

## Optical control of a rhodopsin-based switch

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**ABSTRACT**

A preliminary result supports the feasibility of using visible light to modulate the membrane potential of a cell. Human embryonic kidney cells (HEK293) were transfected with vertebrate rhodopsin and a gradient inward rectifying potassium (GIRK) channel. Whole cell patch clamp recordings of HEK293 cells exposed to 9-cis retinal showed that illumination increases the potassium current compared with recordings obtained in the dark. When combined with a rapid scanning device, this approach has the potential to control the activity of many neurons.

**2. INTRODUCTION**

Although optical microscopy is primarily a tool that is used for observation, several methods (e.g. uncaging and chromophore assisted light inactivation) have evolved that allow optical control of cellular events. It would be ideal to have an optical technique that could stimulate individual neurons in a population with high temporal resolution. An optical method would overcome several of the limitations of either intracellular or extracellular electrodes to manipulate nerve cell activity.

One possible method for creating a light modulated ionic switch that could control membrane potential would be to mimic the assembly of components in the *Drosophila* visual system. Phototransduction in *Drosophila* is the fastest G protein coupled cascade in part because a scaffolding protein, InaD, brings together the rhodopsin, two ion channels (TRP and TRPL), phospholipase C (PLC) and protein kinase C (PKC).<sup>1,2</sup> The optically stimulated, G protein based response leads to activation of the cation channels within about 20 ms of stimulation and the effect deactivates in about 100 ms.<sup>3</sup> The importance of InaD is demonstrated by the abnormal amplitude, latency and deactivation observed in the light response in *Drosophila* mutants that lack InaD.<sup>1</sup>

In *Drosophila*, light of the appropriate frequency can cause the isomerization of 11-cis-retinal to all-trans-retinal and the subsequent conformational changes trigger a G protein based response.<sup>3</sup> The G protein amplified response, which occurs over six orders of magnitude without saturation, persists as long as rhodopsin remains catalytically active. Feedback mechanisms, however, produce photoreceptor adaptation which acts to control the gain.<sup>4</sup> Although the response in *Drosophila* is more variable than vertebrates, tightly controlled deactivation kinetics, which requires phosphorylation of rhodopsin as well as arrestin binding, play an important role in assuring a reproducible response to light.

The charge redistribution associated with rhodopsin's conformational changes in response to pulses of light has been measured using patch clamp recordings of the early receptor currents (ERC) in both single and fused giant cells functionally expressed in HEK293 cells;<sup>5-7</sup> the ERC is similar in waveform and kinetics to that found in photoreceptors. Furthermore, various kinds of TRP channels have also been expressed in heterologous expression systems suggesting that by functionally coupling rhodopsin and the TRP channel in the same heterologous expression system it should be possible to reconstitute the key elements of *Drosophila's* light-activated switching system.<sup>8</sup> Other G protein activated ion channels have also been expressed in these systems,<sup>9</sup> suggesting that if they were coupled to rhodopsin via the appropriate G protein, they could also be activated by light. For example, a recent study has shown that hippocampal neurons in which GIRK channels were over expressed could be hyperpolarized by activating endogenous G protein coupled receptors using glutamate;<sup>10</sup> using a current clamp, GIRK activation hyperpolarized the cell by 11-14 mV and increased the threshold current for firing action potentials by a factor of two.

When designing a molecularly based, light mediated switch for controlling action potentials, it might seem reasonable to attempt to incorporate the mechanisms responsible for either vertebrate or invertebrate vision. The response of chromophores in the visual pigments of vertebrates differ from invertebrates because activated rhodopsin, known as metarhodopsin, is not stable and decays to produce all-trans retinal and opsin (opsin and 11-cis-retinal subsequently recombine to form rhodopsin). Biochemicals in the membranes of invertebrates, however, extend the lifetime of metarhodopsin and stimulation of light at the appropriate wavelength can convert activated rhodopsin back into the ground state.

## **2a. *Drosophila* rhodopsins**

The *Drosophila* compound eye is composed of 750 ommatidia with each ommatidium containing a bundle of 8 photoreceptor cells and 12 auxiliary cells. Each photoreceptor cell contains a microvillar structure, called a rhabdomere that contains the visual pigment. The rhabdomeres of the first six photoreceptor cells, R1—R6, contain the major visual pigment, the blue-absorbing (480 nm) Rh1 rhodopsin (*ninaE*). The R7 cells express UV-absorbing visual pigments (either Rh3 or Rh4), while the R8 cells express either Rh5 or Rh6.

The six rhodopsins are sensitive in wavelengths ranging from the ultraviolet (331 nm) to the orange (520 nm).<sup>11,12</sup> Each rhodopsin can be activated by absorbed light over a broad range of wavelengths (i.e., full width at half maximum is approximately 100 nm). As a consequence, there is an overlap in spectral sensitivity among the rhodopsins. In response to light, rhodopsin is photo-converted to metarhodopsin. For five of the six *Drosophila* rhodopsins (Rh1 - Rh5), photo-activation causes a bathochromic shift in the absorption maxima (towards the red end of the spectrum), for Rh6, however, the shift is hypsochromic (towards the blue end).

*Drosophila* metarhodopsin can be photo-converted back to rhodopsin by absorption of a photon at a shifted wavelength. Figure 1 illustrates the photoactivation cycle for Rh1 and illustrates the role of receptor specific kinase (RK) in phosphorylating metarhodopsin and RdgC phosphatase in dephosphorylating both rhodopsin and metarhodopsin;<sup>13</sup> Vinos et al. has demonstrated that the time to 85% deactivation in wild type *Drosophila*, about 0.17 sec, is extended to 1.78 sec for *rdgC* mutants.<sup>14</sup> As illustrated in Figure 1, the bistability of rhodopsin produces a cycle after metarhodopsin is phosphorylated by a receptor kinase (RK) to  $M_p$ . To complete the cycle,

phosphorylated rhodopsin becomes dephosphorylated rhodopsin via rdgC phosphatase (this also occurs in metarhodopsin, but to a lesser extent). At steady-state the amount of R and M present depends upon the spectrum of light and the relative absorption coefficients of these two states. Extinction coefficients of R to M of about 1.6 and conversion times between the two Rh1 states of about 1 min have been measured.

The absorption spectra of Rh1-Rh6 rhodopsin and metarhodopsin have recently been quantitatively characterized using microspectrophotometry applied to *Drosophila* eyes.<sup>11</sup> The maxima of the absorption spectra for rhodopsin (R) and metarhodopsin (M) for these six rhodopsins are given in Table 1. Salcedo et al. showed that the absorption spectra for the six rhodopsins and metarhodopsins were accurately fit by a log-normal function. Using their published values, it is possible to directly compare the absorption spectra for rhodopsin and metarhodopsin for Rh1 and Rh2 (Fig. 2a), Rh1 and Rh6 (Fig. 2b) and Rh2 and Rh6 (Fig. 2c). Unlike the other five rhodopsins, Rh6 metarhodopsin absorbs at a shorter wavelength than Rh6 rhodopsin.

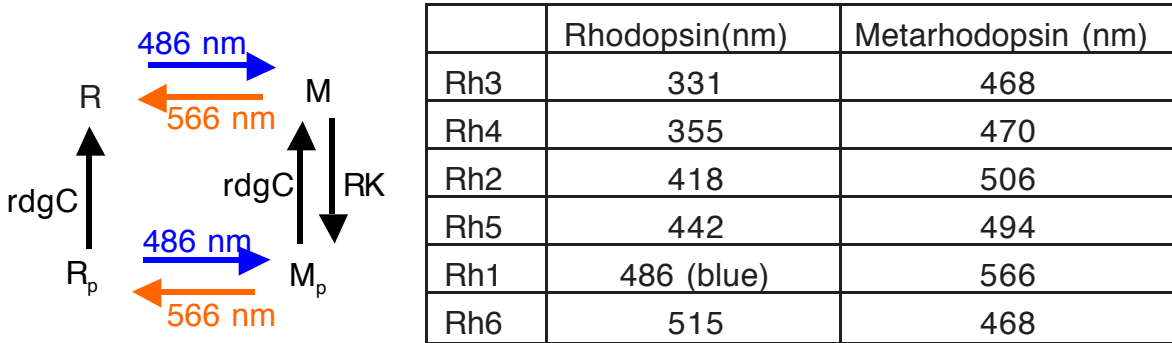


Fig. 1 Photoactivation cycle of R to M.

Table 1 Absorption maxima of rhodopsin and metarhodopsin for Rh1-Rh6.

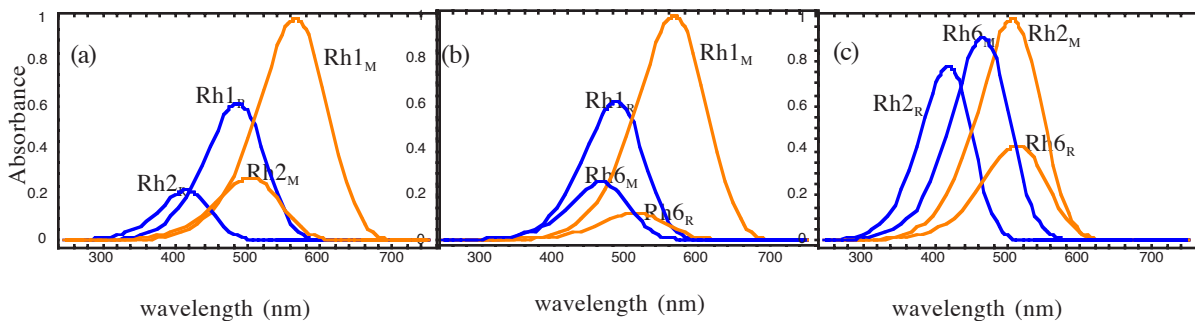


Fig. 2. Absorption spectra of rhodopsin (R) and metarhodopsin (M) for Rh1, Rh2, and Rh6. In (a) and (b), the spectra are normalized to Rh1 metarhodopsin. In (c) the spectra are normalized to Rh2 metarhodopsin.

Salcedo et al. have shown that subsequent absorption of light at the shifted maxima can convert nearly 100% of metarhodopsin back to rhodopsin.<sup>11</sup> When an excessive amount of metarhodopsin is produced, however, it has been shown that a prolonged depolarization afterpotential (PDA) can occur in fly's eyes (even after the light stimulus is turned off). During the PDA, *Drosophila* photoreceptors become refractory to further stimulation. In wild type *Drosophila*, about 20% conversion of rhodopsin to metarhodopsin is required to trigger a PDA;<sup>12</sup> in fly mutations lacking arrestin, there is an abnormal inactivation of metarhodopsin and the PDA is elicited with only a fraction of the amount of activated rhodopsin. It has also been shown that a PDA can be almost instantly terminated using a subsequent light stimulus at the correct wavelength to photoconvert the metarhodopsin.<sup>3,11</sup> ERG recordings from wild type *Drosophila* resulting from repetitive light stimulation show that a strong initial pulse of light at 570 nm elicits a depolarization that is maintained during the stimulus and terminates and repolarizes when the stimulus is turned off.<sup>11</sup> When the same intensity pulse with a wavelength near the peak of the absorption spectrum for Rh1 (470 nm) is used, a significant quantity of rhodopsin is activated and a PDA is produced. Subsequent stimulation at 570 nm photoconverts metarhodopsin and the PDA ends.

It should be possible to exploit the bistability of *Drosophila* rhodopsin to create a switch. Although the mechanisms in *Drosophila* appear to be straightforward, it would be too complicated to express all of the constituents in a single construct. In the absence of some of the key components from *Drosophila*, it is reasonable to assume that the activation and deactivation kinetics and perhaps the sensitivity of the construct will not be the same as the *Drosophila* eye.

## 2b. Hyperpolarizing and depolarizing switch

Because of the differences in the absorption spectra between the six *Drosophila* rhodopsins it should be possible to create a *depolarizing* construct that is excited at a different wavelength than the *hyperpolarizing* construct. *Depolarization* could be obtained by using *Drosophila* rhodopsin to activate a transient receptor potential (TRP) channel, which is a nonselective cation channel,<sup>3,8</sup> (*Drosophila* rhodopsin couples to the Gq pathway). This construct could use Rh1.

To construct a *hyperpolarizing* switch, it is necessary to have ionic flow in the opposite direction to the TRP channel; a gradient inward rectifying potassium (GIRK) channel is one possibility. GIRK channels are activated by G-protein coupled receptors via G-protein  $\beta\gamma$  subunits in a membrane delimited pathway.<sup>9</sup> Furthermore, Ehrenguber et al. have shown that over expression of these channels types in hippocampal neurons and simultaneous activation via G-coupled receptors hyperpolarized the cell and inhibited action potential firing.<sup>10</sup> Because the GIRK channels are mainly activated via the Gi/o pathway and the invertebrate rhodopsin from *Drosophila* works via phospholipase C, a Gq-like pathway, the invertebrate rhodopsin is not expected to be highly effective at activating the GIRK channel. By contrast, effective coupling is achieved in the vertebrate rhodopsin which works via transducin, a Gi-like protein. To activate the *hyperpolarizing* construct at a different wavelength, this construct could be coupled to another rhodopsin e.g Rh2. From Fig. 2a and Table 1, it may be observed that the hyperpolarizing construct can be maximally stimulated at 418 nm. Furthermore, light at 506 nm can be used to photoconvert Rh2 metarhodopsin back to Rh2 rhodopsin. Figure 3 provides a schematic that illustrates the two constructs.

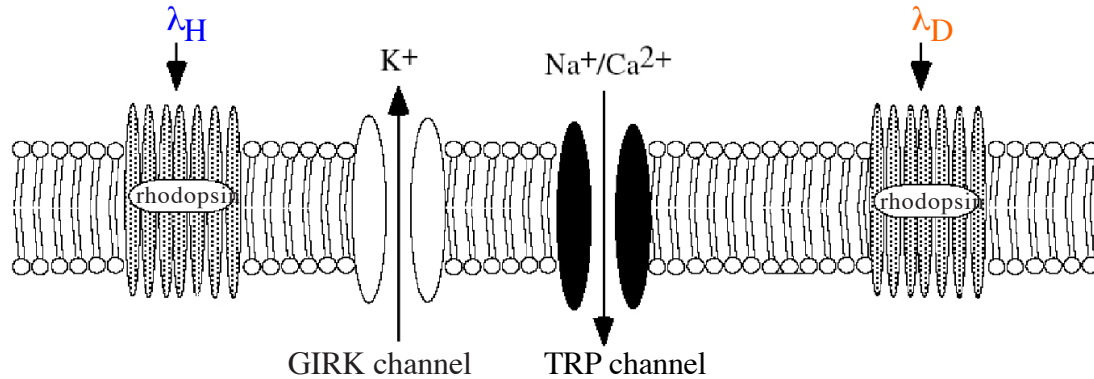


Fig. 3 Co-expression of the hyperpolarizing (GIRK channel) and depolarizing (TRP channel) constructs, activated by two different wavelengths  $\lambda_H$  and  $\lambda_D$  respectively.

### 3. METHODS

Only a hyperpolarizing construct was produced. Human embryonic kidney cells (HEK293) were transfected with vertebrate (rat) rhodopsin and GIRK channels (using cDNAs encoding GIRK2 subunits). To positively identify the transfected cells, the cells were also transfected with GFP. Whole cell recordings of HEK293 cells were obtained using a patch clamp amplifier (EPC-9; HEKA, Lambrecht). In order to assure that a whole cell patch was established, a series of voltage ramps (from  $-100$  mV to  $+50$  mV) were applied and the current was measured. To deliver monochromatic light with rapid switching (ms) between wavelengths, a broadband source with a series of band pass filters (DG5, Sutter) was chosen. The source was coupled to an inverted microscope (DMIRB, Leica, Inc.).

Figure 4 presents a flow chart of the various molecular biology procedures as well images of HEK293 cells; the GFP fluorescence illustrates positively transfected cells. Figure 5 is an illustration of the whole-patch clamping of cells and also presents the circuit diagram for the whole-cell patch. Figure 6 shows the voltage waveform applied using whole-cell patch clamping and Figure 7 defines the direction of positive current flow.

Once it was determined that a whole cell patch was established, all of the lights were turned off, and  $5 \mu\text{l}$  of 9-cis retinal was added to the cell medium. After an additional two minutes of absolute darkness, the computer monitor was turned on and a series of voltage ramps were applied to the cell. The measured current served as a control. Subsequently, the cells were exposed to broadband, white light. After an exposure period of about 500 ms, the series of voltage ramps were repeated and the current was measured.

Establish heterologous expression system

Mammalian cell line: **HEK293** (human embryonic kidney) - very low density of channels

↓

Establish channel constructs

Express channels into mammalian plasmid vector: **pcDNA3.1(-)**.

↓

Transfect cells

Insert nucleic acid into virus: **cytomegalovirus**.  
 Transfect HEK293 cells with construct and GFP for visualization of transfected cells.

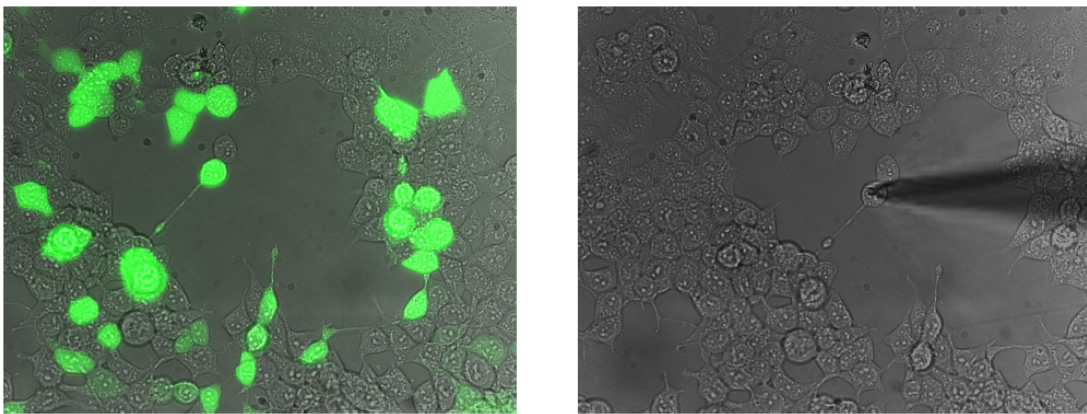


Fig. 4. Flowchart showing steps required for the transfection of HEK293 cells with ion channels. Images of HEK293 cells transfected with GFP, rhodopsin and GIRK channels. Image of the pipette used for the whole-cell recording.

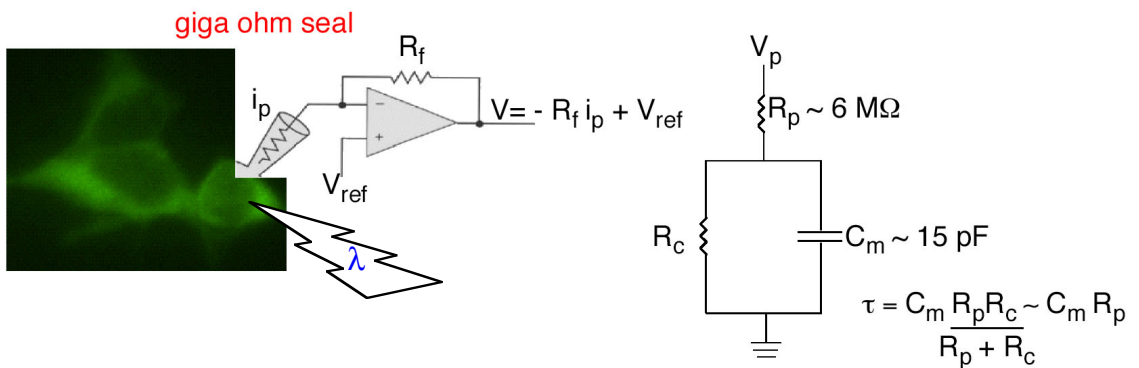


Fig. 5. Whole-cell patch clamp and circuit diagram for the pipette ( $V_p$ ,  $i_p$  and  $R_p$ ) and cell membrane ( $C_m$ ).

## 4. RESULTS

Figure 6 shows the voltage ramp that was used to analyze the current flow in whole-cell recordings of HEK293 cells and Figure 7 defines the direction of positive current flow. Figure 8 shows the current versus voltage recorded from a HEK293 cell that did not express GIRK channels (a) and after positive transfection with GIRK channels (b). Figure 8b illustrates the rectification that is observed with GIRK channels, i.e. the channels permit more  $K^+$  to flow in under hyperpolarization and limits efflux of  $K^+$  during depolarization. Once the channel opens, there is steep voltage dependence and the channel has a fast transition ( $< 1$  ms) from current flow to rectification. As expected, it was observed that the transition voltage strongly depends upon extracellular  $K^+$ .

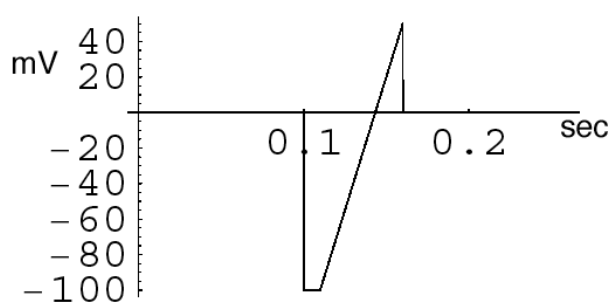


Fig 6. Waveform used to assess current flow through ion channel.

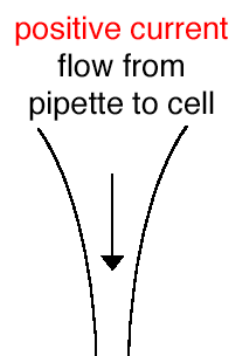


Fig 7. Definition of positive current flow through pipette.

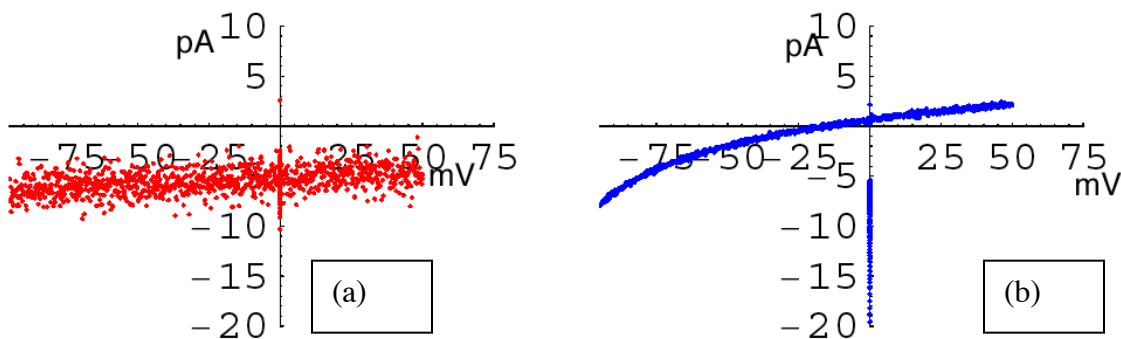


Fig 8. Whole-cell recording of in the absence (a) and presence (b) of the GIRK channel.

Figure 9 shows whole-cell recordings of a single HEK293 cell expressing G-protein coupled receptors and GIRK channels. Figs. 9a and 9b show a series of ten recordings of the current versus time before (a) and after (b) illumination with light. The control (Fig. 9a) was taken with the cells kept in the dark and Fig. 9b shows the current flow 500 ms after illumination of the cells with white light. The average of these ten recordings are shown as a function of the applied voltage ramp (c) both before (control) and after illumination (light). It can be observed that illuminating the cell with light increases the potassium current.

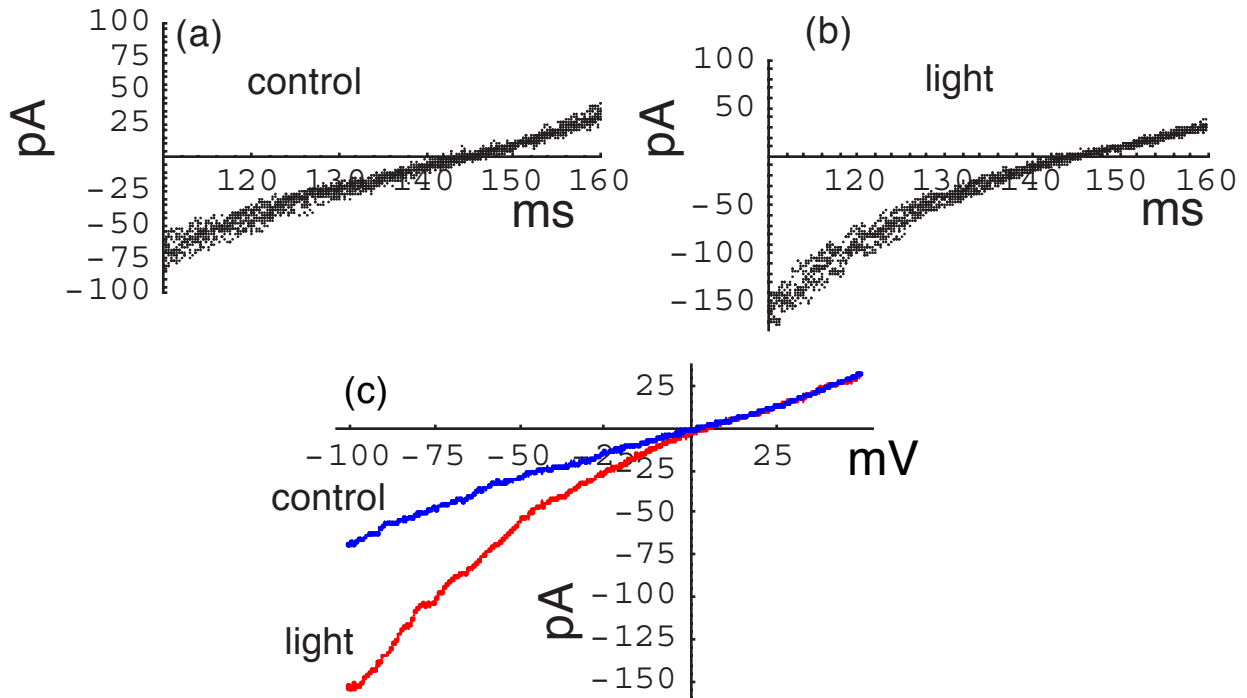


Fig. 9. Whole-cell recordings of a HEK293 cell expressing G-protein coupled receptors and GIRK channels (expression of GIRK2 and rhodopsin). Voltage ramps from  $-100$  mV to  $50$  mV before (control) and  $500$  ms after illumination of the cells with white light (light). A series of ten recordings of the current versus time are shown before (a) and after (b) illumination with light. The average of these ten recordings are shown as a function of the applied voltage ramp (c) both before (control) and after illumination (light).



## 5. DISCUSSION and CONCLUSIONS

Figures 8b and 9c illustrate the measured current flow through the gradient inward rectifying potassium (GIRK) channel. As expected, a good fit to the current flow may be obtained using the Boltzmann equation, Eq. 1:

$$I (V_m) = \frac{g_{\max} (V_m - E_K)}{1 + e^{\alpha (V_m - E_{1/2})}} \quad (1)$$

where:  $g_{\max}$  and  $V_m$  are the maximum conductance and membrane potential, respectively for the channel and  $E_K$  is the Nernst potential and  $E_{1/2}$  is the membrane potential at half the conductance. Figure 9c indicates that when the construct is illuminated with broadband light, the probability of channel opening is increased and the greater current flow is observed. In order to assess the statistical significance of this result, the cell sample must be increased from unity.

Although the data in Fig. 9 demonstrate the possibility of hyperpolarizing the cell, it should also be possible to create a depolarizing construct. From the data in Table 1 and Fig 2., it would be possible to elicit depolarization with a rapid light pulse at  $\lambda_1$  chosen to activate Rh1 rhodopsin that is coupled to a TRP channel. If the wavelength is chosen such that  $\lambda_1 \sim 500$  nm, Rh1 will absorb photons and become activated, but Rh2 will not be maximally activated. Therefore, it should be possible to activate only the depolarizing construct. Similarly, a subsequent light pulse delivered after a defined delay time with  $\lambda_2 \sim 600$  nm, will deactivate Rh1 metarhodopsin, but will not activate Rh2 rhodopsin. Therefore, it would be possible to activate the *depolarizing* construct without activating the *hyperpolarizing* construct. In fact, because the absorption maximum of Rh1 is near the peak of the absorption curve for Rh2 metarhodopsin, activation of Rh1 rhodopsin will also help to photoconvert any Rh2 metarhodopsin. It would also be feasible to create the hyperpolarizing construct using Rh6 instead of Rh2. Conversely, if the *hyperpolarizing* construct were formed with Rh1 and the *depolarizing* construct with Rh2 (or Rh6), it would be possible to stimulate only the *hyperpolarizing* construct and not depolarize the cell.

Assuming that the TRP channel has been coupled to Rh1, then stimulation of the *hyperpolarizing* construct requires activation of Rh2 rhodopsin. Because the absorption spectra of Rh2 rhodopsin and metarhodopsin overlap the absorption spectrum for Rh1 rhodopsin, activation of the *hyperpolarizing* construct with  $\lambda_1 \sim 410$  nm would moderately activate Rh1 metarhodopsin; more significantly, deactivation of Rh2 at  $\lambda_2 \sim 500$  nm would activate the *depolarizing* construct. Therefore, activation and deactivation of the *hyperpolarizing* construct will stimulate the *depolarizing* construct (or vice versa if the coupling of the *depolarizing* construct is to Rh2). Similar conclusions may be drawn for Rh2 and Rh6 rhodopsin.

In order for a light activated method of controlling membrane potential to be fully useful, it is essential that the activation and deactivation kinetics are controllable. An ideal technique for controlling nerve cell activity would be capable of switching nerve cells on or off within milliseconds. It should be capable of imposing depolarization or hyperpolarization of many millivolts for periods lasting from milliseconds to many seconds and to nearly simultaneously control the activity of hundreds or thousands of nerve cells in a single region.

It would be advantageous to couple this technique with the measurement of membrane potential using voltage-sensitive dyes or intrinsic fluorescence or to create another molecular construct

whose fluorescence or absorbance changed in response to changes in membrane voltage.<sup>15</sup> A scanning device that could both generate and measure different wavelengths of light in rapid sequence over a large area of neural tissue would make it possible to nearly simultaneously monitor and manipulate the activity of many individual neurons.

The preliminary data are supported by a recent paper that demonstrated that it is possible to elicit membrane depolarization in *Xenopus* oocytes and hippocampal neurons using selected components of the molecular machinery responsible for light transduction in *Drosophila*.<sup>16</sup> To test which of the several components would be required to produce a light modulation, this group systematically added mRNAs that coded genes for a select group of relevant proteins and using whole cell recordings measured the current elicited from a light stimulus. Of the ten proteins that they tested, ((1) NinaE; (2) NinaA; (3) arrestin 2; (4) G $\alpha$ ; (5) G $\beta$ ; (6) G $\gamma$  subunits; (7) NorpA; (8) TRP channel; (9) TRPL channel; and (10) InaD), they determined that only three components, NinaE, Gq $\alpha$ , and arrestin 2, were required in order to produce light activated currents in *Xenopus* oocytes. Furthermore, by expressing these three components in rat hippocampal neurons, they were able to elicit action potentials. This group was able to show that light elicited membrane depolarization, but that the temporal kinetics of this response differed considerably from those found in *Drosophila*. In oocytes, they observed long latencies to peak response from 2.3 to 6.7 seconds and in hippocampal neurons, a delay period of nearly 20 seconds was observed between the initial application of a light stimulus and the firing of action potentials; it was further observed that these delay times were not consistent. This group recognized the discrepancies between their method and intact photoreceptors and conjectured that additional "... catalytic, structural or regulatory components or fine adjustment of its stoichiometry may be necessary to speed its response kinetics to photoreceptor timescales...".

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