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Report

Light Activation of Channelrhodopsin-2 in Excitable Cells of *Caenorhabditis elegans* Triggers Rapid Behavioral Responses

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Summary

For studying the function of specific neurons in their native circuitry, it is desired to precisely control their activity. This often requires dissection to allow accurate electrical stimulation [1] or neurotransmitter application [2], and it is thus inherently difficult in live animals, especially in small model organisms. Here, we employed channelrhodopsin-2 (ChR2), a directly light-gated cation channel from the green alga *Chlamydomonas reinhardtii* [3], in excitable cells of the nematode *Caenorhabditis elegans*, to trigger specific behaviors, simply by illumination. Channelrhodopsins [3, 4] are 7-transmembrane-helix proteins that resemble the light-driven proton pump bacteriorhodopsin [5], and they also utilize the chromophore all-*trans* retinal, but to open an intrinsic cation pore. In muscle cells, light-activated ChR2 evoked strong, simultaneous contractions, which were reduced in the background of mutated L-type, voltage-gated Ca^{2+}-channels (VGCCs) and ryanodine receptors (RyRs). Electrophysiological analysis demonstrated rapid inward currents that persisted as long as the illumination. When ChR2 was expressed in mechanosensory neurons, light evoked withdrawal behaviors that are normally elicited by mechanical stimulation. Furthermore, ChR2 enabled activity of these neurons in mutants lacking the MEC-4/MEC-10 mechanosensory ion channel [6]. Thus, specific neurons or muscles expressing ChR2 can be quickly and reversibly activated by light in live and behaving, as well as dissected, animals.

Results and Discussion

As we have previously shown, ChR2 is permeable by mono- (e.g., Na^+, K^+, H^+) and some divalent cations, including Ca^{2+}, and enables strong and rapid membrane depolarization after illumination in mammalian cells or in *Xenopus* oocytes [3]. To maximize the effect, we engineered a gain-of-function (gf) mutant of ChR2, namely H134R. H134 corresponds to D96 in bacteriorhodopsin, a residue crucial for proton pumping [7]. Expression of ChR2_H134R(gf) in oocytes and HEK293T cells caused larger stationary photocurrents in comparison to ChR2(wt) (not shown). To monitor expression, we fused YFP to the C terminus, obtaining ChR2(gf)::YFP. This protein mediated large photocurrents in HEK-cells (Figure 1A), with inward rectification, rapid light-induced opening (τ = 0.96 ± 0.12 ms, n = 5), and rapid closing in the dark (τ = 20.9 ± 2.2 ms, n = 5), just as ChR2(wt) [3, 8]. Likewise, ChR2(gf)::YFP-photocurrents rapidly desensitized (τ = 6.8 ± 1.9 ms, n = 5) to a still large steady-state photocurrent, which did not further inactivate, even after repeated illumination (Figure 1B). Recovery of initial peak photocurrents in the dark proceeded with a time constant τ = 6.4 ± 0.9 s (n = 3), as similarly observed in oocytes [3] and rat neurons [8]. Current-clamp measurements in HEK-cells expressing ChR2(gf)::YFP demonstrated a large and fast light-induced membrane depolarization (Figure 1C).

To test whether ChR2(gf)::YFP could function in *C. elegans*, we expressed it either in muscles or in mechanosensory neurons. Transgenic animals, raised in the presence of all-*trans* retinal, were illuminated (450–490 nm) for defined times and filmed to quantify body contractions, or they were observed for behavioral responses. We always used several controls: (1) the same transgenic animals raised without retinal (expressing equal amounts of ChR2, but in a nonfunctional state), and wild-type animals, not expressing any transgene, raised in the (2) presence or (3) absence of retinal.

ChR2 Can Evoke Contraction of *C. elegans* Muscle Cells In Vivo

ChR2(gf)::YFP, expressed in body wall and egg-laying muscles from the *myo-3* promoter (transgene *zxEx17 [pmyo-3::ChR2(gf)::YFP]*), was detectable at the plasma membrane (Figure 2A, Figure S1A in the Supplemental Data available with this article online) and in intracellular structures colocalizing with a marker for the endoplasmic reticulum (ER; Figures S1A–S1C). When exposed to light, *zxEx17* animals reproducibly showed strong and simultaneous contractions of (apparently) all muscle cells, causing a readily visible shrinking of the body (Figure 2B; Movie S1). Often, adult animals expelled eggs, as a result of contraction of vulval muscles, of increased internal pressure, or of both (Movie S2). Contraction was maximal (to circa 87% of initial body length) circa 500–600 ms after illumination began (Figure 2C) and was sustained for the time the illumination continued (even up to 1 min; Figure 2C, inset). When light was turned off, muscles relaxed within circa 1 s (Figure 2D). Importantly, none of the controls (as described above) exhibited any contraction (Figure 2C), verifying that these were due to functional ChR2 containing

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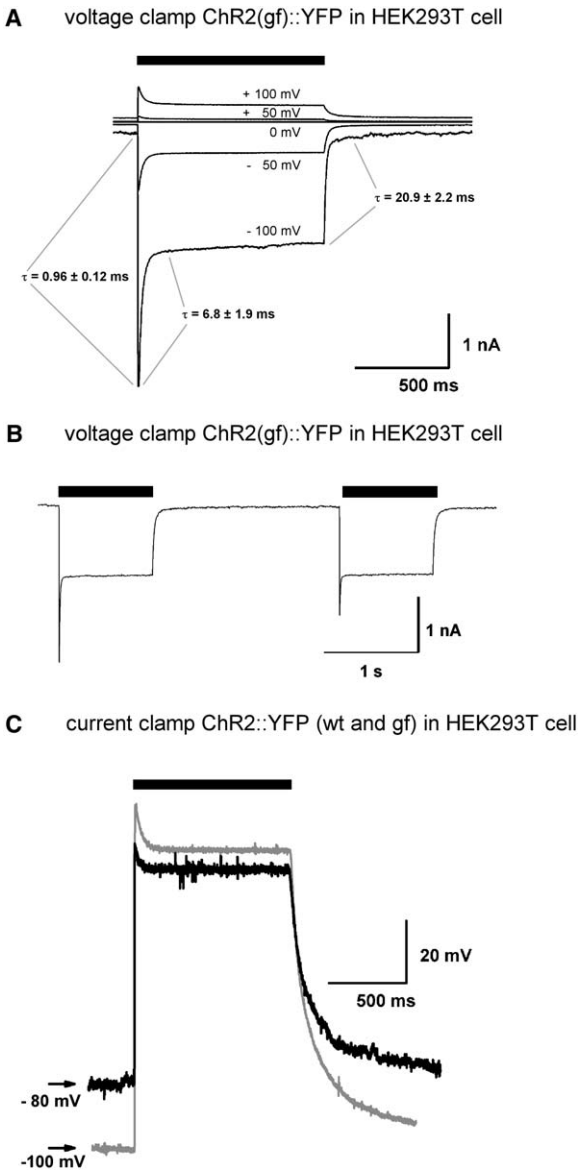

Figure 1. Photocurrents and Membrane-Potential Changes in HEK293T Cells Expressing Wild-Type and Gain-of-Function ChR2::YFP

(A) Photocurrents in a voltage-clamped HEK293T cell expressing ChR2(gf)::YFP, after defined illumination by 1 s laser pulses (442 nm, HeCd-laser) at holding potentials as indicated, demonstrating inward rectification. Time constants for the indicated rise phase and decay phases were deduced from five separate experiments.

(B) Repeated light stimulation of a voltage-clamped HEK293T cell expressing ChR2(gf)::YFP demonstrates a reduction in peak, but not steady-state, photocurrents. Peak currents were recovered in consecutive trials with a time constant $\tau = 6.4 \pm 0.9$ ms, n = 5.

(C) Light-induced depolarization of two HEK293T cells expressing either ChR2(wt)::YFP (black trace) or ChR2(gf)::YFP (gray trace) in current-clamp mode. Initial membrane potentials are indicated. Periods of illumination are indicated by black bars in all panels.

retinal. Shorter illuminations caused less-pronounced contractions and earlier relaxation (Figure 2E).

Muscle contraction is normally triggered by gating of nicotinic acetylcholine receptors (nAChRs) and the resulting Na^+-influx [2]. Because *C. elegans* lacks voltage-gated Na^+-channels [9], action potentials cannot be exerted, and nAChR-mediated depolarization elicits contraction directly via voltage-gated Ca^{2+}-channels (VGCCs) and the resulting cytosolic Ca^{2+} entry. ChR2-evoked contractions should depend both on Ca^{2+} influx directly via ChR2 [3] and on ChR2-mediated depolarization/VGCC activation. Thus, we assayed ChR2 function in a mutant affected for K^+-currents that repolarize the cells after excitation (*slo-2(nf100)*; [10]). SLO-2 encodes a widely expressed K^+-channel, activated by depolarization but also dependent on Ca^{2+} and Cl^- ions [11]. Yet, *slo-2(nf100)*; *zxEx17* animals showed wild-type rates of contraction and relaxation (Figures 2C and 2D). Therefore, either SLO-2 cannot efficiently counteract ChR2-mediated depolarization, or Ca^{2+} influx directly through ChR2 [3], potentially even from the ER (Figure S1), may contribute to light-evoked muscle contractions. Although depolarization/VGCC-mediated Ca^{2+} influx may be increased in *slo-2* mutants, Ca^{2+} entry directly through ChR2 should not be affected.

Mutations of L-Type VGCCs and The Ryanodine Receptor Reduce Light-Evoked Muscle Contractions

To address the role of Ca^{2+} influx through ChR2 in muscle contraction, we investigated the contribution of other cytosolic entry routes for Ca^{2+}. ChR2-mediated contractions (transgene *zxEx17*) were measured in mutants of the L-type VGCC encoded by *egl-19(n582)* (strong reduction-of-function), the P/Q-type VGCC encoded by *unc-2(lj1)* (loss-of-function), and the RyR gene *unc-68(kh30)* (reduction-of-function). EGL-19 represents the major VGCC in muscle [12]. UNC-2 appears to function mainly in neurons [13], though *unc-2* mRNA was found also in muscle [14]. UNC-68 is a sarcoplasmic Ca^{2+} channel largely responsible for Ca^{2+}-induced Ca^{2+} release (CICR) in muscle and thus for contraction [15].

We observed a dramatic reduction (by 62%) of ChR2-evoked contractions in *unc-68(kh30)* RyR mutants (Figure 2F; $p < 0.00004$ over all time points, t test). Thus, as expected, CICR through the RyR is a major determinant of muscle contraction, also when triggered by ChR2 gating and not cholinergic transmission. Reduction of EGL-19 function in *n582* mutants showed a less prominent, but significant, reduction of contractions (by 19%; Figure 2F; $p < 0.05$). In contrast, *unc-2(lj1)* mutants were not affected. Therefore, the major source of Ca^{2+} that normally triggers CICR in response to depolarization may be EGL-19, though we cannot exclude that other, unknown VGCCs may be expressed in muscle and contribute to Ca^{2+} influx. Light-evoked contractions were not abolished in *egl-19(n582)* mutants, which could be explained by residual function of EGL-19 or other VGCCs. Alternatively, cytosolic Ca^{2+} influx directly through ChR2 may contribute to contraction by triggering CICR. To clarify this further, we will study mutants in other VGCCs, as well as double (or multiple) mutants between these and *n582*. Furthermore, restricting ChR2 to the ER by retention signals will allow for directly assessing cytosolic Ca^{2+} entry through ChR2.

Characterization of ChR2-Photocurrents in *C. elegans* Body Muscle

We measured ChR2-mediated photocurrents in body-muscle cells of *zxEx22* (*pmyo-3::ChR2(gf)::YFP; lin-15*$^+$)

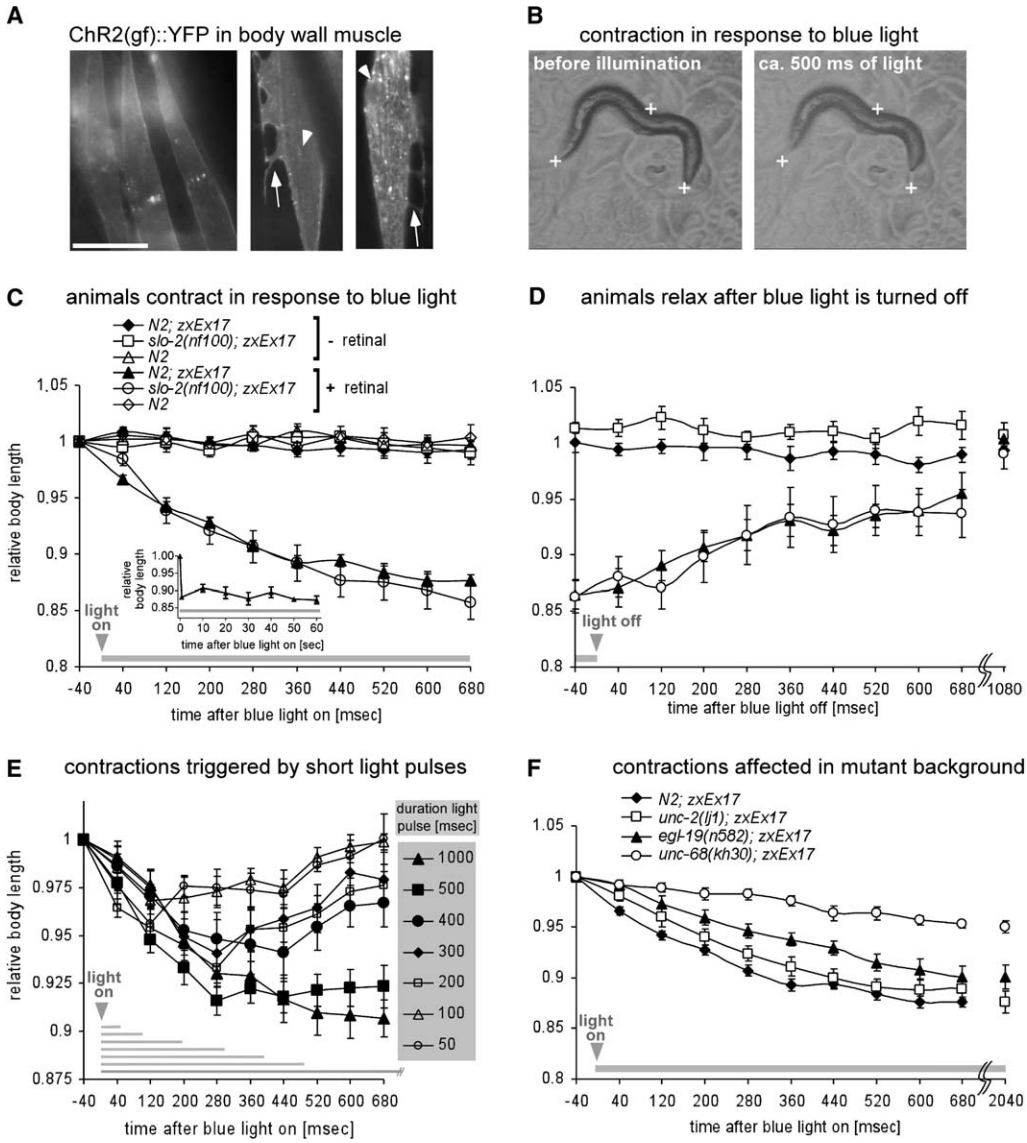

Figure 2. ChR2(gf)::YFP Expression in *C. elegans* Body Muscles Permits Light-Evoked Contractions

(A) ChR2(gf)::YFP was expressed in body muscles (transgene *zxEx17[pmyo-3::ChR2(gf)::YFP]*). Expression is seen at the plasma membrane of three muscle cells (left panel) and muscle arms (middle and left panels, arrows) and in intracellular structures (arrowheads); see Figure S1 for ER localization. The scale bar represents 15 μm.

(B) A *zxEx17* animal, cultivated in the presence of retinal, exhibits simultaneous contraction of body muscles upon illumination with blue light (450–490 nm). The left panel is just before blue light is turned on; the right panel is circa 500 ms after blue light was turned on. Crosses are fixed points in both images, for orientation.

(C) Contractions of *zxEx17* animals were measured in single frames taken from movies (12.5 Hz frame rate) while blue light was turned on for circa 2 s. The last frame before light was turned on was taken as starting point ("−40 ms"); the length of the individual animal was set to 1, and the relative length in the consecutive nine frames was measured (Supplemental Experimental Procedures). Six sets of eight animals of different genotypes (N2, wild-type, n = 24, and *slo-2(nf100)* animals containing *zxEx17*; N2 animals without any transgene) and growth conditions (in presence or absence of retinal) were analyzed, and the observed values were averaged. Inset shows averaged contractions of five *N2; zxEx17* animals observed during long-term illumination; the contractions were measured after 680 ms and consecutive 10 s intervals during 1 min of constant illumination.

(D) Relaxation of the animals in (C) after blue light was turned off. Always the last frame before light was turned off was taken as starting point ("−40 ms"), and body length (relative to the length before light was turned on) in the consecutive nine frames, and, for comparison, in the 15th frame ("1080 ms"), was measured.

(E) Shortening the times of illumination (measured every 80 ms for the first 680 ms during illuminations of 1000, 500, 400, 300, 200, 100, and 50 ms) caused less-pronounced contractions and earlier relaxation of *N2; zxEx17* animals (n = 5 for each illumination interval).

(F) Light-evoked contractions were measured in mutants expressing the *zxEx17* transgene: *unc-68* (*kh30*; RyR), *egl-19* (*n582*; L-type VGCC), or *unc-2* (*lj1*; P/Q-type VGCC); n = 8 for each mutant. When compared to the wild-type (n = 24), all data points later than 40 ms of *egl-19* and *unc-68* mutants were significantly different ($p < 0.05$ and $p < 0.001$, respectively; t test). In (C) through (F), error bars represent standard error of the mean (SEM), and duration of illumination is indicated by gray bars.

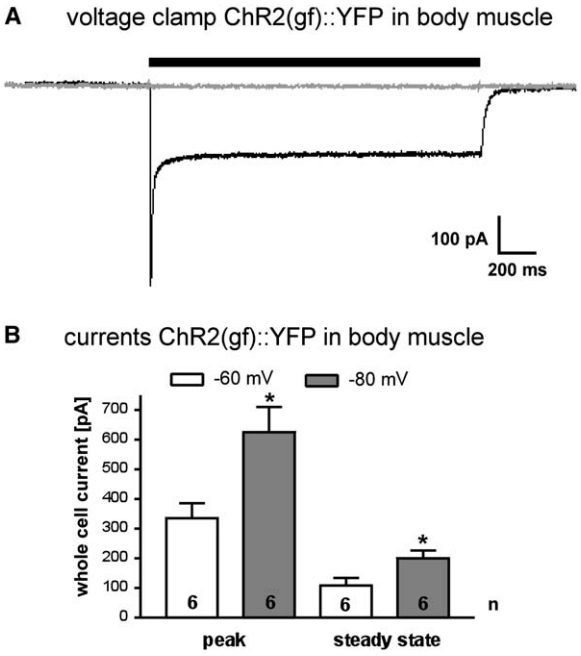

Figure 3. ChR2(gf) Photocurrents in *C. elegans* Muscle Cells

(A) Photocurrents were measured in a whole-cell voltage-clamped *C. elegans* body-muscle cell expressing the *zxEx22* transgene (*pmyo-3::ChR2(gf)::YFP*) at −60 mV holding potential (black trace). The gray current trace is from a *zxEx22* animal cultivated in the absence of retinal. The black bar indicates exposure to blue light.

(B) Statistical analysis of peak and steady-state photocurrents measured at −60 and −80 mV holding potentials in dissected muscle cells (n = 6 for each condition), as described in (A). Error bars represent SEM.

animals under whole-cell voltage clamp (Figure 3A), following an established protocol [2]. ChR2(gf)::YFP-expression and light-evoked body contractions in *zxEx22* animals were comparable to those in *zxEx17* animals. At −60 mV holding potential, light induced initial peak inward currents of 335 ± 51 pA that quickly dropped to a steady-state current of 109 ± 26 pA (Figure 3B; n = 6) and remained unchanged as long as illumination lasted (Figure 3A). At −80 mV, currents were significantly larger (Figure 3B; peak current, 625 ± 85 pA; steady-state current, 200 ± 26 pA; n = 6; $p < 0.05$, t test). In muscles of animals raised in the absence of retinal, no photocurrents occurred (Figure 3A).

Membrane potentials of wild-type muscles and those expressing the *zxEx22* transgene were similar (not shown). Under comparable experimental conditions, endogenous miniature postsynaptic currents (mPSCs) measured in *C. elegans* muscle are circa 25 pA [1], and acetylcholine-evoked currents are circa 700 pA [2]. Thus, ChR2-evoked steady-state photocurrents fall in between. It is unknown what currents occur in a nondissected animal's (hyper-)contracted muscle. The fact that we measured moderate currents yet strong contractions may indicate that Ca^{2+} influx from the ER (not detectable by whole-cell patch clamp) through ChR2 could contribute to contractions directly, or by triggering CICR, consistent with our other findings.

ChR2 Activation in Mechanosensory Neurons Evokes Behavioral Responses

Next, we tested whether ChR2 could depolarize neuronal cells, namely the mechanosensory neurons ALM, PLM, AVM, and PVM. Here, we expressed it from the *mec-4* promoter (*zxEx18[pmec-4::ChR2(gf)::YFP]*). ChR2(gf)::YFP was detectable at plasma membranes (cell bodies and processes) and in membranous intracellular compartments (Figure 4A). The mechanosensory cells respond to touch of anterior and posterior body by evoking, through downstream neurons, withdrawal or acceleration responses, respectively [16]. If all mechanosensory neurons are stimulated simultaneously, i.e., by tapping the culture dish, withdrawals predominate. We thus expected that simultaneous light activation of all mechanosensory cells by ChR2 would evoke withdrawals. Analyzing light responses is complicated by the fact that *C. elegans* avoids strong light, most likely as a result of (noxious) heat caused by light absorption [17], though a specific light response was also reported [18]. However, when *zxEx18* animals were illuminated five consecutive times for circa 1 s with blue light, individuals raised in the presence of retinal showed withdrawals (and sometimes accelerations) significantly more often (circa 72% in first, and 35% in fifth, trials; Figure 4B; Movie S3) than the same transgenic animals raised without retinal (circa 10% and 16%, respectively; Figure 4B). This response was typically observed within the first 500 ms of illumination. Control animals did not show significant responses (Figure 4B). Thus, ChR2 can indeed evoke specific activity in neurons.

Animals expressing ChR2 apparently exhibited habituation to the light stimulus (Figure 4B). The tap (and the touch) response is also subject to habituation (Figure 4C; [19]); however, it is unclear whether this is mediated by the mechanosensory ion channel [20], during signal transduction within the touch-receptor cells [21, 22], or even later. Given that ChR2 does not desensitize in touch neurons, and signal transduction downstream of membrane depolarization induced by ChR2 and mechanosensory channels is similar, our experiments could indicate that functional habituation in touch cells is mediated, at least in part, downstream of mechanosensory channels. In other cell types (Figure 1B; [3, 8]), steady-state ChR2 photocurrents did not inactivate, and ChR2-evoked muscle contraction did not decrease even during prolonged illumination (>1 min; Figure 2F). Desensitization of peak photocurrents (Figure 1B; [3]) should not be critical because we included 10 s dark intervals between stimuli. However, ChR2 may behave differently in touch neurons (from which we did not record currents), and repeated light stimuli caused stronger habituation than repeated touch (Figures 4B and 4C).

ChR2 Evokes Responses in Touch-Receptor Neurons Lacking Functional Mechanosensory Channels

Finally, we assayed ChR2-evoked withdrawal behavior in *mec-10(e1515)* mutants (and *mec-4(u253)*; not shown) [6], which are defective in MEC-4/MEC-10 mechanosensory ion channels and almost nonresponsive to touch (Figure 4C). In gentle-touch assays [23], wild-type and *zxEx18* animals, reacted 95%–97% of the time (Figure 4C) and showed habituation in consecutive trials

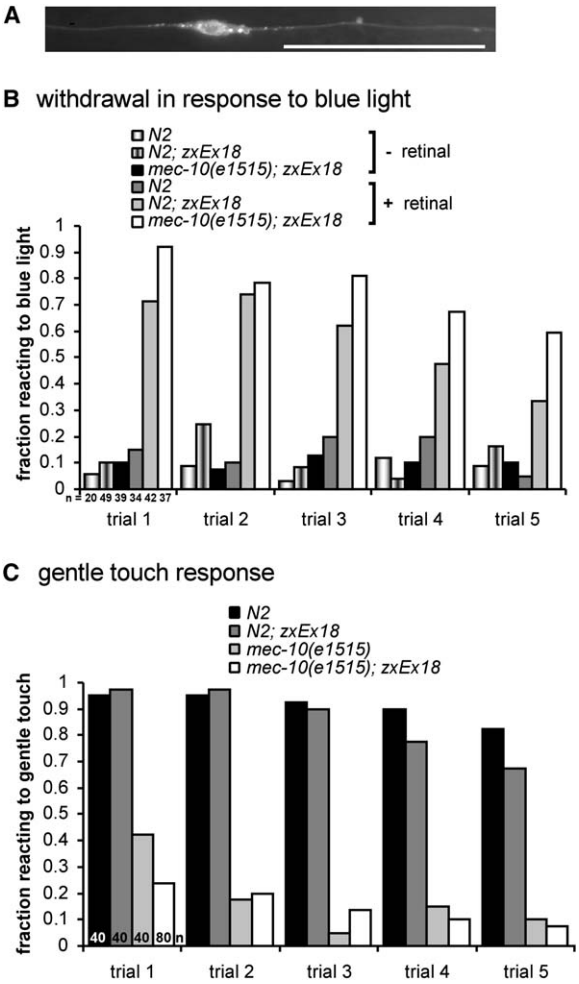

Figure 4. ChR2(gf) Expressed in Mechanosensory Neurons Mediates Light-Evoked Withdrawal Behavior

(A) Expression of a *pmec-4::ChR2(gf)::YFP* transgene (*zxEx18*) in touch-receptor neurons is found at the plasma membrane of cell bodies and mechanosensory projections and in intracellular membranous compartments. Shown is a posterior touch cell, PLM. Anterior is right, and the scale bar represents 10 μm.

(B) *zxEx18* animals were exposed to blue light, and the fraction of individuals that responded with a marked withdrawal or acceleration (i.e., for at least half a body length) within the first 500 ms was plotted. These genotypes were compared: N2 (wild-type), without and with *zxEx18*, and *mec-10(e1515); zxEx18* mutants. All genotypes were tested after cultivation in absence or presence of retinal. For each animal, five assays were conducted in 10 s intervals, and the results for each trial, for all animals of each genotype and condition, were averaged, where 0 = no response and 1 = response (thus, no meaningful statistical analysis is possible).

(C) N2 and *mec-10(e1515)* mutants, either without or with *zxEx18*, were subjected to a gentle touch-response assay with an eyelash. All animals were tested five consecutive times with 10 s intervals; responses were counted as 1, no response as 0, and they were averaged. All assays in (B) and (C) were performed blind; number of animals tested in each assay is indicated.

(70%–85% reacting in fifth trials). In contrast, only 22%–42% of *e1515* mutants responded in first trials and less than 10% in fifth trials. However, upon light stimulation, *e1515; zxEx18* animals responded >90% of the time, whereas only 10% of control animals did (Figure 4B). This demonstrated that *e1515* mutant touch cells were functional with respect to signaling to downstream interneurons. Interestingly, *mec-10(e1515); zxEx18* animals appeared to react more robustly to the light stimulus (circa 90% of first trials) than wild-type animals (circa 70%). This could suggest that normal function of the MEC-4/MEC-10 channel keeps the cells in a partially habituated state. As a result of reduced function of this channel in *e1515* mutants, these neurons could be even more prone to signal to downstream interneurons in response to membrane depolarization/Ca^{2+} influx.

Conclusions

By expression of a mutated ChR2(gf)::YFP fusion-protein, we could elicit strong responses to blue light in excitable cells (muscles and neurons) of live and dissected *C. elegans*. ChR2(wt)::YFP also caused strong photocurrents and robust depolarization (Figure 1C) of HEK293T cells, as previously shown for oocytes [3] and rat neurons [8]. However, for strong light effects in *C. elegans*, high expression and retinal availability appeared more important than using ChR2(gf). Further studies will explore the feasibility of increasing current amplitude and shifting ion selectivity and spectral sensitivity of ChR2 by mutation, a guide to which might be provided by related studies on other microbial-type rhodopsins [24]. Potentially, even several forms of ChR2, triggered by different wavelengths, could be simultaneously used in different cells of the same animal.

Altogether, we could show that ChR2 is uniquely suited for activating certain neurons in specific circuits in live nematodes. Whereas previously, functions of specific neurons were often elucidated by laser ablation and observing effects on the animal's behavior [25], it is now possible to specifically activate a particular neuron (or group of neurons), given that a specific promoter is available to drive ChR2 expression. As we have shown in *C. elegans* muscle and other expression systems, ChR2 is rapidly opened and conducts cations in an essentially nondesensitizing manner, as long as the cell is illuminated. In the dark, ChR2 rapidly closes, thus permitting a high degree of control (see also [8]). Utilizing ChR2 is thus advantageous over an earlier approach used to trigger neuronal activity in *C. elegans*, i.e., expression of a mammalian TRPV1 channel in chemosensory neurons and exposing the animals to capsaicin, a strong activator of this channel [26]. Although this approach evoked cellular activity and withdrawal responses, the stimulus could not be rapidly removed. Another approach to photo-trigger neurons in culture and in live *Drosophila* utilized ATP-gated ion channels ($P2X_2$-receptors) and caged ATP that could be photoactivated [27]. Also in this system, a cofactor (which may become limiting in long-term manipulations) has to be acutely applied (or even injected). More importantly, $P2X_2$ receptors require chemical activation, and thus open on a slower timescale (>several milliseconds) than light-stimulated ChR2 (essentially below 1 ms; Figure 1A and [3]). Again, our method appears advantageous.

Use of ChR2 will allow elucidating functional neuronal circuits in *C. elegans*, e.g., the circuit of command interneurons and the various motorneuron classes controlling forward or backward locomotion. For cells that do not trigger obvious behaviors, Ca^{2+} imaging in

downstream neurons could reveal integration into specific circuits. ChR2 could also be used in electrophysiological experiments to evoke neurotransmitter release, which is usually done by electrically stimulating the nerve cord and poses significant experimental difficulties [1]. By fusing ChR2 to appropriate signal sequences, we may guide it to specific subcellular sites, e.g., pre- or postsynaptic specializations. Finally, the use of lasers to activate ChR2 may provide an even higher degree of spatial and temporal resolution.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures, three movies, and two figures and are available with this article online at: http://www.current-biology.com/cgi/content/full/15/24/2279/DC1/.

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