

Channelrhodopsin-2–XXL, a powerful optogenetic tool for low-light applications

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Channelrhodopsin-2 (ChR2) has provided a breakthrough for the optogenetic control of neuronal activity. In adult *Drosophila melanogaster*, however, its applications are severely constrained. This limitation in a powerful model system has curtailed unfolding the full potential of ChR2 for behavioral neuroscience. Here, we describe the D156C mutant, termed ChR2-XXL (extra high expression and long open state), which displays increased expression, improved subcellular localization, elevated retinal affinity, an extended open-state lifetime, and photocurrent amplitudes greatly exceeding those of all heretofore published ChR variants. As a result, neuronal activity could be efficiently evoked with ambient light and even without retinal supplementation. We validated the benefits of the variant in intact flies by eliciting simple and complex behaviors. We demonstrate efficient and prolonged photostimulation of monosynaptic transmission at the neuromuscular junction and reliable activation of a gustatory reflex pathway. Innate male courtship was triggered in male and female flies, and olfactory memories were written through light-induced associative training.

Identifying causal relationships between neuronal activity and animal behavior is a fundamental goal of neuroscience. Crucially, this task requires testing whether defined neuronal populations are sufficient for eliciting behavioral modules. The development of light-gated ion channels that can be genetically targeted to specific cells has provided a unique solution to this challenge. In pioneering work, such optogenetic effectors or actuators were originally used as multicomponent approaches (1–3). The introduction of Channelrhodopsin-1 (ChR1) (4) and especially ChR2 as a light-sensitive cation channel (5) dramatically advanced the field by providing an efficient and straightforward single-component strategy for stimulating neuronal activity (6, 7).

Besides cell-specific targeting of appropriate effector elements, precise neuronal control by optogenetics demands efficient light delivery to the neurons of interest. For behavioral studies, photostimulation is ideally accomplished in intact, freely moving organisms and accompanied by functional readouts. The combination of a rich, well-characterized behavioral repertoire and elegant molecular genetics has contributed to *Drosophila's* strong impact on behavioral neurogenetics (8, 9). However, low light transmission through the pigmented cuticle presupposes high light intensities for using ChR2 in flies. This obstacle greatly complicates the experimental setup for freely moving animals, and the required light energies can cause heat damage when stimulation is applied over extended time periods. Moreover, limited cellular availability of *all-trans*-retinal (hereafter retinal for short) demands adding high retinal concentrations as a dietary supplement. If optical access to target cells is not provided by a translucent body wall (e.g., as in nematodes, zebrafish, and *Drosophila* larvae), an alternative solution is the implantation of an optical fiber directly into the brain. Although this approach has been used successfully in mammals (10), such an invasive procedure is infeasible for the study of intact small organisms.

Due to these restrictions in *Drosophila*, ChR2 has not reached the popularity attained in other organisms, and instead the field has turned mainly to thermogenetic neuronal stimulation (11–13). As with all techniques, there are also drawbacks to using temperature as a stimulus, such as undesired background activity

and a multitude of temperature-sensitive cellular processes and behavioral responses. Photo-liberation of caged ATP, combined with genetic targeting of ATP-gated ion channels, has been introduced as a different optogenetic technique in *Drosophila* (3, 14). However, its applications are constrained by invasive, time-consuming procedures for injection of caged ATP and a limited experimental time window.

Here, we introduce improved ChR2 variants as an alternative approach to address these shortcomings in *Drosophila*. Compared with wild-type ChR2 (ChR2-wt), expression of these mutants in target cells led to strongly enhanced photocurrents. We provide the first report, to our knowledge, of ChR2-T159C (15, 16) in flies and describe a ChR2 variant, ChR2-XXL (extra high expression and long open state), that is characterized by an extended open-state lifetime, elevated cellular expression, enhanced axonal localization, and reduced dependence on retinal addition. As a consequence, this mutant does not require dietary retinal supplementation to depolarize cells, evoke synaptic transmission, and activate neuronal networks at very low irradiance. These features enabled behavioral photostimulation in freely moving flies using diffuse low-intensity light.

Results

Biophysical Characterization of ChR2 Variants. It was recently demonstrated that the efficiency of ChR2 expression depends on retinal availability and the exact amino acid sequence of the apoprotein [i.e., channelopsin-2 (Chop2)] (16). In *Xenopus* oocytes, Chop2 is degraded more rapidly without its chromophore than in its

Significance

Controlling neuronal activity in live tissue is a long sought-after goal in the neurosciences. Channelrhodopsin-2 (ChR2) is a microbial-type rhodopsin that can be genetically expressed to depolarize neurons with light. Thereby, this “optogenetic tool” delivers cellular specificity and elegant options for studying the neuronal basis of behavior in intact organisms. Unfortunately, low-light transmission through pigmented tissue greatly complicates light delivery to target cells and curtails experiments in freely moving animals. This study introduces a ChR mutant, ChR2-XXL, that gives rise to the largest photocurrents of all ChRs published so far and increases light sensitivity more than 10,000-fold over wild-type ChR2 in *Drosophila* larvae. As a result, behavioral photostimulation is evoked in freely moving flies using diffuse, ambient light.

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retinal bound state (ChR2). Moreover, certain point mutations of Chop2 (T159Y/F/W) help to protect the apoprotein from degradation and thereby enable retinal binding several days after Chop2 translation. In fact, when threonine 159 is replaced by aromatic amino acids [tyrosine (Y), phenylalanine (F), or tryptophan (W)], expression levels of Chop2 (“unloaded” protein) are comparable with ChR2 (“chromophore-loaded” protein). However, only retinal-bound ChR2-T159Y/F/W variants are able to mediate a significant light-gated conductance (16). In contrast, for the related mutant T159C, protein expression levels and photocurrents are greatly enhanced compared with the wt variant both in the presence and in the absence of added retinal (15, 16). This observation has been interpreted as an enhanced binding affinity of Chop2-T159C to the endogenous retinal of oocytes (16).

The hypothesized hydrogen bond between residues C128 and D156 of transmembrane helices 3 and 4 is a crucial determinant of the lifetime of ChR2’s conducting state (Fig. 1 *A* and *B*) (17, 18). In the course of investigating this structural feature, we discovered that substituting aspartic acid with cysteine at position 156 resulted in exceptionally high protein expression in addition to a long open state. ChR2-D156C, recently studied in terms of primary photoreactions (ps to ns range) (19), was therefore termed ChR2-XXL. Intracellular localization and expression levels were analyzed by expressing yellow fluorescent protein (YFP) fusion constructs of ChR2-wt and ChR2-XXL in *Xenopus* oocytes. Confocal fluorescence images were recorded, and signal intensity was quantified (Fig. 1 *C* and *D*). Subsequently, electrophysiological recordings were used to characterize light-induced photocurrents (Fig. 1*E*). ChR2-XXL showed very strong expression, as determined by YFP fluorescence (no added retinal, 100 ± 8 a.u. SD, $n = 6$; plus retinal, 141 ± 13 a.u., $n = 6$), which indicates increased affinity for retinal (ChR2-wt, no added retinal, 24 ± 2 a.u. SD, $n = 6$; plus retinal, 65 ± 9 a.u., $n = 6$). Accordingly, very large photocurrents were recorded without retinal supplementation, and the photocurrents were further

increased when retinal was added to the medium (Fig. 1 *C* and *E*). Adding retinal to oocytes expressing ChR2-XXL resulted in photocurrents of up to $-50 \mu\text{A}$ at -100 mV (Fig. 1*E*) (no added retinal, $12 \pm 3 \mu\text{A}$ SD, $n = 6$; plus retinal, $38 \pm 10 \mu\text{A}$, $n = 6$), rendering these currents the largest of all ChR variants published so far (ChR2-wt, no added retinal, $0.2 \pm 0.2 \mu\text{A}$ SD, $n = 6$; plus retinal, $2 \pm 0.5 \mu\text{A}$, $n = 6$) (Table S1) (15, 16).

After excitation, the channel closes with a time constant of about 76 s at 22 °C (Fig. 1*F*). A deceleration was also observed for the opening of ChR2-XXL with a time constant of ~ 5 ms (Fig. 1*F*), compared with 0.2 ms for ChR2-wt (5). As expected, the slower channel closing is accompanied by a strongly increased light sensitivity (ChR2-XXL, $3.0 \pm 0.3 \mu\text{W}/\text{mm}^2$ SD half-saturating light intensity; ChR2-wt, $0.7 \pm 0.1 \text{ mW}/\text{mm}^2$; ChR2-T159C, $1.1 \pm 0.2 \text{ mW}/\text{mm}^2$). For this reason, exposure of oocytes to blue light before measurements had to be absolutely avoided. Importantly, the extraordinarily high photocurrent amplitude mediated by ChR2-XXL clearly distinguishes it from other mutants possessing an extended open state (C128S, C128T, C128A, and D156A) but that do not greatly exceed maximal current amplitudes of ChR2-wt (Fig. S1 and Table S1) (17, 18, 20). Interestingly, ChR2-C128A and D156A also express more efficiently in oocytes than ChR2-wt although strong expression is not a general feature of slow mutants (Fig. S1). It is therefore conceivable that additional factors (such as, e.g., increased single channel conductance and/or open probability) contribute to the large photocurrent amplitude of ChR2-XXL.

Illumination with different wavelengths revealed that the action spectrum of ChR2-XXL was only slightly red-shifted compared with that of ChR2-wt, with a maximum excitation wavelength at ~ 480 nm, in good agreement with its previously published absorption spectrum (19). In comparison, the recently published variant red-activatable ChR (ReaChR) (21, 22) displayed a wider action spectrum (Fig. 1*G*).

Expression and Function at the *Drosophila* Neuromuscular Junction. The ability of ChR2 variants to elicit large photocurrents in oocytes correlates with their efficacy of membrane depolarization (5) and neuronal stimulation (6, 7, 15, 23). We therefore turned to *Drosophila* to test the performance of ChR2-XXL in an intact, complex biological system. To this end, ChR2-wt (consisting of amino acids 1–315 as originally published) (5, 24), ReaChR, and the highly efficient T159C mutant (15, 16), which had previously not been introduced to *Drosophila*, served as references. ChR2-T159C was chosen for comparison because in oocytes it mediates the largest photocurrents of all heretofore published ChR2 variants, exceeding those of the gain-of-function H134R mutant, the fast E123T/T159C double mutant, and variants possessing an extended open-state lifetime (Table S1) (6, 15, 17, 18, 20). Transgenes were expressed under control of the bipartite GAL4/UAS system for cell-specific targeting (25), and light-triggered outputs of increasingly complex neuronal networks were investigated.

First, we examined photostimulation of monosynaptic transmission at the glutamatergic neuromuscular junction (NMJ) of third instar larvae. ChR2 variants were driven in presynaptic motor neurons (Fig. 2*A*) (*ok6-GAL4* driver) (26), and the postsynaptic muscle response to light-evoked neurotransmitter release was evaluated. Beginning with a simple and optically accessible setting, we assessed the contraction and consequential immobilization of relatively translucent larvae upon exposure to blue light (Fig. 2*B*). When retinal was not added to the food (“0 mM retinal”), larvae expressing ChR2-wt, ChR2-H134R, or ChR2-T159C displayed no discernible reaction to photostimulation using light intensities up to $0.6 \text{ mW}/\text{mm}^2$ at 460 nm. Strikingly, ChR2-XXL mediated a pronounced response without retinal supplementation and with nearly three orders of magnitude lower light intensity. When retinal was added (100 μM), light application induced reversible muscle paralysis in all genotypes. Gradual adaptation of motor neurons to small stationary photocurrents enabled larvae to resume crawling despite ongoing photostimulation (27). As a consequence, the duration of immobilization scaled

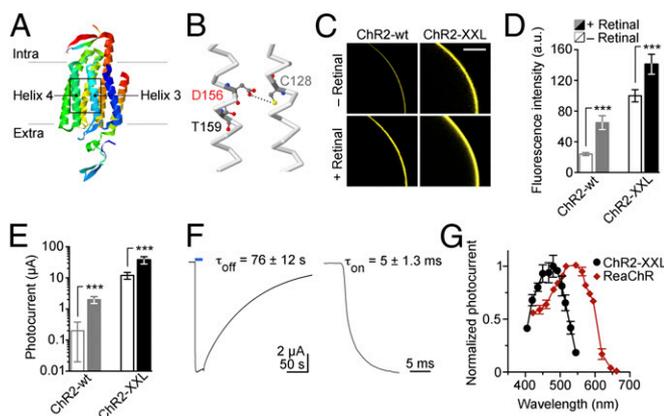


Fig. 1. Characterization of ChR2-XXL in *Xenopus* oocytes. (*A*) Structural illustration of a ChR based on PDB ID code 3UG9 (46). (*B*) Enlarged boxed region of transmembrane helices 3 and 4 showing the hydrogen bond (dotted line) between residues C128 and D156 of ChR2. (*C*) Representative confocal images showing retinal-dependent expression of ChR2-wt and ChR2-XXL C-terminally tagged with YFP. (Scale bar: 300 μm .) (*D*) Comparisons of fluorescence intensities describe enhanced expression of ChR2-XXL both with (filled bars) and without (open bars) retinal supplementation. (*E*) Consistent with an increased retinal affinity of the mutant, steady-state photocurrent amplitudes of ChR2-XXL were greatly increased irrespective of retinal addition. (*F*) Example photocurrent and on-kinetics in response to 20-s (Left; 473 nm, $8 \text{ mW}/\text{mm}^2$, $2 \times 10^{18} \text{ photons}\cdot\text{cm}^{-2}\cdot\text{s}^{-1}$) or 5-ns light pulses (Right; 473 nm, pulse energy density $13 \text{ mJ}/\text{mm}^2$), respectively. Average values for time constants of channel opening (τ_{on}), closing (τ_{off}) and data points in figures are given as mean \pm SD. Statistical comparisons were performed with the two-tailed Student *t* test ($***P \leq 0.001$). (*G*) Action spectra of photocurrents mediated by ChR2-XXL and ReaChR.

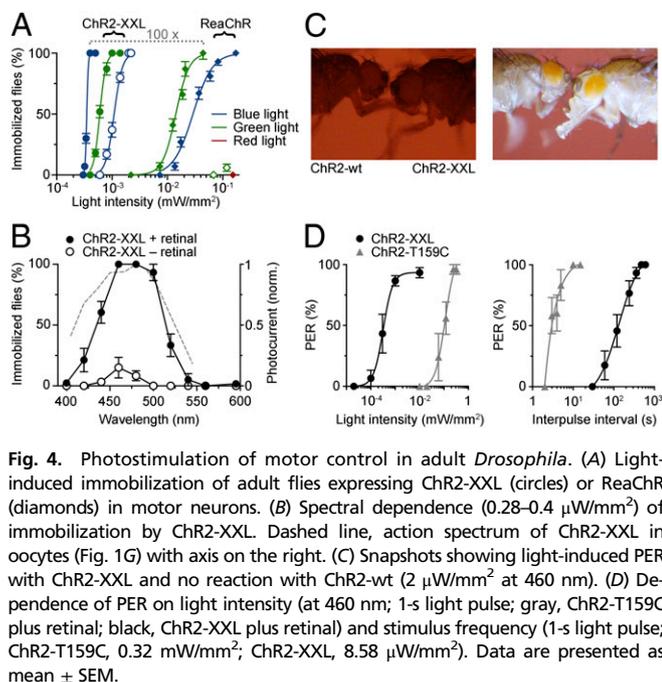


Fig. 4. Photostimulation of motor control in adult *Drosophila*. (A) Light-induced immobilization of adult flies expressing ChR2-XXL (circles) or ReaChR (diamonds) in motor neurons. (B) Spectral dependence (0.28–0.4 $\mu\text{W}/\text{mm}^2$) of immobilization by ChR2-XXL. Dashed line, action spectrum of ChR2-XXL in oocytes (Fig. 1G) with axis on the right. (C) Snapshots showing light-induced PER with ChR2-XXL and no reaction with ChR2-wt (2 $\mu\text{W}/\text{mm}^2$ at 460 nm). (D) Dependence of PER on light intensity (at 460 nm; 1-s light pulse; gray, ChR2-T159C plus retinal; black, ChR2-XXL plus retinal) and stimulus frequency (1-s light pulse; ChR2-T159C, 0.32 mW/mm²; ChR2-XXL, 8.58 $\mu\text{W}/\text{mm}^2$). Data are presented as mean \pm SEM.

In both sexes, *fru* is expressed in a network of \sim 2,000 cells comprising sensory, central, and motor neurons (33). We used a *GAL4* insertion into the *fru* locus (*fru-GAL4*) (34) to drive ChR2-XXL in this sex-specific neuronal network.

Activation of ChR2-XXL in *fru*-expressing neurons triggered distinct behavioral modules of male courtship normally controlled by sensory input, internal states, and experience (Fig. 5). Similar to previous work using the temperature-sensitive cation channel dTRPA1 (*Drosophila* Transient Receptor Potential A1) (35), courtship behaviors could be elicited in fully intact flies with no need for decapitation (36). Strikingly, however, the courtship ritual induced by ChR2-XXL appeared to unfold in reverse (Fig. 5B and Movie S6).

Under natural courting conditions, the male will exhibit a characteristic action pattern of consecutive behaviors. This series includes tapping the female's abdomen, then encircling or following her while extending and vibrating one wing to produce a distinctive "love song," followed by proboscis extension, licking, abdomen bending, and attempted copulation (32).

When a brief light pulse (2s, 30 $\mu\text{W}/\text{mm}^2$ at 460 nm) was used to stimulate *fru* neurons via ChR2-XXL, male flies responded with abdominal bending, which persisted well after the end of the light pulse. Proboscis extension and unilateral wing extension

followed with a delay, and frequently all three behaviors overlapped (Fig. 5B). Moreover, unilateral wing vibration was capable of producing the characteristic pulsatile features of the male courtship song (Fig. 5C) (32, 35, 36). In females, photoactivation of a dormant motor program elicited certain aspects of male courtship although abdomen bending was not observed and song production lacked male-specific precision (Fig. 5D and E) (36).

With ChR2-XXL, bilateral wing extension always preceded unilateral wing extension. Bilateral wing use is likely suppressed during normal courtship although it can be evoked through strong artificial stimulation of the *fru* network (35, 36). When dTRPA1 is expressed in *fru* neurons, a gradual increase in temperature evokes the normal temporal sequence of courtship behaviors and bilateral follows unilateral wing extension (35).

Taken together, these observations indicate that the biophysical properties of ChR2-XXL (Figs. 1F and 3B) partially reversed the normal sequence of courtship modules by triggering rapid onset and gradually decreasing activation of the *fru* network following a brief light pulse. Conversely, this result suggests that progression from one courtship behavior to the next under physiological conditions is driven by increasing activation of the *fru* network, possibly through a gradual buildup of sensory inputs. This interpretation is consistent with the notion that progressively higher activation thresholds separate consecutive courtship modules (35).

Triggering Associative Olfactory Memory. Having tested hardwired, innate, and relatively stereotypic behavior, our final set of experiments focused on experience-dependent, plastic behavior: i.e., learning. Adult and larval fruit flies can be trained to associate an odor stimulus with either a reward or a punishment (37). In *Drosophila* larvae, ChR2-wt has been used to activate aminergic neurons simultaneously with odorant stimulation. When specific groups of dopaminergic neurons, determined by the *TH-Gal4* strain (38), are activated, flies acquire an aversive memory for that odor (24). This finding has been confirmed in adult flies using a thermogenetic TRP channel (12) and UV-dependent liberation of caged ATP in combination with the expression of an ATP-dependent ion channel (14). Optogenetic memory induction using ChR2-wt (or variants thereof) has, however, not been reported for adult fruit flies, most likely due to insufficient photostimulation.

We therefore expressed ChR variants in a large proportion of dopaminergic neurons using *TH-Gal4* (38). In the most commonly used aversive olfactory conditioning procedure, an odor is presented simultaneously with electric shocks; a second odor is presented without electric shock punishment. The animals learn to avoid the odor associated with punishment in a subsequent choice situation (39). We modified this learning paradigm and substituted the electric shock punishment with blue-light illumination of \sim 1 $\mu\text{W}/\text{mm}^2$. Those flies that expressed ChR2-XXL in *TH-Gal4*-positive dopaminergic neurons acquired a clear aversive short-term memory for the odor paired with the optogenetic activation of dopaminergic neurons whereas the

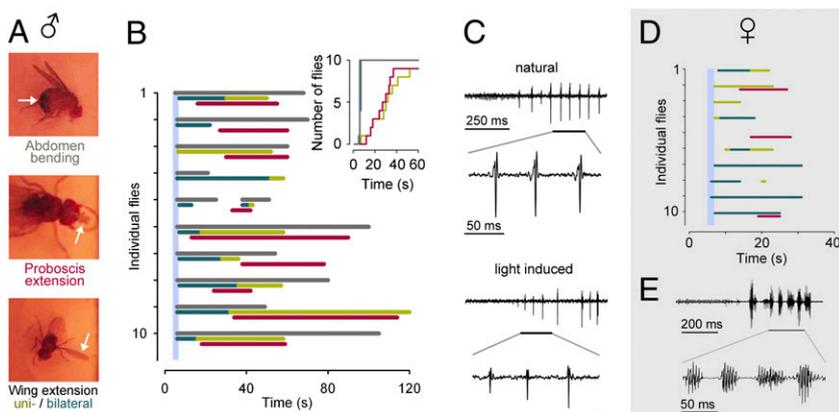


Fig. 5. Light-triggered male courtship behavior. (A) Examples of male courtship modules. (B) Ethogram of courtship behaviors (gray, abdomen bending; dark green, bilateral wing extension; light green, unilateral wing extension; red, proboscis extension) evoked by ChR2-XXL in 10 individual male flies (plus retinal) following an \sim 2-s light pulse (vertical blue line; 30 $\mu\text{W}/\text{mm}^2$ at 460 nm). (Inset) Data as a cumulative plot of courtship behaviors. (C) Example of photostimulated courtship song produced by unilateral wing vibration of a male fly (*Lower*). The pulse components are similar to the natural courtship song (produced by a male of the same genotype courting a wild-type female in red light (*Upper*)). (D) Ethogram of courtship behaviors evoked by ChR2-XXL in 10 individual female flies (plus retinal) following an \sim 2-s light pulse (vertical blue line; 30 $\mu\text{W}/\text{mm}^2$ at 460 nm). (E) Example of photostimulated courtship song. The pulse components lack male-specific precision.

genetic control strains behaved indifferently toward the two odors (Fig. 6A). The artificial induction of an aversive associated memory was independent of dietary retinal supplementation (Fig. 6A), in accordance with the results in motor neurons (Figs. 2B and 4A). All fly strains were able to learn to associate the respective odors with electric shock punishments (Fig. 6B). ChR2-T159C, however, failed to drive the respective dopaminergic neurons such that any light-induced change in behavior could be observed, even when retinal was supplemented (Fig. 6A). Because ChR2-T159C requires significantly higher light intensities to exert an effect *in vivo* (Fig. 2B), we temporally paired the presentation of an odor with stronger light stimulation (~ 0.3 mW/mm²). When combined with retinal supplementation, this protocol resulted in a very slight learning effect (Fig. S3). In conclusion, ChR2-XXL efficiently activated modulatory neurons that mediate the punishment information during associative learning. Moreover, the optogenetically written memory was just as strong as the memory induced using electric shocks as unconditioned stimuli.

Discussion

Ultimately, the field of neuroscience strives to provide a scientific description of how the nervous system generates behavior. Building on major advances in recent decades (9), the ability to control neuronal activity in intact, behaving animals promises to deliver significant progress toward this goal. At present, the optogenetic effector ChR2 is arguably the most auspicious choice for this task. However, the difficulty of activating neurons via ChR2-wt in freely moving, opaque animals has hampered its utility for behavioral neuroscience.

Whereas ChR2-wt can be used in translucent larvae fed high concentrations of retinal (24, 27, 28), it functions inefficiently in central brain neurons of intact adult flies (however, for examples of sensory neurons, see refs. 30 and 40). As a consequence, ChR2 has found only limited application in *Drosophila*, and the community has become somewhat disconnected from technological progress of optogenetic effectors. This development is particularly regretful considering the appeal of *Drosophila* for behavioral studies and the scientific potential of a synergy with optogenetics.

To date, several ChR variants have been introduced to *Drosophila*. ChR2-H134R (6) mediates enhanced photocurrents in larvae (27) but has failed to deliver major improvements in adult flies. The long open state of ChR2-C128S (17, 18) has been exploited to investigate the fly visual system using restrained animals and requiring comparable irradiance as ChR2-wt (41). A

combination of H134R and ChR2-C128T has been used for laser activation of sensory neurons in restrained flies (42). Very recently, it has been demonstrated that several red-shifted ChRs, ReaChR (which has a broad action spectrum extending well into blue) (Fig. 1G) and Chrimson, deliver important improvements over ChR2-wt for stimulating peripheral and central neurons of adult flies, possibly aided by the improved cuticle penetration of long-wavelength light (21, 22, 43). However, both variants require considerably higher light intensities than ChR2-XXL (Figs. 2B and 4A and Table S1). We observed no major improvement of using long-wavelength light to activate ReaChR. Instead, ReaChR was more responsive to green and blue light stimulation than to red in our *in vivo* experiments, which is in line with its action spectrum in oocytes and supports previous findings in flies and cultured neurons (21, 43). In summary, ChR2-XXL is most appropriate when maximum light sensitivity is required: e.g., for optically poorly accessible cells, functional genetic screens, especially with weak enhancer lines (44), and during long-term photostimulation experiments where heat damage must be prevented.

Current alternatives to ChR2 have notable drawbacks. UV-dependent liberation of caged ATP is part of a multicomponent strategy, which enables artificial activation of neurons in adult *Drosophila*. However, this technique cannot be efficiently used for large numbers of animals (3, 14). Thermogenetic effectors, such as dTRPA1 (11, 12) or the mammalian cold-sensitive TRPM8 channel (13), have been very successfully used in *Drosophila*. Although of undisputed utility, these tools come at the cost of undesired off-target effects, are unsuited for studying temperature-dependent processes (such as, e.g., temperature-preference behavior) (45), and are of limited use in warm-blooded animals. Moreover, acute temperature shifts can induce troublesome movement artifacts during live imaging experiments. Therefore, optogenetic approaches are of obvious value, especially when the effectors can be engaged complementarily: e.g., to stimulate separate neuronal populations using spectrally separated ChRs or using temperature and light with little danger of cross-talk.

In the present study, we introduce a ChR2 variant with beneficial functional properties for low light applications. (i) ChR2-XXL possesses an extended open-state lifetime. This feature contributes to higher light sensitivity than the strongly expressed, but fast, ChR2-T159C variant, because slow channel closure will promote the accumulation of channels in the open state and thereby saturate responsiveness at low light intensities (17). (ii) ChR2-XXL exhibits elevated expression in both vertebrate and invertebrate cells. This property likely confers much larger photocurrent amplitudes than other slow mutants (17, 18, 20). The combination of these characteristics delivers a clear improvement for eliciting neuronal activity in intact animals.

Functionality of ChR2-XXL does not require retinal supplementation (Figs. 1E, 2B, 4A, and 6). Besides simplifying the design of experiments, this important feature strongly influences protein expression. When channelopsin-2 (Chop2) is not bound to its cofactor, the protein is more prone to degradation than in its retinal-bound state (ChR2) (16). We hypothesize that Chop2-XXL has a higher affinity for retinal, which leads to increased protein stability in the presence of endogenous and supplemented retinal.

Accordingly, we observed a significant increase in protein levels of membrane-bound ChR2-XXL over ChR2-wt in spherical *Xenopus* oocytes (Fig. 1C and D). In highly polarized *Drosophila* motor neurons, ChR2-XXL displayed enhanced expression in axonal projections under conditions where ChR2-wt localization is restricted to cell bodies (Fig. 2C and D). When combined with spatially restricted illumination, such broad expression could be exploited to facilitate photoactivation of distinct subcellular compartments, such as individual synapses.

The long open-state lifetime of ChR2-XXL contributes to its exceptionally high light sensitivity, albeit at the cost of temporal precision. This optogenetic effector is therefore most suited for applications where light delivery is the limiting factor, such as in studies of freely moving animals. In cases where higher temporal precision of stimulus termination is required and alternative

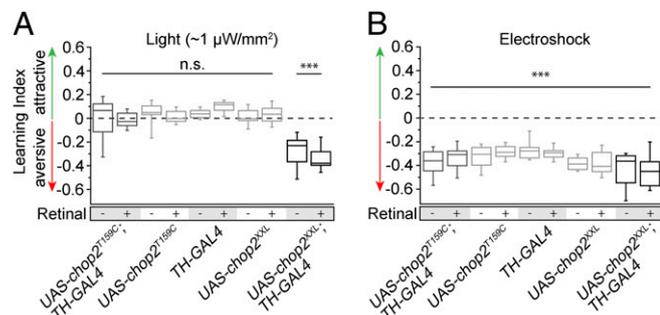


Fig. 6. Light-controlled induction of associative olfactory learning. (A) In adult *Drosophila*, activation of dopaminergic neurons with light induced an odor-associated aversive memory via ChR2-XXL (*UAS-chop2^{XXL}; TH-GAL4*), without a requirement for retinal supplementation. Neither control strains (*UAS-chop2^{T159C}*, *UAS-chop2^{XXL}*, *TH-GAL4*) nor flies expressing ChR2-T159C in dopaminergic neurons (*UAS-chop2^{T159C}; TH-GAL4*) showed an aversive memory after pairing odor and light stimulation. (B) All genotypes associated odors with electric shock punishment, irrespective of retinal addition. $n = 8$ per experimental group. Learning indices were tested for significant negative differences from 0 using one-tailed Student *t* test with Bonferroni correction for multiple tests ($***P \leq 0.001$).

variants, such as ChR2-T159C or ReaChR, do not provide sufficient light sensitivity, other measures can be taken. Adjusting light power such that the action potential threshold is barely crossed will narrow the time window of neuronal firing well below the photocurrent decay time constant of ChR2-XXL. Similarly, omitting retinal also accelerates recovery following photostimulation (Movie S3). The temporal precision of ChR2-wt, afforded by its fast current decay, is without doubt an attractive biophysical property. However, the popularity of thermogenetic effectors in *Drosophila* neurobiology illustrates that many *in vivo* applications do not in fact require highest temporal resolution. Instead, the usefulness of ChRs for *in vivo* studies has been limited by insufficient light delivery to target cells.

Strong photostimulation with ChR2-XXL might depress synaptic transmission during prolonged and uninterrupted light application by preventing, e.g., repolarization-dependent recovery from Ca²⁺ channel inactivation. Correspondingly, larvae expressing the XXL variant in motor neurons appeared to gradually relax while remaining immobilized during continuous irradiation (Movie S7). It may therefore be advantageous to use pulsed light during long-term photostimulation (27).

Conveniently, ChR2-XXL is not activated by long-wavelength light, and, therefore, red foil can simply be used when

stimulation is not desired (Movies S1 and S3). Combined with its efficient photostimulation capacity, we anticipate that the properties of this powerful optogenetic tool will be of interest to the *Drosophila* community and other biologists wishing to control behavior in an intact animal.

Materials and Methods

SI Materials and Methods provides details of experimental procedures. Electrophysiological recordings of photocurrents were made in two-electrode voltage-clamp mode from oocytes in Ringer's solution (pH 7.6) at a holding potential of -100 mV, and current-clamp mode from ventral longitudinal muscle 6 of *Drosophila* larvae (28). For associative olfactory learning, groups of ~ 100 flies (4–7 d old) were trained as described by Tully and Quinn (39) with modifications. To optogenetically substitute the electric shocks with blue light of ~ 1 $\mu\text{W}/\text{mm}^2$, transparent training tubes were used that were equipped with 12 blue-light diodes evenly inserted into the tube surface.

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Supporting Information

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SI Materials and Methods

Molecular Biology and Genetics. Oocytes. cDNA of C-terminally truncated wild-type channelopsin-2 (*chop2*), encoding amino acids 1–315 (1), was cloned into oocyte expression vectors, based on the plasmid pGEMHE 22, a derivative of pGEM3z (Promega). Mutations were inserted via site-directed mutagenesis (Quik-Change kit; Stratagene). The red-activatable ChR (*ReaChR*) sequence was synthesized by combining two GeneArt Strings DNA Fragments (Life Technologies, Thermo Fisher Scientific), ordered according to the published amino acid sequence (2), with *Drosophila* codon use. The DNA was inserted into the pGEMHE vector between *Bam*HI and *Xho*I restriction sites with a YFP attached to its C-terminal end (*ReaChR::YFP*) and confirmed by sequencing. *Nhe*I-linearized plasmid DNA was used for the in vitro generation of cRNA with the AmpliCap-MaxT7 High Yield Message Maker Kit (Epicentre Biotechnologies).

Drosophila. ChR mutants for fly transgenesis were PCR-amplified from plasmid templates for oocyte expression [forward primer, 5'-TCTAGAatcaacatggattatggagcgcctgagtg-3'; reverse primer, 5'-ACCGGTttagtgccggccgggtaccgcgcca-3' (restriction sites capitalized and Kozak sequence underlined)] and inserted via *Xba*I and *Age*I into the expression vector pJFRC7 (3), whose multiple cloning site was previously extended into *Not*I-*Stu*I-*Kpn*I-*Xho*I-*Xba*I-3xFlag-*Age*I. Inserts were verified by restriction analyses and DNA sequencing.

For expression in *Drosophila*, ChR2 variants were not fused to photoproteins. Previously published ChR2-wt flies (4) carried the truncated protein *UAS-chop2*³¹⁵ (1). Flies carrying *UAS-chop2-T159C* and *UAS-chop2-D156C* were generated by targeted PhiC31 recombinase-mediated insertion of either transgene into the genomic P[acman] landing site *attP-9A/VK18* carried on the second chromosome (5) at BestGene Inc. *UAS-ReaChR*^{VK5} (6) was obtained from the Bloomington *Drosophila* Stock Center (no. 53749). Animals were raised at 25 °C in the dark. For dietary supplementation with retinal, aliquots from a 50-mM stock (250 mM for olfactory learning) of *all-trans*-retinal (dissolved in ethanol) were mixed into the food slurry just before egg laying to yield a final concentration of 100 μM (250 μM for olfactory learning).

The following genotypes were used for expression in motor neurons: *ok6-GAL4/+; UAS-chop2*^{wt/+}, *UAS-chop2*^{T159C/ok6-GAL4}, *UAS-chop2*^{D156C/ok6-GAL4}, *ok6-GAL4/+; UAS-ReaChR/+*. The following were used in gustatory cells: *Gr5a-GAL4/+; UAS-chop2*^{wt/+}, *Gr5a-GAL4/ UAS-chop2*^{T159C}, *Gr5a-GAL4/ UAS-chop2*^{D156C}. The following were used in *fru* neurons: *UAS-chop2*^{D156C/+; fru-GAL4/+. The following were used in dopaminergic neurons: *UAS-chop2*^{T159C/+; TH-Gal4/+}, *UAS-chop2*^{D156C/+; TH-Gal4/+}; *UAS-chop2*^{T159C/+; UAS-chop2^{D156C/+; TH-Gal4/+}.}}

Electrophysiology. Oocytes. Oocytes were injected with 20 ng of RNA of *chop2* variants, or *ReaChR* C-terminally tagged with YFP. Oocytes were incubated in medium containing 10 μM *all-trans*-retinal, as indicated. Two electrode voltage-clamp recordings of photocurrents were made in Ringer's solution (pH 7.6) at a holding potential of –100 mV. Photocurrent amplitudes, half-saturating light intensities, and action spectra were taken from stationary currents. A diode pumped solid-state laser was used for longer illumination times (473 nm, 8 mW/mm², 2 × 10¹⁸ photons·cm⁻²·s⁻¹) and a pulsed laser for 5-ns flashes (473 nm, pulse energy density 13 mJ/mm²).

For action spectra of ChR2-XXL (ChR2 extra high expression and long open state) and *ReaChR* (*n* = 3 each), light of different wavelengths was obtained by narrow bandwidth interference filters (Edmund Optics) together with a PhotoFluor II light source (89

North). The wavelength was further confirmed with a spectrometer (Ocean Optics). Equal photon flux was set for each wavelength. The action spectrum of ChR2-XXL was normalized to photostimulation at 480 nm. Because of some “rundown” in *ReaChR*-expressing cells (maximally to 50% during one measurement), these recordings were made at –60 mV, and 517 nm light stimulation was measured before every single wavelength as a reference and for normalization.

Drosophila NMJ. Semi-intact preparations and data acquisition were performed essentially as previously reported (7). In brief, current-clamp recordings were made from ventral longitudinal muscle 6 in abdominal segments A3 and A4 at room temperature using a microelectrode (filled with 3M KCl) in combination with an Axoclamp 900A amplifier (Axon Instruments/Molecular Devices). The extracellular solution (HL3.1) (8) contained 70 mM NaCl, 5 mM KCl, 5 mM MgCl₂, 10 mM NaHCO₃, 5 mM D-(+)-trehalose, 115 mM sucrose, 5 mM Hepes, 1.5 mM CaCl₂; pH was adjusted to 7.2. Excitatory postsynaptic potentials (EPSPs) were evoked with 0.5-s light pulses using the following intensities (μW/mm² at 460 nm): 5, 16, 29, 60 (ChR2-T159C); 5 (ChR2-XXL). The decay of EPSP frequencies was measured using a 5-s sliding window (Fig. 3C).

Imaging. Oocytes. For documentation of ChR2 expression in oocytes, 12-bit images were acquired under identical settings with a confocal laser-scanning microscope (LSM 5 Pascal; Carl Zeiss) equipped with a Zeiss Plan-Neofluar 20×/0.5 objective. Fluorescence signal intensity was determined by quantification of mean gray levels across the entire image of a confocal slice. The mean gray value of an untransfected oocyte in the same dish was subtracted from each measurement. Image analysis was performed using the ImageJ software package (<http://imagej.nih.gov/ij/index.html>).

Drosophila. For imaging, larvae of all genotypes were fed 100 μM retinal. ChR2 expression was coimaged with HRP (goat anti-HRP-Cy3, 1:250) as previously described (7). In brief, larvae expressing ChR2-wt, ChR2-T159C, or ChR2-XXL were stained in the same vial under identical conditions using a commercially available antibody (mouse anti-ChR2 supernatant 1:1, 15E2, mfd Diagnostics; secondary antibody Alexa 488 goat anti-mouse 1:250, Invitrogen), and confocal imaging was performed using equal settings. In the example images, a Gaussian blur (1 px radius) was applied following background subtraction and before normalizing fluorescence intensities.

Light stimulation. Larval immobilization. Blue light was used to stimulate late third instar larvae (light from a mercury lamp passed through a GFP excitation band-pass filter), and a red LED (peak 623 nm) was used for *ReaChR*. Measurements reflect the time between light-induced immobilization of crawling larvae and resumed movement (defined as anterior displacement of posterior end) during ongoing irradiation (*n* ≥ 5 animals per genotype and light intensity). No visible response was scored as <1 s of immobilization. In several examples, long-term photostimulation of larvae was performed: with ChR2-T159C, an individual was immobilized for approximately 4 h (0.1 mW/mm²) and resumed crawling after several seconds following the end of the light stimulus; with ChR2-XXL, two larvae were immobilized for approximately 6 h (50 μW/mm²) and resumed crawling a few minutes after the end of the light stimulus.

Adult immobilization. Five to 10 flies (1–15 d after eclosion) were placed in a vertically positioned Petri dish (8 cm diameter), which was homogeneously irradiated with light from a mercury lamp

(filtered appropriately) for 1 min. For ReaChR, high-power LEDs were used to provide sufficient light intensity (at 626 nm and without retinal addition, on average five ReaChR flies were placed in a 500- μ L PCR tube to ensure homogeneous light application). The flies were then tapped down, and the immobilized individuals were counted. Flies were kept in the dark for 5 min before switching to the next filter, and at least five groups were measured for each data point. For action spectra, irradiance was adjusted to deliver $7.8\text{--}8.4 \times 10^{17}$ photons \cdot cm $^{-2}$ \cdot s $^{-1}$ at each wavelength, and measurements proceeded from short to long wavelengths.

Proboscis extension reflex. Flies (2-4 d after eclosion) were fixed on a glass slide using nail polish and left for 1 h in the dark before the experiment. ChR2-T159C was activated with blue light (mercury lamp), and ChR2-XXL was activated with white LEDs (for white-light sources, the intensity at 460 nm was measured after passing light through a GFP excitation bandpass filter). In between light stimulation, ChR2-T159C and ChR2-XXL were exposed to only very low light intensities or kept in the dark, respectively. To analyze the dependence of photostimulated proboscis extension reflex (PER) on light intensity, four to five light pulses (each lasting \sim 1 s) were applied at each intensity and separated by 20 s (ChR2-T159C) or 5 min (ChR2-XXL). For ChR2-T159C, light intensities were (in mW/mm 2 at 460 nm): 0.01, 0.02, 0.06, 0.12, 0.24, and 0.32 (5 min rest in between different intensities, $n = 5$ flies); for ChR2-XXL (in μ W/mm 2): 0.02, 0.09, 0.23, 0.94, 8.58 (10 min rest in between different intensities, $n = 6$ flies).

For measurements of PER frequency with ChR2-T159C, partial proboscis extensions were also scored. Partial proboscis extensions were never observed with ChR2-XXL. Four to five light pulses (\sim 1-s duration) were applied at each frequency. For ChR2-T159C (0.32 mW/mm 2 , $n = 8$ flies), pulse intervals were 1 s, 2 s, 3 s, 4 s, 9 s, and 14 s (5 min rest between intervals); for ChR2-XXL (8.58 μ W/mm 2 , $n = 10$ flies): 29 s, 59 s, 119 s, 239 s, 359 s, 479 s, and 599 s (10 min rest between intervals).

Courtship. Males and females were collected \leq 6 h after eclosion and aged for at least 3 d in isolation or in groups, respectively. Individual flies were placed in an arena and left in the dark for 30 min before photostimulation with an \sim 2-s light pulse (30 μ W/mm 2 at 460 nm; light from a mercury lamp passed through a GFP excitation bandpass filter). Videos were recorded using red light for offline analysis of courtship behaviors. Abdomen bending was defined as a male curling its abdomen beyond 90 degrees.

Courtship songs were amplified, digitized, and recorded in Audacity (<http://audacity.sourceforge.net>) at a sampling frequency of 44.1 kHz. Audio traces were low-pass filtered at 1 kHz and high-pass filtered at 90 Hz (9). Photostimulation of individual flies in

a compact, soundproofed recording arena was performed with an LED (\sim 2-s light pulse; white light 2 μ W/mm 2 at 460 nm for males and blue light 30 μ W/mm 2 for females). Wing vibration was visually inspected during audio recordings. Photostimulation of sine wave song components was observed in only 1 out of 13 ChR2-XXL-expressing flies.

Associative learning. For associative olfactory learning, groups of \sim 100 flies (4-7 d old) were trained as described by Tully and Quinn (10) with modifications: i.e., four experiments were performed simultaneously in a modified learning apparatus (11). A constant airflow of \sim 167 mL/min in each training tube assured a constant odor flow inside the training tubes. The relative humidity was 65–75%, and experiments were carried out at 25 $^{\circ}$ C and diffuse red light conditions. Ten minutes before each experiment, flies were transferred to empty fly culture vials. The odorants 4-methylcyclohexanol (CAS 589-91-3; Sigma) and 3-octanol (CAS 589-98-0; Sigma), diluted in mineral oil (CAS 8042-47-5; Sigma) at a ratio of 1:750 or 1:500, respectively, were used. The odors were applied using plastic cups of 5-mm diameter that contained 60 μ L of the diluted odorants. Training started 1 min after transferring the flies into the training tubes. Each odor was presented for 1 min with a 1-min break between two odor applications. One odor [conditioned stimulus + (CS+)] was temporally paired with 12 electric shocks of 90 V (1.25-s shock and 3.75-s interpulse interval, DC) applied through an electrifiable grid covering the inside of the tubes. The second odor [conditioned stimulus – (CS–)] was presented without shocks.

To optogenetically substitute the electric shocks with blue light of \sim 1 μ W/mm 2 , transparent training tubes were used that were equipped with 12 blue-light diodes (peak wavelength 468 nm) evenly inserted into the tube surface. To apply high light intensities of \sim 0.3 mW/mm 2 , four powerful blue-light diodes were positioned around the transparent training tube, and heat was dissipated using cooling grids. Equivalent to the electric shock training, 12 pulses of illumination (1.25-s illumination and 3.75-s interpulse intervals) were applied. After another minute of either training, the flies were transferred to the T-maze part of the apparatus with both odors presented from each side, and flies were tested for odor preference for 2 min. Subsequently, the flies were counted and a preference index was calculated by subtracting the number of flies on the side of the CS– from the number of flies on the side of the CS+, divided by the total number of flies. Learning indices were calculated by averaging two reciprocal experiments in which each odor was used as CS+ or CS–, respectively. In Fig. 6 and Fig. S3, box plots of learning indices show medians and interquartile ranges and whiskers show minimum and maximum values.

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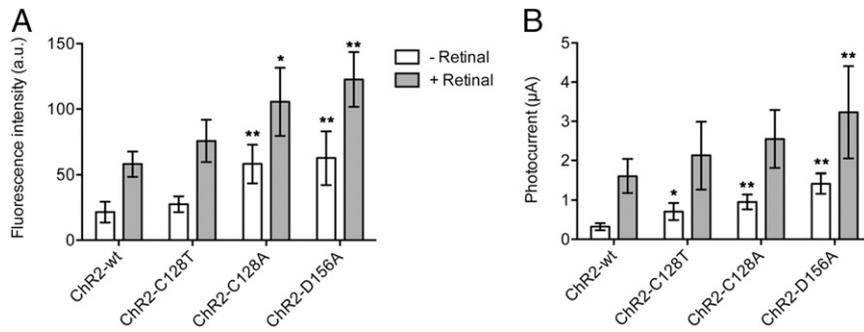


Fig. S1. Expression and photocurrents of slow ChR2 mutants in oocytes. (A) Measuring fluorescence intensities of YFP-tagged ChR2 variants describes increased expression of C128A and D156A compared to wt (Mann–Whitney rank sum test vs. wt no added retinal: C128T, $P = 0.222$; C128A, $P = 0.008$; D156A, $P = 0.008$; vs. wt plus retinal: C128T, $P = 0.056$; C128A, $P = 0.032$; D156A, $P = 0.008$). (B) Steady-state photocurrent amplitudes of slow variants are slightly increased over wt, scaling roughly with expression levels (Mann–Whitney rank sum test vs. wt no added retinal: C128T, $P = 0.016$; C128A, $P = 0.008$; D156A, $P = 0.008$; vs. wt plus retinal: C128T, $P = 0.31$; C128A, $P = 0.056$; D156A, $P = 0.008$). Asterisks denote significance to ChR2-wt at equal retinal concentrations ($*P \leq 0.05$; $**P \leq 0.01$). Data ($n = 5$ for each group) are presented as mean \pm SD.

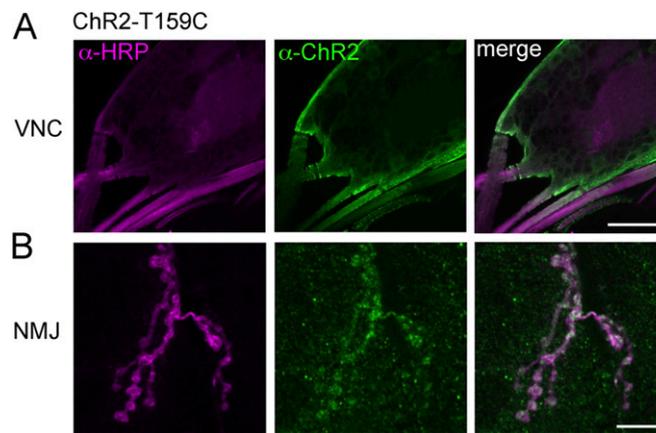


Fig. S2. Localization of ChR2-T159C in larval motor neurons. Antibody staining against ChR2 (green) and HRP (magenta). (A) ChR2-T159C was present in motor neuron axons leaving the ventral nerve cord (VNC), and (B) could be detected at the neuromuscular junction (NMJ) (shown is a single optical slice). (Scale bars: A, 30 μ m; B, 10 μ m.)

Table S1. Biophysical properties of channelrhodopsin variants

Variant	Photocurrent amplitude (rel. to wt)	EC ₅₀ , mW/mm ²	λ _{max} , nm	τ _{on} , ms*	τ _{off}	Source
ChR2-XXL	~19–60× [†]	0.003	480	5	76 s	This study (1)
ChR2-wt	—	0.7	470	0.2	10 ms	
Slow mutants						
C128T	Similar	0.03	480	5.7	2 s	Refs. 2 and 3 and this study
C128A	~0.3–1.6×	0.01	480	7.9	39–52 s	
C128S	~0.3×	0.01	480	13	28–106 s	
D156A	~1–2×	0.01	480	5	>150 s	
ChR2-T159C (TC)	~2–10×	Similar to wt	470* [‡]	~2× slower than wt [§]	20 ms	(4, 5)
ChETA (E123T; ET)	Similar [‡]	Less sensitive than wt [¶]	500	~2.5× faster than wt [§]	5 ms	(6)
ChR2-ET/TC	~2.5×	Similar to wt [¶]	505* [‡]	Similar to wt [§]	8 ms	(4)
CatCh	~3×	0.7	474	0.6	16 ms	(7)
ChR2-H134R (HR)	~3×	Similar to wt	450 –470* [‡]	Similar to wt [§]	15 ms	(4, 8, 9)
ChR1–2 chimera						
ChEF	~4×	~2× more sensitive than wt	490	Similar to wt	~2× longer than wt	(9)
ChIEF	~3×	Similar to wt	450	Similar to wt	Similar to wt	(9)
Red-shifted ChRs						
ReaChR	>HR, similar to ChIEF [‡]	~10× more sensitive than wt [¶]	530** and 630	~30× longer than wt ^{††}	137 ms	(10)
Chrimson [‡]	<wt	Similar to wt [¶]	590	1.2× slower than wt [§]	Similar to wt	(11)

Values refer to stationary photocurrents in oocytes under respective experimental conditions unless indicated otherwise (7).

*Related to photocurrent peak.

[†]Depending on retinal concentration.

[‡]Measurements from neurons.

[§]Flash-to-peak current time.

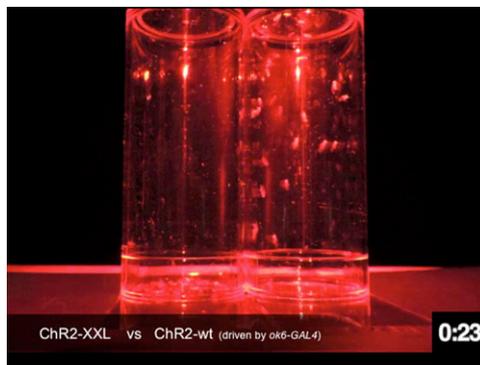
[¶]Approximation based on τ_{off}.

^{||}Measurements from HEK293 cells.

**Primary and secondary steady-state spectral peaks; 530 nm delivers a stronger response in flies (Fig. 4A) (12) and in cultured neurons (11).

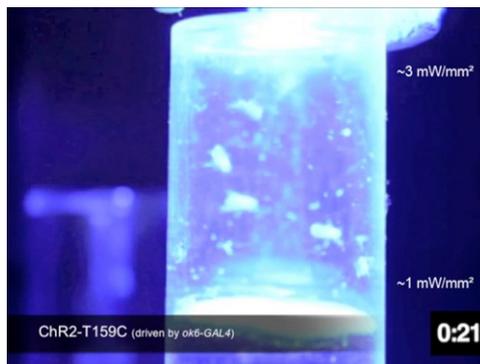
^{††}Approximation based on comparison with ChR2-wt in ref. 9.

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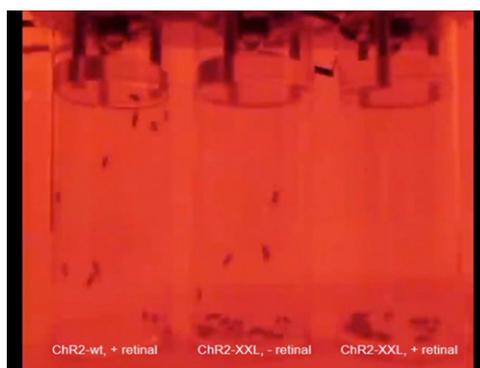
Movie S1. Light-induced stimulation of motor neurons in adult flies with ChR2-XXL. Activation of ChR2-XXL in motor neurons (driven by *ok6-GAL4*, plus retinal; *Left*) with white light LEDs ($2.5 \mu\text{W}/\text{mm}^2$ at 460 nm) induced reversible immobilization of adult flies. In contrast, flies expressing ChR2-wt (plus retinal; *Right*) displayed no response.

[Movie S1](#)



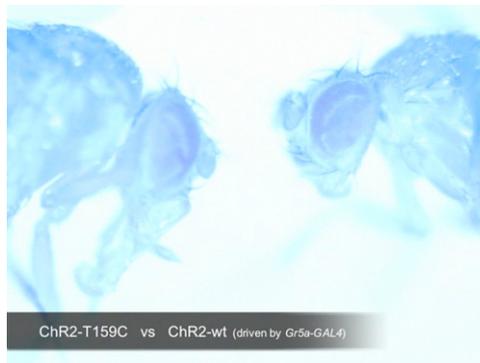
Movie S2. Inefficiency of ChR2-T159C in motor neurons of adult flies. Even with high light intensities ($\sim 3 \text{ mW}/\text{mm}^2$ at the top and $\sim 1 \text{ mW}/\text{mm}^2$ at the bottom of the vial), flies expressing ChR2-T159C in motor neurons (driven by *ok6-GAL4*, plus retinal) displayed no discernible response to photostimulation.

[Movie S2](#)



Movie S3. Accelerated recovery from photostimulation when retinal is not supplemented. The video is displayed at low resolution and sped up ten times. It shows adult flies expressing ChR2 variants in motor neurons (driven by *ok6-GAL4*). (*Left* vial) ChR2-wild type, plus retinal; (*Center*) ChR2-XXL, no retinal supplementation; (*Right*) ChR2-XXL, plus retinal. A weak blue light pulse of $\sim 2 \text{ s}$ ($4 \mu\text{W}/\text{mm}^2$) elicits no clear response from flies whereas $\sim 5\text{-s}$ -long light application has a drastic effect on ChR2-XXL-expressing flies, which are reversibly immobilized. Flies without retinal supplementation recover more rapidly, and even increasing irradiance duration and intensity (about 40-fold; $\sim 140 \mu\text{W}/\text{mm}^2$) fails to immobilize flies via ChR-wt.

[Movie S3](#)



Movie S4. Photostimulation of the PER with ChR2-T159C. Light from a mercury lamp was passed through a GFP excitation band-pass filter (~ 1 s, 0.2 mW/mm² at 460 nm) to activate ChR2-T159C (driven by *Gr5a-GAL4*; plus retinal; *Left*). For comparison, a fly expressing ChR2-wt (plus retinal; *Right*) shows no response.

[Movie S4](#)



Movie S5. Photostimulation of the PER with ChR2-XXL. White light LEDs (~ 1 s, 2 μ W/mm² at 460 nm) activated ChR2-XXL (driven by *Gr5a-GAL4*; plus retinal; *Right*). In contrast, a fly expressing ChR2-T159C (plus retinal; *Left*) shows no response.

[Movie S5](#)



Movie S6. Light-triggered courtship behaviors. Example of a male fly expressing ChR2-XXL in *fru* neurons (plus retinal). A mirror was used to inspect the fly from different angles. Examples of behavioral modules are indicated when they appeared for the first time. An ~ 2 -s light pulse (20 μ W/mm² at 460 nm) seemed to evoke a reversed courtship ritual, beginning with forceful abdomen bending and ending with unilateral wing extension.

[Movie S6](#)

