

# Remote Control of Neuronal Activity with a Light-Gated Glutamate Receptor

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DOI 10.1016/j.neuron.2007.05.010

## SUMMARY

The ability to stimulate select neurons in isolated tissue and in living animals is important for investigating their role in circuits and behavior. We show that the engineered light-gated ionotropic glutamate receptor (LiGluR), when introduced into neurons, enables remote control of their activity. Trains of action potentials are optimally evoked and extinguished by 380 nm and 500 nm light, respectively, while intermediate wavelengths provide graded control over the amplitude of depolarization. Light pulses of 1–5 ms in duration at ~380 nm trigger precisely timed action potentials and EPSP-like responses or can evoke sustained depolarizations that persist for minutes in the dark until extinguished by a short pulse of ~500 nm light. When introduced into sensory neurons in zebrafish larvae, activation of LiGluR reversibly blocks the escape response to touch. Our studies show that LiGluR provides robust control over neuronal activity, enabling the dissection and manipulation of neural circuitry in vivo.

## INTRODUCTION

The link between the activity of neurons and higher-order brain function is classically based on three types of studies: the anatomical determination of wiring, the experimental disruption of function by lesions or transient local block of activity, and the correlation of neuronal firing with specific sensory, motor, or behavioral events. While

these approaches have yielded important information about brain function, they are correlative and would be powerfully complemented by a method that can impose specific spatiotemporal patterns of activity on targeted neurons.

Driving behavior by manipulating neurons in the intact nervous system is a difficult challenge. Every brain region has complex circuits, each containing many neuron types. While electrical or magnetic techniques can be used to stimulate single cells or local groups of cells, they cannot as of yet target specific neuronal cell types. Caged chemical transmitters can be released rapidly (in microseconds) by a flash of light to activate a small group of cells (Callaway and Katz, 1993; Gillespie et al., 2005), but since most transmitters act on many neuronal cell types, this approach cannot achieve complete selectivity. Such selectivity can be obtained, however, by introducing foreign receptor/channels under the control of cell-type-specific promoters, where the receptors bind nonnative ligands (Zemelman et al., 2003). Uncaging nonnative ligands permits rapid and selective remote control over activity and has been shown to trigger dramatic behaviors in *Drosophila* (Lima and Miesenbock, 2005). An alternative approach has been to express in neurons foreign channels that are naturally light sensitive (Zemelman et al., 2002; Nagel et al., 2003; Melyan et al., 2005). Among these, the cation channel from unicellular green algae, channelrhodopsin-2, has been shown to function with fast kinetics in neurons (Boyden et al., 2005; Li et al., 2005; Ishizuka et al., 2006; Zhang et al., 2006, 2007; Zhang and Oertner, 2007; Petreanu et al., 2007; Arenkiel et al., 2007; Wang et al., 2007) and has been utilized in vivo in chick spinal cord, *C. elegans*, mouse retinae and brain, and *Drosophila* (Li et al., 2005; Nagel et al., 2005; Bi et al., 2006; Arenkiel et al., 2007; Schroll et al., 2006;

Zhang et al., 2007). Halorhodopsin, a naturally light-sensitive chloride pump from an archaeobacterium, was demonstrated recently to reversibly silence neurons in culture and in vivo (Han and Boyden, 2007; Zhang et al., 2007).

Much advantage would be gained from a general approach that would enable the optical control of any channel or receptor of interest out of the large repertoire that is native to neurons. Such an approach would, in principle, make it possible to sculpt neuronal activity in a physiological manner in the regions of the cell to which the channels naturally localize. A general approach of this kind has emerged from the demonstration of photo-switchable chemical ligands that are attached covalently to a channel of interest. The first version of this was made by Lester and colleagues in 1980 by fusing an acetylcholine analog to a photoisomerizable azobenzene and attaching this to a nicotinic receptor/channel, thereby conferring upon the channel light-dependent gating (Lester et al., 1980), which was later used to study the kinetics of nAChRs in rat myoballs (Chabala and Lester, 1986).

With the emergence of crystal structures of channels and receptors and with advances in pharmacology, it has become possible to design tethered ligands and azobenzene linkers with very specific geometry, so that photoisomerization of the linker presents or withdraws the ligand from the binding site in a predictable manner and on the fast timescale characteristic of azobenzene photoisomerization. The ligand in these photoswitches can operate either as a pore blocker (Banghart et al., 2004) or an allosteric ligand for an ionotropic receptor (Volgraf et al., 2006).

We recently described the latter technique, in which an ionotropic glutamate receptor subtype 6 (iGluR6) is genetically and chemically engineered, rendering it light sensitive (Volgraf et al., 2006). A chemical photoswitch, consisting of a glutamate analog on a photoisomerizable linker, is attached to an introduced cysteine on the ligand-binding domain of iGluR6. In response to ~380 nm illumination, the linker changes geometry, positioning the glutamate analog in the ligand-binding site and activating the channel. The geometry is reversed and the channel deactivated by ~500 nm light. This earlier work was carried out in non-neuronal (HEK293) cells. We investigate here the ability of this system to manipulate the firing of neurons in culture and in vivo.

We find that, in response to illumination, the light-gated iGluR6 (LiGluR) rapidly generates large (hundreds of pA) currents, yielding substantial (tens of mV) depolarizations in hippocampal neurons. Millisecond flashes of light evoke action potentials (APs) or subthreshold voltage changes that mimic fast excitatory postsynaptic potentials (EPSPs). The ability to excite neurons with LiGluR compares favorably with the responses of channelrhodopsin-2 (ChR2) in that LiGluR currents are ~5-fold larger, are stable during extended periods of illumination, and deactivate more quickly because deactivation is light driven, thus enabling cells to be reliably fired at higher frequencies. In addition,

LiGluR has the unique property that, once activated by a brief pulse of light, the channel will remain open for minutes in the dark, until a pulse of deactivating light closes it, thus enabling long depolarizations and trains of APs to be evoked with minimal light exposure. We demonstrate the utility of this unique property in manipulating the behavior of zebrafish larvae that express LiGluR and are labeled with the chemical photoswitch. Since the single mutation (L439C) does not alter the function of iGluR6 in response to glutamate (Volgraf et al., 2006), LiGluR also opens up the possibility for knockin animals that would be predicted to have synapses that operate normally but could be endowed with the ability to also be activated orthogonally and remotely by light.

## RESULTS

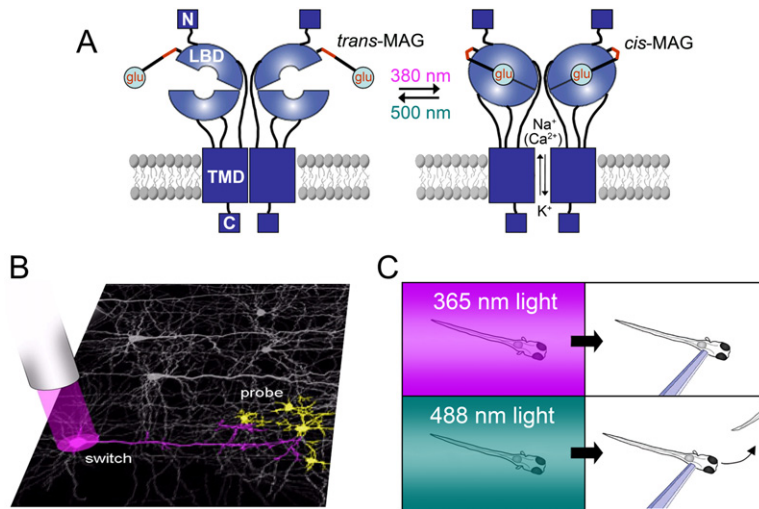
The chemical photoswitch MAG (consisting of: maleimide for attachment to an introduced cysteine, a photoisomerizable azobenzene moiety, and a glutamate analog as an agonist; for chemical structure, see Volgraf et al., 2006) was covalently attached to an introduced cysteine at residue 439 on the outer surface of the ligand-binding domain of the kainate receptor, iGluR6. As shown previously, under visible illumination (~500 nm), MAG is mainly in its *trans* form, with minimal activation of the receptor (Volgraf et al., 2006). Irradiation at long-wave UV (~380 nm) induces *trans* to *cis* photoisomerization and positions the glutamate in the binding pocket, thus activating the receptor (Figure 1A). Photoswitching of MAG leads to the opening and closing of the cation-selective pore of iGluR6(L439C). Our goal was to determine the properties of optical excitation of LiGluR-expressing cultured neurons and to apply the method in vivo to manipulate zebrafish behavior (Figures 1B and 1C).

### Reproducible Bouts of Light-Evoked AP Firing in LiGluR-Expressing Neurons

We examined the ability of light to excite cultured postnatal hippocampal neurons, which were transfected with either iGluR6(L439C) and EYFP or with a GFP fusion construct of iGluR6(L439C), and then labeled with MAG. Current-clamp recordings were performed on the neurons expressing fluorescent protein (Figure 2C).

The timing and duration of firing of the neurons could be set by alternating between wavelengths that activate (~380 nm) and deactivate (~500 nm) the receptor (Figure 2A). The amplitude of depolarization and the frequency of the evoked APs were reproducible and similar to trains evoked by current injection (data not shown). Neurons that were not transfected did not respond to light, despite being exposed to MAG (Figure 2B). This lack of effect can be attributed to the absence of a cysteine in native GluRs at a location that would permit MAG attachment in the correct geometry for its glutamate end to reach the binding pocket.

Photostimulation of LiGluR-expressing neurons could be maintained for as long as seals held in patch clamp



**Figure 1. Study of Neuronal Circuits with Optical Switches**

(A) The light-gated glutamate receptor is based on the reversible photoisomerization of the tethered agonist maleimide-azobenzene-glutamate (MAG) between its *trans* configuration under 500 nm light and its *cis* configuration under 380 nm light. MAG is covalently attached by the maleimide moiety to a cysteine introduced into the ligand-binding domain (LBD) of the receptor. Photoswitching is driven by the reversible binding of the glutamate moiety of MAG, which is presented to the ligand-binding site in the *cis* configuration and withdrawn in *trans*. MAG binding under ~380 nm light activates the receptor and opens its cation-selective channel, resulting in membrane depolarization. TMD, transmembrane domain; C, C terminus; N, N terminus.

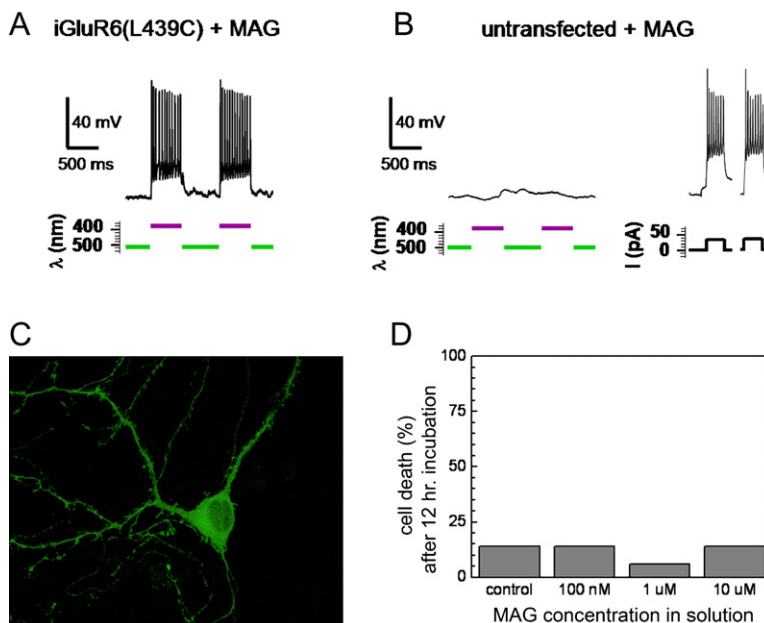
(B) In combination with calcium- or voltage-sensitive dyes to measure neuronal activity, the light-gated glutamate receptor can be

used as a remote, noninvasive neuronal switch for the electrode-free, all-optical analysis of neural circuits.

(C) When expressed in vivo, the light-gated glutamate receptor provides a tool for behavioral studies and circuit analysis. In the zebrafish larva, a touch-evoked escape response is assayed following activation of LiGluR (violet) or deactivation (blue).

(up to 45 min), without any indication of toxicity due to illumination or MAG exposure. Cultured hippocampal neurons were often patch-clamped 2 or more hours after MAG conjugation, indicating that MAG is not toxic over a short period of time. We also examined neuronal survival following 12 hr of continuous exposure to several concentrations of MAG. This is much longer than the standard 15 min labeling time that we employed for our recordings. Staining for dead cells using a Live-Dead viability/cytotoxicity assay (Molecular Probes, kit L-7013), we found there to be no difference in cell death between

neurons exposed to MAG and controls that were cultured in parallel (Figure 2D). This is consistent with our earlier observation that a model of MAG, which contains the (2*S*, 4*R*)-4-substituted glutamate and a linker resembling half of the azobenzene tether, has an apparent affinity of 180  $\mu$ M (Volgraf et al., 2006). Thus, the typical labeling concentration of 10  $\mu$ M MAG will activate iGluR6 only minimally. Activation of other iGluRs will be minimal because similarly substituted glutamate analogs have been shown to be selective kainate receptor agonists (Pedregal et al., 2000).



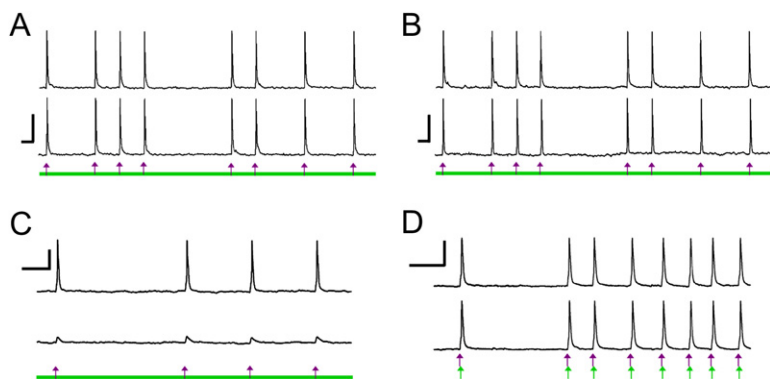
**Figure 2. Photostimulation Yields Reproducible Trains of AP Firing**

(A) A neuron transfected with iGluR6(L439C) and labeled with MAG is illuminated at 380 nm for 500 ms, yielding reproducible depolarizations that trigger trains of APs, which fire at a frequency that is characteristic of the cell. Illumination at 500 nm turns the response off and permits repolarization. (Illumination was with a monochromator at low intensity.)

(B) Untransfected neuron has no response to light, despite exposure to MAG, but does fire repetitively in response to current injection.

(C) Hippocampal neurons transfected with iGluR6(L439C)-GFP are easily identified by fluorescence.

(D) MAG has no deleterious effect on neurons. Untransfected neurons incubated for 12 hr in MAG (as opposed to standard 15 min labeling) show no increase in cell death compared to control.



when the illumination intensity is attenuated with neutral density filters, subthreshold EPSP-like responses (bottom trace). (D) LiGluR can be activated with a brief pulse at 374 nm and deactivated with a brief pulse at 488 nm to fire the neuron, while the interval between APs is kept dark to minimize irradiation. Light pulses are 2 ms in duration.

### Photoswitching in Milliseconds Generates APs and Mock EPSPs

EPSPs mediated by native iGluRs are triggered by very brief (millisecond long) and synchronous glutamate binding events at groups of receptors in postsynaptic membranes. Ideally, an engineered system for triggering neuron activity would operate on the same timescale. Indeed, brief (1–5 ms) pulses of light at 5.5–6 mW/mm<sup>2</sup> (from an 8 mW 374 nm diode laser, attenuated with neutral density filters) evoked currents that triggered reproducible patterns of APs (Figure 3). Light-evoked patterns of firing were repeatable within a neuron (Figure 3A) and in different neurons (Figure 3B). Furthermore, the amplitude of the responses could be easily reduced by attenuating illumination intensity using neutral density filters, in order to induce EPSP-like depolarizations (Figure 3C, lower trace).

Rather than continuously illuminating the cells while switching back and forth between two wavelengths, we also evoked patterned AP firing using only brief pairs of light pulses (374 nm to activate, followed by 488 nm to deactivate) while otherwise keeping the cell in the dark (Figure 3D).

Because rapid stimulation of neurons is often employed in studies of synaptic plasticity, we were interested in determining the response of neurons to light pulses delivered at high frequencies. We found that APs followed optical stimulation reliably up to 50 Hz (Figures 4A and 5A), higher than the frequency reported for ChR2 under similar culture conditions (Boyden et al., 2005; Li et al., 2005). We attribute this performance to the fact that LiGluR evokes larger currents of consistent amplitude and undergoes faster, light-driven deactivation (see the Supplemental Data and Figure S1 available with this article online). While perfect correlation between light stimulus and action potential firing is less likely at frequencies above 50 Hz, we found that subthreshold depolarizations were reliably evoked up to the highest frequency tested of 100 Hz (Figure 4B), demonstrating that the kinetics of light-gating are quite rapid. We found that other measures of fidelity, such as excess APs, latency between stimulus and AP, and jitter (Figure 5B–D),

### Figure 3. Designed Temporal Firing Patterns

Millisecond-timescale pulses of laser illumination are sufficient to significantly depolarize neurons and to trigger APs. Scale bars, 40 mV and 100 ms. In all panels, arrows indicate timing but not duration of light pulses.

(A) A train of 1 ms pulses of 374 nm light (arrows) reliably triggers the same temporal pattern of AP firing in a neuron.

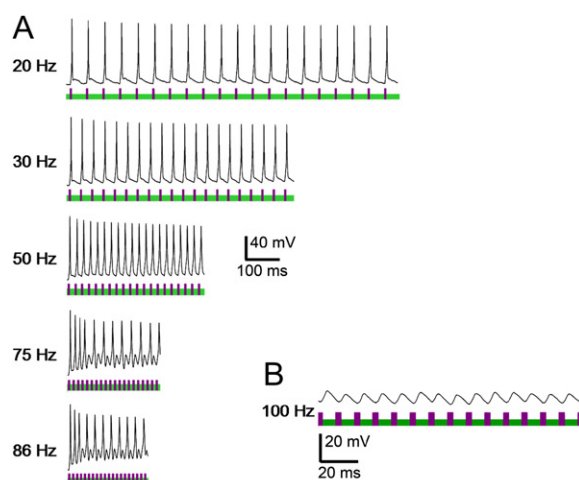
(B) Reproducible firing is triggered in two different neurons by the same pattern of 374 nm light pulses (arrows). Light pulses are 1 ms in the upper trace and 3 ms in the lower trace.

(C) In the same cell, a train of 3 ms pulses of 374 nm light produces APs (top trace) or,

were on par with, or better than, reported values for glutamate photouncaging (Shoham et al., 2005; Yoshimura et al., 2005) and ChR2 (Boyden et al., 2005).

### Light Evokes Depolarization and AP Firing in a Wavelength-Dependent Manner

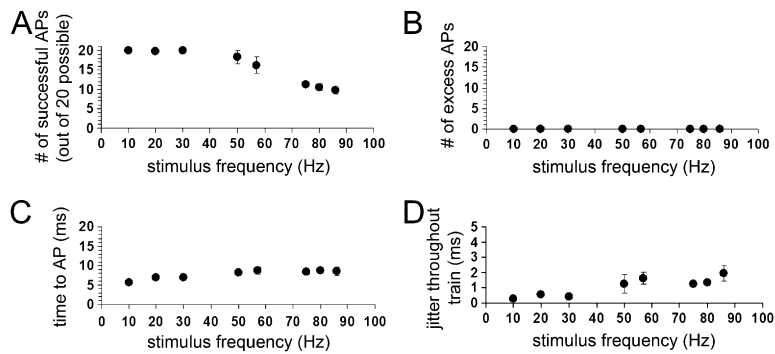
To characterize the amplitude of depolarization evoked by illumination at different wavelengths, we examined iGluR6(L439C) in HEK293 cells labeled with MAG under whole-cell current clamp. Light-induced channel opening evoked large steady-state depolarizations. By taking advantage of the fact that the photostationary state of MAG (i.e., the relative proportion of azobenzene in *cis* and *trans*



### Figure 4. Hippocampal Neurons Follow Pulsed Photostimulation of LiGluR Even at High Frequencies

(A) Trains of 5 ms laser pulses at 374 nm reproducibly trigger APs up to 50 Hz. At 75 Hz, 12 APs are evoked by 20 stimuli, and at 86 Hz, 11 APs are evoked.

(B) Subthreshold depolarizations are reproducible at very high frequencies (in this case, 100 Hz) because both channel activation and deactivation are light driven.



**Figure 5. Fidelity of Neuron Firing**

Neurons are stimulated 20 times by 5 ms pulses of 374 nm light. Values in all panels are mean  $\pm$  SEM. In some cases, error bars are smaller than the symbols.

(A) Stimuli delivered up to 30 Hz reliably evoke APs. At higher frequencies, fewer APs are evoked, with substantial drop-off at >60 Hz.

(B) No excess APs occurred during stimulation at the frequencies tested. (Excess APs are defined as more than one AP per stimulus, or those that occur more than 30 ms after the stimulus.)

(C) Latency between stimulus onset and AP peak increases with higher-frequency stimulation and may account for the fewer APs produced at high frequencies in (A).

(D) The latencies within a train of 20 stimuli vary more at higher frequencies. (Jitter is the standard deviation of the latencies.)

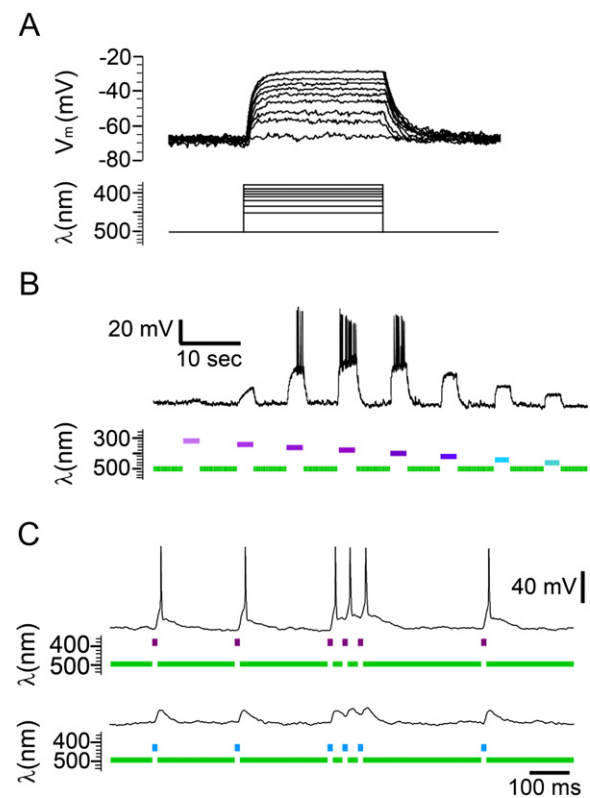
configurations) can be precisely varied by illumination wavelength, it was possible to produce steady-state depolarizations whose amplitudes depended on wavelength (Figure 6A). Similar graded depolarizations could be evoked in cultured postnatal hippocampal neurons that were transfected with iGluR6(L439C) and exposed to MAG. As seen in the HEK cells, the amplitude of depolarization depended on wavelength, with a maximum at  $\sim$ 380 nm. The largest depolarizations evoked trains of APs (Figure 6B). We used the wavelength dependence to adjust the size of EPSP-like waveforms that were triggered by brief pulses of light, so that, for example, pulses of light at 380 nm generated superthreshold depolarizations and evoked APs, while EPSP-like responses were induced in the same cell by pulses of light of the same duration but at the off-peak wavelength of 430 nm (Figure 6C). Thus, the amplitude of brief excitatory events evoked by pulses of light can be controlled either by modifying the intensity of illumination at 380 nm (Figure 3C) or by adjusting wavelength.

### Protracted Excitation in the Dark

We find that our azobenzene photoswitch is robust, yielding reproducible responses for tens of minutes in hippocampal neurons under continuous illumination, alternating between 380 nm and 500 nm at intensities of 5 mW/mm<sup>2</sup> or more. These recordings typically ended only upon loss of the seal, and recordings were equally stable with and without illumination. However, behavioral experiments may require activity to be manipulated over a much longer timescale, where photodestruction of MAG or phototoxicity to cells could become a concern. To reduce this problem, we explored the property of thermal bistability of MAG in an attempt to generate sustained trains of firing in the dark.

Depending on how azobenzene is derivatized, its higher-energy *cis* configuration is stable for seconds to minutes in the dark (Pozhidaeva et al., 2004). For MAG, the half-life is  $17.65 \pm 0.03$  min (Gorostiza et al., 2007). Thus, depolarization induced by a brief pulse of 374 nm

light is followed by sustained excitation in an ensuing period during which there is no illumination (Figure 7). This sustained excitation in the dark can then be rapidly

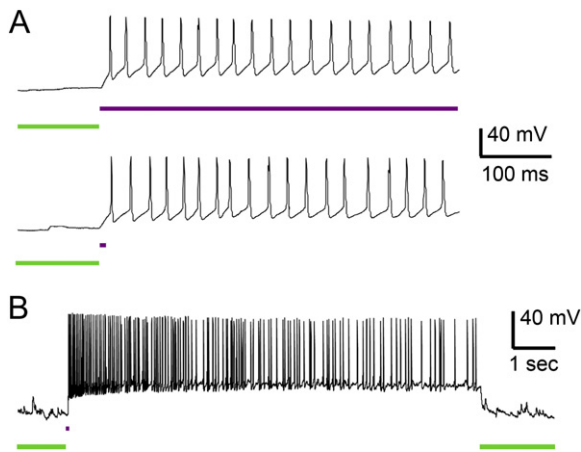


**Figure 6. Wavelength-Dependent Depolarization**

(A) In current-clamped HEK cells expressing LiGluR, light induces depolarization in which amplitude depends on illumination wavelength. In this way, the membrane potential can be accurately and stably controlled across a wide range.

(B) Illumination at a range of wavelengths depolarizes neurons. Some depolarizations are large enough to reach threshold and trigger APs.

(C) Light-pulse stimulation with 380 nm light evokes APs while 430 nm light induces subthreshold, EPSP-like responses in the same cell.



**Figure 7. Brief Pulses of Illumination Followed by Dark Evoke Sustained Firing at Intrinsic Frequency of the Cell**

Due to the stability of the *cis* state of MAG, LiGluR activation by a short pulse of 374 nm light yields a long-lasting depolarization that can trigger sustained trains of APs in the dark. The depolarization and AP train can be turned off with a short pulse of light at 488 nm. (A) Sustained excitation of hippocampal neurons occurs under either continuous 374 nm light (top trace) or when a brief pulse of 374 nm light is followed by dark (bottom trace). (B) After a 5 ms pulse of 374 nm light, excitation is sustained for 10 s in the dark before being turned off by 488 nm light. There is no obvious repolarization in the dark over the 10 s period.

extinguished by a brief pulse of 488 nm light (Figure 7B). This molecular memory of MAG makes it possible to trigger extended periods of excitation with minimal irradiation, during which time the cell fires at its characteristic frequency. This is an advantageous property of LiGluR compared to ChR2 (Figure S1D).

### Transgenic Expression of LiGluR in Zebrafish Enables Reversible Control of Behavior

While LiGluR in cultured hippocampal neurons allows robust optical control over activity, the approach presents an assortment of challenges for implementation *in vivo*. For example, the receptor might not express at high enough levels to influence activity *in vivo*, or the expression itself might perturb behavior. Although we have shown that MAG is not toxic in cultured hippocampal neurons, it could have a toxic effect on living animals. Delivery of MAG into the nervous system and penetration of 380 nm light through tissue are also potential difficulties.

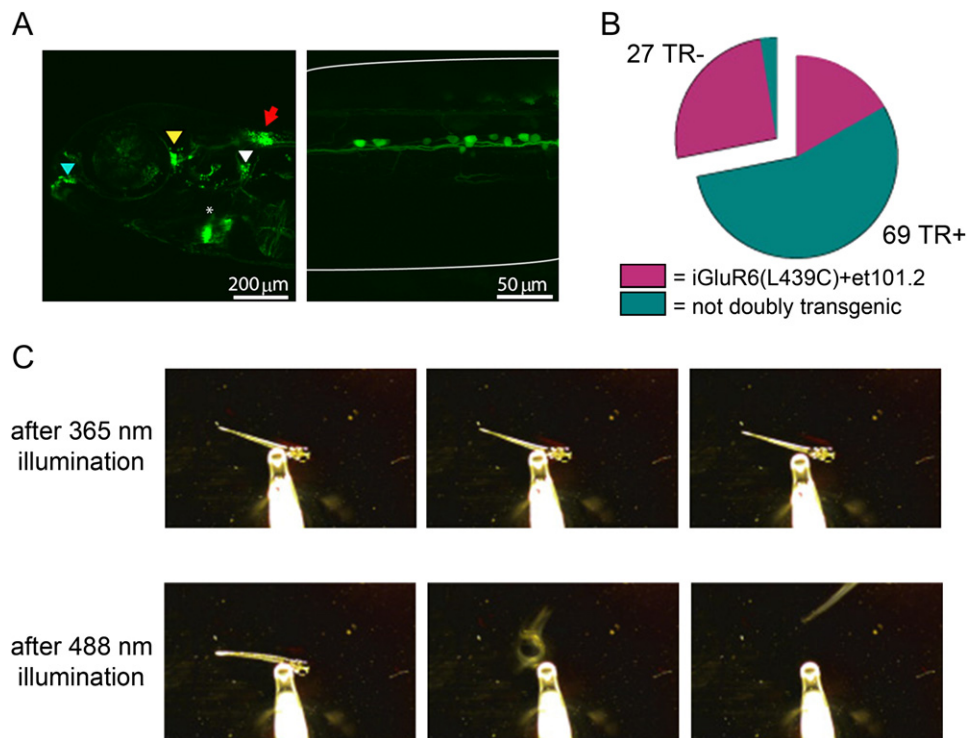
To address these issues, we introduced the iGluR6(L439C) gene into zebrafish, drove its expression in different cell types, and examined the effect of light on behavior. The iGluR6(L439C) gene was placed under the control of a UAS promoter, *UAS:iGluR6(L439C)* transgenic fish were made, and these were crossed to different lines of transgenic fish carrying the GAL4 transcription factor. GAL4 binds UAS promoter elements in the genome and drives the expression of flanking genes. The GAL4 lines are enhancer trap lines, generated as part of a large-scale enhancer trap screen (Scott et al., 2007), in which GAL4 was inserted in the chromosome near enhancers that

produce transcription in specific subsets of cells. We first used *et101.2:GAL4*, which drives expression of UAS-linked transgenes in small subsets of neurons in the head and trunk, including the trigeminal ganglion, vagal ganglion, hindbrain, and a small population of spinal neurons that appear to be Rohon-Beard neurons (Figure 8A).

Because the expression in this line is generally localized to neurons involved in touch sensation (trigeminal ganglion, hindbrain, and Rohon-Beard neurons) (Kimmel and Westerfield, 1990; Sagasti et al., 2005), we tested the ability of wide-field, whole-animal illumination to influence the touch response. This is an escape response evoked by mechanical pressure on the animal's side. For comparison, we also examined two other behaviors: free swimming, which requires an intact motor system, and the optomotor response (chasing of visual objects), which requires the coordinated function of motor and visual systems (Neuhauss et al., 1999; Orger et al., 2000).

The carriers for *UAS:iGluR6(L439C)* and *et101.2:GAL4* used here were each hemizygous for their transgene. Thus, when crossed, 25% of the progeny were predicted to carry both transgenes and therefore to express iGluR6(L439C) in the GAL4 pattern. We found that all the larvae resulting from this cross exhibited normal swimming behavior and responded with a strong optomotor response to visual motion at 6 days after fertilization (6 dpf), suggesting that the transgenes, alone and in combination, do not perturb the animal's development or CNS function. Moreover, similarly to wild-type, all larvae from the cross responded to mechanical stimulation with a fast escape response. This response could be reliably evoked by gently touching their heads, trunks, or tails with a thin probe (the tip of a pipet).

Larvae from this cross (6 or 7 dpf) were incubated with 125  $\mu$ M MAG and 5% DMSO in E3 embryo medium for 30 min and then washed multiple times in fresh E3 embryo medium. This treatment alone did not affect larval survival or behavior (swimming, touch response, and optomotor response). However, after illumination with 365 nm light for 15 min at low intensity (0.04 mW/mm<sup>2</sup>) with a handheld UV lamp, the behavior of some of the larvae was altered. While 72% of the larvae (69 of 96 fish) displayed a normal touch response, the remaining 28% (27 of 96 fish) did not perform an escape movement in response to touch (Figure 8B), although they still exhibited spontaneous swimming behavior. The touch responses of 22 of these nonresponders were restored by illumination with 488 nm light for 30 s. Figure 8C shows an example of an individual fish's behavior following illumination with long-wave UV light (no touch response) and then subsequent illumination with blue light (fully executed escape) (see also Movies S1 and S2). The inhibition of the touch response by UV and its restoration by blue light was repeated two additional times in the same group of animals. When more intense UV light was used at the peak activating wavelength (380 nm, 1 mW/mm<sup>2</sup>), the behavior was reversibly blocked by 10 s of illumination. Preventing scattering of light at the air-water interface should allow



**Figure 8. Activation of LiGluR in the *et101.2* Expression Pattern of Zebrafish Larvae Reversibly Blocks the Touch Response**

(A) Lateral views of the head (left panel) and trunk (right panel) showing the pattern of expression of Kaede in larvae (7 dpf) from a cross of *UAS:Kaede* and *et101.2:GAL4*. In the head, expression is in the trigeminal ganglion (yellow triangle), vagal ganglion (white triangle), and hindbrain (red arrow). Expression is also scattered in the mouth (blue triangle) and heart (asterisk). In the trunk, expression is in a small set of dorsal neurons in the spinal cord, which appear to be Rohon-Beard neurons.

(B) Statistics of light response in 96 larvae show that ~25% lose the touch response following 365 nm light. The majority of larvae (25 out of 27) that lost the touch response (TR-) following 365 nm light were subsequently found to express both iGluR6(L439C) and GAL4 (magenta). Larvae that did not express both the GAL4 driver and iGluR6(L439C) (teal) were overwhelmingly insensitive to 365 nm light (TR+). Some larvae (16 out of 69) were positive for both the GAL4 driver and iGluR6(L439C) but retained the touch response following 365 nm light, suggesting some variability in expression, MAG labeling, or illumination.

(C) Representative zebrafish larva expressing both *UAS:iGluR6(L439C)* and *et101.2:GAL4*. The response to touch is lost following illumination at 365 nm (top) and regained after illumination at 488 nm (bottom).

even greater speeds of switching behavior at this light intensity.

Since the behavioral assays were carried out on all animals resulting from the cross, this functioned as a blind analysis. Genotyping was done following the behavioral experiment to determine the correspondence between expression of iGluR6(L439C) and light-altered behavior. The genotyping revealed that the majority of the light-sensitive larval fish (25 of 27) were in fact doubly transgenic for *et101.2:GAL4* and *UAS:iGluR6(L439C)* (Figure 8B). Thus, 92.5% of the light-sensitive fish expressed iGluR6(L439C) in the *et101.2* pattern.

Reversible disruption of touch response was observed for touch to both the head and the trunk (Figure 8C and Movie S3), consistent with expression in trigeminal ganglion neurons, hindbrain, and Rohon-Beard neurons (Kimmel and Westerfield, 1990; Liu and Fetcho, 1999; Sagasti et al., 2005).

To confirm that the light-evoked perturbation mediated by iGluR6(L439C) is specific to the neural circuit in which it

is expressed, we also crossed *UAS:iGluR6(L439C)* with three other GAL4 lines that drive expression in different sets of cells. The line *et101.1:GAL4* expresses broadly throughout the nervous system, as well as in the heart (Figure S2A), and illumination with 365 nm light resulted in loss of the touch response and also total paralysis, as seen by loss of swimming behavior. This occurred in 26% of the larvae (9 out of 34), consistent with the expectation that ~25% will carry *UAS:iGluR6(L439C)* and *et101.1:GAL4*. Another line, *et101.4:GAL4*, which drives expression exclusively in the heart (Figure S2B), had no perturbation of swimming and touch responses after illumination at 365 nm (0 out of 32 fish). Finally, we examined the cross of *UAS:iGluR6(L439C)* with the *Ath5:GAL4*; *UAS:Kaede* promoter fusion, which expresses only in retinal ganglion cells (Masai et al., 2005; see Figure S2C). Among the progeny expressing the fluorescent marker Kaede [~50% of which also are predicted to carry the *UAS:iGluR6(L439C)* transgene], 365 nm light had no effect on the touch response (0 out of 15 fish).

These experiments show that it is possible to use light to reliably, reproducibly, and reversibly manipulate the activity of LiGluR-expressing neurons in vivo and thereby to modify zebrafish behavior. The nature of the behavioral manipulation depends on the identity of the LiGluR-expressing neurons, making it possible to dissect the role of neurons and neural circuits in behavior. The degree of specificity of such analysis will grow as new enhancer trap GAL4 lines and promoter-GAL4 fusion lines are developed.

## DISCUSSION

Our engineered receptor, LiGluR, consists of an ionotropic glutamate receptor (iGluR6) with an introduced cysteine residue (L439C) for covalent attachment of a chemical photoswitch (MAG). In response to light, LiGluR generates large, stable currents in hippocampal neurons, providing robust optical control over neuron depolarization and AP firing. Individual APs are driven by 1–5 ms pulses of light, which is on the physiological timescale of synaptic activation of glutamate receptors. Thus, millisecond-long flashes of light can drive designed temporal trains of single APs, while longer periods of illumination cause the cell to fire at its characteristic frequency.

Because the MAG photoswitch is covalently attached to the receptor, excitation is confined to the illumination volume, providing spatial resolution in which individual cells (and potentially specific regions within a given cell) can be selectively stimulated. Light-activation and cell viability are maintained during experiments lasting several hours, and a 12 hr incubation of neurons with MAG shows no sign of toxicity to cells. Importantly, exposure to MAG does not affect hippocampal neurons that are not expressing LiGluR. There are several reasons for this specificity. First, native receptors lack a point of attachment for MAG that is close enough (1–2 nm) to the binding pocket. Second, the agonist moiety of MAG is similar to kainate-selective agonists, and so free MAG should be less likely to activate AMPA receptors and NMDA receptors. Even kainate receptors will not be significantly activated by free MAG at the concentration we used for labeling (10  $\mu$ M), which is 18-fold lower than the  $EC_{50}$  of a similar tether model on iGluR6 (Volgraf et al., 2006). The only neurons that become responsive to light are ones that express iGluR6(L439C) and are exposed to MAG. While, in our experiments, cultured neurons were transfected randomly, it should be possible to drive the expression of iGluR6(L439C) in select neurons by the use of cell-type-specific promoters. Since LiGluR retains the ability to be activated by free glutamate, it could be used in knockin animals to gain orthogonal control over the native glutamate receptors.

LiGluR has several favorable properties that lend themselves to tight control over remote excitation. LiGluR generates steady currents, resulting in stable depolarizations that can fire neurons at constant rates. By illuminating with different wavelengths of light, it is possible to evoke

either small or large depolarizations. Because LiGluR is both activated and deactivated by light, the timing of excitation can be precisely defined. This fast bidirectional switching makes it possible to fire neurons reliably at high frequencies. In addition, because azobenzene is bistable and will reside for minutes in the higher-energy *cis* state, which activates the channel, a brief pulse of light can trigger a large depolarization that lasts for an extended period of time in the dark until deactivation is triggered by illumination at the longer wavelength. This means that long trains of firing can be evoked with LiGluR by brief pulses of light, minimizing illumination time and possible photodamage to cells. Because the half-life of spontaneous (thermal) isomerization of *cis* (activated) back to *trans* (deactivated) is  $\sim$ 18 min, very long bouts of firing are possible. This could be particularly useful for many types of behavioral experiments and in fact was essential to our touch-response experiments in zebrafish larvae, because their behavior is easily perturbed by both UV and visible light (Ren et al., 2002; Risner et al., 2006). In this way, we were able to activate LiGluR with 365 nm light and then apply the touch stimulus several minutes later, while observing behavior, under low-intensity ambient light conditions.

Our transgenic zebrafish larvae do not show any sign of problems with MAG delivery, toxicity, or interference with native circuitry. The fish larvae behave normally both before and after incubation with MAG. Expression of iGluR6(L439C) in sensory neurons is robust, and the expression itself does not appear to alter behavior. While the duration of UV illumination and concentrations of DMSO and MAG were not tested on zebrafish larvae at lower values, the exposure levels that were used did not appear to cause any complications.

Optical activation of LiGluR in specific neurons in zebrafish larvae is capable of preventing an escape reflex when the fish are touched with a pipet tip along the head or trunk. While trigeminal ganglion neurons in the head and Rohon-Beard neurons in the trunk have been implicated in touch-evoked escape (Sagasti et al., 2005; Cox and Fetcho, 1996), we test their function, using genetically targeted, reversible manipulation of neuronal activity. The larvae become unresponsive to touch, possibly because illumination of the whole animal simultaneously activates the sensory portion of the escape circuitry on both sides of the animal, which might interfere with making a directed turn away from the local stimulus. Alternatively, the larvae may become unresponsive to touch because the local stimulus cannot be detected over the elevated activity in the sensory cells or because of habituation after prolonged activation ( $>$ 15 min). Further studies are needed to deduce the exact mechanism.

In conclusion, we have shown that a simple approach of chemically re-engineering a receptor can endow it with the ability to be controlled by light in a selective manner, even in the complex environment of a neuron and in the sensory circuitry of live zebrafish larvae. We have used the approach to optically control AP firing with a light-gated



excitatory ionotropic receptor, but in principle it should be possible to extend the approach to inhibitory ionotropic receptors and to other classes of neuronal membrane proteins, such as adhesion proteins, growth factor receptors, and enzymes, as long as a tethered ligand is able to modulate function. Thus, neural activity can be controlled with light in a way that uses native proteins and closely mimics native activity.

## EXPERIMENTAL PROCEDURES

### Photoswitch Synthesis and Generation of L439C Mutant of iGluR6

Synthesis of MAG and introduction of cysteine L439C in iGluR6 were carried out as described (Volgraf et al., 2006). Amino acid residues are numbered from the starting methionine.

### Cell Culture and Transfection

Dissociated postnatal rat hippocampal neurons (P0–P5) were plated on poly-L-lysine-coated glass coverslips at a density of  $1 \times 10^5$  cells per 12 mm coverslip. Cells were cultured in MEM supplemented with 5% fetal bovine serum, B27 (Invitrogen), GlutaMAX (Invitrogen), and serum extender (BD Biosciences). Ara-C (4  $\mu$ M) was added after 4 DIV. Cells were transfected by the calcium phosphate method using 0.8  $\mu$ g of DNA per coverslip. Cells were either transfected with a fusion construct of GFP and iGluR6(L439C) or cotransfected with iGluR6(L439C) and EYFP at a 3:1 ratio.

HEK293 cells were plated at approximately  $6 \times 10^6$  cells per coverslip on 25 mm poly-L-lysine-coated glass coverslips and maintained in DMEM with 5% fetal bovine serum, 0.2 mg/mL streptomycin, and 200 U/mL penicillin. Cells were cotransfected with 4  $\mu$ g iGluR6(L439C) and 200 ng EYFP using lipofectamine 2000 (Invitrogen).

### Conjugation of MAG Compounds In Vitro

To conjugate MAG to iGluR6(L439C) in hippocampal neurons for patch-clamping experiments, the compound was diluted to 10  $\mu$ M in a solution containing (in mM) 150 NMDG-HCl, 3 KCl, 0.5 CaCl<sub>2</sub>, 5 MgCl<sub>2</sub>, 10 HEPES, and 5 glucose, pH 7.4. For HEK cells, MAG was diluted to 10  $\mu$ M in a solution of (in mM) 135 NaCl, 5.4 KCl, 0.9 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, 10 HEPES, and 0.3 mg/mL Concanavalin A type VI (Sigma), pH 7.6. The cells were incubated with MAG in the dark for 10–15 min and then rinsed in extracellular recording solution (described below).

### Whole-Cell Patch-Clamping

Patch-clamp recordings used an Axopatch 200A amplifier in the whole-cell mode. Recordings were carried out 1–8 days after transfection in hippocampal neurons and 36–48 hr after transfection in HEK cells. Cells were current-clamped or voltage-clamped at about –65 mV. Pipettes had resistances of 2–5 M $\Omega$  and were filled with a solution containing, for neurons (in mM), 135 K-gluconate, 10 NaCl, 10 HEPES, 2 MgCl<sub>2</sub>, 2 MgATP, 1 EGTA, pH 7.4; and for HEK cells (in mM), 145 CsCl, 5 EGTA, 0.5 CaCl<sub>2</sub>, 1.0 MgCl<sub>2</sub>, and 10 HEPES, pH 7.2. The extracellular recording solution for hippocampal neurons was (in mM) 138 NaCl, 1.5 KCl, 1.2 MgCl<sub>2</sub>, 2.5 CaCl<sub>2</sub>, 10 glucose, and 5 HEPES, pH 7.4; and for HEK cells was (in mM) 135 NaCl, 5.4 KCl, 0.9 MgCl<sub>2</sub>, 1.8 CaCl<sub>2</sub>, and 10 HEPES, pH 7.6.

Illumination was applied using a TILL Photonics Polychrome monochromator through the side port of the IX70 inverted microscope of the physiology rig (Olympus) and using either a 40 $\times$  or 60 $\times$  objective. Fast photoswitching experiments were carried out with a custom shuttered laser illumination setup mounted on a large breadboard. A 488 nm, 20 mW argon-ion laser (Laser Innovations) and a 374 nm, 8 mW Cube laser (Coherent) were combined with a dichroic mirror z405RDC (Chroma) and coupled into a P600-2-UV/vis optical fiber (Ocean

Optics), using a 10 $\times$ , 0.25 NA, 16.5 mm WD objective (Newport). The fiber was connected into the IX70 microscope (Olympus) through the Laser B port using a custom-made adaptor. Fast shutters (Uniblitz UHS1T2-100 driven by VMM-T1 controllers, Vincent Associates) were placed at the output of each laser to control the illumination pulses via software trigger. Light intensity measured at the 40 $\times$  or 60 $\times$  objective was 5.5–6 mW/mm<sup>2</sup>.

Electrophysiological data were recorded with pClamp software, which was also used to automatically control the monochromator and laser shutters by means of digital signals and sequencing keys.

### Generation of Zebrafish Transgenic Lines

To make the *UAS:iGluR6(L439C)* transgenic construct, the iGluR6(L439C) open reading frame was amplified by PCR and inserted downstream of the E1b promoter in a 14xUAS:eGFP construct (Koster and Fraser, 2001) and upstream of a SV40 polyadenylation sequence. This expression cassette was inserted between the Tol2 recognition sequences in the pT2KXIG $\Delta$ in vector (Kotani et al., 2006). Wild-type TL embryos were injected at the one-cell stage with a solution of 25 ng/ $\mu$ l *UAS:iGluR6(L439C)* DNA, 50 ng/ $\mu$ l transposase mRNA (prepared using the Ambion mMMESSAGE mMACHINE T7 kit), and 0.04% Phenol Red.

F<sub>1</sub> embryos were pooled and screened by PCR for the transgene: forward primer (5') ggcttgaggatgggaatatg and reverse primer (3') gggttcaagggtgtgggtatacc. F<sub>0</sub> founder animals giving rise to *UAS:iGluR6(L439C)*-positive offspring were then mated to wild-type TL fish to create stable lines.

To generate lines *et101.2:GAL4*, *et101.1:GAL4*, and *et101.4:GAL4*, the heat-shock promoter in *hsp(1.5kb):GAL4* (Scott et al., 2007) was replaced with the insertion of a linker containing an I-SceI meganuclease site, a P1-PspI meganuclease site, the basal E1b promoter (Argenton et al., 1996), and a KpnI restriction site, respectively, from 5' to 3'. This *e1b:GAL4* enhancer trapping construct was used to generate expression lines as described (Scott et al., 2007). Successful enhancer traps were identified by crossing to *UAS:Kaede* and visual screening under a dissecting microscope and then outcrossed to generate stable lines.

### Labeling and Illumination of Zebrafish Larvae

MAG was first diluted to 2.5 mM in 40  $\mu$ l of DMSO and preactivated by UV light (365 nm) for 1 min. The medium E3 was then added to reach the final concentration of 125  $\mu$ M MAG, 5% DMSO. Twenty larvae were bathed in 800  $\mu$ l of the labeling solution for 30 min at 28.5°C. The larvae were then removed from the labeling solution to a well containing fresh E3 and were washed three times. After a 30 min recovery period, the larvae were examined to confirm that they were all responsive to touch. The larvae were illuminated under a UV lamp (365 nm, 0.04 mW/mm<sup>2</sup> for 15 min). All the larvae were tested for touch response within 15–20 min. Single larvae were successively transferred to the center of a 35 mm diameter Petri dish, and their response to touch was assayed under a Zeiss Lumar fluorescent stereomicroscope equipped with a 0.8 $\times$  magnification objective. If a larva failed to show a response to repetitive touches with a pipet tip, it was illuminated under blue light (488 nm, 1.6 mW/mm<sup>2</sup> for 30 s) through the objective. The recovery of the touch response was tested within a minute after blue illumination.

### Supplemental Data

The Supplemental Data for this article can be found online at <http://www.neuron.org/cgi/content/full/54/4/535/DC1/>.

### ACKNOWLEDGMENTS

We are grateful to H. Fiumelli, F. Tombola, H. Lee, and S. Pautot for help with neuronal cultures and patch-clamping; to A. Johnson

(Coherent, Inc.) for the loan of a 374 nm diode laser; to R. Staples (Olympus, Inc.) for help with the laser setup; and to David Raible (University of Washington) for expert advice on the labeling pattern in our GAL4 zebrafish line. We also thank Georg Nagel for kindly providing ChR2. This work was supported by predoctoral fellowships from the National Science Foundation (to S.S.), the ACS Medicinal Chemistry Division (to M.V.), and UC Berkeley (to O.T.); by postdoctoral fellowships from the Human Frontier Science Program (to F.D.B. and P.G.), the Nanotechnology Program of the Generalitat de Catalunya (to P.G.), an OIF Marie Curie Fellowship (to C.W), the NIH (to E.K.S.), and the Japan Society for the Promotion of Science (to R.N.); and by grants from the Lawrence Berkeley National Laboratory, the Human Frontier Science Program, and the National Institutes of Health. Work in H.B.'s lab was supported by a Sandler Opportunity Award and a Byers Award (to H.B.).

Received: October 18, 2006

Revised: March 10, 2007

Accepted: May 4, 2007

Published: May 23, 2007

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