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Optogenetic user's guide to Opto-GPCRs

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1. ABSTRACT

Optogenetics has taken biomedical research by storm. The power and precision at which light-gated ion channels control cellular excitability in diverse biological systems has convinced researchers of an optical future. Growing interest in optical methods has sparked the development of multiple new optogenetic tools, which allow precise control of numerous cellular processes. Among these new tools are the light-activatable G-protein coupled receptors (GPCRs) or Opto-GPCRs. The extent of the GPCR family, which in humans alone encompasses approximately 800 different proteins, and the immense therapeutic potential of Opto-GPCRs predict a big future for this juvenile field. Here the different approaches taken to design Opto-GPCRs are reviewed, outlining the advantages and disadvantages of each method for physiological and potential clinical application.

2. INTRODUCTION

Over the last decade optogenetics has revolutionized neuroscience (1, 2). The optogenetic technology, as the name suggests, is based on targeted genetic introduction ("genetics") of light-sensing, ion-translocating membrane proteins that can be rapidly and reversibly activated by light ("opto"). Exogenous expression of "traditional" optogenetic tools, including Channelrhodopsin-2 (ChR2) from the green alga *Chlamydomonas rheinhardtii* and halorhodopsin (eNphR) from the archaebacteium *Natromonas pharaonis* has allowed scientists to bidirectionally steer the membrane potential and thus the activity of targeted neuronal populations, elucidating their roles in neural circuits, behavior and disease (3, 4). These microbial opsins, although structurally similar to mammalian opsins, are not G-protein coupled, but form direct depolarizing ion channels and hyperpolarizing ion pumps.

Neuronal circuits are, however, not simple on or off, but they are extensively modulated, in particular by G-protein coupled receptors (GPCRs), acting through G proteins and cell-specific intracellular signaling pathways, which were until recently not accessible to optogenetic manipulation. GPCRs are activated by diverse stimuli including neurotransmitters, peptides, cations, odor components and light. With few exceptions they stimulate intracellularly associated heterotrimeric G-protein complexes that fall into four families based on alpha-subunit similarity: G_i, G_s, G_q and G_{12}. G_i and G_s alpha-subunits act on adenylyl cyclase, causing a reduction or an increase in intracellular cAMP, respectively, while G_q subunits stimulate phospholipase C and and G_{12} subunits stimulate monomeric G proteins of the Rho family. Although broadly categorized into only these four families, the signaling pathways within each family are highly variable. This variety is a consequence of several factors, such as (i) multiple G-protein subtypes with diverse subunit assemblies – the human genome comprises at least 16 G(alpha), 5 G(beta) and 12 G(gamma) subunits (5), (ii) the cell-specific protein complement that specifies the intracellular pathway, (iii) the cytosolic G protein regulators (RGS-proteins) that modify G protein activity (iv) the cellular state (e.g. depolarization, intracellular Ca^{2+} and Mg^{2+} levels) (6, 7) and (v) GPCR desensitization by cell-specific G-protein coupled receptor kinases (GRKs) and arrestins acting on the active receptor. Owing to the extreme

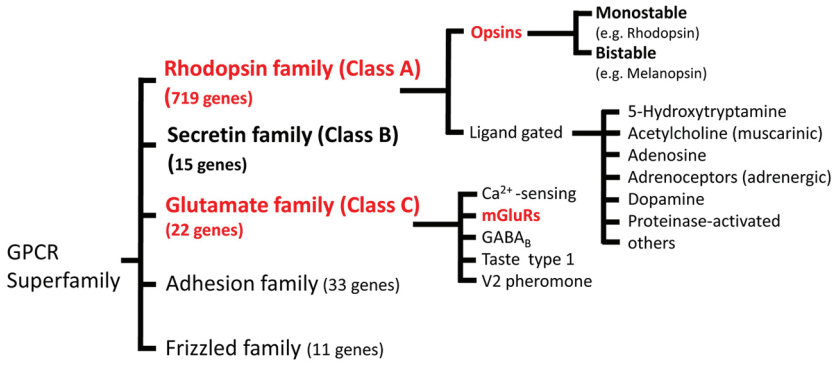

Figure 1. Phylogenetic classification of human GPCRs using the GRAFS system based on sequence homology and functional similarity. GPCRs represent the largest superfamily of receptors in the human genome and are divided into five families, comprising the three classical main classes A, B, and C. GRAFS stands for Glutamate, Rhodopsin, Adhesion, Frizzled, Secretin. By far the largest and best understood family is the Rhodopsin class that includes all classical GPCRs as well as hundreds of olfactory receptors. Metabotropic glutamate receptors (mGluRs) belong to Class C. Some structural elements including seven transmembrane domains are common to all families, but the members of any family exhibit no sequence similarity to those of other families.

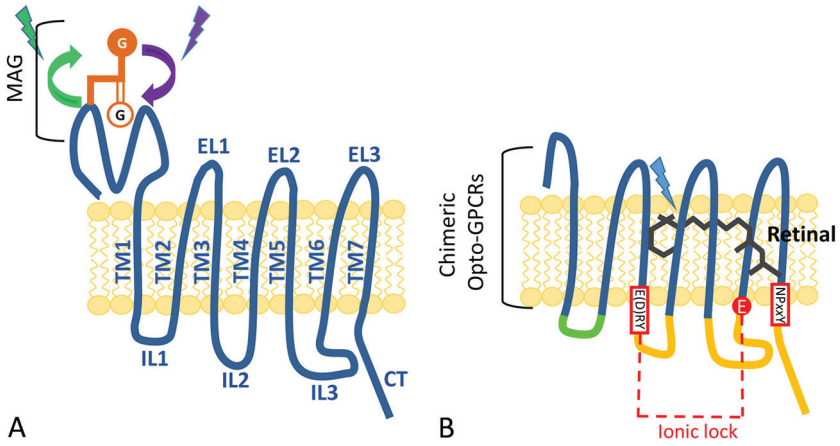

Figure 2. A sketch highlighting the two main areas of Opto-GPCR engineering, exemplified for a class A GPCR (A) photoswitchable ligands (PTLs) where the synthetic ligand is covalently attached close to the ligand binding domain *via* a photoisomerizable tether and (B) engineered opsins where the intracellular domains have been substituted with the corresponding domains of the target GPCR. While the use of PTLs require an accurate structural model of the ligand binding site in order to attach the PTL in a functional position, engineering chimeric GPCRs is simplified by highly conserved structural motifs, or functional variants of these motifs, located at the splice sites between the transmembrane helices (TM1-7) and the intracellular loops (IL1-3) and the C-terminus (CT), respectively. This conserved motifs include the "ionic lock" formed by the E(D)RY site at the cytoplasmic border of TM3 linked to a glutamate residue (E) at the junction of IL3 and TM7, stabilizing the inactive state of the receptor, and the highly conserved NP*x*Y (*x*PK*x*Y in mGluRs) motif at the C-terminal end of TM7. Replacing IL1 is typically not required.

cell-type and cell-state specific effects of various GPCRs, they represent one of the most sought after drug targets for the pharmaceutical industry (8). The potential benefit of a comprehensive understanding of specific GPCR signaling pathways in health and disease has fueled the development of a novel category of optogenetic tools, light-activatable GPCRs or Opto-GPCRs that can tap into cell-specific signaling pathways (9-11).

GPCRs constitute the largest family of membrane proteins in the human genome, with approximately 800 members (12). Based on structural and functional similarity, mammalian GPCRs are divided into five families or classes, with class A comprising the light-sensitive opsins and class C the metabotropic glutamate receptors (mGluRs), on which we mainly focus in this review (Figure 1) (13).

All GPCRs share a highly conserved tertiary structure and ultimately a similar mode of receptor activation, although virtually no sequence homology exists between classes (14, 15). All GPCRs comprise seven transmembrane helices (TM1-7) connected via intra- (ILs) and extracellular loops (ELs) of varied length (Figure 2). Structural alignment of the three major GPCR classes A, B and C revealed a particularly good consensus across the intracellular halves of the TM domains (16, 17) exhibiting a set of conserved micro-domains that are

important in signal transduction (18). These include the highly conserved E(D)RY motif at the junction of TM3 with IL2 and the NP*xx*Y (*x*PK*x*Y in mGluRs) motif at the junction of TM7 and the C-terminus (CT) required for the structural integrity of the receptor (Figure 2) (16, 18, 19). The crystal structures of the active states of class A GPCRS, including rhodopsin and the adrenergic receptor (14, 17), provided important insights into conformational changes that occur upon activation. All crystallized GPCRs appear to share the same activation scheme: an "ionic lock" formed between the arginine residue of the E(D)RY motif (e.g. R_{135} in rhodopsin and R_{668} in mGluR6) and a glutamate residue at the border of IL3 and TM6 (e.g. E_{247} in rhodopsin and E_{775} in mGluR6) stabilizes the inactive state. In mGluRs, the "ionic lock" further involves interactions between a threonine residue in TM6 (e.g. T_{785} in mGluR6) and a glycine residue in TM7 (e.g. G_{827} in mGluR6) as well as interactions between a lysine residue in TM3 (e.g. K_{665} in mGluR6), a glutamate residue in TM6 (e.g. E_{775} in mGluR6) and a serine residue in IL1 (e.g. S_{646} in mGluR6). Reorganization of the hydrogen-bonding networks among TM3, TM5, TM6 and TM7 and disruption of the "ionic lock" upon receptor activation leads to a large outward movement of TM6 exposing the G protein binding sites on IL2, IL3 and the CT (14, 17). It is believed that the degree of TM6 movement and expansion of the space between TM6 and TM7 upon disruption of the "ionic lock" is related to the strength of activation (20).

The most striking differences between class A and class C GPCRs are the position of TM5(15) and the identity of the longest IL, which determines G protein selectivity and is IL3 in class A GPCRs and IL2 in class C GPCRs (21, 22). Class A GPCRs contain an additional highly conserved 8^{th} helix that directly follows TM7 and lies parallel to the cytoplasmic membrane surface. The 8^{th} helix starts with the conserved NR(K)Q sequence and its proximal part is considered to play a role in the receptor's conformational switch as well as arrestin binding to control activity. For light-activated class A GPCRs (opsins) the integrity of the retinal binding pocket is crucial for proper function. The binding pocket comprises a conserved lysine residue (e.g. K_{296} in rhodopsin) in TM7 that is covalently linked to the chromophore retinal *via* a Schiff base and stabilized by a negative counterion in TM3 (e.g. E_{113} for rhodopsin). Absorption of a photon isomerizes *cis*-retinal to the all-*trans* form inducing helix opening and exposure of the cytoplasmic G-protein binding sites. The negatively charged residue in the E(D)RY motif (e.g. E_{134} in rhodopsin) additionally, stabilizes the inactive opsin molecule.

In this review I discuss the three main approaches that have been taken to optogenetically control cell-specific GPCR signaling pathways by light, with emphasis on vertebrate GPCRs: (1) optochemical light switches tethered to the ligand binding domains of class C GPCRs, (2) exogenous expression of unmodified opsins (23, 24) and (3) chimeric GPCR proteins that combine the light switch of an opsin with the intracellular G protein binding domains of a non-opsin target GPCR (11, 21, 25).

3. OPTOCHEMICAL LIGHT-SWITCHES: SYNTHETIC PHOTOSWITCHABLE THETHERED LIGANDS (PTLS) ATTACHED TO GENETICALLY MODIFIED GPCRS

The PTL technology, which chemically introduces a photoswitchable ligand to the ligand binding domain of a receptor, was developed in ionotropic receptors (26, 27) and was recently extended to mGluRs (10), class C GPCRs that are allosterically regulated by glutamate binding to a large extracellular clamshell ligand-binding domain (28). mGluRs respond to spatially confined, temporal patterns of synaptic and extrasynaptic glutamate to regulate neuronal excitability, transmitter release and synaptic plasticity. The mGluRs are classified into three groups (group I-III) based on receptor structure and physiological activity (28). They are ubiquitously distributed at excitatory and inhibitory nerve terminals as well as associated glial processes, making it difficult to determine cell-type specific functions using pharmacology. Levitz and colleagues successfully engineered group II (mGluR2 and mGluR3) and group III (mGluR6) mGluRs into Opto-GPCRs, which couple to the G_i pathway and modulate adenylyl cyclase activity (10). The PTL consists of a photoisomerisable azobenzene group (A) to which the glutamate is attached in either D- or L-stereoisoform *via* a malemeide (M) group. This particular PTL assembly is referred to as MAG (malemeide-azobenzene-glutamte). To engineer a light-activatable mGluR, refered to as LimGluR (light-agonized and light-antagonized mGluR), MAG was covalently attached to a genetically engineered mGluR, where a cysteine residue for attachment has been introduced close to the glutamate binding pocket through a point mutation (10). For each mGluR the ideal position for MAG attachment was determined by screening cysteine substitutions around the glutamate binding pocket (10). MAG was designed with an mGluR homology model based on the crystal structure of mGluR3 and can act as agonist or antagonist, depending if the tether is short (MAG-0) or long (MAG-1). MAG-0 promotes clamshell closure and G protein activation, whereas MAG-1 prevents clamshell closure and inactivates the mGluR. To toggle the synthetic glutamate in and out of the mGluR clamshell, the conformation of the azobenzene moiety can be switched between the *cis* and *trans* form using two different wavelengths (Figure 2A). 380 nm promotes the *cis* conformation, which shortens the MAG molecule and in the case of MAG-0 positions the synthetic glutamate into the mGluR ligand-binding pocket or in the case of MAG-1 prevents clamshell closure. 500 nm light drives the azobenzene moiety into its extended

trans configuration, retracting MAG from the clamshell. LimGluRs have proven to be fast (millisecond kinetics) and to support multiple rounds of on/off switching by 380 nm and 500 nm light (10). Levitz and colleagues showed that LimGluR2 and LimGluR3 can be used to modify cellular excitability in cultured hippocampal neurons by modulating excitatory and inhibitory currents and pre-synaptic transmitter release (paired pulse facilitation) (10). LimGluR2 was further shown to function *in vivo*, modifying the behavioral escape response of zebrafish larvae (10).

The PTL-approach can principally be applied to any ligand-gated GPCR. The major disadvantages of first-generation MAG-PTLs in view of clinically oriented applications are (i) their very low light sensitivity ($I_{activation}$ ~10^{18} photons cm^{-2} s^{-1}) in combination with short-wavelength activation (380 nm), which collectively bear the risk of phototoxicity and tissue damage, (ii) the need for repeated PTL supplementation to the tissue as a consequence of their very short half-life lasting only hours in tissue. As the azobenzene photoswitch is well understood, one line of development focused on shifting the azobenzene absorption spectrum to longer wavelengths (29). Several red-shifted PTLs have been engineered (Figure 3), with one variant (MAG_{460}) that is activated by blue light (460 nm). MAG_{460} has the further advantage to spontaneously relax back to the inactive *trans*-state, making inactivation by a second wavelength superfluous (30). Recently, *to*Cl-MAG, a variant activated by even longer wavelengths (yellow and red light) was developed. However, *to*Cl-MAG still requires inactivation by a second wavelength (blue light) (31). Other red-shifted MAG versions with an enhanced two-photon absorption cross-section (MAG_{2P} and $MAGA_{2P}$) that spontaneously isomerize back to the *trans*-state were specifically developed for 2-photon photoswitching (32). A second direction of development focused on increasing the PTL's half-life in tissue. One improved version, DENAQ, has a tissue stability of several days and has been successfully used in pilot experiments to recover vision in the blind mouse (33). Other advantages of DENAQ for physiological and clinical applications include: (i) activation by "less harmful", longer-wavelength light (~ 500 nm), (ii) a ~100-fold increased light-sensitivity compared to MAGs and (iii) it spontaneous inactivation (29). Although these optimized PTLs have not yet been used in combination with GPCRs, they should in principle be fully compatible.

4. EXOGENOUS EXPRESSION OF NATURALLY LIGHT-SENSITIVE GPCRS

Fully genetically encoded opsins, which do not require the addition of an exogenous ligand or cofactor are, not surprisingly, alternative candidates for controlling neuronal activity. Animal opsin-based pigments, as opposed to the microbial opsin-based traditional optogenetic tools (e.g. ChR2 and eNphR), are class A GPCRs and have been successfully used to "artificially" activate specific G-protein families in the target cells, both *in vitro* and *in vivo*. As retinal is sufficiently available in neuronal tissue, expressed opsins are *per se* functional in the brain. Additionally, opsins profit from an integral signal amplification cascade inherent to GPCRs. In contrast to the traditional optogenetic tools, a single photon can activate multiple G-proteins, each in turn triggering large changes in second messengers, which renders animal opsins 2-3 log units more light sensitive than non-GCPR based optogenetic tools (9).

Animal opsins couple to all major G-proteins including the G_q, G_s and G_i types. One of the most frequently used opsins is vertebrate rhodopsin (vRh) naturally expressed by the rods of the retina. vRh naturally activates the G-protein transducin (G_t), which belongs to the G_i family. Activated G_t stimulates phosphodiesterases, which hydrolyze cGMP molecules and thereby open downstream cyclic nucleotide-gated cannels (34).

Several studies showed that heterologous expression of vRh in neurons is sufficient to modulate endogenous conductances through G_i activation (23, 24, 35). Li and colleagues showed that vRh activation can be used to modulate neuronal excitability and synaptic transmission in cultured hippocampal neurons (23). They functionally differentiated between the postsynaptic G_i-pathway, activating G protein inward rectifying potassium channels (GIRKs) and reducing neuronal excitability, and the presynaptic, dendritic G_i pathway inhibiting voltage-gated Ca^{2+} channels and reducing transmitter release (25). Using the embryonic chick spinal cord as an *in vivo* model, Li and colleagues also showed that vRh activation can be used to re-synchronize activity in surgically disconnected cord halves. In a later publication the same lab showed that cerebellar Purkinje cell targeted vRh can manipulate motor coordination in the mouse acting through G_i (24). And recently, vRh targeted to the retinal ON-bipolar cells successfully coupled to G_i and restored light-responsiveness in the blind mouse retina (35).

Despite the clear advantages of vRh over LimGluRs, such as no need for ligand supplementation and increased light-sensitivity, there also exist drawbacks: vRh has a slow deactivation kinetics (36) and suffers from a rapid response rundown due to bleaching by light (37). Color opsins from the cone photoreceptors of the retina, i.e. short- and long-wavelength mouse opsins, are bleached less rapidly and were therefore used as alternatives to vRh (38). Vertebrate cone opsins successfully modulated anxiety behavior in mice by repetitively activating G_i signaling in serotonin 1A (5-HT1A) receptor domains in the dorsal raphe nucleus (DRN) (38). Although not shown so far, different opsins with different wavelength sensitivities

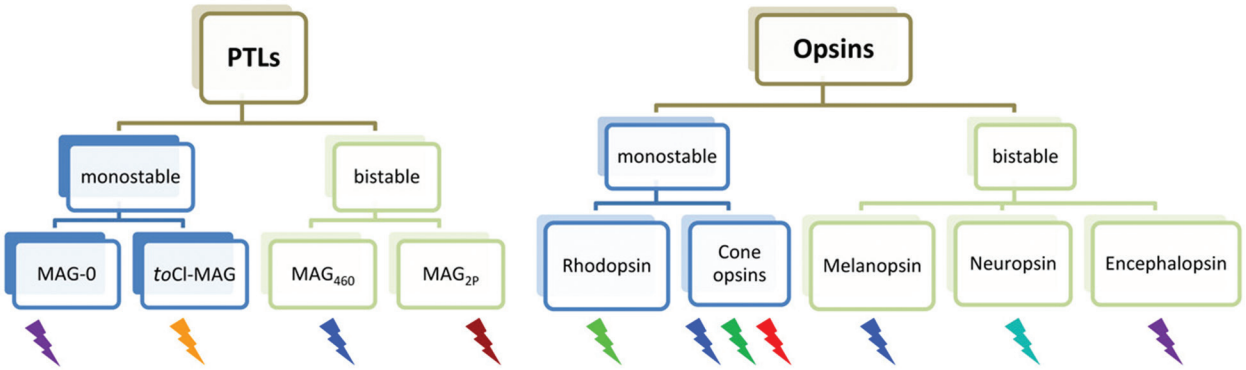

Figure 3. Overview of used light-switches to construct Opto-GPCRs. The light-switches have been grouped in terms of their origin (PTLs or opsins) and in terms of their thermal stability (monostable *vs* bistable). The activation wavelengths are given as colored flashes underneath the hierarchy.

(OPN1SW, OPN1MW, OPN1LW) could in principle be used to independently activate G_i pathways in different target cell types.

With the exception of vRh and vertebrate cone opsins, the majority of GPCR opsins found in the animal kingdom are bistable, possessing intrinsic retinal isomerase activity that turns inactive *trans* retinal to the active *cis* conformation. As a consequence, bistable opsins spontaneously relax back to the light-activatable ground state. In other words, bistable opsins are bleach-resistant to light and therefore reusable with no response rundown, which makes them potentially advantageous tools. In vertebrates, three bistable opsins are known: melanopsin (OPN4), encephalopsin (OPN3) and neuropsin (OPN5) (Figure 3). Blue-light sensitive vertebrate melanopsin is found in about 1% of intrinsically light-sensitive retinal ganglion cells and couples to a G_q-type G protein, mediating mainly non-image forming vision, i.e. pupil responses to light and photoentrainment of the circadian rhythm (39, 40). Multiple studies have shown that ectopic expression of melanopsin renders mammalian cells photosensitive (41-43). Lin and colleagues successfully introduced melanopsin into the retinal ganglion cells of blind mice lacking photoreceptors (41). The melanopsin expressing ganglion cells provided an enhancement of visual function such that the pupillary light reflex and light-avoidance behavior returned to near normal (41). Tsunematsu and colleagues introduced melanopsin into hypothalamic orexin neurons to control sleep and wakefulness *in vivo* (43). Short (4 s) blue-light activation supplied by an optical fiber induced a transition from slow-wave-sleep to long-lasting wakefulness for several tens of seconds even after the light was switched off (43).

The recently discovered blue-green light sensitive vertebrate encephalopsin is found in the brain and retina and believed to be at least partly involved in the regulation of the circadian melatonin production (44). The UV-sensitive vertebrate neuropsin is found throughout the body, but also in retinal ganglion, amacrine and horizontal cells where it was shown to couple to the Gi pathway (45). Both opsins present interesting vertebrate light antennas for future engineering. Also invertebrate opsins comprise a versatile collection of bistable pigments with a great variety of spectral sensitivities ranging from UV to infrared (46), however, their use in vertebrate tissue bears the risk of potential immunogenicity, which is perhaps the reason that most studies so far have focused on the use of vRh, vertebrate cone opsins and vertebrate melanopsin.

Of course there also exist drawbacks when using unmodified opsins for activation of a specific G protein cascade in a target cell type. First, ectopically expressed opsins do not always couple to their native G-proteins when expressed in a host system (23). They may have weak activity or couple to alternative or even multiple signalling pathways, depending on the available G-protein complement within the host cell. Second, whilst heterologous expression of vRh, cone opsin and melanopsin proved that functional pathways exist within most target cell types that can couple opsin activation to a membrane channel, response kinetics were often slow. This is traced back to a multitude of factors involved in the complex G protein signaling cascade, such as the lack of targeted subcellular transport and availability of GPCR modulator proteins, such as RGS-proteins, GRKs and arrestins. With ectopically expressed opsins the question therefore always remains of how much of the observed signal is intrinsic to the individual cell and how much depends on the way in which the cell is excited.

5. CHIMERIC GPCRS WITH AN OPSIN LIGHT ANTENNA AND INTRACELLULAR COMPONENTS OF A TARGET GPCR

5.1. Opsins with C-terminal GPCR targeting sequences

To target an opsin GPCR to the same subcellular location as a native GPCR, where all downstream

components of the G protein cascade are available and assembled in proximity, the CT of the opsin can be replaced with that of the target GPCR. The CT of GPCRs contains glycosylation sites important for subcellular protein trafficking and palmitoylatable cysteine residues important for targeting of the GPCR to lipid rafts within the cell membrane for assembly with downstream transducer and effector molecules. Oh and colleagues tagged vRh with the CT domain of the 5-HT_{1A} receptor in order to manipulate the anxiety circuitry by light (47). They demonstrated that vRh-5-HT_{1A} receptors were efficiently trafficked in a 5-HT_{1A} specific manner to the distal dendritic processes of hippocampal neurons. Importantly, the light-activation properties of tagged vRh remained unchanged. Similar results were recently presented for tagged cone opsin (38) and tagged melanopsin, containing the CT of the serotonin 2c receptor (5-HT_{2c}) that were expressed in the dorsal raphe nucleus (DRN) in mice. Indeed, repetitive activation of the G_i pathway in 5-HT neurons with CT-tagged cone opsin regulated anxiety behavior in mice (38). Activation of the G_q pathway in the same cells with tagged melanopsin unraveled a so far unknown autoregulatory negative feedback mechanism that also modulates anxiety behaviors (48).

Although CT-tagged opsins are trafficked to a specific subcellular domain and are able to recruit G protein cascades, they may still activate promiscuous G proteins within the target cells, as G-protein selectivity is mediated almost exclusively by IL2 and IL3 of the GPCR (22, 49-54). Additionally, even though the CT contains beta-arrestin binding and GRK phosphorylation sites for activity modulation, ILs have been shown to also affect cascade modulators (49). In other words, to activate a particular intracellular pathway with native kinetics in the target cell, the Opto-GPCR should additionally to the CT contain the ILs of the target GPCR.

5.2. Chimeras between different Class A GPCRs

Activation of native and endogenously regulated G protein pathways was the obvious next step of Opto-GPCR development. Pioneering engineering attempts in the 1980ies and 1990ies showed proof of principle that functional chimeras between class A GPCRs can be constructed and that G-protein selectivity is conferred by the receptor that contributes the ILs, in particular IL3 (22, 49-54). In 1988 Kolbika and colleagues constructed a set of functional chimeras between the *alpha2*- and $beta_2$-adrenergic receptors ($alpha_2$AR and $beta_2$AR) (50) and Cotecchia and colleagues shortly after extended the adrenergic receptor engineering to a chimera between *alpha*$_1$AR and $beta_2$AR, where they swapped the IL3 of $beta_2$AR with that of *alpha*$_1$AR (22). Other chimeras between class A GPCRs were successfully constructed that replaced the ILs and the CT of the first receptor with the corresponding domains of the second GPCR. All of them indicated the pivotal role of IL3 and contributions of IL2 for G protein specificity. Chronologically, these included chimeras between the muscarinic cholinergic receptor subtypes M1 or M2 and $beta_1$AR (54), chimeras between D1 and D2 dopamine receptors (53), chimeras between A_{2a} and A_1 adenosine receptors (51) and chimeras between $beta_2$AR and the thrombin receptor (52). Engineering of above constructs was facilitated by the sequence homology found within class A GPCRs, particularly when receptor class subtypes are combined (12). Suitable cutting sites between TMs and ILs were determined by sequence alignment of the two receptors constituting the chimera.

The first class A chimeric Opto-GPCR was engineered in 2005 by Khorana's group (25). Based on vRh they exchanged all the ILs and the CT by those of $beta_2$AR (25). As in the previous studies this changed G-protein selectivity from G_t of vRh to G_s specific for $beta_2$AR signaling. In their *in vitro* experiments Khorana and colleagues showed that optical stimulation of the chimeric vRh-$beta_2$AR induced cAMP levels comparable to pharmacological stimulation of wild-type $beta_2$AR. Airan and colleagues later successfully used the same vRh- $beta_2$AR chimera to modulate behavior *in vivo* in freely moving mice (11). After the same principle they additionally engineered a chimeric vRh-$alpha_1$AR that couples to G_q and upregulated inositol triphosphate to similar levels achieved in pharmacological studies activating wild-type $alpha_1$AR (11). Precise photostimulation of vRh-$beta_2$AR and vRh-$alpha_1$AR, now referred to as optoXRs, expressed in the *Nucleus accumbens* resulted in native signaling kinetics and drove conditioned place preference. Analogously, Li and colleagues recently engineered a chimeric vRh receptor based on another class A GPCR receptor, the adenosine A2A receptor (A_{2A}R), which they named optoA_{2A}R (55). OptoA_{2A}R was shown to activate in an A_{2A} receptor specific manner the G_S pathway with cell-specific selectivity of signaling: when A_{2A}R was expressed in the hippocampus, downstream signaling was mediated by c-Fos, whereas when A_{2A}R was expressed in the *Nucleus accumbens*, downstream signaling was mediated by p-MAPK (55). On a behavioral level, hippocampal activation of optoA_{2A}R targeted to the glutamatergic terminals triggered p-CREB signaling and impaired spatial memory performance, while optoA_{2A}R activation in the *Nucleus accumbens* triggered MAPK phosphorylation and modulated locomotor activity (55).

The elegant studies by Airan *et al.* (11) and Li *et al.* (55) demonstrated that the diversity of GPCR signaling *in vivo* is vastly greater than can be accounted for by the four families of G proteins to which GPCRs couple. Of course the intracellular domains of the GPCR play an important role, but additionally the available cell-type specific complement of GPCR interacting partners that strongly modify the response has to also be considered (49). Factors promoting signaling

diversity include the variety of G-protein alpha subunits within each G-protein family, the promiscuity of many GPCRs activating multiple G-protein types (56), GPCR localization into specific subcellular compartments and signaling nanodomains with particular effectors, assembly of preformed GPCR-G protein-effector complexes and heteromultimerization into complexes with specialized properties and unique profiles of interaction with regulatory proteins (57, 58). Therefore, Opto-GPCRs able to recruit the native, cell-specific complement of intracellular effectors are considered favorable over the use of opsins *per se* or CT-tagged versions, particularly when the causal impact of biochemical signaling is investigated in a behaving animal or when function should be recovered in a regenerative context.

5.3. Chimeras across GPCR classes

All Opto-GPCR chimeras discussed so far were chimeras between members of class A GPCRs. The next step of development was to engineer chimeric GPCRs between different GPCR classes, which is complicated by the lack of sequence homology.

Pioneering engineering attempts resulted in functional chimeras between the Class A *beta*$_2$AR and the Frizzled-2 (59) and Frizzled-1 (60) receptors. In both chimeras the ILs and the CT of *beta*$_2$AR have been replaced by those of Frizzled-2 and Frizzled-1, respectively. Suitable cutting and ligation sites between *beta*$_2$AR and Frizzled were primarily based on comparison of secondary and tertiary protein structures that indentified the borders of intracellular and extracellular domains as well as primary sequence alignment at the N- and C-terminal ends of any particular domain. *beta*$_2$AR-Frizzled-2 and *beta*$_2$AR-Frizzled-1 displayed agonist specificity of *beta*$_2$AR and downstream signaling specificity of Frizzled.

We recently engineered a functional Opto-GPCR between a class A and a class C GPCR (9). We based our engineering on vertebrate melanopsin, a bistable opsin and replaced IL2, IL3 and CT of melanopsin by those of the class C GPCR mGluR6, our target receptor. As opposed to previously engineered Opto-GPCRs, we were the first to use a bistable opsin to avoid response run-down due to opsin bleaching and we indeed achieved sustained and reproducible Opto-mGluR6 signaling *in vivo* (9). As we targeted the retinal ON-bipolar cells of blind mice lacking photoreceptors, the aim was to turn bipolar cells into “replacement photoreceptors” by changing their sensitivity from glutamate (the native neurotransmitter) to light. We therefore based our engineering on the native ON-bipolar cell specific glutamate receptor mGluR6, which is naturally activated by glutamate released from the photoreceptors and signals through G_i (61). As in class A GPCRs, the CTs of mGluRs mediate important regulatory functions such as protein trafficking, alternative splicing, activity regulation by phosphorylation, modulatory protein-protein interactions and even to some degree G protein coupling and affinity (49, 56, 62). And IL2 and IL3 of mGluRs play similar detrimental roles for G protein coupling and selectivity as in class A GPCRs (21, 63-65). As IL3 of class A and IL2 of class C GPCRs are both the longest ILs within the protein, their concomitant presence within the Opto-GPCR protein should be avoided in order to circumvent steric hindrance (63). We replaced IL2, IL3 and the CT of melanopsin with the corresponding domains of mGluR6 to create the light-activatable Opto-mGluR6 receptor. The exchange of IL1 did not further improve Opto-mGluR6 function (9).

Despite the highly conserved tertiary structures of GPCRs, mGluRs share very little sequence homology with class A GPCRs. This impedes homology modeling to determine splice sites for IL exchange. We tested a variety of potential splice sites for IL2 and IL3 whilst constructing Opto-mGluR6 and realized that they led to no major functional differences (9). One exception is the highly conserved “E(D)RY” motif at the N-terminus of IL2 of all GPCRs. This “E(D)RY” motif should be preserved in engineering attempts as it is part of the “ionic lock” and plays an important role in receptor activation (63, 66) (Figure 2B).

6. PERSPECTIVES

Optical control of native GPCR signaling pathways using Opto-GPCRs provides the unique opportunity to examine or rehabilitate specific synaptic or circuit functions in a targetable and temporally precise manner. In combination with viral delivery and cell-type specific promoters the Opto-GPCR technology holds great promise for the development of cell-tailored therapeutics. As it was shown that *in vivo* expression of synthetic Opto-GPCRs competes with the expression of their native counterparts, GPCR defects could in principal be treated by functional replacement with a synthetic Opto-GPCR.

As the GPCR structure-function relationships are being continuously resolved and understood, essentially every target GPCR can in theory be engineered into an Opto-GPCR.

The properties of the light-switches that have been used so far to construct Opto-GPCRs are summarized in Figure 3. The different opsin classes with different spectral sensitivities potentially allow combinatorial stimulation in different subcellular domains, of different GPCRs within one cell type or GPCRs on different cell types targeted by different promoters. There exist over 1000 opsins that form a natural resource potentially minable for optical tools (36). If non-vertebrate light-switches are used in regenerative studies, their potential antigenicity should, however, be kept in mind and carefully evaluated.

All of the presented technologies have their advantage and disadvantages and they should all be considered candidates for a specific purpose.

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