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

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Chu B, Liu CH, Sengupta S, Gupta A, Raghu P, Hardie RC. Common mechanisms regulating dark noise and quantum bump amplification in *Drosophila* photoreceptors. *J Neurophysiol* 109: 2044–2055, 2013. First published January 30, 2013; doi:10.1152/jn.00001.2013.—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, 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 *ninaC* (no inactivation no afterpotential; encoding myosin III) and an associated protein, retinophilin (*rtp*). Dark noise was similarly increased in heterozygote mutants of diacylglycerol kinase (*rdgA/+*). Dark noise in *ninaC*, *rtp*, and *rdgA/+* mutants was greatly suppressed by mutations of the G_q α-subunit (*Gαq*) and the major light-sensitive channel (*trp*) but not rhodopsin. *ninaC*, *rtp*, and *rdgA/+* mutations also all facilitated residual light responses in *Gαq* and PLC hypomorphs. Raising cytosolic Ca^{2+} in the submicromolar range increased dark noise, facilitated activation of transient receptor potential (TRP) channels by exogenous agonist, and again facilitated light responses in *Gαq* hypomorphs. Our results indicate that RTP, NINAC, INAD, and diacylglycerol kinase, 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.

TRP channels; phototransduction; retinophilin; INAD; NINAC

PHOTOTRANSDUCTION IN *DROSOPHILA* is mediated by a 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 characterized by high sensitivity, rapid kinetics, and wide dynamic range, in part achieved by the ultracompartmentalization inherent in the microvillar design of the photoreceptors (Fain et al. 2010; Hardie and Postma 2008; Yau and Hardie 2009). Photoisomerization of 1 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 (guanine nucleotide-binding protein) or PLC mutants, bump amplitudes are reduced severalfold, 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 retinal degeneration A (*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 maximize sensitivity, photoreceptors must minimize 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), and dark noise is dominated by small (~2 pA) bumplike events at rates of 2–3/s. These are eliminated in mutants of the G_q 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 2007; Takeshima et al. 2000). 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 on cytosolic Ca^{2+} in the submicromolar range. We confirm that dark noise in wild-type photoreceptors as well as the enhanced dark noise in various mutant backgrounds is G_q protein-dependent. We show that it is 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.

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Importantly, we also show that the various mutations that increase dark noise (*ninaC*, *rtp*, and *rdgA*) all increase the amplitude of the small quantum bumps in *Gαq* and no receptor potential A (*norpA*; gene encoding PLC) 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:

$Gαq^1$, a hypomorph of the *Gαq* expressing ~1% of wild-type protein levels (Scott et al. 1995);

$norpA^{P16}$, PLC hypomorph (Pearn et al. 1996);

$rdgA^1$ (also known as $rdgA^{BS12}$), severe allele of *rdgA* encoding DGK (Masai et al. 1993);

$rdgA^{KS60}$, near protein-null allele of *rdgA* used for validating RDGA antibody (Masai et al. 1993);

$ninaE^{I17}$, null mutant for the rhodopsin of R1–6 (Rh1);

rtp, null mutant of RTP, generated by combining the $Df(3R)rtp^1$ 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 Na^+-Ca^{2+} exchanger (NCX; Wang et al. 2005);

pCalX, flies overexpressing wild-type *calx* gene under the *ninaE* promoter enhancing NCX activity in the photoreceptors approximately five- to eightfold (Wang et al. 2005);

$ninaC^{P235}$, a null mutant of the NINAC myosin III, and $ninaC^{I1501E}$, a transgene with a point mutation in the COOH terminus on $ninaC^{P235}$-null background (Montell and Rubin 1988; Wes et al. 1999). Seven further transgenic mutants of *ninaC* (mutagenized transgenes on null $ninaC^{P235}$ background) were tested, but none showed a dark noise phenotype. These were: $ninaC^{KD}$ (deletion in kinase domain); $ninaC^{45KR}$ (point mutation in kinase domain); $ninaC^{\Delta 132}$ (lacking P132 cytosolic isoform); $ninaC^{\Delta C1}$ and $ninaC^{\Delta C2}$ (lacking calmodulin binding domains); and $ninaC^{1015.4}$ (4-amino acid deletion in myosin domain) and $ninaC^{174PS}$ [mutations of 2 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 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 $MgCl_2$, 1 NAD, and 0.4 Na-GTP, pH 7.15. For manipulation of NCX equilibrium, the intracellular pipette was adjusted to contain either 10 or 20 mM Na-gluconate (and 130 or 120 mM K-gluconate, respectively). Extracellular NaCl was substituted with LiCl (see individual figures), and solutions were applied from a closely positioned puffer pipette. Linolenic acid (LNA) was kept as a 20 mM DMSO stock at −20°C and diluted 1:1,000 in appropriate bath solution. All chemicals were obtained from Sigma-Aldrich. Whole cell voltage clamp recordings were made at room temperature (20 ± 1°C) at −70 mV (including correction for −10-mV junction potential) using electrodes of resistance approximately 10–15 MΩ. Series resistance values were generally <30 MΩ. Series compensation of >80% was applied for macroscopic responses but not for sampling quantum bumps and dark noise (when series resistance errors are negligible). Data were collected and analyzed using Axopatch 200 or 2D amplifiers and pCLAMP8, 9, or 10 software (Molecular Devices, Union City, CA). Quantum bumps and spontaneous dark events were analyzed using the Mini Analysis Program (Jaejin Software, Leonia, NJ) with a threshold criterion of 0.5 pA. Event rates were analyzed automatically, but for event amplitudes and waveforms, all events were individually scrutinized before acceptance. Photoreceptors were stimulated via green light-emitting diode; intensities were calibrated in terms of wild-type effectively absorbed photons by counting quantum bumps at low intensities in wild-type cells.

Western immunoblotting. Heads from flies aged 0–24 h posteclosion were prepared by decapitating flies cooled on ice. Samples were homogenized in 2× SDS-PAGE sample buffer followed by boiling at 90°C for 1 min. Samples were separated using SDS-PAGE and electroblotted onto supported nitrocellulose membrane (Hybond-P or Hybond-C Extra; GE Healthcare). The uniformity of transfer onto membranes was checked by staining with Ponceau S. Following blocking in 5% nonfat milk (Santa Cruz Biotechnology, Santa Cruz, CA), blots were incubated overnight at 4°C in appropriate dilutions of primary antibodies [rabbit anti-α-tubulin (1:1,000 dilution; ab15246; Abcam), rabbit anti-Gαq (1:3,000 dilution), rabbit anti-RTP (1:500 dilution), and rabbit anti-RDGA (1:500 dilution)]. In each case, antibody specificity was confirmed by lack of staining in respective mutants. Immunoreactive protein was visualized after incubation in appropriate dilution of secondary antibodies. The anti-rabbit IgG ECL HRP-Linked Secondary Antibodies (GE Healthcare) were used with 1:5,000 dilution for RTP and α-tubulin blot and 1:10,000 for Gαq. The RDGA blot was probed with 1:10,000 dilution of anti-rabbit IgG coupled to horseradish peroxidase (Jackson ImmunoResearch Laboratories). For Gαq, RTP, and α-tubulin, bands were visualized by the Amersham ECL Plus detection regents (GE Healthcare) and quantitated by G:BOX iChemi imaging system (Syngene). For RDGA, the blots were developed with ECL (GE Healthcare) using LAS 4000 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 DGK, indicating that DGK, which converts diacylglycerol (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 $rdgA^1$/+ heterozygotes (Fig. 1). These had normal morphology and sensitivity to light (Fig. 2*C*); however, immediately after establishing the whole cell configuration in $rdgA^1$/+ photoreceptors, we detected a conspicuously high rate of spontaneous events in complete darkness, consisting of small, quantum bumplike inward currents (Fig. 1*A*). For quantitative analysis, we used event detection software to calculate the rates and amplitudes of dark events over the 1st 100 s after establishing the whole cell configuration. Despite cell-to-cell variation (Fig. 1*F*), event rates in $rdgA^1$/+ were on average four times faster than in wild-type (9.5 ± 0.8 events per second, mean ± SE, n =20), and dark event amplitudes were 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 scatterplot of event rates vs. amplitudes for

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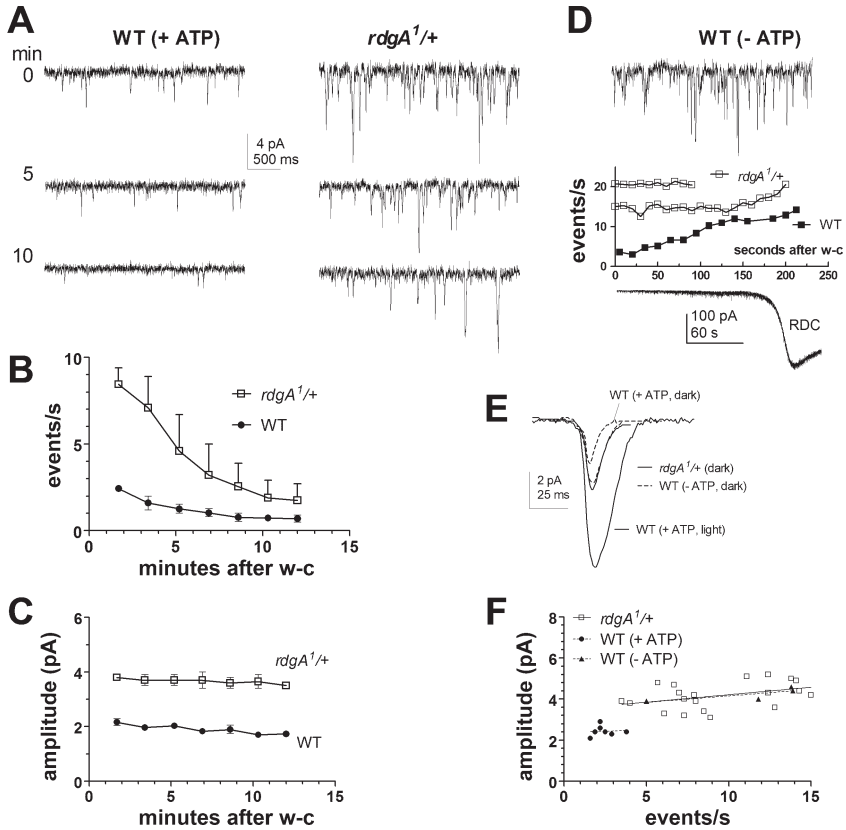

Fig. 1. Increase in dark noise in heterozygote mutants of diacylglycerol kinase (*rdgA*/+) or by ATP depletion. *A*: spontaneous noise recorded in wild-type (WT) and $rdgA^1$/+ photoreceptors with normal electrode solution (+ATP) in complete darkness at specified time [minutes after establishing whole cell (w-c) recording configuration]. Dark events were larger and more frequent in $rdgA^1$/+ 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 ± SE, $rdgA^1$/+ $n = 2$, 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 1st 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 2 $rdgA^1$/+ 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 $rdgA^1$/+ and WT (−ATP) were ~2 times larger than events in WT (+ATP) but smaller than light-induced quantum bumps. *F*: summary scatterplot of event rate against mean event amplitude for $rdgA^1$/+ cells ($n = 20$) and WT (with ATP, $n = 8$; without ATP, $n = 4$) obtained during the 1st 100 s of w-c recording. All $rdgA^1$/+ and WT (−ATP) photoreceptors are distinct from miniature events in WT control (+ATP); regression line shows no correlation between event rate and amplitude.

all cells showed no overlap in distributions (Fig. 1*F*). As in wild-type, the dark event rate (but not amplitude) gradually subsided over a recording period of 10–15 min (Fig. 1, *B* and *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 *Gαq*-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 reexamined 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 per second within ~2 min, after which the events fused to form a noisy inward current, heralding the onset of the full RDC (Fig. 1*D*, *bottom*). 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 $rdgA^1$/+ 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 events per second was observed from the moment of establishing the whole cell configuration until the onset of the full RDC (Fig. 1*D*). 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 $ninaC^{\Delta 174}$ mutants lacking the rhabdomeric p174 isoform of the NINAC protein (Hofstee et al. 1996). NINAC is a multifunctional nonconventional 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 MATERIALS AND METHODS). Apart from $ninaC^{P235}$ and $ninaC^{\Delta 174}$, the only other line reproducing the dark noise phenotype was $ninaC^{I1501E}$, a point mutation in the COOH-terminal PDZ-domain binding motif that has been reported to anchor NINAC p174 to the INAD scaffolding protein (Wes et al. 1999). $ninaC^{I1501E}$ photoreceptors displayed high levels of spontaneous dark events (Fig. 2) that were indistinguishable from those in $ninaC^{P235}$-null mutants in terms of both amplitude (mean, 3.8 ± 0.2 pA; $n = 13$; 2-tailed unpaired *t*-test, $P = 0.1$) and event rates (mean, 6.9 ± 0.7 events per second; *t*-test, $P = 0.7$). None of the other physiological phenotypes of the *ninaC*-null or $ninaC^{\Delta 174}$ mutants, which include reduced sensitivity and a prolonged response decay (Hofstee et al. 1996; Porter et al. 1995), were reproduced in $ninaC^{I1051E}$ (Fig. 2*C*).

We recently reported that dark noise was also substantially increased in mutants (*rtp*) of a novel rhabdomeric protein, RTP (Mecklenburg et al. 2010), which coimmunoprecipitates with

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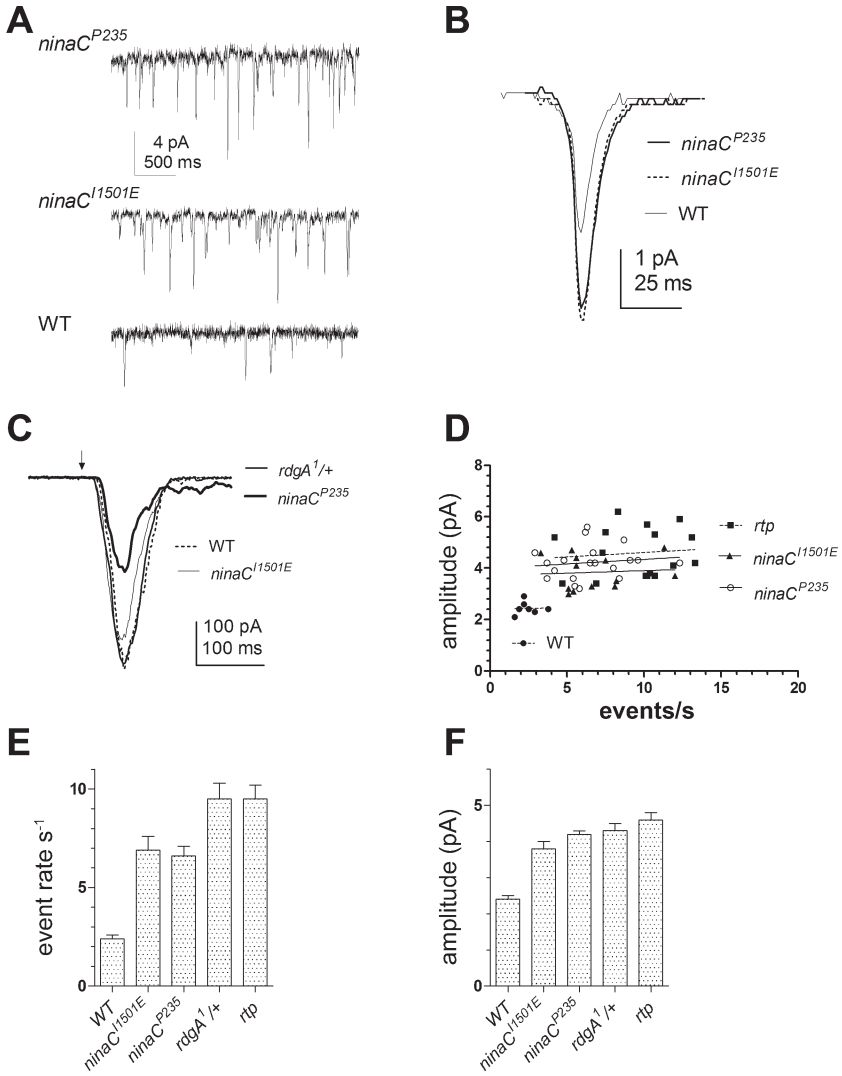

Fig. 2. High rates of dark noise in *ninaC* (no inactivation no afterpotential C; gene encoding myosin III) mutants. *A*: a high rate of spontaneous dark events was observed in $ninaC^{P235}$ and $ninaC^{I1501E}$ mutants relative to WT (*bottom* trace). *B*: the average dark event waveforms (≥100 events 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 $ninaC^{I1501E}$ and $rdgA^1/+$ were similar to WT. As previously described, $ninaC^{P235}$ has reduced sensitivity and a deactivation defect (Hofstee et al. 1996). *D*: summary scatterplots of event rate against mean event amplitude (regression line shows no correlation) for: $ninaC^{P235}$ (n = 19), $ninaC^{I1501E}$ (n = 13), WT (n = 8), and retinophilin (*rtp*; n = 16; data from Mecklenburg et al. 2010). *E* and *F*: summary bar graphs showing mean event rate (*E*) and dark event amplitude (*F*) in *ninaC* alleles, $rdgA^1/+$ and *rtp* mutants. Seven further alleles of *ninaC* (see MATERIALS AND METHODS) had low levels of dark noise similar to WT (data not shown).

NINAC (Venkatachalam et al. 2010). Comparison of dark noise in *rtp* mutants (Fig. 2, *D–F*, *rtp* data replotted from Mecklenburg et al. 2010) indicated that it was very similar to that in $ninaC^{P235}$, $ninaC^{I1501E}$, and $rdgA^1/+$. Because RTP protein was undetectable in Western blots of $ninaC^{P235}$-null mutants (Mecklenburg et al. 2010; Venkatachalam et al. 2010), we had previously 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 $ninaC^{I1501E}$ point mutant (Fig. 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.

Gαq, RTP, and 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, Gαq, and RTP in heads from various mutant backgrounds. As shown in Fig. 3, DGK was normally expressed in $ninaC^{P235}$, $ninaC^{I1501E}$, and *rtp* mutants; Gαq was expressed normally in *rdgA*/+, $ninaC^{P235}$, $ninaC^{I1501E}$, and *rtp* mutants. RTP was expressed normally in $rdgA^1/+$ and essentially eliminated in $ninaC^{P235}$ 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 1 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 $ninaC^{P235}$/+ heterozygotes (which have similarly reduced RTP protein levels). We found no significant increase in dark event rate (3.1 ± 0.4 events per second, n = 12) or amplitude (2.3 ± 0.1 pA) above wild-type levels ($P \geq 0.15$ for rate, and $P \geq 0.25$ for amplitude).

Dark noise is Gαq-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 *Gαq*-hypomorphic mutants (Elia et al. 2005; Hardie et al. 2002). To test whether the same is true for the enhanced noise in $rdgA^1/+$, *ninaC*, and *rtp* photoreceptors, we introduced each genotype into a $Gαq^1$-hypomorphic background. Indeed, dark noise was almost completely eliminated in $rdgA^1/+;Gαq^1$, $ninaC^{P235},Gαq^1$, and $Gαq^1;rtp$ double mutants. In all double mutants, the mean amplitude of any rare residual dark events was approxi-

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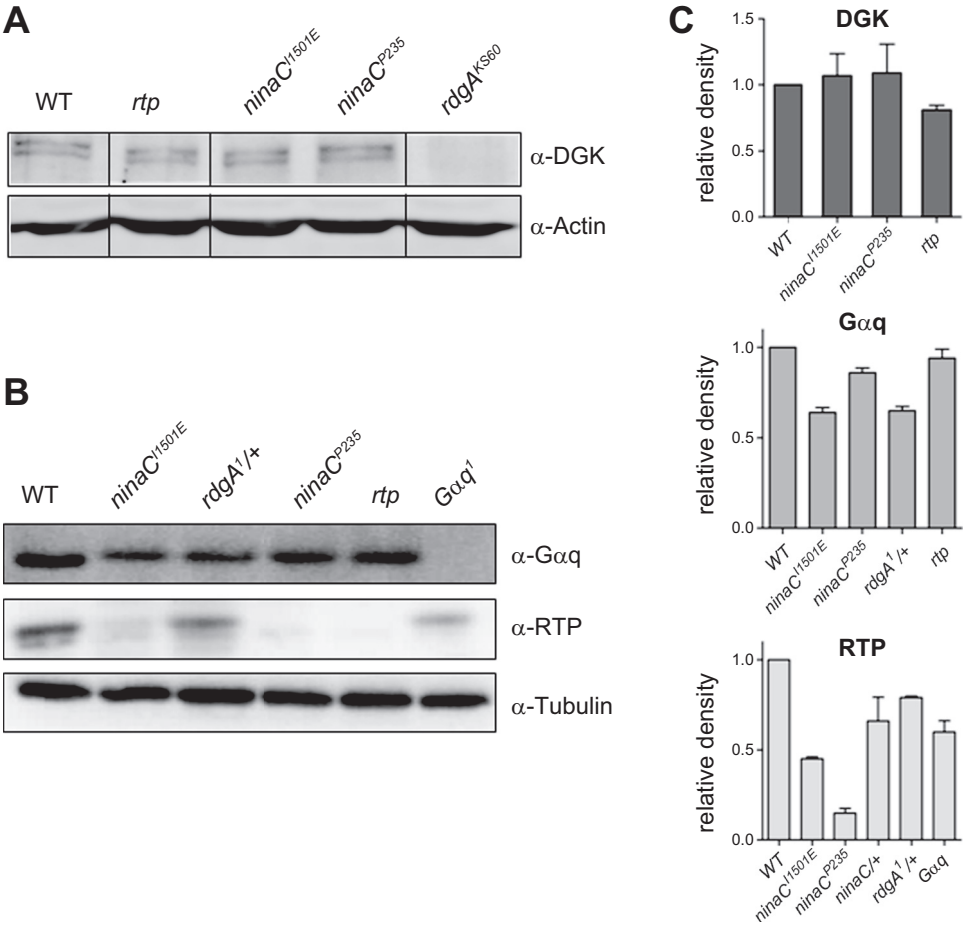

Fig. 3. Western blots of diacylglycerol kinase (DGK), G_q α-subunit (Gαq), and RTP. Representative Western blots in wild-type, wild-type (w^{1118}), $ninaC^{I1501E}$, $rdgA^1/+$, $ninaC^{P235}$, *rtp*, and $G\alpha q^1$ probed with antibodies for DGK (*A*) and Gαq and RTP (*B*) and loading controls of either actin (*A*) or tubulin (*B*). Gαq, RTP, and DGK cannot be reliably detected in $G\alpha q^1$, *rtp*, or $rdgA^{KS60}$ respective null/near null controls. *C*: quantitation (relative density) of Western blots relative to WT controls normalized to tubulin/actin standards (means ± SE, $n = 3$).

mately halved to ~2 pA compared with respective single mutants (Fig. 4, *A* and *B*).

In vertebrate rods, the G protein (transducin) can be activated with greatly reduced efficiency by bleached opsin (reviewed in Fain 2001). To ask whether G_q protein activation in the dark in *Drosophila* was truly spontaneous or whether it might result from rare activation by rhodopsin molecules in a nonactivated state, we investigated null mutants of the rhodopsin (*Rh1*) gene, *ninaE* (O'Tousa et al. 1985). Recordings from $ninaE^{I17}$ mutants also revealed small, spontaneous miniature events in complete darkness that were indistinguishable from those seen in wild-type photoreceptors (Fig. 4, *D–F*). To test whether rhodopsin is also dispensable for the enhanced dark noise production in $rdgA^1/+$, we generated $rdgA^1/+;ninaE^{I17}$ double mutants and found that the phenotype (high rate of spontaneous dark events of larger amplitude) was similar to $rdgA^1/+$ controls (Fig. 4, *D–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 *Gαq*-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 Ca^{2+}-permeable cation channels, TRP and TRPL (Hardie and Minke 1992; Niemeyer et al. 1996; Reuss et al. 1997), both activated downstream of Gαq and PLC. To test which are responsible for dark noise, we recorded from *trp*- and *trpl*-null mutants to isolate the respective channels (Fig. 5*A*). 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 trp^{343} mutants lacking the highly Ca^{2+}-permeable TRP channels, with at most 0.1 events per second ($n = 6$). We also generated $rdgA^1/+;trp^{343}$ and $ninaC^{P235};trp^{343}$ double mutants; both again resulted in greatly reduced frequency of dark events (Fig. 5, *B* and *D*), whereas their mean amplitude was reduced to ~2 pA. We also perfused wild-type and $rdgA^1/+$ heterozygote photoreceptors with La^{3+}, which completely blocks all TRP channel activity (Hardie and Minke 1992; Niemeyer et al. 1996; Reuss et al. 1997). La^{3+} (80 μM) effectively eliminated most dark events within seconds, leaving only occasional small, residual events presumably mediated by TRPL channels (Fig. 5*C*).

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 wild-type and *trpl* mutants can be interpreted as the summed opening of 2–3 TRP channels, whereas 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).

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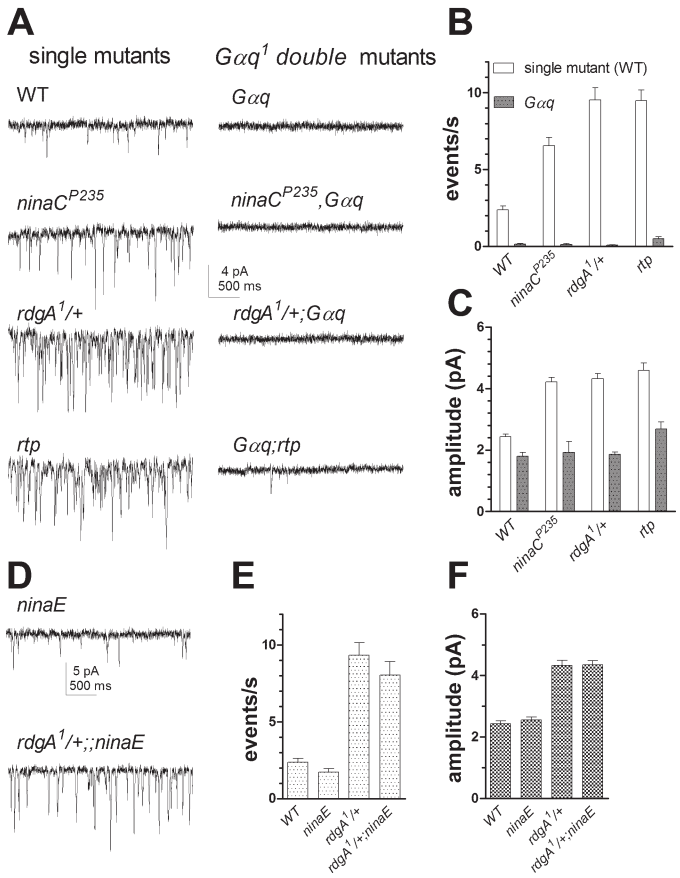

Fig. 4. Dark noise is eliminated in $G\alpha q^1$ but not in rhodopsin (*ninaE*) mutants. *A*: example traces of dark noise. *Left* in WT and single mutants: $ninaC^{P235}$, $rdgA^1/+$, and *rtp*. *Right*, same genotypes on $G\alpha q^1$-hypomorphic background: dark events were virtually eliminated, leaving only very occasional events (e.g., $G\alpha q^1$;*rtp*). *B* and *C*: summary bar graphs showing event rates (*B*) and amplitude (*C*) in $G\alpha q^1$ (n = 5–8), $ninaC^{P235}$;$G\alpha q^1$ (n = 4–19), $rdgA^1/+$;$G\alpha q^1$ (n = 11–20), and $G\alpha q^1$;*rtp* (n = 15) along with single mutant (or WT) controls. *D*: example traces of dark noise events in $ninaE^{I17}$ and $rdgA^1/+$; $ninaE^{I17}$ mutants. *E* and *F*: summary bar graphs showing mean event rate (*E*) and amplitude (*F*) of spontaneous dark events in WT, $ninaE^{I17}$, and $rdgA^1/+$ on WT (n = 8) and $ninaE^{I17}$ backgrounds (n = 11).

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 NCX 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 Fig. 6, *calx* mutants invariably displayed a high frequency of spontaneous miniature events with rates at least twice as fast as in wild-type (mean, 6.0 ± 1.4 events per second; 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 $G\alpha q^1$;*calx* double mutant. Conversely, overexpression of CalX in *pCalX* 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 (Fig. 6*B*). This is consistent with the known inhibition of the light-sensitive channels by Ca^{2+} (Gu et al. 2005; see also Fig. 6*E*) and 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 ($[Na]_i/[Na]_o$) and thereby manipulate intracellular Ca^{2+} ($[Ca]_i$) according to the NCX equilibrium (Gu et al. 2005; Wang et al. 2005):

$$[Ca]_i = [Ca]_o \frac{[Na]_i^3}{[Na]_o^3} e^{-\frac{EF}{RT}} \quad (1)$$

where E is potential difference in volts, R is the gas constant, T is temperature in kelvin, and F is Faraday's constant.

In wild-type photoreceptors recorded with 10 mM $[Na]_i$ and 120 mM $[Na]_o$ (i.e., control bath, predicted $[Ca]_i$ at −70 mV, 55 nM), the dark event rate of 3.1 ± 0.2 events per second (n = 16; Fig. 6, *C* and *E*) was slightly above controls (*t*-test, P = 0.03) without added internal $[Na^+]_i$ in the electrode. As $[Na]_o$ was lowered (substituted for Li^+), the rate of dark events increased two- to threefold as $[Ca]_i$ was raised (nominally) from 55 nM to 1.5 μM (Fig. 6, *C–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_{50} of ~1 μM as previously reported (Gu et al. 2005). Nominal Ca^{2+} concentrations beyond 1 μM were not tested due to deteriorating signal-to-noise ratio that prevented discrete events from being reliably resolved.

Ca^{2+} sensitizes TRP channels. In principle, there are several mechanisms by which Ca^{2+} could increase the rate of dark events. For example, we previously speculated that Ca^{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. 2012); however, exogenously applied polyunsaturated fatty acids (PUFAs) such as 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^{2+} manipulated, as above, by exploiting the NCX equilibrium (Fig. 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 *trp* and *trpl* mutants. In *trp* mutants, where only TRPL channels are present, the time taken to activate a criterion current was ~50 s and independent of $[Ca]_i$ in the range tested. By contrast, the time required to activate TRP channels (in *trpl* mutants) was very sensitive to imposed cytosolic $[Ca]_i$, accelerating from ~80 s at (nominally) 100 nM to ~20 s at concentrations >1 μM with an EC_{50} of ~300 nM. A similar sensitization to LNA by raised

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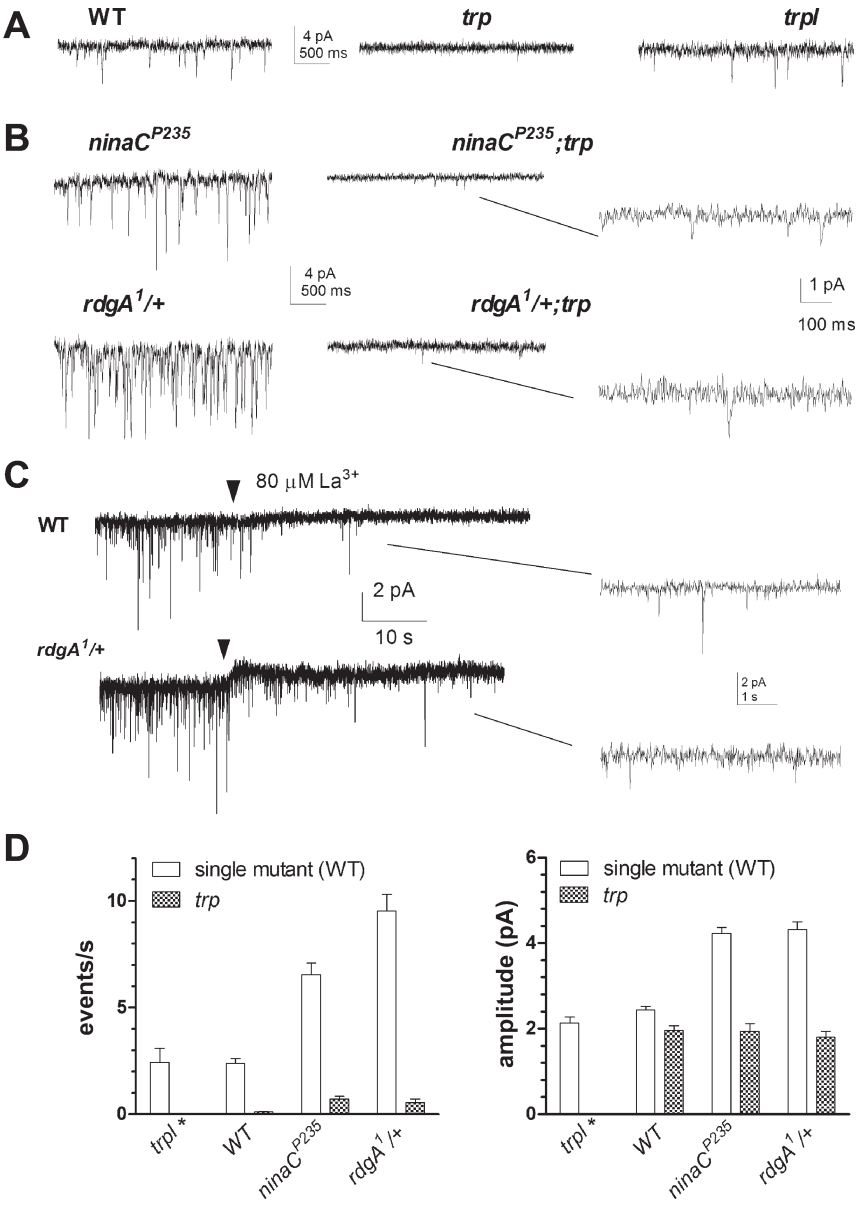

Fig. 5. Dark noise is mediated by transient receptor potential (TRP) channels. *A*: spontaneous dark noise was present in WT (*left*) and *trp*-like (*trpl*; *right*) photoreceptors but almost entirely absent in *trp* mutants (*middle*). *B*: high rate of dark noise seen in $rdgA^1/+$ and $ninaC^{P235}$ mutants (*left*) was abolished on *trp* mutant background (*middle*). *Right*: closer inspection of favorable recordings revealed infrequent residual dark bumps presumably mediated by TRPL channels. *C*: 80 μM La^{3+} (perfusion onset marked by arrowhead) rapidly suppressed dark noise in WT (n = 3) and $rdgA^1/+$ (n = 2) photoreceptors, although some miniature dark bumps were retained. *D*: bar graph (means ± SE) for event rates (*left*) and bump amplitude (*right*) in *trpl* (n = 3; *no dark events were detected in *trpl*;*trp* double mutants), WT (n = 8), *trp* (n = 6), $ninaC^{P235}$ (n = 19), $ninaC^{P235}$;*trp* (n = 5), $rdgA^1/+$ (n = 21), and $rdgA^1/+$;*trp* (n = 4).

Ca^{2+} was also observed in a $norpA^{P24}$;*trpl* double mutant indicating that the effect was mediated independently of PLC and most likely at the level of the channel (Fig. 7).

The ability to facilitate dark events and LNA-induced channel activity by raising Ca^{2+} in this manner contrasts with the effect of Ca^{2+} on the wild-type light response, where raising $[Ca]_i$ via the NCX 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 Ca^{2+} in $G\alpha q^1$ 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 $G\alpha q^1$, the response to light was facilitated two- to threefold by $[Ca]_i$ in a narrow, submicromolar (approximately 100–400 nM) range similar to the Ca^{2+} dependence of dark event rate (Fig. 7, *D–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 Ca^{2+}-dependent inhibition. At higher concentrations, Ca^{2+}-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 Gαq mutant backgrounds. As previously reported, hypomorphic *Gαq* and *norpA* mutants show a severalfold reduction in quantum bump amplitude (Hardie et al. 2002) and variable reduction (up to ~1,000-fold in $G\alpha q^1$) 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 $G\alpha q^1$ and $norpA^{P16}$ hypomorphs would be similarly enhanced in these backgrounds.

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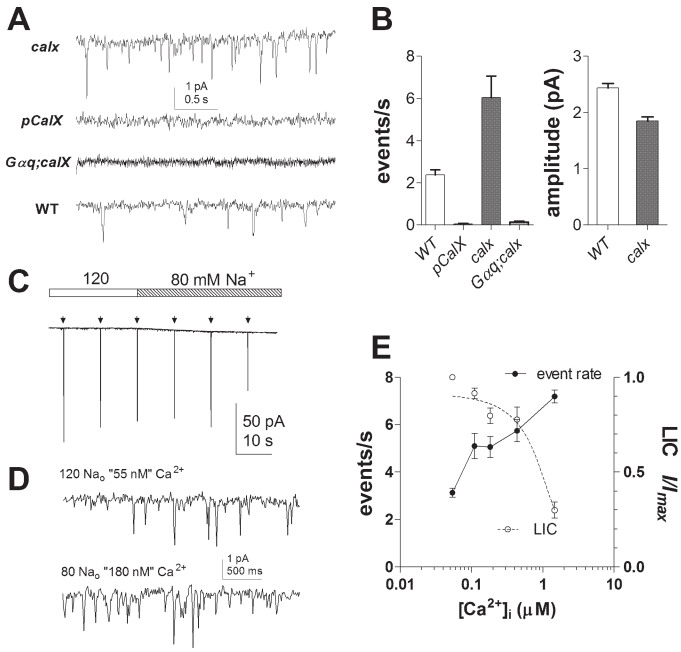

Fig. 6. Ca^{2+} dependence of dark noise. *A*: relative to WT (*bottom* trace), dark noise was increased in *calx* mutants (severe-loss-of-function mutants of CalX, the Na^+-Ca^{2+} exchanger; *top*) but virtually absent in *pCalx* photoreceptors overexpressing *calX* (2nd trace) and in *Gαq*;*calx* double mutants (3rd trace). *B*: summary of dark event rate and amplitude in WT (n = 8), *pCalX* (n = 6), *Gαq*;*calx* (n = 11), and *calx* (n = 6). Amplitudes for *pCalx* and *Gαq*;*calx* are not shown due to negligible event count. *C*: responses to brief (5-ms) flashes (~100 effective photons; arrows) in WT photoreceptors were slightly (~20%) reduced as the control bath solution containing 120 mM Na^+ was substituted for 80 mM Na^+ [10 mM intracellular Na ($[Na]_i$) predicted intracellular Ca^{2+} ($[Ca]_i$) at −70 mV, 55 nM, and 180 nM, respectively]. *D*: on expanded scale, the rate of spontaneous dark events was increased following perfusion with 80 mM extracellular Na ($[Na]_o$). *E*: Ca^{2+} dependence of dark noise (event rate) and macroscopic light-induced current (LIC). Nominal $[Ca^{2+}]$ was calculated from *Eq. 1* with internal solution containing either 10 or 20 mM $[Na]_i$ (holding potential of −70 mV); peak responses (I/I_{max}) were normalized to responses immediately before Na^+ substitution. Mean ± SE, n = 7–18 cells per data point.

In agreement with an earlier study (Hardie et al. 2002), we confirmed that quantum bump amplitudes in $rdgA^1$/+;$G\alpha q^1$ and $rdgA^1$/+,$norpA^{P16}$ were increased ~2-fold relative to the respective $G\alpha q^1$ or $norpA^{P16}$ single mutants. New here, we found that bump amplitudes in both $ninaC^{P235}$,$G\alpha q^1$ and $G\alpha q^1$;*rtp* (Fig. 8) as well as $norpA^{P16}$;$ninaC^{P235}$ and $norpA^{P16}$;*rtp* (Fig. 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 $G\alpha q^1$ 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 $G\alpha q^1$ double mutants with respect to $G\alpha q^1$ single mutants (Fig. 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 $G\alpha q^1$ successfully generate a bump and conversely that in *Gαq* 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 that 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 bumplike events occurring at rates of approximately 2–3/s. Previous studies indicated that these dark events reflect spontaneous G protein activations (Hardie et al. 2002) and that a suprastoichiometric 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 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, RTP, the INAD scaffolding protein, DGK, and a Ca^{2+}-dependent threshold for channel activation. Our results further indicate that all of 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 signaling 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; Henderson et al. 2000), and although PLC has measurable basal activity, it is less than ~1/1,000th 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 could be accelerated in various mutant backgrounds by ATP depletion or 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 *Gαq* 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 in $rdgA^1$/+ heterozygotes under ATP-deprived conditions). Assuming 2×10^6 G proteins per cell (Hardie and Raghu 2001), this represents an average lifetime (per G protein molecule) of ~30 h. 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

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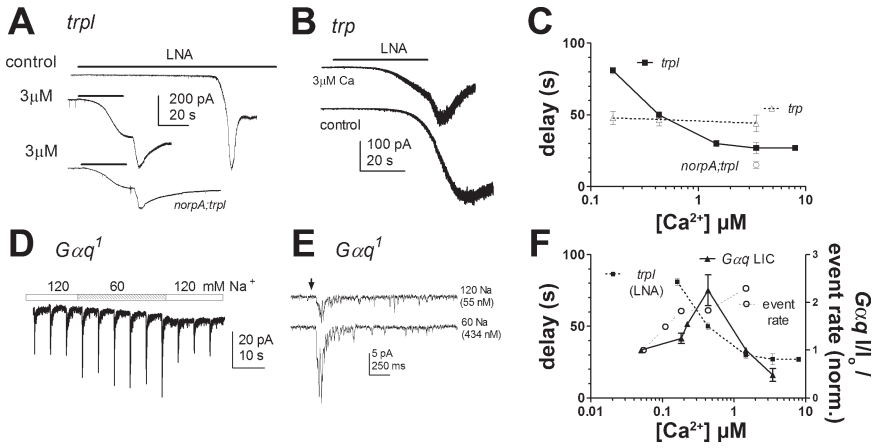

Fig. 7. Ca^{2+} dependence of TRP channel sensitivity to LNA and light response in *Gαq* mutants. *A*: activation of TRP channels (in *trpl* mutant photoreceptor) by perfusion of 20 μM linolenic acid (LNA; bars): under control conditions (*top*; 1 mM $[Na]_i$, 120 mM $[Na]_o$) and with $[Ca]_i$ nominally at 3 μM (*middle*; perfusion with 60 mM Na; 20 mM $[Na]_i$). Activation was greatly accelerated at the higher $[Ca]_i$. Activation in no receptor potential A (*norpA*; gene encoding PLC);*trpl* (*norpA;trpl*) under the same conditions (*bottom*) was similarly fast. *B*: by contrast, activation of TRPL channels (in *trp* mutants) occurred with a similar delay under both conditions. In both cases (*A* and *B*), the high Ca^{2+} also induced Ca^{2+}-dependent inhibition of the TRP (or TRPL) channels, and on reperfusion with 120 mM Na (end of bar) extrusion of Ca^{2+} by the CalX exchanger relieved inhibition inducing an increase in current. *C*: dose-response (mean ± SE, n = 4–6 cells per point) expressed as perfusion time (delay) required to activate a criterion (10-pA) current, plotted against nominal Ca^{2+} concentration (*Eq. 1*). *D*: responses to brief (0.5-ms) flashes (~3×10^5 WT effective photons) in $Gαq^1$ hypomorphs were reversibly increased ~2-fold during perfusion with 60 mM $[Na]_o$ (10 mM $[Na]_i$), nominally increasing Ca^{2+} from 55 to 434 nM. *E*: example responses to light flash (arrow) before and during perfusion with 60 mM $[Na]_o$. *F*: dose-response function for LIC in $Gαq^1$ [solid line; peak response normalized (norm.) to value before perfusion with low $[Na]_o$; n = 3–5 cells per data point]. The Ca^{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 Fig. 5*E*. All showed significant facilitation in a similar submicromolar range.

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 the COOH terminus of NINAC ($ninaC^{I1501E}$), required for its interaction with the INAD scaffolding protein (Wes et al. 1999), fully and uniquely reproduced the $ninaC^{P235}$-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.

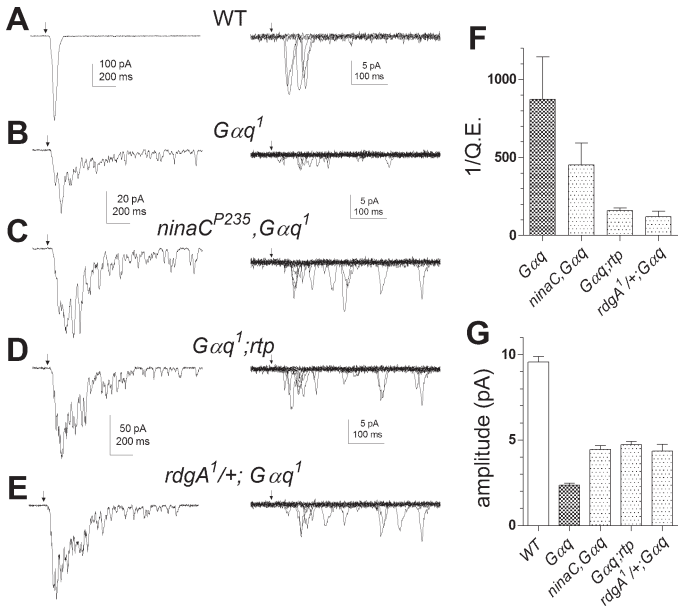

Fig. 8. Facilitation of light responses in *Gαq* by $ninaC^{P235}$, *rtp*, and *rdgA*/+ mutations. *A–E*, *left*: responses to brief flashes (arrow) containing ~100 effective photons in WT and ~7×10^4 photons in *Gαq* mutants, $ninaC^{P235}$, *Gαq*, *Gαq;rtp*, and *rdgA*/+;*Gαq* double mutants. *Right*: 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 $Gαq^1$ were increased on the $ninaC^{P235}$ *rtp* and *rdgA*/+ backgrounds (note different scales). *F* and *G*: bar graphs showing reciprocal of quantum efficiency (*F*) relative to WT (1/Q.E.) and bump amplitude (*G*) in WT (n = 11), $Gαq^1$ (n = 6), $ninaC^{P235}$,$Gαq^1$ (n = 8), $Gαq^1$;*rtp* (n = 14), and *rdgA*/+;$Gαq^1$ (n = 3) double mutants.

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 *ninaC*, *rdgA*, and *trp* mutations on both spontaneous dark noise and bump amplification in *Gαq* and *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 G_qα-PLC complexes inactivate before generating sufficient excitatory messenger(s) to activate any channels. However, if DGK activity is reduced (in $rdgA^1$/+ heterozygotes), then PLC products [e.g., DAG, protons, and phosphatidylinositol 4,5-bisphosphate (PIP_2) reduction] can now overcome threshold more readily, resulting in increased probability of generating a dark event. On one hand, the similar effects seen in *ninaC* and *rtp* mutants might be explained if a NINAC-RTP-INAD complex is required to maximize effective DGK activity (e.g., by incorporating DGK itself within the INAD

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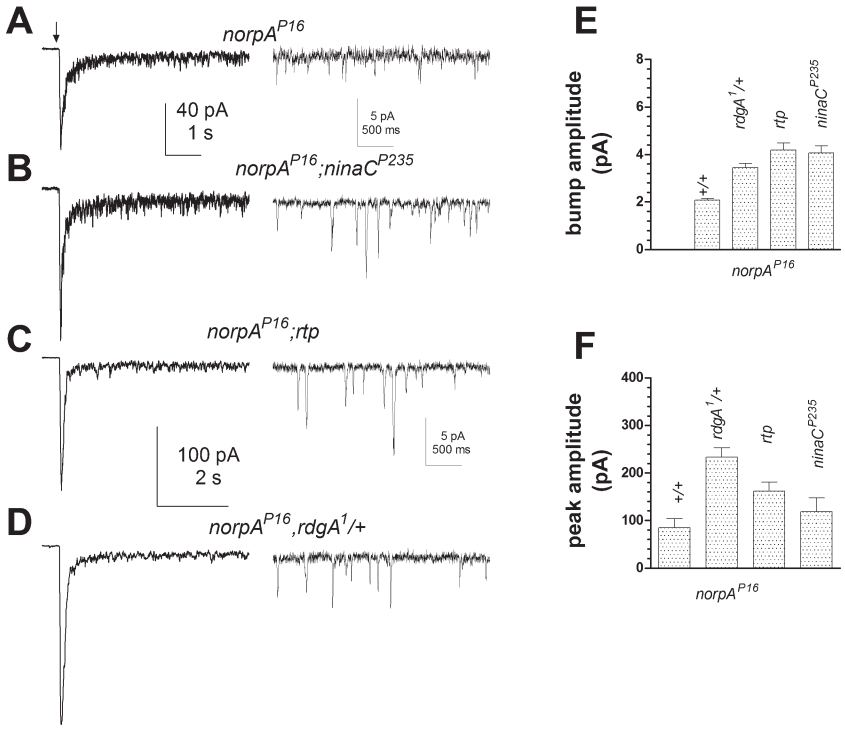

Fig. 9. Facilitation of light responses in PLC hypomorph *norpA*^{P16} by *rdgA*^{1}/+, *ninaC*^{P235}, and *rtp* mutations. *A–D*: responses in *norpA*^{P16} hypomorph were enhanced on *ninaC*^{P235}, *rdgA*^{1}/+, and *rtp* backgrounds. *Left*: macroscopic responses elicited by flashes (arrow) containing ~7.5 × 10^5 WT effective photons. *Right*: representative samples of quantum bumps in darkness after decay of the current to baseline. *E* and *F*: bar graphs summarizing the effect of *rdgA*/+, *ninaC*^{P235}, and *rtp* backgrounds on bump amplitude (*E*) and peak response to flashes containing ~7.5 × 10^5 WT effective photons (*F*) compared with single *norpA*^{P16} mutant controls (labeled +/+). (*P16*, n = 6–14; ≥50 bumps in each cell; double mutants, n = 5–19 cells).

complex). However, because available evidence indicates that DGK is localized 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. 2012; 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 Franze 2012), and the proton released by the PLC reaction rather than by DAG (Huang et al. 2010). Since DGK product (PA) is a potent positive regulator of PIP 5-kinase (Cockcroft 2009; Jenkins et al. 1994), *rdgA*^{1}/+ mutants might have significantly impaired ability to rapidly resynthesize PIP_2, potentially also accounting for the increased sensitivity and dark noise phenotypes in *rdgA*^{1}/+ (reviews: Hardie 2012; Raghu and Hardie 2009).

Ca^{2+} dependence of spontaneous dark noise. Importantly, we also found that dark event rate, but not amplitude, was increased by raising cytosolic Ca^{2+} via manipulating NCX (Wang et al. 2005). Conversely, dark noise was almost eliminated in photoreceptors overexpressing CalX, which presumably results in a lower $[Ca]_i$. We again consider it unlikely that $[Ca]_i$ affects the spontaneous rate of G protein activation, and therefore these results suggest the existence of an effective Ca^{2+}-dependent threshold, which must be reached for successful generation of a dark event. The failure of raising Ca^{2+} to increase dark event amplitude is presumably explained by the competing effect of Ca^{2+}-dependent inhibition of the light-sensitive channels, which has an IC_{50} of 1 μM (Gu et al. 2005).

Positive feedback by Ca^{2+} 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 Ca^{2+} might act to facilitate channel activation, namely PLC and the channels themselves (see also Katz and Minke 2012). PLC is a Ca^{2+}-dependent enzyme (Rhee 2001), and light-induced PLC activity in *Drosophila* eyes is facilitated by Ca^{2+} in the submicromolar 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 Ca^{2+} on a submillisecond time scale, 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 Ca^{2+} with an EC_{50} of ~300 nM, downstream of PLC (Fig. 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 one hand by hydrolyzing more PIP_2 and on the other by lowering the threshold of PIP_2-derived excitatory messenger required to activate the channels.

Concluding remarks. 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 *Gαq*^{1} 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 $G_q\alpha$-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 sensitivity to excitatory

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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 while maximizing the detectability of light-evoked quantum bumps, which require sequential activation of five 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: on 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 buildup 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 (Pumir et al. 2008; Song et al. 2012). Importantly, they also predict that single G protein activations lead to the generation of only small bumps with much reduced probability or Q.E. (Pumir et al. 2008).

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

B.C. performed most electrophysiological experiments and wrote paper; C.-H.L., Western blot analysis and genetics; S.S., genetics, designing, and performing crosses; A.G., generation of RDGA antibody and Western blot analysis; P.R., generation of RDGA antibody and Western blot analysis; R.C.H., electrophysiology, experimental design, and wrote paper.

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