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Optical control of neuronal ion channels and receptors

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Key words: receptors, ion channels, neuron, synapse, optogenetics, optopharmacology, photopharmacology; caged compounds, photoswitch

28 **Abstract**

29 Light-controllable tools provide powerful means to manipulate and interrogate brain function with
30 low invasiveness and high spatiotemporal precision. Although optogenetics permits neuronal
31 excitation or inhibition at the network level, other technologies such as optopharmacology (also
32 known as photopharmacology) have emerged that provide molecular level control by endowing
33 light-sensitivity to endogenous biomolecules. In this Review, we discuss the challenges and
34 opportunities of photocontrolling native neuronal signaling pathways, focusing on ion channels
35 and neurotransmitter receptors. We describe existing strategies for rendering receptors and
36 channels light-sensitive and provide an overview of the neuroscientific insights gained from such
37 approaches. At the cross roads of chemistry, protein engineering and neuroscience,
38 optopharmacology offers great potential in understanding the molecular basis of brain function
39 and behavior, with promises for future therapeutics.

40

41

42 **Introduction**

43 Ion channels and neurotransmitter receptors are the linchpin of brain function. They control
44 neuronal membrane potential and excitability as well as synaptic transmission and neuronal
45 communication¹⁻⁴. Neuronal ion channels and neurotransmitter receptors are involved in many
46 neurological and psychiatric disorders, and are thus major drug targets; an analysis in 2011
47 showed that more than 40% of all then-commercialized drugs targeted an ion channel or a
48 surface receptor⁵. Thus, there is enormous interest in understanding how these proteins work
49 and are regulated both for better comprehension of brain function and for therapeutic purposes.

50 Neuronal ion channels and receptors show great diversity, each family comprising
51 numerous members (or subtypes) with distinct distribution and properties. This heterogeneity
52 largely derives from the multigenic and multimeric nature of these protein families, resulting in a
53 wide variety of subunit assemblies. As examples, 77 genes encode potassium channel subunits
54 in humans, whereas 19 subunits encode pentameric GABA type A receptors (GABA_ARs) which
55 mediate the bulk of synaptic inhibition^{6,7}. The physiological relevance of such plurality is largely
56 unknown: whether individual ion channel or receptor subtypes carry out specific tasks or whether
57 redundancy prevails is unknown. Hopefully this diversity can be exploited for new and better
58 medicines. So far, research in electrophysiology, molecular biology, genetics, biochemistry,
59 pharmacology and structural biology have provided much information on the structure and
60 function of ion channels and receptors. However, none of these approaches combines the
61 proper molecular and spatiotemporal precision that is required to probe specific ion channel and
62 receptor subpopulations in live systems. Consequently there is great need for novel approaches
63 to manipulate rapidly and precisely different brain proteins with high molecular, cellular and
64 network control level.

65 Light permits superior spatiotemporal resolution and combined to genetic and pharmacology
66 allows precise manipulation of protein targets with defined molecular composition^{8,9}.
67 Optogenetics has driven huge advances in causal understanding of the neural circuits
68 underlying behaviour, and confers high temporal precision, on-off control of neuronal activity and
69 cell-type specificity¹⁰. However, classical optogenetics lacks the ability to probe the role of
70 specific receptors or signaling pathways in the control of physiology and behaviors.
71 Optopharmacology (also known as photopharmacology or optochemical genetics or synthetic
72 optogenetics) aims at filling this gap, by endowing light sensitivity to specific protein targets and
73 thus providing a molecular control and understanding of brain function.

74 Starting with photoreactive ligands in the late 1970s¹¹⁻¹⁴, the field of optopharmacology
75 boomed in recent years with the development of new methods directly tagging 'blind' proteins
76 (i.e. insensitive to light) with light-sensitive protein modules or synthetic chromophores¹⁵⁻¹⁸. Such
77 photoactuators allow the manipulation and interrogation of target proteins, and even individual
78 constitutive subunits, with unprecedented precision. Progress has been particularly applicable
79 and striking in neuroscience, where conventional in vivo pharmacology displays many inherent
80 limitations (slow diffusion, off targets, poor spatial confinement, and slow reversibility). In the
81 present review, we present current strategies to render neuronal receptors and ion channels
82 light responsive, and their applications for biophysical and neurophysiological studies.
83 Optopharmacology covers multiple scales from the molecular, synaptic, cellular up to the circuit
84 level. We also discuss practicalities and challenges for in vivo implementation in behaving
85 animals and translational potentialities in the clinic. Optopharmacological approaches are poised
86 to illuminate our understanding of the molecular and functional diversity of key brain
87 biomolecules with important consequences on neuronal signaling in the normal and diseased
88 brain.

89

90 **Optopharmacological approaches**

91 Three types of approaches have been developed to design of light-controllable receptors and ion
92 channels (**Fig. 1**): chemical approaches, which rely exclusively on exogenous photosensitive
93 chemicals (no modification of the target protein); genetic approaches, which require genetic
94 modification of the protein of interest; and hybrid approaches, which are two-component systems
95 involving both genetic and chemical modifications. As described below (and see **Table 1**), each
96 of these approaches has its own requirements, and concomitant advantages and limitations.
97 Choosing the right tool depends on the biological question and the amenability to genetic
98 modification of the target. Altogether, they provide a large toolset for precise manipulation of
99 membrane proteins and their associated signaling pathways with broad applicability in molecular
100 and neuronal studies.

101 ***Chemical approaches***

102 Photochemical approaches target native (that is unmodified, wild-type) proteins and were the
103 first to emerge in the late 1960s. The general idea is to confer light-sensitivity to a freely
104 diffusible ligand, rather than the target protein. Chemists have developed various
105 photochemicals for the control of neurotransmitter receptors and other signaling molecules,

106 including caged compounds, photoswitches and photolabels (**Fig. 1a**). Such chemical
107 approaches have one crucial advantage: they bestow light sensitivity to endogenous proteins
108 without requiring gene modification, and therefore act on native tissues and signaling molecules.
109 They may lack precise molecular target specificity, however.

110 *Caged compounds.* Caged compounds contain a photo-removable protecting group that is
111 cleaved upon light stimulation, resulting in a rapid release of biologically active molecules¹⁵. The
112 compound is inert (that is caged) in darkness, whereas uncaging following illumination triggers a
113 concentration jump of the molecule. Diffusible caged ligands are arguably the most widely used
114 photochemicals in neuroscience¹⁵. Several desirable properties of caged compounds explain
115 such wide use^{15,19,20}. They are inherently biologically inactive before photolysis while full
116 biological activity is recovered by irradiation. Their rate of uncaging is usually much faster than
117 most biological processes that are being studied and photolysis uses biocompatible wavelengths
118 not absorbed by proteins or DNA. Finally, the chromophoric byproducts are usually non-toxic.
119 Most neurotransmitters have been caged, including glutamate²¹⁻²⁴, GABA²⁵⁻²⁹, dopamine³⁰ and
120 serotonin³¹. Moreover, most of these have been made two-photon sensitive, allowing fine spatial
121 manipulation down to the dendritic spine level³². Caged molecules with refined pharmacological
122 spectra have also been developed, such as caged nicotinic³³⁻³⁶ and glycine³⁷ receptor agonists,
123 caged NMDA^{38,39} and kainate⁴⁰, or caged neuropeptide receptor agonists and antagonists⁴¹⁻⁴³,
124 enabling the control of specific receptor types or subtypes.

125 *Photoswitchable ligands.* Photoswitchable ligands (PLs, also known as photochromic ligands
126 (PCLs)) contain a photo-isomerizing group that, unlike caged compounds, have the major
127 advantage of being able to alternate reversibly between an active and an inactive form with two
128 different wavelengths of light^{18,44,45}. This results in a reversible on and off control of protein
129 targets. In some rare cases, PLs can covalently attach to an ideally positioned endogenous
130 reactive amino acid on the target wild-type receptor or ion channel^{11,14,46}. The use of two different
131 colors of light to drive the active and inactive states of molecular photoswitches means that
132 activation/deactivation (or inhibition/disinhibition) steps can be precisely time-controlled⁴⁷. PLs
133 have been designed for a huge variety of neurotransmitter receptors and ion channels^{8,17,18,48,49}
134 (**Table 1**). Examples include blockers of voltage-gated potassium^{45,50,51}, sodium^{52,53} and
135 calcium⁵⁴ channels, activators of GIRK channels⁵⁵, modulators of delayed rectifiers⁵⁶, ATP-
136 sensitive⁵⁷ and two-pore domain⁵⁶ K⁺ channels, activators of TRPV1 channels⁵⁸, agonists and
137 antagonists of ionotropic glutamate receptors (iGluRs)^{44,59-62}, agonists^{11,63} and pore blockers¹² of
138 nicotinic acetylcholine receptors (nAChRs), an agonist of adenosine receptors⁶⁴, an antagonist

139 of GABA_A receptors⁶⁵, and allosteric modulators of metabotropic glutamate receptors
140 (mGluRs)⁶⁶⁻⁷⁰ and of GABA_A^{71,72} receptors.

141 The most commonly used synthetic photoswitch is azobenzene (**Fig. 2**). Azobenzenes have
142 many advantageous attributes^{73,74}: they are small (length <10 Å) and easy to synthesize, display
143 high quantum yield (0.2-0.5) and minimal photobleaching, and can be rapidly and reversibly
144 switched between a bent, *cis* form and an extended, *trans* configuration using two different
145 colors, classically near-ultraviolet (UV) (360–400 nm) and blue-green light (480–550 nm).
146 Usually, the *trans* form is the most stable isomer (in darkness or visible light), whereas the *cis*
147 form gradually relaxes back to *trans* configuration in darkness with kinetics ranging from
148 milliseconds to days depending on the chemical substituents⁷⁵. ‘Bridged’ azobenzenes having a
149 two-carbon link between ortho-positions provide an exception whereby the *cis* configuration is
150 thermodynamically favored^{76,77}. In the case of slow relaxation lifetimes (in the hour timescale),
151 excellent bi-stability is reached without the need for long-duration light exposure. Rather brief
152 pulses of light are sufficient to rapidly toggle the azobenzene between its two states. To avoid
153 UV irradiation and thus limit phototoxicity and enhance biocompatibility, red-shifted azobenzenes
154 were recently developed^{51,78-84}. Red shift usually comes at the expense of faster spontaneous
155 *cis*-to-*trans* photorelaxation kinetics (ms to s; but see ref. ⁸⁵). Thus, whereas ideally one photo-
156 isomer of the azobenzene is fully pharmacologically inactive while the other is active, in practice
157 both isomers are active to some extent (for diffusible photoswitches at least), resulting in ten to
158 hundred-fold maximal differences in activity between the two isomers (250-fold in ref. ⁸⁶). In
159 addition, since the absorption spectra of the two isomers overlap to some degree, the
160 photostationary state ratio between the two isomers is classically around 95:5 (refs. ^{87,88}).
161 Remarkably, many pharmacological agents can be converted into photoswitchable drugs by
162 substituting an azobenzene-related structural motif for a genuine azobenzene moiety (termed
163 ‘azologization’; ref. ^{65,66,74,89}). Other photochromic chemicals besides azobenzenes include the
164 spiropyrans⁹⁰ and dithienylethenes⁹¹, but their use as photoswitches in biology remains limited.

165 *Photo-affinity labels*. Photo-affinity labels (PALs) contain a photoreactive group that is converted
166 to a reactive, alkylating entity upon photostimulation. PALs reversibly bind to receptors in
167 darkness but bind permanently after photo-activation; hence, they can ‘trap’ receptors or ligand-
168 activated ion channels in a permanently activated (or inhibited) state⁹²⁻⁹⁴.

169 *Chromophore-assisted laser inactivation*. Finally, chromophore-assisted laser inactivation (CALI)
170 relies on a synthetic dye (such as malachite green, fluorescein or eosin) that releases short-lived
171 reactive oxygen species upon photostimulation (mostly singlet oxygen), leading to damage and

172 irreversible inactivation of nearby proteins through amino acid side chain oxidation and protein
173 crosslinking^{95,96}. CALI can be targeted to specific endogenous membrane proteins using
174 antibodies⁹⁷.

175 **Genetic approaches**

176 Genetic approaches require the incorporation of a protein-based, light-sensitive module in the
177 target protein, resulting in a light-responsive receptor that is fully encoded genetically, without
178 any exogenous co-factors. Genetic and hybrid approaches have one essential superiority over
179 chemical approaches: they allow photosensitization with molecular and cellular specificity, a
180 crucial feature when it comes to dissecting discrete signaling pathways in complex circuits.

181 Various natural photoreceptors from animals, plants and microorganisms have been fused
182 to mammalian cytoplasmic signaling proteins and enzymes to render them photocontrollable⁹⁸,
183 but only in a few cases have they been fused to membrane receptors or ion channels. Natural
184 photoreceptors include opsins, which contain an endogenous retinal photoswitch (**Fig. 1b**), and
185 the light-oxygen-voltage (LOV)-sensing domains of flavoproteins, and cryptochromes (including
186 cryptochrome-2 (CRY2)), which are both from plants. Purely genetic methods are easier to use
187 than hybrid approaches, especially *in vivo*, since light-sensitivity is inherent to the protein itself
188 with its natural co-factor (virtually any mammalian cell produces enough quantities of retinal or
189 flavin photosensors).

190 *Opsins*. Opsins belong to the G-protein coupled receptor (GPCR) superfamily and can be
191 engineered as chimeric receptors with intracellular loops of (light-insensitive) neuromodulatory
192 GPCRs to produce light-controllable receptors (opto-XRs)⁹⁹. Although restricted to GPCRs, opto-
193 XR engineering has proved highly versatile enabling light control of signaling via adrenergic<sup>100-
194 102</sup>, adenosine¹⁰³, opioid^{104,105}, glutamatergic¹⁰⁶, dopaminergic¹⁰⁷, serotonergic¹⁰⁸⁻¹¹⁰ and even
195 orphan¹¹¹ receptors (**Table 1**).

196 *LOV domains and CRY2*. LOV domains contain a flavin mononucleotide chromophore, whereas
197 CRY2 use a flavin adenine dinucleotide chromophore. Blue light triggers large conformational
198 changes in these plant domains (**Fig. 1b**) — protein unfolding in LOV domains and dimerization
199 with CIB1 for CRY2 — that can be exploited to modulate protein-protein interactions and protein
200 localization with subcellular precision⁹⁸. These plant domains are relatively bulky protein
201 modules (~12-19 kDa) with inherently slow on and off kinetics. Consequently, they have been
202 mostly used to control slow cell parameters such as neurite outgrowth, intracellular signaling
203 cascades, gene expression or protein localization, and less often to manipulate rapid signaling

204 events such as neurotransmission¹¹². Ion channels can be indirectly photosensitized by fusing
205 LOV domains to partner proteins such as K⁺ channel toxins¹¹³ or the intracellular Ca²⁺ sensor
206 protein STIM1 that interacts with Ca²⁺ release-activated channels¹¹⁴. LOV domains can also be
207 fused to GTPases, thereby resulting in photocontrol of voltage-gated calcium channels¹¹⁵ or
208 dendritic spine size¹¹⁶⁻¹¹⁸. CRY2 has been successfully used as optogenetic devices to inhibit G
209 protein signaling in specific microdomains¹¹⁹ and to control neurotransmitter release using an
210 engineered botulinum neurotoxin¹²⁰. In only a few examples have LOV or CRY2 domains been
211 directly fused to the membrane protein itself, as demonstrated with potassium channels¹²¹,
212 tyrosine-kinase receptors^{122,123} or AMPA receptors (AMPA receptors) to control their abundance at
213 excitatory synapses¹²⁴.

214 *CALI*. Genetically-encoded fusion proteins can also be used in the CALI approach (**Fig. 1b**), as
215 tags to anchor the photosensitizer¹²⁵ or protein modules directly acting as singlet oxygen
216 generators¹²⁶.

217 **Hybrid approaches**

218 Hybrid approaches exploit the chemical and genetic methodologies to precisely manipulate and
219 control protein function with optochemical tools that are genetically anchored to or incorporated
220 in the desired membrane target.

221 *Unnatural amino acids*. One hybrid strategy relies on unnatural amino acids (UAAs), which can
222 be inserted at virtually any position into the target protein, including buried sites, using genetic
223 code expansion¹²⁷. This methodology hijacks the translational machinery through the
224 reassignment of a stop codon (usually amber) driven by a suppressor tRNA that is
225 aminoacylated with the UAA of interest^{128,129}. Light-responsive UAAs are diverse in
226 photochemical nature and have proved powerful tools to manipulate membrane receptors and
227 ion channels¹³⁰⁻¹³² (**Fig. 1c**). Amber stop codon suppression was used in particular to design
228 nAChRs harboring caged tyrosine^{133,134} and inwardly rectifying potassium channels incorporating
229 caged cysteine¹³⁵. As with diffusible caged compounds, caged amino-acid side-chains enable
230 time-controlled changes, abrupt and irreversible, in protein structure and function. UAAs carrying
231 photocrosslinking or photoswitchable moieties have also been incorporated into various
232 receptors and ion channels^{131,132} (see below). An advantage of photosensitive UAAs (and UAAs
233 in general) is that they can be inserted at any desired location in the target protein, including
234 solvent-inaccessible sites, although this UAA incorporation usually results in decreased protein
235 expression¹³².

236 *Photoswitchable tethered ligands.* Photoswitchable tethered ligands (PTLs) provide another
237 powerful and popular strategy to confer light responsiveness to receptors and ion channels. The
238 approach relies on the covalent attachment of a photoswitchable ligand either at close proximity
239 to its binding pocket through a single cysteine substitution^{136,137}, or remotely through
240 bioconjugation to larger protein tags (as in the case of photoswitchable orthogonal remotely
241 tethered ligands (PORTLs))¹³⁸⁻¹⁴⁰. In both cases, different wavelengths of light are used to
242 present or withdraw the pharmacologically active head group to or from its binding pocket (**Fig.**
243 **1c**). Bi-anchoring chemical photoswitches (nano-tweezers) are additional optopharmacological
244 tools that can control membrane proteins by evoking light-driven mechanical forces between two
245 cysteine-substituted protein segments¹⁴¹⁻¹⁴³ (**Fig. 1 c**).

246 PTLs in the form of tethered photoswitchable azobenzene-coupled ligands has proven
247 particularly efficient in photocontrolling neuronal receptors and ion channels^{8,17,48,49}. They are
248 tripartite molecules, with a central photoreactive azobenzene chromophore asymmetrically
249 substituted on one ring with a pharmacologically-active head group and on the other ring with a
250 cysteine-reactive moiety, usually a maleimide (for an example see **Fig. 2d**). The conjugation of
251 thiol groups of cysteine residues with maleimides is rapid (minutes time scale), highly selective
252 and irreversible, enabling permanent installation of photoswitchable effectors on the protein
253 target. Engineered light-controllable receptors and channels with PTLs include voltage-gated
254 potassium channels^{136,144}, two-pore domain potassium channels¹⁴⁵, ionotropic and metabotropic
255 glutamate receptors^{137,146,147}, nAChRs^{14,148}, GABA_ARs^{149,150}, purinergic P2X receptors¹⁵¹ and
256 dopamine receptors¹⁵² (**Table 1**). The kinetics of photocontrol with PTLs are greatly accelerated
257 because they are present in high local concentrations and cannot diffuse away¹⁵³. Another
258 advantage of such tethered ligands, when compared to freely diffusible photoswitches, is that
259 they provide excellent separation in biological activity between the *cis* and *trans* forms. Thus, in
260 the ideal realization, PTLs become truly binary photochemical switches for ion channels and
261 receptors. It also requires minimal modification (single amino acid substitution) of the target
262 protein. Perhaps the most distinctive advantage of PTLs for optical neurophysiology is that they
263 provide control over receptors with subtype specificity. For instance, the entire GABA_AR family
264 (α 1-6) was made photocontrollable using the PTL strategy¹⁵⁰. However, the PTL approach is
265 probably restricted to sites that are accessible to the external solvent for proper protein
266 conjugation, excluding intra-cellular and transmembrane protein domains.

267

268 **Probing receptors and ion channels**

269 Photo-responsive elements introduced at strategic locations in proteins or as ligands have been
270 used to drive changes in protein structure and function with unsurpassed precision. All core
271 biophysical features of receptors and ion channels — including ligand binding, activation and
272 gating kinetics, and ionic permeation — can be controlled using optopharmacology. Light
273 actuators can act as all-or-none switches, allowing direct channel opening or receptor activation,
274 or as regulators, modulating the activity of receptors or ion channels activated by their cognate
275 stimuli (**Fig. 2a**). Therefore, light can mimic agonists (full, partial or inverse), antagonists
276 (competitive or non-competitive), channel blockers and openers, or allosteric modulators (opto-
277 NAMs and opto-PAMs). Grading light intensity and/or wavelength confers another level for
278 refined control of the protein target (**Fig. 2b**).

279 *Mapping structural properties.* Photoaffinity labeling using photocrosslinking ligands (PALs, **Fig.**
280 **1a**) has been extensively used to map ligand binding sites on receptors and channels with
281 atomic details^{16,131}. This chemical approach allows direct identification of the amino acid residues
282 that contribute to a ligand binding site without a priori knowledge of the site of interaction.
283 Examples include the agonist-binding sites of nAChRs¹⁶ or the binding sites for general
284 anesthetics within transmembrane cavities of pentameric GABA_ARs^{154,155}. A photoactivatable
285 cross-linking UAA — classically azido-phenylalanine (AzF) or benzoyl-phenylalanine (BzF) —
286 moiety can also be genetically inserted into a protein. These UAAs crosslink upon UV
287 illumination and have been used to map the ligand-receptor interactions of several neuronal
288 GPCRs, including the neurokinin-1 receptor¹⁵⁶, cortico-releasing factor receptors type 1
289 (CRFR1)¹⁵⁷ and calcitonin gene-related peptide receptors (CGRPR)¹⁵⁸. Based on similar
290 covalent attachment to a nearby residue, photocrosslinking UAAs have been employed to trap
291 interacting proteins or to map subunit-subunit interfaces and their dynamics during channel
292 gating or receptor activation¹³². BzF crosslinking revealed a state-dependent interaction between
293 the voltage-gated K⁺ channel KCNQ1 and the β-subunit KCNE1^{159,160}. BzF and AzF also
294 enabled the capture of distinct functional states of iGluRs, including desensitized states of
295 AMPARs¹⁶¹, and inhibited¹⁶² or potentiated¹⁶³ states of NMDA receptors (NMDARs). Generally,
296 the irreversible nature of photocrosslinking allows the accumulation of trapped proteins in a
297 given conformational state, resulting in strong functional phenotypes.

298 *Controlling channel properties.* Azobenzene-based photoswitchable UAAs (PSAAs) provide
299 alternative ways to photocontrol biophysical attributes of target proteins. Introduction of PSAAs
300 within different NMDAR subunits and domains enabled rapid, reversible and allosteric regulation
301 of receptor channel open probability, agonist sensitivity and deactivation kinetics (**Fig. 2c**), as

302 well as modulation of ionic conductance and Mg^{2+} pore block¹⁶⁴, without directly interfering with
303 natural gating mechanisms. In that respect, PSAAs differ from most PTLs, which act as
304 surrogate receptor agonists¹³⁷ and abolish the need for natural agonist activation. Therefore, in
305 native settings, PSAAs can preserve natural patterns of receptor activation, whereas PTLs can
306 take over immediate control of receptor activation and signaling. As examples, fast
307 photoswitching using PTLs makes it possible to mimic synaptic activation profiles¹⁴⁷, whereas
308 tuning agonist deactivation kinetics using PSAAs¹⁶⁴, could be useful to assess how this gating
309 parameter sculpts the time window for synaptic plasticity and integration.

310 Pushing the photocontrol even further, photoswitchable nano-tweezers, which covalently
311 attach to two engineered cysteine residues, fully substitute the channel natural gating machinery
312 by triggering forceps-like motions on transmembrane helices upon azobenzene isomerization.
313 This technology permits direct light-dependent opening and closure of ligand-gated ion channels
314 such as P2X receptors^{141,142} or acid-sensing ion channels (ASIC)¹⁴¹. Opto-tweezers of various
315 sizes also provide useful molecular rulers to probe conformational rearrangements involved in
316 pore dilation and control of ion fluxes^{142,143}.

317 *Temporal and molecular precision.* Generally, optopharmacology offers tools of great precision
318 for the biophysical study of receptors and channels. In the temporal domain, caged agonists
319 permit rapid ligand delivery (<25 μ s) useful to probe receptor onset kinetics^{22,25,38,165,166}. Similarly,
320 fast light switching using PTLs allows submillisecond control over ligand binding and
321 unbinding¹⁵³, surpassing conventional techniques of studying receptors and channels that are
322 based on mechanical fast-perfusion systems and issues related to ligand diffusion. At the
323 molecular level, individual subunit accuracy is achievable. Using PALs as agonist analogs, the
324 contribution of sequential binding events to channel gating in multimeric complexes can be
325 dissected, as performed on tetrameric cyclic-nucleotide-gated channels⁹². Similarly, using PTLs
326 conjugated to specific subunits, the influence of individual subunit occupancy on receptor gating
327 can be resolved, as shown on homomeric and heteromeric iGluRs¹⁵³ (**Fig. 2d**) and mGluRs¹⁶⁷
328 glutamate receptors. Small single crosslinking groups and azobenzene groups seem to be
329 remarkably efficient photo-devices to gain real-time control of large molecular complexes and
330 their functionalities.

331

332 **Interrogation of neuronal physiology**

333 Optopharmacology opened new windows for the understanding of neuronal physiology by

334 allowing the control of ion channels and receptors in locations that are poorly accessible to
335 electrophysiological or pharmacological manipulations, such as sub-cellular compartments or
336 pre- vs. post-synaptic sites. Historically, caged neurotransmitters were the first photochemical
337 tools to provide major impact on neurobiology, especially caged glutamate and GABA^{19,168}.
338 Neurotransmitter uncaging has two important advantages for neuronal physiology. First, the
339 kinetics of release are very rapid (submillisecond time scale)^{22,25,38} and can mimic the time-
340 course of pre-synaptic neurotransmitter release. Second, the measured downstream effect
341 bypasses (and therefore is unambiguously dissociated from) upstream signal production. Hence,
342 laser uncaging enables stereotyped input to be applied at will to any part brain region or part of a
343 neuron in a highly reproducible manner^{168,169}. Glutamate uncaging can produce connectivity
344 maps between nearby but also distant neurons^{169,170}, a task that is nowadays routinely achieved
345 using channelrhodopsin¹⁰.

346 *Receptor and channel trafficking and subcellular compartmentalization.* Understanding how ion
347 channels and receptors traffic and compartmentalize in neurons has greatly benefited from
348 optopharmacological techniques. Specific receptors and ion channels can be manipulated or
349 isolated in a compartment-specific fashion using appropriate photoresponsive ligands and
350 illuminations patterns⁸⁸. Two-photon uncaging of glutamate or GABA permits functional mapping
351 of neurotransmitter receptor locations and densities individual-spine resolution^{28,171}. In brain
352 slices, the expression of functional AMPARs was found to tightly correlate with spine size and
353 geometry in pyramidal neurons, with mushroom spines containing the largest numbers of
354 receptors²³ (**Fig. 3a**). ANQX, a PAL antagonist that irreversibly silences surface AMPARs, was
355 used to investigate the trafficking of native AMPARs; this approach revealed that AMPAR
356 insertion at synapses occurs primarily through lateral diffusion from extrasynaptic sites⁹³.
357 Subcellular mapping of receptors using two-photon uncaging and subunit-specific PTLs also
358 revealed that $\alpha 5$ subunit-containing GABA_ARs are distributed evenly along dendrites of CA1
359 pyramidal neurons, whereas $\alpha 1$ -containing GABA_ARs are concentrated at (inhibitory)
360 synapses¹⁵⁰. Strikingly, in the same neurons no current was detected following local uncaging of
361 nAChR agonist, whereas large responses were detected on nearby CA1 interneurons, mostly at
362 perisomatic sites¹⁷². Similar experiments performed in the medial habenula showed that in
363 addition to concentrating close to (or at) the soma³⁵, nAChRs also populate axons³⁶, raising
364 questions about the source of acetylcholine and their physiological role. In the axon initial
365 segment (AIS) of gerbil auditory neurons, the contributions of specific ion channel conductances
366 to neuronal firing were dissected using AAQ as a light-sensitive channel blocker.
367 Hyperpolarization and cyclic-nucleotide-gated (HCN) channels located in the AIS, but not in the

368 soma or dendrites, were found to strongly influence excitability by controlling spike threshold and
369 probability¹⁷³ (**Fig. 3b**).

370 *Investigation of spine and dendritic function.* Analysis of spine and dendritic function has been
371 extensively studied using two-photon uncaging of glutamate²⁰. Although the z resolution of two-
372 photon uncaging (~1.6 μm) is larger than a spine head, laser light power can be tuned such that
373 the currents evoked by photolysis of caged compounds are the same as a single quantum
374 release²³. Direction of 60 such photochemical quanta at a small spine at 1-2 Hz induced
375 structural LTP lasting over 90 minutes (ref.²⁴) (**Fig. 3c**). Coupling two-photon uncaging with
376 genetically encoded fluorescence indicators allows imaging of: the insertion of AMPARs
377 associated with structural LTP¹⁷⁴; the local life-time of activation of CaMK-II¹⁷⁵; the spread of
378 RAS induced by LTP¹⁷⁶; the role of Rho during LTP^{177,178}; and long-distance ERK signaling¹⁷⁹
379 (for review see ref.¹⁸⁰). Structural LTP was also induced at single spines by directly photo-
380 agonizing synaptic light-sensitive NMDARs (LiGluNs) using PTLs¹⁴⁷ (**Fig. 3c**). Inversely, photo-
381 antagonizing LiGluNs optically prevented the induction of LTP at individual spines following
382 glutamate uncaging, demonstrating that caged compounds and PTLs can potentially be used in
383 concert¹⁴⁷.

384 Although one photon uncaging has contributed to our understanding on dendritic
385 integration¹⁸¹, two-photon uncaging at many individual spines along dendritic segments has
386 added a new level of detail by showing that forward-propagating dendritic spikes are initiated by
387 as few as 20 synchronous quanta (release events) on the same dendritic branch¹⁸². Pairing two-
388 photon calcium imaging with two-photon uncaging at single and multiple spines also allowed
389 detailed study of Ca^{2+} dynamics. The size of the spine neck was found to be crucial for the
390 retention of Ca^{2+} (ref.¹⁸³). Moreover, the sequence at which excitatory synapses are activated
391 turned up to be crucial for controlling the size of the local Ca^{2+} signal and determining how
392 neurons respond¹⁸⁴, confirming an old hypothesis of 'directional selectivity'¹⁸⁵.

393 *Control of synaptic physiology.* Synaptic physiology can also be controlled with cell type and
394 micro-domain specificity using genetic and hybrid approaches. For instance, photo-inactivation
395 of SNARE proteins fused with genetically encoded singlet oxygen generators fully disrupted
396 presynaptic vesicular release at targeted synapses in a spatially and temporally precise manner
397 in rat hippocampal slices and in behaving worms¹²⁶. Because it targets synaptic proteins, this
398 approach - known as inhibition of synapses with CALI (InSynC) - has a broad applicability in
399 understanding the contribution of individual synapses to the control of neural circuits and
400 behaviors.

401 PTLs and opto-XRs have the potential to probe the role of receptors and ion channels in
402 distinct synaptic compartments (including presynaptic, postsynaptic, and extrasynaptic
403 compartments) and in a cell-specific fashion (**Fig. 4**). For instance, a retrograde signal that
404 enhances recovery from presynaptic depression was revealed at the *Drosophila* neuromuscular
405 junction upon specific photo-activation of post-synaptic light-controllable kainate receptors
406 (LiGluRs)¹⁸⁶. The probability of neurotransmitter release can also be optically manipulated
407 through presynaptic expression of light-sensitive mGlu2 receptors (LimGluR2) in hippocampal
408 neurons, thereby affording precise control over short-term plasticity¹⁴⁶ (**Fig. 4a**). Synaptic and
409 extra-synaptic GABAergic inhibition was independently controlled using α 1- and α 5-containing
410 isoforms of light-sensitive GABA_ARs (LiGABARs), respectively, offering ways to evaluate the
411 impact of phasic vs. tonic inhibition in a neural circuit¹⁵⁰ (**Fig. 4b**). Similarly, NMDAR-mediated
412 currents can be precisely controlled at synapses using specific isoforms of LiGluNs. In
413 organotypic hippocampal slices, photo-antagonism of GluN2A-containing receptors during LTP
414 induction is sufficient to reversibly prevent morphological and functional changes, enabling 'time-
415 locked' gating of LTP¹⁴⁷. Finally, glial cell activity and gliotransmission were also manipulated
416 using PTLs. Exploiting the relatively high conductance and calcium permeability of LiGluRs,
417 light-evoked calcium elevations in cultured cortical astrocytes were shown to trigger non-
418 vesicular glutamate release¹⁸⁷, demonstrating the utility of LiGluR-mediated optopharmacological
419 approaches for studying electrically silent cells (**Fig. 4c**).

420 *Multi-chromophore approaches.* Synthetic chromophores offer the possibility of flexible
421 manipulation of wavelength absorption through spectral tuning, a property exploited in the
422 confocal imaging of various colors without any channel cross-talk. Pairing two-photon uncaging
423 of glutamate at 720 nm with blue light activation of ChR2 allows all-optical induction of structural
424 LTP¹⁸⁸ as caged glutamate does not absorb blue while ChR2 is not significantly activated by 720
425 nm light. Another possibility is to pair a blue-light-sensitive caged GABA with caged glutamate.
426 The poor two-photon cross-section of caged GABA prevents any measurable uncaging from
427 irradiation at 720 nm, thus single spine signaling of glutamate and GABA have been probed
428 using this approach^{189,190}. An elegant study using two-color actuation paired circuit-specific
429 expression of ChR2 in dopamine neurons with two-photon uncaging of glutamate on D1 targets
430 to unravel the timing window for the effects of dopamine on single spine plasticity in the nucleus
431 accumbens¹⁹¹. Recently, a new caging chromophore was developed that enables the
432 photochemical control of several important signaling molecules such as glutamate¹⁹², GABA¹⁹³,
433 and cyclic nucleotides^{194,195} with excellent chromatic independence, enabling two-color uncaging
434 and actuation with many wavelengths of light¹⁹⁶. Moreover, taking advantage of the broad

435 spectral properties of photoswitches as well as orthogonal strategies for their attachment,
436 manipulation of multiple receptor subpopulations simultaneously and independently is now
437 achievable ('multiplexing', ref. ^{138,139}). This opens interesting perspectives for studying potential
438 cross-talk between different neurotransmitter systems.

439

440 **In vivo optopharmacology**

441 One fundamental goal of modern neuropharmacology is to link causally changes in receptor
442 activity in given neuronal pathways with alterations of circuit physiology and ultimately behavior.
443 The utility of classical pharmacology in vivo is limited, because local drug delivery is slow,
444 imprecise, and hardly compatible with electrophysiology. In contrast, optochemical technologies
445 are, in principle, able to mimic the timing, amplitude and spread of naturally occurring
446 modulatory signals. In addition, the advent of *in vivo* optogenetics in the past decade¹⁰ has
447 pushed the advances of complementary technologies including multimodal fibers combining
448 optical, electrical and chemical interrogation. These permit local delivery of light and chemicals
449 in precisely defined brain regions, while simultaneously recording of neuronal activity^{197,198}.
450 Controlling the brain's own receptors and channels with light in the behaving animal is thus now
451 in reach.

452 Small organic, diffusible photochemicals have profoundly impacted neurophysiology
453 research in vitro (see above), but come with one major inherent drawback when it comes to in
454 vivo applications: they must be supplied continuously. In frog tadpoles or fish larvae, photoswitch
455 application and photocontrol are relatively easy, because these animals are transparent and the
456 photoswitch can be simply added to the swimming water⁶⁶. Diffusible opto-PAMs and opto-
457 NAMs of mGluRs⁶⁶⁻⁶⁸, and photoswitchable activators of GIRK channels⁵⁵, drove light-dependent
458 motility behavior in zebrafish larvae. A rhodamine-based photocontrollable TRPA1 agonist
459 (optovin) also enables rapid and reversible motor activity in paralyzed zebrafish¹⁹⁹. Similarly,
460 light-dependent perturbation of behavior in the nematode *Caenorhabditis elegans* was induced
461 by feeding the animals with photoswitchable ligands of nAChRs⁶³.

462 In rodents, reliable and quantitative application of diffusible photochemicals in nervous
463 tissue is more challenging. However, proof-of-concept for in vivo two-photon glutamate uncaging
464 has been demonstrated²⁰⁰. Two-photon uncaging of glutamate applied from the pial surface led
465 to single spine stimulation (assessed using calcium imaging and patch-clamp recordings) in
466 superficial cortical layers. The concentration of caged glutamate was estimated to be

467 approximately homogenous up to 200 μm below the surface, and these in vivo observations of
468 synaptic effects confirmed previous reports from brain slices²³. Further demonstration of the
469 feasibility of controlling neuromodulatory mechanisms in freely behaving rodents using diffusible
470 photochemicals was achieved in recent studies using photosensitized mGluRs drugs, which
471 were either 'azologized'^{68,69} or contained a photo-releasable protecting group²⁰¹..

472 Unlike other non-tethered small photochemicals, which are associated with diffusion-related
473 issues, quaternary ammonium photoswitchable blockers (including AAQ, QAQ and BENAQ)
474 ^{45,50,52,202-205} cross membranes and accumulate within cells, where they block voltage-gated ion
475 channels, and photosensitize neural tissue for days after a single infusion²⁰³. Such long-lasting
476 photosensitization of neurons enables restoration of visual functions in blind mice²⁰⁶ and control
477 of pain signaling in rats⁵². Another alternative to get around the diffusion issue is to immobilize
478 the photochemical at proximity to its target receptor, for instance using an antibody-based CALI
479 approach. The latter strategy was successfully implemented in mice to inactivate irreversibly
480 synaptic GluA1-containing AMPARs using specific eosin-conjugated antibodies locally delivered
481 either in the hippocampus or the lateral habenula, resulting in light-induced erasing of contextual
482 fear memory⁹⁷ or avoidance learning²⁰⁷, respectively.

483 The most straightforward means of limiting diffusion is to have the photochemical contained
484 within (or anchored to) the membrane protein itself, as in genetic methods. Ideally, the
485 engineered receptors should be controllable with kinetics that mimic those of neuronal
486 excitability and transmission, while preserving the properties of endogenous receptors (including
487 sensitivity to endogenous stimuli, coupling to signaling pathways, expression patterns and
488 levels, subcellular trafficking and recycling, and so on). In practice, these requirements have
489 proved difficult to combine. For instance, opto-XRs, which contain a naturally occurring retinal
490 group in their agonist-binding pocket, are insensitive to their cognate ligands^{101,102,105}. Hence,
491 opto-XRs cannot fully replace endogenous receptors, and require ectopic expression. This
492 results in mixture of receptor populations (exogenous and endogenous) which potentially
493 compete for subcellular targeting¹⁰⁸ or express at non-physiological levels.

494 Nonetheless, opto-XRs have proved useful for triggering specific intracellular GPCR
495 signaling pathways with high spatial and temporal precision (**Fig. 5a**). Photoactivation of opto-
496 β 2ARs, when expressed in principal neurons of the basolateral amygdala, modulates neuronal
497 activity and induces anxiety-like behavioral states¹⁰². Importantly, these opto- β 2ARs showed
498 similar signaling (cAMP production and MAP kinase activation) and dynamics (internalization) to
499 endogenous β 2ARs. Likewise, opto-MORs closely match wild-type μ -opioid receptors in terms of

500 signaling and trafficking, and can promote opposite behavioral responses (reward or aversion)
501 depending on which brain nuclei is targeted¹⁰⁵. Placing the C-terminal domain of the
502 serotonergic (5-HT_{1A} or 5-HT_{2c}) receptor onto vertebrate opsins yields chimeric opto-XRs that
503 signal and distribute at the sub-cellular level like their wild-type counterparts^{109,110}. Expressing
504 these chimeric receptors in the dorsal raphe nucleus of awake mice reveals how activation of 5-
505 HT_{1A} or 5-HT_{2c} receptors can reduce anxiety, although via different mechanisms. In general,
506 opto-XRs provide valuable optomolecular tools to study signaling cascades in vivo, yet how their
507 activation mimics that of endogenous receptors remains unsettled.

508 In hybrid approaches, single-cysteine or -UAA substitutions only perturb minimally target
509 proteins. Hence, the modified receptor or ion channel usually behaves like its wild-type
510 counterpart (for example, its sensitivity to endogenous neurotransmitters is unaffected), but is
511 photocontrollable^{147,150,208,209}. Nevertheless, implementing hybrid approaches to study brain
512 proteins in animals has been challenging, because of the dual requirement for gene and
513 photochemical delivery (**Fig. 5b**). In vivo use of PTLs (attached to an engineered cysteine) was
514 pioneered in zebrafish larvae²¹⁰ using LiGluRs. Since then, several studies have reported the
515 use of this technology in behaving zebrafish^{146,147,211,212}. In mice, the PTL approach was originally
516 implemented in the eye — a compartmentalized organ with ideal access to light and drug
517 delivery²¹³ — with the aim of restoring visual function^{214,215}. In most of these studies, however,
518 LiGluRs were utilized as binary on/off switches of action potential firing, as in classical
519 optogenetics, and not to interrogate receptor function. An arsenal of light-activatable and light-
520 inhibitable iGluRs and mGluRs of various subtypes is available^{17,49} but remains to be exploited
521 fully in vivo to understand how different GluR subtypes contribute to neural circuit operation and
522 behavior. Results using light-regulated NMDARs on zebrafish and highlighting the role of
523 GluN2A-containing receptors in the formation of sensory topographic maps have set a
524 precedent for this kind of work¹⁴⁷.

525 In recent years, cysteine-anchored PTLs were deployed in the mouse brain in vivo, first in
526 superficial cortical layers using LiGABARs¹⁵⁰ and LiGluRs²⁰⁸ and, more recently, in deep brain
527 areas and behaving mice using LinAChRs²⁰⁹. Importantly, even though free cysteines are
528 present on other extracellular sites on cells, this approach to photosensitizing receptors has
529 proved to be remarkably specific both in fish and mice, with wild-type neurons and animals being
530 unaffected by light after PTL treatment^{146,150,208,209}. Photoswitches can be applied topically to the
531 cortex after craniotomy and duratomy, but to be targeted deeper in the brain they must be
532 infused locally using cannula. Systemic photoswitch application is likely problematic, owing to

533 the strong lability and widespread reactivity of the maleimide group, but recent development of
534 self-labeling tags^{138,139} may help reduce off-target effects and fast degradation. Although the first
535 generation of PTLs required near-UV light to operate, they could be efficiently photocontrolled up
536 to 350 μm deep below the brain surface¹⁵⁰. Recently developed red-shifted and two-photon
537 sensitive azobenzenes^{84,216-219} should facilitate in vivo photocontrol with deeper penetrating
538 wavelengths of light.

539 Two options are available to express the mutated subunit: viral expression and transgenic
540 knock-in mice. A molecular replacement strategy using knock-in animals is the optimal option is
541 terms of ensuring unperturbed protein density and localization¹⁵⁰. Although viral transduction is
542 cheaper and quicker than transgenesis, expression levels may be altered (although not
543 systematically; see ref. ^{147,209}). Nonetheless, viral transduction has one important added value,
544 which is to afford circuit-specific targeting. For instance, LinAChRs over-expressed in the ventral
545 tegmental area (VTA) enabled acute and reversible inactivation of postsynaptic cholinergic
546 transmission, while leaving pre-synaptic nAChRs outside VTA afferents untouched²⁰⁹ (**Fig. 5b**).
547 Hence, LinAChRs could reveal, in real time, the cholinergic tone that is broadcasted, and its
548 impact, on VTA dopamine neuron firing in vivo²⁰⁹. On the other hand, light-controllable $\alpha 1$ -
549 containing GABA_ARs expressed in knock-in mice permitted direct photocontrol of visually-
550 evoked responses and revealed the contribution of such receptors in shaping gamma
551 oscillations in awake animals¹⁵⁰.

552 Because of constant protein turnover, PTL-labeled receptors are expected to decline in
553 abundance with time. Experimental evidence on LiGluRs indicates that this may not be a major
554 issue for long-lasting behavioral assays. Indeed, light-mediated responses in mice expressing
555 LiGluRs were maintained for one (ref. ²⁰⁸) up to fourteen (ref. ²¹⁴) days after a single bolus supply
556 of photoswitchable ligand demonstrating stable labeling and surface expression of the tagged
557 receptors for prolonged periods of time.

558 Incorporation of photosensitive UAAs for in vivo studies of neuronal proteins remains in its
559 infancy, although proof-of-concept experiments in various organisms including rodents^{135,220-223}
560 indicate that feasibility is in reach. In brain slices, a photocontrollable inwardly rectifying
561 potassium (Kir2.1) channel incorporating a caged cysteine in the channel pore region was
562 successfully expressed in the mouse neocortex¹³⁵, allowing acute light-induced suppression of
563 neuronal firing. With its site flexibility and genetic encodability, UAA-based photocontrol
564 combines several advantages; however, the system requires multiple components to be
565 delivered — including the orthogonal tRNA/synthetase pair and the mutated target protein of

566 interest — and so its in vivo implementation is technically difficult. The bioavailability of the UAA
567 poses another hurdle. Recent successful attempts to address these challenges include the
568 generation of transgenic mice incorporating, in their genome, the necessary tRNA- and
569 synthetase-encoding genes needed for amber stop codon suppression^{221,223}. In principle,
570 photoresponsive UAAs provide flexible means not only for optical control of protein activities but
571 also for the identification of interacting partners within signaling complexes (using photo-
572 crosslinking UAAs; ref. ¹³¹).

573

574 **Concluding remarks and perspectives**

575 With its high flexibility and versatility, optopharmacology yielded a large repertoire of light-
576 tunable ion channels and neurotransmitter receptors allowing fast and remote control over
577 specific neuronal signaling pathways at specific locations within a cell, a network or an organism.
578 By establishing causal relationships between a protein activity and a cellular or behavioral
579 output, optopharmacology appears unrivaled to unlock brain molecular logic. It also holds strong
580 potential for identifying new therapeutic targets in neurological disorders (Box 1). It is thus
581 possible to explore the role and signaling mechanisms of numerous receptor and ion channel
582 subtypes in their natural environment in normal and diseased states. Virtually any protein,
583 membranous or soluble, is amenable to optopharmacological engineering provided that its
584 activity is controllable by interacting ligands or binding partners. The greatest molecular
585 specificity can be obtained from light-activated chemicals that are tethered to specific proteins
586 (such as PTLs). On the other hand, approaches that rely solely on synthetic ligands (such as
587 caged compounds and PLs) have the major advantage not to require genetic modifications of
588 the target protein. Both approaches require synthetic chemistry, however, which can be arduous
589 and costly. Naturally occurring photosensitive proteins, usually from plants of microbes and
590 which bypass the need for chemical synthesis, offer an alternative. They can be easily spliced
591 onto mammalian proteins enabling light-controlled of proteins sitting on the cell surface or
592 intracellularly. Booming genome sequencing from various organisms (including non-animal)
593 augurs bright days for biotechnological applications using native light-sensitive modules.
594 Neuroscience through perfected optogenetics and optopharmacology should greatly benefit from
595 these advances. However, one serious limitation remains to implementing light-based
596 approaches in behaving animals: the necessity to deliver light to the cells of interest often
597 requires invasive surgery. Novel approaches based on transcranial acoustic²²⁴ or magnetic²²⁵
598 stimuli to remotely manipulate neurons and biochemical pathways may overcome this hurdle, yet

599 face many technical obstacles. Undoubtedly light-based approaches such as optopharmacology
600 will continue shaping new horizons to tackle fundamental processes in brain physiology and
601 pathology.

602

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1140

1141 **Box 1 | Applications in biomedicine**

1142 Photocontrollable drugs and receptors may open up exciting new opportunities for light-guided
1143 therapeutic interventions^{206,226}. Light delivered at the appropriate wavelength and intensity could
1144 potentially trigger signals at specific time and places and with accurate dosing pattern, thereby
1145 reducing off-target effects. This concept has shown promise in animal models for two
1146 neurological applications: vision restoration and pain management.

1147 ***Vision restoration***

1148 Degenerative retinal diseases are characterized by progressive loss of photoreceptors,
1149 ultimately leading to blindness. One therapeutic strategy for advanced retinal degeneration, after
1150 photoreceptors have been lost completely, is to photosensitize remaining retinal neurons.
1151 Photoswitchable blockers of voltage-gated ion channels^{45,50-52} have been tested for their ability to
1152 photosensitize the blind retina in animal models of retinal degeneration^{202-205,227}. The first
1153 generation elicited robust light responses in blind retinas *ex vivo*, and restored the pupillary light
1154 reflex and light-avoidance behavior in blind mice²⁰². Newly developed, improved photoswitches
1155 that fully operate in the visible spectrum and that quickly relax back to *trans* in darkness were
1156 later designed, eliminating the need for two different wavelengths of light (including potentially
1157 damaging UV light) for on and off control. These molecules photosensitized blind retinas from
1158 mice and rats²⁰³⁻²⁰⁵, and restored visual fear conditioning behaviors in blind mice²⁰³. One
1159 photoswitch, named BENAQ, photosensitized blind retinas for almost a month following a single
1160 intraocular injection of low micromolar photoswitch solution²⁰⁵. Interestingly, certain
1161 photoswitches do not photosensitize all retinal neurons indiscriminately²⁰⁴, and therefore permit
1162 restoration of complex visual processing in the blind retina, such as ON and OFF light
1163 responses²²⁷.

1164 Completely restoring visual functions may still require cell-specific targeting of retinal
1165 neurons, for instance using optogenetic gene therapy. A phase II/II clinical trial with
1166 channelrhodopsin-2 for advanced retinitis pigmentosa is currently underway (clinical trial
1167 #NCT02556736)²²⁸. Meanwhile, other optogenetic technologies based on engineered
1168 mammalian receptors, which may be less immunogenic, are being preclinically evaluated.
1169 Expression of LiGluRs (included red-shifted versions) in retinal ganglion cells or ON-bipolar cells
1170 restored light sensitivity to blind retinas from mice and dogs^{213,214}. A combination of two
1171 orthogonal systems, LiGluR and SNAG-mGluR2 (a light-controllable mGluR with a remotely-
1172 tethered photoswitch) improved ON/OFF responses and enhanced visual acuity²¹⁵. Finally, the
1173 mGluR-melanopsin chimera opto-mGluR6, which, unlike LiGluRs does not require photoswitch

1174 delivery, is another interesting alternative to restore ON responses in blind mouse retinas with
1175 moderate light levels¹⁰⁶.

1176 ***Pain management***

1177 Many ion channels and receptors expressed at the periphery and/or centrally are involved in
1178 nociception²²⁹, and represent obvious targets for focused and photo-titrated analgesia. Photo-
1179 controllable drugs potentially useful for controlling pain signaling include TRPV1 agonists and
1180 antagonists^{58,230}, TRPA1 agonists¹⁹⁹, μ opioid receptor agonists²³¹, GABA_AR potentiators⁷¹,
1181 blockers of voltage-gated sodium channels^{52,232} and mGluR modulators^{66-69,201}.
1182 Photochemical^{41,231} or optogenetic¹⁰⁵ regulators of opioid signaling could potentially provide
1183 analgesia at peripheral targets, while avoiding brain pathways linked to addiction.
1184 Photoswitchable allosteric modulators of mGluRs - one an opto-PAM of mGlu4⁶⁹, and the other
1185 an opto-NAM of mGlu5⁶⁸ - function as photoreversible analgesics in behaving mice, at peripheral
1186 nerve endings and in the amygdala^{68,69}. Photoreversible local anesthetics (QAQ and QENAQ)
1187 also demonstrated spatio-temporal and phototitrable control of pain signaling in behaving
1188 rodents^{52,232}. In addition, QAQ and QENAQ only enter and silence TRPV1-expressing (that is,
1189 pain-sensing) neurons, minimizing undesirable off-target effects on other sensory neurons.

1190 ***Outlook***

1191 A major hurdle for implementing optopharmacology in clinical settings is light delivery to precise
1192 locations in the body, the eye being an obvious exception. Recent advances in preclinical
1193 developments have generated light-delivery devices that are miniaturized, injectable,
1194 programmable and wirelessly controlled, affording efficient remote photocontrol and minimal
1195 damage to neural tissue²³³⁻²³⁷. Concomitant local drug delivery is also possible with wireless
1196 opto-fluidic probes that combine a micro-LED with soft microfluidic delivery systems²³⁸. In
1197 parallel, photochemicals that respond to deeper-penetrating red or infra-red light⁸⁴ have been
1198 developed, possibly eliminating the need for LED implantation. Despite rapid technological
1199 advances, optopharmacological approaches are still far from ready to use in the clinic.
1200 Tolerability and reduced invasiveness to liberate the subject's mobility remain significant
1201 challenges for clinical success.

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1204

1205 **Figure Legends**

1206 **Fig. 1 I: Main strategies to endow light-sensitivity to neuronal receptors and ion channels.**

1207 **a I** Chemical approaches are based exclusively on modified ligands that are rendered
1208 responsive to light (hv). No modification of the protein target is required. With caged compounds,
1209 light triggers the release of biologically-active ligands. Photoswitchable ligands reversibly
1210 alternate between active and inactive forms using two different wavelengths of light. Photolabels
1211 bind a target receptor and upon light irradiation form a covalent link with the protein binding site.
1212 Contrasting with photoswitchable ligands, caged compounds and photolabels act irreversibly.

1213 **b I** Genetic approaches are based on the insertion of genetically encoded light-sensitive protein
1214 modules in the target protein of interest. Color coding: light and dark grey represent two
1215 different states (e.g. inactive and active) of the protein. Light-sensitivity is endowed by
1216 photosensitive co-factors (such as retinal or flavin) that are endogenously present in mammalian
1217 cells. Opsin chimeras consist in the fusion between a light-sensitive opsin (grey) and a light-
1218 insensitive G-protein coupled neurotransmitter receptor (black). Illumination triggers the
1219 conversion from 11-*cis* to 11-*trans* retinal, which causes conformational change in the fusion
1220 protein and its activation. In LOV-domain chimeras, light triggers unfolding of the LOV domain,
1221 which directly or indirectly modulates protein function. In CALI, light activates the flavin
1222 chromophore and generates reactive oxygen species, which results in irreversible inactivation of
1223 nearby proteins (shown in white with dashed borders).

1224 **c I** Hybrid or chemogenetic approaches are based on a photosensitive synthetic chemical and its
1225 genetic attachment to or incorporation within the target protein. Different types of light-sensitive
1226 unnatural amino-acids (UAAs) can be incorporated into proteins: caged, photocrosslinking (i.e.
1227 photolabels), or photoswitchable (i.e. alternating between two configurations upon illumination
1228 with different wavelengths) as depicted. Photoswitchable tethered ligands can be covalently
1229 attached to receptors in two ways: either to a cysteine-substituted site usually through
1230 maleimide-sulfhydryl chemistry (as depicted), or to a self-labeling protein tag (not depicted),
1231 resulting in both cases in reversible control of protein activity. In nanotweezers, a bis-maleimide
1232 photoswitch bridges two cysteine mutants. Light-induced conformational changes of the
1233 photosensitive moiety exert mechanical forces on the protein, potentially triggering its activation
1234 in the absence of ligand. Note that caged compounds, photolabels and CALI are unidirectional
1235 while photoswitches (both synthetic and natural) allow for bidirectionality.

1236

1237 **Fig. 2 | Photocontrol of ion channel and receptor biophysics and pharmacology.**

1238 **a |** With appropriate optopharmacological tools, light can be used to directly activate receptors or
1239 ion channels (agonist), to inhibit them (antagonist or negative allosteric modulator (NAM)), or to
1240 positively modulate their function (positive allosteric modulator (PAM)).

1241 **b |** Activity of the target receptor or ion channel can be adjusted in a graded manner using
1242 different light intensity and/or wavelength. The graph illustrates various levels of photo-
1243 antagonism associated with changes in light intensity or wavelength.

1244 **c |** Photomodulation of NMDA receptors (NMDARs) using photoswitchable amino acids
1245 (PSAAs). Left: Chemical structures of *trans* and *cis* PSAA in the context of a protein. The
1246 azobenzene moiety is highlighted in shaded grey. Right, upper part: Schematic representation of
1247 the mechanism of NMDAR photomodulation. The PSAA is incorporated in the GluN1 subunit
1248 (dark grey), close to the GluN2 subunit (light grey). GluN1 binds glycine (orange), whereas
1249 GluN2 binds glutamate (red). Right, lower part: Illumination with 365 nm light isomerizes PSAA
1250 to *cis*, a conformational change sufficient to destabilize glycine binding (that is, leading to a
1251 decrease in glycine affinity). This change results in a reduction of current amplitude during
1252 agonist applications (Glu, glutamate; Gly, glycine) (left trace) and in an acceleration in
1253 deactivation kinetics upon glycine washout (right trace; current normalized). I, current amplitude;
1254 $I_{norm.}$, normalized current amplitude; t, time.

1255 **d |** Photocontrol of kainate receptors using photoswitchable tethered ligands (PTLs). Left:
1256 Chemical structures of the PTL maleimide azobenzene glutamate (MAG) in the *trans* and *cis*
1257 configurations. The azobenzene moiety is highlighted in shaded grey. Right, upper part:
1258 Schematic representation of heteromeric GluK2/K5 kainate receptors. MAG is covalently
1259 attached to an engineered cysteine residue on the GluK2 subunit (light grey). Under 380 nm
1260 light, MAG adopts its *cis* configuration allowing the glutamate moiety to dock in the agonist
1261 binding pocket. Right, lower part: Heteromeric kainate receptors conjugated with two PTLs can
1262 be directly activated with 380 nm light which isomerizes the photoswitch to *cis*, and deactivated
1263 with 500 nm light which reverts the photoswitch to *trans* (left trace). In such conditions, no or little
1264 desensitization is observed. In contrast, when two agonists (pale red) are pre-bound selectively
1265 to GluK5 subunits, photoswitching leads to full receptor occupancy and almost complete
1266 receptor desensitization (right trace). Part **c** is adapted from ref. ¹⁶⁴. Part **d** is adapted from ref.
1267 ¹⁵³.

1268

1269 **Figure 3: Optopharmacology for subcellular neuronal studies.**

1270 **a.** Functional receptor mapping. Left: Chemical structure of MNI glutamate and photorelease of
1271 glutamate following either one-photon (1P) or two-photon (2P) illumination. Right: Two-photon
1272 uncaging of glutamate at different spots along the dendrite for functional glutamate receptor
1273 mapping. Receptor activity is revealed using whole-cell patch-clamp recordings in voltage-clamp
1274 mode. I, current amplitude; t, time.

1275 **b.** Compartment-specific blockade of HCN channels using the photoswitch AAQ. Left: Chemical
1276 structures of *trans* and *cis* AAQ. Middle: Schematic representation of photoreversible HCN
1277 blockade using AAQ. Right: Probability of spiking is increased when HCN channels are blocked
1278 in the axon initial segment (AIS), but probability of spiking is decreased when these channels are
1279 blocked in the soma. Neuronal excitability is measured using whole-cell patch-clamp recordings
1280 in current-clamp mode. V_m , membrane potential; t, time.

1281 **c.** Single-spine structural LTP induced using two-photon (2P) uncaging of glutamate or one-
1282 photon (1P) activation of light-controllable NMDA receptors (LiGluN) expressed in transfected
1283 neurons (green). The left and middle cartoons show how illumination leads to LiGluN activation,
1284 while the right cartoon depicts the resulting long-lasting changes in dendritic spine morphology.
1285 The graph on the right illustrates the evolution of the spine volume as a function of time before
1286 and after LTP induction (red arrow). Part **a** is adapted from ref. ²³. Part **b** is adapted from ref. ¹⁷³.
1287 Part **c** is adapted from ref. ²⁴ and ref. ¹⁴⁷.

1288

1289 **Figure 4: Optopharmacology for cell- and receptor-specific interrogation of synaptic**
1290 **physiology.** In all panels, the cell selectively expressing the photosensitized receptors is shown

1291 in green. **a** | Optical control of neurotransmitter release using light-controllable metabotropic
1292 glutamate receptors (LimGluRs) expressed in axon terminals. Photo-antagonizing LimGluR with
1293 380 nm light decreases neurotransmitter release and post-synaptic currents (bottom traces). I,
1294 current amplitude; t, time.

1295 **b** | Optical control of post- and extra-synaptic currents. Phasic (synaptic) and tonic (extra-
1296 synaptic) GABA_A receptor-mediated inhibitory currents (bottom traces) can be photo-
1297 antagonized under 380 nm light using α 1- and α 5-containing light-inhibitable GABA_A receptors
1298 (LiGABARs), respectively. I, current amplitude; t, time.

1299 **c** | Optical control of gliotransmission using light-activatable glutamate receptors (LiGluRs)
1300 expressed in astrocytes. When LiGluRs are activated with pulses of 380 nm light, intracellular

1301 calcium concentration increases (bottom trace) triggering non-vesicular release of glutamate.
1302 $\Delta F/F$, changes in calcium-dependent fluorescence; t, time. Part **a** is adapted from ref. ¹⁴⁶. Part **b**
1303 is adapted from ref. ¹⁵⁰. Part **c** is adapted from ref. ¹⁸⁷.

1304

1305 **Fig. 5 IOptopharmacology for behavioral studies**

1306 **a I** In vivo optical manipulation of GPCR signaling with opto-XRs. A guide cannula for
1307 concomitant photocontrol and electrical recording is depicted. Inset: Light-stimulation of opto-
1308 β 2AR activates downstream $G\alpha s$ pathway in transduced neurons, leading to an increase in
1309 intracellular cAMP and Ca^{2+} concentrations, to the phosphorylation of ERK, and eventually to an
1310 increase in cellular excitability.

1311 **b I** In vivo optical control of neurotransmitter receptors and associated behaviors with PTLs. The
1312 cannula guide allows for local delivery of the photoswitch and light as well as for electrical
1313 recordings. Inset: Schematic showing conditional expression of LinAChRs in dopamine (DA)
1314 neurons of the ventral tegmental area (VTA) using a Cre-dependent expression system (AM,
1315 unpublished data). In this scenario, LinAChRs are absent in other neurons of the VTA or in
1316 cholinergic afferents from extra VTA regions, allowing acute disruption of nicotinic transmission
1317 at the post-synaptic level. The cell selectively expressing LinAChRs is shown in green. In the
1318 original study²⁰⁹, LinAChRs were non-selectively expressed in both DA and non-DA cells of the
1319 VTA. Top right: Spontaneous activity of VTA DA neurons is reduced under 380 nm light, when
1320 LinAChRs are photo-antagonized. Bottom right: Behavioral experiment using the nicotine-
1321 induced conditional place preference test. Preference to nicotine is reversibly disrupted under
1322 380 nm light. Part **a** is adapted from ref. ¹⁰². Part **b** is adapted from ref. ²⁰⁹.

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1325

1326	Glossary
1327	<i>Cis-to-trans</i> isomerization
1328	Photostationary state
1329	Metabotropic and ionotropic receptors
1330	Singlet oxygen generators
1331	Genetic code expansion
1332	Allosteric modulation
1333	
1334	Acronyms (in addition to acronyms of optopharmacological tools listed in Table 1)
1335	CALI: Chromophore-Assisted Laser Inactivation
1336	CRY2: Cryptochrome 2
1337	FAD: Flavin Adenine Dinucleotide
1338	FMN: Flavin Mononucleotide
1339	LOV: Light-Oxygen-Voltage
1340	MAG: Maleimide Azobenzene Glutamate
1341	Opto-NAM: photoswitchable negative allosteric modulator
1342	Opto-PAM: photoswitchable positive allosteric modulator
1343	Opto-XR: light-controllable G-protein coupled receptor
1344	PAL: PhotoAffinity Label
1345	PL (or PCL): Photoswitchable Ligand (or PhotoChromic Ligand)
1346	PORTL: Photoswitchable Orthogonal Remotely Tethered Ligands
1347	PSAA: photoswitchable unnatural amino acid
1348	PTL: Photoswitchable Tethered Ligand
1349	UAA: Unnatural Amino Acid
1350	

1351 **Table 1 | Main optopharmacological tools used in neurophysiology.**

1352 All methods, either purely chemical, purely genetic or hybrid, have strengths and weaknesses.
 1353 Key advantages of each approach are amply discussed in the Main Text, whereas main
 1354 drawbacks are listed in the far right column. Choosing one approach or the other depends on the
 1355 biological problem at hand, and on the genetic tractability of the target protein.

1356

Tool	Name (full definition)	Action	Refs	Limitations
<i>Chemical</i>				
Caged compounds	MNI-Glu (4-methoxy-7-nitroindoliny- caged-L-glutamate)	Activates glutamate receptors in UV light	²³	<ul style="list-style-type: none"> - Requires chromophore delivery - Irreversibility - High concentrations required (potential off target effects, in vivo use limited) - Should be inert and stable in darkness
Diffusible Photoswitches	AAQ (acrylamide azobenzene quaternary ammonium)	Blocks voltage-gated K ⁺ and HCN channels in dark or green light	45,50,173,203	<ul style="list-style-type: none"> - Requires chromophore delivery - Activity difference between the two isomers can be limited (no all-or-none effects) - Require UV illumination (although red-shifted versions)
	DENAQ (diethyl aminoazobenzene quaternary ammonium)	Blocks voltage-gated K ⁺ and HCN channels in dark	51,203,204	
	BENAQ (benzyl ethyl aminoazobenzene quaternary)	Blocks voltage-gated K ⁺ and HCN channels in dark	51,203,204	

	ammonium)			available)
	QAQ (quaternary ammonium azobenzene quaternary ammonium)	Blocks voltage-gated K^+ , Na^+ and Ca^{2+} channels of TRPV1-expressing neurons in dark or green light	45,52,232	
	QENAQ (quaternary ammonium aminoazobenzene quaternary ammonium)	Voltage-gated K^+ , Na^+ and Ca^{2+} channels in dark	232	
	LOGO (light-operated GIRK-channel opener)	Activates GIRK channels in dark	55	
Photo-affinity labels	ANQX (6-azido-7-nitro-1,4-dihydroquinoxaline-2,3-dione)	Inactivates AMPA receptors in UV light	93	- Requires chromophore delivery - Requires UV light
Genetic				
Opto-XRs	Opto- α 1AR Opto- β 2AR	Activates adrenergic receptors (α 1 and β 2) in blue light	100-102	- Requires gene delivery - Fusion proteins do not bind cognate neurotransmitters
	Opto-A2A	Activates adenosine receptors (A2A) in blue light	103	- Applicability restricted to GPCRs
	Opto-MOR	Activates opioid receptors (μ) in blue light	104,105	

	Opto-mGluR6	Activates metabotropic glutamate receptors (mGlu6) in blue light	¹⁰⁶	
	Opto-D1R	Activates dopamine receptors (D1) in blue light	¹⁰⁷	
	CT-5HT	Activates serotonergic receptors (5HT2c and 5HT1a) in blue light	¹⁰⁸⁻¹¹⁰	
	Opto-GPR1–183	Activates orphan receptors in blue light	¹¹¹	
LOV or CRY2 domains	Lumitoxin	Blocks voltage-gated K ⁺ channels (Shaker, Kv1.1, Kv1.2) in dark	¹¹³	- Requires gene delivery - Slow on and off kinetics (min - hours)
	BLINK1 (blue-light-induced K ⁺ channel 1)	Activates small viral K ⁺ channels Kcv in blue light	¹²¹	- Relatively large protein domain (>12 kD)
CALI	InSynC (inhibition of synapses with CALI)	Inactivate SNARE proteins (VAMP2 and Synaptophysin) with blue light	¹²⁶	- Requires gene delivery - Irreversibility - Inactivation only (no activation)
Hybrid				
Tethered photoswitches	SPARK (synthetic photoisomerizable azobenzene-regulated K ⁺	Blocks voltage-gated (Shaker, Kv1.3, Kv3.1, Kv3.4, Kv7.2) and	^{136,144}	- Requires gene + photoswitch delivery

	channel)	calcium-activated (SK2) K ⁺ channels in dark		- Stability of the <i>cis</i> configuration
	TREKLight (light-gated TREK channel)	Blocks two-pore domain (K2P) K ⁺ channels (TREK1) in dark	145	- Restricted to solvent-accessible sites (usually extracellular)
	LiGLuR (light-gated ionotropic glutamate receptor)	Activates kainate receptors (GluK2) in UV light (alternative version using blue, yellow or red light exist)	80,81,137	- Potential for unspecific cysteine conjugation (yet no adverse effect observed so far, and see PORTL for alternative, orthogonal bioconjugation)
	LiGluN (light-gated NMDA receptor)	Activates or inhibit NMDA receptors (GluN1, GluN2A, GluN2B) in UV light	147	- Requires UV illumination (although red-shifted versions available)
	LimGluR (light-gated metabotropic glutamate receptor)	Activates or inhibit metabotropic glutamate receptors (mGlu2, mGlu3 and mGlu6) in UV or blue light	146,218	
	SNAG-mGluR (SNAP-tagged-azobenzene-glutamate receptor)	Activates metabotropic glutamate receptors (mGlu2, mGlu7, mGlu8) in UV or blue light	138,139	
	LinAChR (light-controlled nicotinic acetylcholine receptor)	Activates or inhibit nicotinic acetylcholine receptors (β 2 and β 4) in UV light	148	

	LiGABAR (light-controlled GABA type A receptor)	Antagonize GABA _A receptors (α 1-6) I, UV or dark	149,150	
	LiDAR (light-controlled dopamine receptor)	Inhibit dopamine receptors (D1 and D2) in UV light	152	
	Light-activated P2X receptors	Activates P2X receptors in UV or green light	151	
Nanotweezers	Light-controlled P2X receptors and Acid-sensing ion channels (ASICs)	Activates P2X receptors or ASICs in UV or green light	141-143	<ul style="list-style-type: none"> - Requires gene + photoswitch delivery - Specificity and toxicity not determined in vivo - Applicability to other receptor types unknown - May hinder ion flow and kinetics
UAAs	PSAA (azobenzene-based photoswitchable amino acid)	Any (photoswitch)	164	<ul style="list-style-type: none"> - Requires gene + UAA delivery - Only UV-sensitive probes available
	AzF (azido-phenylalanine)	Any (photocrosslinker)	131,162	<ul style="list-style-type: none"> - Expression level of target protein potentially affected
	BzF (benzoyl-phenylalanine)	Any (photocrosslinker)	131,161	

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Fig 1

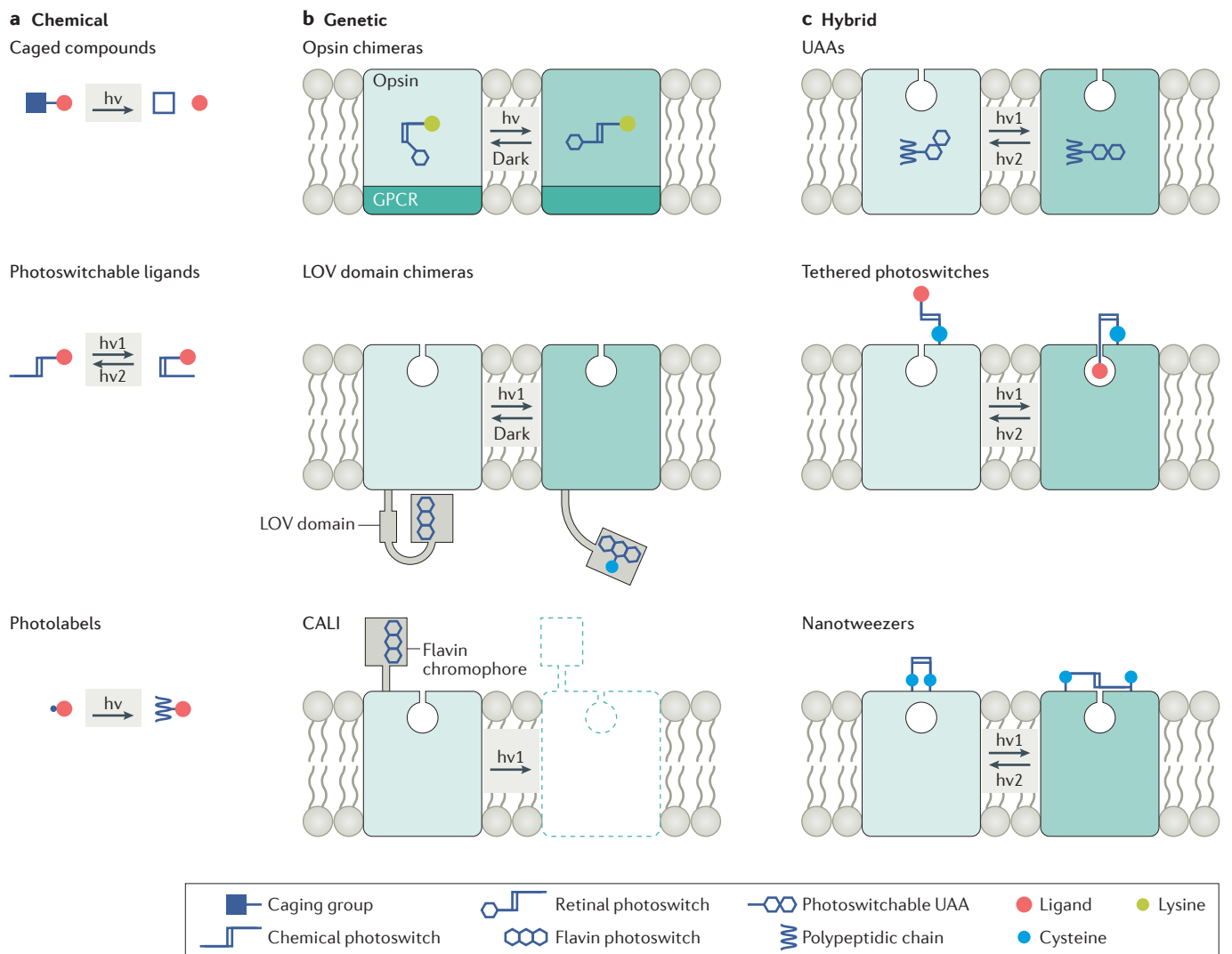


Fig 2

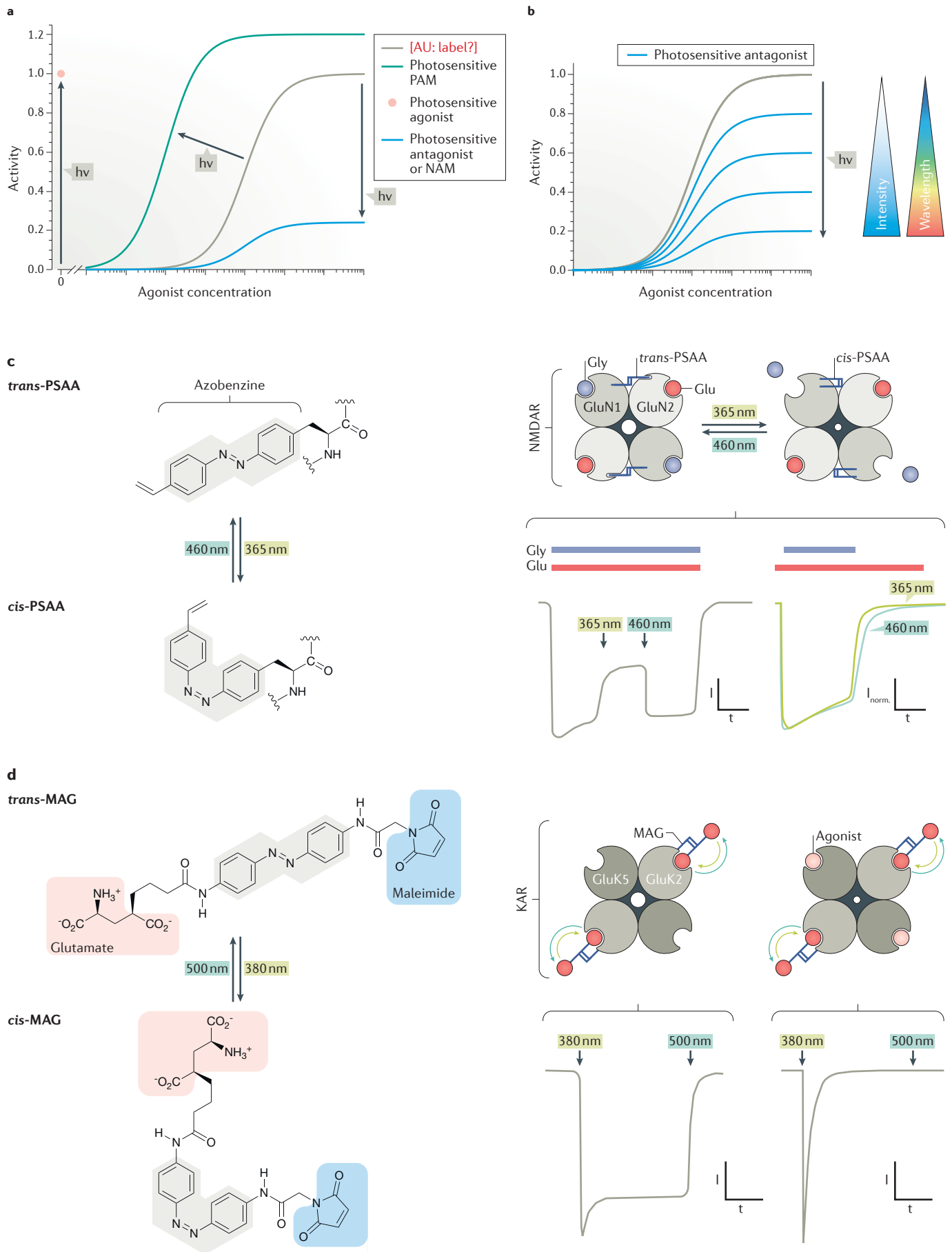
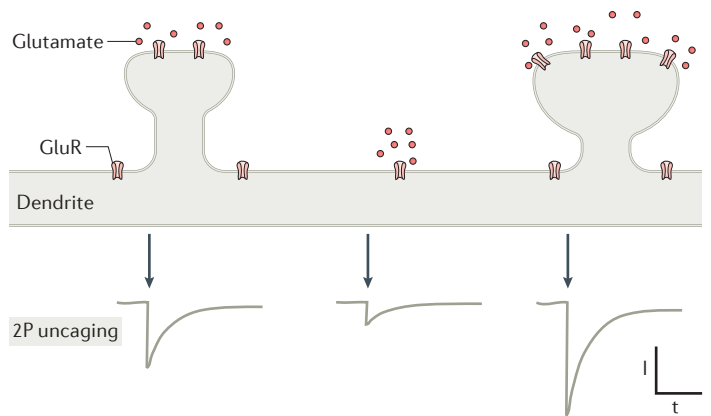
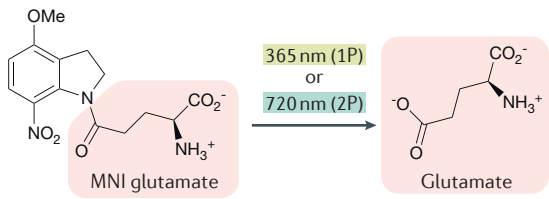
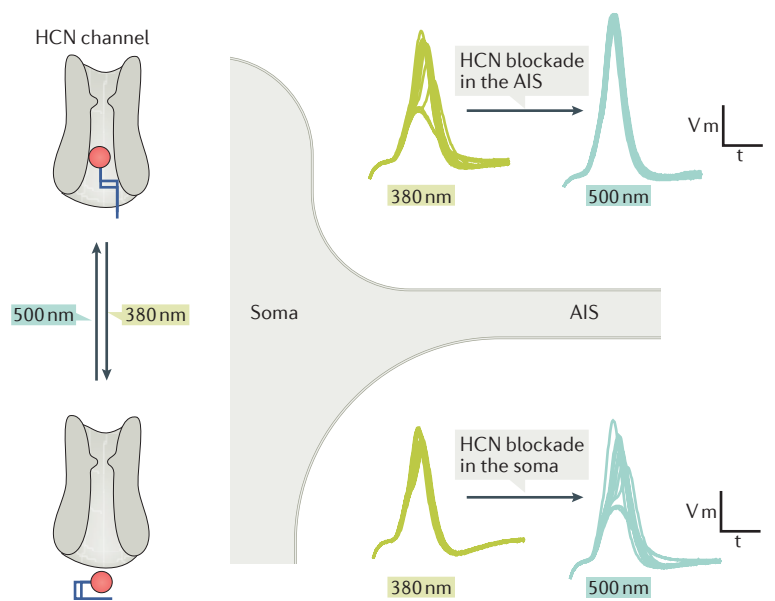
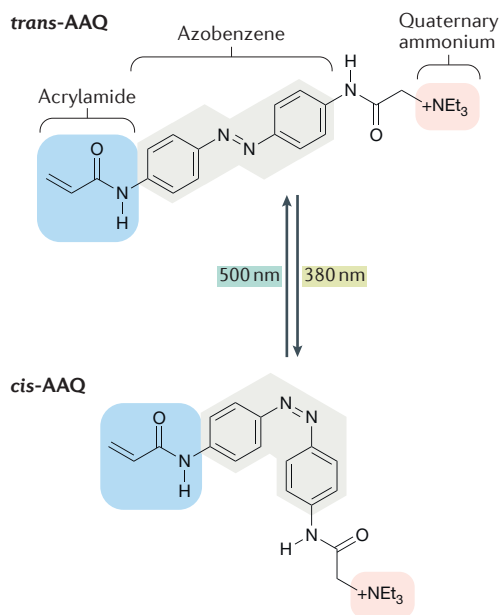


Fig 3

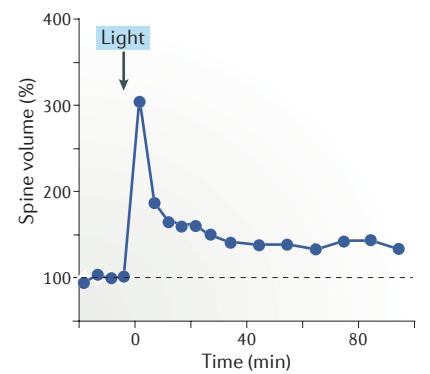
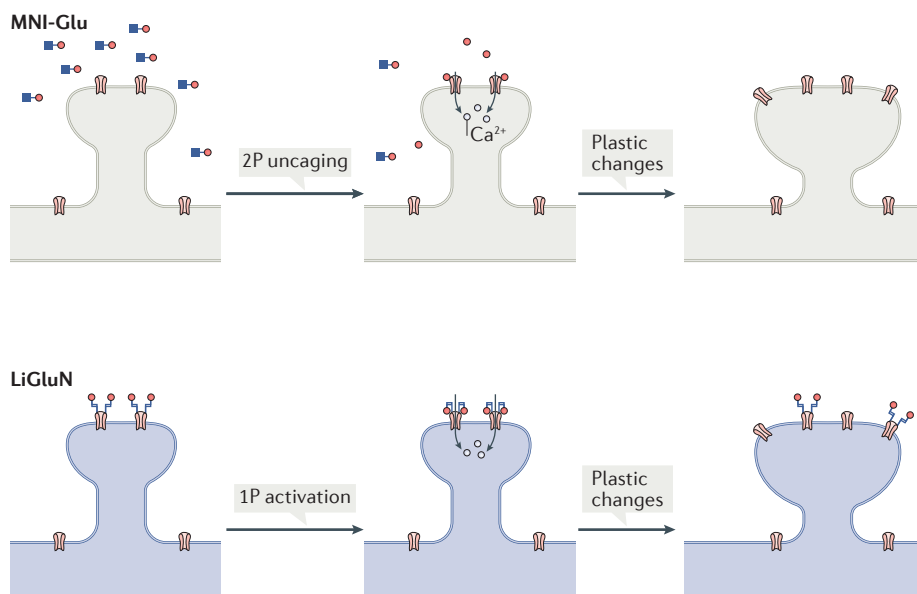
a Functional receptor subcellular mapping



b Compartment-specific blockade



c Induction of LTP at single dendritic spines



[Au:OK?]

Fig 4

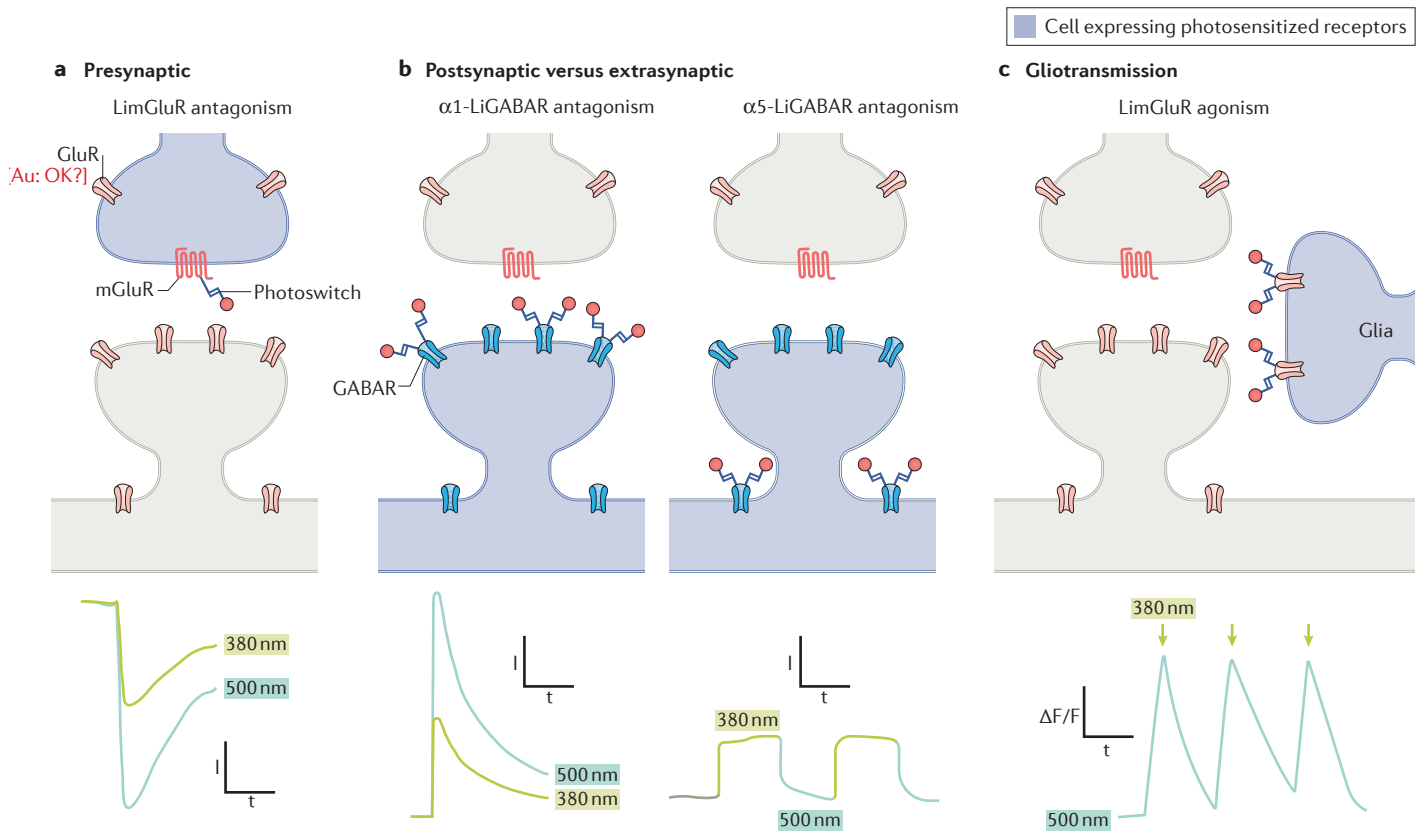


Fig 5

