effect appeared to
be largely due to an increase in Q.E. as bump amplitudes were unaffected or slightly
reduced – again presumably because of the competing effect of Ca2+ dependent
inhibition. At higher concentrations, Ca2+ dependent inhibition dominated, resulting in
suppression of the light-induced currents as found in wild-type photoreceptors.
Mutations that enhance dark noise also facilitate light responses in norpA and \(G \alpha q\) mutant backgrounds.

As previously reported, hypomorphic \(G \alpha q\) and norpA mutants show a several-fold reduction in quantum bump amplitude (Hardie et al. 2002), and variable reduction (up to ~1000-fold in \(G \alpha q^I\)) in effective Q.E. (Hardie et al. 2002; Scott et al. 1995). This implies that normal bump generation and amplification requires the activation of multiple G-proteins. Since dark events were amplified ~2-fold in \(rdgA^1/+, ninaC\) and \(rtp\) mutants, we asked whether light-evoked quantum bumps in \(Gaq^I\) and norpA\(^{P16}\) hypomorphs would be similarly enhanced in these backgrounds.

In agreement with an earlier study (Hardie et al. 2002), we confirmed that quantum bump amplitudes in \(rdgA^1/+;Gaq^I\) and \(rdgA^1/+;norpA^{P16}\) were increased ~2-fold relative to the respective \(Gaq^I\) or norpA\(^{P16}\) single mutants. New here, we found that bump amplitudes in both \(ninaC^{P235};Gaq^I\) and \(Gaq^I;rtp\) (Figure 8), as well as \(norpA^{P16};ninaC^{P235}\) and \(norpA^{P16};rtp\) (Figure 9) were also all similarly increased, supporting roles for DGK, NINAC and RTP in regulating bump amplification as well as noise suppression. The average waveform and amplitude of quantum bumps in \(Gaq^I\) and norpA\(^{P16}\) double mutants were indistinguishable from dark events in each respective single mutant (i.e. \(rdgA^1/+\), \(rtp\) or \(ninaC\)) genotype. Significantly, Q.E. was enhanced in all three \(Gaq^I\) double mutants with respect to \(Gaq^I\) single mutants (Figure 8).

Improvement in Q.E. in these flies implies that \(rdgA^1/+\), \(ninaC\) and \(rtp\) mutations all resulted in a higher probability that activated G-proteins in \(Gaq^I\) successfully generate a bump – and conversely that in \(Gaq\) hypomorphs many (single) G-protein activations fail to generate a quantum bump (see also Hardie et al. 2002). This suggests that reduction/removal of DGK, NINAC and RTP lowers the effective threshold which G-proteins and PLC must overcome for bump generation. The increase in bump amplitude can be explained similarly: lowering the threshold for channel activation allows more channels to be recruited per bump.
Discussion

Spontaneous, dark noise in photoreceptors sets a fundamental limit to absolute visual thresholds (Aho et al. 1988; Rieke and Baylor 1996) and in Drosophila is manifest as small (~2 pA) quantum bump-like events occurring at rates of ~2-3 s⁻¹. Previous studies indicated that these dark events reflect spontaneous G-protein activations (Hardie et al. 2002), and that a supra-stoichiometric Gβ/Gα subunit ratio (~2-fold excess of Gβ) is required to keep the rate even this low (Elia et al. 2005). Our results strengthen and extend this conclusion, by showing that dark events are generated independently of rhodopsin, but are Gαq dependent in a variety of mutant backgrounds and are mediated predominantly by the major light-sensitive channel (TRP). Our results suggest that spontaneous G-protein activations are at least ~10 times more frequent than indicated by the wild-type dark noise, but that potential dark noise is normally suppressed by mechanisms involving NINAC, retinophilin (RTP), the INAD scaffolding protein, diacylglycerol kinase and a Ca²⁺ dependent threshold for channel activation. Our results further indicate that all these mechanisms regulating dark noise are also involved in light-induced quantum bump amplification.

Spontaneous G-protein activation

Little information is available on spontaneous G-protein activation rates in vivo in any system (e.g. Siekhaus and Drubin 2003). Photoreceptors, with their high density of signalling components, would seem well-suited for studying spontaneous G-protein activation. However, dark noise in vertebrate photoreceptors is dominated by spontaneous activation of phosphodiesterase, or spontaneous thermal isomerization of rhodopsin (Luo et al. 2011; Rieke and Baylor 1996; 2000). In Drosophila, thermal isomerizations are rare (< 1 min⁻¹, Henderson et al. 2000), and whilst PLC has measurable basal activity, it is less than ~1/1000⁰ of the G-protein activated PLC activity and does not normally overcome threshold for channel activation (Hardie et al. 2004). Fly photoreceptors thus afford a rare opportunity to study spontaneous G-protein activation rates. In wild-type photoreceptors, the spontaneous dark event rate of 2-3 s⁻¹
could be accelerated in various mutant backgrounds, by ATP depletion or by raising cytosolic Ca\(^{2+}\). It seems unlikely that these diverse manipulations directly affected the G-protein activation rate, and hence the wild-type dark event rate probably greatly underestimates the underlying rate of spontaneous G-protein activation.

This implies that most single G-protein activation events normally fail to overcome threshold for channel activation. A similar conclusion was also reached in a recent independent study (Katz and Minke 2012) and first proposed in an earlier study showing that Q.E. is greatly reduced in \(G_\alpha q\) hypomorphs (Hardie et al. 2002). We suggest that the true rate of spontaneous G-protein activation is of the order of the maximum we observed (~ 20 s\(^{-1}\) in \(rdgA^1/+\) heterozygotes under ATP-deprived conditions). Assuming 2\(\times\)10\(^6\) G proteins per cell (Hardie and Raghu 2001), this represents an average lifetime (per G-protein molecule) of ~30 hours. Heterotrimeric G-proteins would thus seem rather stable, albeit much less so than the visual pigment rhodopsin, which can have a theoretical half-life of hundreds of years (Baylor et al. 1980)!

**Dark-noise suppression by DGK, NINAC and RTP**

Increased dark noise in \(ninaC\) and \(rtp\) mutants had been reported previously (Hofstee et al. 1996; Mecklenburg et al. 2010), and attributed to loss of RTP, since RTP protein is absent in \(ninaC^{P235}\) null mutants (Mecklenburg et al. 2010). Here, we found that a point mutation in NINAC’s C-terminal (\(ninaC^{I1501E}\)), required for its interaction with the INAD scaffolding protein (Wes et al. 1999), fully, and uniquely reproduced the \(ninaCI1501E\) null mutation in increasing dark noise, without mimicking any other electrophysiological phenotypes of the null mutant. NINAC and RTP protein are both expressed at near normal levels in \(ninaC^{I1501E}\) (Venkatachalam et al. 2010), leading us to propose that incorporation of RTP in the INAD complex via attachment to NINAC is required for suppressing noise. Also new here, we report a dark noise phenotype, similar to that in \(ninaC^{I1501E}\) and \(rtp\), in \(rdgA^1/+\) heterozygotes expressing reduced levels of DGK.
In principle, DGK, NINAC and RTP might maintain a low rate of dark events by suppression of spontaneous G-protein activation. However, there is no obvious precedent for such a mechanism, and it would be difficult to reconcile the equivalent effect of \textit{ninaC}, \textit{rdgA} and \textit{trp} mutations on both spontaneous dark noise and bump amplification in \textit{Ga}q and \textit{norpA} hypomorphs with such an explanation. We suggest instead that DGK and RTP/NINAC modulate the downstream effects of spontaneous G-protein activations, either by regulating the availability of excitatory second messenger(s) that lead to channel openings or by modulating the sensitivity of the channels to excitatory messenger.

Thus, following spontaneous G-protein activations, we propose that most \textit{Gq}α-PLC complexes inactivate before generating sufficient excitatory messenger(s) to activate any channels. However, if DGK activity is reduced (in \textit{rdgA}^{+/+} heterozygotes), then PLC’s products (e.g. DAG, protons and PIP_{2} reduction) can now overcome threshold more readily, resulting in increased probability of generating a dark event. On the one hand, the similar effects seen in \textit{ninaC} and \textit{rtp} mutants might be explained if a NINAC/RTP/INAD complex is required to maximise effective DGK activity (e.g. by incorporating DGK itself within the INAD complex). However, because available evidence indicates that DGK is localised outside the rhabdomere (Masai et al. 1997), and because both PLC and TRP are integral components of the INAD scaffolding complex (Chevesich et al. 1997; Huber et al. 1996; Shieh and Zhu 1996; Tsunoda et al. 1997), it can also be speculated that interactions between INAD/NINAC and RTP may be involved in regulating either PLC activity or the sensitivity of the TRP channel to excitatory messenger(s).

At face value the role of DGK in dark noise suppression supports a role for DAG (and/or downstream PUFA metabolites) in channel activation as previously discussed (Hardie et al. 2003; Leung et al. 2008; Lev et al. 2011; Raghu et al. 2000). A detailed discussion of the mechanism of excitation is beyond the scope of this study; however, a recent study suggested that the light-sensitive channels may be activated by the combination of PIP_{2}
depletion – possibly acting via mechanical effects on the lipid bilayer (Hardie and Franz 2012)- and the proton released by the PLC reaction rather than by DAG (Huang et al. 2010). Since DGK’s product (PA) is a potent positive regulator of PIP 5-kinase (Cockcroft 2009; Jenkins et al. 1994), rdgA1/+ mutants might have significantly impaired ability to rapidly resynthesise PIP2, potentially also accounting for the increased sensitivity and dark noise phenotypes in rdgA1/+ (reviews: Hardie 2011; Raghu and Hardie 2009).

Ca²⁺ dependence of spontaneous dark noise

Importantly, we also found that dark event rate, but not amplitude, was increased by raising cytosolic Ca²⁺ via manipulating Na⁺/Ca²⁺ exchange (Wang et al. 2005). Conversely, dark noise was almost eliminated in photoreceptors overexpressing CalX, which presumably results in a lower Caᵢ. We again consider it unlikely that Caᵢ affects the spontaneous rate of G-protein activation and therefore these results suggest the existence of an effective Ca²⁺ dependent threshold, which must be reached for successful generation of a dark event. The failure of raising Ca²⁺ to increase dark event amplitude is presumably explained by the competing effect of Ca²⁺ dependent inhibition of the light-sensitive channels, which has an IC₅₀ of 1 μM (Gu et al. 2005).

Positive feedback by Ca²⁺ is a key feature of phototransduction in microvillar photoreceptors (Hardie 1991), but the underlying mechanisms are poorly understood. There are at least two targets where Ca²⁺ might act to facilitate channel activation: namely PLC and the channels themselves (see also Katz and Minke 2012). PLC is a Ca²⁺ dependent enzyme (Rhee 2001), and light-induced PLC activity in Drosophila eyes is facilitated by Ca²⁺ in the sub-micromolar range both in vitro (Running Deer et al. 1995) and in vivo (Hardie 2005). However, TRP (but not TRPL) channels can be facilitated by caged Ca²⁺ on a sub-millisecond timescale suggesting a direct effect on the channels themselves (Hardie 1995). In the present study, we found that the sensitivity of TRP (but not TRPL) channels to exogenous agonist (LNA) was enhanced by Ca²⁺ with an EC₅₀ of ~ 300 nm, downstream of PLC (Figure 8), supporting a previous suggestion that Ca dependent positive feedback functions by lowering the threshold for channel
activation (Hardie et al. 2002). Both mechanisms (facilitation of TRP channels or PLC) clearly have the potential to increase the probability that an activated G-protein/PLC complex may activate the channels: on the one hand by hydrolysing more PIP$_2$, and on the other by lowering the threshold of PIP$_2$ derived excitatory “messenger” required to activate the channels.

Concluding remarks (was 190)

Our results indicate roles for DGK and INAD/NINAC/RTP in suppressing dark-noise. Importantly, all mutations that enhanced dark noise also increased Q.E. and bump amplitude in $G_{\alpha q}$ and norpA$^{P16}$ hypomorphs, suggesting common roles in regulating bump amplification. We propose that DGK, NINAC and RTP (via INAD) all function to reduce the probability that single activated G$_{\alpha q}$/PLC complexes overcome a Ca$^{2+}$ dependent threshold for channel activation. The downstream mechanisms require further investigation, but probably include regulation of the TRP channel’s sensitivity to excitatory messenger, as well as the generation and/or degradation of excitatory messenger. The Ca$^{2+}$ dependent threshold seems critical to minimize dark noise from spontaneous (single) G-protein activation, whilst maximizing the detectability of light-evoked quantum bumps, which require sequential activation of 5 or so G-proteins and PLC molecules within the finite but variable latency period of 20-100 ms (Hardie et al. 2002). More specifically, under physiological conditions, low Ca$_i$ in the dark sets a high threshold for channel activation: one the one hand this suppresses generation of dark events by single, spontaneous G-protein activations; on the other hand, it also delays light-induced quantum bump initiation until sufficient G-protein and PLC molecules are activated to allow build up of excitatory “messenger” throughout most of the microvillus. Once the first channel opens, Ca$^{2+}$ influx rapidly floods the entire microvillus lowering the threshold for the remaining channels, which can then be activated by what were previously only subthreshold (but finite) levels of excitatory messenger, thus generating an amplified full size quantum bump. Computational models based on this conceptual model (postulated by Hardie et al. 2002; Henderson et al. 2000) successfully recreate quantum bump kinetics, amplification and latency distributions.
Importantly, they also predict that single G-protein activations lead to the generation of only small bumps with much reduced probability or quantum efficiency (Pumir et al. 2008).

**Acknowledgements.**

The authors thank Drs B Minke, B Katz and J. O’Tousa for comments on an earlier version of the manuscript. We also thank Dr Craig Montell for supplying his collection of transgenic *ninaC* alleles. This research was supported by the Biotechnology and Biological Sciences Research Council (BBSRC Grant BB/G006865/1 to RCH, and BBSRC doctoral award to BC) and the Cambridge-Nehru Trust (SS).

**Abbreviations**

*ninaC* no inactivation no after potential C (gene encoding myosin III)

*inaD* inactivation no after potential D (gene encoding INADscaffolding protein)

*trp* transient receptor potential

PLC phospholipase C

DGK diacylglycerol kinasse

RDGA retinal degeneration A (gene encoding DGK)

*norpA* no receptor potential A (gene encoding PLC)
References


Hardie RC. Inhibition of phospholipase C activity in *Drosophila* photoreceptors by 1,2-bis(2-aminophenoxo)ethane N,N,N',N'-tetraacetic acid (BAPTA) and di-bromo BAPTA. *Cell Calcium* 38: 547-556, 2005.


Figure legends

Figure 1 Increase in dark noise in *rdgA<sup>+/+</sup>* heterozygotes or by ATP depletion

* A* Spontaneous noise recorded in wild-type (WT) and *rdgA<sup>+/+</sup>* photoreceptors with normal electrode solution (+ATP) in complete darkness at specified time (minutes after establishing whole-cell (w-c) recording configuration). Dark events were larger and more frequent in *rdgA<sup>+/+</sup>* mutants. In both cases, the frequency, but not amplitude of spontaneous events subsided over the recording period, as plotted in *B*, the amplitude and *C*, event rate of spontaneous dark events (mean ±SEM *rdgA<sup>+/+</sup>* n = 2, WT n=3). *D top*: spontaneous dark noise recorded immediately (< 10 s) after establishing the w-c mode in WT photoreceptor with electrode containing no nucleotide additives (-ATP). The plot shows event rate increased rapidly during the first few minutes (n=4, error bars not shown), before events fused to form rundown current (RDC: example trace shown below). Also plotted (open symbols) are event rates in two *rdgA<sup>+/+</sup>* photoreceptors recorded without ATP. *E* averaged dark event waveforms (average of ≥ 60 events in a representative cell of each genotype, aligned by the rising phase) in *rdgA<sup>+/+</sup>* and WT (- ATP) were approximately two times larger than events in WT (+h ATP), but smaller than light-induced quantum bumps. *F* Summary scatter plot of event rate against mean event amplitude for *rdgA<sup>+/+</sup>* cells (n = 20), WT (with ATP, n = 8; without ATP, n = 4), obtained during the first 100 seconds of w-c recording. All *rdgA<sup>+/+</sup>* and WT (-ATP) photoreceptors are distinct from miniature events in WT control (+ATP); regression line show no correlation between event rate and amplitude.
Figure 2 High rates of dark noise in *ninaC* mutants

A a high rate of spontaneous dark events was observed in *ninaC*\(^{P235}\) and *ninaC*\(^{d501E}\) mutants relative to WT (bottom trace). B the average dark event waveforms (≥ 100 events in a representative cell of each genotype, aligned by the rising phase) in both *ninaC* alleles are larger than in WT (see also F). C Macroscopic responses to brief flashes (~100 photons, arrow) in *ninaC*\(^{d501E}\) and *rdgA*\(^{1/+}\) were similar to wildtype. As previously described *ninaC*\(^{P235}\) has reduced sensitivity and a deactivation defect (Hofstee et al. 1996) D Summary scatter plots of event rate against mean event amplitude (regression line shows no correlation) for: *ninaC*\(^{P235}\) (n = 19), *ninaC*\(^{d501E}\) (n = 13), WT (n = 8), and *rtp* (n=16 data from Mecklenburg et al. 2010). E/F Summary bar graphs showing mean event rate (E) and dark event amplitude (F) in *ninaC* alleles, *rdgA*\(^{1/+}\) and *rtp* mutants. 7 further alleles of *ninaC* (see materials and methods) had low levels of dark noise similar to wild-type (not shown).

Figure 3 Western blots of DGK, G\(\alpha_q\) and RTP

Representative Western blots in wild-type, wild-type (*w\(^{1118}\)*), *ninaC*\(^{d501E}\), *rdgA*\(^{1/+}\), *ninaC*\(^{P235}\), *rtp* and *Gaq*\(^{1}\) probed with antibodies for: A) DGK and B) G\(\alpha_q\)-subunit and RTP; loading controls either actin (A) or tubulin (B). G\(\alpha_q\), RTP and DGK cannot be reliably detected in *Gaq*\(^{1}\), *rtp* or *rdgA*\(^{K860}\) respective null/near null controls. C) Quantitation (relative density) of Western blots relative to wild-type controls normalised to tubulin/actin standards (mean ± SEM, n = 3).

Figure 4 Dark noise is eliminated in *Gaq*\(^{1}\), but not in rhodopsin (*ninaE*) mutants

A Example traces of dark noise: *left* in WT and single mutants - *ninaC*\(^{P235}\), *rdgA*\(^{1/+}\) and *rtp*. Right same genotypes on *Gaq*\(^{1}\) hypomorphic background: dark events were virtually eliminated, leaving only very occasional events (e.g *Gaq*\(^{1}\);*rtp*) B&C Summary bar graphs showing event rates (B) and bump amplitude (C) in *Gaq*\(^{1}\) (n = 5-8), *ninaC*\(^{P235}\) *Gaq*\(^{1}\) (n = 4-19), *rdgA*\(^{1/+}\);*Gaq*\(^{1}\) (n = 11-20) and *Gaq*\(^{1}\);*rtp* (n = 15) along with single mutant (or WT) controls. D example traces of dark noise events in *ninaE*\(^{117}\) and *rdgA*\(^{1/+}\);*ninaE*\(^{117}\) mutants. E&F) Summary bar graphs showing mean event rate (E) and amplitude (F) of spontaneous dark events in WT, *ninaE*\(^{117}\) and *rdgA*\(^{1/+}\) on WT (n = 8) and *ninaE*\(^{117}\) backgrounds (n = 11),

Figure 5 Dark noise is mediated by TRP channels
A Spontaneous dark noise was present in WT (left) and \textit{trpl} (right) photoreceptors, but almost entirely absent in \textit{trp} mutants (middle). \textit{B} High rate of dark noise seen in \textit{rdgA}+/+ and \textit{ninaC}p235 mutants (left) was abolished on \textit{trp} mutant background (middle). \textit{Right}: closer inspection of favourable recordings revealed infrequent residual dark bumps presumably mediated by TRPL channels. \textit{C} 80 µM La3+ (perfusion onset marked by arrow) rapidly suppressed dark noise in WT (n = 3) and \textit{rdgA}+/+ (n = 2) photoreceptors, although some miniature dark bumps were retained. \textit{D} Bar graph (mean ± SEM) for event rates (left) and bump amplitude (right) in \textit{trpl} (n = 3, * no dark events were detected in \textit{trpl};\textit{trp} double mutants), WT (n = 8), \textit{trp} (n = 6), \textit{ninaC}p235 (n = 19), \textit{ninaC}p235;\textit{trp} (n = 5), \textit{rdgA}+/+ (n = 21) and \textit{rdgA}+/+;\textit{trp} (n = 4).

\textbf{Figure 6} Ca2+ dependence of dark noise

\textit{A} Relative to WT (lower trace), dark noise was increased in \textit{calx} mutants (top) but virtually absent in \textit{pCalX} photoreceptors overexpressing \textit{calX} (2\textsuperscript{nd} trace) and in \textit{Gαq;calx} double mutants (3\textsuperscript{rd} trace). \textit{B} Summary of dark event rate and amplitude in WT (n = 8), \textit{pCalX} (n = 6) \textit{Gαq;calx} (n = 11); and \textit{calx} (n = 6). Amplitudes for \textit{pCalX} and \textit{Gαq;calx} not shown due to negligible event count \textit{C} responses to brief (5 ms) flashes (~ 100 effective photons, arrowheads) in WT photoreceptors were slightly (~ 20%) reduced as the control bath solution containing 120 mM Na\textsuperscript{+} was substituted for 80 mM Na\textsuperscript{+} (10mM Na\textsubscript{i}, predicted Ca\textsubscript{i} at -70 mV, 55 nM and 180 nM, respectively). \textit{D} on expanded scale, the rate of spontaneous dark events was increased following perfusion with 80 mM Na\textsuperscript{+}. \textit{E} Ca2+ dependence of dark noise (event rate), and macroscopic light-induced current (LIC). Nominal [Ca\textsuperscript{2+}] calculated from Equation 1, with internal solution containing either 10 or 20 mM Na\textsubscript{i}; holding potential of -70 mV; peak responses (\textit{I}/\textit{I}_{\text{max}}) were normalised to responses immediately before Na\textsuperscript{+} substitution. Mean ± SEM n= 7-18 cells per data point.

\textbf{Figure 7} Ca2+ dependence of TRP channel’s sensitivity to LNA, and light response in \textit{Gαq} mutants

\textit{A} Activation of TRP channels (in \textit{trpl} mutant photoreceptor) by perfusion of 20 µM linolenic acid (LNA, bars): \textit{top} under control conditions (1mM Na\textsubscript{o}, 120 mM Na\textsubscript{i}) and \textit{middle} with Ca\textsubscript{i} nominally at 3µM (perfusion with 60 mM Na; 20 mM Na\textsubscript{i}). Activation was greatly accelerated at the higher [Ca\textsubscript{i}]. Activation in \textit{norpA;trpl} under the same conditions \textit{lower} was similarly fast. \textit{B} By contrast activation of TRPL channels (in \textit{trp} mutants) occurred with a similar delay under
both conditions. In both cases (A & B) the high Ca\textsuperscript{2+} also induced Ca\textsuperscript{2+} dependent inhibition of the TRP (or TRPL) channels and on reperfusion with 120 mM Na (end of bar) extrusion of Ca\textsuperscript{2+} by the CalX exchanger relieved inhibition inducing an increase in current.

C Dose response (mean ± SEM n= 4-6 cells per point) expressed as perfusion time (delay) required to activate a criterion (10 pA) current, plotted against nominal Ca\textsuperscript{2+} concentration (Eq. 1). D Responses to brief (0.5 ms) flashes (~3 x10\textsuperscript{5} WT effective photons) in \textit{Gaq}\textsuperscript{1} hypomorphs were reversibly increased ~2-fold during perfusion with 60 mM Na\textsubscript{o} (10 mM Na\textsubscript{i}), nominally increasing Ca\textsuperscript{2+} from 55 to 434 nM. E Example responses before and during perfusion with 60 mM Na\textsubscript{o}. F Dose response function for light induced current (LIC) in \textit{Gaq}\textsuperscript{1} (solid line, peak response normalised to value before perfusion with low Na\textsubscript{o}, n = 3-5 cells per data point). The Ca\textsuperscript{2+} dependence of TRP channel activation by LNA is replotted from C (trpl LNA) for comparison, along with the increase in dark event rate (normalized to value before perfusion) from Figure 5E. All showed significant facilitation in a similar submicromolar range.

**Figure 8 Facilitation of light responses in \textit{Gaq} by \textit{ninaC}\textsuperscript{P235}, \textit{rtp} and \textit{rdgA}/+ mutations.**

A-E Left: responses to brief flashes (arrow) containing ~100 effective photons in WT and ~7 x 10\textsuperscript{4} photons in \textit{Gaq} mutants, \textit{ninaC}\textsuperscript{P235}, \textit{Gaq}, \textit{Gaq;rtp} and \textit{rdgA}/+;\textit{Gaq} double mutants. Right: quantum bumps induced by brief flashes (arrows) in the same fly strains (superimposed traces, selected for single bumps). Both macroscopic responses and quantum bumps in \textit{Gaq}\textsuperscript{1} were increased on the \textit{ninaC}\textsuperscript{P235} \textit{rtp} and \textit{rdgA}/+ backgrounds (note different scales). F,G Bar graphs showing reciprocal of quantum efficiency (F) relative to WT (1/Q.E.) and bump amplitude (G), in: WT (n = 11), \textit{Gaq}\textsuperscript{1} (n = 6), \textit{ninaC}\textsuperscript{P235}, \textit{Gaq}\textsuperscript{1} (n = 8), \textit{Gaq}\textsuperscript{1};\textit{rtp} (n = 14), and \textit{rdgA}/+;\textit{Gaq}\textsuperscript{1} (n = 3) double mutants.

**Figure 9 Facilitation of light responses in PLC hypomorph \textit{norpA}\textsuperscript{P16} by \textit{rdgA}/+, \textit{ninaC}\textsuperscript{P235} and \textit{rtp} mutations.**

A-D Responses in \textit{norpA}\textsuperscript{P16} hypomorph were enhanced on \textit{ninaC}\textsuperscript{P235}, \textit{rdgA}/+ and \textit{rtp} backgrounds. Left: Macroscopic responses elicited by flashes containing ~7.5 x 10\textsuperscript{5} WT effective photons: right: Representative samples of quantum bumps in darkness after decay of the current to baseline. E,F Bar graphs summarising the effect of \textit{rdgA}/+, \textit{ninaC}\textsuperscript{P235} and \textit{rtp} backgrounds on bump amplitude (E) and peak response to flashes containing ~7.5 x 10\textsuperscript{5} WT effective photons (F), compared to single \textit{norpA}\textsuperscript{P16} mutant controls (labelled +/-). (\textit{P16}, n = 6-14; ≥50 bumps in each cell; double mutants n= 5-19 cells).
A

WT  rtp  ninaC H56HE  ninaC p225  rdgA K989

α-DGK  α-Actin

B

WT  ninaC H56HE  rdgA/+/  ninaC p225  rtp  Gαq

α-Gαq  α-RTP  α-Tubulin

C

DGK

relative density

Gαq

relative density

RTP

relative density
Figures A, B, C, and D show recordings of currents in different genetic backgrounds. Figure A shows recordings for the genotype norpA<sup>P16</sup>. Figures B, C, and D show recordings for the genotypes norpA<sup>P16</sup>;ninaC<sup>P235</sup>, norpA<sup>P16</sup>;rtp, and norpA<sup>P16</sup>,rdgA<sup>1</sup>/+. Figure E shows a bar graph of bump amplitude for the genotypes norpA<sup>P16</sup>, rdgA<sup>1</sup>/+<sup>/+</sup>, rtp, and ninaC<sup>P235</sup>. Figure F shows a bar graph of peak amplitude for the genotypes norpA<sup>P16</sup>, rdgA<sup>1</sup>/+<sup>/+</sup>, rtp, and ninaC<sup>P235</sup>.