Biophysical constraints of optogenetic inhibition at presynaptic terminals

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We investigated the efficacy of optogenetic inhibition at presynaptic terminals using halorhodopsin, archaerhodopsin and chloride-conducting channelrhodopsins. Precisely timed activation of both archaerhodopsin and halorhodopsin at presynaptic terminals attenuated evoked release. However, sustained archaerhodopsin activation was paradoxically associated with increased spontaneous release. Activation of chloride-conducting channelrhodopsins triggered neurotransmitter release upon light onset. Thus, the biophysical properties of presynaptic terminals dictate unique boundary conditions for optogenetic manipulation.

Testing whether a particular projection pathway is involved in a defined behavioral or physiological process often requires prolonged inhibition of specific axonal projections during behavioral trials or electrophysiological recordings. The hyperpolarizing effects of light-gated chloride pumps, proton pumps and chloride channels, in action potential firing in neuronal somata have been widely characterized. Although light-driven ion pumps attenuate synaptic transmission when activated in presynaptic terminals, the direct effects of these tools on presynaptic terminal function have not been characterized in detail. We therefore asked whether inhibitory optogenetic tools can be used to achieve sustained, efficient silencing of neurotransmitter release from presynaptic terminals of long-range axonal projections.

We first investigated the effects of the most widely used inhibitory optogenetic tools, archaerhodopsin and halorhodopsin, on the activity of presynaptic terminals. To record presynaptic terminal activity, we coexpressed a synaptophysin-GCaMP6 fusion protein (SyGCaMP6s) with the membrane trafficking–enhanced variants of archaerhodopsin and halorhodopsin (eArch3.0-mCherry or eNpHR3.0-mCherry, respectively) in cultured rat hippocampal neurons. The fluorescence labeling of SyGCaMP6s colocalized with the endogenous presynaptic protein synapsin 1 (Fig. 1a), confirming its presynaptic localization, and mCherry fluorescence was observed both in soma and neurite membranes (Supplementary Fig. 1). Two-photon calcium imaging of spontaneous activity in these neurons showed a pattern of regularly occurring network bursts and individual-bouton calcium dynamics (Supplementary Video 1). We reasoned that optogenetic inhibition would reduce the amplitude and/or frequency of presynaptic calcium transients measured by SyGCaMP6s. Surprisingly, a 5-min-long activation of eArch3.0 caused a significant increase in SyGCaMP6s fluorescence that recovered only partially following the termination of yellow light illumination (Fig. 1b–d and Supplementary Video 2).
expressing a cytoplasmic GaMP6s also showed increased somatic and dendritic fluorescence upon eArch3.0 activation (Supplementary Video 3). In contrast, neurons expressing SyGaMP6s alone or with eNpHR3.0-mCherry showed no detectable change in fluorescence upon illumination (Fig. 1c,d and Supplementary Videos 1 and 4). Maximal SyGaMP6s fluorescence during eArch3.0 activation was 16.47 ± 3.84 fold (mean ± s.e.m.) higher than the maximal fluorescence recorded during the baseline period (Fig. 1e).

To examine whether the eArch3.0-induced increase in presynaptic [Ca2+]i leads to increased neurotransmitter release, we recorded from hippocampal neurons expressing eArch3.0 and SyGaMP6s (Fig. 1f–h). Activation of eArch3.0 as described above led to a gradual three-fold rise in the rate of excitatory postsynaptic currents (EPSCs; Fig. 1g,h). Both eNpHR3.0-expressing and control neurons showed no change from baseline during illumination, consistent with the calcium imaging data (Fig. 1f–h). These findings indicate that eArch3.0 activation leads to a paradoxical increase in presynaptic [Ca2+]i, and neurotransmitter release.

We next tested whether activation of proton or chloride pumps in presynaptic terminals effectively inhibits action potential-evoked neurotransmitter release. We injected adult mice with AAVs coexpressing eArch3.0 or eNpHR3.0 with SyGaMP6s into the whisking thalamus, which projects to the primary somatosensory (S1) barrel cortex. In acute coronal slices, we observed mCherry-labeled thalamocortical projections terminating in S1 (Fig. 2a). Electrical microstimulation of these thalamocortical fibers in the external capsule produced reliable evoked excitatory and inhibitory postsynaptic currents (eEPSCs and eIPSCs) in S1 layer 4 (L4) neurons (Fig. 2b and Supplementary Fig. 2a), which were attenuated by activation of both eArch3.0 and eNpHR3.0 with 200-ms light pulses (Fig. 2c and Supplementary Fig. 2c), leading to an increase in the paired-pulse ratio (Fig. 2d and Supplementary Fig. 2d). Notably, in both eArch3.0- and eNpHR3.0-expressing slices, we observed strong rebound-like synaptic responses after light offset that were attenuated when step-like termination of light was replaced by a more gradual decrease in light power.

Figure 2 Effects of eArch3.0 and eNpHR3.0 on evoked and spontaneous release in thalamocortical terminals. (a) The experimental setup. The bipolar stimulation electrode was placed in the thalamocortical fiber tract while whole-cell voltage-clamp recordings were performed in L4 of barrel cortex. Yellow circle indicates area of light application for opsin activation. (b) Example traces of EPSCs evoked by electrical microstimulation (eEPSCs) with and without light application to the presynaptic terminals. Individual traces are overlaid with their mean. Black ticks indicate time of electrical stimulation. (c,d) Quantification of eEPSCs before and during brief light application (200 ms, 590 nm, 2 mW mm−2). (e) eEPSC amplitude relative to baseline and paired-pulse ratio during 5 min constant illumination. (f) eEPSC amplitude, n = 10; paired-pulse ratio, n = 9. (h) eEPSC amplitude, n = 14; paired-pulse ratio, n = 14. (i) SyGaMP6s imaging of presynaptic boutons during the same light application protocol. (j) Quantification of average fluorescence during baseline, light and post-light periods shown in i. eArch3.0, n = 9; eNpHR3.0, n = 6. (k,m) Effect of constant illumination on spontaneous EPSC (k) and IPSC (m) rates (z-scores). Insets depict sample eArch3.0 traces from baseline (top), light (middle) and post-light (bottom) periods (scale bars, 40 pA, 200 ms). (l,n) Quantification of EPSC and IPSC rates from k and m. (i) eArch3.0, n = 13; eNpHR3.0, n = 10. (n) eArch3.0, n = 13; eNpHR3.0, n = 10. Error bars and shading indicate s.e.m. *P < 0.05; see Supplementary Table 1 for statistics.
(Supplementary Fig. 3). These results indicate that brief illumination of axonal terminals expressing eArch3.0 or eNpHR3.0 can attenuate synaptic transmission but is associated with strong rebound responses that can be avoided by ramp-like light termination.

To evaluate the potential of eArch3.0 and eNpHR3.0 for sustained inhibition of synaptic transmission, we recorded responses of S1 L4 neurons to microstimulation of thalamocortical fibers during 5 min of constant illumination (Fig. 2c,h). Microstimulation-evoked EPSCs and IPSCs in L4 neurons were attenuated during 5 min of eArch3.0 activation (Fig. 2e and Supplementary Fig. 2e). Inhibition of presynaptic release by eNpHR3.0 was maximal within 50 ms of light onset and its efficacy—although still evident—was approximately halved after 150 ms (Fig. 2h and Supplementary Figs. 2i and 4), likely contributing to the increase in paired-pulse ratio observed with eNpHR3.0 (Fig. 2g).

We next asked whether presynaptic [Ca²⁺]i and spontaneous release are affected in the acute slice preparation by optogenetic hyperpolarizing tools in a manner similar to that observed in our cultured neuron recordings. Presynaptic SyGCaMP6s fluorescence in thalamocortical boutons increased during activation of eArch3.0, but not eNpHR3.0 (Fig. 2i,j). Consistently, the rate of spontaneous EPSCs and IPSCs increased during activation of eArch3.0, but not eNpHR3.0, in thalamocortical terminals (Fig. 2k–n). These results indicate that activation of eArch3.0 increases presynaptic [Ca²⁺]i and spontaneous neurotransmitter release. Since thalamocortical projections are glutamatergic, the increase in spontaneous IPSCs indicates that local-circuit feedforward inhibition [12,13] might be recruited by this rise in spontaneous excitatory input.

For a more detailed understanding of the paradoxical effects of eArch3.0 on presynaptic function, we first tested whether the light-evoked increase in SyGCaMP6s signal is due to influx of extracellular Ca²⁺. Removal of extracellular Ca²⁺ strongly attenuated the light-induced elevation in SyGCaMP6s fluorescence (Fig. 3a–c) and eliminated the increase in EPSC rates (Fig. 3d,e). The light-induced increase in the SyGCaMP6s signal and EPSC rates were not action potential–dependent (Fig. 3f–i). In cultured neurons, eArch3.0 photocurrents strongly adapted during 5 min of light application and this adaptation was greatly reduced by intracellular pH stabilization via addition of l-lactate to the recording medium (Supplementary Fig. 5). This led us to hypothesize that intracellular alkalization contributes to eArch3.0 effects on synaptic function. Indeed, pH-sensitive dye imaging showed that eArch3.0 activation led to an increase in intracellular pH, which was attenuated by inclusion of l-lactate in the recording medium (Supplementary Fig. 6a–c). eArch3.0-expressing cells recorded in l-lactate medium displayed attenuated Ca²⁺ influx (Fig. 3g) and reduced EPSC rates during illumination (Fig. 3h,i). These findings are consistent with previous work showing that intracellular alkalization triggers calcium influx in neurons [14,15] (Supplementary Fig. 6d).

Finally, we tested whether light-gated chloride channels can inhibit synaptic transmission via shunting of action potential–mediated release. In cultured hippocampal neurons expressing GtACR1 (ref. 6), light-gated photocurrents allowed robust inhibition of action potential firing (Supplementary Fig. 7a,b). We next expressed GtACR1 in the whisking thalamus and recorded from S1 L4 neurons as described above. Illumination of GtACR1-expressing presynaptic terminals evoked strong, short-latency EPSCs that were similar in amplitude to the postsynaptic responses evoked by microstimulation of thalamocortical inputs to the same cells (Supplementary Fig. 7c,d). When light and microstimulation were coapplied with varying delays, light administration did not attenuate microstimulation-evoked EPSCs (Supplementary Fig. 7e,f). Increased expression levels or improved axonal targeting of these channels may provide more efficient shunting of synaptic release but would most likely not eliminate the light onset–associated responses.

Our findings uncover the biophysical constraints of optogenetic inhibition at presynaptic terminals. We show that while light-gated proton and chloride pumps can effectively attenuate neurotransmission, sustained proton pump activity in synaptic terminals induces a pH-dependent calcium influx that leads to increased spontaneous release at the target circuit. Consistent with previous reports of a depolarized chloride reversal potential in presynaptic terminals [16–18], we found that activation of light-gated chloride channels triggers neurotransmitter release upon light onset, potentially limiting the utility of these tools for temporally precise manipulation of synaptic release. Our data therefore suggest that eNpHR3.0 is the most suitable existing tool for synaptic terminal silencing, although its use should be carefully controlled to account for changes in chloride reversal potential [19] and strong light–off rebound responses. We expect that further optimization of light-gated potassium channels [20] will allow improvements in the efficacy of fast optogenetic inhibition of neurotransmitter release.

METHODS

Methods and any associated references are available in the online version of the paper.

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS

M.M. and O.Y. designed the study. M.M. performed all electrophysiology and imaging experiments. R.L. prepared neuronal cultures and viral vectors. M.P. and S.R. helped with cloning and viral injections. M.M. and O.Y. analyzed and interpreted the results and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS
Cloning of SyGCaMP6s expression vectors. SyGCaMP6s was fused in-frame to synaptophysin and ligated into the Cre-dependent pAAV-Ihsyn-DIO-WPRE backbone. A Cre-independent version of this expression plasmid was generated by treatment of the DIO vector with Cre recombinase (NEB) in vitro.

Production of recombinant AAV vectors. HEK293 cells were seeded at 25–35% confluency. The cells were transfected 24 h after with plasmids encoding AAV rep, cap and a vector plasmid for the RAAV cassette expressing the relevant DNA using the PEI method. Cells and medium were harvested 72 h after transfection, pelleted by centrifugation (300g), resuspended in lysis solution (in mM: 150 NaCl, 50 Tris–HCl, pH 8.5 with NaOH) and lysed by three freeze-thaw cycles. The crude lysate was treated with 250 U benzonase (Sigma) per ml of lysozyme at 37°C for 1.5 h to degrade genomic and unpackaged AAV DNA before centrifugation at 3000g for 15 min to pellet cell debris. The virus particles in the supernatant (crude virus) were purified using heparin-agarose columns, eluted with soluble heparin, washed with phosphate buffered saline (PBS) and concentrated by Amicon columns. Viral suspension was aliquoted and stored at −80°C. Viral titers were measured using real-time PCR. AAV vectors used for intracranial injections had genomic titers ranging between 1.49 × 10^11 and 3.28 × 10^11 genome copies per milliliter (gc/ml). AAV vectors used for neuronal culture transduction had titers ranging between 1.08 × 10^10 and 3.15 × 10^10 gc/ml.

Primary hippocampal neuron culture and viral transduction. Primary cultured hippocampal neurons were prepared from male and female P0 Sprague–Dawley rat pups (Envigo). CA1 and CA3 were isolated, digested with 0.4 mg ml^-1 papain (Worthington) and plated onto glass coverslips precoated with 1:30 Matrigel (Worthington) and A-21206; Thermo Fisher Scientific) and then washed twice with PBS, dipped 1:500 in 5% NDS, PBS; 711-175-152; Jackson ImmunoResearch Laboratories a donkey anti-rabbit Cy5- or Alexa488-conjugated secondary antibody (diluted washes in PBS, coverslips were incubated for 2 h at room temperature (RT) with either −70 mV or 0 mV to measure EPSCs or IPSCs, respectively. To calculate [Ca^2+], fluorescence was excited using 488-nm and 555-nm laser diodes, respectively. Alexa488 and mCherry fluorescence was excited using 488-nm and 639-nm laser diodes, respectively. SyGCaMP6s and Cy5 fluorescence was excited using a two-photon microscope (Ultima IV, Bruker) equipped with a 12-bit monochrome CCD camera (QImaging QIClick R-F-M-12). Bosorilsicate glass pipettes (Sutter Instrument BF100-58-10) with resistances ranging from 3–7 MΩ were pulled using a laser micropipette puller (Sutter Instrument Model P-2000). For hippocampal neuron cultures, electrophysiological recordings from neurons were obtained in Tyrode's medium (in mM: 150 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, 10 HEPES; 320 mOsm; pH adjusted to 7.35 with NaOH), AChOH Tyrode medium (in mM: 125 NaCl, 25 acetic acid, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, 10 HEPES; 320 mOsm; pH adjusted to 7.35 with NaOH) or 1-lactate Tyrode's medium (in mM: 100 NaCl, 50 1-lactate, 4 KCl, 2 MgCl₂, 2 MgCl₂, 10 glucose, 10 HEPES; 320 mOsm; pH adjusted to 7.35 with NaOH), where indicated. The recording chamber was perfused at 0.5 ml min^-1 and maintained at 32°C.

Electrophysiological methods. Whole-cell patch clamp recordings were performed under visual control using oblique illumination on a two-photon laser scanning microscope (Ultima IV, Bruker) equipped with a 12-bit monochrome CCD camera (QImaging QIClick R-F-M-12). Bosorilsicate glass pipettes (Sutter Instrument BF100-58-10) with resistances ranging from 3–7 MΩ were pulled using a laser micropipette puller (Sutter Instrument Model P-2000). For hippocampal neuron cultures, electrophysiological recordings from neurons were obtained in Tyrode's medium (in mM: 150 NaCl, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, 10 HEPES; 320 mOsm; pH adjusted to 7.35 with NaOH), AChOH Tyrode medium (in mM: 125 NaCl, 25 acetic acid, 4 KCl, 2 MgCl₂, 2 CaCl₂, 10 glucose, 10 HEPES; 320 mOsm; pH adjusted to 7.35 with NaOH) or 1-lactate Tyrode's medium (in mM: 100 NaCl, 50 1-lactate, 4 KCl, 2 MgCl₂, 2 MgCl₂, 10 glucose, 10 HEPES; 320 mOsm; pH adjusted to 7.35 with NaOH), where indicated. The recording chamber was perfused at 0.5 ml min^-1 and maintained at 29°C. Pipettes were filled using standard intracellular solution (in mM: 135 potassium glutamate, 4 KCl, 2 NaCl, 10 HEPES, 4 EGTA, 4 Mg-ATP, 0.3 Na-GTP; 280 mOsm kg^-1; pH adjusted to 7.3 with KOH) or an intracellular solution allowing EPSC and IPSC recording (in mM: 120 cesium glutamate, 1 NaCl, 1 MgCl₂, 1 CaCl₂, 10 HEPES, 11 EGTA, 5 QX-314; 280 mOsm kg^-1; pH adjusted to 7.3 with CsOH). For acute brain slice experiments, whole-cell patch-clamp recordings were obtained from layer 4 neurons of the barrel cortex, in regions showing robust axonal expression (mCherry fluorescence). During voltage clamp experiments neurons were clamped at either −70 mV or 0 mV to measure EPSCs or IPSCs, respectively. To calculate the paired-pulse ratio, pairs of electrical microstimulation pulses were delivered at 100-ms inter-pulse intervals. To aid in visualization, Alexa Fluor 350 (Invitrogen, 100 µM) was included in some of the patch pipettes. Whole-cell voltage-clamp recordings were performed using a MultiClamp 700B amplifier, filtered at 8 kHz and digitized at 20 kHz using a Digidata 1440A digitizer (Molecular Devices).

Two-photon Ca^2+ and BCECF imaging. Imaging of the genetically encoded calcium sensor SyGCaMP6s was performed using a two-photon microscope (Ultima IV, Bruker) equipped with a 20× objective (NA 0.5). SyGCaMP6s was excited with a mode-locked Ti:sapphire laser tuned to 940 nm (Chameleon, Coherent) using a dwell time of 2.4 µs per pixel. Fluorescence was detected by a GaSP photomultiplier (Hamamatsu) after passing through a 525/50 nm filter. Images of 256 × 256 pixels (1,024 × 1,024 pixels) area were recorded at 0.33 Hz to minimize photobleaching. To record intracellular pH in cultured neurons, we used the pH-sensitive dye 2′,7′-bis-(2-carboxyethyl)-5′-(and-6)-carboxyfluorescein (BCECF). In these experiments, hippocampal neuron cultures expressing eArch3.0 or eNpHR3.0 were incubated for 10 min in BCECF-AM (TEFLABS) loading solution (in mM: 150 NaCl, 4 KCl, 2 MgCl₂, 2 MgCl₂, 10 d-glucose, 10 HEPES, 1 × 10⁻³ BCECF-AM; 320 mOsm; pH adjusted to 7.35 with NaOH). Fluorescence of BCECF was excited at the pH-sensitive two-photon wavelength of 795 nm.
Stimulation and drug application. For activation of thalamocortical fibers, an extracellular stimulating electrode (concentric bipolar Pt/Ir electrode, 33 G; FHC) was placed in the external capsule in close proximity to visually identified mCherry-expressing fiber bundles. Fibers were activated by short, bipolar electrical pulses (400 μs, <100 μA). Opsins were activated using a 590-nm light emitting diode (LED; M590L2-C2; Thorlabs) delivered through the microscope illumination path, which included a custom dichroic to reflect the 590-nm activation wavelength while collecting green fluorescence emission. Light power densities were calculated by measuring the light transmitted through the objective using a power meter (Thorlabs PM100A with S146C sensor) and dividing by the illumination area, calculated from the microscope objective field number and magnification. For activation of thalamocortical fibers, mCherry-expressing fiber bundles. Fibers were activated by short, bipolar electrical pulses (33 G; FHC) was placed in the external capsule in close proximity to visually identified fibers. Fibers were activated by short, bipolar electrical pulses (400 μs, <100 μA). Opsins were activated using a 590-nm light emitting diode (LED; M590L2-C2; Thorlabs) delivered through the microscope illumination path, which included a custom dichroic to reflect the 590-nm activation wavelength while collecting green fluorescence emission. Light power densities were calculated by measuring the light transmitted through the microscope objective field number and magnification. TTX (1 μM; T-550, Alomone), d-AP5 (25 μM; ab120003; Abcam) and CNQX (10 μM; C-141, Alomone) were bath applied where indicated.

Data analysis and statistical methods. During whole-cell recordings, pClamp 10 software (Molecular Devices) was used for acquisition. Data were analyzed using custom scripts written in Matlab (Mathworks). EPSCs and IPSCs were automatically detected during periods in which cells were held at negative and positive membrane potentials, respectively. Holding current traces were filtered at 400 Hz using a fifth-order lowpass Butterworth filter and deflections in the filtered trace were detected by threshold crossings of a 4-ms sliding window variance calculation. A suitable variance threshold was determined by recordings in the presence of synaptic blockers (25 μM t-AP5, 10 μM CNQX). To quantify postsynaptic current amplitudes evoked by electrical microstimulation, holding current traces were filtered with a Savitzky-Golay 11 point, second order, Welch window function filter and the maximal change in holding current within 5 ms (eEPSCs) and 10 ms (eIPSC) after electrical microstimulation was determined. For display purposes, only electrical microstimulation artifacts were manually removed. Fiji (based on ImageJ; US National Institutes of Health) and Matlab were used for image analysis. Colocalization of SyGCaMP6s fluorescence with anti-synapsin 1 staining was repeated three times and quantified using Manders split coefficients and Costes P-value. 87.7% of the SyGCaMP6s signal above threshold significantly colocalized with 87.5% of the synapsin 1 staining above threshold (thresholded Manders M1 and M2: 0.877 and 0.875; Costes P-value: 1.00).

For SyGCaMP6s and BCECF fluorescence analysis, images were smoothed by convolution with a Gaussian function (σ = 0.29 μm) and fluorescence background, measured from a manually defined region with no neurons, was subtracted to correct for bleed-through of fluorescence and tissue autofluorescence excited by the opsin activation light (590 nm). Motion correction was performed using the image stabilizer ImageJ plugin (http://www.cs.cmu.edu/~kangli/code/Image_Stabilizer.html). Pixels above threshold, defined by Renyi’s entropy method, were used for further analysis. Fluorescence traces are expressed as relative mean fluorescence change, ∆F/F0 = (F − F0)/F0, where F0 is the background-corrected minimal fluorescence during the baseline period. In case of Ca2+-free recordings, F0 is defined as the background-corrected minimal fluorescence after 2 mM Ca2+ reconstitution. In case of t-lactate recordings, F0 is defined as the background-corrected minimal fluorescence before t-lactate application. No temporal filtering was performed on the fluorescence transients for analysis. In imaging experiments, all numbers (n) refer to the number of imaged regions (each including neurites of multiple cells); in electrophysiological recordings, n refers to the number of neurons. All values are indicated as mean ± s.e.m. Significance was determined at a significance level of 0.05 using paired two-tailed t tests in Figures 2c, d, f, g and Supplementary Figures 2c, d, g, h and 7d, one-way ANOVA in Figure 1e, h with Bonferroni post hoc tests, and repeated-measures ANOVA with Bonferroni post hoc tests for all other comparisons. Data shown in Figure 1d, e, g, h were log transformed before statistical comparison to compensate for unequal variance. A Huynh-Feldt correction was performed on data shown in Figure 2e, h, i and Supplementary Figures 2i, 3c and 4b to correct for violations of sphericity. No statistical tests were run to predetermine sample size, but sample sizes were similar to those commonly used in the field. Blinding and randomization were not performed; however, automated analysis was used whenever possible. Supplementary Table 1 contains detailed descriptions of all of the statistical tests used in this study.

A Supplementary Methods Checklist is available.

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