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Optogenetic approaches for dissecting neuromodulation and GPCR signaling in neural circuits

Skylar M Spangler[1,2,3,4] and Michael R Bruchas[1,2,3,4]

Optogenetics has revolutionized neuroscience by providing means to control cell signaling with spatiotemporal control in discrete cell types. In this review, we summarize four major classes of optical tools to manipulate neuromodulatory GPCR signaling: opsins (including engineered chimeric receptors); photoactivatable proteins; photopharmacology through caging — photoswitchable molecules; fluorescent protein based reporters and biosensors. Additionally, we highlight technologies to utilize these tools *in vitro* and *in vivo*, including Cre dependent viral vector expression and two-photon microscopy. These emerging techniques targeting specific members of the GPCR signaling pathway offer an expansive base for investigating GPCR signaling in behavior and disease states, in addition to paving a path to potential therapeutic developments.

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Introduction

G-protein coupled receptors (GPCRs) are critical for neuromodulation. GPCRs modulate neuronal excitability by signaling through the heterotrimeric G-protein families (G_s, $G_{i/o}$, G_q $G_{12/13}$, among others), which can couple to channels or enzymes via direct beta-gamma subunit interactions or amplify intracellular signaling pathways (Figure 1). G_q couples to phospholipase C, cleaving phosphatidylinositol into IP_3 and diacylglycerol (DAG), mobilizing the release of calcium from intracellular stores. This is known to facilitate depolarization, in addition to synaptic release in terminals, also dependent on calcium. $G_{i/o}$ signaling via beta-gamma interactions positively regulates G-protein coupled inward rectifying potassium channels (GIRKs) to hyperpolarize the cell, as well as negatively couples to calcium channels to inhibit synaptic release. G_s signaling utilizes coupling to adenylyl cyclase to amplify the levels of cAMP in a neuron, which can influence excitability through cyclic-gated nucleotide channels (CNGA2) or via enzymatic kinase mediated pathways. GPCRs signal through other intracellular pathways through G-proteins and arrestin to modulate neuron and glial activity independent of ionic changes in the cell.

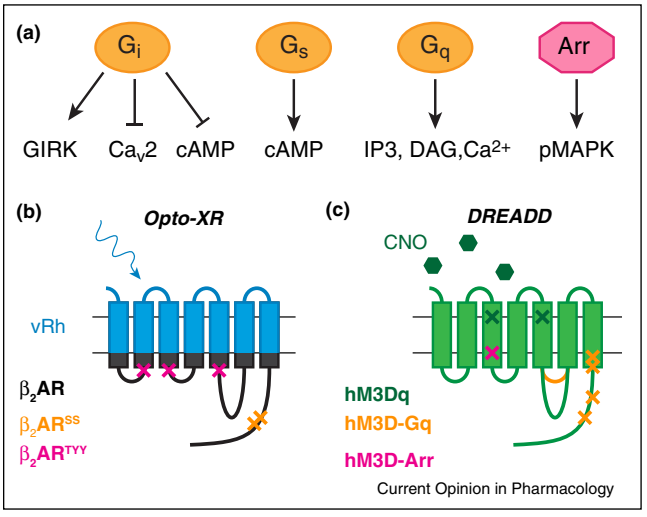
Many neurotransmitters activate GPCRs that initiate intracellular pathways with complex temporal and spatial effects within a neuron. In the brain, cellular, receptor, and signaling heterogeneity can cloud discrete conclusions. Therefore, GPCR-based discoveries have slowly matured, due to the intrinsic limitations of pharmacological manipulations. These approaches lack both receptor subtype specificity and cellular resolution. Though development of optogenetics provided neuroscientists with the ability to probe neural circuits with discrete spatiotemporal control, most optogenetic applications have focused on using light-sensitive ion channels. To achieve more naturalistic control of neuromodulator signaling, GPCR-based optogenetic and chemogenetic tools were created. These techniques allow for the selective interrogation of specific signaling and cell types in neural circuits with high spatial and temporal resolution *in vivo*. These tools (used independently or in a multiplexed fashion) offer powerful means for neuromodulation in the brain.

Here we provide a description of recent advances in the development of genetically targeted tools to interrogate neuromodulation, more specifically GPCR signaling in the brain (outlined in Table 1). This review is not meant to be exhaustive; here we focus on recently developed approaches used to characterize GPCR bias, or define either specific endogenous GPCR intracellular pathways, each with potential to be used in the brain to aid in the development of novel therapeutic treatments. For more comprehensive reviews, see Refs. [1–6]. We also compare both optical and chemical techniques. Additionally, we will discuss technological advancements for utilizing these tools *in vivo*. Finally, we discuss future development and application of optogenetic and chemogenetic tools.

Optogenetics: control or measurement of cell signaling with light

Optogenetics describes the technology in which cells are modified genetically to express light sensitive proteins

Figure 1



Schematic representations of GPCR signaling in neurons. **(a)** GPCRs signal through both heterotrimeric G-proteins and arrestins. G_i activates G protein-coupled inwardly-rectifying potassium channels (GIRK) to hyperpolarize the cell and inhibits voltage gated calcium channels (Ca_V2) to inhibit synaptic release. G_i and G_s respectively inhibit or amplify cyclic AMP (cAMP) production. G_q couples to phospholipase C to generate IP_3 and DAG which in turn regulate intracellular calcium stores. Arrestin signaling is predominantly mediated through phosphorylation of MAP kinases. **(b)** Schematic representation of Opto-β2AR depicting mutations for G-protein (Opto-2AR-SS) or arrestin (Opto-β2AR-LYY) bias. **(c)** Schematic representation of Designer Receptors Exclusively Activated by Designer Drugs depicting mutations for G-protein (hM3D-Gq) or arrestin (hM3D-Arr) bias. *(IP_3, Inositol triphiosphate; MAPK, mitogen activated protein kinase).*

allowing for either measuring or controlling cellular signaling with light. Optogenetics debuted in neuroscience as the direct activation of ion channels to regulate neuronal excitability with discrete spatiotemporal control [7].

Current advances in optogenetics target specific members of canonical GPCR signaling pathways with both natural and engineered optically sensitive proteins. Additionally, endogenous receptors are targeted by photoactivatable ligands, a method called photopharmacology [4,8]. Furthermore, other optogenetic approaches utilize genetically encoded reporters of protein-protein interactions or the production of second messengers [9].

Opsins

Naturally occurring opsins

Opsins are photosensitive GPCRs bound to chromophores. The first GPCR specific optogenetic tools were naturally occurring opsins [10]. Animal opsins are advantageous because they do not require exogenous chromophores (called retinals) to be added to the brain. Naturally occurring opsins are diverse in their spectral and signaling properties, often requiring little mutation or special tuning.

Vertebrate visual opsins: Visual opsins are conopsin and rhodopsin, the latter is classified as a weak candidate for optogenetics due to slow kinetics and bleaching. Conopsins have spectrally diverse excitation spectra. Developing tools from conopsin could in theory allow for the combinatorial activation of two neuronal cell populations. In the eye, conopsins couple to Gα transducin, part of the $G_{i/o}$ family of G-proteins, and thus optogenetic tools based on conopsin will signal to $G_{i/o}$. Thus far, all human

Table 1

Recently developed optogenetic tools

Tool	Mechanism	Targeted pathway	Activation	Deactivation/ dissociation
Opsins [14•,143]	Conformational change	G-protein and arrestin	NIRW-UV	Dark or bistable
Opto-Chimeras [1,20]	Conformational change	G-protein and arrestin	Red-blue	Dark or bistable
BLUF photoreceptors [35,37]	Conformational change	Cyclic nucleotides	Blue	Dark
LOV domains [42,44,48]	Proximity	P13K	Blue	Dark
	Conformational change	GTPase, MAPK	Blue	Dark
Cryptochromes [3,51]	Proximity	Kinase, GTPase, PI3K, MAPK, transcription *in vivo*	Blue	Dark
Phytochromes [56•,59,144]	Conformational change	cAMP	NIRW	NIRW
	Proximity	ERK, PI3K, GTPase	Red	NIRW
Caged Ligands [145,146]	Uncaging	GPCR	UV	Irreversible
Photoswitchable molecules [4,67]	Uncaging	GPCR, DAG	UV (red)	Green (blue)
Photoswitchable tethered ligands [73,74]	Uncaging	GPCR	UV	Green
Resonance Energy Transfer (RET) [75]	Proximity, sensor	Kinase activity, 2nd messengers, inter-and intramolecular interactions	Red-blue	Target protein dependent
Genetically encoded calcium indicators (i.e. GCaMP) [5,82]	Sensor	Calcium	Red and green	Calcium dependent
Fluorescent Protein Exchange (FPX) [80•]	Sensor	Calcium, PIP2, PKA activation, ERK activation	Red or green	Target domain dependent

conopsins have been characterized in neuronal cultures, with some work in brain tissue [11]. The use of human blue conopsin (vLWO; vertebrate long wavelength opsin) and mouse red conopsin (vSWO; vertebrate short wavelength opsin) for modulating neural circuits has been validated brain slices [12]. Photostimulation of conopsin modulates GIRK currents, a typical GPCR coupling event in neural circuits. Additionally, unlike rhodopsin, conopsins can be repeatedly activated without a noticeable desensitization of the response [12]. Recently, conopsin hyperpolarized DRN neurons in brain slice, and a chimera (see below) expressed in the DRN was able to modulate anxiety behavior [12].

Melanopsin, a non-visual opsin, has also successfully engaged neural circuits [13]. Melanopsins are activated by blue light, but unlike the visual opsins, are bistable and thus can be deactivated by yellow light. Though melanopsin has the potential to couple $G_{i/o}$ to regulate GIRK channels in heterologous systems, when examined in neurons it is almost exclusively G_q-coupled. Recently two variants of melanopsin have been characterized for transient and sustained G_q activation [14•]. Stimulation of ectopic melanopsin in pyramidal neurons mimics G_q modulation of channels [15].

G_s signaling can be activated by photo-stimulating certain non-mammal opsins. Jellyfish opsin signals through G_s to induce translocation of adenylyl cyclase, increase the production of cAMP, and increase phosphorylation of ERK [16]. A natural guanylyl cyclase fused to an opsin, was recently discovered in fungus [17]. This cyclase, termed Guanylyl Cyclase Rhodopsin (BeCyclOp), does not produce cAMP, unlike previously engineered guanylyl cyclase optogenetic tools. BeCyclOp has a functional spectrum broader than vertebrate opsins, with maximum cGMP production in green (530 nm) light and low activity in red and violet light. This cyclase was also functionally expressed in *Caenorhabditis elegans* muscle cells, demonstrating activity comparable to photo-stimulation from channel-based optogenetics [18•].

Opto-XRs

GPCR signaling in neural circuits may also be modulated though intracellular signaling that may not be dependent solely on generic Gα subunit coupling dynamics. Therefore, to more closely mimic endogenous signaling, chimeric optogenetic tools have been engineered using opsins with intracellular loops and C-terminal tail of GPCRs endogenously expressed in the central nervous system. The first of this family of 'Opto-XRs' were adrenergic receptors Opto-α1AR and Opto-β2AR (Figure 1b). [19,20]. The full profile of Opto-β2AR signaling was recently validated as mimicry of most of the endogenous receptor's properties. This study was also the first to express Opto-β2AR *in vivo* to demonstrate real time behavioral responses in endogenous neural circuits (anxiogenesis) [21••,22••]. Additionally, the first prototypes of G-protein and arrestin biased chimeras have been developed and characterized *in vitro* [21••]. This study reported diverse signaling dynamics were possible with Opto-XRs mutated either in the canonical GPCR DRY motif (Optoβ2-LYY, Addgene), known to be involved in G-protein coupling [23], or in the c-terminal serines (Optoβ2-SS, available in Addgene) known to be phosphorylation sites of G-protein coupled receptor kinase (GRK; Figure 1b) [24]. These prototypes differed in canonical ERK signaling, densensitization and internalization patterns. However, future studies and additional prototypes are warranted in this regard, as crystal structures, critical residues for G-protein and arrestin bias, and interactions continue to be identified [25].

Another chimera, Opto-A_{2A}R, from bovine rhodopsin chimera and the Adenosine 2A receptor, was also characterized *in vitro* and *in vivo* [26•]. Opto-A_{2A}R mimicked endogenous A_{2A}R by increasing cAMP production and differentially recruiting phosphorylation of only CREB in the hippocampus and only MAPK in the nucleus accumbens (NAc). In the same behavioral paradigm, stimulation of Opto-A_{2A}R in the hippocampus impaired memory consolidation, while stimulation in the NAc did not affect memory but increased locomotor activity. This demonstrates that Opto-A_{2A}R mimics differential A2A signaling in endogenous brain regions which leads to also different behaviors [26•].

OMOR, comprised of rat rhodopsin with intracellular components of the mu opioid receptor (MOR), mimics endogenous opioid signaling *in vitro* through $G_{i/o}$ protein and arrestin mediated signaling, by inhibiting production of cAMP, coupling to GIRK and stimulating ERK phosphorylation. In neuronal slices, saturating concentrations of mu agonist, DAMGO, prevented a subsequent response to photo-stimulation of OMOR-induced GIRK currents suggesting this chimeric receptor accessed the same intracellular pools of downstream effectors. Lastly, OMOR also mimicked MOR's ability to induce either a preference or aversion depending on the endogenous neural circuit where it was expressed [27••]. This study comprehensively validated the use of OMOR as a proxy for MOR a variety of preparations.

Additional, chimeric GPCRs have been engineered with melanopsin and conopsins and have been utilized *in vivo* [12,15,28••,29]. Opto-mGluR6, which is human melanopsin with the intracellular components of metabotropic glutamate receptor mGluR6, was used to restore vision through ON bipolar cells in blind mice [28••]. Other non-rhodopsin chimeras are partial chimeras that contain only some of the intracellular domains serotonin receptors. Herlitze and colleagues used the C-terminal tail of serotonin receptors to target opsins to the appropriate signaling domains. This method was also used to create tools

from vertebrate conopsins ($G_{i/o}$ coupled) and human melanopsin (Gq coupled) with the c-tail of 5HT1A and 5-HT2C, respectively. Both tools modulated dorsal raphe circuits involved in anxiety behavior. Recently, another partial melanopsin–serotonin chimera, created only with the intracellular loops of 5-HT2A, was reported to transiently hyperpolarize cells, but unreliable expression prevented quantification of the effects [15].

One exciting possibility with these chimeric Opto-XR approaches is the ability to mimic spatial and temporal properties of neuromodulator signaling at synapses or *in vivo*. For example, tuning the amount of response via a photo-stimulation event to mirror the release and activation kinetics of a monoamines or neuropeptides will allow us to better understand how these signals are transmitted in time, over small distances, in genetically defined cell types, and neural circuits. In addition, future approaches to define the 2-photon emission spectra of these opsin tools will allow for more advanced imaging studies *in vitro* in brain sections alongside sensing of signal transduction (i.e. GCaMP, or FLIM-based methods; see below). This would allow the investigator to perturb signaling in a native system while capturing neuronal ensemble dynamics, or GPCR signaling in real time. Advances along these avenues are likely to occur as additional Opto-XRs and native GPCR opsins become characterized and validated side-by-side against their native receptor counterparts. Additional work will be needed to validate the expression and localization profiles of these non-native receptor tools, and they may not recapitulate endogenous GPCR trafficking, and may need chaperone sequences added in some cases. These photo-sensitive GPCR constructs could also be used for subcellular optogenetics to investigate bias based on GPCR localization. Localization of signaling is now at the forefront of GPCR research. Gautam *et al.* demonstrated that Gβγ subunits not only translocate to intracellular membranes upon GPCR activation, but regulate cytoplasmic calcium concentrations [30,31]. Additionally, there is recent and increasing evidence for the sustain propagation of G-protein-mediated signaling within endosomes [32,33]. Photoactivatable proteins are another powerful tool to investigate subcellular GPCR signaling cascades.

Photoactivatable proteins

In addition to activation of GPCRs, there are other optogenetic tools for the activation/inhibition of 2nd messengers. This subset of tools includes unmodified proteins and engineered/chimeric tools containing naturally derived domains from non-mammalian species. These tools regulate downstream signaling through two mechanisms: allostery and proximity (Figure 2). For a more comprehensive review of these tools, see Ref. [3,34].

Flavoproteins

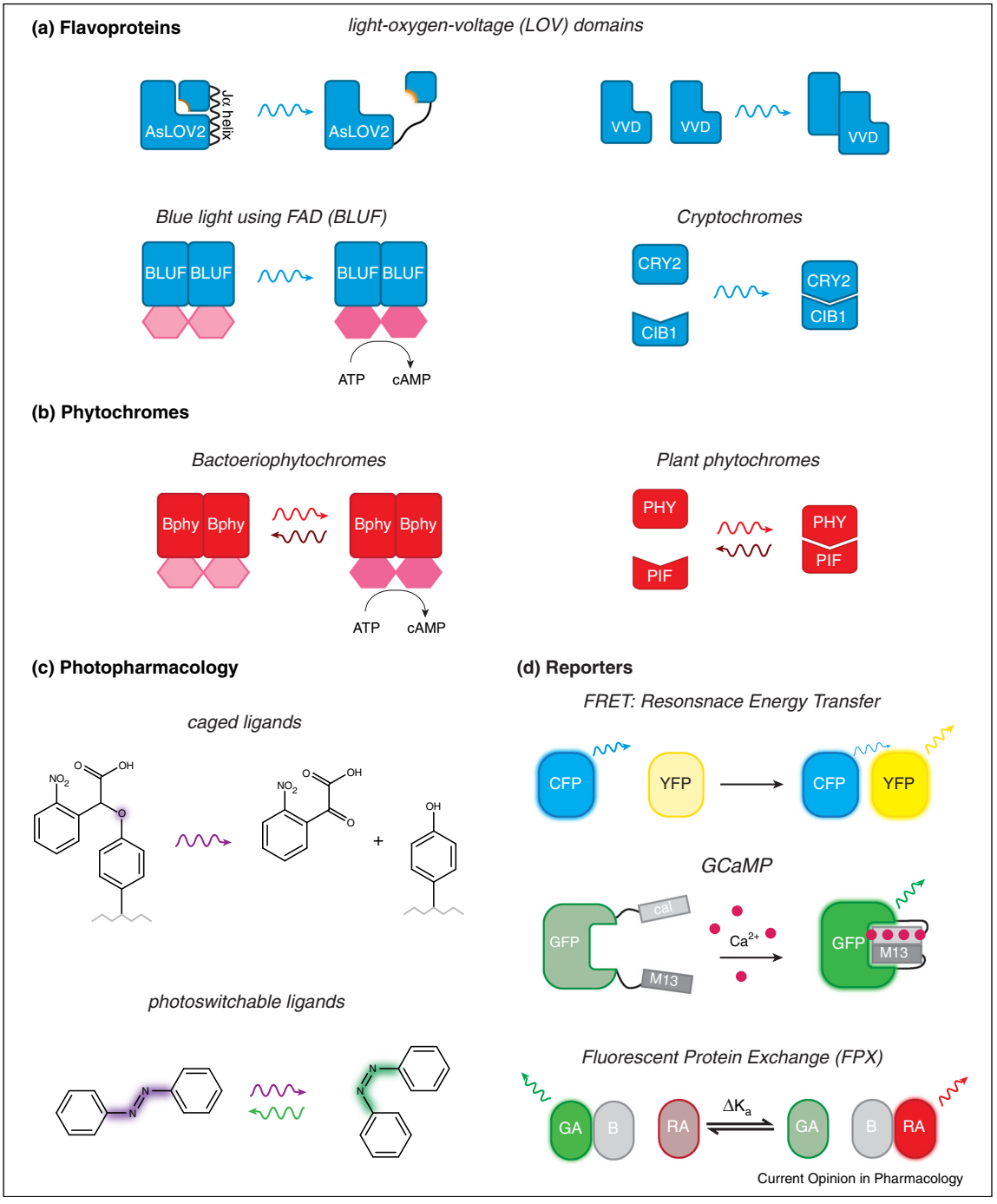
Flavoproteins are a diverse set of proteins that contain blue light sensing domains that use either flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) chromophores, both produced in mammalian cells. These include blue light using FAD (BLUF), light-oxygen-voltage (LOV) domains, and cryptochromes (Figure 2a). Flavoprotein domains used for optogenetic tools either initiate enzymatic activity, dimerize, or change in conformation, all in response to light.

Photoreceptors containing BLUF domains modulate cyclic nucleotide production (for a complete review on tools to modulate cAMP signaling see Ref. [35]); Pioneering studies used naturally derived flagellate Photoactivated Adenylyl Cyclase (PACα) to modulate behavior in non-mammalian model organisms [36–39]. Recently, investigators used PAC in rodent neuronal cultures to define the cAMP-PKA pathway that modulates axonal branching, a study capitalizing on the subcellular spatiotemporal control of optogenetics [40]. BLUF domains are the primary way to optogenetically manipulate cAMP aside from GPCRs, though the precise mechanism of activation is unknown. Excitingly, the crystal structure of a smaller version of PAC was characterized from cyanobacteria *Oscillatoria acuminate* (OaPAC), providing insights on its mechanism of light activation [41••].

LOV domains uniquely offer two mechanisms for tool development, conformational change and dimerization (for review see Ref. [42]). Light induced conformational change in the AsLOV2 domain, includes the unfolding of the Jα helix, which reveals the c-terminus [43]. In the 'unmasking' approach, enzymatic or binding domains are fused to the c-terminus [42] (Figure 2a, upper left). Unmasking was used to design a photoactivatable GTPase (PA-Rac1), which was used in brain with some success (NAc cocaine) [44,45]. Recently, AsLOV2 was also used as the foundation for light-induced protein dissociation, an approach called LOV2 trap and release of protein (LOVTRAP). The creators of LOVTRAP generated a small protein (ZDark) with high affinity to the coiled Jα helix, thus protein association only occurs in the dark. In the proof of concept study, LOVTRAP was used to sequester proteins from their signaling domain, but this approach can be extended to diffusible partners [46••]. Dimer association can be achieved with the Vivid (VVD) LOV domains that do not contain the Jα helix [47,48]. Recently, VVD domains were tuned to create Magnets, a family of photoswitches with dissociation constants ranging on the time scale of seconds to minutes. The authors demonstrated the utility of magnets by creating a photoactivatable phosphatidylinositol 3-kinase with the ability to change the morphology of COS-7 cells [49••].

Cryptochromes are another class of proteins able to create dimers. CRY2-CIB1 heterodimers are widely used in

Figure 2



Schematics of Non-GPCR optogenetic tools **(a)** Blue light sensitive flavoproteins induce conformational changes (LOV and BLUF domains, left panel) or protein interactions (LOV domains and cryptochromes, right panel) **(b)** Red sensitive phytochromes induce both conformational changes (bacteriophytochromes, left panel) or photoswitchable protein interactions (plant phytochromes, right panel). **(c)** Photopharmacology techniques: peptides may be caged by the addition of a photolabile carboxynitrobenzyl moiety (CNB) to tyrosine (top), or photoswitchable ligands provide reversible caging through photoisomerization of azobenzene (bottom). **(d)** Ratiometric optogenetic reporters: FRET sensors report protein interactions (top). GCaMP, is a genetically encoded calcium sensor (middle). Fluorescent Protein Exchange sensors rely on the change of affinity of a fluorescence enhancing monomer (bottom). (AsLOV2, avena sativa phototropin 1 LOV domain; Bphy, *Rhodobacter sphaeroides* bacteriophytochrome domain; CIB1, CRY-interacting bHLH1 (helix-loop-helix 1); CRY2, cryptochrome 2; CFP, cyan fluorescent protein; NIRW, near infrared window; PIF, phytochrome interaction factor; PHY, phytochrome domain; YFP, yellow fluorescent protein).

optogenetic tools to modulate protein-protein interaction and regulate protein localization [50,51]. CRY2-CIB1 technology was specifically used to inhibit G-protein signaling by recruiting regulator of G-protein signaling 4 (RGS4) to the plasma membrane, providing both the groundwork for tools to optically control G-protein activation with subcellular precision, and new insights into GPCR localized signaling [52••]. Interestingly, CRY2 domains also have the ability to form homodimers. This has been used recently to develop CRY2olig: Light Induced Co-clustering (LINC), which can be used as a mode of activation or inhibition through sequestration. Limitations to LINC are similar to those of co-immunoprecipitation, for the binding partners of the target protein may be sequestered as well. Another consideration is that oligomerization through LINC currently does not have the capability to localize clustering to a specific subcellular domain.

Phytochromes

Tools engineered with phytochromes enhance temporal control of signaling because they are bistable; they are sensitive to both red and neared infrared wavelengths of light (650–750 nm). Near red wavelengths are ideal for *in vivo* models due to the deceased amount of photo toxicity and increase penetrance in tissue [53]. However, red-shifted proteins do have the risk of thermal activation [54]. Phytochromes are derived from plant, fungi and bacteria; and like LOV domains they can regulate signaling through conformational changes and dimerization (Figure 2b).

Bacteriophytochromes are an extremely attractive platform for *in vivo* applications since not only are they the most sensitive to far red wavelengths, but their chromophore (biliverdin) is present in all mammalian cells [53,55•]. Bacteriophytochromes are enzymatic photoreceptors. Though the exact details of conformational changes that lead to the activation of bacteriophytochromes is unknown, two groups have used crystal structures to engineer optogenetic tools to regulate cyclic nucleotides in eukaryotic cells. Light-activatable phosphodiesterase (LAPD) was based on structure similarities of the regulatory domains of human phosphodiesterase, PDE2, and the light sensing domain of a bacteriophytochrome histidine kinase, PaBPhy. Fusion of the phosphodiesterase and light sensitive domains of these proteins generated a red light-activatable phosphodiesterase that deactivates in far red light [56•]. LAPD was functional in whole zebrafish embryos, but has yet to be used in a behavioral assay. Also guided by structure, Ryu *et al.* [55•] generated a near infrared window light activated adenylyl cyclase (Ilac) by fusing domains of a bacteriophytochrome diguanylate cyclase and bacterial adenylyl cyclase. The authors used this tool to induce locomotion in wildtype *C. elegans* through the production of cAMP, an experiment not possible using PAC, due to confounding blue light avoidance in wildtype in *C. elegans* [38,57].

Optogenetic tools derived from plant phytochromes take advantage of the heterodimerization of the PHY domain with PIF3 or PIF6 [58,59]. These domains can be used to associate known interacting partners, or to translocate a protein of interest to its functional intracellular domain. Phytochrome heterodimers may be preferred over flavoproteins due to their red-shifted sensitivity; however, the chromophore PCB is not endogenous in mammalian cells, limiting its application in neuroscience. Additional genetic engineering would be required to enable cells to synthesize PCB [60]. Phytochromes have been used to generate optogenetic tools for the regulation of phosphoinositides and ERK [61,62]. It is likely that future iterations of phytochrome domains will begin to be used in cultured neurons and in brain tissue as they become refined and more well accepted. Phytochromes offer several advantages for dissecting subdomains of neuronal signaling, and for uncovering the complexity of the intracellular compartments involved in neuromodulation.

Photopharmacology

Studies using classic pharmacological approaches are limited: drugs cannot target a subset of constitutively expressed proteins with spatiotemporal precision. However, spatiotemporally precise drug delivery can be achieved by integrating optical methods, an approach called photopharmacology (Figure 2c). Photopharmacology offers subcellular resolution while potentially preserving properties of the native receptors, including activation and deactivation kinetics, trafficking, and levels of expression [63–65].

For several years, neuroscientists have practiced photopharmacology with caged-ligands [66]. Caged ligands contain a photosensitive moiety that render the ligand inert. These ligands become biologically active only milliseconds after UV photolysis. Recently, Banghart and colleagues from the Sabatini group have used this technology to target opioid receptors. Endogenous opioid agonists were modified at the terminal tyrosine with a photoactivatable chromophore. These ligands offered the spatiotemporal control needed to study neuropeptide signaling in brain tissue [64] (Figure 2c). In a later study, the same group caged opioid antagonist naloxone to study the deactivation kinetics of opioid signaling *in vitro* [65].

Though photo-caging is a powerful tool for delivering ligands, it is largely irreversible in most instances. Reversible activation of ligands is achieved through the use of proteins containing photolabile domains, also known as photoswitches. The most common photoswitch is azobenzene, originally sensitive to UV light; red shifted versions have developed to allow for use *in vivo* [67,68] (Figure 2c). A photoswitchable small molecule mu opioid

agonist fentanyl has been developed as well using this approach [69•]. Diffusible photoswitches have also been developed to allosterically modulate metabotrophic glutamate receptors [70,71••]. Additional, neuropeptide photoligand mimics are in development and offer exciting extensions of this early work. These modified neuropeptides, however, have yet to be applied *in vivo*. Azobenzene technology can also be extended to second messengers. Recently, photoswitchable DAG was developed and used to control synaptic transmission in hippocampal mouse slices and *C. elegans in vivo*, illustrating the potential of photoswitches to modulate specific signaling pathways [72•].

Diffusible photosensitive ligands allow for spatial activation, but, like traditional pharmacological methods, lack genetic specificity. Genetic targeting can be achieved with Photoswitchable tethered ligands (PTL). PTLs are photosensitive ligands that will covalently bind to an engineered protein [4]. This approach was developed for metabotropic glutamate receptors (LimGluR), but could be translated to another GPCRs [73]. Although engineering GPCRs for PTL requires minimal mutation, the design of PTLs entails modeling based on crystal structures, possibly limiting their extension to less studied receptors [74]. Nevertheless, the PTL approach is an attractive technology as minimal mutations may preserve properties of the native receptor and allow for integration of other mutations, including those that generate bias.

Reporters

Not only can optogenetic approaches use light to induce changes in the cell, they can also report localization, intramolecular or intermolecular interactions, or the state of the cell (voltage, pH, presence of second messengers) (Figure 2d). These are usually fluorescent proteins (FP) containing intrinsic chromophores. Optogenetic reporters are useful for real-time imaging. Optogenetic reporters are advantageous over chemical probes because through mutagenesis their spectral properties can be changed.

Most commonly used are Resonance Energy Transfer (RET) techniques. The RET approach examines inter and intramolecular interactions by utilizing the overlap of emission and excitation of light-emitting proteins [75]. In this technique, light emitted from a donor protein excites an acceptor FP within 100 Å [75]. The donor proteins of RET are either bioluminescent (BRET), or fluorescent [in the case of FRET (Förster Resonance Energy Transfer)]. Interactions in RET are determined by analyzing intensity. Classically, this is of the relative intensity expressed as a ratiometric output, but RET can also be determined by the exponential decay of the intensity of the donor, fluorescent lifetime imaging (FLIM), which is independent of relative protein expression [76]. Recently, a FRET-FLIM PKA sensor expressed in neurons was able to report endogenous adenosine-A2A receptor activity, demonstrating the value of this technology to observe GPCR signaling in the brain [77].

Other optogenetic reporters are comprised of single fluorescent proteins. Previously, interacting domains of interest were fused to independent halves of a single FP, creating a fully functional protein based on proximity. However, this approach is irreversible, posing challenges for some applications. Alford and colleagues have developed a method using heterodimers: one monomer weakly fluoresces due to a quenched chromophore; the second monomer enhances the fluorescence (Figure 2d). Originally, there were two sets of AB heterodimers for either red (RA and RB) or green (GA and GB) fluorescence [78,79]. However, the enhancing partners (monomer B) bound both RA and GA. Through optimization of the monomer B, the authors created a sensor that would fluoresce green or red depending on the preferential affinity of monomer B, a concept coined Fluorescent Protein Exchange (FPX) [80•]. By fusing the monomers to various protein domains, the author created sensors for MAP kinase activity and a variety of second messengers (Ca2+, PIP2, PKA activation *via* CaMP). Furthermore, FPX sensors can be expressed single polypeptides, reducing confounds of expressing multiple proteins.

Classically, G_q-mediated calcium increases via neuromodulator receptors was detected by variety of dyes including Fura-based compounds that cannot be targeted to genetically defined neuronal populations. Recently, genetically encoded calcium sensors (GECI) have revolutionized neuroscience with the ability to detect calcium transients as a proxy for action potential firing [5]. GCaMP-type GECIs are the most utilized and optimized for *in vivo* applications, although in some cases they have been used in primary cultures [81]. Reporters in the GCaMP family are circularly permutated FPs fused to calmodulin (CaM) and a Ca2+/calmodulin binding domain, M13 [82]. In the absence of Ca^{2+}, the intrinsic chromophore is exposed and therefore quenched by the cytosol. In the presence of Ca^{2+}, CaM and M13 interact, protecting the chromophore from quenching, therefore allowing the FP to fluoresce more effectively (Figure 2d) [82]. The original GCaMP fluoresces green but now they are available in other colors [83]. Though GCaMP has been extensively used to uncover the specific contributions of neuronal ensembles to behavior or evoked circuit activity, its value as a tool to investigate endogenous GPCR signaling through pharmacological approaches is only beginning to be realized [84]. In the next several years, we are likely to see the use of this tool broadened to neuromodulator based questions and *in vivo* drug screening approaches. Developments in voltage indicators such will also allow for fine temporal precision of measuring neuromodulatory effects on membrane potential [85,86].

Chemogenetics: control of cell signaling with biologically inert molecules

Chemogenetic tools allow for selective modulation of GPCR signaling in specific tissues or cell types. Like optogenetic tools, chemogenetic tools enhance the spatial resolution of GPCR signaling; Chemogenetic tools are also more easily adaptable for behavioral applications (Table 2). For a more extensive review on these tools see Refs. [87–89]. The most widely used chemogenetic tools are modified GPCRs (see below). In addition to GPCRs, kinases have also been chemically engineered. By mutating ATP-binding sites allowing for the acceptance of bulky ATP analogues, Shokat and colleagues were able to develop methods to selectively inhibit kinase activity or identify substrates [90–92]. Both of these techniques have been used in neuroscience applications, though not directly related to GPCRs [93]. Mitogen activated protein kinases, commonly downstream of both G-protein and arrestin signaling pathways through GPCRs have also been a subject to this engineering [94]. However, due to the toxicity of thiophosphates, identification of kinase substrates has limited use *in vivo*.

The pioneering chemogenetic GPCRs were termed RASSLs, Receptor Activated Solely by Synthetic Ligands. RASSLs tended to have limited use *in vivo*, due to either low affinity of RASSL ligands, endogenous receptor activation, or constitutive activity [95]. The newest generation of chemogenetic GPCRs, DREADDs (Designer Receptors Exclusively Activated by Designer Drugs), were designed to only respond to *biologically inert* synthetic compounds [96]. Through directed molecular evolution of the human muscarinic receptor, the Roth group engineered a family of minimally mutated muscarinic receptors that are only activated by clozapine-N-oxide (CNO), but not endogenous ligands, including acetylecholine. Because of the diversity of muscarinic receptors, DREADDs were originally either G_q (hM3Dq) and $G_{i/o}$ coupled (hM4Di) (Figure 1c).

Currently, hM3Dq and hM4Di are widely used *in vivo* to excite and silence neuronal populations, and to modulate gliotransmission [97–99]. Furthermore, the development of DREADDs has been pivotal in selectively modulating GPCR signaling specifically in the brain, notably signaling through pathways that are not directly coupled to

Table 2

Comparison of optogenetic and chemogenetic tools

Tool	Advantage	Disadvantage	Mitigations
Photoactivable proteins	Microsecond temporal resolution [49••]	Costly equipment	Spectral tuning
	Subcellular Spatial resolution [52••]	Potential thermal activation (long wavelengths) [54]	Use of 2-photon to reduce phototoxicity [114]
	Abundant in nature [10]	Phototoxicity (short wavelength)	
	Diversity in wavelength and dynamic range [144]	Less naturalistic	
	High specificity of signaling proteins/pathway	Invasive surgery	
Opto-XRs	Receptor specific signaling	Costly equipment	Newer devices to increase mobility and minimize damage in behavior [117]
	Microsecond temporal resolution	Chimeras	Use of shorter wavelengths to decrease phototoxicity and increase penetrance
	Subcellular spatial resolution	Invasive surgery	
	Fine control of dosage	Restrictive actuators	
	Diversity of tools		
	Greater potential for multiplexing		
DREADDs	Minimally modified	Low temporal resolution [89,97]	Spatial resolution:
	Potential for chronic activation over weeks [89]	Lower spatial resolution	Use to target larger brain structure [118]
	Minimal equipment required	'Generic' GPCR signaling	Target two pathways with one drug [111••]
	Non-restrictive	Lack of diversity	
	Non-invasive stimulation	Potential for constitutive activity [123]	Pharmacology:
	Many available transgenic lines [88]		Titration of CNO
		Pharmacological limitations	Alternative agonists for muscarinic DREADDs available [109•]
		SalB: not water soluable and retains low KOR affinity [112]	
		CNO: can metabolize into clozapine [108], not completely biologically inert [122]	

excitability through ionic changes. CNO-induced activation of hM3Dq has been validated in its mimicry of acetylecholine-induced M3 receptor activation, aiding in the development of an arrestin-biased and recent Gq-biased DREADD, all of which have been characterized for use *in vivo* [100,101,102•] (Figure 1c). Additionally, hM4Di has the ability to inhibit vesicle release at terminals, similar to endogenous G_i-coupled receptors in the brain [99]. Thus, targeting hM4Di to axons offers a tool to silence synapses, independent of the excitability of the cell [103•]. Unlike, hM3Dq and hM4di, G_s-coupled DREADDs (rM3Ds) are chimeras, similar to Opto-β2AR, except the extracellular domain is a G_q DREADD as opposed to rhodopsin [104]. The value of DREADDs is demonstrated by the discovery of novel PKA-dependent GPCR signaling in consummatory behaviors. In an exploratory study, activation of rM3Ds in AgRP neurons increased food intake, through activation of PKA [105••]. Additionally, G_i-coupled postsynaptic receptors modulated binge alcohol behavior, through inhibition of PKA signaling [106•]. DREADDs have also been used to elucidate sustained G_q-mediated neuronal inactivation through JNK MAP kinase [107]. These experiments highlight the ability of DREADDs to elucidate molecular mechanisms of neuromodulation through G-protein activation.

Though DREADDs demonstrate cell behaviors akin to endogenous GPCRs, their translational potential is limited by their ligands. Controversy over the metabolization of CNO to pharmacologically active clozapine in humans and guinea pigs inspired a structure-activity relationship (SAR) analysis of DREADDs aimed to identify alternative agonists [108]. This study yielded 'Compound 21', which is not metabolized in the same pathways as CNO, eliminating the risk for conversion to clozapine [109•]. This study also identified perlapine, a sleep-inducing hypnotic drug, as a highly selective novel agonist for hM3dq [110]. Lack of diversity in DREADDs and their ligands, also limits the potential of multiplexing. However, Alrdin-Kirk and colleagues took advantage of the shared ligand by pairing hM3Dq and rM3Ds to maximally activate dopamine neurons [111••]. In addition to the G_q-coupled excitation by hM3Dq, the inhibitory effects of G_i-coupled dopamine autoreceptors were counteracted by G_s signaling through rM3Ds. With these tools the authors were able to define a cAMP induced mechanism for Graft Induced Dyskinesia in Parkinsonian patients, a phenomenon with no known mechanism and great clinical relevance [111••]. Alternatively, chemogenetic multiplexing can now be achieved with the use of KORD (Kappa Opioid Receptor DREADD) [112]. KORD's design was driven by the structure of KOR combined with molecular modeling of Salnorvin B (SalB), a low affinity inert ligand of KOR. Because it is pharmacologically distinct, KORD can be used in concert with muscarinic DREADDs to gain bidirectional control of the same neuron [111••,112]. KORD is also kinetically unique from hMD4i DREADDs, exerting shorter lasting effects. KORD has played a pivotal role in expanding the chemogenetic toolbox for modulation of neural circuits.

Applicability of optogenetic and chemogenetic tools

Optogenetic and chemogenetic tools have undoubtedly revolutionized interrogation of specific GPCR signaling, especially in the brain. Both genetic approaches have inherent limitations; thus applicability of each toolset depends on experimental design. It is important to note that both approaches have been used to simulate the endogenous signaling of the other techniques model GPCRs: hMD3q has been used to recapitulate melanopsin signaling in the retina and melanopsin for muscarinic signaling in pyramidal neurons of the cortex [15,113].

Optogenetics provides precise spatial (subcellular) and temporal (microsecond) control of signaling. Temporal control is enhanced with bistable tools (ligands, receptors, effectors) that allow for inactivation by absorption at a second wavelength. Because of the range of spectral properties optogenetic tools from ultraviolet to near infrared wavelength, there is flexibility in the number of available tools for a specific target. Additionally, since photosensitive proteins are abundant in nature, there are many unexplored avenues for tool development.

Currently, there are optogenetic tools available for each step of the GPCR signaling pathway, from ligand binding to downstream signaling events and deactivation. There are some considerations in using optogenetics, although recent advances in technology have addressed some of these concerns. Biophysical considerations include the risk of dark state activity, whether basal enzymatic activity, dimerization or incomplete caging, and limitations of wavelength required for stimulation. Some opotogenetic tools require blue or UV light, short wavelengths with potentially toxic effects and shallow tissue penetration. The use of UV light can be avoided either by delivering two or more photons of lower energy, as in 2-photon microscopy, a method especially useful for commonly UV sensitive photoactivatable ligands. Two-photon microscopy also enhances the spatial resolution, as focusing two beams restricts stimulation to a confined three dimensional space [114]. Furthermore, near IR wavelengths combined with cranial windows allow for the stimulation and imaging of deep brain structures *in vivo* [115]. Previously, *in vivo* actuators greatly limited behavioral approaches because they were invasive, restrictive, and lacked spatial precision. However, the latest optogenetic devices minimally damage the brain, liberate the subject's mobility and precisely deliver light [116,117].

Compared to optogenetics, chemogenetic approaches are generally less technically challenging to use, for they are

less invasive, less restrictive and cost-effective. Chemogenetic tools require no specialized equipment for actuation; Because of its high blood brain barrier penetrance, CNO can be administered through local or systemic injection [99,103•,118]. CNO is also water soluble, allowing for chronic exposure through dilution in animal drinking water for extended periods of time (*i.e.* 2 weeks), without decreases in behavioral effects [89,119–121]. However, some recent studies highlight the need for care and optimization when using DREADD receptors *in vivo*, including the selection of proper control groups. Previous concerns about the biological activity of CNO were limited to only humans and guinea pigs, however a recent study demonstrated that CNO also exerts behavioral effects on rats that do not express DREADDs [122]. Another group also demonstrated biologically activity of DREADDs in the absence of CNO [123]. These studies highlight the importance of experimental design, including proper controls.

Chemogenetic tools enable the activation of GPCR pathways with spatial resolution, though significantly less so than optogenetic tools. Both tools can be similarly expressed through stereotaxic injections (see below). Optogenetics, however, allows for precise subcellular stimulation of GPCR signaling, not available in chemogenetics [34]. Nevertheless, chemogenetic tools allow for modulation of larger brain areas that cannot be feasibly illuminated through optogenetic approaches [118]. Chemogenetic tools share limitations with classic pharmacological methods: these tools potentially have off-target effects and lack precise temporal control. In addition to the slower kinetics of activation, compared to optogenetic tools, CNO has a slow wash out rate, potentially lasting for at least 2 hours [89,97,104]. Therefore, chemogenetics are more suited for investigating slow, long-lasting behavioral effects. In summary, chemogenetic and optogenetic tools differ in their feasibility and spatiotemporal resolution. Each toolset has a set potential strengths and limitations, determined by experimental design. Both tools can be used complimentary to each other to dissect receptor specific from generic G-protein signaling [22••].

Expression methods

The resolution of optogenetic and chemogenetic tools is defined not only by stimulation but also expression. These tools are expressed either virally or through transgenic animal approaches. Viral delivery is the most widely used route for expressing transgenes [124]. Viral delivery requires less commitment of resources needed to generate and maintain a genetic line. Additionally, viral expression is limited to the site of infection, increasing spatial resolution. Adeno-associated viruses (AAV) and lentiviruses are most commonly used [21••,27••,124]. Preference of viral vector can depend on desired specificity of expression. AAVs spread farther from the injection site, due to their small size, and lentiviruses typically infect smaller regions, but can carry larger cargo when needed. Fine spatial resolution can theoretically be achieved by encoding elements to ensure proper targeting to synapses and other neuronal compartments. Recent efforts have utilized both c-terminal and n-terminal domains for this purpose and further developments will enhance specificity to select neuronal compartments [12,29,103•].

As their name implies, genetically targeted technologies gain resolution through genetic identity. Lentiviruses have larger packaging capabilities, permitting the use of transcriptional promoters to target expression neuronal types, an approach usually not available with AAVs [125]. Cell-type specific promoters, however, may not a drive sufficient amount of expression [126]. To overcome this, viruses dependent on DNA recombination, especially through Cre recombinase, have been developed [127]. Recombinases usually are driven by cell-type specific promoters in the genome of a transgenic line or in an independent virus [128]. More discrete populations of neurons can be targeted using intersectional approaches, utilizing other recombinases such as FLP and DRE [129,130]. Recombinase technology can also target neurons that project to the injection site through the use of Canine-adenovirus (CAV) [131]. CAV viruses have the ability to infect neurons through retrograde axonal transport [132]. DREADDs have been successfully expressed *in vivo* through this method [133,134]. Another, very promising retro-AAV (rAAV2) was recently developed, that will open even more possibilities for retrograde expression [135]. Finally, recombinases are also subject to optogenetic stimulation, photoactivatable Cre-recombinase is continually being optimized for the spatial control gene regulation *in vivo* [136–138]. These approaches of expression allow for further control of discrete cell type manipulations within neural circuits offering even greater specificity and reductionism of neuromodulator pathways *in vitro* and *in vivo*.

Development

There are many unexplored avenues of optogenetic and chemogenetic tool development. These techniques can be developed to model other GPCR signaling components, such as the $G_{11/12}$ signaling family. Additionally, the spatial resolution of DREADDs would significantly increase with photoactivatable ligands. Tuning and optimization through mutation can also allow for the development of new tools with different signaling dynamics, including signaling bias, kinetics, spectral properties and subcellular localization [21••,139]. Many optogenetic tools are still in the conceptual stages and have yet to be optimized for expression in neurons or *in vivo*. In a recent proof of concept report, light induced secretion was developed using UV8R, a plant photoreceptor [140•]. Impressively, the authors used this method to optically induce secretion of a reporter protein at specific dendritic

branch points [140•]. Validating this approach with functional signaling proteins will significantly expand the neuroscience optogenetic toolbox for GPCRs. Full characterization of existing tools and their ability to simulate endogenous *in vivo* signaling will be crucial as subcellular localization and cell-type specific expression can define GPCR bias *in vivo*. Overall, *in vitro* and *in vivo* validation of current optogenetic tools will aid the community in selecting the most applicable tools [141].

Developing advances in many fronts of biological technology will foster the creation of many genetically targeted approaches. Genome sequencing has dramatically increased the diversification of the available library of genomic sequences from biological organisms. Currently, tens of thousands of sequences for opsins and photoactivatable proteins have been reported [10]. Natural proteins hold potential as resources for opsin based tools with unique spectral properties with minimal mutagenesis [10]. Advances in technology, such as 2-photon stimulation and imaging have revitalized the development of optogenetic tools sensitive to short wavelengths, such as the recently discovered bistable vertebrate G_i coupled neuropsin [142]. Structural biology, through high resolution crystal structures and developing modeling software will continue to inspire the development of optogenetic and chemogenetic tools far into the foreseeable future.

Concluding remarks

Optogenetics and chemogenetics have revolutionized the study of neuronal circuitry and have great potential in dissecting the role of specific GPCR signaling pathways. Multiplexing within or between tools enables bidirectional neuromodulatory control of signaling pathways in a single neuronal population. In addition, multiplexing of optogenetic actuators and reporters of different spectral occupancy can further define pre- and post-synaptic signaling involved in behavior and disease states. These tools also have translational potential, providing reductionist approaches for understanding drugable neural circuits that may lead to novel chemical entities for drug discovery.

Conflict of interest statement

Nothing declared.

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