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3 Challenges and opportunities for optochemical genetics

"The trick then is not to use the clumsy and inefficient techniques of classical organic chemistry by themselves but to make use of Nature's tools."

Francis Crick, 1999 [1]

3.1 Introduction

The transmembrane proteins that underlie neural processing are now known at a level of detail that has greatly increased our understanding of these sophisticated molecular machines. Starting with MacKinnon's seminal structure of a potassium channel, several voltage-gated ion channels and ionotropic receptors have been revealed with atomic resolution (Figure 3.1) [2, 3, 4, 5, 6]. This has been complemented by structures of G-protein coupled receptors, adding opsins and metabotropic receptors to the ever-increasing repertoire of transmembrane proteins elucidated with structural biology [7, 8, 9, 10]. As a consequence of this structural revolution and recent advances in pharmacology, Nature's molecular machines can now be manipulated with relative ease. This can be done, for instance, via synthetic on-off switches or tuning elements that are attached to the signaling protein of interest to allow for its orthogonal control with non-natural input signals. Amongst these signals, light is particularly useful, since it is unmatched in terms of temporal and spatial precision and techniques for the delivery and control of light are highly developed.
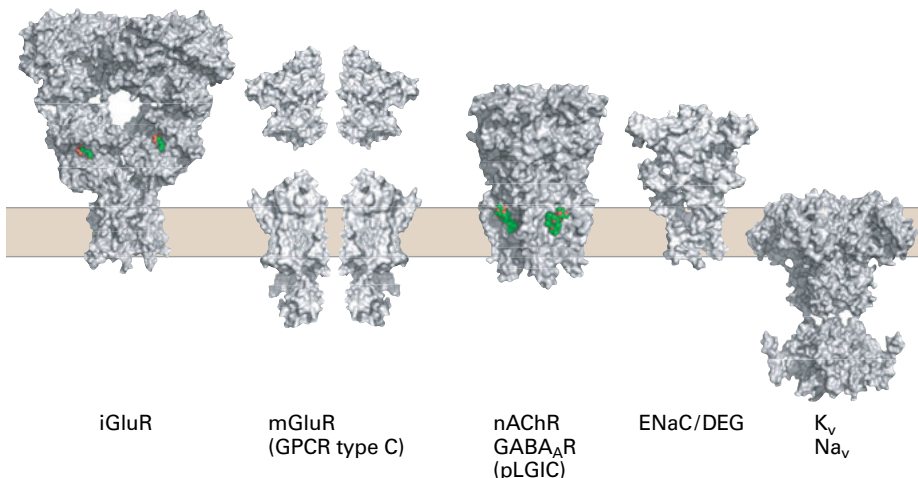

iGluR	mGluR (GPCR type C)	nAChR GABA$_A$R (pLGIC)	ENaC/DEG	K$_v$ Na$_v$

Figure 3.1: A selection of transmembrane receptors that have been characterized in atomic detail.

The resulting semisynthetic photoreceptors are particularly useful for applications in neuroscience since they can be used to control neuronal firing patterns and to mimic or block synaptic signals. This kind of approach was proposed as a method of choice for unraveling neural systems by Francis Crick in his 1999 Kuffler lecture [1] – a challenge that was met shortly thereafter giving rise to a rapidly developing field, now termed "optogenetics". Like their naturally light-sensitive counterparts, the opsin channels, pumps and GPCRs, semisynthetic photoreceptors work in animals and can be applied to the restoration of vision, the optical control of touch sensation and the dissection of neural circuits underlying behavior. The integration of synthetic photoswitches into mammalian receptor proteins has the unique advantage of enabling native signals to be optically controlled, thereby providing an additional ability to elucidate the physiological mechanisms by which the regulation of excitability and synaptic transmission controls circuits and behavior. The new functional dimension of chemistry to this field led us to term the approach "Optochemical Genetics (OCG)" [11].

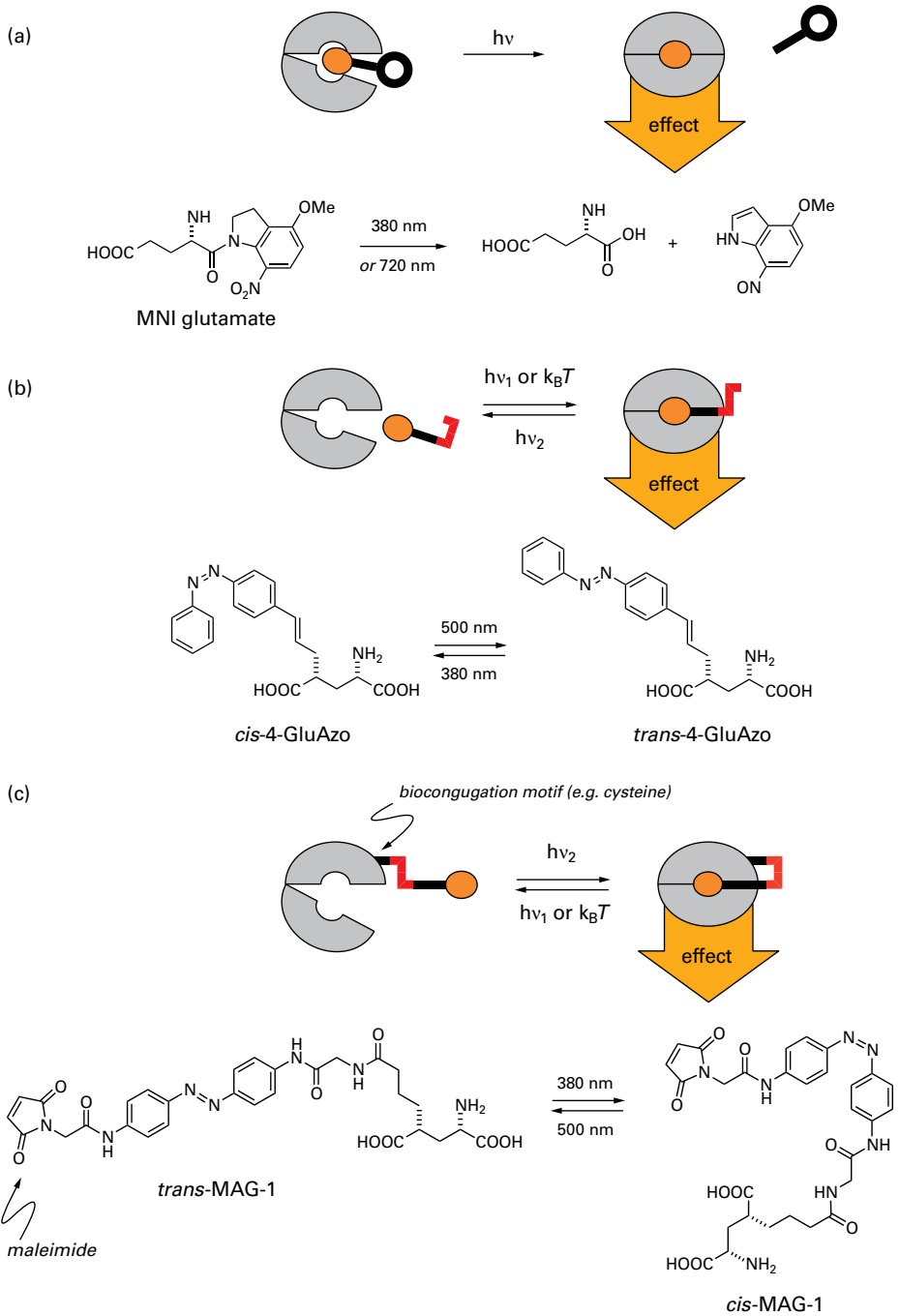## 3.2 Photosensitizing receptors

Three general chemical strategies have emerged to directly or indirectly endow receptors and channels with light-sensitivity. The simplest and oldest approach employs so-called caged ligands (CL) [12] (Figure 3.2A). Here, a ligand is endowed with a protecting group (the "cage") that masks a functional group crucial to the ligand-receptor interaction and thus renders it ineffective. Photochemical cleavage of this protecting group then sets the active ligand free and triggers the desired biological effect. While this approach has been employed with much success, especially in neuroscience (*e.g.*, in the form of caged glutamate), there are certain functional disadvantages associated with caged ligands. Uncaging is a unidirectional process and it is difficult, if not impossible, to "stuff the beast back into the cage". Unless one is interested in tonic effects, one is limited to uncaging in a small volume with two-photon illumination to enable diffusion, uptake, and/or enzymatic destruction to mimic the physiological rise and fall in synaptic concentration. In addition, uncaging produces byproducts, *i.e.*, the remnants of the protecting group, which can be toxic.

Some of these shortcomings can be overcome with a second approach that we call the photochromic ligand (PCL) approach (Figure 3.2B) [11]. Herein, the ligand carries a photoswitchable side-chain that can be switched between two (or more) configurations but is not cleaved off upon irradiation. As the photoswitch toggles between different states, the efficacy of the ligand changes, triggering the desired biological effect in a reversible fashion. The PCL can even be an agonist in one form and an antagonist in the other. PCLs have all the advantages of small-molecule drugs, including their ease of application and fast tissue distribution. As with drugs, selectivity between

receptor subtypes can be a challenge, but this can often be overcome through systematic variation of the molecule.

Of course, there are situations where receptor-subtype selectivity and cellular targeting is highly desirable. In this case, a third approach, which we call the photoswitched tethered ligand (PTL) approach, can be employed (Figure 3.2C) [11]. Here, the ligand is *covalently* attached to its receptor in a site-directed manner through a tether that contains a photoswitch. As the photoswitch toggles between extended and bent forms, the local concentration, position and/or efficacy of the ligand changes, thereby activating, antagonizing or modulating an allosteric domain, or blocking an effector domain of the signaling protein in a reversible fashion. Importantly, PTL-gated proteins can be genetically encoded, since the point of attachment can be an engineered cysteine residue or any other encodable chemical motif that allows for specific bioconjugation. Since the PTL is covalently tethered, its local concentration at the site of attachment is very high in the active form of the photoswitch, which means that the affinity of the ligand is not a major concern. In fact, low-affinity ligands have the advantage of ensuring that photoswitching can rapidly remove the ligand from the binding site.

The CL and the PCL strategies are akin to "chemical genetics" [13]. Chemical genetics attempts to address every protein target with a selective small-molecule ligand. Although such pharmacological control can have a rapid onset once the chemical reaches its target, diffusion barriers often slow the onset of drug action considerably and make washout difficult, especially *in vivo*. This limitation is overcome by allowing for long equilibration times to get the caged molecule into place and then rapidly optically controlling its function in the CL and PCL approaches. The PTL approach is essentially a variant of optogenetics, since it combines a genetically encodable receptor with light to precisely control neural activity. As opposed to conventional optogenetics with opsins, in which the retinal photoswitch is biologically available in vertebrates, the synthetic PTL is not endogenously produced, but needs to be supplied by a chemist. This poses the additional burdens of synthesis and delivery, while providing the advantage of controlling native channels and receptors and providing the elegant negative control where the genetic component is expressed, but it is left inert to light, because the photoswitch is withheld. For therapeutic applications, the PCL approach has the appeal of avoiding the need to implement gene therapy, while the PTL approach shares with conventional optogenetics the advantage of genetically constrained cell-specific targeting. It is also extremely useful in the functional dissection of closely related receptor subtypes, since selectivity is achieved through covalent attachment to genetically engineered isoforms and high affinity ligands are not required (Figure 3.3) [14, 15].

(a)

MNI glutamate

380 nm *or* 720 nm

(b)

$h\nu_1$ or $k_B T$

$h\nu_2$

effect

500 nm
380 nm

cis-4-GluAzo   *trans*-4-GluAzo

(c)

bioconjugation motif (e.g. cysteine)

$h\nu_2$

$h\nu_1$ or $k_B T$

effect

380 nm
500 nm

trans-MAG-1

maleimide

cis-MAG-1

◄ **Figure 3.2:** Strategies for photosensitization. (a) The caged ligand approach (**CL**); (b) the Photochromic Ligand Approach (**PCL**) with an azobenzene switch; and (c) the Photoswitched Tethered Ligand Approach (**PTL**) using an azobenzene switch. A corresponding glutamate derivative is shown in each case.
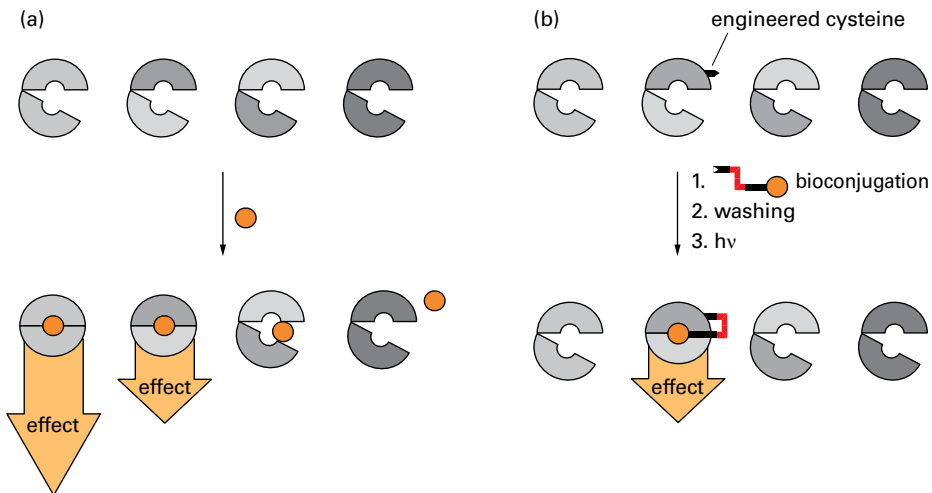

Figure 3.3: The PTL approach to selective pharmacology. (a) Selectivity between receptor subtypes is difficult to achieve. (b) In OCG, selectivity can be achieved through genetic engineering of a bioconjugation site. In addition, the photoactivatable ligand can have low affinity to the receptor subtype.

3.3 PCL and PTL development and applications

Optochemical genetics had an early forerunner with Erlanger's and Lester's PCLs and PTLs for neuromuscular nicotinic acetylcholine receptors [16, 17, 18, 19], but only came of age in 2004 when we introduced the synthetic azobenzene-regulated potassium channel (SPARK) [20]. Incidentally, this was the second system that could be used to control action potentials in neurons following Miesenböck's pioneering "ChARGe" system [21]. Although SPARK was overshadowed shortly after its introduction by the advent of channel- and halorhodopsins [22, 23, 24, 25, 26], OCG has undergone a rapid development, providing alternative ways to optically control neural activity and a singular approach for the remote control of native synaptic signals. Since 2004, PTLs have been developed for a number of channels and receptors, including voltage-gated potassium channels (*e.g.*, SPARK) [14, 20, 27], two-pore-domain potassium channels [15], kainate receptors (LiGluR) [28, 29, 30, 31, 32, 33], potassium-selective glutamate receptors (HyLighter) [34], metabotropic glutamate

receptors (LimGluR) [35], GABA$_A$-receptors [36], and neuronal nicotinic acetylcholine receptors (LinAChR) [37]. Conversely, photochromic ligands (PCLs) are available for K$_v$-channel [38, 39, 40, 41, 42], Na$_v$-channels [42], Ca$_v$-channels [42], kainate receptors [43, 44, 45], AMPA-receptors [46], NMDA-receptors [47], GABA$_A$-receptors [36, 48], and neuromuscular nicotinic acetylcholine receptors [16, 17, 18, 19]. A photochromic agonist for AMPA receptors, ATA [46], and its effect on mouse layer 2/3 cortical neurons is shown in Figure 3.4.
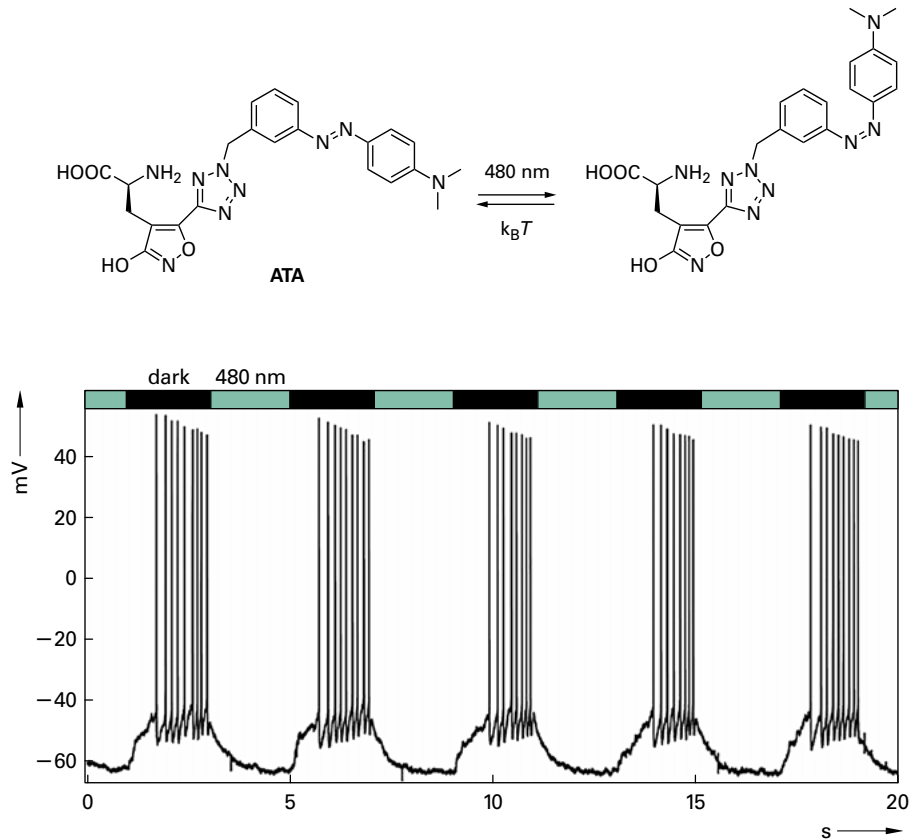

Figure 3.4: ATA, a photochromic agonist of AMPA-receptors, as an example of a PCL that can control mouse cortical neurons.

Synthetic photoswitches of both types work well in dissociated hippocampal and Purkinje neurons; cortical, hippocampal and cerebellar slices; intact dorsal root ganglia; flat-mounted retinas; and *in vivo* in the zebrafish central nervous system and the rodent eye. In terms of its applications to biological and clinical problems, the optochemical approach has been used to selectively block specific potassium chan-

nels and dissect their functional role [14], elucidate the role of auxiliary channel subunits [15], control calcium exocytosis [49], analyze the spinal circuitry of zebrafish larvae and control their escape behavior [29, 50], control heart rate in the leech *Hirudo medicinalis* [38], restore visual responses in blind mice [51, 52], and optically control pain sensation in rodents [42].

3.4 Advantages and disadvantages of PCLs and PTLs

When compared with the conventional tools of optogenetics, *i.e.*, rhodopsins, halorhodopsins and channelrhodopsins, PCLs and PTLs show certain functional advantages and disadvantages. PTLs constitute two-component systems that require synthetic compounds, few, if any, of which are presently commercially available. The currently used reactive maleimides have to penetrate tissues to find their protein targets, something that occurs readily in larval zebrafish and the rodent eye, but waits to be demonstrated in the mammalian brain. Selectivity is not a major concern since accessible surface cysteines are rare and the PTLs undergo affinity labeling, concentrating them at the intended sites. However, the instability of the maleimides in the sera of target organisms potentially poses problems, which could be overcome, for instance with SNAP-tags [53] or other genetically encoded bioconjugation motifs. On the other hand, the currently used maleimides PTLs operate on native receptors that are only modified by the substitution of a single cysteine with little or no effect on protein expression or function. Because the receptors are full length, they are expected to undergo their normal protein interactions and, therefore, be targeted precisely in the neuron and maintain native signaling specificity.

PCLs represent one-component systems that lack the selectivity and precision provided by genetic targeting but they are as easy to apply as regular drugs and molecular probes. Their usefulness in dissecting neural circuitry might be somewhat limited but their medical impact could be significant, since they do not require genetic manipulation (and ultimately human gene therapy) to function. Indeed, first applications in vision restoration and pain management have already surfaced. Like caged ligands (and PTLs), they require a commercial source to gain in popularity.

In principle, the action spectra of PCLs and PTLs could cover a much wider range than the opsins, since they are made by organic synthesis and their photophysical and functional features are not limited by biosynthetic pathways. It should be possible, for instance, to extend the action spectra into the deep red or near infrared range, as originally proposed [1]. It should also be possible to develop synthetic photoswitches with much higher extinction coefficients or useful two-photon cross sections. Both bistable and fast-relaxing photoswitches, which mirror the development of step-function opsins [54] and very fast relaxing optogenetic tools, such as ChETA [55], have been explored but need to be investigated more systematically. Singlet-oxygen production via intersystem crossing does not seem to be a big concern with azo-

benzenes, which are currently the most popular photoswitches in OCG. Other types of photoswitches, however, should be explored.

3.5 Conclusion

In summary, OCG adds an extra dimension to optogenetics, further increasing our ability to control neuronal activity with light. Like conventional optogenetics, it benefits from the huge advances that have been made in light delivery and viral transfection. Despite the comparatively small number of laboratories that have joined the effort to date, OCG has enjoyed rapid growth and has already made the transition from proof of concept to applications in neuroscience and preclinical investigations. Given the enormous advances in structural biology mentioned in the introductory paragraph and recent progress in the design and synthesis of photoswitches, it is likely that this pace is going to increase. OCG employs the techniques of classical organic chemistry, which were apparently held in so little esteem by Francis Crick. However, as suggested in his lecture [1], it does not use these techniques *by themselves* but rather *in conjunction with* Nature's magnificent tools, benefiting from advances in both areas. As such, it has enabled the optical control of neural systems and entire animals, just as a leash is able to (inefficiently) control the behavior of a dog, a sophisticated being only slightly modified through genetic manipulation (Figure 3.5).

Figure 3.5: An attempt to control Paula, a complex system, with a clumsy and inefficient technique.

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