A light-gated, potassium-selective glutamate receptor for the optical inhibition of neuronal firing

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Genetically targeted light-activated ion channels and pumps make it possible to determine the role of specific neurons in neuronal circuits, information processing and behavior. We developed a K⁺-selective ionotropic glutamate receptor that reversibly inhibits neuronal activity in response to light in dissociated neurons and brain slice and also reversibly suppresses behavior in zebrafish. The receptor is a chimera of the pore region of a K⁺-selective bacterial glutamate receptor and the ligand-binding domain of a light-gated mammalian kainate receptor. This hyperpolarizing light-gated channel, HyLighter, is turned on by a brief light pulse at one wavelength and turned off by a pulse at a second wavelength. The control is obtained at moderate intensity. After optical activation, the photocurrent and optical silencing of activity persists in the dark for extended periods. The low light requirement and bi-stability of HyLighter represent advantages for the dissection of neuronal circuitry.

Understanding the neural circuitry underlying the behavior of organisms is a fundamental challenge for both basic and clinical neuroscience. The exploration of neuronal network function has been advanced considerably through the development of new tools for the non-invasive, reversible and precise spatio-temporal manipulation of nerve cell activity using light. Caged and photochromic neurotransmitter ligands, first invented decades ago and improved considerably since then, were recently joined by engineered ion channels that are controlled by covalently attached synthetic photoswitched tethered ligands (PTLs), natural photoreceptors from vertebrates and microorganisms, were determined using X-ray crystallography and suggested that closure of the clamshell-like LBD in all of these receptors forces the opening of the transmembrane pore. The pore of sGluR0 is K⁺ selective and its re-entrant pore loop has a GYG amino acid motif typical of the K⁺ channel selectivity filter. The idea that K⁺ selectivity can exist in a prokaryotic glutamate receptor supports a functional resemblance and evolutionary connection of iGluRs and K⁺ channels. We engineered chimeric ionotropic glutamate receptors that possess the LBD of iGluR6, including the PTL attachment site that renders it light sensitive in LiGluR, and combined this with the membrane-spanning domain, and thus the pore, of sGluR0. One of the chimeras functioned as a light-gated K⁺ channel that responded with maximal activation at moderate intensity.
This lack of agonism is consistent with the
3
5
b
5
K
m
m
membrane potential (0.16 mV ms
for photoswitch attachment in a single HEK cell during ramp changes in

Chimeras F and G used the
iGluR6-p0-C S1-TM1 junction and varied in the TM2-S2 junction.

low light intensity and provides the advantageous properties of sustained photoresponses in the dark and light-induced closure.

RESULTS
sGluR0 and light-gated, K⁺-selective chimeras
We first asked whether the K⁺-selective sGluR0 could be converted into a tool for optical silencing of neuronal activity. We expressed a mammalian signal peptide–modified and codon-optimized version of sGluR0 in HEK293 cells. The transfected cells had small glutamate-induced currents (peak currents of 130 ± 37 pA measured at +60 mV, n = 5). We tested whether sGluR0 could be activated by a soluble test compound (the tether model) that has been shown to mimic the maleimide azobenzene glutamate (MAG) PTL and activate iGluR6 (ref. 5). No activation of sGluR0 was detected with 0.5–3 mM tether model (Supplementary Fig. 1). This lack of agonism is consistent with the observation that the ligand-binding motif of sGluR0 differs from that of the iGluRs and that the ligand docks in an opposite orientation, which would yield a steric clash with the tether. Another problem of attempting to hyperpolarize cells with a light-gated sGluR0 is that sGluR0 activates very slowly (time constant of activation of ~290 ms), too slowly for the highly temporally precise inhibition of action potential firing. Taken together, the small and slowly activating currents of sGluR0 and the lack of agonism by the MAG tether model indicated that another strategy was needed to create a light-gated inhibitory channel.

We turned to the notion of designing a K⁺-selective, light-gated channel by combining the MAG photoswitching of the iGluR6 LBD with the K⁺ pore of sGluR0. A chimeric protein may combine the rapid activation of iGluR6 by glutamate and photoswitching with the K⁺ selectivity of sGluR0. AMPA and kainate receptor LBDs have already been shown to gate pores (and pore loops) of members of other iGluR families, including of the otherwise nonfunctional delta receptors. However, although the iGluR6 LBD has been shown to gate the sGluR0 pore and the LBD of the NR1 NMDA receptor can gate several K⁺ channel pore loops (not including that of sGluR0), none of these are K⁺-selective, indicating that selectivity depends on more than just the K⁺ channel pore loop. Furthermore, transplanting the GYG motif to iGluRs does not render them K⁺-selective. We therefore designed an alternative set of chimeras with the hope of obtaining coupling while preserving K⁺ selectivity.

In the chimeric receptors, different portions of the iGluR6 pore were replaced by the corresponding codon-optimized portion of the sGluR0 pore (we refer to these as iGluR6-p0). We sought to transplant both transmembrane helices and the re-entrant pore loop of sGluR0 (TM1–TM2; Fig. 1c) while retaining the native pore-LBD

Figure 1 Chimeras of the iGluR6 LBD and the sGluR0 K⁺-selective pore. (a) Mammalian ionotropic glutamate receptors are modular proteins with an N-terminal domain (NTD), LBD (formed by segments S1 and S2) and transmembrane channel (formed by transmembrane regions 1–3 (TM1–3) and the pore region (P)). Glutamate binding induces closure of the LBD and opening of a nonselective cation pore. (b) sGluR0 is a prokaryotic glutamate receptor that lacks an NTD and has a K⁺-selective pore. (c) The iGluR6-p0 chimeras have the pore of sGluR0 (TM1, P with its K⁺ selectivity filter signature GYG motif and TM2) inserted into iGluR6. Chimeras A–E differed at the junction between the S1 of the iGluR6 LBD and the N-terminal end of TM1 of sGluR0. Chimeras F and G used the iGluR6-p0-C S1-TM1 junction and varied in the TM2-S2 junction.

Figure 2 Glutamate gates K⁺ currents in iGluR6-p0 chimeras. (a) Representative, glutamate-induced outward currents recorded in whole-cell mode at −20 mV after exposure to concanavalin A to block desensitization for iGluR6-p0-A (top left), iGluR6-p0-B (top right), iGluR6-p0-C (middle left), iGluR6-p0-D (middle right), iGluR6-p0-E (bottom left) and iGluR6-p0-G (bottom right). For iGluR6-p0-A, C, D, E and G, currents were observed in 60–100% of cells, whereas no currents were observed for iGluR6-p0-F and only one cell gave measurable current for iGluR6-p0-B. Black scale bars represent 100 pA and 10 s. Gray horizontal bars above traces indicate perfusion of 1.5 mM glutamate. (b) Average outward currents (± s.e.m.) of iGluR6-p0 chimeras induced by 1.5 mM glutamate. The largest currents were observed for iGluR6-p0-C. The numbers of observations are shown in parentheses. (c) Leak-subtracted current for iGluR6-p0-C with added L439C mutation revealed high K⁺ selectivity of iGluR6-p0-C. The s.e.m. values are the same size as the symbols (n = 5 at 4 and 145 mM KC1, n = 3 at 40 mM KC1).
Fig. 3 Photo-control of K⁺ currents. (a) The PTL MAG0 is anchored to a cysteine residue introduced into iGluR6-p0-C (L439C). Illumination at 380 nm (violet) converts the azobenzene of MAG from trans to cis and illumination at 500 nm (green) drives the reverse isomerization, thereby conferring light sensitivity by reversibly presenting and withdrawing glutamate to and from its binding site. MAG0 consists of a glutamate, a photosomerizable azobenzene core and a maleimide group that attaches to the cysteine. (b) Whole-cell currents at −20 mV in a HEK293 cell after labeling with MAG0 and concanavalin A. Reversible photocurrents are 17.7% of the size of glutamate-evoked currents. Insets, photocurrent activation and deactivation at high, medium and low light intensities. (c) Steady-state current amplitude was saturated above 1 mW mm⁻². Receptor activation by ultraviolet light (filled symbols) and deactivation by green light (open symbols) were normalized to currents at highest power. (d) Reducing light intensity below 5 mW mm⁻² slowed the rate of current activation by light.

liners of iGluR6. In the absence of crystal structures, we estimated the locations of the ends of the helices in sGluR0 and iGluR6 using sequence comparison to two K⁺ channels with known structures, KcsA and Kv1.2, and hydropathy analysis (Supplementary Fig. 2). Several excision sites were chosen and a total of seven unique chimeras were generated and tested.

Our first set of chimeras, iGluR6-p0-A, B, C, D and E, varied only in the most N-terminal residues of the transplanted pore (Fig. 1c). When functionally testing chimeras in HEK293 cells, we recorded large, stable glutamate-induced outward currents at −20 mV for iGluR6-p0-A, C, D and E (Fig. 2a). Because iGluR6-p0-C gave particularly large outward currents (941 ± 239 pA, n = 8; Fig. 2a,b), we focused on this variant for further modifications. The iGluR6-p0-C chimera was refined into two additional variant chimeras, iGluR6-p0-F and G, in which residues in the C-terminal pore-LBD linker were replaced with residues from sGluR0. No glutamate-induced responses were detected for iGluR6-p0-F and the currents of iGluR6-p0-G (303 ± 130 pA, n = 8) were substantially smaller than those of iGluR6-p0-C (Fig. 2a,b). The latter observation agrees with a previously proposed functional interaction of residues in this linker and the LBD in iGluR6 (ref. 41).

Photoswitching iGluR6-p0

Having found that iGluR6-p0-C had the largest glutamate-induced currents of the iGluR6-p0 chimera, we combined it with the MAG attachment site of the best photoswitching version of LiGluR, a leucine to cysteine substitution at position 439 (ref. 42). HEK293 cells expressing iGluR6-p0-C-L439C had a mean glutamate-induced current of 745 ± 252 pA. This final construct, termed HyLighter, had reversal potentials of glutamate-induced currents of −79.7 ± 3.0, −26.5 ± 0.0 and −1.0 ± 2.7 mV when the external K⁺ concentration was adjusted to 4, 40 and 145 mM KCl, respectively (Fig. 2c,d). Analysis of these reversal potentials with the Goldman-Hodgkin-Katz equation indicates a K⁺:Na⁺ permeability ratio of ~100:1. Current-voltage traces (Fig. 2c) exhibited the same kind of outward rectification in the presence of extracellular Na⁺ that was seen previously in sGluR0 and attributed to Na⁺ block of inward K⁺ current (ref. 32), indicating that the sGluR0 pore maintains its character when controlled by the iGluR6 LBD in iGluR6-p0-C.

We tested the ability of HyLighter to be photo-controlled by MAG0, the shortest MAG PTL (Online Methods; Fig. 3a). After labeling with 40–60 μM MAG0 for 20 min, we measured glutamate-induced currents in the dark, followed by alternating illumination at 380 nm to drive isomerization of MAG0 to the cis-state and 500 nm to drive isomerization to the trans-state. The photocurrents were 15.2 ± 4.0% (n = 4) of the size of the glutamate-evoked currents. Maximal photocurrents were reached at modest light intensities (Fig. 3b,c). For example, illumination with 380 nm at 0.7 mW mm⁻² resulted in 95.8 ± 1.9% of current achieved with maximal light intensity (14.3 mW mm⁻²), whereas illumination with 500 nm at 0.9 mW mm⁻² resulted in 97.3 ± 1.0% of the current seen at 20.0 mW mm⁻² (n = 4). The time course of the photocurrents was intensity dependent. At maximal light intensity, half-maximal photocurrents were reached in 28.5 ± 2.8 ms at 380 nm and half-maximal deactivation was reached in 42.1 ± 1.7 ms at 500 nm (n = 4; Fig. 3d). The slower off-speed could be a result of a stabilization of MAG0 in the cis-state by the binding of its glutamate end to the LBD binding pocket. Such stabilization could also slow thermal cis to trans isomerization when the glutamate end of MAG is bound in the receptor. Indeed, we detected no change in current over tens of minutes in LiGluR (ref. 37). Using kinetic modeling (Online Methods) and taking into account the fact that unbound cis-MAG1 has a lifetime of ~25 min (ref. 37), we found that a stabilization of a few kJ mol⁻¹, equivalent to the energy of a hydrogen bond, was sufficient to increase cis-state lifetime by one order of magnitude. This prolonged activated state in the dark combined with the ability to rapidly turn HyLighter off is a unique feature among proteins capable of hyperpolarizing cells with light (see below).

Optical suppression of neuronal firing and behavior

In cultured hippocampal neurons expressing HyLighter under the control of the CMV promoter, brief light pulses at 380 nm triggered photocurrents with a mean amplitude of 92.8 ± 13.2 pA in whole-cell voltage-clamp mode (at a holding potential of −45 mV, n = 3; Fig. 4a). In whole-cell current-clamp mode, the same light pulse evoked hyperpolarizations of 10.2 ± 3.1 mV (Fig. 4a). Addition of the endoplasmic reticulum–forward trafficking motif of Kir2.1 (ref. 143), in combination with the SYN1 promoter, increased currents to 225 ± 28 pA and hyperpolarizations to 15.8 ± 2.0 mV. Once the photocurrent or photo-hyperpolarization was triggered by the 380-nm light pulse, it remained constant in the dark until deactivation was induced by a light pulse at 500 nm (Fig. 4a). Photocurrents could be repeatedly activated and deactivated for hundreds of cycles without deterioration (Supplementary Fig. 3).
Fig. 4 - We generated transgenic PC21,22 expressing HyLighter, following labeling with MAG. Brief (100 ms) pulses of 380-nm light (violet bars) activated the channels, which remained open in the dark and were then deactivated by 500-nm light (green bars). (b) Action potential firing evoked by a 250-pA pulse was silenced by 380-nm light (violet bar). The silencing persisted until HyLighter was turned off by 500-nm light (green bar). (c) Action potential firing evoked by a four-pulse train of 50-pA depolarizing current injections was silenced by a single light pulse. Arrow indicates brief (350 ms) illumination at 380 nm. (d) Inhibition of action potential firing after a single 380-nm light (violet arrows) pulse persisted for seconds to minutes, until HyLighter is switched off by a pulse of 500-nm light (green arrows). Four-pulse depolarizing current trains were given every 7.75 s. Note that different neurons are depicted in c and d.

**DISCUSSION**

Light-controlled systems provide many advantages for artificially manipulating biological function in vivo with high spatial and temporal resolution and precise control of signal strength. Proteins that bind natural or synthetic photoswitches have made it possible to probe neuronal networks1,2 by asking how turning on and off the activity of specific cells alters information processing and behavior. Although a number of light-controlled, genetically targetable excitatory systems promoter were crossed to fish from an enhancer trap line in which the GAL4 transcription factor is expressed in spinal cord motor neurons (line Gal41020e, Fig. 6a)2,4. Escape responses were triggered by a mechanical stimulus to the dish and we monitored behavior by observing the motions of the free tail in head-embedded fish larvae. The fish were first labeled with the MAG photoswitch at 5 d post fertilization, as previously described2,12. Bouts of mechanical stimulation were administered in the absence of illumination, following illumination of the tail with 390-nm light (0.5 mW mm⁻²) to activate HyLighter and following illumination at 500 nm (0.5 mW mm⁻²) to deactivate HyLighter. Illumination at 390 nm reduced the probability of an escape response (P < 0.01, n = 14; Fig. 6b) and this effect was reversed by illumination at 500 nm. The same illumination had no effect on the probability of escape responses in siblings from the same crosses that did not express HyLighter (P > 0.31, n = 16).

**Figure 4** Optical inhibition of neuronal activity in dissociated cultured hippocampal neurons. (a) Photocurrent measured in whole-cell voltage clamp (top) and photo-hyperpolarization measured in whole-cell current clamp (bottom) recorded at −45-mV membrane potential in a hippocampal neuron expressing HyLighter, following labeling with MAG. Brief (100 ms) pulses of 380-nm light (violet bars) activated the channels, which remained open in the dark and were then deactivated by 500-nm light (green bars). (b) Action potential firing evoked by a 250-pA pulse was silenced by 380-nm light (violet bar). The silencing persisted until HyLighter was turned off by 500-nm light (green bar). (c) Action potential firing evoked by a four-pulse train of 50-pA depolarizing current injections was silenced by a single light pulse. Arrow indicates brief (350 ms) illumination at 380 nm. (d) Inhibition of action potential firing after a single 380-nm light (violet arrows) pulse persisted for seconds to minutes, until HyLighter is switched off by a pulse of 500-nm light (green arrows). Four-pulse depolarizing current trains were given every 7.75 s. Note that different neurons are depicted in c and d.

**Figure 5** Silencing of activity in cultured hippocampal slice. Action potentials triggered by current injections (up to 400 pA) are reliably silenced by activation of HyLighter at 390 nm (violet bar) and released from inhibition by deactivation of HyLighter at 500 nm (green bar).
Allosteric control of an ionotropic glutamate receptor with an
photoswitch under illumination could be used to pattern photoswitches. Indeed, the delivery of the light sources, it may be possible to deliver MAG through the same portal
lighting could be used to pattern photoconjugates. This is an advantage over light
activation of HyLighter with illumination at 390 nm of neurons in the tail before the stimulus (*P < 0.01, n = 14). The escape response recovered after illumination at 550 nm (which deactivates HyLighter) to a level no different from the initial control (P > 0.57, n = 14). Error bars are s.e.m.

Author Contributions
H.J. designed chimeric proteins, conducted experiments in HEK293 cells and neuronal cultures, contributed to experiments in brain slices and zebrafish, analyzed data and wrote the manuscript. S.S. conducted experiments in brain slices, analyzed data and wrote the manuscript. C.W. conducted experiments in zebrafish, analyzed data and wrote the manuscript. D.T. developed photoswitching methodology and provided photoswitches. E.Y. designed chimeric proteins, developed photoswitching methodology, analyzed data and wrote the manuscript.

Competing Financial Interests
The authors declare no competing financial interests.

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ONLINE METHODS

Synthesis of MAG photoswitches. MAG0, MAG1 and tether model synthesis and chemical analysis were described previously6,18.

Protein construction and site-directed mutagenesis. A DNA segment coding for the sGluR0 pore was synthesized with mammalian codon optimization according to the supplier’s recommendation (Epoch Biosci). The α-splice isoform of iGluR6 was obtained from K. Partin (Colorado State University). An initial iGluR6-p6-p0-variant was created by cloning the sGluR0 pore into iGluR6 using introduced sites for Bsp611 and BstEII restriction enzymes (New England Biolabs). To generate iGluR6-p0-A to G, regions flanking the sGluR0 pore were extended by PCR and 5'-phosphorylated oligonucleotides with corresponding overhangs. For testing, residues 32 to the last residue of iGluR6-p0-A to G were subcloned into pcDNA3.1 containing a viral signal peptide (K. Kainanen, University of Helsinki)17. eGFP-tagged iGluR-p0-C-L439C was obtained by introducing the L439C substitution and subcloning into the pEGFP-N1 vector containing the native iGluR6 signal peptide and a WPR element for optimized expression. iGluR-p0-C-L439C-GFP-WPRE was then subcloned into pcDNA3.1 containing the human SYN1 promoter. Restrictions sites and point substitutions were introduced by site-directed mutagenesis (Quickchange XL, Stratagene). Residues are numbered according to the start methionine of wild-type iGluR6. Codon-optimized sGluR0 cDNA with a modified signal peptide was obtained from E. Gouaux (Oregon Health and Science University)22.

HEK293 and neuron culture and transfection. HEK293 cells were maintained in DMEM (Invitrogen) with 5% fetal bovine serum (vol/vol, Sigma), seeded on glass coverslips coated with poly-lysine (Sigma) and transfected with glutamate receptors and eYFP in pEGFP-N1 at a ratio of 20:1 with Lipofectamine 2000 (Invitrogen). Dissociated postnatal rat hippocampal neurons (postnatal days 0–5) were prepared and transfected using calcium phosphate as described previously21.

MAG conjugation to HEK293 cells and neurons. To conjugate MAG0 in HEK293 cells, we diluted MAG0 to 40–60 μM in extracellular recording solution containing 145 mM NaCl, 4 mM KCl, 1 mM MgCl2, 2 mM CaCl2, and 10 mM HEPES (pH 7.4). After illumination of MAG with 380-nm light (0.19 mW mm−2) for 2 min, cells were incubated for 20–25 min at 20–25 °C in the dark and then rinsed with extracellular recording solution. Prior to recording, the HEK293 cells were incubated in an extracellular solution containing 9.3 mg ml−1 concanavalin A type VI (Sigma) to block desensitization of iGluR6. Concanavalin was applied only to HEK293 cells and never to neurons. For neurons, MAG was diluted in a solution containing 150 mM NMDG-HEPES, 3 mM MgCl2, 0.5 mM CaCl2, 5 mM MgCl2, 10 mM HEPES and 5 mM glucose (pH 7.4). After illumination of MAG with 380-nm light for 2 min, cells were incubated for 20–25 min at 20–25 °C in the dark and then rinsed with extracellular recording solution.

Electrophysiology and light switching in cultured cells. Whole-cell patch-clamp recordings were performed with an Axopatch 200A amplifier (Molecular Devices) 36–72 h after transfection for HEK cells and 2–5 d after transfection for neurons. Pipettes had resistances of 3–5 MΩ and were filled with a solution containing 140 mM KCl, 5 mM EGTA, 0.5 mM CaCl2, 1.0 mM MgCl2 and 10 mM HEPES (pH 7.4) for HEK cells or 135 mM potassium gluconate, 10 mM NaCl, 10 mM HEPES, 2 mM MgCl2, 2 mM MgATP and 1 mM EGTA (pH 7.4) for neurons. For HEK cell recordings, perfusion with extracellular solution (see above) was continuous, with 1.5 mM glutamate added as indicated. For reversal potential measurements, extracellular Na+ was replaced by K+. For neuron recordings, 25 μM 6,7-dinitroquinoxaline-2,3-dione and 10 mM glucose was added to the extracellular solution. Illumination was applied using a 300-W wavelength-switching system with 1–2 ms switching time (DG-4P, Sutter Instruments) connected to the back illumination port of the microscope (IX70, Olympus). Light intensities were determined using a power meter (Newport). Filters for 380- and 500-nm illumination (Semrock) had bandwidths of 34 and 25 nm, respectively, and were directed to a 20x objective (Olympus) using a total reflectance mirror (Edmund Optics). Electrophysiological data was recorded with pClamp software (Molecular Devices), which was also used to automatically control the illumination device. Only neurons with a resting potential ≤−50 mV were analyzed.

Preparation of cultured hippocampal slices. Hippocampi were obtained from postnatal Sprague-Dawley rats (postnatal days 6 and 7), cut into 400-μm slices and cultured on 0.4-μm Millicell culture inserts (Millipore) in Neurobasal-A medium (Gibco) supplemented with 20% horse serum (vol/vol), insulin, ascorbic acid, GlutaMAX (Gibco), penicillin/streptomycin, HEPES and Ara-C. Slices were transfected 1 d after isolation by Biolistic gene transfer using a BioRad Helios Gene Gun and gold microcarriers coated with both Hydrolight-GFP-WPRE (in pcDNA3.1 containing human SYN1) and cytotoxic tdTomato DNA (to aid in the visualization of the transfected cells).

MAG conjugation and hippocampal slice electrophysiology. Electrophysiological recordings were obtained from slices after 6–9 d in vitro. Just before recording, slices were incubated at 20–25 °C for 30 min with MAG0 (250 μM) diluted in NMDG-labeling solution (150 mM NMDG-HCl, 3 mM KCl, 0.5 mM CaCl2, 5 mM MgCl2, 10 mM HEPES and 5 mM glucose, pH 7.4). Slices were rinsed twice in labeling solution before recording. Whole-cell patch-clamp recordings were performed on an upright Zeiss AxioExaminer using an Axopatch 200B amplifier (Molecular Devices). Pipettes had resistances of 3–5 MΩ and were filled with the neuron pipette solution containing (135 mM potassium-glucuronate, 10 mM NaCl, 10 mM HEPES, 2 mM MgCl2, 2 mM MgATP and 1 mM EGTA, pH 7.4). The slices were perfused with aCSF consisting of 119 mM NaCl, 2.5 mM KCl, 1.3 mM MgSO4, 1 mM NaH2PO4, 26.2 mM NaHCO3, 11 mM glucose and 2.5 mM CaCl2. The aCSF was continually circulated and bubbled with 95% O2/5% CO2. The light used for photoswitching was from a DG-4 (Sutter Instruments) coupled to the microscope and projected onto the sample through a digital micromirror device (Mosaic System, Photonic Instruments) through a 4× objective. Light intensity at the sample was approximately 20 mW mm−2 at 390 nm and 40 mW mm−2 at 500 nm. In many cases, the illumination area was smaller than the neuron and distal processes were not subject to photostimulation.

Generation of stable zebrafish transgenic line. To make the UAS::iGluR-p0-C-L439C-GFP transgenic construct, iGluR-p0-C-L439C-GFP was subcloned in between the Tol2 recognition sequences in the pT2KXGdin vector40, Gal4p1020 embryos22,44 were injected at the one-cell stage with a solution of 25 ng μl−1 UAS::iGluR-p0-C-L439C-GFP DNA, 50 ng μl−1 transposase mRNA and 0.04% Phenol Red (vol/vol). F1 embryos were screened by fluorescence and used for experiments 5 d post fertilization.

MAG labeling and photostimulation on zebrafish larvae. MAG1 and MAG0 were diluted to 5 mM in DMSO and pre-activated by ultraviolet light (365 nm) for 5 min. The E3 medium was then added to reach a final concentration of 100 μM MAG. We bathed 5-d-old larvae in the labeling solution for 30 min at 28.5 °C. The larvae were then washed three times with fresh E3 medium. Following a 1-h recovery period, all spontaneously swimming larvae were embedded in agar and their tails were freed. The light source used for photoswitching was a DG-4 (Sutter Instruments) coupled to an upright Zeiss AxioExaminer epifluorescence microscope (the light power was 0.5 μW mm−2 at 390 nm and 500 nm). Escape responses were induced by mechanical taps to the dish at a 10-s inter-stimulus interval. Ultraviolet light pulses (1–5-second duration) illuminating the tail were immediately followed by the mechanical stimulus. Motion of the tail was monitored under low-light conditions at 60 frames per s using a CCD camera coupled to the side port of the AxioExaminer microscope and a 5× objective. The tracking of the tail position was performed using a custom-made script written in Matlab 2007 (Mathworks).

Kinetic model. We describe MAG with a two-state model in which the high energy cis-state is separated from the low-energy trans-state by a single energy barrier. In the dark after ultraviolet illumination, the only existing reaction is the thermally activated cis→trans isomerization. This process can be described by an Arrhenius-type equation49,50,  \[ \frac{1}{T} = \frac{E_c}{A_0 e^{-k_B T}} \], where \( T \) denotes cis-state lifetime, \( A_0 \) denotes attempt frequency (1019 s−1) for azobenzenes49,50, \( E_c \) denotes activation energy (that is, the energy barrier) and \( k_B T \) denotes thermal energy. \( E_c \) can be determined to be ≈75 kJ mol−1 by inserting a τ of 25.47 min−1 into the Arrhenius equation after rearranging. This value agrees well with that of other
azobenzene photoswitches\textsuperscript{49,50}. Finally, the Arrhenius equation allows estimating that increasing $E_A$ by less than 10% (6 kJ mol$^{-1}$) will result in an increased lifetime of one order of magnitude.

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