Optogenetics at the presynapse

Optogenetics at the presynapse

Rost, Benjamin R.; Wietek, Jonas; Yizhar, Ofer

Rost, Wietek, J., Yizhar, O., & Schmitz, D. (2022). Optogenetics at the presynapse. Nature Neuroscience, 25(8), 984–998. https://doi.org/10.1038/s41593-022-01113-6

Published Version: https://doi.org/10.1038/s41593-022-01113-6

Optogenetics at the Presynapse

Benjamin R. Rost[1,2,8,*], Jonas Wietek[3,4,8], Ofer Yizhar[3,4*], Dietmar Schmitz[1,2,5,6,7]

Affiliations:

[1] German Center for Neurodegenerative Diseases (DZNE), 10117 Berlin, Germany.

[2] Charité-Universitätsmedizin Berlin, Corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Neuroscience Research Center, 10117, Berlin, Germany.

[3] Department of Brain Sciences, Weizmann Institute of Science, Rehovot 76100, Israel

[4] Department of Molecular Neuroscience, Weizmann Institute of Science, Rehovot 76100, Israel.

[5] Bernstein Center for Computational Neuroscience, 10115 Berlin, Germany.

[6] Einstein Center for Neurosciences Berlin, 10117 Berlin, Germany.

[7] Max-Delbrück Center for Molecular Medicine in the Helmholtz Association, Berlin, Germany.

[8] equal contribution

*Corresponding authors: ofer.yizhar@weizmann.ac.il; benjamin.rost@dzne.de

Abstract

Optogenetic actuators enable highly precise spatio-temporal interrogation of biological processes ranging from the subcellular level to cells, circuits, and behaving organisms. While their application in neuroscience has traditionally focused on the control of spiking activity at the somatodendritic level, the scope of optogenetic modulators for direct manipulation of presynaptic functions is growing. Presynaptically localized opsins combined with light stimulation at the terminals allow for light-mediated neurotransmitter release, presynaptic inhibition, induction of synaptic plasticity, and specific manipulation of individual components of the presynaptic machinery. Here, we describe presynaptic applications of optogenetic tools in the context of the unique cell biology of axonal terminals, discuss their potential shortcomings, and outline future directions for this rapidly developing research area.

Keywords:

optogenetics, axons, synapses, neurotransmitter release, presynaptic inhibition, synaptic plasticity, neuronal networks

Optogenetics refers to the expression of light-sensitive proteins in defined cell populations, allowing the use of light to manipulate cellular physiology. Optogenetic tools have opened up entirely new experimental approaches for neurosciences, due to their targeting specificity, simple and temporally precise controllability by light, and to a rapidly diversifying range of applications[1-3]. Paired with subcellular targeting strategies[4], photocontrolled molecular actuators complement electrophysiological, pharmacological, and genetic approaches that often lack subcellular precision and fine temporal control. Direct optogenetic manipulation of the presynapse offers unparalleled experimental opportunities: First, tailor-made optogenetic tools enable precise control of the specialized synaptic machinery that ensures the fidelity of neurotransmission; while presynaptic physiology has traditionally been probed with molecular or pharmacological methods, often combined with electrophysiology or imaging techniques, now, optogenetics enables acute and more temporally precise manipulations of presynaptic function both *in vitro* and *in vivo*. Second, the complex wiring of the brain is best understood by probing functional connectivity directly at the level of specific synapses; while light-driven excitation of axon terminals can reveal synaptic connections between two neuronal populations, optogenetic synaptic inhibition enables us to decipher the role of synapses in signal propagation, network oscillations, computation, and behavior. Third, presynaptic optogenetics enables graded modulation of neurotransmitter release efficiency. Such dynamic control of transmitter release creates an experimental opportunity to address the role of synaptic activity in information processing[5], the function of physiological neuromodulation, and the consequences of synaptic dysfunction in neurodegenerative diseases.

Although undeniably incomplete, the three points above indicate the range of questions, from the molecular to the behavioral level, that presynaptic optogenetics can potentially address. Currently, presynaptic optogenetics offers the most comprehensive toolset compared to alternatives such as chemogenetics or constitutively active, genetically-encoded tools (Table 1). However, with a variety of optogenetic tools at hand, researchers must consider both the biophysical nature of the actuators and the unique physiology of the axon and the presynapse in their experimental design. In this Review, we first outline the main classes of photoreceptors that can be applied at the presynapse, and strategies for their presynaptic targeting. We then consider optogenetic tools for eliciting or inhibiting neurotransmitter release, as well as other optogenetic manipulations of the presynaptic machinery. Finally, we discuss critical technical aspects and future applications of presynaptic optogenetics.

Biophysics of optogenetic tools

Rhodopsins

Rhodopsins encompass the largest group of optogenetic tools of various different functions (Fig. 1A-D). They share a general architecture composed of a heptahelical membrane protein (opsin) in which a retinal chromophore is embedded and covalently linked to the protein. Light absorption induces retinal isomerization, conformational rearrangements and amino acid protonation changes of the rhodopsin, thereby altering the rhodopsin's activation state. While retinal is sufficiently available in vertebrates, it has to be supplemented for most invertebrate model systems like Drosophila and C. elegans. The spectral sensitivity of rhodopsins can range from UV to infrared (Fig. 1A-D), and therefore permits spectral multiplexing with other light-sensitive tools. However, it is important to consider that even red light-absorbing rhodopsins display activation by blue light. Thus, one should consider the potential crosstalk in the design of spectrally multiplexed optogenetic experiments using different actuator and sensor combinations.

Microbial (type I) rhodopsins comprise one of the two groups of rhodopsins used in optogenetics. Originating from various phyla (prokaryotic and eukaryotic microbes, algae, fungi and even viruses)[6], type I rhodopsins have evolved various functions, which can be utilized for manipulating cellular physiology. In most microbial opsins the covalently-bound retinal cofactor izomerizes from the all-*trans* to the 13-*cis* configuration following photon absorption[7]. This photoreaction is cyclic, which enables repeated activation, depending on the time required to complete the photocycle. Photocycles of type I rhodopsins can exhibit side reactions into parallel photocycles with slower kinetics[8], which can impair tool efficacy during repetitive or long-term activation[9, 10]. Historically, most microbial rhodopsins have been identified from genomes of cultured organisms, but recent advances in assembling marine meta-genomes strongly contribute to the growing number of newly discovered rhodopsins[6].

Channelrhodopsins (ChRs) are light-gated, passively conducting ion channels that are subdivided based on their cation- and anion-conductivity (CCRs and ACRs, respectively, Fig. 1A). CCRs and ACRs display no selectivity to specific cations or anions, respectively. CCRs usually conduct smaller cations better than larger ones, whereas ACRs conduct larger anions (less electronegative) better than smaller ones (more electronegative). Consequently, CCRs with high proton conductance could cause intracellular acidification upon sustained illumination[11]. However, the conductivity ratios for different ions varies among CCRs and there have been attempts to change them in favor of certain ions (e.g. Na^+, Ca^{2+}) by rational mutagenesis[11]. ACRs, which were first engineered by targeted mutagenesis of CCRs[12, 13] and later found in nature with higher efficiency[14], can be exploited to suppress AP firing by shunting inhibition. However, pan-neuronal ACR activation can elicit spiking instead of the desired inhibition, due to elevated chloride concentrations in the axon, which can be avoided by restricting ACR expression to the somatodendritic region[15-17].

Light-driven ion pumps (Fig. 1B) share a similar architecture and the same retinal isomerization with ChRs, but they actively transport ions across the cellular membrane in one direction. To date, outward-directed H^+- and Na^+- pumps, and inward-directed Cl^-- and H^+-pumps have been described[6, 18]. Except for the inward-directed H^+-pumps, their activity induces hyperpolarization, effectively increasing the rheobase and thereby suppressing AP generation. However, due to their low transport ratio (one-ion-pumped-per-one-photon-absorbed), efficient inhibition requires dense membrane expression and high light power. Similar to ChRs, activating light-driven pumps causes local changes in ion concentration, which may cause undesired effects, like changes in pH or ion concentration[19, 20].

Enzyme rhodopsins are a group of non-electrogenic type I microbial rhodopsins (Fig. 1C). Among these are rhodopsin-coupled phosphodiesterases, histidine kinases and guanylyl cyclases (RhGCs). RhGCs were used to induce depolarization by co-expression with cyclic nucleotide gated ion channels[21, 22]. While the group of enzyme rhodopsins is still relatively unexplored, the functionalization of enzymatic activity at targeted membranes bears great potential for future applications.

Animal (type II) rhodopsins are specialized G-protein-coupled receptors (GPCRs) that are activated by light rather than biochemical ligands, and use retinal as a chromophore (Fig. 1D). Although they share no sequence homology with type I rhodopsins[6], type II rhodopsins have the same heptahelical architecture and the retinal binding pocket as microbial rhodopsins. In visual type II rhodopsins (such as rod and cone opsins), 11-*cis* retinal isomerizes to all-*trans* upon photon absorption, which triggers G-protein signaling through conformational changes of the receptor. In contrast to microbial rhodopsins, the covalent bond between retinal and opsin is broken upon illumination and all-*trans* retinal has to be externally re-isomerized. If the necessary enzymes for the re-isomerization are not available, functional visual rhodopsins cannot regenerate, i.e. they bleach and can no longer convey signals[23]. In contrast, non-visual type II rhodopsins, which exist in both vertebrates and invertebrates[24], can perform the re-isomerization of the bound chromophore, rendering them bistable. Therefore, these bistable, non-bleaching type II rhodopsins have a major advantage when expressed ectopically in neurons or other excitable cells.

By tapping into endogenous GPCR-mediated signaling cascades, type II rhodopsins have gained considerable interest as tools for modulating neuronal functions. Pioneering work by the laboratories of Shichida and Khorana demonstrated that replacing parts[25] or the entire intracellular signaling interface[26] of bovine rhodopsin with domains from adrenergic or muscarinic acetylcholine receptors allow light-regulated receptor signaling *in vitro*. Using this chimeric approach (later termed optoXRs), photocontrol of adrenergic signaling has been demonstrated *in vivo*[27]. Additional neuromodulatory GPCRs have been developed using similar approaches, including the 5-HT1a, mμ-opioid, D1, D2, and GPCR class A orphan receptors[28-31]. However, the use of rod-based optoXRs in neurons has been

limited due to bleaching-induced decrease in receptor activation over time[23]. Moreover, ectopically expressed optoXRs show slow off-kinetics because non-visual cells lack rhodopsin kinases and arrestins that are necessary for rapid receptor inactivation[32]. Finally, since visual rhodopsins have a broad excitation spectrum, they are less compatible with multiplexed applications. Instead, the non-bleaching vertebrate and invertebrate bistable GPCR rhodopsins (optoGPCRs) are increasingly utilized because they can be activated repeatedly. Some optoGPCRs display spectrally distinct stable states that enable photochromic switching between the active and inactive forms of the protein[24]. While many potential optoGPCRs have been described, mainly in spectroscopic studies, their application as modulators of presynaptic function has only recently been explored (see below). For most optoGPCRs, the G-protein specificity has not been tested in detail. However, promiscuous G-protein coupling of exogenously-expressed optoGPCRs should be considered to exclude activation of undesired signaling cascades. In the future, chimeras of optoGPCRs and endogenous GPCR signal domains promise to combine high coupling specificity with the non-bleaching properties of bistable opsins.

Blue-light receptors (BLRs)

Other types of photoreceptors utilized for optogenetic applications are proteins that use flavins as chromophores, namely flavin adenine mononucleotide/dinucleotide (FMN/FAD) and riboflavin. All three flavins are sufficiently available in mammalian tissues[33]. Their blue absorption spectrum (up to ~500 nm) makes them favorable for multiplexing with green to red-light sensitive tools. While deep tissue penetration is limited in the blue range, it can be achieved with two-photon activation[34, 35]. Unlike rhodopsins, BLRs are soluble proteins that control various effector protein functions[36]. Importantly, since most BLRs display residual dark activity, they should be considered as light-dependent analogue activity modulators[36], in contrast to rhodopsins that are usually not active in the absence of light (but see Karapinar *et al*., 2021[37]). Since termination of BLR signaling relies on thermal relaxation, these photoreceptors do not allow precise temporal control. Switchable control of effector domains can potentially be achieved by using near-infrared sensitive phytochromes as photoreceptors, but most phytochrome-based tools use chromophores that are not available in mammalian tissues (Phytochromobilin, Phycocyanobilin), making them less suitable for most optogenetic applications.

The BLR family is subdivided into light-oxygen-voltage (LOV) domains, blue light sensors utilizing FAD (BLUF) domains and cryptochromes (Fig. 1E-G). LOV domains are relatively small (110 amino acids) and usually bind FMN non-covalently. Typically found in phototropins of higher plants and micro-algae, the slightly fluorescent LOV domains are linked at their C-terminus to the effector domains (e.g., kinases). Photon absorption leads to a structural transition into the signaling state and activation of the effector domain. Light-induced unfolding of the C-terminal Jα-helix of the AsLOV2 domain from *Avena sativa*

was exploited to mask protein epitopes fused to Jα in the dark, which became accessible upon illumination. By fusing two bacterial binding peptides to Jα, an improved light-inducible dimer (iLID) system was designed with over 50-fold increase in dimerization during illumination[38]. This system was further optimized for applications with high effective protein concentrations at synapses[39]. iLID-based approaches allow tightly controlled heterodimerization, including the photo-activated split protein complementation system for Botulinum neurotoxin B[40], described below.

In BLUF domains (Fig. 1F), flavin chromophores are also non-covalently bound, but the activation mechanism differs from that of LOV domains. Instead of oxidation state changes and adduct formation, BLUF domain activation only causes non-covalent electronic bonding changes. Despite these subtle protein alterations, which have not yet been completely elucidated[41], BLUF domains have a long-lasting signaling state (seconds to minutes). In nature, BLUF domains are commonly found either without effectors or fused to domains that control cyclic nucleotide turnover. Among the most utilized members of the BLUF-coupled cyclases is the photoactivated adenylyl cyclase (PAC) from the bacterium *Beggiatoa* (bPAC)[42]. The >300-fold increase in its cyclase activity following illumination allows for tightly light-controlled cAMP production. Moreover, any residual dark activity can be further reduced by membrane anchors or directed mutagenesis[43].

Cryptochromes (CRY, Fig. 1G) are found in plants and animals. While highly homologous to photolyase proteins, these proteins lack the ability to interact with DNA. Although FAD is bound to the CRY N-terminal photolyase homology region (PHR), cryptochromes can still harbor secondary antenna chromophores. Contrary to photolyases, there is no consensus to date about the activation mechanism of CRY[44]. However, CRY can form light-induced homooligomers via their PHRs or heterodimers via CRY-interacting basic-helix–loop–helix proteins (CIBs)[45]. Therefore, CRY:CRY or CRY:CIB oligomerization has been used for various dimerization, oligomerization, clustering, or colocalization approaches when fused to different proteins of interest.

For a deeper mechanistic and application overview of BLRs, the reader is referred to a detailed review by Losi *et al*.[36] and the BLR database optobase.org[46].

Photosensitizers

Genetically encoded photosensitizers (Fig. 1H,I) are derived either from GFP or the LOV2 domain of *Arabidopsis thaliana* phototropin 2[47]. In response to illumination they generate reactive oxygen species (ROS) rather than fluorescence[48]. Singlet oxygen ($^1O_2$) oxidizes cysteine-, histidine-, methionine-, tryptophan- and tyrosine side chains, thereby disrupting protein function in a range of 20 - 150 nm[49]. Thus, photosensitizers fused to a protein enable spatiotemporally precise chromophore-assisted light inactivation (CALI) *in situ*. GFP and related fluorophores generate ROS upon bleaching, although at a low quantum yield. The first specifically engineered, genetically encoded photosensitizer was the dimeric

KillerRed, which showed significantly improved ROS production compared to EGFP[50]. Mutagenesis of KillerRed yielded the spectrally shifted KillerOrange[51] and the monomeric photosensitizers SuperNova and SuperNova green[52, 53], which are more suitable for fusion with target proteins and allow differential CALI with spectrally distinct illumination. MiniSOG (mini Singlet Oxygen Generator) is a modestly fluorescent flavoprotein engineered from phototropin 2[54], which under blue light efficiently generates ROS. Originally developed as a probe for correlative light and electron microscopy, miniSOG and its improved variants are versatile tools for CALI applications due to their small size[49, 55]. Still, photosensitizers are difficult to use compared to other optogenetic tools, because it is necessary to carefully evaluate the degree of specific protein inactivation relative to nonspecific tissue damage.

Applying optogenetic tools requires consideration of both the biophysical characteristics of the actuator and the specific physiology of the cells. Important biophysical properties of optogenetic tools include operational light sensitivity, activation spectrum, dark activity, and kinetics. Even slight variations of these properties can have considerable consequences for complex behavioral readouts[56]. Optogenetic actuators are increasingly combined with fluorescent sensors to monitor various physiological processes, and sensor properties must therefore be considered in parallel. For a detailed description of optogenetic sensors for interrogating synaptic signaling, the reader is referred to recent reviews[57-61] and the fluorescent biosensor database (https://biosensordb.ucsd.edu)[62]. The effects of optogenetic interrogations on the cellular physiology and network activity also depend on the localization of the tool within the cell and the specific physiology within this subcellular compartment. In this regard, the axon has proven particularly challenging due to its length, specific protein targeting, and unique physiology.

Targeting optogenetic actuators to the axon and presynapse

Most optogenetic tools originate from phylogenetically distant species and lack the trafficking signals used by mammalian cells. This often leads to ineffective membrane localization, intracellular aggregation, and cell toxicity at high expression levels[63]. Addition of ER export and Golgi trafficking signals from the potassium channel Kir2.1 enhances somatic and also axonal surface expression of rhodopsins[15, 64]. Still, expression-enhanced opsins accumulate only passively in the axon, and it was therefore suggested to allow more than one month for sufficient expression in long-range projections[65]. Presynaptic proteins are synthesized in the soma and trafficked over considerable time and distance[66]. Different transport mechanisms exist for synaptic vesicle proteins, active zone components and presynaptic membrane proteins[66], which may be 'hijacked' to deliver optogenetic tools to axon terminals. Synaptophysin, the most abundant protein on both glutamate-containing and GABA-containing vesicles is particularly useful for targeting optogenetic actuators and fluorescent proteins to synaptic vesicles[67, 68]. Proteins fused to synaptophysin are either

located in the presynaptic cytosol or face the synaptic vesicle lumen (Fig. 2C). Importantly, overexpressing synaptophysin seems not to affect synaptic transmission in rodent neurons[67, 69].

Efficient targeting of optogenetic tools to the axonal plasma membrane has proven more difficult. Axonal membrane proteins have to pass the axon initial segment at the boundary of the somatodendritic and the axonal compartment[70]. No general signal sequence for axonal trafficking or presynaptic anchoring has been identified for membrane proteins. Nonetheless, two mechanisms are known to facilitate axonal localization: Unidirectional membrane insertion followed by preferential endocytosis in the somatodendritic compartment (Fig. 2A)[71], and axon-directed transport via specialized vesicle carriers (Fig. 2B)[66]. Dendritic endocytosis is mediated at least in part by Myosin VI followed by anterograde axonal transport, a mechanism referred to as transcytosis[72]. These findings inspired strategies for axonal localization of membrane-spanning optogenetic actuators. Fusion of the ChR2 C-terminus to a myosin VI-binding domain was shown to increase ChR2-YFP expression in axons and decrease its expression in dendrites[73]. Another method utilized an intracellular neurexin 1α tag previously established for presynaptic targeting of hM4D[74] to facilitate axonal expression of ChR2 and ArchT in songbirds[75, 76], but this strategy was never benchmarked against non-targeted constructs. Hamada *et al*. recently combined the presynaptic mGluR2 targeting sequence for specific axonal enrichment with a proteolytic motif for reducing somatodendritic expression[77]. The resulting ChR2-mGluR2-PA showed stronger expression in long-range projections and reduced light-evoked responses in the soma. However, it remains unclear whether axonal targeting motifs significantly improve the performance of optogenetic tools at presynaptic terminals. Further work is required to systematically evaluate presynaptic targeting strategies of optogenetic actuators.

Presynaptic applications of optogenetic tools

Light-evoked neurotransmitter release

Photostimulation of CCR-expressing axons evokes synaptic transmission even when axons are severed from the somata in acute slice preparations[78]. For 'Channelrhodopsin-assisted circuit mapping' (CRACM), CCRs are expressed in one brain region, and functional connectivity is later assessed by local illumination in the target region. Mechanistically, the photocurrent depolarizes the axonal membrane and elicits an AP. At the terminals the AP causes Ca^{2+} influx via voltage-gated channels, which triggers neurotransmitter release (Fig. 3A). Light-evoked postsynaptic currents confirm that functional synapses exist between the CCR-expressing and the postsynaptic neuron population. CRACM is applied widely to study the connectivity of genetically defined neurons, offering a technically easier and higher throughput alternative to paired recordings or selective presynaptic electrical stimulation[79,

[80]. In combination with input cell-specific genetic knockout or knockdown strategies, CRACM is also useful for studying the effects of protein loss on transmitter release[81, 82]. However, light-evoked APs propagate not only towards the synapse, but also antidromically to the soma (Fig. 3D), inducing transmitter release from collaterals, which complicates the interpretation of *in vivo* CRACM experiments[65, 83]. In slices, CRACM can be refined by abolishing spiking using Na^+-channel blockers (Fig. 3B), while enhancing the light-driven depolarization and transmitter release by blocking K^+-channels[79] (Fig. 3C). However, application of such drugs is not feasible in living animals. Therefore, control experiments have been proposed for meaningful behavioral experiments involving CCRs, for example, blocking antidromic APs at the soma by local injection of lidocaine[84], or specific blockage of postsynaptic receptors in the target region as internal control[85].

Compared to electrical stimulation, photostimulation of presynaptic terminals induces vesicle fusion with a higher probability, resulting in robust postsynaptic responses. Several reasons may account for this: AP broadening due to long-lasting depolarization by ChR2[86] may increase presynaptic Ca^{2+}-influx and thus increase transmitter release[87, 88]. In addition, transmitter release is positively modulated by subthreshold voltage deflection that can travel down the axon into the presynapse[89], so the photocurrent might directly increase release probability. The elevated release probability resulting from direct illumination of CCR-expressing terminals can be advantageous for determining functional connectivity[80, 86, 90]. On the other hand, it can result in synaptic depression during high-frequency firing by depletion of the readily releasable pool[91]. Jackman *et al.* found that direct photostimulation of hippocampal Schaffer collateral synapses in CA1 caused atypical synaptic depression[80] (Fig. 3E). Direct illumination of presynaptic boutons should therefore be avoided when studying short-term plasticity. The same study[80] also described an effect of the AAV serotype on release properties. While short-term plasticity was similar for electrical and optical stimulation when ChR2 was expressed transgenically or via AAV9, expression via AAV1, 5 or 8 resulted in artificial synaptic depression. Notably, repetitive optical stimulation can induce long-term depression, which has been used experimentally to silence long-range projections[92-94]. In other synapses, high-frequency optical stimulation can lead to long-term potentiation, for example at cortico-striatal synapses[95].

Taken together, optical excitation of axons has proven highly valuable for identifying functional long-range connections between distant circuits, but shows protocol-, pathway- and cell type-specific effects. Thus, carefully designed controls are required when it is used to study synaptic plasticity or the functional interaction of distant brain areas *in vivo*.

Optogenetic inhibition of neurotransmitter release

Optogenetic inhibition of neurotransmitter release is an important complementary approach to optogenetic excitation, because it circumvents issues arising from uncontrolled

antidromic APs *in vivo*. Three experimental strategies have emerged for presynaptic optogenetic inhibition (Fig. 4): i) hyperpolarization of axon terminals to inhibit AP propagation and decrease presynaptic Ca^{2+} influx, ii) inhibition of transmitter release by GPCRs, and iii) destruction of the release machinery. Importantly, since effective optogenetic inhibition of transmission is more difficult to confirm than optogenetic excitation, tool expression and performance should be carefully validated in every experimental setting (see Box 1 and Fig. 5).

Optogenetic inhibition of axons has been achieved with two different classes of electrogenic pumps (Figs. 1B, 4A): the outward-directed proton pumps Arch3[96], ArchT[97], and Jaws[98], and the inward directed chloride pump halorhodopsin (NpHR)[99]. Presynaptic inhibition by light-driven pumps has been applied for suppressing propagation of spontaneous network oscillations between brain regions[100, 101] and for *in vivo* silencing of specific synaptic connections to study their role in behavior[84, 102-105]. Pioneering work has demonstrated that continuous activation of these pumps in terminals reduces spontaneous and electrically-evoked synaptic release but does not affect spiking at the non-illuminated somata[84, 103-105]. The reduction of transmission occurs instantaneously and disappears within seconds after termination of illumination (Figs. 4D, 5B). However, while rapid silencing is ideal for closed-loop experiments, minute-long ion pumping may cause unintended effects: First, prolonged activity of H^+-pumps significantly changes intra- and extracellular pH[106], and Cl^--pump activity leads to a depolarizing shift in the reversal potential of $GABA_A$-mediated currents[20]. Such effects exacerbate in small compartments with a large surface-to-volume ratio[107]. In axon terminals, sustained activation of ArchT rapidly increased cytosolic pH to 8[108]. Preventing alkalinization abolished synaptic silencing, indicating that proton pumps suppress evoked release in terminals primarily by intracellular alkalinization and not by hyperpolarization. Second, cytosolic alkalinization triggers Ca^{2+} influx, which dramatically increases the frequency of spontaneous transmitter release[19, 105]. Such aberrant spontaneous release may activate local interneurons, causing undesired effects on network activity[105]. Third, the silencing efficacy of NpHR decreases from ~50% during the first seconds to ~20% within one minute, and rebound spiking after a step-like termination of light (Fig. 5B)[19]. Considering these effects, it seems advisable to minimize pump activation to short intervals of <1 min, and to apply gradual light off-ramps[19]. The strong Cl^--mediated hyperpolarization in dendrites and somata by ACRs rendered them a reasonable alternative for axonal silencing by shunting. However, due to the elevated intracellular chloride concentration in axons of mature neurons[109-111], ACRs actually depolarize axons and trigger transmitter release[15, 16]. Inhibition of transmitter release by light-gated potassium channels[112, 113] has not been successfully demonstrated, probably due to insufficient axonal trafficking. The effects of the recently-described K^+-ChRs in axons have not been described so far[114].

Presynaptic $G\alpha_{i/o}$-coupled receptors are native inhibitors of synaptic release. They inhibit release via the βγ subunits of the heterotrimeric $G_{i/o}$-protein, primarily by lowering the opening probability of voltage-gated calcium channels (VGCCs)[115-117]. Because of the non-linear dependence of vesicle fusion on presynaptic Ca^{2+}, a modest decrease of Ca^{2+}-influx significantly reduces neurotransmitter release[118, 119]. In addition, βγ subunits can inhibit release by directly interfering with the release machinery[120]. Many of the type II rhodopsins couple to $G\alpha_{i/o}$ or the related transducin ($G\alpha_t$), suggesting that ectopically expressed light-sensitive GPCRs in neurons might confer light-gated presynaptic inhibition, provided that these rhodopsins efficiently traffic to axon terminals (Fig. 4B). As early as 2005, Stefan Herlitze's group demonstrated light-triggered reduction of release by rat rhodopsin[121]. This approach, however, has not been widely adopted, probably due to the strong photobleaching of visual rhodopsins in neurons[23]. Bistable type-II GPCR opsins (optoGPCRs) from both vertebrates and invertebrates can be resistant to photobleaching[24]. Currently, the best-established optoGPCRs for presynaptic inhibition are lamprey parapinopsin (*Lc*PPO)[122] and mosquito OPN3[123]. In neurons, *Lc*PPO and a surface trafficking-enhanced version of mosquito OPN3 (eOPN3) inhibit presynaptic Ca^{2+} influx via VGCCs, which suppresses neurotransmitter release[124, 125]. In line with their action at the presynaptic terminal, activation of these opsins increases the paired-pulse ratio of evoked postsynaptic currents, and decreases frequency, but not amplitude of spontaneously occurring release events. *Lc*PPO and eOPN3 also activate G-protein-coupled inward rectifying K^+ (GIRK) channels, which hyperpolarizes the soma and therefore reduces AP firing[125-127]. $G\alpha_{i/o}$-mediated signaling of *Lc*PPO and eOPN3 was confirmed by the sensitivity of their effects to the $G\alpha_{i/o}$ blocker pertussis toxin. Both *Lc*PPO and eOPN3 were shown to silence transmitter release *in vivo*, demonstrating the potential of presynaptic optoGPCRs for studying the behavioral role of specific long-range projections. *In vivo* expression of eOPN3 in nigrostriatal dopaminergic projections (Fig. 5A) and their light-induced unilateral inhibition caused ipsiversive rotational locomotion of mice[125]. In head-fixed awake mice, activation of eOPN3 inhibited release from visual thalamocortical inputs, which suppressed V1 unit activity in response to visual stimulation (Fig. 5C)[125]. *Lc*PPO effectively suppressed GABAergic inputs to the lateral hypothalamus, causing disinhibition and enhanced unit activity[124]. Remarkably, *Lc*PPO and eOPN3 have very different spectral properties. *Lc*PPO is a switchable opsin that is efficiently activated by light <420 nm and inactivated by wavelength ranging from 450 - 600 nm[126], although activation with 488 nm was also reported[124]. In contrast, eOPN3 is a non-switchable opsin. It is activated by light of the entire visible range (400 - 630 nm), and inactivates spontaneously within minutes[123, 125]. Due to the intrinsic amplification via G-proteins, light activation of both opsins is extremely effective, with an EC_{50} of 2 to 3 µW mm^{-2} s^{-1}[125, 127], an irradiance approx. 3 orders of magnitude lower than that required for inhibition with NpHR or Arch[9, 96, 128]. Due to its high light sensitivity over a broad wavelength range, eOPN3 can efficiently inhibit synaptic

release even with longer wavelength and low intensity light, allowing manipulation of larger tissue volumes, while avoiding tissue heating. eOPN3 and *Lc*PPO also differ with respect to their activation by two-photon (2P) illumination. *Lc*PPO is reliably activated by 2P illumination at 700 nm, which decreases to <20% at 1000 nm. While potentially allowing effective 2P-induced presynaptic inhibition by *Lc*PPO, cross-activation or inactivation of *Lc*PPO will be difficult to avoid in multiphoton imaging experiments using blue or green indicators. In contrast, eOPN3 is mostly insensitive to 2P activation at 920 nm, allowing crosstalk-free 2P Ca^{2+}-imaging experiments in parallel.

Optogenetic tools that destroy the release machinery can be used to chronically suppress release for hours and days (Fig. 4C,D). The first tool based on this approach was 'Inhibition of Synapses with CALI' (InSynC). Light activation of the photosensitizer miniSOG (Fig. 1H) attached to the cytosolic ends of synaptic vesicles produces reactive oxygen species (ROS)[129]. ROS generated under constant illumination of miniSOG oxidize synaptic proteins, impairing AP-evoked release within minutes in neuronal cultures, while also increasing spontaneous release. At neuromuscular junctions of *C. elegans*, InSynC activation over tens of seconds to minutes caused paralysis of the worms, and locomotion recovered after 24 hours. Importantly, expression of the construct alone had no adverse effects on synaptic release. Proteolytic cleavage of SNARE proteins by botulinum or tetanus neurotoxins efficiently eliminates synaptic transmission. Based on the *As*LOV2-derived iLID dimerization system[38], Liu *et al.* developed a vesicle-attached, photocontrolled split protein complementation system for Botulinum neurotoxin B (vPA-BoNT), which specifically cleaves VAMP2, a core SNARE protein[40] (Fig. 4C). Optimizing for a low affinity of the split BonT-B and the iLID components reduced dark activity of vPA-BoNT. While VAMP2 levels in vPA-BoNT-expressing cultures were still reduced by ~25%, this reduction did not impair baseline synaptic function. Photoactivation of vPA-BoNT lowered spontaneous miniature EPSC frequency in dissociated cultures, reduced evoked transmitter release in acute mouse brain slices by 50%, and decreased swimming frequency of transgenic *C. elegans* by 50%. Due to the size of its genetically-expressed components, the vPA-BoNT system requires co-application of two AAVs.

The long-lasting disruption of neurotransmission by InSynC or vPA-BoNT comes at the cost of poor temporal resolution, with onset taking minutes and recovery dependent on protein synthesis (Fig. 4D). Repeated silencing with InSynC or vPA-BoNT is only feasible on very long timescales, and the consequences of the destruction of presynaptic components are unclear. ROS-mediated unspecific protein damage by CALI may have detrimental effects on synapse architecture and cellular viability[55, 130]. SNARE protein cleavage by botulinum neurotoxins, while more specific in comparison, was shown to induce axonal sprouting of motor neurons[131]. It remains to be investigated whether such effects occur following implementation of InSynC or vPA-BoNT. Still, photocontrolled chronic synaptic silencing may be extremely useful when investigating long-lasting effects,

such as 'synaptic engrams' of a given learning task[132]. Recently, a CRY2:CIB-based approach (Fig. 1G) for non-proteolytic optogenetic interference with synaptic release has been published[133]. The opto-vTRAP system clusters synaptic vesicles by light-induced complexation of CIBN fused to the cytosolic N-terminus of VAMP2 and cytosolic Cry2. In acute brain slice recordings from Schaffer collateral synapses in hippocampus CA1, activation of opto-vTRAP with continuous blue light reduced EPSCs by 50% within 15 min, which recovered within 30 min after light termination. Vesicle aggregation apparently decreased the number of release-competent vesicles without affecting vesicular release probability, thereby not affecting short-term plasticity as determined by the paired-pulse ratio.

The three classes of optogenetic presynaptic inhibitors operate on very different time-scales (Fig. 4D): Destructive optogenetic inhibitors require minutes of continuous illumination, but their effect is long-lasting; optogenetic pumps inhibit release within milliseconds, but they should not be activated for more than a few minutes[19]; effects of optoGPCRs unfold within a few hundred milliseconds, and last for seconds to minutes. In contrast to pumps and destructive inhibitors, optoGPCRs require very little light for their activation. Their repeated activation with short light flashes is likely to extend the effect duration, and switchable optoGPCRs such as *Lc*PPO allow control over the recovery of transmission. OptoGPCRs might be too slow for applications that require ultrafast closed-loop suppression of release, but otherwise seems ideal for most forms of presynaptic inhibition on physiological time scales. Of note, high-frequency trains of APs can affect GPCR-mediated inhibition of presynaptic Ca^{2+} influx by βγ subunits[134], which should be experimentally tested when optoGPCRs are applied (Fig. 5D and Box I). Presynaptic optogenetic inhibition offers superior spatio-temporal specificity compared to chemogenetic approaches or constitutively active, genetically-encoded toxins (Table 1). On the other hand, chemogenetic inhibition can be achieved by systemic agonist administration, and Tetanus Toxin does not require any hardware for activation. Given the variety of tools at hand, users should consider the temporal domain in which transmitter release needs to be inhibited and whether specific properties of the presynapse or the tool's properties constrain the experimental setting. Users should aim to validate the performance of their selected optogenetic tool and consider possible side effects, plasticity mechanisms and compensatory circuit rebalancing effects, and design appropriate controls. Although the ideal presynaptic inhibitor that provides reversible suppression of transmitter release at one specific synapse between two genetically-defined classes of neurons is not yet available[107], the available optogenetic tools for presynaptic inhibition are remarkably advanced and offer a wide range of functionalities to interfere with transmitter release.

Presynaptic potentiation by light

Long-lasting modifications of synaptic strength can shape the neuronal landscape underlying memory engrams. There is ample evidence that the structural and functional mechanisms of postsynaptic long-term potentiation (LTP) are associated with memory-guided behavioral performance[135]. In comparison, the role of presynaptic LTP, found at various synapses throughout the CNS, in learning and behavior output remains unclear[136]. Presynaptic LTP can be triggered by high frequency firing and is expressed as a persistent increase of transmitter release[137]. At hippocampal mossy fiber synapses, high-frequency firing increases cAMP levels, which via protein-kinase A and the guanine nucleotide exchange factor Epac2[138-140] increases vesicular release probability and activates novel release sites[141]. It is difficult to assess the effects of presynaptic LTP in living animals, since high-frequency stimulation of axons could entail unintended off-target effects. Recent work established optogenetic induction of presynaptic potentiation by directly elevating cAMP levels in axon terminals[69]. For this, the photoactivated adenylyl cyclase bPAC was attached to the cytosolic C-terminus of synaptophysin. Blue-light activation of the resulting 'synaptoPAC' triggered a rapid increase of transmission at mossy fiber synapses in CA3, which decayed with a time course similar to electrically-induced LTP. Transmission at hippocampal CA3–CA1 Schaffer collateral synapses was not enhanced by synaptoPAC activation, indicating that optogenetic induction of cAMP only potentiates transmitter release from terminals that are predisposed to undergo presynaptic plasticity. SynaptoPAC is likely to be a useful tool to mimic presynaptic plasticity at genetically defined synapses in living animals, and may help elucidate the behavioral role of presynaptic potentiation[142].

Optogenetic control of presynaptic organelles and proteins

Other than modulating synaptic vesicle exocytosis, optogenetics also allows manipulation of additional processes within the presynaptic compartment: pHoenix, a fusion construct comprised of the light-driven proton pump Arch3 and the vesicle protein synaptophysin, enables light-regulated acidification of synaptic vesicles[67]. Following pharmacological inhibition of V-ATPases and depletion of neurotransmitters from the vesicles, light-controlled vesicle re-acidification by pHoenix initiates neurotransmitter uptake. Visualization of intravesicular pH by pHluorin showed rapid acidification preceding neurotransmitter accumulation, and titration of the vesicular neurotransmitter filling state revealed preferential exocytosis of completely filled vesicles over partially filled vesicles. The pHoenix concept was further adopted for lysosomes[67], and may serve as a framework for future optogenetic manipulations of other organelles.

Optogenetic oligomerization enables studies on the effect of position and mobility of presynaptic Ca^{2+} channels and other proteins on synaptic transmission: Blue light-induced oligomerization has been demonstrated for presynaptic Ca^{2+}-channels N-terminally fused to cryptochrome 2 (CRY2olig)[143]. The reversible sequestration of channels in

nanodomain-like clusters altered presynaptic Ca^{2+} flux and glutamate release in a channel subtype-specific manner.

Finally, acute optogenetic ablation of specific proteins allows testing their role in transmission, while avoiding long-term functional compensation or toxicity as might occur in genetic knockout or knockdown models. Reactive oxygen production by presynaptically targeted photosensitizers inactivates presynaptic proteins[129]. Selective photo-inactivation of synaptic proteins was achieved with GFP-derived photosensitizers (Fig. 1I) that generate less ROS than miniSOG, leading to a smaller radius of protein inactivation. Accordingly, specific damage of synaptophysin by the photosensitizer "supernova" allowed to selectively study autophagy of synaptophysin[144]. Remarkably, synaptic transmission was only compromised when autophagy was blocked, illustrating that efficient inhibition of synapses with CALI requires pronounced damaging of the release machinery.

Experimental constraints

Optogenetics in neurons requires the introduction of foreign proteins into highly specialized cells that form a most sensitive organ. Other than considering the biophysical constraints of the actuators, researchers must control for unintended off-target effects by opsin expression and photostimulation[63]. These include overexpression artifacts[145], dark activity effects[146], off-target effects within cells[19, 105, 147] and the network[148], and effects of tissue heating following illumination[149]. Many of these side-effects can be minimized by avoiding high protein load and excessive light, both of which can cause unphysiologically strong intracellular signals. Development of improved actuators with higher light sensitivity, minimal dark activity and optimized intracellular trafficking will allow safer and more robust application of optogenetic manipulations. Additionally, future tool design will benefit from more subtle gene expression and targeting strategies, by either choosing appropriate promoters for expression of the optogenetic tools, use of knock-in mice, or utilizing CRISPR-Cas for tagging endogenous proteins with actuators[150, 151].

Discussion and outlook

The unique physiology and intricate trafficking machinery of axons have hampered optogenetic applications in presynaptic terminals for many years. However, a better understanding of the interactions between the unique intracellular environment of the axon and the biophysical properties of optogenetic actuators has led to the development of optogenetic tools specifically adapted for presynaptic applications.

The combination of several recent technological advancements, including better subcellular targeting for exclusive localization in the axon and more sophisticated gene expression systems for activity- and connectivity-dependent control of tool expression[152, 153], will improve spatial and temporal optogenetic control. However, there are still

limitations when considering specific synapses between two groups of neurons as exclusive targets for optogenetic manipulations. Current retrograde or anterograde viral expression systems allow genetic targeting of all input or target cells of a given region, but lack synapse specificity. Beyond more refined genetic targeting strategies, functional tool complementation by the pre- and postsynaptic compartment as 'transsynaptic optogenetics' would enable us to investigate synapses between a selected source population of neurons that target onto one defined target population[107]. Intersectional, synapse-specific optogenetics may therefore employ two components that are expressed separately, one on the pre- and the other on the post-synaptic compartments, and reconstitute functionally over the synaptic cleft[154].

We further expect the development of optogenetic tool pairs that offer bidirectional control of transmission, preferentially via a single vector, in analogy to the bidirectional control of somatodendritic excitability by the recently published BiPOLES[155]. The combination of inhibitory presynaptic optoGPCRs with excitatory CCRs could enable such bidirectional control of transmitter release. Such tools will allow the activation of axons via CCRs in the target region and inactivation of transmitter release locally via the optoGPCR. When continuing to optically elicit APs while locally suppressing release, one could then control for unwanted excitatory effects by back-propagating APs outside of the target region. However, this requires that the excitation spectra of the opsins are well separated and that CCR activation does not inactivate the optoGPCR.

Several optogenetic tools to manipulate other cell-biological processes exist, but these have not yet been implemented in the presynapse. Such applications include oligomerization of the clathrin light chain by CRY2olig to impair endocytosis[156], repositioning of organelles by transient light-controlled attachment to motor proteins[157], and intracellular phase transitions of protein domains containing intrinsically disordered protein regions by a CRY2-based optoDroplet system[158]. Applying these tools at the presynapse may require further optimizations, but these examples illustrate the feasibility and the potential power of such novel approaches.

In the future, presynaptic optogenetics will likely be combined with other methods covering the entire spectrum of modern neuroscience, from ultrastructural analysis using electron microscopy and super-resolution microscopy to *in vivo* large-scale network readouts using high-density electrode arrays, live cell imaging, and complex behavioral analysis. In some experimental settings, chemogenetic tools can provide a valid alternative or complementary addition to optogenetics, especially when targeting widely dispersed presynaptic terminals and when the temporal precision is not a limiting factor. However, chemogenetics does not cover the range of potential manipulations provided by optogenetics (Table 1) and has other system-inherent limitations. Genetically-encoded ultrasound activated tools[159] may emerge as a possible addition to the toolbox, but their development does not allow routine implementation at this stage. In summary, presynaptic

optogenetics offers neuroscientists diverse experimental approaches at unsurpassed spatio-temporal resolution, and the ongoing developments are likely to provide exciting new insights in the future.

Figure Legends

Figure 1: Overview on optogenetic actuators. Left: Chromophore photoreaction. Middle: schematized architecture and function. Right: selected absorption or activation spectra scaled to maximum chromophore absorption or actuator response (from top to bottom): Chrimson[160], TsChR[161], A1ACR1[162], GtACR2[14], NsXeR[163], KR2[164], NpHR[165], Arch3.0[166], CaRhGC[22], neoR[167], CrHKR1[168], mosOPN3[123], LcPPO[169], LOV (PtAu1a)[170], BLUF (RsBlrB)[171], AtCRY2[172], SuperNova Green[53], miniSOG[54], and KillerRed[173]. Abbreviations used: AC (adenylyl cyclase), ACR (anion-conducting ChR), ATP (adenosine triphosphate), BLUF (blue-light using FAD), bPAC (bacterial photoactivated adenylyl cyclase), cAMP (cyclic adenosine monophosphate), CCR (cation-conducting ChR), cGMP (cyclic guanosine monophosphate), CIB (cryptochrome-interacting basic-helix-loop-helix protein), CRY (cryptochrome), FAD (flavin adenine dinucleotide), FMN (flavin mononucleotide), FP (fluorescent protein), GC (guanylyl cyclase), GTP (guanosine triphosphate), HKR-GC (histidine kinase rhodopsin coupled guanylyl cyclase), iLID (improved light-induced dimer), LOV (light-oxygen-voltage-sensing (domain)), miniSOG (miniature singlet oxygen generator), P (phosphorylation site), PDE (phosphodiesterase), POI (protein of interest), RhG/AC (rhodopsin gyanyly/adenylyl cyclase), RhoPDE (rhodopsin phosphodiesterase), and SspB/SSRA (binding element partner A/B (from *E. coli*)).

Figure 2: Axonal transport and presynaptic targeting of optogenetic actuators. The axon initial segment (AIS) demarcates the boundary of the axon and the somatodendritic compartment and acts as a gatekeeper by repelling cargo vesicles containing proteins for the somatodendritic compartment. Major mechanisms to enrich proteins in the axon are: A, Preferential endocytosis from the dendritic membrane, illustrated here for an opsin tagged with the neurexin 1α (Nrx1α) C-terminus. B, Directed anterograde axonal transport of cargo vesicles along microtubules by kinesins. Such cargo vesicles include synaptic vesicle precursors (SVP), which carry vesicular proteins such as synaptophysin, vesicular neurotransmitter transporters, and associated active zone components. SVPs also transport axon-targeted proteins that have been endocytosed from dendritic membranes. Note that dendritic endocytosis and fast axonal anterograde transport by KIF1A has been demonstrated for Nrx1α[72, 174], but not specifically for opsins fused to the Nrx1α C-terminus. Calcium channels are transported in separate vesicles (CaV), probably by different kinesin isoforms. Membrane proteins can also diffuse passively along the axon, whereby opsins lacking a specific axonal targeting sequence can passively reach axonal terminals over longer periods of time. C, Synaptic vesicle targeting of actuators by fusion to synaptophysin allows for manipulating the intravesicular H^+ concentration by proton pumps or for increasing cAMP in the presynaptic cytosol by photoactivated adenylyl cyclases.

Figure 3: Concept and pitfalls of light-evoked neurotransmitter release by CCR stimulation.

A, Light-triggered currents activate voltage-gated Na^+ channels (Na_v), which amplifies depolarization and opens voltage-gated Ca^{2+} channels (VGCCs) at the terminal. Thereby CCR-driven depolarization elicits transmitter release from the presynaptic bouton, but also APs that back-propagate towards the soma. B, Probing synaptic connectivity in acute slice preparations allows blocking Na_v by tetrodotoxin (TTX), which abolishes the antidromic AP

(left). However the depolarization provided by CCRs can be insufficient to activate VGCCs due to shunting by voltage-gated K^+ channels (K_v). C, Additional application of 4-aminopyridine (4-AP) to block K_v then ensures sufficient light-triggered depolarization for activation of VGCCs. D, Local illumination in one brain region can elicit antidromic APs and transmitter release in other brain regions due to divergent axonal arborizations, exemplified here for noradrenergic projections originating from the locus coeruleus (LC), with photostimulation in the amygdala. E, Over-bouton illumination increases synaptic release probability compared to over-axon illumination or electrical stimulation, which modifies the short-term plasticity of transmission. Synaptic responses drawn according to Jackman *et al.*, 2014[80], and scaled to the first EPSP amplitude.

Figure 4: Different principles of optogenetic inhibition of transmitter release.
A, Optogenetic hyperpolarization (shown here for the chloride pump NpHR) reduces the open probability of VGCCs after an AP, which reduces transmitter release. B, Light-activated GPCRs (optoGPCRs) can effectively inhibit transmitter release by activating heterotrimeric G-proteins containing the $G\alpha_{i/o}$ subunit. The membrane-anchored βγ subunits physically interact with presynaptic P/Q or N-type VGCCs and reduce their open probability. In addition, βγ subunits can directly interfere with SNARE-mediated vesicle fusion. C, Permanent inactivation of the synaptic release machinery by vesicle-bound photoactivated botulinum toxin B (vPA-BoNT). *left:* Two fragments of BoNT-B are fused to the iLID photodimerizer subunits, with one part bound to the synaptic vesicle protein synaptophysin (syp) and the second part present separately in the cytosol. In the dark, the toxin remains inactive, and binding of Ca^{2+} to synaptotagmin (syt) triggers SNARE-mediated fusion of synaptic vesicles. *right:* Light-induced association of ILID components recruits the cytosolic component of vPA-BoNT to the synaptic vesicle. The reconstituted toxin cleaves the SNARE protein VAMP2, which abolishes vesicle fusion. D, Time-course and effect size of the different kinds of presynaptic optogenetic inhibition, plotted on a logarithmic time scale. Underlying parameters for a simple kinetic model are provided in the table. Blue bars indicate illumination (0.5 s for Arch/NpHR, mOPN3 and parapinopsin (*Lc*PPO), 5 min for InSync/vPA-BoNT). Green bar indicates inactivating light flash for *Lc*PPO.

Figure 5: Validation of optogenetic presynaptic inhibition.
A, Histological verification of optogenetic actuator expression following *in vivo* experiments. The figure shows coronal sections of a mouse brain in which eOPN3-mScarlet was unilaterally expressed in substantia nigra pars compacta (SNc) by AAV injection. Fluorescence is visible both in the somata in the SNc (left) and the fibers in the striatum (STR, right), the target region of the dopaminergic projections (unpublished, Yizhar laboratory). B, EPSC recordings in acute brain slices allow quantifying the optogenetic suppression of transmitter release. Shown here is the inhibitory effect of NpHR at thalamocortical synapses. Paired pulse ratio of evoked EPSC amplitudes is increased by presynaptic inhibition. Note the rebound spike after light termination (arrow) and the reduced silencing efficiency under continuous illumination compared to the instant effect of a 200 ms light flash[19]. C, *In vivo* recordings of unit activity can reveal successful optogenetic inhibition of a major excitatory input. eOPN3 was expressed in the lateral geniculate nucleus (LGN) that projects to V1. Units were recorded in V1, while animals were presented with a drifting grid pattern. Activation of eOPN3 in V1 strongly reduced unit

716 activity in response to the moving grid[125]. D, Example of increased short-term facilitation
717 during tonic presynaptic inhibition. Schaffer collaterals were stimulated with 10 pulses at
718 25 Hz before (black) and after (green) eOPN3 activation. Transmission was constantly
719 reduced by eOPN3, but normalized EPSC amplitudes showed increased facilitation relative
720 to the first amplitude in presence of presynaptic inhibition[125].
721

722 ***Table1: Methods for manipulating transmitter release from axon terminals***

	Optogenetics	Chemogenetics	Constitutively active, genetically-encoded toxins
Principle	Photostimulation of opsins	Pharmacological activation of designer receptors	Neurotoxin expression
Stimulation of transmitter release at terminals	CCR-mediated depolarization of axons and terminals.	Local terminal stimulation not possible, but DREADD-driven APs induced by somatic depolarization (e.g. CNO: hM3D; Varenicline: PSAM^4-5HT3)	N/A
Inhibition of transmitter release at terminals	Illumination of terminals expressing inhibitory opsins reduces transmission by hyperpolarization (Arch/ NpHR), GPCR-mediated inhibition (mOPN3/ *Lc*PPO), destruction of SNAREs (InSync/ vPA-BoNT), or repositioning of vesicles (opto-vTRAP).	Local agonist application (CNO: hM4D[175]; salvinorin B: KORD[176]) at terminals expressing inhibitory DREADDs triggers GPCR-mediated presynaptic inhibition	Tetanus Toxin or Botulinum Neurotoxin-mediated cleavage of SNARE proteins abolishes neurotransmitter release at all terminals of the expressing neuron
Other manipulations of presynaptic function	SynaptoPAC: increase of intraterminal cAMP. Cry2-Cav2.1 Clustering of presynaptic Ca^{2+} channels. pHoenix: acidification of synaptic vesicles.	N/A	N/A
Experimental requirements for *in vivo* applications	Injection of virus or breeding; implantation of light guide; transcranial photostimulation (rare)	Injection of virus or breeding; local drug perfusion or systemic drug application	Injection of virus or breeding
Multiplexing (excitation + inhibition; targeting different axon populations)	Possible, but limited due to spectral overlap	Possible when combining different DREADD systems (e.g. hM3D and KORD[176])	Not shown

On kinetics	ms - min	s - min	days (expression of toxin)
Inactivation and off kinetics	Opsin inactivation after light-off (ms - s) and recovery of terminals (ms - days)	Drug washout (min - h) and receptor inactivation (s-min)	Protein turnover in inducible systems (days)
Highlights	High temporal precision for activation; presynaptic inhibition on very different temporal scales (s-h)	Hardware free with systemic drug delivery (but this lacks synapse specificity). Efficient presynaptic silencing (h)	Hardware free. Highly efficient for chronic silencing
Limitations	Bleaching of opsins; tissue heating and photo-damage; homeostatic adaptation	Receptor desensitization; homeostatic adaptation	No temporal control, no synapse specificity
Examples for transgenic mouse lines (Cre- or Tet-conditional, publicly available lines only)	Cre-conditional NpHR (Ai39 / Jax: 014539); Cre+tet-conditional Jaws (Ai79D / Jax: 023529); Cre-conditional Arch3 (Ai35D / Jax: 012735); Cre-conditional ArchT (Ai40D / Jax: 021188)	Tet-conditional hM4D (Jax: 024114); Cre-conditional hM4D (Jax: 029040)	Tet-conditional Tetanus toxin (Jax: 010713 & 023757)

723

724

BOX 1: Validating presynaptic optogenetic inhibition

The performance of optogenetic tools at axon terminals depends on various parameters, including expression levels (determined by vector design and the virus preparation), method of light delivery, and physiological characteristics of the particular synapse under investigation. It is therefore important to validate the tool's function in the targeted projection pathway, especially when it is applied to manipulate transmitter release *in vivo*. Whereas the consequences of CCR activation at excitatory synapses are relatively easy to detect (e.g. by recording postsynaptic activity), it is typically more challenging to assess the efficacy of presynaptic optogenetic silencing. Indeed, in early 2022, we conducted an online survey in which 51% of 161 participants reported failed or inconclusive experiments with presynaptic optogenetic inhibition (see Supplementary Information).

Here we propose control experiments for validating the performance of optogenetic tools at presynaptic terminals. We focus on presynaptic optogenetic silencing, but such controls should also be applied for other optogenetic, chemogenetic or constitutively active, genetically encoded tools expressed for manipulating synaptic activity. Performing all control experiments is in most cases beyond the experimental scope, but we recommend verifying the anatomically correct expression of the tools in every single animal used for behavior or *in vivo* recordings (I), and performing at least one experiments confirming the tool's functionality (II). In addition, the interplay of short-term plasticity and presynaptic inhibition should be considered (III).

Confirming expression:

Expression of the optogenetic actuator at the axonal terminals can be validated by fluorescence microscopy, either by detecting signals from a fluorophore fused to the optogenetic actuator or by immunofluorescence staining. Fluorescence readouts at the somata can be combined with antibody staining for cell-type specific markers, while fluorescence in the target region confirms axonal expression (Fig. 5A).

Confirming presynaptic inhibition:

Postsynaptic recordings of synaptic transmission: Presynaptic inhibition can be assessed by monitoring the effect on evoked or spontaneous transmitter release. While challenging *in vivo*, this might be performed *ex vivo* with similar illumination time and intensity, to investigate the effect under comparable conditions. In some cases, neurotransmitter release might be visualized by combining optogenetic stimulation with imaging of genetically encoded fluorescent transmitter probes*, but in most cases, transmission will be determined by electrophysiological recordings of postsynaptic currents. Such recordings revealed the rapid inhibitory effect of NpHR on synaptic transmission (Fig. 5B)[19].

Imaging presynaptic Ca^{2+} transients: Activation of optoGPCRs has been shown to partially suppress presynaptic Ca^{2+} influx. Therefore, functional inhibition might be analyzed by *ex*

vivo or *in vivo* Ca^{2+} imaging at axon terminals*. One should keep in mind that a mild suppression of presynaptic Ca^{2+} influx is sufficient for a strong reduction of transmitter release, due to the non-linear dependency of exocytosis (power law exponent typically in the range of 2-5) on the intracellular Ca^{2+} concentration.

Determining the effect on postsynaptic network activity: In vivo electrophysiological recordings of postsynaptic unit activity or network oscillations, or imaging of neuronal activity* can reveal changes in the strength of synaptic inputs during optogenetic manipulation of incoming axons. Due to a high variability in network activity, such recordings require multiple recording trials of the activity before and after light, and should be supported by control recordings in the absence of optogenetic actuator activation (Fig. 5C).

*CAVE: avoid cross-activation of fluorescent probes and optogenetic tools.

Presynaptic modulation and short-term plasticity

GPCRs that couple to $G\alpha_{i/o}$ exert multiple effects at presynaptic terminals, namely inhibition of Ca^{2+} influx, reduction of cAMP levels, and interference with the SNARE machinery. Reducing presynaptic Ca^{2+} influx by GPCR signaling might not just simply lower synaptic gain, but can introduce a high-pass filter on transmission[5]. In this scenario, transmitter release is efficiently blocked by tonic GPCR activity during sparse firing. However, when neurons fire at high frequencies, intracellular Ca^{2+} transiently accumulates. Moreover, strong membrane depolarization during high-frequency AP bursts can relieve βγ-subunit-mediated inhibition of Ca^{2+} channels[134]. Even if presynaptic inhibition greatly reduces initial transmitter release in an AP burst, these effects may transiently elevate transmission (relative to the initial AP) over the course of the AP train and shift the short-term dynamics of transmission toward facilitation[5]. This may pose a problem if tonic synaptic silencing is required for neurons firing at very high frequencies, as charge transfer may not be reduced effectively during bursts of activity *in vivo*. Indeed synaptic facilitation was observed after eOPN3-mediated inhibition of transmitter release[125], however, absolute EPSC amplitudes evoked by 25 Hz stimulation under light were always significantly smaller than under control conditions (Fig. 5D). Similarly, activation of NpHR increased the paired-pulse ratio of two EPSCs at thalamocortical fibers[19] (Fig. 5B). It should be noted that such mechanisms are synapse- and tool-specific. Presynaptic GPCRs can also reduce the synaptic gain by mechanisms not affecting the vesicular release probability and without altering short-term plasticity[177]. Thus, it is recommended to test the performance of presynaptic inhibitory tools within the physiological range of spike frequencies of the targeted neurons. Alternatively, inhibition by vesicle trapping and SNARE protein-cleavage was shown not to alter paired-pulse behavior as a measure of short-term plasticity[40, 133], and might therefore present an alternative if high temporal resolution is not required.

Declaration of interest:

The authors declare no competing financial interest.

Acknowledgements:

We are grateful to the many participants in our online survey on presynaptic silencing tools. We thank Inbar Saraf-Sinik, Yoav Livneh, Stella-Amrei Kunde, Mauro Pulin and Felicitas Brüntgens for fruitful discussions and comments on the manuscript. Figures were in part generated using BioRender.com. Studies in the authors' laboratories are supported by grants from the Deutsche Forschungsgemeinschaft (DFG; German Research Foundation) SPP 1926 (B.R.R, O.Y.), EXC-2049 – 390688087, SPP 1665, SFB 1315, SFB 958 (to D.S.), and by the European Research Commission (ERC CoG PrefrontalMap 819496 and H2020-RIA DEEPER 101016787 to O.Y., ERC SyG BrainPlay 810580 to D.S.), the Israel Science Foundation (ISF 3131/20) to O.Y.. J.W. is supported by the EMBO ALTF 378-2019 and Amos de Shalit-Minerva fellowship. O.Y. is supported by the Joseph and Wolf Lebovic Charitable Foundation Chair for Research in Neuroscience.

References:

1. Fenno, L., Yizhar, O. & Deisseroth, K. The development and application of optogenetics. *Annu Rev Neurosci* **34**, 389-412 (2011).
2. Zhou, X.X., Pan, M. & Lin, M.Z. Investigating neuronal function with optically controllable proteins. *Front Mol Neurosci* **8**, 37 (2015).
3. Kim, C.K., Adhikari, A. & Deisseroth, K. Integration of optogenetics with complementary methodologies in systems neuroscience. *Nat Rev Neurosci* **18**, 222-235 (2017).
4. Rost, B.R., Schneider-Warme, F., Schmitz, D. & Hegemann, P. Optogenetic Tools for Subcellular Applications in Neuroscience. *Neuron* **96**, 572-603 (2017).
5. Abbott, L.F. & Regehr, W.G. Synaptic computation. *Nature* **431**, 796-803 (2004).
6. Rozenberg, A., Inoue, K., Kandori, H. & Beja, O. Microbial Rhodopsins: The Last Two Decades. *Annu Rev Microbiol* **75**, 427-447 (2021).
7. Govorunova, E.G., Sineshchekov, O.A., Li, H. & Spudich, J.L. Microbial Rhodopsins: Diversity, Mechanisms, and Optogenetic Applications. *Annu Rev Biochem* **86**, 845-872 (2017).
8. Kuhne, J. *et al.* Unifying photocycle model for light adaptation and temporal evolution of cation conductance in channelrhodopsin-2. *Proc Natl Acad Sci U S A* **116**, 9380-9389 (2019).
9. Mattis, J. *et al.* Principles for applying optogenetic tools derived from direct comparative analysis of microbial opsins. *Nat Methods* **9**, 159-172 (2012).
10. Wietek, J. & Prigge, M. Enhancing Channelrhodopsins: An Overview. *Methods Mol Biol* **1408**, 141-165 (2016).
11. Vierock, J., Grimm, C., Nitzan, N. & Hegemann, P. Molecular determinants of proton selectivity and gating in the red-light activated channelrhodopsin Chrimson. *Sci Rep* **7**, 9928 (2017).
12. Wietek, J. *et al.* Conversion of channelrhodopsin into a light-gated chloride channel. *Science* **344**, 409-412 (2014).
13. Berndt, A., Lee, S.Y., Ramakrishnan, C. & Deisseroth, K. Structure-guided transformation of channelrhodopsin into a light-activated chloride channel. *Science* **344**, 420-424 (2014).
14. Govorunova, E.G., Sineshchekov, O.A., Janz, R., Liu, X. & Spudich, J.L. Natural light-gated anion channels: A family of microbial rhodopsins for advanced optogenetics. *Science* **349**, 647-650 (2015).
15. Mahn, M. *et al.* High-efficiency optogenetic silencing with soma-targeted anion-conducting channelrhodopsins. *Nat Commun* **9**, 4125 (2018).
16. Messier, J.E., Chen, H., Cai, Z.L. & Xue, M. Targeting light-gated chloride channels to neuronal somatodendritic domain reduces their excitatory effect in the axon. *Elife* **7** (2018).
17. Malyshev, A.Y. *et al.* Chloride conducting light activated channel GtACR2 can produce both cessation of firing and generation of action potentials in cortical neurons in response to light. *Neurosci Lett* **640**, 76-80 (2017).
18. Inoue, K. Diversity, Mechanism, and Optogenetic Application of Light-Driven Ion Pump Rhodopsins. *Adv Exp Med Biol* **1293**, 89-126 (2021).
19. Mahn, M., Prigge, M., Ron, S., Levy, R. & Yizhar, O. Biophysical constraints of optogenetic inhibition at presynaptic terminals. *Nat Neurosci* **19**, 554-556 (2016).

20. Raimondo, J.V., Kay, L., Ellender, T.J. & Akerman, C.J. Optogenetic silencing strategies differ in their effects on inhibitory synaptic transmission. *Nat Neurosci* **15**, 1102-1104 (2012).
21. Gao, S. *et al.* Optogenetic manipulation of cGMP in cells and animals by the tightly light-regulated guanylyl-cyclase opsin CyclOp. *Nat Commun* **6**, 8046 (2015).
22. Scheib, U. *et al.* Rhodopsin-cyclases for photocontrol of cGMP/cAMP and 2.3 A structure of the adenylyl cyclase domain. *Nat Commun* **9**, 2046 (2018).
23. Bailes, H.J., Zhuang, L.Y. & Lucas, R.J. Reproducible and sustained regulation of Galphas signalling using a metazoan opsin as an optogenetic tool. *PLoS One* **7**, e30774 (2012).
24. Koyanagi, M. & Terakita, A. Diversity of animal opsin-based pigments and their optogenetic potential. *Biochim Biophys Acta* **1837**, 710-716 (2014).
25. Yamashita, T., Terakita, A. & Shichida, Y. Distinct roles of the second and third cytoplasmic loops of bovine rhodopsin in G protein activation. *J Biol Chem* **275**, 34272-34279 (2000).
26. Kim, J.M. *et al.* Light-driven activation of beta 2-adrenergic receptor signaling by a chimeric rhodopsin containing the beta 2-adrenergic receptor cytoplasmic loops. *Biochemistry* **44**, 2284-2292 (2005).
27. Airan, R.D., Thompson, K.R., Fenno, L.E., Bernstein, H. & Deisseroth, K. Temporally precise in vivo control of intracellular signalling. *Nature* **458**, 1025-1029 (2009).
28. Masseck, O.A. *et al.* Vertebrate cone opsins enable sustained and highly sensitive rapid control of Gi/o signaling in anxiety circuitry. *Neuron* **81**, 1263-1273 (2014).
29. Tichy, A.M., Gerrard, E.J., Sexton, P.M. & Janovjak, H. Light-activated chimeric GPCRs: limitations and opportunities. *Curr Opin Struct Biol* **57**, 196-203 (2019).
30. Morri, M. *et al.* Optical functionalization of human Class A orphan G-protein-coupled receptors. *Nat Commun* **9**, 1950 (2018).
31. Tichy, A.-M., So, W.L., Gerrard, E.J. & Janovjak, H. Structure-guided optimization of light-activated chimeric G-protein coupled receptors. *bioRxiv*, 2021.2012.2013.472518 (2021).
32. Scott, K. & Zuker, C. Lights out: deactivation of the phototransduction cascade. *Trends Biochem Sci* **22**, 350-354 (1997).
33. Huhner, J., Ingles-Prieto, A., Neususs, C., Lammerhofer, M. & Janovjak, H. Quantification of riboflavin, flavin mononucleotide, and flavin adenine dinucleotide in mammalian model cells by CE with LED-induced fluorescence detection. *Electrophoresis* **36**, 518-525 (2015).
34. Homans, R.J. *et al.* Two photon spectroscopy and microscopy of the fluorescent flavoprotein, iLOV. *Phys Chem Chem Phys* **20**, 16949-16955 (2018).
35. Kennedy, M.J. *et al.* Rapid blue-light-mediated induction of protein interactions in living cells. *Nat Methods* **7**, 973-975 (2010).
36. Losi, A., Gardner, K.H. & Moglich, A. Blue-Light Receptors for Optogenetics. *Chem Rev* **118**, 10659-10709 (2018).
37. Karapinar, R. *et al.* Reverse optogenetics of G protein signaling by zebrafish non-visual opsin Opn7b for synchronization of neuronal networks. *Nat Commun* **12**, 4488 (2021).
38. Guntas, G. *et al.* Engineering an improved light-induced dimer (iLID) for controlling the localization and activity of signaling proteins. *Proc Natl Acad Sci U S A* **112**, 112-117 (2015).

907 39. Zimmerman, S.P. *et al.* Tuning the Binding Affinities and Reversion Kinetics of a Light
908 Inducible Dimer Allows Control of Transmembrane Protein Localization. *Biochemistry* **55**,
909 5264-5271 (2016).
910 40. Liu, Q. *et al.* A Photoactivatable Botulinum Neurotoxin for Inducible Control of
911 Neurotransmission. *Neuron* **101**, 863-875 e866 (2019).
912 41. Fujisawa, T. & Masuda, S. Light-induced chromophore and protein responses and
913 mechanical signal transduction of BLUF proteins. *Biophys Rev* **10**, 327-337 (2018).
914 42. Stierl, M. *et al.* Light modulation of cellular cAMP by a small bacterial photoactivated
915 adenylyl cyclase, bPAC, of the soil bacterium Beggiatoa. *J Biol Chem* **286**, 1181-1188 (2011).
916 43. Yang, S. *et al.* PACmn for improved optogenetic control of intracellular cAMP. *BMC*
917 *Biol* **19**, 227 (2021).
918 44. Wang, Q. & Lin, C. Mechanisms of Cryptochrome-Mediated Photoresponses in Plants.
919 *Annu Rev Plant Biol* **71**, 103-129 (2020).
920 45. Liu, H. *et al.* Photoexcited CRY2 interacts with CIB1 to regulate transcription and floral
921 initiation in Arabidopsis. *Science* **322**, 1535-1539 (2008).
922 46. Kolar, K., Knobloch, C., Stork, H., Znidaric, M. & Weber, W. OptoBase: A Web Platform
923 for Molecular Optogenetics. *ACS Synth Biol* **7**, 1825-1828 (2018).
924 47. Hilgers, F. *et al.* Genetically Encoded Photosensitizers as Light-Triggered Antimicrobial
925 Agents. *Int J Mol Sci* **20** (2019).
926 48. Trewin, A.J. *et al.* Light-induced oxidant production by fluorescent proteins. *Free*
927 *Radic Biol Med* **128**, 157-164 (2018).
928 49. Westberg, M., Bregnhoj, M., Etzerodt, M. & Ogilby, P.R. No Photon Wasted: An
929 Efficient and Selective Singlet Oxygen Photosensitizing Protein. *J Phys Chem B* **121**, 9366-
930 9371 (2017).
931 50. Onukwufor, J.O. *et al.* Quantification of reactive oxygen species production by the red
932 fluorescent proteins KillerRed, SuperNova and mCherry. *Free Radic Biol Med* **147**, 1-7 (2020).
933 51. Sarkisyan, K.S. *et al.* KillerOrange, a Genetically Encoded Photosensitizer Activated by
934 Blue and Green Light. *PLoS One* **10**, e0145287 (2015).
935 52. Takemoto, K. *et al.* SuperNova, a monomeric photosensitizing fluorescent protein for
936 chromophore-assisted light inactivation. *Sci Rep* **3**, 2629 (2013).
937 53. Riani, Y.D., Matsuda, T., Takemoto, K. & Nagai, T. Green monomeric photosensitizing
938 fluorescent protein for photo-inducible protein inactivation and cell ablation. *BMC Biol* **16**,
939 50 (2018).
940 54. Shu, X. *et al.* A genetically encoded tag for correlated light and electron microscopy
941 of intact cells, tissues, and organisms. *PLoS Biol* **9**, e1001041 (2011).
942 55. Makhijani, K. *et al.* Precision Optogenetic Tool for Selective Single- and Multiple-Cell
943 Ablation in a Live Animal Model System. *Cell Chem Biol* **24**, 110-119 (2017).
944 56. Baleisyte, A., Schneggenburger, R. & Kochubey, O. Optogenetic stimulation of medial
945 amygdala GABA neurons with kinetically different channelrhodopsin variants yield opposite
946 behavioral outcomes. *bioRxiv*, 2021.2006.2030.450543 (2021).
947 57. Kavalali, E.T. & Jorgensen, E.M. Visualizing presynaptic function. *Nat Neurosci* **17**, 10-
948 16 (2014).
949 58. Lin, M.Z. & Schnitzer, M.J. Genetically encoded indicators of neuronal activity. *Nat*
950 *Neurosci* **19**, 1142-1153 (2016).
951 59. Gobbo, F. & Cattaneo, A. Neuronal Activity at Synapse Resolution: Reporters and
952 Effectors for Synaptic Neuroscience. *Front Mol Neurosci* **13**, 572312 (2020).

60. Sabatini, B.L. & Tian, L. Imaging Neurotransmitter and Neuromodulator Dynamics In Vivo with Genetically Encoded Indicators. *Neuron* **108**, 17-32 (2020).
61. Shen, Y., Nasu, Y., Shkolnikov, I., Kim, A. & Campbell, R.E. Engineering genetically encoded fluorescent indicators for imaging of neuronal activity: Progress and prospects. *Neurosci Res* **152**, 3-14 (2020).
62. Greenwald, E.C., Mehta, S. & Zhang, J. Genetically Encoded Fluorescent Biosensors Illuminate the Spatiotemporal Regulation of Signaling Networks. *Chem Rev* **118**, 11707-11794 (2018).
63. Allen, B.D., Singer, A.C. & Boyden, E.S. Principles of designing interpretable optogenetic behavior experiments. *Learn Mem* **22**, 232-238 (2015).
64. Gradinaru, V. *et al.* Molecular and cellular approaches for diversifying and extending optogenetics. *Cell* **141**, 154-165 (2010).
65. Britt, J.P., McDevitt, R.A. & Bonci, A. Use of channelrhodopsin for activation of CNS neurons. *Curr Protoc Neurosci* **Chapter 2**, Unit2 16 (2012).
66. Rizalar, F.S., Roosen, D.A. & Haucke, V. A Presynaptic Perspective on Transport and Assembly Mechanisms for Synapse Formation. *Neuron* **109**, 27-41 (2021).
67. Rost, B.R. *et al.* Optogenetic acidification of synaptic vesicles and lysosomes. *Nat Neurosci* **18**, 1845-1852 (2015).
68. Granseth, B., Odermatt, B., Royle, S.J. & Lagnado, L. Clathrin-mediated endocytosis is the dominant mechanism of vesicle retrieval at hippocampal synapses. *Neuron* **51**, 773-786 (2006).
69. Oldani, S. *et al.* SynaptoPAC, an optogenetic tool for induction of presynaptic plasticity. *J Neurochem* **156**, 324-336 (2021).
70. Leterrier, C. The Axon Initial Segment: An Updated Viewpoint. *J Neurosci* **38**, 2135-2145 (2018).
71. Sampo, B., Kaech, S., Kunz, S. & Banker, G. Two distinct mechanisms target membrane proteins to the axonal surface. *Neuron* **37**, 611-624 (2003).
72. Ribeiro, L.F. *et al.* SorCS1-mediated sorting in dendrites maintains neurexin axonal surface polarization required for synaptic function. *PLoS Biol* **17**, e3000466 (2019).
73. Lewis, T.L., Jr., Mao, T. & Arnold, D.B. A role for myosin VI in the localization of axonal proteins. *PLoS Biol* **9**, e1001021 (2011).
74. Stachniak, T.J., Ghosh, A. & Sternson, S.M. Chemogenetic synaptic silencing of neural circuits localizes a hypothalamus-->midbrain pathway for feeding behavior. *Neuron* **82**, 797-808 (2014).
75. Xiao, L. *et al.* A Basal Ganglia Circuit Sufficient to Guide Birdsong Learning. *Neuron* **98**, 208-221 e205 (2018).
76. Kearney, M.G., Warren, T.L., Hisey, E., Qi, J. & Mooney, R. Discrete Evaluative and Premotor Circuits Enable Vocal Learning in Songbirds. *Neuron* **104**, 559-575 e556 (2019).
77. Hamada, S. *et al.* An engineered channelrhodopsin optimized for axon terminal activation and circuit mapping. *Commun Biol* **4**, 461 (2021).
78. Petreanu, L., Huber, D., Sobczyk, A. & Svoboda, K. Channelrhodopsin-2-assisted circuit mapping of long-range callosal projections. *Nat Neurosci* **10**, 663-668 (2007).
79. Petreanu, L., Mao, T., Sternson, S.M. & Svoboda, K. The subcellular organization of neocortical excitatory connections. *Nature* **457**, 1142-1145 (2009).
80. Jackman, S.L., Beneduce, B.M., Drew, I.R. & Regehr, W.G. Achieving high-frequency optical control of synaptic transmission. *J Neurosci* **34**, 7704-7714 (2014).

81. Barthet, G. *et al.* Presenilin-mediated cleavage of APP regulates synaptotagmin-7 and presynaptic plasticity. *Nat Commun* **9**, 4780 (2018).
82. Ben-Simon, Y. *et al.* A Combined Optogenetic-Knockdown Strategy Reveals a Major Role of Tomosyn in Mossy Fiber Synaptic Plasticity. *Cell Rep* **12**, 396-404 (2015).
83. Lee, C., Lavoie, A., Liu, J., Chen, S.X. & Liu, B.H. Light Up the Brain: The Application of Optogenetics in Cell-Type Specific Dissection of Mouse Brain Circuits. *Front Neural Circuits* **14**, 18 (2020).
84. Stuber, G.D. *et al.* Excitatory transmission from the amygdala to nucleus accumbens facilitates reward seeking. *Nature* **475**, 377-380 (2011).
85. McCall, J.G. *et al.* Locus coeruleus to basolateral amygdala noradrenergic projections promote anxiety-like behavior. *Elife* **6** (2017).
86. Zhang, Y.P. & Oertner, T.G. Optical induction of synaptic plasticity using a light-sensitive channel. *Nat Methods* **4**, 139-141 (2007).
87. Sabatini, B.L. & Regehr, W.G. Control of neurotransmitter release by presynaptic waveform at the granule cell to Purkinje cell synapse. *J Neurosci* **17**, 3425-3435 (1997).
88. Geiger, J.R. & Jonas, P. Dynamic control of presynaptic Ca(2+) inflow by fast-inactivating K(+) channels in hippocampal mossy fiber boutons. *Neuron* **28**, 927-939 (2000).
89. Alle, H. & Geiger, J.R. Combined analog and action potential coding in hippocampal mossy fibers. *Science* **311**, 1290-1293 (2006).
90. Cruikshank, S.J., Urabe, H., Nurmikko, A.V. & Connors, B.W. Pathway-specific feedforward circuits between thalamus and neocortex revealed by selective optical stimulation of axons. *Neuron* **65**, 230-245 (2010).
91. Regehr, W.G. Short-term presynaptic plasticity. *Cold Spring Harb Perspect Biol* **4**, a005702 (2012).
92. Nabavi, S. *et al.* Engineering a memory with LTD and LTP. *Nature* **511**, 348-352 (2014).
93. Creed, M., Pascoli, V.J. & Luscher, C. Addiction therapy. Refining deep brain stimulation to emulate optogenetic treatment of synaptic pathology. *Science* **347**, 659-664 (2015).
94. Klavir, O., Prigge, M., Sarel, A., Paz, R. & Yizhar, O. Manipulating fear associations via optogenetic modulation of amygdala inputs to prefrontal cortex. *Nat Neurosci* **20**, 836-844 (2017).
95. Pascoli, V., Turiault, M. & Luscher, C. Reversal of cocaine-evoked synaptic potentiation resets drug-induced adaptive behaviour. *Nature* **481**, 71-75 (2011).
96. Chow, B.Y. *et al.* High-performance genetically targetable optical neural silencing by light-driven proton pumps. *Nature* **463**, 98-102 (2010).
97. Han, X. *et al.* A high-light sensitivity optical neural silencer: development and application to optogenetic control of non-human primate cortex. *Front Syst Neurosci* **5**, 18 (2011).
98. Chuong, A.S. *et al.* Noninvasive optical inhibition with a red-shifted microbial rhodopsin. *Nat Neurosci* **17**, 1123-1129 (2014).
99. Gradinaru, V., Thompson, K.R. & Deisseroth, K. eNpHR: a Natronomonas halorhodopsin enhanced for optogenetic applications. *Brain Cell Biol* **36**, 129-139 (2008).
100. Nitzan, N. *et al.* Propagation of hippocampal ripples to the neocortex by way of a subiculum-retrosplenial pathway. *Nat Commun* **11**, 1947 (2020).
101. Yamamoto, J. & Tonegawa, S. Direct Medial Entorhinal Cortex Input to Hippocampal CA1 Is Crucial for Extended Quiet Awake Replay. *Neuron* **96**, 217-227 e214 (2017).

1045 102. Mangieri, L.R. *et al.* A neural basis for antagonistic control of feeding and compulsive
1046 behaviors. *Nat Commun* **9**, 52 (2018).
1047 103. Spellman, T. *et al.* Hippocampal-prefrontal input supports spatial encoding in working
1048 memory. *Nature* **522**, 309-314 (2015).
1049 104. Tye, K.M. *et al.* Amygdala circuitry mediating reversible and bidirectional control of
1050 anxiety. *Nature* **471**, 358-362 (2011).
1051 105. Lafferty, C.K. & Britt, J.P. Off-Target Influences of Arch-Mediated Axon Terminal
1052 Inhibition on Network Activity and Behavior. *Front Neural Circuits* **14**, 10 (2020).
1053 106. Ferenczi, E.A. *et al.* Optogenetic approaches addressing extracellular modulation of
1054 neural excitability. *Sci Rep* **6**, 23947 (2016).
1055 107. Wiegert, J.S., Mahn, M., Prigge, M., Printz, Y. & Yizhar, O. Silencing Neurons: Tools,
1056 Applications, and Experimental Constraints. *Neuron* **95**, 504-529 (2017).
1057 108. El-Gaby, M. *et al.* Archaerhodopsin Selectively and Reversibly Silences Synaptic
1058 Transmission through Altered pH. *Cell Rep* **16**, 2259-2268 (2016).
1059 109. Turecek, R. & Trussell, L.O. Presynaptic glycine receptors enhance transmitter release
1060 at a mammalian central synapse. *Nature* **411**, 587-590 (2001).
1061 110. Szabadics, J. *et al.* Excitatory effect of GABAergic axo-axonic cells in cortical
1062 microcircuits. *Science* **311**, 233-235 (2006).
1063 111. Baldi, R., Varga, C. & Tamas, G. Differential distribution of KCC2 along the axo-somato-
1064 dendritic axis of hippocampal principal cells. *Eur J Neurosci* **32**, 1319-1325 (2010).
1065 112. Bernal Sierra, Y.A. *et al.* Potassium channel-based optogenetic silencing. *Nat Commun*
1066 **9**, 4611 (2018).
1067 113. Alberio, L. *et al.* A light-gated potassium channel for sustained neuronal inhibition.
1068 *Nat Methods* **15**, 969-976 (2018).
1069 114. Govorunova, E.G. *et al.* Kalium rhodopsins: Natural light-gated potassium channels.
1070 *bioRxiv*, 2021.2009.2017.460684 (2021).
1071 115. Herlitze, S. *et al.* Modulation of Ca2+ channels by G-protein beta gamma subunits.
1072 *Nature* **380**, 258-262 (1996).
1073 116. Ikeda, S.R. Voltage-dependent modulation of N-type calcium channels by G-protein
1074 beta gamma subunits. *Nature* **380**, 255-258 (1996).
1075 117. Wu, L.G. & Saggau, P. Adenosine inhibits evoked synaptic transmission primarily by
1076 reducing presynaptic calcium influx in area CA1 of hippocampus. *Neuron* **12**, 1139-1148
1077 (1994).
1078 118. Schneggenburger, R. & Neher, E. Intracellular calcium dependence of transmitter
1079 release rates at a fast central synapse. *Nature* **406**, 889-893 (2000).
1080 119. Dodge, F.A., Jr. & Rahamimoff, R. Co-operative action a calcium ions in transmitter
1081 release at the neuromuscular junction. *J Physiol* **193**, 419-432 (1967).
1082 120. Zurawski, Z., Yim, Y.Y., Alford, S. & Hamm, H.E. The expanding roles and mechanisms
1083 of G protein-mediated presynaptic inhibition. *J Biol Chem* **294**, 1661-1670 (2019).
1084 121. Li, X. *et al.* Fast noninvasive activation and inhibition of neural and network activity
1085 by vertebrate rhodopsin and green algae channelrhodopsin. *Proc Natl Acad Sci U S A* **102**,
1086 17816-17821 (2005).
1087 122. Kawano-Yamashita, E. *et al.* Activation of Transducin by Bistable Pigment
1088 Parapinopsin in the Pineal Organ of Lower Vertebrates. *PLoS One* **10**, e0141280 (2015).

123. Koyanagi, M., Takada, E., Nagata, T., Tsukamoto, H. & Terakita, A. Homologs of vertebrate Opn3 potentially serve as a light sensor in nonphotoreceptive tissue. *Proc Natl Acad Sci U S A* **110**, 4998-5003 (2013).
124. Copits, B.A. *et al.* A photoswitchable GPCR-based opsin for presynaptic inhibition. *Neuron* (2021).
125. Mahn, M. *et al.* Efficient optogenetic silencing of neurotransmitter release with a mosquito rhodopsin. *Neuron* **109**, 1621-1635 (2021).
126. Eickelbeck, D. *et al.* Lamprey Parapinopsin ("UVLamP"): a bistable UV-sensitive optogenetic switch for ultrafast control of GPCR pathways. *Chembiochem* (2019).
127. Rodgers, J. *et al.* Using a bistable animal opsin for switchable and scalable optogenetic inhibition of neurons. *EMBO Rep*, e51866 (2021).
128. Zhang, F. *et al.* Multimodal fast optical interrogation of neural circuitry. *Nature* **446**, 633-639 (2007).
129. Lin, J.Y. *et al.* Optogenetic inhibition of synaptic release with chromophore-assisted light inactivation (CALI). *Neuron* **79**, 241-253 (2013).
130. Xu, S. & Chisholm, A.D. Highly efficient optogenetic cell ablation in C. elegans using membrane-targeted miniSOG. *Sci Rep* **6**, 21271 (2016).
131. Schiavo, G., Matteoli, M. & Montecucco, C. Neurotoxins affecting neuroexocytosis. *Physiol Rev* **80**, 717-766 (2000).
132. Humeau, Y. & Choquet, D. The next generation of approaches to investigate the link between synaptic plasticity and learning. *Nat Neurosci* **22**, 1536-1543 (2019).
133. Won, J. *et al.* Opto-vTrap, an optogenetic trap for reversible inhibition of vesicular release, synaptic transmission, and behavior. *Neuron* **110**, 423-435 e424 (2022).
134. Brody, D.L. & Yue, D.T. Relief of G-protein inhibition of calcium channels and short-term synaptic facilitation in cultured hippocampal neurons. *J Neurosci* **20**, 889-898 (2000).
135. Takeuchi, T., Duszkiewicz, A.J. & Morris, R.G. The synaptic plasticity and memory hypothesis: encoding, storage and persistence. *Philos Trans R Soc Lond B Biol Sci* **369**, 20130288 (2014).
136. Monday, H.R., Younts, T.J. & Castillo, P.E. Long-Term Plasticity of Neurotransmitter Release: Emerging Mechanisms and Contributions to Brain Function and Disease. *Annu Rev Neurosci* **41**, 299-322 (2018).
137. Yang, Y. & Calakos, N. Presynaptic long-term plasticity. *Front Synaptic Neurosci* **5**, 8 (2013).
138. Fernandes, H.B. *et al.* Epac2 Mediates cAMP-Dependent Potentiation of Neurotransmission in the Hippocampus. *J Neurosci* **35**, 6544-6553 (2015).
139. Weisskopf, M.G., Castillo, P.E., Zalutsky, R.A. & Nicoll, R.A. Mediation of hippocampal mossy fiber long-term potentiation by cyclic AMP. *Science* **265**, 1878-1882 (1994).
140. Nicoll, R.A. & Schmitz, D. Synaptic plasticity at hippocampal mossy fibre synapses. *Nat Rev Neurosci* **6**, 863-876 (2005).
141. Orlando, M. *et al.* Recruitment of release sites underlies chemical presynaptic potentiation at hippocampal mossy fiber boutons. *PLoS Biol* **19**, e3001149 (2021).
142. Kees, A.L., Marneffe, C. & Mulle, C. Lighting up pre-synaptic potentiation: An Editorial for "SynaptoPAC, an optogenetic tool for induction of presynaptic plasticity" on page 324. *J Neurochem* **156**, 270-272 (2021).
143. Heck, J. *et al.* Transient Confinement of CaV2.1 Ca(2+)-Channel Splice Variants Shapes Synaptic Short-Term Plasticity. *Neuron* **103**, 66-79 e12 (2019).

144. Hoffmann, S. *et al.* Light-Activated ROS Production Induces Synaptic Autophagy. *J Neurosci* **39**, 2163-2183 (2019).
145. Miyashita, T., Shao, Y.R., Chung, J., Pourzia, O. & Feldman, D.E. Long-term channelrhodopsin-2 (ChR2) expression can induce abnormal axonal morphology and targeting in cerebral cortex. *Front Neural Circuits* **7**, 8 (2013).
146. Henss, T. *et al.* Optogenetic tools for manipulation of cyclic nucleotides functionally coupled to cyclic nucleotide-gated channels. *Br J Pharmacol* (2021).
147. Otchy, T.M. *et al.* Acute off-target effects of neural circuit manipulations. *Nature* **528**, 358-363 (2015).
148. Li, N. *et al.* Spatiotemporal constraints on optogenetic inactivation in cortical circuits. *Elife* **8** (2019).
149. Stujenske, J.M., Spellman, T. & Gordon, J.A. Modeling the Spatiotemporal Dynamics of Light and Heat Propagation for In Vivo Optogenetics. *Cell Rep* **12**, 525-534 (2015).
150. Willems, J. *et al.* ORANGE: A CRISPR/Cas9-based genome editing toolbox for epitope tagging of endogenous proteins in neurons. *PLoS Biol* **18**, e3000665 (2020).
151. Pausch, P. *et al.* CRISPR-CasPhi from huge phages is a hypercompact genome editor. *Science* **369**, 333-337 (2020).
152. Kim, C.K. *et al.* A Molecular Calcium Integrator Reveals a Striatal Cell Type Driving Aversion. *Cell* **183**, 2003-2019 e2016 (2020).
153. Yook, J.S., Kim, J. & Kim, J. Convergence Circuit Mapping: Genetic Approaches From Structure to Function. *Front Syst Neurosci* **15**, 688673 (2021).
154. Prakash, M. *et al.* Selective control of synaptically-connected circuit elements by all-optical synapses. *Commun Biol* **5**, 33 (2022).
155. Vierock, J. *et al.* BiPOLES is an optogenetic tool developed for bidirectional dual-color control of neurons. *Nat Commun* **12**, 4527 (2021).
156. Taslimi, A. *et al.* An optimized optogenetic clustering tool for probing protein interaction and function. *Nat Commun* **5**, 4925 (2014).
157. van Bergeijk, P., Adrian, M., Hoogenraad, C.C. & Kapitein, L.C. Optogenetic control of organelle transport and positioning. *Nature* **518**, 111-114 (2015).
158. Shin, Y. *et al.* Spatiotemporal Control of Intracellular Phase Transitions Using Light-Activated optoDroplets. *Cell* **168**, 159-171 e114 (2017).
159. Rabut, C. *et al.* Ultrasound Technologies for Imaging and Modulating Neural Activity. *Neuron* **108**, 93-110 (2020).
160. Oda, K. *et al.* Crystal structure of the red light-activated channelrhodopsin Chrimson. *Nat Commun* **9**, 3949 (2018).
161. Klapoetke, N.C. *et al.* Independent optical excitation of distinct neural populations. *Nat Methods* **11**, 338-346 (2014).
162. Govorunova, E.G. *et al.* RubyACRs, nonalgal anion channelrhodopsins with highly red-shifted absorption. *Proc Natl Acad Sci U S A* **117**, 22833-22840 (2020).
163. Shevchenko, V. *et al.* Inward H(+) pump xenorhodopsin: Mechanism and alternative optogenetic approach. *Sci Adv* **3**, e1603187 (2017).
164. Inoue, K. *et al.* A light-driven sodium ion pump in marine bacteria. *Nat Commun* **4**, 1678 (2013).
165. Feroz, H. *et al.* Light-Driven Chloride Transport Kinetics of Halorhodopsin. *Biophys J* **115**, 353-360 (2018).

166. AzimiHashemi, N. *et al.* Synthetic retinal analogues modify the spectral and kinetic characteristics of microbial rhodopsin optogenetic tools. *Nat Commun* **5**, 5810 (2014).
167. Broser, M. *et al.* NeoR, a near-infrared absorbing rhodopsin. *Nat Commun* **11**, 5682 (2020).
168. Luck, M. *et al.* A photochromic histidine kinase rhodopsin (HKR1) that is bimodally switched by ultraviolet and blue light. *J Biol Chem* **287**, 40083-40090 (2012).
169. Koyanagi, M. *et al.* Bistable UV pigment in the lamprey pineal. *Proc Natl Acad Sci U S A* **101**, 6687-6691 (2004).
170. Heintz, U. & Schlichting, I. Blue light-induced LOV domain dimerization enhances the affinity of Aureochrome 1a for its target DNA sequence. *Elife* **5**, e11860 (2016).
171. Jung, A. *et al.* Structure of a bacterial BLUF photoreceptor: insights into blue light-mediated signal transduction. *Proc Natl Acad Sci U S A* **102**, 12350-12355 (2005).
172. Banerjee, R. *et al.* The signaling state of Arabidopsis cryptochrome 2 contains flavin semiquinone. *J Biol Chem* **282**, 14916-14922 (2007).
173. Bulina, M.E. *et al.* A genetically encoded photosensitizer. *Nat Biotechnol* **24**, 95-99 (2006).
174. Neupert, C. *et al.* Regulated Dynamic Trafficking of Neurexins Inside and Outside of Synaptic Terminals. *J Neurosci* **35**, 13629-13647 (2015).
175. Armbruster, B.N., Li, X., Pausch, M.H., Herlitze, S. & Roth, B.L. Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc Natl Acad Sci U S A* **104**, 5163-5168 (2007).
176. Vardy, E. *et al.* A New DREADD Facilitates the Multiplexed Chemogenetic Interrogation of Behavior. *Neuron* **86**, 936-946 (2015).
177. Burke, K.J., Jr., Keeshen, C.M. & Bender, K.J. Two Forms of Synaptic Depression Produced by Differential Neuromodulation of Presynaptic Calcium Channels. *Neuron* **99**, 969-984 e967 (2018).