Identification of key factors that reduce the variability of the single photon response

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Rod photoreceptors mediate vision in dim light. Their biological function is to discriminate between distinct, very low levels of illumination, i.e., they serve as reliable photon counters. This role requires high reproducibility of the response to a particular number of photons. Indeed, single photon responses demonstrate unexpected low variability, despite the stochastic nature of the individual steps in the transduction cascade. We analyzed individual system mechanisms to identify their contribution to variability suppression. These include: (i) cooperativity of the regulation of the second messengers; (ii) diffusion of cGMP and Ca^{2+} in the cytoplasm; and (iii) the effect of highly localized cGMP hydrolysis by activated phosphodiesterase resulting in local saturation. We find that (i) the nonlinear relationships between second messengers and current at the plasma membrane, and the cGMP hydrolysis saturation effects, play a major role in stabilizing the system; (ii) the presence of a physical space where the second messengers move by Brownian motion contributes to stabilization of the photoresponse; and (iii) keeping Ca^{2+} at its dark level has only a minor effect on the variability of the system. The effects of diffusion, nonlinearity, and saturation synergize in reducing variability, supporting the notion that the observed high fidelity of the photoresponse is the result of global system function of phototransduction.

modeling | rhodopsin | deactivation | phosphorylation

In vertebrate retinal rod photoreceptors, light-activated rhodopsin R^* activates dozens of G proteins ($\text{T} \rightarrow \text{T}^*$) during its random lifetime, by catalyzing GDP/GTP exchange on the G protein α -subunit. Each T^* molecule associates, one-to-one, with a catalytic subunit of the effector, forming an active $\text{T}^*\text{-E}$ complex, denoted by E^* . Molecules of E^* hydrolyze the second messenger, cGMP. Reduction of the cGMP level induces its dissociation from the cGMP-gated cation channels, causing channel closure and suppression of the inward current. This current suppression is the experimentally measured electrophysiological response, which is usually normalized by the dark current j_{dark} , yielding the relative current suppression (1)

$$I(t) = 1 - j_{\text{tot}}(t)/j_{\text{dark}}. \quad [1]$$

The mechanism of R^* deactivation contains several random components, including the random number of phosphorylations by rhodopsin kinase (RK), and the random sojourn time at each phosphorylation level. These steps regulate the number of generated E^* molecules and, hence, the response. Because of this randomness the responses are expected to be inherently variable, in the sense that any two rhodopsin photoisomerizations yield different responses. The system would be stable if these responses are statistically close. A measure of stability is the coefficient of variation (CV), defined as the ratio of the standard deviation to the mean, calculated over a large number of signaling events and their corresponding responses. However, the measured single photon response (SPR) is highly reproducible, i.e., the coefficient

of variation of the current response and its time integrals, is relatively low, about one half of what would be theoretically expected from this series of reactions in the test tube (2–4). For example the CV of the peak current amplitudes in toad is reported to be about 20% (5), and for a mouse, the CV of the area integral (integral of the relative current suppression, over the whole time course of the phenomenon), is about 36% (4). The mechanisms that ensure such a low variability of the SPR are not completely understood, and are the object of current experimental and theoretical investigation (2–10).

We have recently shown in ref. 10 that the randomness of the sojourn times of R^* in each of its phosphorylation states is the dominant contributor to the variability of the response. At the same time, the number of R^* phosphorylation sites has negligible influence on variability suppression. We also showed that the cascade in the cytoplasm of the rod outer segment (ROS) with complex geometry greatly reduces variability (9).

Here we selectively examined the contribution of each of the components of the transduction cascade and the cGMP replenishing machinery to variability suppression. Designing experiments that can selectively exclude these components is a major challenge. Experimentally, only the photocurrent can be measured in intact rods at physiologically relevant light intensities, and though biochemical steps can be measured, these studies suffer from the necessity of isolating the mechanism in question, often in homogenized tissue, and assays are not sensitive enough to demonstrate changes at low light intensities. We accomplished this dissection virtually, by means of the space resolved mathematical model (9, 11), which is capable of tracking and selectively including or excluding each component.

Our analysis shows that the positive cooperativity (Hill coefficient $n_H > 1$) of the relationship between cGMP, Ca^{2+} , and the photocurrent, the diffusion of the second messengers in the cytoplasm, and the nonlinear relationship between cGMP hydrolysis and the number of activated effector molecules, are the main variability suppressors of the single photon response. Moreover, they act synergistically to yield low variability of the response.

Methods

The mathematical model introduced in refs. 10 and 12, populated by the parameters in refs. 10 and 13, simulates several components of the SPR. These include the stochastic effects of the activation/deactivation cascade (10), and biophysical and electrophysiological processes taking place in the transduction part of the cascade. Among these are the effects of sophisticated ROS geometry, the diffusion of the second messengers cGMP, and Ca^{2+} in the cytoplasm, and their role in generating the photocurrent with high coopera-

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tivity. The model here is used as a tool to selectively include and or exclude one or several of these components to gain insights from the behavior of a virtually modified ROS. Below is a list of the processes and/or components that were systematically included and/or excluded from the numerical simulations:

- i. The nonlinear relationships regulating the dynamics of the second messengers cGMP and Ca^{2+} , and ultimately current suppression. The nonlinearity arises from Hill's type laws linking powers of $[\text{Ca}^{2+}]$ and $[\text{cGMP}]$ on the lateral boundary of the ROS to the current through the cGMP-gated channels (9), and in the production of cGMP by Ca^{2+} regulated guanylyl cyclase (GC), respectively. The presence of these nonlinear relations is labeled by (N), whereas their exclusion is labeled by (n).
- ii. Ca^{2+} feedback in the cytoplasm is mediated by Ca^{2+} regulation of GC that regenerates cGMP (14). The SPR with clamped Ca^{2+} exhibits a larger amplitude and a delayed peak time, and is slower than WT SPR (3). The presence of Ca^{2+} feedback is labeled by (F) and its absence (Ca^{2+} clamped) is labeled by (f).
- iii. Diffusion of the cGMP and Ca^{2+} in the highly compartmentalized cytoplasm of the ROS. This biophysical effect links the biochemical components of the cascade (9), and is labeled by (D). Its absence, as in a well-stirred system, is labeled by (d).
- iv. Local cGMP hydrolysis by the $\text{T}^+ \cdot \text{E}^+$ complex. This mechanism passes the variability of the activated $\text{T}^+ \cdot \text{E}^+$ along to the second messengers and ultimately to current suppression. The cGMP drop is largely localized in the interdiscal space adjacent to the activated disk, reducing the possibility of further cGMP hydrolysis and thus leading to a saturation effect. For this reason this mechanism is labeled by (S) for "saturation." If instead in the hydrolysis term cGMP is kept at its dark value, this saturation effect is not present and we label it by (s).

A mathematical description of each of these components is in [SI Appendix](#). Variability is monitored at two levels. The first is by the CV of the activated effector E^+ (not experimentally accessible in intact rods), and the second is by the CV of the photocurrent [the output of the system that can be experimentally measured (2–4), downstream of the transduction process]. The first bears the random elements of the deactivation cascade (10), and the second is mitigated by the cytoplasm, the physical medium where the second messengers diffuse (9). The stochastic and biochemical aspects of R^+ deactivation are described in (10). The technical aspect of the nonlinear couplings between second messengers and current and Ca^{2+} feedback are explained in refs. 9, 11, and 12. Details of both are given in [SI Appendix](#). The variability of the activated effector E^+ is simulated by the CV of the functionals:

$$\text{E}_{\text{int}}^*(t) = \int_0^t \text{E}^*(s)ds, \quad \text{E}_{\text{area}}^* = \int_0^\infty \text{E}^*(t)dt. \quad [2]$$

Here $\text{E}^*(s)$ is the total number of molecules of E^+ in the activated disk at time s , the first integral $\text{E}_{\text{int}}^*(t)$ is the total activity of E^+ up to time t , and the second integral E_{area}^* is the total activity of E^+ over the entire time course of the process. The variability of the current suppression is measured and simulated by the CV of the functionals (3, 4)

$$\text{I}_{\text{int}}(t) = \int_0^t \text{I}(s)ds, \quad \text{I}_{\text{area}} = \int_0^\infty \text{I}(t)dt. \quad [3]$$

The first integral $\text{I}_{\text{int}}(t)$ is the total relative charge suppression up to time t , and the second integral I_{area} is the total relative charge suppression over the time course of the SPR. Random choices are made of the sojourn times of R^+ in its phosphorylation states, according to their exponential distribution; the ensuing deactivation is implemented according to the statistical scheme presented in (10) and the output E^+ , as a function of time, is fed into the spatiotemporal model described in refs. 9 and 11 to compute the relative current suppression $\text{I}(t)$ and the values of the functionals in Eq. 3. This operation is done on the full model in refs. 9 and 10, containing all features of diffusion (D), Ca^{2+} feedback (F), nonlinear coupling of current to Ca^{2+} and cGMP, local cGMP hydrolysis by E^+ (S), and nonlinear regulation of cGMP synthesis by guanylyl cyclase activating proteins (GCAPs) and Ca^{2+} (N), denoted by DFSN. This procedure is then repeated with one or more of these components removed. When one of these elements is mathematically removed from the model, it is denoted by the same letter in lower case. For example, dFSN means that the effects of diffusion have been eliminated from the model, making it globally well-stirred, while the remaining features are kept operative. After 5,000 iterations of this process, for each of the indicated cases, the CVs of E_{area}^* and I_{area} for both WT and serine triple mutant mouse (3-P mutant mouse), that shows significantly slower recovery (15), are computed and shown in

Table 1. CV of the functional I_{area} in WT mouse

	FSN	FSn	FsN	fSN	fsN	fSn	Fsn	fsn
D	0.37	0.43	0.41	0.38	0.48	0.41	0.55	0.55
d	0.50	0.54	0.51	0.51	0.52	0.53	0.55	0.55

CV for models with (D) or without (d) diffusion plotted against presence or absence of other stabilizing mechanisms, Ca^{2+} feedback (F), saturation effects (S), and nonlinearity (N).

Tables 1 and 2. The CVs of $\text{E}_{\text{int}}^*(t)$ and $\text{I}_{\text{int}}(t)$ for both WT and 3P-mutant mouse are computed as functions of time, and reported in Figs. 1 and 2. One can also compute the CV of other functionals, such as the current I at its peak value $\text{I}(t_{\text{peak}})$. Tables for this functional, both for WT and mutant mice with different numbers of rhodopsin phosphorylation sites removed, are presented in [SI Appendix](#), Tables S7–S10. The numerical values of the parameters involved are determined as in refs. 10 and 13 and are reported in a parameter table in [SI Appendix](#), Table S1.

Results and Discussion

In the experimental literature, the variability of the SPR is measured only in terms of the relative current suppression $\text{I}(s)$, its integral $\text{I}_{\text{int}}(t)$, and the area integral I_{area} , introduced in Eqs. 1 and 3, as these are the only experimentally accessible quantities in intact rods (2–4, 16). Our $\text{CV}(\text{I}_{\text{area}})$ of a virtual WT mouse reproduces the experimental $\text{CV}(\text{I}_{\text{area}})$ of a WT mouse: The DFSN yields $\text{CV} = 37\%$ (Table 1) vs. $\approx 36\%$ reported in ref. 4. A more detailed comparison between virtual and experimental data is in ref. 10. However, often an explanation of variability suppression is provided in terms of the variability of the activated complex E^+ and the number of steps to R^+ deactivation (2, 4, 7, 16). The underlying assumption is that the variability of E^+ and its functional is essentially passed along one-to-one to that of the current and its functional. However, careful consideration of rod structure and function clearly shows that this notion is unrealistic. First, the activation of a single R^+ results in highly localized cGMP hydrolysis in the small part of cytoplasm adjacent to the activated disc. The hydrolysis results in an immediate drop in local cGMP concentration, which affects local phosphodiesterase activity. Second, to affect the current, this drop in the cGMP has to reach the outer membrane. Third, the relationship between cGMP concentration near the channel and the current is nonlinear. Fourth, Ca^{2+} regulates cGMP synthesis, and the suppression of the current in the course of the SPR results in the immediate reduction of Ca^{2+} concentration at the membrane, next to closed channels. Fifth, this local drop in Ca^{2+} spreads to the GCAP-GC system by diffusion through the rod cytoplasm, which has an intricate shape. Sixth, the effect of Ca^{2+} concentration on GC activity is nonlinear. Because all these events take place within the time course of the SPR, the change of E^+ cannot directly translate into current suppression. These nonlinear relations surface rather naturally from Hill's type laws linking cGMP and Ca^{2+} to the photocurrent, the Ca^{2+} feedback, and the second order reactions involved in the cGMP hydrolysis by E^+ .

In view of this frequent and improper identification of CV (E_{area}^*) with $\text{CV}(\text{I}_{\text{area}})$, it is natural to ask under what circumstances one might expect such a one-to-one correspondence between $\text{CV}(\text{E}_{\text{area}}^*)$ and $\text{CV}(\text{I}_{\text{area}})$. Our analysis shows that for these two CVs to be equal, two conditions must be simultaneously met: hydrolysis of cGMP by E^+ occurs at constant rate, i.e., $[\text{cGMP}] = [\text{cGMP}]_{\text{dark}}$, and the Hill's relations linking the photo-

Table 2. CV of the functional I_{area} in serine triple mutant mouse

	FSN	FSn	FsN	fSN	fsN	fSn	Fsn	fsn
D	0.38	0.44	0.42	0.35	0.44	0.39	0.57	0.57
d	0.51	0.55	0.51	0.48	0.51	0.52	0.57	0.57

CV for models with (D) or without (d) diffusion plotted against presence or absence of other stabilizing mechanisms, Ca^{2+} feedback (F), saturations effects (S), and nonlinearity (N).

Fig. 1. Time course of variability suppression in a WT mouse containing all, some, or no stabilizing mechanisms. Plotted is the CV of the area integral $\int_0^t E^*(s)ds$ of the activated complex E^* on the rod disc surface, that has largest variability (brown dashed curves), and of the current suppression $\int_0^t (s)ds$ as functions of time, for the models that contain all the stabilizing mechanisms, diffusion (D), Ca^{2+} feedback (F), saturation effects (S), and nonlinearity (N) (DFSN, red), or remove them one at a time (DFSn, purple diamond; DFsN, dashed red; DfsN, green circle; dFSN, blue triangle; or dfsn, light brown square).

current current to [cGMP] are linear. In short, if all sources of nonlinearity are removed from the system. For well-stirred systems (dfsn), a rigorous mathematical proof of this fact is presented in [SI Appendix](#).

In view of the unrealistic nature of such a linear rod outer segment, and given that, as shown in ref. 10, the number of steps to R^* deactivation does not act as a variability suppressor, one is led to search for a stabilizing mechanism in other components of the process. In ref. 9 we identified the diffusion of the second messengers in the cytoplasm as a major variability suppressor; here we separate the components of the transduction cascade to identify further stabilizing mechanisms. The main results, extracted from Tables 1 and 2 and Figs. 1 and 2 are as follows:

- i. The cooperative, nonlinear relations between second messengers and current, and their mutual nonlinear links through GC (N), play a major role in stabilizing the system. That is, when the (nonlinear) Hill's type relations are replaced by their linear approximations (n), the resulting system exhibits a considerably higher CV.

Fig. 2. Effect of removing three phosphorylation sites on rhodopsin (3P) on the time course of variability suppression (serine triple mutant mouse). CV of the area integral $\int_0^t E^+(s)ds$ of the activated complex E^+ (brown dashed curves), and of the current suppression $\int_0^t I(s)ds$ as functions of time, for the models that contain all the stabilizing mechanisms, diffusion (D), Ca^{2+} feedback (F), saturation effects (S), and nonlinearity (N) (DFSN red), or remove them one at a time (DFS_n, purple diamond; DF_SN, dashed red; DF_SN, green circle; dFSN, blue triangle; or dfs_n, light brown square).

- ii. By keeping $[GMP] = [cGMP]_{\text{dark}}$ in the hydrolysis of cGMP by the T^*E^* complex increases the CV of the relative photocurrent suppression. Thus, local hydrolysis of cGMP producing saturation effects acts as a variability suppressor.
- iii. The presence of a physical space distributed system where the second messengers move by Brownian motion (D), contributes to stabilization of the photoresponse. That is, when the space resolved model of refs. 9, 11, and 12 is replaced by a lumped or well-stirred one, where the spatial component and diffusion play no role (d), the CV of the photocurrent functionals is considerably higher.
- iv. There is a cooperative (although not additive) effect of the three features: (D), (N), and (S). Indeed, each of them alone (Dns), (dNs), or (dnS) has a moderate effect on the CV of the photocurrent, whereas (D) with at least one of (S) and (N) or both, significantly reduce variability, pointing to a system behavior.
- v. Clamping Ca^{2+} at its dark level (f), while changing the photoresponse (higher amplitude and slower recovery) has a minor effect on the variability of the system. That is if $[Ca^{2+}]$ is kept at its dark value $[Ca^{2+}]_{\text{dark}}$ throughout the process, the CV of the relative photocurrent suppression, is essentially the same as the CV of the relative current suppression, when Ca^{2+} feedback is permitted (F). This result is in agreement with the experimental data presented in refs. 2 and 3.
- vi. These results continue to hold for mutant mice, where one or more of the R^* phosphorylation sites are mutated or made inoperative (15, 17,18). Simulations are reported for a virtual 3-P mutant mouse, the mutant where three phosphorylation sites have been mathematically removed, which is compared to the experimental data shown in ref. 15. The simulated CV of other virtual mutant mice is reported in [SI Appendix](#).

We showed in ref. 10 that if the transduction functions of the cytoplasm are in their WT state, the number of phosphorylation sites does not act as a variability suppressor. On the one hand, this result suggests that the response stabilizing mechanisms reside elsewhere; on the other hand, it raises the question as to whether genetically manipulated ROS, which have fewer sites for phosphorylation, exhibit stabilization mechanisms, downstream of activation/deactivation, that could be different than in WT.

Therefore, we simulated the photoresponse of virtually modified mice expressing rhodopsin with 1–5 phosphorylation sites. Table 2 and Fig. 2 report the simulations for the representative case of 3-P mutant mouse, that shows significantly slower recovery (15). Simulations for 4–5P virtual mutant mice are presented in *SI Appendix*.

In all cases, although the numerical values of the CV functionals are slightly different, the patterns are very similar. First the largest CV is exhibited by the effector area integrals $E_{\text{int}}^*(t)$ and E_{area}^* . The last two columns in Tables 1 and 2 show the same CV, regardless of the presence or absence of diffusion, in WT and transgenic mice. A comparison of Figs. 1 and 2 shows that asymptotically as $t \rightarrow \infty$, the CV(I_{area}) is the same as that of the area integral E_{area}^* of the activated complex E^* . Thus for the models (DFsn), (dFsn), (Dfsn), and (dfsn), the CV of E_{area}^* is passed along, asymptotically as $t \rightarrow \infty$, to that of I_{area} . This analysis identifies the nonlinearities arising from the Hill's relations, and localized hydrolysis of cGMP by E^* , as key players in the variability suppression of $I_{\text{int}}(t)$ as $t \rightarrow \infty$.

The removal of diffusion, keeping one or all, nonlinearities active (dFSN), (dFsN), and (dFSn), yields $CV(I_{area})$ of the order of 50%, regardless of Ca^{2+} clamping, demonstrating only a moderate CV suppression (Tables 1 and 2). Thus, diffusion is essential for the nonlinearities to play their stabilizing role. However, diffusion without both the nonlinearities generated by the Hill's laws and saturation effects (dFSn), (Dfsn) yields a CV of the same order (about 55%). In the presence of diffusion, at least