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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

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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

154 limited due to bleaching-induced decrease in receptor activation over time[23]. Moreover,
155 ectopically expressed optoXRs show slow off-kinetics because non-visual cells lack
156 rhodopsin kinases and arrestins that are necessary for rapid receptor inactivation[32]. Finally,
157 since visual rhodopsins have a broad excitation spectrum, they are less compatible with
158 multiplexed applications. Instead, the non-bleaching vertebrate and invertebrate bistable
159 GPCR rhodopsins (optoGPCRs) are increasingly utilized because they can be activated
160 repeatedly. Some optoGPCRs display spectrally distinct stable states that enable
161 photochromic switching between the active and inactive forms of the protein[24]. While
162 many potential optoGPCRs have been described, mainly in spectroscopic studies, their
163 application as modulators of presynaptic function has only recently been explored (see
164 below). For most optoGPCRs, the G-protein specificity has not been tested in detail.
165 However, promiscuous G-protein coupling of exogenously-expressed optoGPCRs should be
166 considered to exclude activation of undesired signaling cascades. In the future, chimeras of
167 optoGPCRs and endogenous GPCR signal domains promise to combine high coupling
168 specificity with the non-bleaching properties of bistable opsins.
169

170 *Blue-light receptors (BLRs)*

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

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

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

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

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

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

222

223 *Photosensitizers*

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

232 KillerRed, which showed significantly improved ROS production compared to EGFP[50].
233 Mutagenesis of KillerRed yielded the spectrally shifted KillerOrange[51] and the monomeric
234 photosensitizers SuperNova and SuperNova green[52, 53], which are more suitable for fusion
235 with target proteins and allow differential CALI with spectrally distinct illumination.
236 MiniSOG (mini Singlet Oxygen Generator) is a modestly fluorescent flavoprotein engineered
237 from phototropin 2[54], which under blue light efficiently generates ROS. Originally
238 developed as a probe for correlative light and electron microscopy, miniSOG and its
239 improved variants are versatile tools for CALI applications due to their small size[49, 55]. Still,
240 photosensitizers are difficult to use compared to other optogenetic tools, because it is
241 necessary to carefully evaluate the degree of specific protein inactivation relative to
242 nonspecific tissue damage.
243 Applying optogenetic tools requires consideration of both the biophysical
244 characteristics of the actuator and the specific physiology of the cells. Important
245 biophysical properties of optogenetic tools include operational light sensitivity, activation
246 spectrum, dark activity, and kinetics. Even slight variations of these properties can have
247 considerable consequences for complex behavioral readouts[56]. Optogenetic actuators are
248 increasingly combined with fluorescent sensors to monitor various physiological processes,
249 and sensor properties must therefore be considered in parallel. For a detailed description
250 of optogenetic sensors for interrogating synaptic signaling, the reader is referred to recent
251 reviews[57-61] and the fluorescent biosensor database (https://biosensordb.ucsd.edu)[62]. The
252 effects of optogenetic interrogations on the cellular physiology and network activity also
253 depend on the localization of the tool within the cell and the specific physiology within this
254 subcellular compartment. In this regard, the axon has proven particularly challenging due
255 to its length, specific protein targeting, and unique physiology.
256

257 ***Targeting optogenetic actuators to the axon and presynapse***

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

271 located in the presynaptic cytosol or face the synaptic vesicle lumen (Fig. 2C). Importantly,
272 overexpressing synaptophysin seems not to affect synaptic transmission in rodent
273 neurons[67, 69].
274 Efficient targeting of optogenetic tools to the axonal plasma membrane has proven
275 more difficult. Axonal membrane proteins have to pass the axon initial segment at the
276 boundary of the somatodendritic and the axonal compartment[70]. No general signal
277 sequence for axonal trafficking or presynaptic anchoring has been identified for membrane
278 proteins. Nonetheless, two mechanisms are known to facilitate axonal localization:
279 Unidirectional membrane insertion followed by preferential endocytosis in the
280 somatodendritic compartment (Fig. 2A)[71], and axon-directed transport via specialized
281 vesicle carriers (Fig. 2B)[66]. Dendritic endocytosis is mediated at least in part by Myosin VI
282 followed by anterograde axonal transport, a mechanism referred to as transcytosis[72]. These
283 findings inspired strategies for axonal localization of membrane-spanning optogenetic
284 actuators. Fusion of the ChR2 C-terminus to a myosin VI-binding domain was shown to
285 increase ChR2-YFP expression in axons and decrease its expression in dendrites[73]. Another
286 method utilized an intracellular neurexin 1α tag previously established for presynaptic
287 targeting of hM4D[74] to facilitate axonal expression of ChR2 and ArchT in songbirds[75, 76], but
288 this strategy was never benchmarked against non-targeted constructs. Hamada *et al*.
289 recently combined the presynaptic mGluR2 targeting sequence for specific axonal
290 enrichment with a proteolytic motif for reducing somatodendritic expression[77]. The
291 resulting ChR2-mGluR2-PA showed stronger expression in long-range projections and
292 reduced light-evoked responses in the soma. However, it remains unclear whether axonal
293 targeting motifs significantly improve the performance of optogenetic tools at presynaptic
294 terminals. Further work is required to systematically evaluate presynaptic targeting
295 strategies of optogenetic actuators.
296

297 ***Presynaptic applications of optogenetic tools***

298

299 *Light-evoked neurotransmitter release*
300 Photostimulation of CCR-expressing axons evokes synaptic transmission even when axons
301 are severed from the somata in acute slice preparations[78]. For 'Channelrhodopsin-assisted
302 circuit mapping' (CRACM), CCRs are expressed in one brain region, and functional
303 connectivity is later assessed by local illumination in the target region. Mechanistically, the
304 photocurrent depolarizes the axonal membrane and elicits an AP. At the terminals the AP
305 causes Ca^{2+} influx via voltage-gated channels, which triggers neurotransmitter release (Fig.
306 3A). Light-evoked postsynaptic currents confirm that functional synapses exist between the
307 CCR-expressing and the postsynaptic neuron population. CRACM is applied widely to study
308 the connectivity of genetically defined neurons, offering a technically easier and higher
309 throughput alternative to paired recordings or selective presynaptic electrical stimulation[79,

310 [80]. In combination with input cell-specific genetic knockout or knockdown strategies,
311 CRACM is also useful for studying the effects of protein loss on transmitter release[81, 82].
312 However, light-evoked APs propagate not only towards the synapse, but also antidromically
313 to the soma (Fig. 3D), inducing transmitter release from collaterals, which complicates the
314 interpretation of *in vivo* CRACM experiments[65, 83]. In slices, CRACM can be refined by
315 abolishing spiking using Na^+-channel blockers (Fig. 3B), while enhancing the light-driven
316 depolarization and transmitter release by blocking K^+-channels[79] (Fig. 3C). However,
317 application of such drugs is not feasible in living animals. Therefore, control experiments
318 have been proposed for meaningful behavioral experiments involving CCRs, for example,
319 blocking antidromic APs at the soma by local injection of lidocaine[84], or specific blockage of
320 postsynaptic receptors in the target region as internal control[85].
321 Compared to electrical stimulation, photostimulation of presynaptic terminals
322 induces vesicle fusion with a higher probability, resulting in robust postsynaptic responses.
323 Several reasons may account for this: AP broadening due to long-lasting depolarization by
324 ChR2[86] may increase presynaptic Ca^{2+}-influx and thus increase transmitter release[87, 88]. In
325 addition, transmitter release is positively modulated by subthreshold voltage deflection
326 that can travel down the axon into the presynapse[89], so the photocurrent might directly
327 increase release probability. The elevated release probability resulting from direct
328 illumination of CCR-expressing terminals can be advantageous for determining functional
329 connectivity[80, 86, 90]. On the other hand, it can result in synaptic depression during high-
330 frequency firing by depletion of the readily releasable pool[91]. Jackman *et al.* found that
331 direct photostimulation of hippocampal Schaffer collateral synapses in CA1 caused atypical
332 synaptic depression[80] (Fig. 3E). Direct illumination of presynaptic boutons should therefore
333 be avoided when studying short-term plasticity. The same study[80] also described an effect
334 of the AAV serotype on release properties. While short-term plasticity was similar for
335 electrical and optical stimulation when ChR2 was expressed transgenically or via AAV9,
336 expression via AAV1, 5 or 8 resulted in artificial synaptic depression. Notably, repetitive
337 optical stimulation can induce long-term depression, which has been used experimentally
338 to silence long-range projections[92-94]. In other synapses, high-frequency optical stimulation
339 can lead to long-term potentiation, for example at cortico-striatal synapses[95].
340 Taken together, optical excitation of axons has proven highly valuable for identifying
341 functional long-range connections between distant circuits, but shows protocol-, pathway-
342 and cell type-specific effects. Thus, carefully designed controls are required when it is used
343 to study synaptic plasticity or the functional interaction of distant brain areas *in vivo*.
344
345
346 *Optogenetic inhibition of neurotransmitter release*
347 Optogenetic inhibition of neurotransmitter release is an important complementary
348 approach to optogenetic excitation, because it circumvents issues arising from uncontrolled

349 antidromic APs *in vivo*. Three experimental strategies have emerged for presynaptic
350 optogenetic inhibition (Fig. 4): i) hyperpolarization of axon terminals to inhibit AP
351 propagation and decrease presynaptic Ca^{2+} influx, ii) inhibition of transmitter release by
352 GPCRs, and iii) destruction of the release machinery. Importantly, since effective
353 optogenetic inhibition of transmission is more difficult to confirm than optogenetic
354 excitation, tool expression and performance should be carefully validated in every
355 experimental setting (see Box 1 and Fig. 5).
356
357 Optogenetic inhibition of axons has been achieved with two different classes of
358 electrogenic pumps (Figs. 1B, 4A): the outward-directed proton pumps Arch3[96], ArchT[97],
359 and Jaws[98], and the inward directed chloride pump halorhodopsin (NpHR)[99]. Presynaptic
360 inhibition by light-driven pumps has been applied for suppressing propagation of
361 spontaneous network oscillations between brain regions[100, 101] and for *in vivo* silencing of
362 specific synaptic connections to study their role in behavior[84, 102-105]. Pioneering work has
363 demonstrated that continuous activation of these pumps in terminals reduces spontaneous
364 and electrically-evoked synaptic release but does not affect spiking at the non-illuminated
365 somata[84, 103-105]. The reduction of transmission occurs instantaneously and disappears
366 within seconds after termination of illumination (Figs. 4D, 5B). However, while rapid
367 silencing is ideal for closed-loop experiments, minute-long ion pumping may cause
368 unintended effects: First, prolonged activity of H^+-pumps significantly changes intra- and
369 extracellular pH[106], and Cl^--pump activity leads to a depolarizing shift in the reversal
370 potential of $GABA_A$-mediated currents[20]. Such effects exacerbate in small compartments
371 with a large surface-to-volume ratio[107]. In axon terminals, sustained activation of ArchT
372 rapidly increased cytosolic pH to 8[108]. Preventing alkalinization abolished synaptic silencing,
373 indicating that proton pumps suppress evoked release in terminals primarily by intracellular
374 alkalinization and not by hyperpolarization. Second, cytosolic alkalinization triggers Ca^{2+}
375 influx, which dramatically increases the frequency of spontaneous transmitter release[19, 105].
376 Such aberrant spontaneous release may activate local interneurons, causing undesired
377 effects on network activity[105]. Third, the silencing efficacy of NpHR decreases from ~50%
378 during the first seconds to ~20% within one minute, and rebound spiking after a step-like
379 termination of light (Fig. 5B)[19]. Considering these effects, it seems advisable to minimize
380 pump activation to short intervals of <1 min, and to apply gradual light off-ramps[19]. The
381 strong Cl^--mediated hyperpolarization in dendrites and somata by ACRs rendered them a
382 reasonable alternative for axonal silencing by shunting. However, due to the elevated
383 intracellular chloride concentration in axons of mature neurons[109-111], ACRs actually
384 depolarize axons and trigger transmitter release[15, 16]. Inhibition of transmitter release by
385 light-gated potassium channels[112, 113] has not been successfully demonstrated, probably
386 due to insufficient axonal trafficking. The effects of the recently-described K^+-ChRs in axons
387 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

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

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

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

466 such as 'synaptic engrams' of a given learning task[132]. Recently, a CRY2:CIB-based approach
467 (Fig. 1G) for non-proteolytic optogenetic interference with synaptic release has been
468 published[133]. The opto-vTRAP system clusters synaptic vesicles by light-induced
469 complexation of CIBN fused to the cytosolic N-terminus of VAMP2 and cytosolic Cry2. In
470 acute brain slice recordings from Schaffer collateral synapses in hippocampus CA1,
471 activation of opto-vTRAP with continuous blue light reduced EPSCs by 50% within 15 min,
472 which recovered within 30 min after light termination. Vesicle aggregation apparently
473 decreased the number of release-competent vesicles without affecting vesicular release
474 probability, thereby not affecting short-term plasticity as determined by the paired-pulse
475 ratio.
476 The three classes of optogenetic presynaptic inhibitors operate on very different
477 time-scales (Fig. 4D): Destructive optogenetic inhibitors require minutes of continuous
478 illumination, but their effect is long-lasting; optogenetic pumps inhibit release within
479 milliseconds, but they should not be activated for more than a few minutes[19]; effects of
480 optoGPCRs unfold within a few hundred milliseconds, and last for seconds to minutes. In
481 contrast to pumps and destructive inhibitors, optoGPCRs require very little light for their
482 activation. Their repeated activation with short light flashes is likely to extend the effect
483 duration, and switchable optoGPCRs such as *Lc*PPO allow control over the recovery of
484 transmission. OptoGPCRs might be too slow for applications that require ultrafast closed-
485 loop suppression of release, but otherwise seems ideal for most forms of presynaptic
486 inhibition on physiological time scales. Of note, high-frequency trains of APs can affect
487 GPCR-mediated inhibition of presynaptic Ca^{2+} influx by βγ subunits[134], which should be
488 experimentally tested when optoGPCRs are applied (Fig. 5D and Box I). Presynaptic
489 optogenetic inhibition offers superior spatio-temporal specificity compared to
490 chemogenetic approaches or constitutively active, genetically-encoded toxins (Table 1). On
491 the other hand, chemogenetic inhibition can be achieved by systemic agonist
492 administration, and Tetanus Toxin does not require any hardware for activation. Given the
493 variety of tools at hand, users should consider the temporal domain in which transmitter
494 release needs to be inhibited and whether specific properties of the presynapse or the
495 tool's properties constrain the experimental setting. Users should aim to validate the
496 performance of their selected optogenetic tool and consider possible side effects, plasticity
497 mechanisms and compensatory circuit rebalancing effects, and design appropriate controls.
498 Although the ideal presynaptic inhibitor that provides reversible suppression of transmitter
499 release at one specific synapse between two genetically-defined classes of neurons is not
500 yet available[107], the available optogenetic tools for presynaptic inhibition are remarkably
501 advanced and offer a wide range of functionalities to interfere with transmitter release.
502
503
504

505 *Presynaptic potentiation by light*
506 Long-lasting modifications of synaptic strength can shape the neuronal landscape
507 underlying memory engrams. There is ample evidence that the structural and functional
508 mechanisms of postsynaptic long-term potentiation (LTP) are associated with memory-
509 guided behavioral performance[135]. In comparison, the role of presynaptic LTP, found at
510 various synapses throughout the CNS, in learning and behavior output remains unclear[136].
511 Presynaptic LTP can be triggered by high frequency firing and is expressed as a persistent
512 increase of transmitter release[137]. At hippocampal mossy fiber synapses, high-frequency
513 firing increases cAMP levels, which via protein-kinase A and the guanine nucleotide
514 exchange factor Epac2[138-140] increases vesicular release probability and activates novel
515 release sites[141]. It is difficult to assess the effects of presynaptic LTP in living animals, since
516 high-frequency stimulation of axons could entail unintended off-target effects. Recent work
517 established optogenetic induction of presynaptic potentiation by directly elevating cAMP
518 levels in axon terminals[69]. For this, the photoactivated adenylyl cyclase bPAC was attached
519 to the cytosolic C-terminus of synaptophysin. Blue-light activation of the resulting
520 'synaptoPAC' triggered a rapid increase of transmission at mossy fiber synapses in CA3,
521 which decayed with a time course similar to electrically-induced LTP. Transmission at
522 hippocampal CA3–CA1 Schaffer collateral synapses was not enhanced by synaptoPAC
523 activation, indicating that optogenetic induction of cAMP only potentiates transmitter
524 release from terminals that are predisposed to undergo presynaptic plasticity. SynaptoPAC
525 is likely to be a useful tool to mimic presynaptic plasticity at genetically defined synapses in
526 living animals, and may help elucidate the behavioral role of presynaptic potentiation[142].
527

528 *Optogenetic control of presynaptic organelles and proteins*
529 Other than modulating synaptic vesicle exocytosis, optogenetics also allows manipulation
530 of additional processes within the presynaptic compartment: pHoenix, a fusion construct
531 comprised of the light-driven proton pump Arch3 and the vesicle protein synaptophysin,
532 enables light-regulated acidification of synaptic vesicles[67]. Following pharmacological
533 inhibition of V-ATPases and depletion of neurotransmitters from the vesicles, light-
534 controlled vesicle re-acidification by pHoenix initiates neurotransmitter uptake.
535 Visualization of intravesicular pH by pHluorin showed rapid acidification preceding
536 neurotransmitter accumulation, and titration of the vesicular neurotransmitter filling state
537 revealed preferential exocytosis of completely filled vesicles over partially filled vesicles.
538 The pHoenix concept was further adopted for lysosomes[67], and may serve as a framework
539 for future optogenetic manipulations of other organelles.
540 Optogenetic oligomerization enables studies on the effect of position and mobility
541 of presynaptic Ca^{2+} channels and other proteins on synaptic transmission: Blue light-
542 induced oligomerization has been demonstrated for presynaptic Ca^{2+}-channels N-terminally
543 fused to cryptochrome 2 (CRY2olig)[143]. The reversible sequestration of channels in

544 nanodomain-like clusters altered presynaptic Ca^{2+} flux and glutamate release in a channel
545 subtype-specific manner.
546 Finally, acute optogenetic ablation of specific proteins allows testing their role in
547 transmission, while avoiding long-term functional compensation or toxicity as might occur
548 in genetic knockout or knockdown models. Reactive oxygen production by presynaptically
549 targeted photosensitizers inactivates presynaptic proteins[129]. Selective photo-inactivation
550 of synaptic proteins was achieved with GFP-derived photosensitizers (Fig. 1I) that generate
551 less ROS than miniSOG, leading to a smaller radius of protein inactivation. Accordingly,
552 specific damage of synaptophysin by the photosensitizer "supernova" allowed to selectively
553 study autophagy of synaptophysin[144]. Remarkably, synaptic transmission was only
554 compromised when autophagy was blocked, illustrating that efficient inhibition of synapses
555 with CALI requires pronounced damaging of the release machinery.
556

557 ***Experimental constraints***

558 Optogenetics in neurons requires the introduction of foreign proteins into highly
559 specialized cells that form a most sensitive organ. Other than considering the biophysical
560 constraints of the actuators, researchers must control for unintended off-target effects by
561 opsin expression and photostimulation[63]. These include overexpression artifacts[145], dark
562 activity effects[146], off-target effects within cells[19, 105, 147] and the network[148], and effects of
563 tissue heating following illumination[149]. Many of these side-effects can be minimized by
564 avoiding high protein load and excessive light, both of which can cause unphysiologically
565 strong intracellular signals. Development of improved actuators with higher light
566 sensitivity, minimal dark activity and optimized intracellular trafficking will allow safer and
567 more robust application of optogenetic manipulations. Additionally, future tool design will
568 benefit from more subtle gene expression and targeting strategies, by either choosing
569 appropriate promoters for expression of the optogenetic tools, use of knock-in mice, or
570 utilizing CRISPR-Cas for tagging endogenous proteins with actuators[150, 151].
571

572 ***Discussion and outlook***

573 The unique physiology and intricate trafficking machinery of axons have hampered
574 optogenetic applications in presynaptic terminals for many years. However, a better
575 understanding of the interactions between the unique intracellular environment of the
576 axon and the biophysical properties of optogenetic actuators has led to the development of
577 optogenetic tools specifically adapted for presynaptic applications.
578 The combination of several recent technological advancements, including better
579 subcellular targeting for exclusive localization in the axon and more sophisticated gene
580 expression systems for activity- and connectivity-dependent control of tool expression[152,
581 153], will improve spatial and temporal optogenetic control. However, there are still

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

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

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

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

621 optogenetics offers neuroscientists diverse experimental approaches at unsurpassed
622 spatio-temporal resolution, and the ongoing developments are likely to provide exciting
623 new insights in the future.
624

625 ***Figure Legends***

626 **Figure 1: Overview on optogenetic actuators.** Left: Chromophore photoreaction. Middle:
627 schematized architecture and function. Right: selected absorption or activation spectra
628 scaled to maximum chromophore absorption or actuator response (from top to bottom):
629 Chrimson[160], TsChR[161], A1ACR1[162], GtACR2[14], NsXeR[163], KR2[164], NpHR[165], Arch3.0[166],
630 CaRhGC[22], neoR[167], CrHKR1[168], mosOPN3[123], LcPPO[169], LOV (PtAu1a)[170], BLUF (RsBlrB)[171],
631 AtCRY2[172], SuperNova Green[53], miniSOG[54], and KillerRed[173]. Abbreviations used: AC
632 (adenylyl cyclase), ACR (anion-conducting ChR), ATP (adenosine triphosphate), BLUF (blue-
633 light using FAD), bPAC (bacterial photoactivated adenylyl cyclase), cAMP (cyclic adenosine
634 monophosphate), CCR (cation-conducting ChR), cGMP (cyclic guanosine monophosphate),
635 CIB (cryptochrome-interacting basic-helix-loop-helix protein), CRY (cryptochrome), FAD
636 (flavin adenine dinucleotide), FMN (flavin mononucleotide), FP (fluorescent protein), GC
637 (guanylyl cyclase), GTP (guanosine triphosphate), HKR-GC (histidine kinase rhodopsin
638 coupled guanylyl cyclase), iLID (improved light-induced dimer), LOV (light-oxygen-voltage-
639 sensing (domain)), miniSOG (miniature singlet oxygen generator), P (phosphorylation site),
640 PDE (phosphodiesterase), POI (protein of interest), RhG/AC (rhodopsin gyanyly/adenylyl
641 cyclase), RhoPDE (rhodopsin phosphodiesterase), and SspB/SSRA (binding element partner
642 A/B (from *E. coli*)).
643

644 **Figure 2: Axonal transport and presynaptic targeting of optogenetic actuators.** The axon
645 initial segment (AIS) demarcates the boundary of the axon and the somatodendritic
646 compartment and acts as a gatekeeper by repelling cargo vesicles containing proteins for
647 the somatodendritic compartment. Major mechanisms to enrich proteins in the axon are:
648 A, Preferential endocytosis from the dendritic membrane, illustrated here for an opsin
649 tagged with the neurexin 1α (Nrx1α) C-terminus. B, Directed anterograde axonal transport
650 of cargo vesicles along microtubules by kinesins. Such cargo vesicles include synaptic
651 vesicle precursors (SVP), which carry vesicular proteins such as synaptophysin, vesicular
652 neurotransmitter transporters, and associated active zone components. SVPs also transport
653 axon-targeted proteins that have been endocytosed from dendritic membranes. Note that
654 dendritic endocytosis and fast axonal anterograde transport by KIF1A has been
655 demonstrated for Nrx1α[72, 174], but not specifically for opsins fused to the Nrx1α C-terminus.
656 Calcium channels are transported in separate vesicles (CaV), probably by different kinesin
657 isoforms. Membrane proteins can also diffuse passively along the axon, whereby opsins
658 lacking a specific axonal targeting sequence can passively reach axonal terminals over
659 longer periods of time. C, Synaptic vesicle targeting of actuators by fusion to synaptophysin
660 allows for manipulating the intravesicular H^+ concentration by proton pumps or for
661 increasing cAMP in the presynaptic cytosol by photoactivated adenylyl cyclases.
662

663 **Figure 3: Concept and pitfalls of light-evoked neurotransmitter release by CCR**
664 **stimulation.**
665 A, Light-triggered currents activate voltage-gated Na^+ channels (Na_v), which amplifies
666 depolarization and opens voltage-gated Ca^{2+} channels (VGCCs) at the terminal. Thereby
667 CCR-driven depolarization elicits transmitter release from the presynaptic bouton, but also
668 APs that back-propagate towards the soma. B, Probing synaptic connectivity in acute slice
669 preparations allows blocking Na_v by tetrodotoxin (TTX), which abolishes the antidromic AP

670 (left). However the depolarization provided by CCRs can be insufficient to activate VGCCs
671 due to shunting by voltage-gated K^+ channels (K_v). C, Additional application of 4-
672 aminopyridine (4-AP) to block K_v then ensures sufficient light-triggered depolarization for
673 activation of VGCCs. D, Local illumination in one brain region can elicit antidromic APs and
674 transmitter release in other brain regions due to divergent axonal arborizations,
675 exemplified here for noradrenergic projections originating from the locus coeruleus (LC),
676 with photostimulation in the amygdala. E, Over-bouton illumination increases synaptic
677 release probability compared to over-axon illumination or electrical stimulation, which
678 modifies the short-term plasticity of transmission. Synaptic responses drawn according to
679 Jackman *et al.*, 2014[80], and scaled to the first EPSP amplitude.
680

681 **Figure 4: Different principles of optogenetic inhibition of transmitter release.**
682 A, Optogenetic hyperpolarization (shown here for the chloride pump NpHR) reduces the
683 open probability of VGCCs after an AP, which reduces transmitter release. B, Light-activated
684 GPCRs (optoGPCRs) can effectively inhibit transmitter release by activating heterotrimeric
685 G-proteins containing the $G\alpha_{i/o}$ subunit. The membrane-anchored βγ subunits physically
686 interact with presynaptic P/Q or N-type VGCCs and reduce their open probability. In
687 addition, βγ subunits can directly interfere with SNARE-mediated vesicle fusion. C,
688 Permanent inactivation of the synaptic release machinery by vesicle-bound photoactivated
689 botulinum toxin B (vPA-BoNT). *left:* Two fragments of BoNT-B are fused to the iLID
690 photodimerizer subunits, with one part bound to the synaptic vesicle protein
691 synaptophysin (syp) and the second part present separately in the cytosol. In the dark, the
692 toxin remains inactive, and binding of Ca^{2+} to synaptotagmin (syt) triggers SNARE-mediated
693 fusion of synaptic vesicles. *right:* Light-induced association of ILID components recruits the
694 cytosolic component of vPA-BoNT to the synaptic vesicle. The reconstituted toxin cleaves
695 the SNARE protein VAMP2, which abolishes vesicle fusion. D, Time-course and effect size of
696 the different kinds of presynaptic optogenetic inhibition, plotted on a logarithmic time
697 scale. Underlying parameters for a simple kinetic model are provided in the table. Blue bars
698 indicate illumination (0.5 s for Arch/NpHR, mOPN3 and parapinopsin (*Lc*PPO), 5 min for
699 InSync/vPA-BoNT). Green bar indicates inactivating light flash for *Lc*PPO.
700

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

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

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

725 ***BOX 1: Validating presynaptic optogenetic inhibition***

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

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

745

746 **Confirming expression:**

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

752

753 **Confirming presynaptic inhibition:**

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

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

764 *vivo* or *in vivo* Ca^{2+} imaging at axon terminals*. One should keep in mind that a mild
765 suppression of presynaptic Ca^{2+} influx is sufficient for a strong reduction of transmitter
766 release, due to the non-linear dependency of exocytosis (power law exponent typically in
767 the range of 2-5) on the intracellular Ca^{2+} concentration.
768 *Determining the effect on postsynaptic network activity: In vivo* electrophysiological
769 recordings of postsynaptic unit activity or network oscillations, or imaging of neuronal
770 activity* can reveal changes in the strength of synaptic inputs during optogenetic
771 manipulation of incoming axons. Due to a high variability in network activity, such
772 recordings require multiple recording trials of the activity before and after light, and should
773 be supported by control recordings in the absence of optogenetic actuator activation (Fig.
774 5C).
775 *CAVE: avoid cross-activation of fluorescent probes and optogenetic tools.
776

777 **Presynaptic modulation and short-term plasticity**

778 GPCRs that couple to $G\alpha_{i/o}$ exert multiple effects at presynaptic terminals, namely inhibition
779 of Ca^{2+} influx, reduction of cAMP levels, and interference with the SNARE machinery.
780 Reducing presynaptic Ca^{2+} influx by GPCR signaling might not just simply lower synaptic
781 gain, but can introduce a high-pass filter on transmission[5]. In this scenario, transmitter
782 release is efficiently blocked by tonic GPCR activity during sparse firing. However, when
783 neurons fire at high frequencies, intracellular Ca^{2+} transiently accumulates. Moreover,
784 strong membrane depolarization during high-frequency AP bursts can relieve βγ-subunit-
785 mediated inhibition of Ca^{2+} channels[134]. Even if presynaptic inhibition greatly reduces initial
786 transmitter release in an AP burst, these effects may transiently elevate transmission
787 (relative to the initial AP) over the course of the AP train and shift the short-term dynamics
788 of transmission toward facilitation[5]. This may pose a problem if tonic synaptic silencing is
789 required for neurons firing at very high frequencies, as charge transfer may not be reduced
790 effectively during bursts of activity *in vivo*. Indeed synaptic facilitation was observed after
791 eOPN3-mediated inhibition of transmitter release[125], however, absolute EPSC amplitudes
792 evoked by 25 Hz stimulation under light were always significantly smaller than under
793 control conditions (Fig. 5D). Similarly, activation of NpHR increased the paired-pulse ratio of
794 two EPSCs at thalamocortical fibers[19] (Fig. 5B). It should be noted that such mechanisms are
795 synapse- and tool-specific. Presynaptic GPCRs can also reduce the synaptic gain by
796 mechanisms not affecting the vesicular release probability and without altering short-term
797 plasticity[177]. Thus, it is recommended to test the performance of presynaptic inhibitory
798 tools within the physiological range of spike frequencies of the targeted neurons.
799 Alternatively, inhibition by vesicle trapping and SNARE protein-cleavage was shown not to
800 alter paired-pulse behavior as a measure of short-term plasticity[40, 133], and might therefore
801 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.

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