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To cite this version:

Sarah Mondoloni, Romain Durand-de Cuttoli, Alexandre Mourot. Cell-Specific Neuropharmacology. Trends in Pharmacological Sciences, 2019, 40 (9), pp.696-710. 10.1016/j.tips.2019.07.007 . hal-02344518

HAL Id: hal-02344518

https://hal.sorbonne-universite.fr/hal-02344518v1

Submitted on 4 Nov 2019

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Cell-specific neuropharmacology

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Key words: optogenetics, chemogenetics, tethered ligand, bump-hole, receptor-ligand pair, photopharmacology

Abstract

Neuronal communication involves a multitude of neurotransmitters and an outstanding diversity of receptors and ion channels. Linking the activity of cell surface receptors and ion channels in defined neural circuits to brain states and behaviors has been a key challenge in neuroscience, since cell-targeting is not possible with traditional neuropharmacology. We review here recent technologies that enable the effect of drugs to be restricted to specific cell types, thereby allowing acute manipulation of the brain's own proteins with circuit specificity. We highlight the importance of developing cell-specific neuropharmacology strategies for decoding the nervous system with molecular- and circuit-precision, and for developing future therapeutics with reduced side effects.

38 **Probing the nervous system with cell-targeted drugs**

39

40 Investigating the function of neurotransmitter receptors and ion channels has greatly
41 benefited from both pharmacological and genetic techniques. Conditional mutagenesis and
42 virally-delivered **shRNAs** enable the manipulation of proteins with molecular specificity and in
43 targeted brain circuits, notably through the use of **cre/lox recombination**. Yet, these
44 techniques do not have sufficient temporal resolution to establish a direct link between the
45 activation of receptors and the modulation of circuits and behavior. In addition, they can lead
46 to developmental alterations that compensate for those induced by the deletion/mutation of
47 the receptor. In contrast, conventional pharmacology offers acute and often reversible control
48 of endogenous proteins, enables graded alterations by varying drug concentration, and can
49 be applied at any time in development. Nevertheless, pharmacology suffers from lack of
50 functional selectivity, since small chemicals affect all types of neurons cannot be cell-targeted.
51 This is especially an issue considering the wide distribution of ion channels and receptors in
52 the brain, and the various functions they have in different cells or networks. Local distribution
53 of drugs to precise brain regions may afford anatomical specificity, but has major drawbacks,
54 such as the difficulty to control dosage and diffusion, and the inability to target specific
55 neuronal types. Making small chemicals photocontrollable using caged compounds or
56 photoswitches permits cellular or even subcellular optical targeting at the single cell level, and
57 improves spatio-temporal control *in vivo* [1,2]. Yet, even with the most sophisticated light
58 source, controlling receptors located on different cell types within the same circuit in an intact
59 brain is elusive with optical techniques alone. The ability to acutely control signaling proteins
60 in a complex environment such as the nervous system in a cellular- and circuit-specific manner
61 and in the behaving animal should accelerate progress in our molecular understanding of
62 brain function (Figure 1A).

63

64 The idea behind cell-specific pharmacology is to combine the acute onset of pharmacology
65 with the cellular and molecular precision of genetics, to achieve acute control of signaling
66 proteins in a cell-specific fashion. To this aim, several chemogenetic strategies have recently
67 been developed, which we classify here in three categories (Figure 1, key figure, Table 1). The
68 receptor-ligand pair approach (Figure 1B) consists in re-engineering proteins to make them
69 sensitive to synthetic ligands. The key outcome is an increase in potency of the synthetic ligand

70 compared to the natural one (Figure 1C). The **tethered**-ligand approach (Figure 1D) relies on
71 the anchoring of a ligand to the cell surface and results in an increased local concentration of
72 the ligand. Anchoring can be performed either to a membrane-embedded element
73 (membrane-tethered) or to a genetically-modified receptor (receptor-tethered). Finally, the
74 last approach is to selectively deliver drugs to the cytoplasm of targeted cells, either using
75 enzyme-prodrug pairs or facilitated diffusion through large ion channels (Figure 1E). Cell-
76 specific neuropharmacology approaches provide the ability to test the function of receptors
77 on specific types of neurons with unprecedented cellular precision (e.g. pre- *vs*. post-synaptic
78 cells, or two different cell types within the same circuit) [3,4]. They also offer a unique
79 opportunity to test the benefits of cell-targeted drugs for neurological and neuropsychiatric
80 disorders. We review here these techniques, with a special focus on surface receptors and ion
81 channels, highlighting their potentials and pitfalls, and the challenges they meet for *in vivo*
82 use in rodents and for clinical applications.
83
84 **The receptor-ligand pair approach**
85
86 One central problem of traditional pharmacology is to identify small molecules that interact
87 with a desired protein target with high specificity. This is especially challenging for proteins
88 that belong to large protein families and therefore share a high degree of homology with other
89 proteins in the cell. To address this shortcoming, a chemo-genetic strategy named **bump-hole**
90 was developed, allowing inhibition of specific alleles of protein kinases [5]. The idea is to
91 genetically create a hole on the catalytic active site of the protein, and to chemically modify
92 the inhibitor, with a corresponding bump (Figure 1B). The synthetic ligand is **orthogonal**: it
93 confers high specificity to the engineered enzyme, without affecting wild-type (WT) kinases.
94 Importantly, the engineered kinase is a non-orthogonal mutant protein, i.e. it can still
95 phosphorylate endogenous substrates. A wide range of kinase-inhibitor pairs were generated,
96 displaying incomparable potency and specificity compared to known inhibitors [5].
97
98 Inspired by this approach, neuroscientists developed orthogonal receptor-ligand pairs, the
99 most widely used being the Designer Receptors Exclusively Activated by Designer Drugs
100 (**DREADDs**) [6,7]. DREADDs are modified G-protein coupled receptors (GPCRs) engineered
101 through directed molecular evolution, that are insensitive to their natural ligands but sensitive

102 to synthetic agonists (Figure 2A). The original DREADDs (hM3Dq and hM4Di) are based on
103 human M3 and M4 muscarinic receptors, which couple to Gq and Gi, respectively [6]. hM3Dq
104 and hM4Di are made insensitive to acetylcholine (ACh), sensitive to the synthetic ligand
105 clozapine-N-oxyde (CNO), and importantly show minimal basal activity in the absence of
106 chemical activation (but see [8]). hM3Dq is classically used for enhancing neuronal activity,
107 while hM4Di is used for neuronal inhibition. Newer DREADDs include GsD that couples to Gs,
108 $hM4D^{nrxn}$ that is axonally targeted and affords pre-synaptic inhibition, and a κ-opioid-derived
109 DREADD (KORD) that operates with salvinorin B [9], a compound distinct from CNO, thus
110 allowing multiplexed and bidirectional modulation of neuronal activity and behavior.
111
112 Receptor-ligand pairs have also been developed for ligand gated ion channels (LGICs, Figure
113 2B). The ligand binding domain (LBD) of the α7 nicotinic acetylcholine receptor (nAChR),
114 referred to as pharmacologically selective actuator module (**PSAM**), was engineered to
115 respond solely to synthetic molecules called pharmacological selective effector molecules
116 (**PSEM**) [10,11]. Activation of PSAM by PSEMs induces either cation, calcium or chloride influx,
117 depending on the ion pore domain (IPD) spliced onto the LBD (serotonin 5HT3, α7-nAChR or
118 glycine receptor IPD, respectively). Hence PSAMs can be used to either drive or suppress
119 neuronal activity, or to increase intracellular calcium, in genetically-targeted neurons.
120 DREADDS, and to a lesser extent PSAMs, have proven extremely valuable tools for the
121 manipulation of circuits with cellular specificity, providing crucial information as to how
122 circuits shape behavior [7,12]. However, because they are no longer sensitive to their cognate
123 ligands, DREADDs and PSAMs are constraint to decode the nervous system at the circuit level,
124 and cannot be used for the molecular dissection of the role of endogenous GPCR or LGIC
125 signaling in the modulation of circuits and behaviors.
126
127 With the advent of optogenetics [13], light-based strategies for controlling brain proteins have
128 emerged, affording improved spatio-temporal resolution over chemical approaches [1].
129 Notably, light-controllable adrenergic, opioid, serotoninergic and glutamatergic GPCRs (**Opto-**
130 **XRs**) have been engineered [14,15]. Opto-XRs are chimeric proteins, usually engineered with
131 a mammalian opsin (e.g. rhodopsin or melanopsin) and the intracellular loops and C-terminal
132 tail of endogenous GPCRs (Figure 2C). In addition to trafficking and signaling like their native
133 counterparts, OptoXRs are photo-activatable, offering the possibility to mimic the spatio-

temporal dynamics of neuromodulator signaling *in vivo*. Nevertheless, Opto-XRs bind retinal and therefore lack responsiveness to endogenous ligands, restricting their use, as with DREADDS, to a circuit-level understanding of brain function.

Dissecting the role of endogenous receptors and channels in behaviors requires maintaining their natural pharmacology and signaling properties. To this aim, two receptor-ligand pair-like approaches have been developed for $GABA_A$ receptors ($GABA_ARs$) and nAChRs. $GABA_AR$ signaling is potentiated by the allosteric modulator zolpidem, yet only when the γ2 subunit is present in the receptor. A single mutation (phenylalanine to isoleucine) at position 77 on the γ2 subunit is sufficient to convert a zolpidem-sensitive into a zolpidem-insensitive receptor (Figure 2D) [16]. In transgenic mice expressing γ2(Ile77), sensitivity to zolpidem can be restored in a tissue-specific fashion using Cre-recombinases that switch γ2(Ile77) to γ2(Phe77). Zolpidem-sensitivity was notably restricted to cerebellar Purkinje neurons, to show that potentiation of GABAergic inhibition in these cells induces motor deficits [17]. Even though motor deficits had been observed after zolpidem administration in WT mice, interpretation was ambiguous considering the profound sedative effects of this compound. Furthermore, mice with a deletion of γ2 in Purkinje cells show no motor deficit, emphasizing the importance of developing cell-specific pharmacology approaches for acute interventions. Nevertheless, it should be noted that this strategy requires triple crosses of mouse lines, making it technologically demanding.

In nAChRs, the strategy was referred to as gain-of-function mutations [18]. The idea is to increase agonist sensitivity in a subtype-specific fashion using site-directed mutagenesis, and to use sub-threshold doses of nicotine for isoform-selective activation (Figure 2E). The leucine residue in position 9’ on the transmembrane (TM) segment M2 is a conserved residue that, when mutated to alanine, serine or threonine in the α subunit, considerably reduces the energy required to open the channel, resulting in a “hypersensitive” mutant with increased agonist sensitivity [18]. Using the Cre-lox technology, expression of such hypersensitive mutants could be restricted to specific neurons such as GABAergic cells of the ventral tegmental area or cholinergic neurons of the medial habenula, unveiling new roles for α4 nAChRs in reward and anxiety, respectively [19,20]. However, it should be noted that these

mutant receptors are also more sensitive to endogenous acetylcholine (ACh), which may result in unintended neuronal adaptations.

The tethered-ligand approach

The overall strategy is to increase local concentration of the drug at the cell surface through covalent attachment [21]. One major advantage of this approach is that once bio-conjugation is achieved, there is no need for reapplication of the drug.

Membrane-tethered ligands

A first example is based on genetically-encoded ligands that are self-embedded in cell membranes. The fusion construct is composed of a peptide linked by a flexible linker to the extracellular side of the membrane, either through a TM segment or a glycosyl-phosphatidyl-inositol (**GPI**) anchor (Figure 3A) [22-27]. The peptide ligand can either be an agonist (t-peptide) or an antagonist (t-toxin). This versatile approach has been successfully applied to activate class B GPCRs [22] and to inhibit specific voltage-gated sodium (Na_v) and calcium (Ca_v) channels as well as nAChRs [24-27]. Toxins can be extremely specific for a particular type of ion channel, enabling blockade of $NA_v1.7$ without affecting $Na_v1.8$ for instance [28]. However, since the ligand is permanently expressed at the membrane, the action of t-peptides and t-toxins is irreversible. T-toxin expression can be placed under the control of a **Tet-on/Tet-off system***,* allowing antagonism to be triggered on and off, yet still with very slow kinetics (days) [24]. Consequently, these tools have been used for chronic inhibition of neuronal activity and genetic dissection of neurophysiological circuits rather than for acute, cell-specific pharmacology. T-toxins were notably used in freely-moving mice, where chronic inhibition of $Ca_v2.1$ and $Ca_v2.2$ allowed probing the role of thalamo-striatal excitatory synaptic transmission in the susceptibility to social stress [27].

An extension of this approach is to include in the construct a photosensitive group to afford reversibility. Notably, the **light-oxygen voltage** (LOV) protein domain from plants was used to produce Lumitoxins, light-controllable membrane-tethered toxins (Figure 2B) [29]. LOV domains incorporate a flavin chromophore, ubiquitously present in mammalian cells, and

197 changes conformation upon illumination with blue light. In darkness, Lumitoxins produce
198 sustained block of voltage-gated potassium (Kv) channels, while illumination results in rapid
199 (seconds) channel unblock. The LOV domain returns to its resting state slowly in darkness,
200 restoring blockade within minutes. Specific Kv homologues such as Kv1.1, Kv1.2 or Shaker can
201 be photosensitized using appropriate membrane-tethered toxins. However, this technique
202 has not yet been extended to other protein families and has not been deployed *in vivo*.
203
204 Another strategy, called Drug Acutely Restricted by Tethering (**DART**), relies on a bacterial
205 enzyme called **HaloTag** for capturing drugs at the cell surface [3]. HaloTag is a self-labeling
206 enzyme that catalyzes the covalent attachment of synthetic molecules containing a HaloTag
207 Ligand (HTL) with very high efficiency and specificity (Box 1). In DART, the HaloTag is expressed
208 at the cell surface through a TM domain. The synthetic ligand is composed of an active drug
209 linked to the HTL through a Poly-Ethylene Glycol (**PEG**) flexible linker. Once infused, it attaches
210 to the HaloTag, resulting in a hundred-fold elevation of drug concentration at the cell surface.
211 This strategy is unique in that it offers acute pharmacological manipulation (seconds to
212 minutes) of native receptors with cellular specificity. However, the effect is only very slowly
213 reversible (days). DART has been applied to AMPA receptors (AMPARs) and metabotropic
214 muscarinic receptors (mAChRs), showing that the method can be applied to different receptor
215 types . Specific inhibition of AMPARs in distinct neuronal populations of the dorsal striatum
216 (D1 versus D2 neurons) revealed that activity of these receptors is causally linked with the
217 akinesia observed in a mouse model of Parkinson's disease. Moreover, antagonism of AMPARs
218 expressed on D2-, but not D1-, neurons had therapeutic effects on motor dysfunction,
219 illustrating the power of targeting drugs to specific cell types.
220
221 ***Receptor-tethered ligands***
222
223 Various strategies have been developed to tether ligands directly to their receptors. In the
224 RECON (REductively Cleavable agONist) approach, a GPCR is N-terminally fused to a **SNAP-**
225 **tag** [30], another type of self-labeling tag. The tethered ligand combines a peptide agonist for
226 either class A or B GPCR, a central PEG linker bearing a disulfide bridge, and a SNAP-tag
227 substrate. This synthetic ligand covalently and specifically attaches to the SNAP-tag, resulting
228 in permanent receptor activation. The disulfide bridge can be cleaved with redox agents,

229 resulting in slow agonist dissociation. This method should be especially useful for studying
230 GPCR activation and internalization in vitro; however, its use for neuropharmacology is
231 elusive.
232
233 Increasing local agonist concentration at the receptor surface does not necessarily require
234 covalent attachment. Another design is to install a **His-tag** on a GPCR, and to use a Metal-
235 complex Agonist Conjugate (**MAC**) that binds with high affinity to the His-tag through
236 coordination tethering (Figure 3D) [31]. Affinity of the synthetic ligand is 10-100 higher for the
237 engineered than for the WT receptor. Interaction between MAC and the His-tag is not
238 covalent, hence activation is reversible. The MAC strategy was used for cell-specific control of
239 class A GPCRs (β2 adrenoreceptors and mAChRs), but its efficacy *in vivo* has not been reported
240 yet.
241
242 One major drawback of tethered ligands is irreversibility of action. One solution to this issue
243 is to incorporate a chemical photoswitch in the ligand, and use light to trigger
244 binding/unbinding. This can be achieved by anchoring a photoswitchable tethered ligand (**PTL**)
245 onto a cysteine-substituted receptor (Figure 3E). The cysteine mutation is incorporated in
246 proximity to a ligand-binding site. The PTL is made of three elements: a **maleimide** moiety,
247 which is a **thiol**-reactive group for attachment to cysteines; a central **azobenzene**
248 photoswitch; and a bioactive ligand (agonist, antagonist or pore-blocker). Light is used to
249 reversibly change the geometry of the photoswitch from elongated to twisted, which triggers
250 binding/unbinding. This opto-chemogenetic strategy allows reversible control of receptors
251 with very high spatial and temporal precision. The PTL approach has proven to be highly
252 versatile. It has been applied to potassium channels [32-34], ionotropic [35-37] and
253 metabotropic [38] glutamate receptors, nAChRs [4,39], $GABA_A$Rs [40,41], dopamine receptors
254 [42] as well as P2X receptors [43]. It was used to probe neurotransmission in various neuronal
255 settings, both *ex* and *in vivo* in zebrafish and mice [1]. In zebrafish, the photoswitch can simply
256 be added to the swimming water, but in mice it has to be locally delivered. Despite this
257 drawback, the PTL approach has been applied in the living mouse, notably for restoring vision
258 to blind mice [44], for manipulating action potential firing [45] and GABAergic inhibition [41]
259 in the visual cortex, or for controlling nicotinic transmission in the ventral tegmental area and
260 addiction-related behaviors [4]. One potential shortcoming of PTLs is their non-selective

261 attachment to endogenous cysteines, even though no adverse effect has been observed so
262 far [4,41].
263
264 More specific bioconjugation (Box 1) can be achieved with the Photoswitchable Orthogonal
265 Remotely Tethered Ligand (**PORTL**) approach, which uses self-labelling enzymes such as **SNAP-**
266 or **CLIP-tags** (Figure 3F) [46-48]. These self-labeling tags can be fused directly to receptors
267 [46,47] or alternatively to nanobodies [48], an interesting alternative for proteins for which
268 incorporation of the tag is prohibited. Light is used to change the geometry of the ligand, and
269 thereby its affinity for the protein. This technology has so far only been applied to
270 metabotropic glutamate receptors, and was used to restore patterned vision in a blind mouse
271 model [49].
272
273 Another alternative for site-specific bioconjugation is the Bio-Orthogonal Ligand Tethering
274 (**BOLT**) technique, which is based on the incorporation of unnatural amino acids (**UAA**) in
275 proteins [50]. The UAA is used as a biorthogonal handle for attachment of a synthetic ligand
276 through **click-chemistry**. BOLT demonstrated selective inhibition of kinases in mammalian
277 cells, and can be made photo-reversible by introducing a photoisomerizable group to the
278 ligand (photoBOLT). Expansion of the genetic code in the mouse is technically challenging[51],
279 but the recent generation of transgenic animals carrying a tRNA synthetase / tRNA pair into
280 their genome should facilitate future use [52].
281
282 **Intracellular delivery approaches**
283
284 Intracellular, cell-specific delivery can also be achieved, allowing targeting not only cell-
285 surface receptors but also enzymes and signaling pathways (Figure 1E). Two different
286 approaches have been described.
287
288 ***The enzyme-prodrug pair approach***
289
290 This approach relies on selective enzyme-substrate pairs to convert an inert prodrug into an
291 active molecule (Figure 1E) [53]. The drug is masked by a disposable blocking group that is
292 hydrolyzed specifically by an exogenous enzyme, but not by native ones. Expression of the

293 specific enzyme in genetically-targeted cells allows unmasking the drug in a cell-specific
294 fashion. The challenges were to develop an ester masking group with high stability towards
295 hydrolysis by endogenous esterases, and to find an esterase that would hydrolyze this ester
296 bond with high efficiency. Screening resulted in the discovery of porcine liver esterase (**PLE**),
297 an enzyme that efficiently hydrolyzes the cyclopropylmethyl carboxyl (**CM**) ester masking
298 group [53]. More recently, another selective enzyme-substrate pair was developed, based on
299 engineered variants of E. coli nitroreductase (**NTR**) and a nitroimidazol (**NM**) masking group
300 [54]. This strategy has proven highly generalizable to various masked small molecules such as
301 fluorophores, calcium indicators, enzyme inhibitors, cAMP analogs or ion channel blockers
302 [53-55]. Notably, the masked compound CM-MK801 was used to confirm, in brain slices, the
303 role of dopamine neuron-expressed NMDA receptors in cocaine-induced plasticity [55]. Both
304 the CM and NM groups were shown to be highly stable in neurons, an important requirement
305 for cell-specificity. That said, care must be taken because drugs that are too membrane
306 permeable may diffuse out of the target neuron and affect nearby cells non-selectively. This
307 limitation can be circumvented, for instance by increasing polarity of the compound [54]. The
308 biggest challenge for future applications remains to use this technology *in vivo*. So far, the
309 limited aqueous solubility of the masked compounds precludes direct brain delivery. In
310 addition, systemic application of CM-masked drugs is prohibited as well because CM is not
311 resistant to esterases expressed in the periphery.
312
313 ***Facilitated diffusion through large ion-channel pores***
314
315 Another approach is based on the selective intracellular diffusion of membrane-impermeant
316 drugs through large ion channels (Figure 1E). The capsaicin TRPV1 and some P2X receptors
317 open a very large pore when activated, allowing permeation of large organic cations [56,57].
318 This biophysical property was ingeniously exploited to facilitate the entry of QX-314, a
319 membrane-impermeant lidocaine derivative, into pain-sensing neurons [58]. QX-314 is
320 normally inert on neurons because it has an intracellular site of action in Na_Vs. However, it can
321 selectively enter nociceptors by diffusing through open TRPV1 channels, which are abundant
322 in these cells but virtually absent in other neurons. QX-314 remains trapped inside pain
323 neurons for hours, resulting in long-lasting local analgesia [58-60]. To gain rapidly reversible
324 control over nociception, we designed a photoswitchable version of QX-314, named **QAQ** [61-

325 63]. QAQ can rapidly and reversibly block blocks Na_vs in nociceptors, switching pain signaling
326 on and off upon illumination with the appropriate wavelength of light.
327
328 Unlike all the methods presented in this review, this strategy does not necessarily require
329 genetic manipulation. Rather, it exploits the sparse distribution of TRPV1 channels or P2X
330 receptors to achieve targeted cell loading, and thus bears potential therapeutic interest. So
331 far, QX-314 and QAQ were co-administered with capsaicin to enable selective cell entry. Yet,
332 recent studies suggest that capsaicin may not always be needed, since TRPV1 channels in
333 central terminals are extensively hyperactive in neuropathic pain models [64]. Similarly, the
334 retina was shown to be highly remodeled in animal models of retinal degeneration [65], with
335 P2X receptors functionally upregulated in retinal ganglion cells [66]. This feature was exploited
336 to deliver photoswitchable blockers of potassium channels [61,67] specifically to OFF-ganglion
337 cells, thereby restoring visual responses to blind retinas [65,66].
338
339 **Concluding remarks**
340
341 Investigating the role of individual receptors and ion channels in particular brain regions
342 requires methods for perturbing protein activity selectively, acutely, reversibly and in a cell-
343 specific fashion, ideally in the behaving animal. Diverse methods are being developed toward
344 this goal. Yet no method is universal, and virtually all have shortcomings, especially when
345 considering their use *in vivo*. One important limitation lies in the limited therapeutic window
346 for selective agonism / antagonism, which is classically in the range 10-100 fold (Table 1).
347 Recent studies demonstrate that with some optimization, very potent agonists with
348 exceptional selectivity (>10 000 fold) can be generated for PSAMs [11] and DREADDs [68],
349 suggesting that such selectivity should be in principle attainable for other designer receptors.
350 Full orthogonality can even be achieved, for instance using light-based methods (after ligand
351 bioconjugation for PTLs and PORTLs). Yet, there is still a need for orthogonal labeling motifs
352 that are efficient at lower doses (Box 1).
353
354 There is also a need for caution and appropriate controls when using chemogenetic
355 approaches, even when reported to be fairly orthogonal. For instance, CNO was originally
356 selected because of its excellent drug-like properties, but recent reports show that *in vivo* it is

357 metabolically back-converted to clozapine, a molecule that not only activates DREADDs but
358 also many other endogenous GPCRs [69]. Importantly, non-CNO analogues with improved
359 selectivity have been developed, notably compound 21 [70] and perlapine [68], circumventing
360 this potential issue.
361
362 Another important limitation to consider is invasiveness. DREADD activators, PSEMs, nicotine
363 and zolpidem have rapid central nervous system (CNS) penetration and distribution in mice
364 and can therefore be applied systemically [7,17,18,71]. But other approaches require local
365 drug delivery in brain tissue through a cannula guide, either because of the instability of the
366 compound in aqueous medium (e.g. maleimide-based PTLs) or because of poor blood brain
367 penetration (e.g. DART), or both. Methods for improved systemic delivery are awaited.
368 Similarly, the optical tools described in this review classically work with visible light (Table 1)
369 and therefore require local light delivery with a chronically implanted optic fiber. Red-shifted
370 chromophores that operate at more deeply-penetrating wavelengths such as near-infra-red
371 light are currently under development [72] and should facilitate remote, transcranial control
372 in the future.
373
374 Another important aspect to consider is receptor-type specificity. Techniques like DART or
375 intracellular delivery methods target native receptors and therefore cannot differentiate
376 between different receptor isoforms expressed on the same cell. Toxin-based methods
377 provide increased selectivity but whether they can be used for acute and reversible control of
378 receptors and ion channels in vivo is unclear. On the other hand, methods that require genetic
379 engineering of receptors, like PTL or PORTL, provide absolute receptor-type specificity and can
380 help dissect the role of individual receptor subtypes in a circuit. However, molecular specificity
381 comes at a cost: the requirement for ectopic expression of the target protein, which may
382 affect expression level and/or patterns. Methods for overcoming this issue include the
383 generation of transgenic knock-in animals [41]- ideally in a tissue-specific fashion- or the use
384 of a subunit replacement strategy [34].
385
386 Finally, these tools bear strong potential not only for research purposes in animal models, but
387 also for therapeutic applications in humans. Indeed, targeting drugs to particular neuronal
388 populations may improve therapeutic efficacy, while decreasing the side effects associated

389 with insufficient selectivity of conventional approaches. For instance, QX-314 or QAQ can
390 selectively block the function of pain-sensing neurons in rodents, while leaving other sensory
391 modalities unaffected [58,59,62], and do not require genetic modification, making them
392 potential drug-like candidates for pain-selective local anesthesia in humans. Approaches
393 requiring genetic manipulation are also being explored to treat CNS diseases in a titrated and
394 cell-specific manner. This includes strategies for restoring the balance of excitation/inhibition
395 selectively in epileptogenic zones, or for treating movement disorder in Parkinson's disease.
396 The DART technology for instance revealed that antagonizing AMPARs expressed on D2
397 neurons of the basal ganglia was much more efficient at improving motor dysfunction than
398 global antagonism through D1 and D2 neurons. Yet it is not clear how this finding can be
399 translated into human therapy. DREADDs and PSAMs are currently progressing into non-
400 human primates [73,74], an important step towards being used as treatment to people. In
401 addition, hM4Di, KORD and new-generation PSAMs can be potently activated by low doses of
402 olanzapine [75], salvinorin B [9] and varenecline [71], respectively, three drugs that are
403 already clinically approved, thus facilitating translation to humans. Despite these important
404 advances, considerable obstacles for implementing such approaches to the clinic remain,
405 notably those associated with gene therapy and, to a lesser extent, drug delivery and
406 selectivity.
407

408 **Table 1: Cell-specific neuropharmacology approaches.**

409 N.R.: not reported; N.A.: not applicable

Tool Name	Receptor	Ligand	On timescale	Off timescale	Therapeutic window (fold)	Application	In vivo application	Refs
Receptor-ligand pairs								
DREADDS	Synthetic GPCR	Synthetic (CNO, perlapine…)	s-min	min-hour	100-10000	On/off control of neuronal activity	Drosophila, mice, rats, monkeys	[6,7]
PSAM/PSEM	Synthetic LGIC	Synthetic (22S, 89S…)	s-min	min-hour	30->10000	On/off control of neuronal activity	Mice	[10,11]
Opto-XR	Opsin-GPCR chimera	Retinal + Blue light	ms-s	ms-s	Fully orthogonal (light)	Activation of G protein signaling	Mice	[14,15]
Zolpidem-insensitive $GABA_AR$	Mutant $GABA_AR$	Synthetic (Zolpidem)	s-min	min-hour	>600	Modulation of $GABA_ARs$	Mice	[17]
Gain of function nAChRs	Mutant nAChR	Exogenous (Nicotine) and endogenous (ACh)	s-min	min-hour	10-100	Activation of nAChR subtypes	Mice	[19,20]
Membrane-tethered ligands								
t-toxins and t-peptides	Native receptors and ion channels	Genetically-encoded toxins or peptides	Always on (days with Tet-on system)	Irreversible (days with Tet-off system)	N.A. (constitutive)	Genetic dissection of circuits	Drosophila, zebrafish, mice	[22-27]
Lumitoxin	Native receptors and ion channels	Genetically-encoded toxins + blue light	min	s	Fully orthogonal (light)	Block of ion channel subtypes	N.R.	[29]
DART	Native AMPA receptors	HaloTag-reactive ligand	s-min	days	30-300	Antagonism of AMPARs and mAChRs	Mice	[3]
Receptor-tethered ligands								
RECON	SNAP-tagged GPCR	SNAP-reactive ligand	s-min	Irreversible (min with redox agent)	N.A.	Activation and internalization of GPCRs	N.R.	[30]
MAC	His-tagged GPCR	Metal complex-agonist conjugate	s-min	min	10-100	Activation of GPCRs	N.R.	[31]
PTL	Cysteine-substituted receptor	Thiol-reactive ligand + UV-visible light	ms-s	ms-s	N.A. (orthogonal after attachment)	On/off control of receptors and ion channels	Zebrafish, mice	[4,32-43]
PORTL	SNAP- or CLIP-tagged GPCR or nanobody	SNAP- or CLIP-reactive ligand + UV-visible light	ms-s	ms-s	N.A. (orthogonal after attachment)	On/off control of receptors	Mice	[46-48]
iBOLT (and photoBOLT)	Receptor mutated with unnatural aminoacid	Click chemistry-reactive ligand (+ UV light)	min-h	Irreversible (min with light)	N.A.	Inhibition of protein activity (reversible with light)	N.R.	[50]
Intracellular delivery								

Enzyme-pro drug pair	Native proteins	Masked drug	min	Irreversible	N.A.	Control of protein activity	N.R.	[53-55]
Facilitated diffusion through large ion channels	Native proteins	Membrane-impermeant and cationic (+UV-visible light)	Min (ms-s with light)	Days (ms-s with light)	N.A.	Block of ion channels (on/off with light)	Mice, rats	[58-60,62,63,65,66]

Acknowledgement: We wish to thank Fabio Marti and Philippe Faure (Sorbonne Université) for their comments on the manuscript. This work was supported by grants from the Fondation pour la Recherche Médicale FRM (Equipe FRM DEQ2013326488), the Brain and Behavior Research Foundation (NARSAD Young Investigator Grant), the Agence Nationale de la Recherche (ANR-JCJC), the Institut National Du Cancer (TABAC-16-022) and the Fondation de France (Fondation Médisite). SM and RDC were recipient of a 4th year PhD fellowship from FRM (FDT201904008060 and FDT20170437427, respectively).

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602 **Glossary**

603

604 **Azobenzene**, chemical photoswitch that can be reversibly isomerized between an elongated
605 *trans* state and a twisted *cis* isomer with short (classically near-UV) and long (blue-green)
606 wavelengths of light, respectively.

607

608 **BOLT**, bioorthogonal ligand tethering. Ligand is conjugated to an UAA through click-chemistry.

609

610 **Bump-hole**, strategy based on the enlargement of binding sites (holes) in proteins (initially
611 enzymes) to make them selective to complementary "bumped" ligands.

612

613 **Click chemistry,** orthogonal bioconjugation reaction that is rapid, biocompatible and high
614 yielding.

615

616 **CM**, cyclopropylmethyl carboxyl. Masking group cleaved selectively by PLE.

617

618 **Cre/lox recombination,** genetic manipulation based on an enzyme, Cre recombinase, and its
619 recognition site, lox P, used for tissue-specific gene expression.

620

621 **DART,** drugs acutely restricted by tethering. Method for capturing drugs at the cell surface
622 using a HaloTag.

623

624 **DREADDs,** Designer Receptors Exclusively Activated by Designer Drugs. Engineered GPCRs
625 that exclusively respond to synthetic ligands.

626

627 **GPI anchor,** glycolipid (Glycosylphosphatidylinositol) that can be attached to the C-terminus
628 of a protein.

629

630 **His-tag,** tag made of 4 to 9 histidine residues (4 in the MAC technology) classically used for
631 purification of recombinant proteins.

632

633 **LOV domain,** light-oxygen voltage domain. Blue light sensor from algae, plants, bacteria and
634 funghi, used to control cellular responses with light.
635
636 **MAC,** metal complex–agonist conjugates. Bifunctional ligand containing a Ni^{2+}-nitrilotriacetic
637 acid (Ni-NTA) moiety for selective coordination tethering to his-tagged receptors.
638
639 **Maleimide**, 1H-pyrrole-2,5-dione. Cysteine-reactive chemical group.
640
641 **NM,** 2-nitro-N-methylimidazolyl. Masking group selectively unmasked with NTR.
642
643 **NTR,** Nitroreductase from *E. Coli* used for selective reduction of NM groups.
644
645 **Opto-XR,** chimeric photocontrollable receptor engineered using opsins and the intracellular
646 loops or N-terminal tail of mammalian GPCRs.
647
648 **Orthogonal**, which does not interfere with native biological processes.
649
650 **PEG**, polyethylene glycol. Flexible polymer that is highly water soluble.
651
652 **PLE**, porcine liver esterase. Exogenous enzyme that efficiently and selectively hydrolyses CM
653 ester substrates.
654
655 **PORTL**, photoswitchable orthogonal remotely tethered ligand. Photoswitchable ligand
656 tethered to a protein or nanobody through SNAP- or CLIP-tag conjugation.
657
658 **PSAM/PSEM**, Synthetic protein (pharmacologically selective actuator module, PSAM) that is
659 selectively activated by synthetic ligands (pharmacologically selective effector molecules,
660 PSEMs).
661
662 **PTL**, photoswitchable tethered ligand. Thiol-reactive ligand incorporating a chemical
663 photoswitch, that photosensitizes cysteine-substituted receptors and ion channels.
664

665 **QAQ**, Quaternary-ammonium azobenzene quaternary-ammonium. Light-sensitive blocker of
666 voltage-gated potassium and sodium channels, used as a photoreversible local anesthetics.
667
668 **shRNAs**, short (or small) hairpin RNA used to silence gene expression.
669
670 **SNAP-, CLIP- and Halo-tags**, Protein-based self-labeling tags, catalyzing the formation of a
671 specific, covalent bond between a labeling molecule and a tag-fused protein of interest.
672
673 **Tethered**, covalently (irreversibly) anchored.
674
675 **Tet-on/off system**, tetracycline (Tet) approach for precise and reversible spatiotemporal
676 control of gene expression.
677
678 **Thiol**, side chain of the amino acid cysteine, which imparts reactivity to maleimides for ligand
679 tethering.
680
681 **UAA**, unnatural amino acid. Synthetic amino acid which can be incorporated in proteins using
682 the Amber stop codon technology.

683 **Figure legends**

684

685 **Figure 1 – Cell-specific neuropharmacology strategies**. A. Cell-specific pharmacology concept.
686 A drug (green) is delivered to the whole organism but targets only specific types of neurons in
687 the brain. The approach allows to evaluate the neurophysiological and behavioral
688 consequences of the manipulation of receptors in discrete circuits of the brain. B. Bump-hole
689 strategy. Top: the ligand (agonist or antagonist, green) acts on wild-type proteins. (Bottom
690 left) The engineered protein contains a "hole" in the binding pocket, while the synthetic ligand
691 (purple) contains a corresponding "bump". The mutant protein is non-orthogonal because it
692 remains sensitive to the endogenous ligand (green). The orthogonal ligand acts specifically
693 and exclusively on the engineered protein. (Bottom right) Both the modified protein and the
694 synthetic ligand are orthogonal. C. Leftward shift in the concentration-response curve for the
695 synthetic ligand (purple) compared to the natural agonist or antagonist (green). D. Tethered-
696 ligand approaches developed for G-protein coupled receptors (GPCRs), ligand-gated (LGICs)
697 and voltage-gated ion channels (VGIC). (Left) The ligand is either embedded in the cell
698 membrane (membrane-tethered) or tethered to the receptor itself (receptor-tethered). E.
699 Intracellular delivery approaches. The drug is inactive in the extracellular space, and gets
700 active only after it has entered targeted cells. Color coding throughout figures A-E: native
701 proteins are shown in light grey and engineered ones in dark grey.

702

703 **Figure 2 – The receptor-ligand pair approach.** A. Designer Receptors Exclusively Activated by
704 Designer Drugs (DREADDs). DREADDs are orthologous receptor-ligand pairs made of a
705 modified human muscarinic receptor (hM), with mutations in transmembrane domains
706 (orange), that is insensitive to its natural agonist acetylcholine (ACh), but is exclusively
707 activated by a synthetic ligand (e.g. clozapine-N-oxide, CNO). B. The Pharmacologically
708 Selective Actuator Module/Effector Molecules (PSAM/PSEM) strategy: PSAM is an orthogonal,
709 engineered ligand binding domain (LBD) of the homopentameric α7-nAChRs that is solely
710 activated by PSEM (and not ACh). Different ion pore domains (IPDs) can be spliced on the
711 PSAM allowing calcium, cation or chloride permeation. C. The Opto-XR approach: Opsin-GPCR
712 chimeras are made of the intracellular domains of mammalian GPCRs (such as β2- and α1-
713 adrenergic receptors, grey) swapped on the transmembrane (TM) domain of mammalian
714 opsin (e.g. rhodopsin, blue). Opto-XRs are not sensitive to endogenous ligands (e.g.

715 norepinephrine, NE). They contain a retinal chromophore (blue) and enable the optical control
716 of intracellular signaling transduction. D. Zolpidem (pink) potentiates GABA signaling by
717 binding to $GABA_A$Rs that contain the γ2 subunit. Substitution of phenylalanine (F) 77 by
718 isoleucine (I) on γ2 induces a loss of response to zolpidem. E. Mutation of the leucine residue
719 in position 9' (L9'), for instance to Alanine (A), leads to a "hypersensitive" nAChR that is
720 activated with subthreshold concentrations of the agonist nicotine.
721
722 **Figure 3 – The tethered ligand approach.** A. t-Toxins and t-Peptides are genetically-encoded
723 ligands permanently tethered to the cell surface through either a glycosylphosphatidylinositol
724 (GPI) anchor (depicted here) or a transmembrane domain (TM). B. Lumitoxin are chimeric
725 proteins composed of a TM, a light-oxygen-voltage (LOV) domain, a linker and a toxin (green).
726 In darkness, Lumitoxin blocks endogenous potassium channels at the cell surface, while
727 illumination with blue light unfolds LOV and relieves blockade. C. The Drug Acutely Restricted
728 by Tethering (DART) strategy is based on the expression of a TM anchor linked to a specific
729 self-labeling protein tag (e.g. HaloTag), allowing the capture of a specific ligand to the cell
730 surface. The ligand is composed of a HaloTag Ligand (HTL), a flexible linker (poly-ethylene
731 glycol, PEG) and a ligand (green). Conjugation results in a 100-fold increased concentration of
732 the ligand at the cell surface. D. Specific binding of a Metal complexe-Agonist Conjugate (MAC)
733 to a His-Tagged GPCR, through coordination between the Ni^{2+}–nitrilotriacetic acid (Ni–NTA)
734 group of the ligand and the His-Tag. E. Photoswichable tethered ligands (PTLs) are composed
735 of a cysteine reactive group (maleimide, grey), a photosensitive azobenzene core (orange),
736 and a ligand (green). PTLs covalently attach to an engineered receptor that contains a single
737 cysteine substitution, near the ligand binding site, thereby affording reversible photocontrol.
738 F. Photoswitchable orthogonal remotely tethered ligands (PORTLs) are composed of a ligand,
739 a photoswitchable azobenzene molecule, a flexible linker and a benzylguanine (BG) or
740 benzylcytosine (BC) group for conjugation to SNAP- or CLIP-Tags, respectively.
741
742 **Figure 4: Intracellular delivery approaches**. A. The enzyme-prodrug pair approach relies on
743 the use of a masked prodrug, and an exogenous enzyme that coverts the prodrug into an
744 active drug. B. The facilitated diffusion approach uses large ion channels such as TRPV1 or P2X
745 receptors for the selective entry of membrane-impermeant drugs. Here the drug is
746 photocontrollable, enabling on and off action at the target protein with two distinct

747 wavelengths of light.

748

749

750 Box 1: Bioconjugation technologies.

751 Bioconjugation reagents are used to link together a small chemical molecule (e.g. a ligand)
752 and a protein of interest (POI). Chemically tagging a protein with low toxicity and high
753 specificity in a complex cellular environment is a challenge. It requires genetic modification of
754 the POI, in order to incorporate a reactive group that will serve as a biorthogonal handle for
755 conjugation. Multiple strategies exist [76]. The smallest and least disruptive genetic
756 modification is the incorporation of a cysteine amino acid on the protein surface through site-
757 directed mutagenesis. Cysteines contain a thiol group that reacts efficiently, rapidly (minutes)
758 and with high selectivity with maleimide groups (Figure I) to form stable, covalent adducts
759 [76]. Cysteine has become the primary choice for site-specific modification of membrane
760 proteins because it is relatively low abundant, often engaged in disulfide bridges, and highly
761 nucleophilic at neural pH [21]. Importantly, due the strong reductive environment of the
762 cytoplasm, cysteine-maleimide conjugation chemistry is restricted to extracellularly-
763 accessible sites on membrane proteins [21]. In addition, because cysteines are naturally
764 present on many endogenous proteins, novel bioconjugation techniques that work inside cells
765 and that are fully bio-orthogonal have been developed. This includes the use of unnatural
766 amino acids (UAA) that contain a double (alkene) or triple bond (alkyne) for bioconjugation
767 with tetrazine-containing ligands through click chemistry [50] (Figure I). Click-chemistry is
768 extremely popular for protein bioconjugation because it relies on chemical groups that are
769 highly selective toward each other -yet remain inert otherwise-, exhibits fast reaction kinetics
770 in aqueous media (minutes) and produces adducts that are very stable [76]. However, UAAs
771 must be incorporated into proteins through Amber codon suppression technology, which
772 remains challenging *in vivo* [52]. The other approaches for orthogonal labeling rely on larger
773 modifications of the POI, such as the incorporation of polypeptide tags. For instance, metal
774 chelation methods using poly-histidine tags (His-tag), which are classically used for protein
775 purification, have been used for non-covalent labeling with Ni-NTA ligands [31]. His-tags are
776 small (4-9 residues), conferring minimal disturbance to the protein, and label probes with high
777 efficiency and selectivity. Nevertheless, labeling is reversible and Ni is toxic to cells, hampering
778 *in vivo* use [76]. Finally, self-labeling domains such as SNAP-, CLIP- or HALO-tags use enzyme-

779 catalyzed reactions for irreversible conjugation of ligands to POI in live cells. The reaction is
780 highly biorthogonal, rapid, irreversible and works intracellularly with low concentration of
781 substrate (nanomolar range) [76], but requires fusion of the POI with a large protein domain
782 (>20 kDa) at the N- or C-terminus, which either is prohibited (as with nAChRs or $GABA_A$Rs for
783 instance) or may affect POI function.
784
785 Box1 Figure I. Representative reactive groups for protein-ligand bioconjugation.
786
787
788

Highlights

Chemogenetic technologies that combine the speed of pharmacology with the cellular precision of genetics are emerging, enabling acute control of neuronal receptors with circuit specificity.

Targeting drugs to specific neuronal types usually requires genetic manipulation of either the target protein or the target cell.

In vivo implementation of these technologies allows unprecedented control brain of circuits at the molecular level, and help unambiguously link the activity of specific receptors to behavioral functions.

Outstanding questions box

Is multiplexing possible? Only some of the methods described here allow for multiplexed interrogation across cell- (DREADDs, PSAM, DART) and receptor-types (PORTL). Notably, CLIP-mGluR2 and SNAP-mGluR7 expressed in the same cell could be labeled and manipulated with orthogonal reactivity and wavelengths, respectively [41]. Nevertheless, novel approaches for concurrent delivery of two or more drugs to distinct cellular populations are needed.

Can DART be made photo-reversible? One current limitation of the DART technology is its slow reversal. In principle, adding a photo-isomerizable linker could help accelerate the off rate, as in the PORTL approach. However, DART and PORTL have two major differences: ligand/receptor stoichiometry and fixed vs. variable distance between the receptor and the anchor. It will be interesting to determine whether DART can be made photo-controllable.

Can fully bio-orthogonal groups be developed for tethered ligands? Most of the functional groups described in this review are not fully orthogonal, causing non-specific labeling in live cells. Others are too large (SNAP, CLIP…) and may affect protein function. Some groups are too lipophilic and have limited bioavailability. And most of them cannot pass the blood brain barrier and therefore have to be delivered locally into the brain. Hence, numerous challenges remain in bio-orthogonal chemistry in live cells.

Click here to access/download;Key Figure;Figure1_Rev.pdf

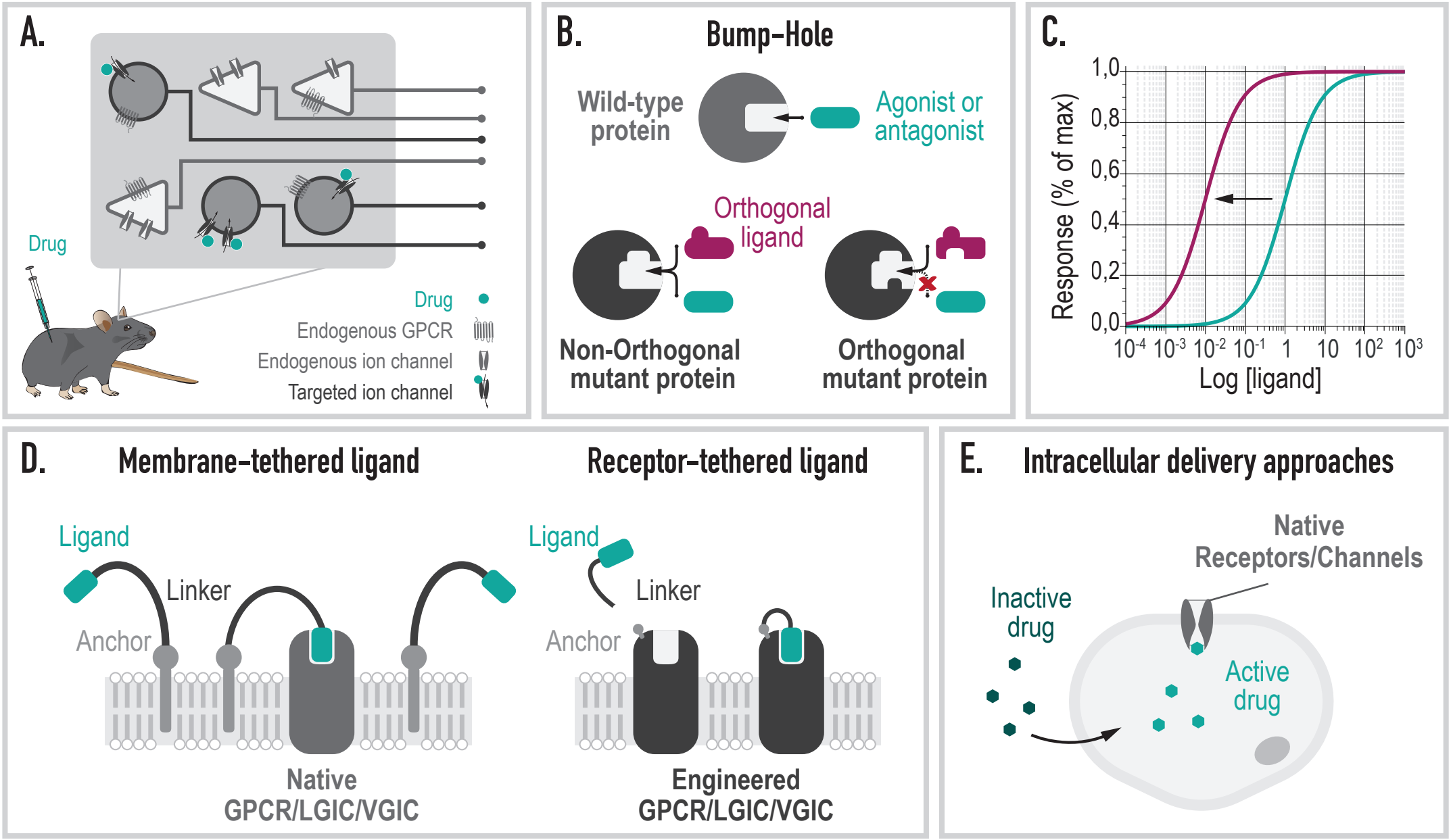

FIGURE 1

Figure 2

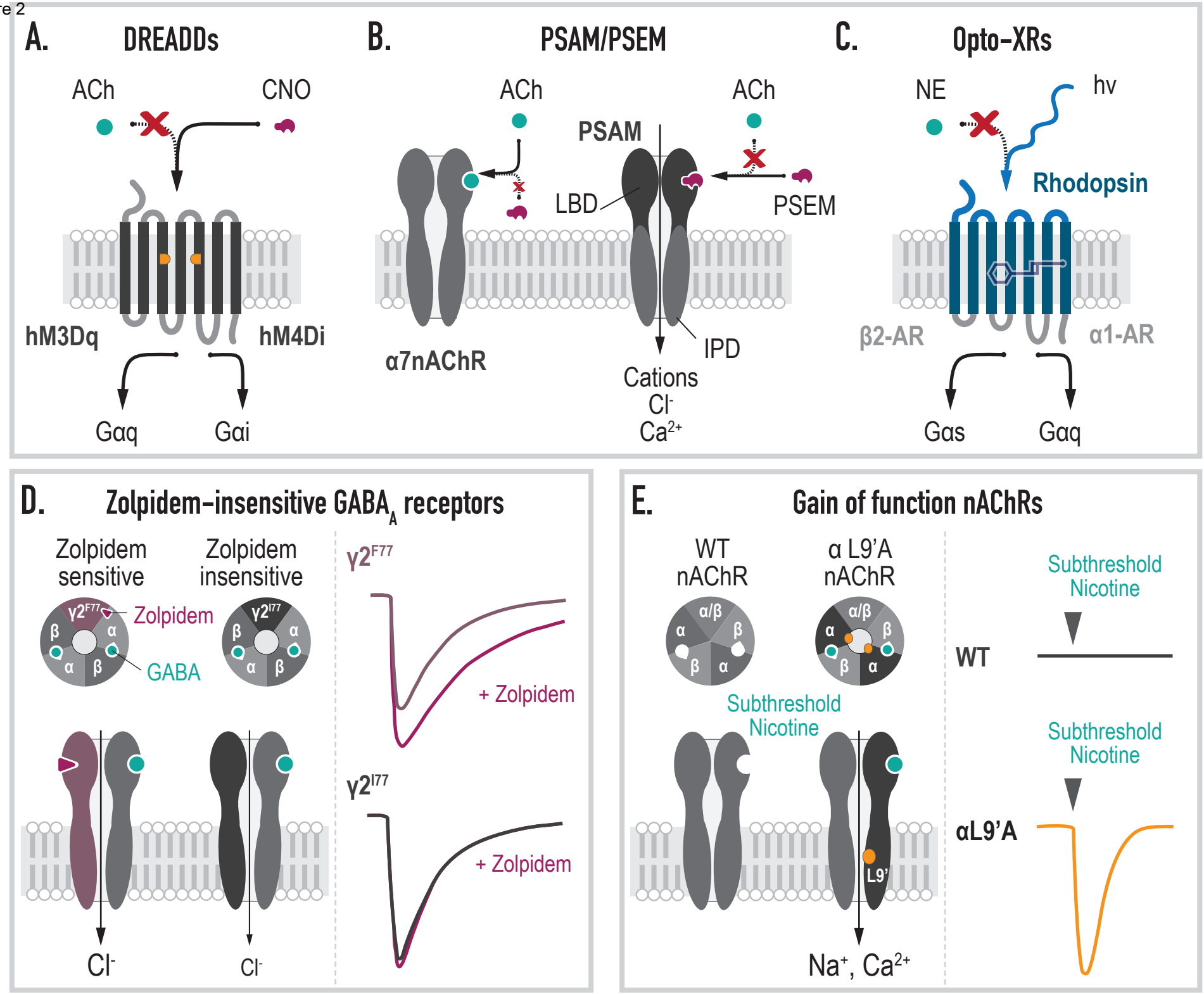

FIGURE 2

Figure 3

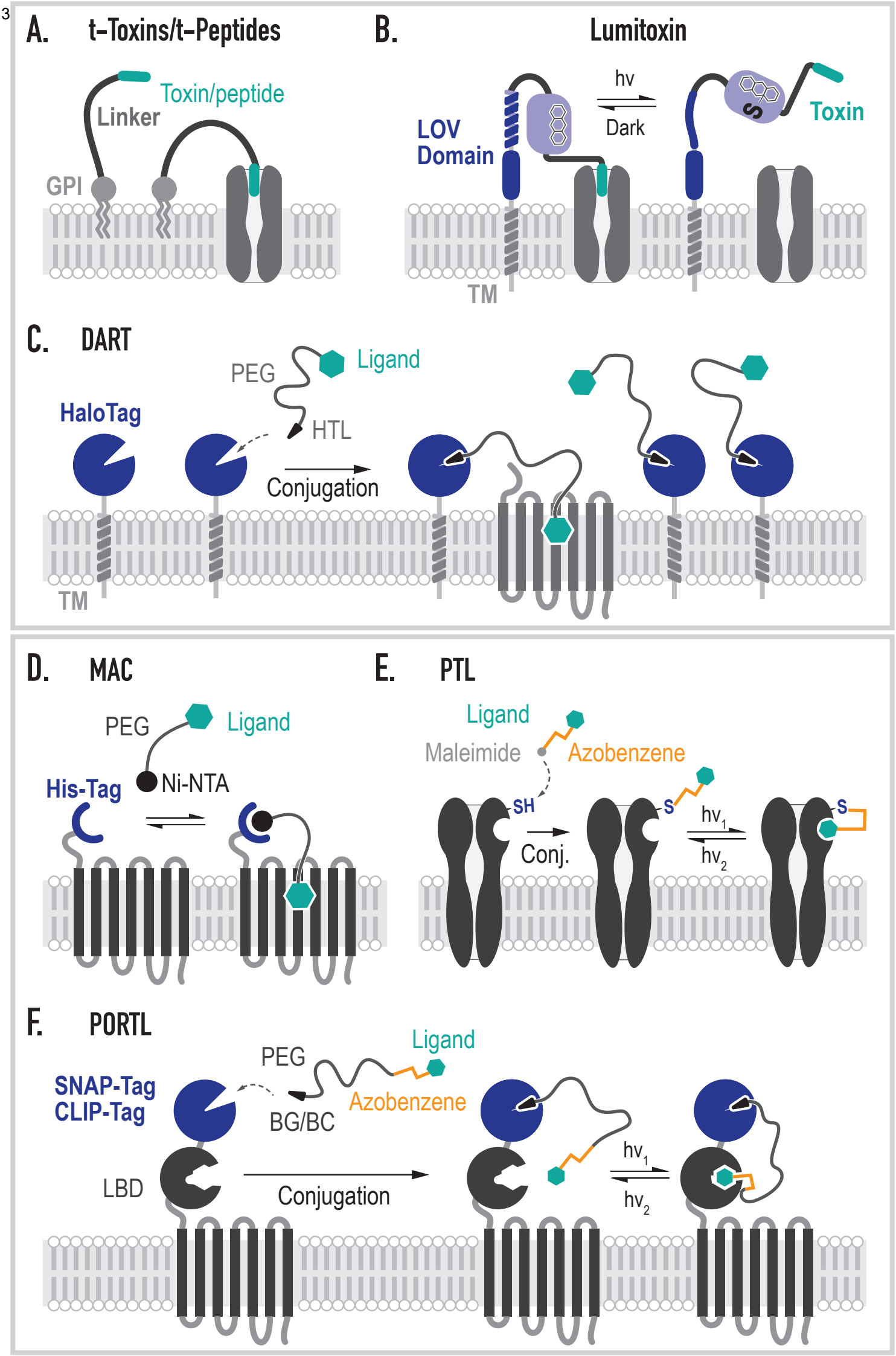

FIGURE 3

Figure 4

FIGURE 4

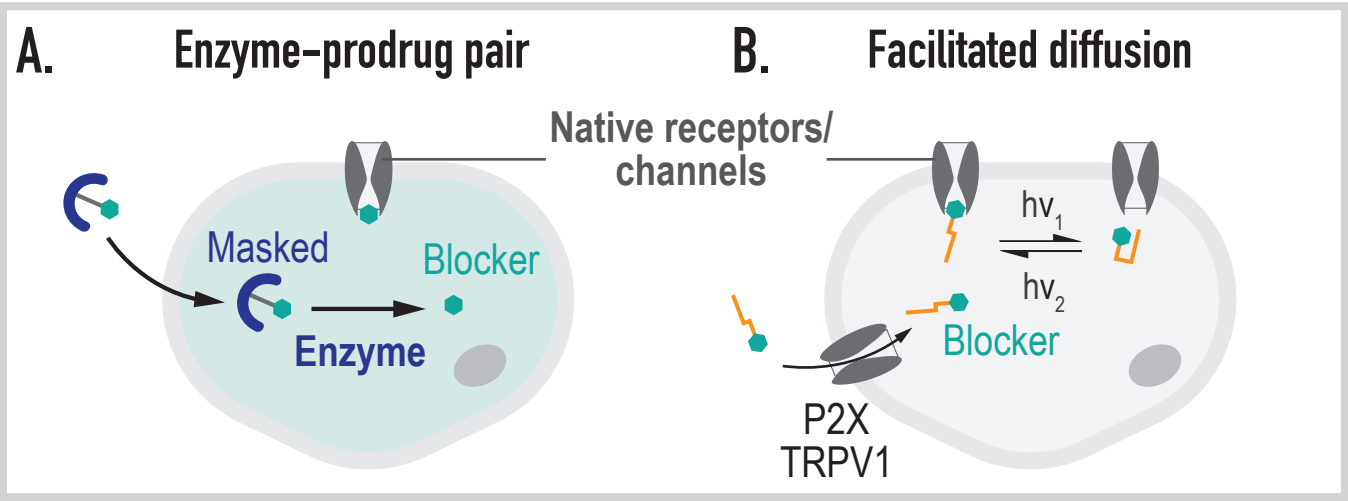

Figure I
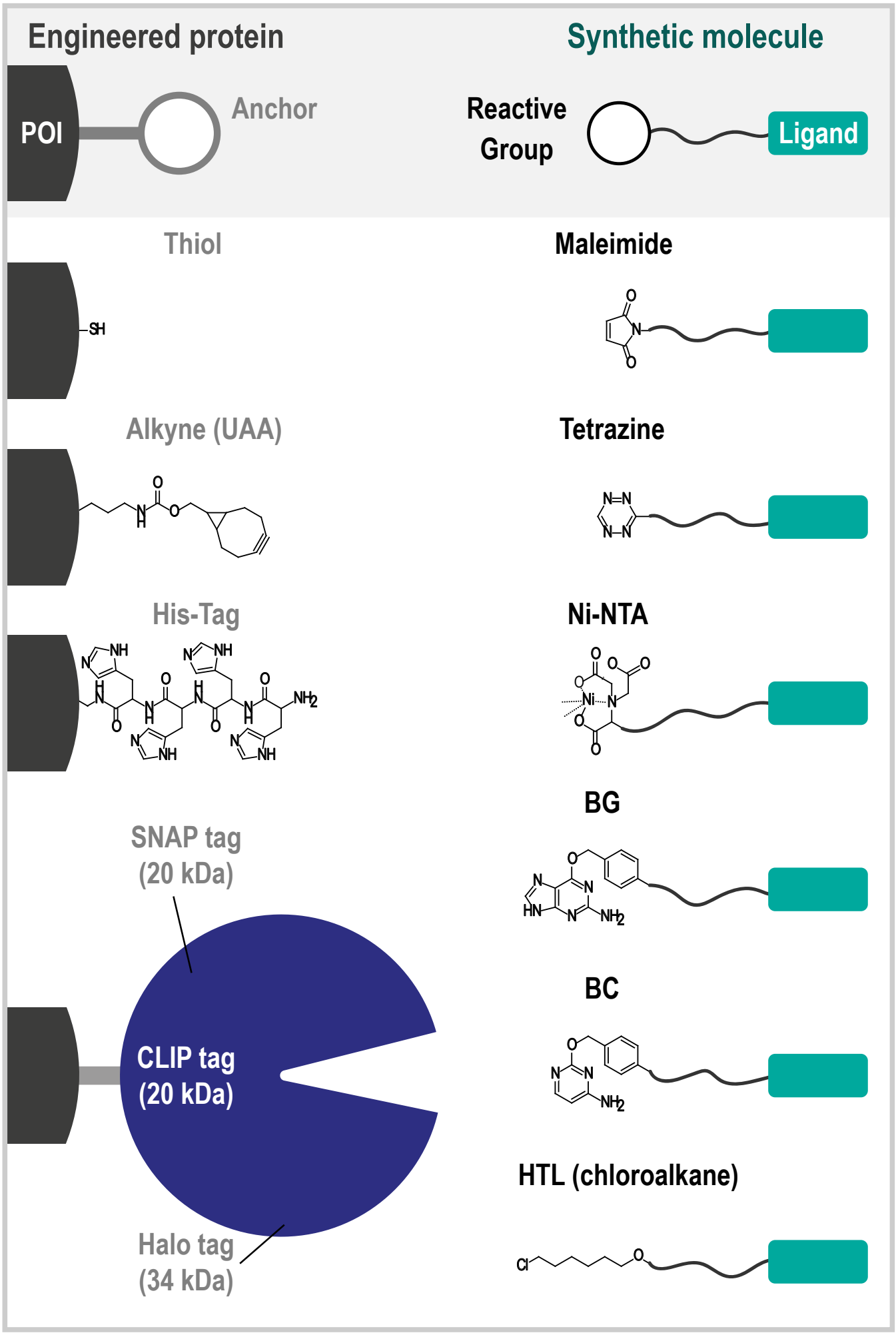
FIGURE - Box 1