

## RGS Expression Level Precisely Regulates the Duration of Rod Photoresponses

Regulators of G protein signaling (RGS) constitute a family of proteins that bind specifically to the activated  $\alpha$  subunits of G proteins ( $G\alpha$ -GTP), acting as GTPase activating proteins, or GAPs, for the rate of GTP hydrolysis. In this issue of *Neuron*, Krispel et al. resolve a long-standing puzzle in phototransduction, establishing that RGS9 “GAPping” of  $G_{t\alpha}$ -GTP is the molecular mechanism underlying the dominant recovery time constant of mouse rod photoreceptors and that a precise level of expression of RGS9 is required for normal photoresponse timing.

### RGS Proteins: GAPs for G Protein Signaling

The human genome contains more than 30 RGS genes comprising six distinct families (Hollinger and Hepler, 2002). The core protein structure is a highly homologous RGS domain that confers binding specificity for GPCR-activated  $G\alpha$ -GTP. This RGS domain is flanked in different subfamilies by domains that confer other features, such as the “disheveled, EGL-10, pleckstrin” (DEP) homology domain, which determines subcellular localization in subfamily R7, likely by binding a SNARE-family-related protein such as R9AP (Martemyanov et al., 2003).

In many GPCR cascades, the receptor ( $R^*$ ) and the effector ( $E^*$ ) are enzymes and act as amplifiers of signaling; in contrast, the intermediate ( $G^* = G\alpha$ -GTP) that activates  $E^*$  has 1:1 stoichiometry with it, and does not amplify (Figure 1A). The instantaneous signal strength at each of the first three cascade steps can be identified with the number of activated protein molecules of each species at a given moment. RGSs modulate the signal strength not by altering the number of molecules activated per unit time as GPCRs do, but rather by acting as GAPs to control the  $G^*$  lifetime. A fundamentally important contribution of Krispel et al.’s investigation is their thoroughly convincing evidence that the concentration determines RGS9 GAP activity in rods (their Figure 3F). To appreciate the broad significance of this result, some background on GPCR signaling in rods is helpful.

### Phototransduction: GPCR Cascade Signaling with Two Time Constants

Krispel et al.’s investigation contributes to a rich tradition of using rods for investigating quantitative aspects GPCR signaling. The initial steps of the rod cascade follow the classic pattern (Figure 1A): the light-activated GPCR, rhodopsin ( $R^*$ ), activates a rod-specific G protein, transducin ( $G_t$ ), whose activated  $\alpha$  subunit ( $G^* = G_{t\alpha}$ -GTP) stoichiometrically activates one catalytic subunit ( $E^*$ ) of the rod phosphodiesterase (PDE) by relieving the inhibition of a  $\gamma$  subunit ( $PDE\gamma$ ). The hydrolysis of cGMP by  $E^*$  decreases the inward cationic current through cGMP-gated channels, leading to membrane hyperpolarization. Signal amplification is a property of the activation phase of the response, and its molecular

basis is now well understood: it is achieved primarily by the actions of  $R^*$  and  $E^*$ , with the cooperative gating of the cyclic nucleotide-gated channels contributing an additional factor (Lamb and Pugh, 1992; Pugh and Lamb, 1993; Leskov et al., 2000). In contrast, problems remain in understanding the molecular mechanisms governing inactivation and recovery. Perhaps none has been more resistant to resolution than the molecular identity of the so-called “dominant recovery time constant” or “rate-limiting step” in recovery. For insight into this problem, it is helpful to consider a schematic of the cascade (Figure 1A). Theoretical models that treat the first three steps of phototransduction as a linear cascade, with the molecular amplifiers  $R^*$  and  $E^*$  inactivating with first-order time constants  $\tau_R$  and  $\tau_E$ , respectively, have provided a good description of the flash responses of rods under a variety of conditions (Nikonov et al., 1998; Hamer, 2000). However successful, such models have an inherent ambiguity: the values assigned to  $\tau_R$  and  $\tau_E$  are interchangeable without altering the predictions of response kinetics. A potential resolution of this ambiguity comes from systems analysis, which shows that in any linear reaction cascade, the longest time constant will be rate limiting and completely govern the decay of the response at sufficiently long times after a brief activation (see, e.g., Nikonov et al., 1998). The proviso for achieving this resolution is that an experiment must be designed that uniquely manipulates the molecular step hypothesized to be dominant or rate limiting. In the special case in which there are only two time constants—which can be symbolized as  $\tau_D$  (“dominant”) and  $\tau_{ND}$  (“nondominant”)—identification of the molecular mechanism underlying  $\tau_D$  also determines the identity of the mechanism underlying  $\tau_{ND}$  by default.

### Pepperberg’s Inactivation Time Constant

Pepperberg et al. (1992) discovered a remarkable feature of amphibian rod light responses: the recovery of the cGMP-mediated current after a series of saturating flashes of increasing intensity was found to be displaced laterally in time, about 2–2.5 s per e-fold increase in intensity; the semilog “Pepperberg plot” of displacement versus intensity readily extracted this critical parameter,  $\tau_C$ , which in mouse is 200–250 ms (Lyubarsky and Pugh, 1996; cf. Figures 2D and 3E of Krispel et al., 2006). Pepperberg et al. argued that  $\tau_C$  estimated the “dominant recovery time constant” (labeled above and hereafter as  $\tau_D$ ) of the disc-associated reactions (steps 1–3 of Figure 1A); they also argued that their data supported the hypothesis  $\tau_D = \tau_R$ , i.e., that the lifetime of  $R^*$  is the dominant or rate-limiting step of inactivation. A controversy developed, however, as doubts were subsequently cast on the hypothesis by investigations characterizing functional properties of  $\tau_D$  and  $\tau_{ND}$ . Notable among these were calcium-clamp experiments: one set showed that  $\tau_D$  is not affected by the decline in intracellular calcium ( $Ca^{2+}_i$ ) that accompanies the light response (Lyubarsky et al., 1996), while others with rapid solution-exchange clamping provided evidence that  $\tau_{ND}$  is sensitive to  $Ca^{2+}_i$  in a manner consistent with the hypothesis that the molecular mechanism underlying

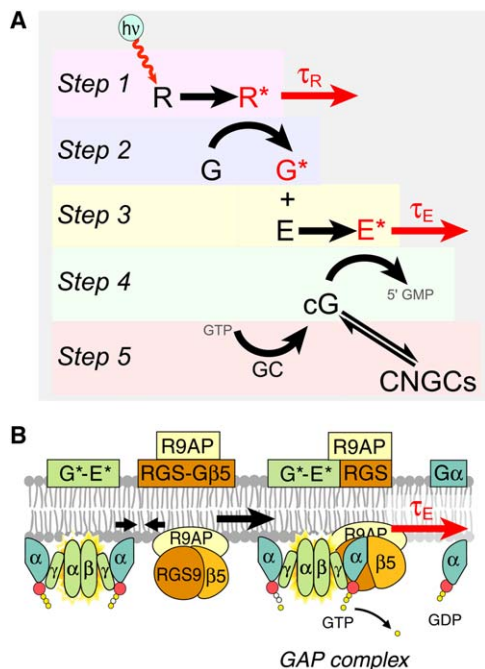


Figure 1. The Phototransduction GPCR Cascade and the Molecular Mechanism of the Rate-Limiting Step of Recovery

(A) Schematic of phototransduction cascade. In step 1, light isomerizes rhodopsin, creating  $R^*$ . Steps 1–3 are similar in many GPCR cascades:  $R^*$  activates a specific G protein, creating the activated intermediate  $G^*$  ( $=G\alpha\text{-GTP}$ ) (step 1); a  $G^*$  stoichiometrically activates one of the two catalytic subunits of the tetrameric phosphodiesterase (PDE), creating  $E^*$  (step 2);  $E^*$  catalyzes the hydrolysis of cG (cyclic GMP) (step 3), which holds open cyclic nucleotide-gated channels (CNGCs) in the dark, and which close as cG is reduced by  $E^*$ ; the resting cG level is set by the balance of synthesis by guanylyl cyclase (GC) and PDE hydrolysis. Models of phototransduction in which  $R^*$  and  $E^*$  inactivation are characterized by first-order decays with time constants  $\tau_R$  and  $\tau_E$ , respectively (red arrows), have been successful in providing quantitative descriptions of rod photoresponses (see text for details). (B) Molecular mechanism underlying  $\tau_E$ :  $E^*$ s activated by  $G^*$  encounter R9AP-RGS9-G $\beta$ 5, leading to formation of the “GAP complex,” in which RGS9 facilitates hydrolysis of GTP after  $G^*$  has bound to and activated  $E^*$ .

$\tau_{ND}$  is Ca-dependent modulation of  $\tau_R$ , the  $R^*$  lifetime (Matthews, 1997). This latter hypothesis was later shown to be quantitatively consistent with the biochemical properties of recoverin’s calcium-dependent inhibition of rhodopsin phosphorylation (Nikonov et al., 2000). The controversy regarding the identity of the molecular mechanism underlying  $\tau_D$  has resisted resolution for almost 15 years, with different phototransduction labs presenting results and arguments in favor of  $\tau_R$  or  $\tau_E$ . A critical experiment was needed to definitively distinguish between the two hypotheses, and it became clear that such an experiment should meet two criteria: (1) it should involve a manipulation established biochemically to target exclusively (and incontrovertibly) the inactivation of  $R^*$  or  $E^*$ ; (2) it should produce robust evidence that this manipulation reduces the Pepperberg constant,  $\tau_D$ . (The rationale for the second criterion is that manipulations that increase  $\tau_D$  could do so by causing the nondominant mechanism to become dominant.) Krispel et al. (2006) have executed and in this issue of *Neuron* present the results of such a critical experiment.

### Resolution of the Controversy of the Rate-Limiting Step of Rod Inactivation

The central result of Krispel et al. (2006) is presented in their Figure 3. The data in this figure establish that in mouse rods overexpressing RGS9 the “Pepperberg constant”  $\tau_D$  is highly reliably reduced from its normal value of 250 ms down to 75 ms. As the manipulation of RGS9 overexpression uniquely targets  $E^*$  (Figure 1B), it meets criterion (1); and as  $\tau_D$  is reduced by RGS9 overexpression, criterion (2) is also met. Thus, it can be unequivocally and definitively concluded that  $\tau_D = \tau_E$ , i.e., that the rate-limiting inactivation step of the rod phototransduction cascade is the “GAPping” of GTP hydrolysis in the quaternary protein complex, G $\beta$ 5-RGS9-(G $\alpha$ -GTP)-PDE $\gamma$  (Figure 1B). Krispel et al. further strengthen this conclusion by showing that a lowered level of RGS9 leads to an increased  $\tau_D$  and that molecular manipulations aimed at speeding  $R^*$  shutoff have no reliable effect on  $\tau_D$ . Thus, the overall recovery time course can be made faster or slower, depending upon whether RGS9 is over- or underexpressed. Above 3-fold overexpression, the effect of RGS9 in reducing the Pepperberg constant saturates, with an asymptotic value  $\tau_D = 75$  ms. The natural interpretation of this result is that, as  $\tau_E$  becomes smaller and smaller, the previously non-rate-limiting decay of  $R^*$  activity becomes dominant: and so, the data yield the estimate  $\tau_R = 75$  ms.

These results are a landmark in phototransduction research because they not only unambiguously identify RGS-mediated GAPping of the  $E^*$  complex as the rate-limiting step for rod response recovery but also because they provide, for the first time, an *experimental* estimate of  $\tau_R$ . From a larger perspective, the meticulous, quantitative analysis by Krispel et al. of the effect of the level of expression of a transgene on a quantitative physiological parameter raises the bar for future targeted gene manipulations and invites novel hypotheses about the role of expression level in determining the function of an RGS.

### RGS Concentration Matters

Experiments utilizing gene knockout approaches generally yield only three levels of expression of the protein product of the targeted gene: normal (wt), null ( $-/-$ ), and one intermediate value ( $+/-$ ). The inferences to be drawn are relatively simple: “this protein is essential for that function” ( $-/-$  versus wt) and/or “normal levels of this protein are required for that function” ( $+/-$  versus wt). In contrast, Krispel et al. (2006) generated transgenic mouse lines with six different levels of RGS9, confirmed normal expression levels of other critical transduction proteins in each line, and then measured  $\tau_D$ , a quantitative characteristic of cascade inactivation, in adequate populations of rods from each line. The result is a powerful dose-response relationship for  $\tau_E$  versus RGS9 in situ, where the other proteins are present in their native concentrations (cf. Krispel et al.’s Figure 3F). A remarkable feature of this dose-response relationship is that mere 2-fold changes in RGS9 expression up or down from its normal level highly reliably alter  $\tau_D$  in a corresponding manner.

How does RGS9 concentration exert such a powerful influence on the effector ( $E^*$ ) lifetime? Two non-mutually exclusive hypotheses can be put forward. First, the speed of the GAP reaction may be governed by the time taken for RGS9-G $\beta$ 5, anchored to the membrane by R9AP, to diffuse to, bind to, and form the effective GAP

complex (Figure 1B). Second, the second-order binding reaction for GAP complex formation may be weak and, thus, driven faster by elevated concentrations of RGS9. Testing these hypotheses will yield new information about the cellular and molecular details of RGS function.

#### **Implications for Non-Retinal Neuronal Timing Circuits and for Therapeutics**

GPCR cascades are implicated in a variety of neuronal circuits with strict timing requirements: for example, the basal ganglia are widely understood to be involved in precision timing of locomotion and other behaviors, and evidence has been presented that a splice variant of the photoreceptor RGS, RGS9-2, acting through dopaminergic D1 and D2 GPCR cascades, modulates psychostimulant-induced locomotion and reward behavior (Rahman et al., 2003). One important implication of the experiments of Krispel et al. is that the expression levels of different RGSs in specific neuronal subpopulations are likely to be tightly regulated in order to achieve precision timing of behavior. Another implication of broad significance is that the expression levels per se of RGSs may become proper targets for therapeutic interventions in diseases of such timing circuits. In sum, the bottom line is simple and powerful: RGS expression levels are critical for timing, and in GPCR signaling “timing is everything” (Shea et al., 2000).

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## **Not Too Excited? Thank Your Endocannabinoids**

Endocannabinoids can mediate neuroprotection, but it is not known how. In this issue of *Neuron*, Monory et al. use mutant mice and localized viral targeting to produce conditional knockouts of the cannabinoid CB1 receptor. They show that protection against kainic acid-induced seizures and cell death is conferred by CB1Rs on hippocampal glutamatergic nerve terminals.

Epileptic seizures reflect states of pathological hyperexcitability and hypersynchronous activity in large neuronal networks. Broadly speaking, seizures arise from an imbalance of two fundamental antagonistic neuronal motive forces—excitation and inhibition—toward excitation. But the underlying mechanisms may be very complex and, in addition to alterations in the strength of excitatory and inhibitory chemical synapses, may involve electrical gap junctions, neuronal network oscillations, and rewiring of the neuronal circuits. The hippocampus is one of the most seizure-prone brain regions, perhaps because it typically rests near the tipping point of the balance and is susceptible to numerous forms of plasticity. Whatever their etiology, seizures are highly disruptive to normal brain functions, and if severe and prolonged, can lead to very bad outcomes, including neuronal cell death. Intrinsic biological mechanisms that protect against seizures are therefore of great theoretical and practical interest.

Endogenous cannabinoids (“endocannabinoids”) are the natural agonists of membrane-bound, G protein-coupled receptors that mediate the actions of drugs, such as marijuana, derived from the cannabis plants. The principal cannabinoid receptor subtype in the CNS, CB1R, is predominantly localized on or near synaptic terminals, and its activation inhibits synaptic transmitter release. The two major endocannabinoids are arachidonyl-ethanolamide (anandamide) and 2-arachidonyl glycerol (2-AG). They are produced by neuronal enzymatic activity and generally serve as intercellular messengers, often traveling in the “retrograde” direction to the incoming synaptic input. CB1Rs are widely dispersed throughout the brain in specific association with well-defined cell types in the different regions, and this accounts for the variety of behavioral effects caused by the exogenous cannabinoids. Several years ago, Panikashvili et al. (2001) reported that experimental closed-head injury produced an elevation in 2-AG and that exogenous administration of 2-AG reduced the brain edema and hippocampal cell death associated with such injuries. This was direct in vivo evidence for a neuroprotective effect of an endocannabinoid. In this issue of *Neuron*, Monory et al. (2006) now ask and answer novel questions about the cellular mechanisms of endocannabinoid-mediated neuroprotection.

In an earlier investigation, Lutz, Marsicano, and colleagues (Marsicano et al., 2003) reported that mice lacking CB1 (*CB1*<sup>-/-</sup>) experienced kainic acid (KA)-induced seizures that were much more severe than those experienced by wt or heterozygotic *CB1*<sup>+/-</sup>