discovering the disease mechanisms and the genetic origins of psychiatric disease.

COMPETING FINANCIAL INTERESTS
The author declares no competing financial interests.


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# How (not) to silence long-range projections with light

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**Inhibitory optogenetic tools prevent action potential generation during illumination. A study explores the possibility of squelching already propagating action potentials locally at axon terminals before they trigger neurotransmitter release.**

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To reveal the brain circuits that underlie a specific behavior, ideally one would switch defined projection pathways on and off at will. Optogenetic silencing of synaptic terminals by local illumination of the target region would be the method of choice. Suppressing spike generation at the cell body does not achieve the same goal because, in that case, all efferent synapses are affected irrespective of their location in the brain. In this issue of *Nature Neuroscience*, Mahn *et al.*[1] sound a warning: they reveal paradoxical effects of some inhibitory optogenetic tools when these are activated in presynaptic boutons. Depending on the opsin expressed and the illumination protocol, effects range from transient inhibition to strong activation of synaptic transmission.

The discovery that microbial rhodopsins can be used to increase or decrease neuronal activity with different colors of light[2] fueled expectations that precise spatiotemporal control over synaptic transmission would be straightforward. Indeed, direct illumination of axonal boutons expressing the cation channel channelrhodopsin-2 (ChR2) stimulates synaptic transmission and has been used to map the excitatory inputs onto cortical pyramidal cells[3] and to reveal synaptic pathways controlling sensation and behavior[4]. To inhibit neuronal activity, two rhodopsin classes have been used: ion pumps that hyperpolarize neurons, such as archaerhodopsin (Arch, which pumps protons out) and halorhodopsin (NpHR, which pumps chloride in)[2,5], and anion-selective channelrhodopsins that increase the chloride conductance of the plasma membrane[6,7].

Constant illumination of the ion pumps is required to maintain a hyperpolarized membrane potential during the desired silencing period. When activated for too long, however, these pumps will significantly affect ion concentration gradients across the cell membrane. Inside the neuron, ion concentrations will change most rapidly where the surface-to-volume ratio is high, as in presynaptic boutons. Even at the soma, accumulation of chloride ions during continuous illumination of NpHR shifts the reversal potential for $GABA_A$ receptors, leading to elevated spike rates shortly after NpHR photoactivation[8]. Sustained illumination of Arch, on the other hand, could increase cytoplasmic pH. Indeed, the strong proton pumping activity of Arch has recently been exploited to acidify synaptic vesicles[9].

Mahn *et al.* systematically explored the biophysical constraints on optogenetic silencing of synaptic transmission, comparing the highly potent rhodopsins eArch3.0, eNpHR3.0 (ref. 10) and GtACR1 (ref. 7). They first demonstrated that activation of either eArch3.0 or eNpHR3.0 at presynaptic boutons attenuated evoked synaptic transmission, as expected. When they looked for effects on spontaneous activity, however, they made the surprising observation that illumination of eArch3.0-expressing boutons actually increased the frequency of synaptic events, whereas illumination of those expressing eNpHR3.0 had no effect (**Fig. 1a**).

What could be going on during illumination of eArch3.0-expressing boutons that could explain the opposite effects on evoked versus spontaneous synaptic transmission? In addition to the expected hyperpolarization, the authors identified a secondary effect of sustained eArch3.0 activation in boutons: the large proton efflux alkalized the boutons, and this, in turn, triggered the opening of pH-sensitive calcium channels. The rise in intracellular calcium increased the probability of spontaneous vesicle fusion and neurotransmitter release. Thus, although eArch3.0 strongly hyperpolarizes presynaptic terminals and prevents fast, depolarization-triggered vesicle fusion, spontaneous vesicle fusion increases via alkalization-mediated calcium influx (**Fig. 1b**).

To further complicate matters, projection neurons often innervate local inhibitory interneurons that also innervate the target neuron. Mahn *et al.* demonstrated that Arch-mediated synaptic excitation of these interneurons can increase tonic inhibition, counteracting the direct Arch-mediated increase in spontaneous excitatory synaptic transmission. Thus, sign inversion at intermediate synapses can occlude direct rhodopsin-mediated effects on synaptic transmission and create the illusion of successful optogenetic inhibition of release. Since the indirect action of eArch3.0 on interneurons alters the local excitation-inhibition balance, the net result of sustained presynaptic eArch3.0 activation on intact neuronal networks is highly unpredictable.

Notably, the paradoxical increase in spontaneous release during eArch3.0 activation was time dependent and did not occur during the first milliseconds of illumination. Brief light pulses attenuated evoked synaptic transmission without changing pH and increasing calcium, albeit not as efficiently as in eNpHR3.0-expressing boutons. However, with both eArch3.0 and eNpHR3.0, a strong synaptic response was evoked when the light was switched off, even after brief light pulses. This rebound excitation has been described[8] and can have multiple causes. In the case of eNpHR3.0, chloride accumulates in the cytosol, causing GABAergic currents to become depolarizing[8]. During prolonged hyperpolarization, voltage-gated sodium and low-threshold calcium channels recover from inactivation, and hyperpolarization-activated channels open. Depending on the type of synapse,

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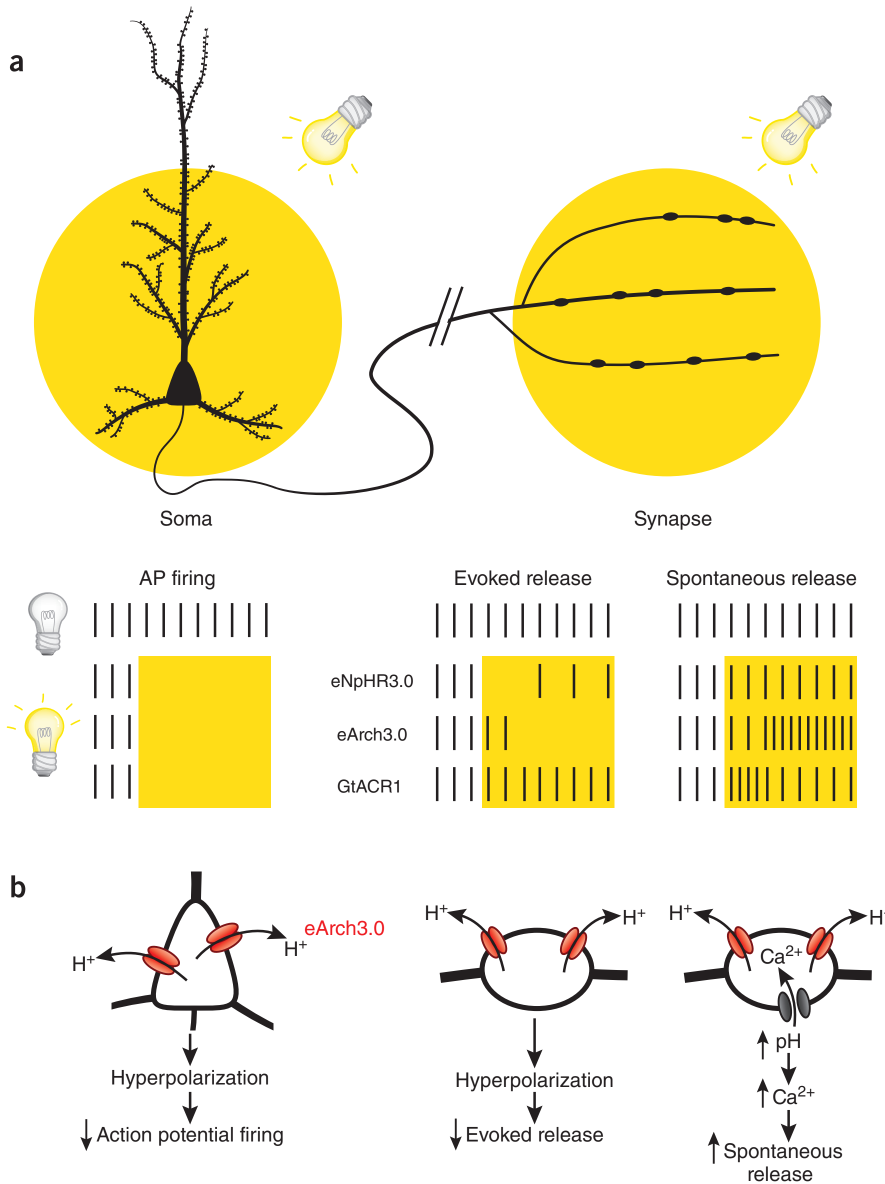


**Figure 1** Optogenetic inhibition works well at the soma but can have unexpected effects when presynaptic terminals are illuminated. (**a**) The chloride pump eNpHR3.0 suppresses release evoked by action potentials and has no effect on spontaneous release. The proton pump eArch3.0 suppresses evoked release, but strongly enhances spontaneous release, making the net result difficult to predict. The chloride channel GtACR1 does not affect evoked release, but it directly stimulates release—not the desired outcome if inhibition is the goal. (**b**) Proposed mechanism for the inhibitory and excitatory actions of eArch3.0. During prolonged illumination, alkalization of the cytoplasm leads to activation of pH-dependent $Ca2^+$ channels, increasing spontaneous release of glutamate.

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combined action of these channels can accelerate repolarization and trigger action potentials following a step-like light offset. As Mahn *et al.* show, rebound excitation is prevented by fading out the light over tens of milliseconds. Thus, paradoxical effects are avoided and synaptic transmission can be suppressed using either eArch3.0 or eNpHR3.0 if short light pulses with gradual off-ramps are applied.

Might suppressing synaptic transmission be more straightforwardly achieved by activating a chloride-conducting channelrhodopsin in presynaptic boutons? Illumination of GtACR1-expressing neurons at the soma had almost no effect on the membrane potential, which at rest is already close to the chloride reversal potential, but effectively inhibits somatic spiking. At synaptic boutons, however, GtACR1 illumination enhanced neurotransmitter release, suggesting that the terminals were being depolarized. This surprising observation may be explained by a higher chloride concentration in the axon and boutons and a positively shifted chloride reversal potential[11]. Whether chloride is naturally elevated in thalamic and potentially other axons or whether this is a consequence of GtACR1 expression remains to be shown. While at first disappointing, these results hint at an alternative use for chloride-conducting channelrhodopsins: measuring chloride gradients in subcellular compartments.

The dream tool for optogenetic silencing would be a fast, high-current potassium channel gated by light. Silencing of synapses using chemogenetic activation of potassium channels[12] demonstrates that such a strategy is viable but lacks the temporal and, to some extent, spatial precision that could be offered by a light-activated channel. Improvements to the available light-gated potassium channel[13] or continued screening for new rhodopsins may soon provide better tools. The recently described light-driven sodium extrusion pump Kr2 (ref. 14) is also promising and may be another contender in the quest for the perfect silencing tool. But even with the ideal inhibitory rhodopsin, users must be on the lookout for unexpected effects. For example, certain serotypes of adeno-associated virus, which are often used as gene-delivery vehicles, directly affect transmission at some synapses[15].

In summary, local optogenetic inhibition of synaptic transmission remains challenging. The biophysical properties of the compartment to be illuminated and the functional characteristics of the selected rhodopsin must be considered when designing experiments. And light sources used for optogenetic inhibition must dim rather than abruptly switch off. Ultimately, the lesson learned from Mahn *et al.* is that preventing an already propagating action potential from releasing transmitter is much harder than preventing its initiation at the soma.

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