The photopharmacology of nicotinic acetylcholine receptors

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Nicotinic acetylcholine receptors (nAChRs) are one of the most abundant classes of receptors present in the mammalian nervous system and play a significant role in synaptic transmission. The development of new tools that can precisely control the function of nAChRs is important for the study of their complex biological processes. It could also lead to new therapeutic treatments for neurological diseases associated with nAChRs. Herein, we present a review of the photopharmacology of nAChRs, where small photochromic ligands are used to control function using the high spatial and temporal precision of light. A survey of the literature shows that, although several diffusible photochromic ligands and photochromic tethered ligands exist, further development of new molecules is required to allow in-depth studies into the role of different nAChR subtypes.

Keywords: nicotinic acetylcholine receptor; photopharmacology; azobenzene; photoswitch; BisQ; optochemical genetics; photochromic ligand; photochromic tethered ligand

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Nicotinic acetylcholine receptors in the neural system

Nicotinic acetylcholine receptors (nAChRs) are ligand-gated ion channels that are highly abundant in the mammalian nervous system, playing a major role in synaptic transmission between neurons and at the neuromuscular junction. These receptors are highly important for movement and cognitive functions, such as memory and reward.
mechanisms. Beside the crucial involvement in nicotine addiction, malfunctions in cholinergic communication often lead to severe diseases like epilepsy, Parkinson’s or Alzheimer’s disease [1, 2]. In general, neuronal nAChRs consist of five subunits, which can be either heteropentamers (combinations of α2 - α10 and β2 - β4) or homopentamers (α7 - α9). Because of the complexity and diversity of cholinergic function, a variety of these nAChR subtype compositions can be found in the brain. These subtypes not only differ in their affinities towards their endogenous ligand acetylcholine (ACh), but also in their kinetic profiles of channel activation and deactivation [3]. For example, homopentameric α7 nAChRs show lower affinity and faster desensitization compared to heteropentameric α4β2 nAChRs, resulting in a different cellular response to the same stimulus. This response is shaped by the different kinetics and ion permeability of individual receptors.

In order to study cholinergic systems, a broad pharmacological toolset has been developed over recent decades [5]. It is now possible to target many receptor subtypes with highly specific agonists and antagonists (Figure 1). However, nAChRs are usually investigated with electrophysiology experiments that use bath application of an agonist or antagonist. These conditions are slow, imprecise and often do not correspond to physiological conditions, since the compound is used at high concentrations and cannot be quickly cleared. These properties stand in contrast to the fast dynamic characteristics of activation of cholinergic signals. Lately several methods have been introduced to overcome these limitations, unfortunately bringing other disadvantages with them. For instance, local pressure pulse application of cholinergic ligands is spatially precise and quick, but nevertheless is only applicable for single cell investigations. When investigating neural circuits, this method meets its limitations. Thus, there is a need for a pharmacological solution that can overcome these restrictions. This is where photopharmacology comes into its own.

Photopharmacology and azobenzene photoswitches

Due to the numerous subtypes and the complex pharmacology of nAChRs, there is a great need for new methods that can reversibly control their function [6]. To achieve the desired dynamic control, an external stimulus is required that can be accurately controlled with high spatial and temporal precision, as well as exhibiting low or negligible toxicity in biological systems. The use of light as the external stimulus fulfills all of these requirements, for it can be efficiently manipulated by adjusting its wavelength and intensity, whilst not interfering with other biological processes [7-9].

To enable the optical control of a protein, the structure of an organic molecule that interacts with the desired target has to be modified to incorporate a functionality that undergoes a transformation when exposed to light. In its most simple incarnation, a photolabile component is introduced resulting in an inactive caged molecule [10]. Upon exposure to light, the cage is removed and the active molecule is released enabling it to affect its biological target. Several caged molecules that
target nAChRs, have been reported over recent years (Figure 2). NPE-Carbachol and CNB-carbachol have the cage covalently attached to the carbachol, while RuBi-nicotine is a metal complex with a non-covalently attached caging moiety. Although they have proved useful for the study of nAChRs, they do suffer from several drawbacks. For example, the light induced removal of the cage can only occur once, therefore reversible control of the desired target cannot be easily achieved. Additionally, the remainder of the cage needs to be compatible with the biological system under investigation. It should be noted that several caged derivatives of choline, the biosynthetic precursor and cleavage product of ACh, have also been reported. However, they were used as tools to investigate the action of acetylcholinesterase (AChE) and not the function of nAChRs.

Another method that exploits the high spatial and temporal precision of light is provided by optogenetics. Rather than using a synthetic organic compound, optogenetics achieves dynamic control of biological functions via photoresponsive proteins, such as rhodopsins, phototropins and phytochromes. These gain their photosensitivity through incorporation of abundant natural chromophores (retinal, flavine mononucleotide, biliverdin). Optogenetics has proven extremely useful for the study of biological processes, especially in neuroscience. Cholinergic systems have been extensively studied using this method. However, the nAChRs themselves have not been rendered photosensitive with mere genetic manipulation and the reliance on natural chromophores alone limits insights into the role of individual subtypes.

The photopharmacology approach involves the incorporation of synthetic photoswitchable ligands into proteins. Upon exposure to a certain wavelength of light, the ligand undergoes a conformational change, which can be reversed using light of another wavelength or by thermal relaxation. If the pharmacological efficacy of the ligand changes upon switching, the target protein is essentially converted into a photoreceptor. Photopharmacology works particularly well in nonlinear systems, such as the nervous system and can enable ultra-fast and highly accurate control of biological functions.

There are several classes of molecular photoswitches reported in the literature, each with their own unique properties and characteristics. Amongst these, the azobenzenes have arguably attracted the most attention from the scientific community, at least as far as biological applications are concerned. This could be due to the fact that they can be synthesized with relative ease but also results from their advantageous geometrical and photophysical properties. Azobenzenes, which belong to the smallest photoswitches, can exist as trans- and cis-isomers, where the trans-isomer is the thermodynamically more stable. When irradiated with light (typically 360-480 nm), the trans-isomer undergoes photochemically induced isomerization to form the cis-isomer. The cis-isomer can be converted back to its trans-isomer using a longer wavelength or via thermal relaxation. Both light induced isomerizations generally occur in the range of picoseconds, whereas the thermal relaxation takes place anywhere in the range of milliseconds to days depending on the substitution pattern of the azobenzene.

The speed of photoswitching prevents intersystem crossing to triplet states, which could result in formation of singlet oxygen under biological conditions. The trans- to cis-isomerization of the azobenzene is accompanied by a considerable change in its geometry. A trans-azobenzene is almost planar and has little or no dipole moment. In contrast, a cis-azobenzene exhibits an angular geometry and has a considerable dipole moment. Analysis of the UV-Vis absorption spectrum of the azobenzene BisQ, photochromic agonist for neuromuscular nAChRs (see below), reveals two distinct absorption bands (Figure 3a). The very intense band...
For example, the addition of an electron donating group in the para position on one aromatic ring creates a class of azobenzenes referred to as ‘aminoazobenzenes’ (Figure 4b). The absorption maxima in these compounds is red-shifted towards the visible region of the spectrum and the thermal relaxation of the cis-isomer is faster. Also, the $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ bands lie closer together. Azobenzenes that contain (280-340 nm) is indicative of the $\pi \rightarrow \pi^*$ transition whereas the weaker band (380-480 nm) is characteristic of the $n \rightarrow \pi^*$ transition. The variation of substituents on the azobenzene core can change the spectral sensitivity and kinetics of the photochemical and thermal isomerization (Figure 4) [32-35]. For example, the addition of an electron-donating group in

Figure 3. Photoswitching characteristics of azobenzenes. a) UV-Vis spectrum of BisQ [31]. Reprinted with permission [28]. The difference in the UV-Vis absorption spectra of the trans- and cis-isomers is clearly visible. b) The structure of trans- and cis-azobenzene showing the putative change in geometry that occurs during the isomerization process. BisQ = [(E)-diazene-1,2-diylidibenzene-3,1-diyl]bis(N,N,N-trimethylmethanaminium).

Figure 4. Examples of azobenzenes that exhibit different photoswitching properties. a) Fotocaine is an ‘azobenzene class’ photoswitch [30]. b) Azo-propofol is an ‘aminoazobenzene class’ photoswitch [30]. c) JB253 is a ‘pseudostilbene class’ photoswitch [30]. d) A tetra-ortho substituted azobenzene that is photoswitched with red and blue light [27]. e) A photoswitch that is isomerized with near-infrared light [29]. f) A bistable arylazopyrazole photoswitch [29]. g) A bistable cyclic azobenzene derivative that is thermodynamically more stable in its cis-form [29]. Fotocaine = 4-(3-[4-{(E)-phenyl diazenyl}(phenyl)propyl]morpholine. Azo-propofol = 4-[{(E)-(4-aminophenyl) diazenyl]-2,6-di(propan-2-yl)phenol. JB253 = N-(cyclohexylcarbamoyl)-4-[(E)-4-(diethylamino)phenyl]diazenyl] benzenesulfonamide.
both a para electron-donating group and a para electron-withdrawing group are called ‘pseudo-stilbenes’ (Figure 4c). Their absorption maxima are further red-shifted with thermal relaxation occurring very quickly. In this regard, the addition of substituents to azobenzene photoswitches enables them to effectively be tuned to give the desired properties for a certain purpose [36]. Very recently, there has been a surge in new azobenzene motifs that offer interesting and remarkable photoswitching properties, such as bistability (i.e. the molecule stays in its excited conformational state without further illumination) and very red-shifted photoswitching wavelengths (Figure 4d-g) [37-40]. Of particular interest to photopharmacology are the red-shifted azobenzenes as they can take advantage of the increased tissue penetration of red light [41].

Although azobenzene photoswitches have dominated photopharmacology thus far, many other photoswitches have been used in biological systems with great success. Some of the photoswitches also exhibit interesting photoswitching properties that could be advantageous for photopharmacology. Among these photoswitches, stilbenes, dithienylethenes (DTEs), spiropyrans and hemithioindigos (HTIs) have received a large amount of attention from the community (Figure 5). Stilbenes are isoelectronic to azobenzenes and undergo photoisomerisation to switch between their cis- and trans-isomers. The main difference between stilbenes and azobenzenes is that the cis-isomer of stilbene is metastable, meaning that it does not thermally convert back to its trans-isomer [42]. Although they are similar to azobenzenes, stilbene photoswitches exhibit two major disadvantages for applications in biological systems. Firstly, strong UV irradiation is required for the trans- to cis-isomerization and secondly, stilbenes suffer from a tendency to irreversibly cyclize and oxidize while in their cis-form preventing reversion back to their trans-isomer [43]. DTE photoswitches are related to stilbenes by the fact that they are effectively cis-stilbenes that are fixed in the cis-conformation by a bridging cyclohexene ring. The photochromism of DTEs arises from their ability to reversibly undergo photochemically induced cyclisation from its open form to its closed form [44]. Typically the open form is converted to the closed form by irradiating with UV light, with the reverse isomerization to the open form occurring with visible light illumination. Both the open and closed forms of the DTE photoswitches are thermally stable [44], this gives them unique opportunities for photopharmacology. Spiropyans are a class of photoswitches that exhibit photochromism via reversible C-O bond cleavage that is induced by UV light. The cleavage of the C-O bond results in a zwitterionic conjugated system that is referred to as the merocyanine form. The reverse reaction back to the spiropyran can occur either thermally or by illumination with visible light [45]. The addition of substituents to the spiropyran motif can result in different equilibria between the spiropyran and merocyanine states, with some substituents resulting in complete reversal of the photochromism usually observed for spiropyran photoswitches [46]. HTIs are another class of photoswitch that exhibit cis- to trans-isomerization upon irradiation with distinct wavelengths of light. Illuminating with 400 nm light drives the cis- to trans-isomerization, with 480 nm light facilitating the reverse isomerization [47]. The photoswitching of hemithioindigos is very fast, occurring on the picosecond time scale and the thermal relaxation of the trans-isomer back to the more stable cis-isomer is very slow [48].

Optical control of nicotinic acetylcholine receptors

Within the field of photopharmacology, two main strategies for ligand function and design are usually implemented. The first strategy is referred to as the photochromic ligand (PCL) approach, whereas the second strategy is called the photochromic tethered ligand (PTL) approach (Figure 6) [23]. The PCL approach consists of a
soluble ligand that is endowed with a photoswitchable moiety, such as an azobenzene. Ideally, the PCL activates the target receptor as one isomer, but not as the other isomer. In the example shown in Figure 6a, the trans-isomer of the PCL is able to bind to the ligand-binding domain of the receptor and opens the channel, whereas the cis-isomer of the PCL cannot bind to the receptor, resulting in closure of the channel. In contrast, the PTL approach involves molecules that incorporate a ligand, an azobenzene tether, and a bio-conjugation motive, allowing the entire construct to be conjugated to a genetically modified protein. In the example shown in Figure 6c, the trans-photoswitch does not reach the binding pocket. When the ligand is photoisomerized, the cis-conformation of the ligand reaches the binding pocket and activates the receptor. If the ligand is an antagonist (Figure 6b, d), the PCL or PTL can compete with the endogenous ligand for the binding pocket, which allows for another type of photocontrol. Ideally, this photoinhibition or activation occurs only in one configuration, enabling normal receptor function in the other configuration. Both, the PCL and the PTL strategies, have their respective advantages and disadvantages. For example, one benefit of a PCL approach is that it does not require any genetic manipulation of the target cell or organism, making it applicable to endogenous receptors. The specific interaction of the PCL with the target receptor dictates if the activation occurs through the binding of the cis-isomer or trans-isomer. Because of the complexity of this binding it is difficult to predict the active form. When using the PTL approach, the attachment site of the photoswitch can be chosen based on the intended binding mode. Based on a three dimensional structural model of the target protein it is possible to computationally model the binding of the ligand to the protein and then screen for suitable attachment sites. In addition, the genetic modification required for PTLs allows the users to specifically target the exact receptor type and subtype that they wish to study. Over recent years, many PTLs have been developed that feature a maleimide as the electrophile for bio-conjugation to genetically engineered cysteines [49]. Maleimide chemistry in vivo bears many problems when the electrophile unspecifically reacts with freely accessible cysteines in the extracellular space or cytoplasm. Therefore alternative bio-conjugation motives like electrophiles that react specifically with certain genetically encoded protein domains such as SNAP or CLIP Tags might be more applicable in the future [50]. Nevertheless, both PCL and PTL strategies have been used to great effect with respect to nAChRs.

**Turning nAChRs into photoreceptors**

In the late 1960s, Erlanger and co-workers recognized that incorporating azobenzene photoswitches into known pharmacophores could enable the precise control of biological function using light. They envisaged that the cis- or trans-isomers of the azobenzene photodrug could have different activities for the biological target, effectively resulting in the function of the target being turned ‘on’ or ‘off’ with different wavelengths of light. In essence, the relative concentration of the ‘active’ and ‘inactive’
compounds could be fine-tuned by applying distinct wavelengths of light. This hypothesis was first successfully applied in 1968, when a PCL was used to optically control the inhibition of the digestive enzyme chymotrypsin. In this study, the authors found that the cis-isomer of their PCL was five times more efficient at inhibiting the effect of chymotrypsin than the trans-isomer was. Shortly after this report, Erlanger and Nachmansohn demonstrated that the PCLs p-phenylazophenyltrimethylammonium (azo-PTA) and N-p-phenylazophenyl-N-phenylcarbamylcholine (azo-Ph-carbachol) were photoswitchable inhibitors of acetylcholine receptors (AChRs) (Figure 7). When tested on the excitable membrane obtained from the Electrophorus electricus electroplax, both azo-PTA and azo-Ph-carbachol were found to function as antagonists in their trans-forms. Exposure to 320 nm UV light with the accompanied trans-to-cis-isomerization, resulted in a large depolarization of the electroplax membrane in the presence of the agonist carbachol. At this time it was not possible to distinguish whether this observation was due to nAChRs or muscarinic acetylcholine receptors (mAChRs). An interesting feature of this study was that azo-PTA and azo-Ph-carbachol were derived from known agonists of nAChRs, such as phenyltrimethylammonium (PTA) (Figure 7) and carbachol (Figure 1). However, the presence of the azobenzene functionality in each of these compounds converts them from agonists to antagonists. This is an example of how the introduction of a photoswitch can have a profound effect on the pharmacology of a compound.

Shortly after the discovery of azo-PTA and azo-Ph-carbachol, Erlanger and Wassermann disclosed the photocromic nAChR agonist BisQ (Figure 7d). BisQ is a PCL that can be considered as the azologue of the known nAChR partial agonist decamethonium, where the 10 membered carbon chain is replaced by an azobenzene moiety. BisQ was evaluated in the electroplax membrane in a similar fashion to their previous studies (Figure 8). The trans-isomer of BisQ was found to be a very potent nAChR agonist, inducing depolarization of the electroplax membrane. Rapid repolarization of the membrane then occurred when irradiating with 360 nm light. Concentration-response studies revealed that trans-BisQ (EC$_{50}$ = 60-80 nM) is 500 times more potent than the AChR agonist carbachol. However, the maximal response to
trans-BisQ is lower (i.e., partial agonist) than that of carbachol at high concentrations. In contrast to trans-BisQ, cis-BisQ showed very low activity, with the authors postulating that a pure sample of cis-BisQ may not exhibit any activity towards nAChRs at all. Studies on the activity of trans-BisQ in the presence of the nAChR antagonist tubocurarine showed that at low concentrations of trans-BisQ, the depolarization was blocked. When the concentration of trans-BisQ was increased, repolarization of the membrane ensued. This occurred both in the presence and in the absence of tubocurarine, therefore the authors speculated, that there are two binding sites for trans-BisQ with only one competing with tubocurarine.

Later, the successful photochromic agonist BisQ was turned into an antagonist by changing the positions of the quarternized amines from the 3,3' to the 2,2' positions \[54\]. This resulted in the PCL 2BQ (Figure 7d), which enabled investigations to further understand antagonist-receptor binding. Studies in Electrophorus electroplaxes revealed that the receptor activation by carbachol could be allowed by the cis- to trans-isomerization of 2BQ, while flash-induced trans- to cis-concentration jumps decreased agonist induced currents within milliseconds \[55\].

In 1969, Siliman and Karlin introduced the idea of covalently attaching an agonist to a nAChR \[56\]. For this, a disulfide bond near the active site of the receptor was reduced with dithiothreitol and the agonist was tethered to the protein. With Erlanger’s knowledge of photochromic agonists, this technique was combined with BisQ yielding QBr (Figure 7). QBr has a very similar structure to BisQ except that a bromine atom replaces one of the trimethylammonium groups. This converts the PCL into a PTL as the reduced disulfides can now react with the benzylic bromide, tethering it to the receptor. The attached agonist could now be presented to the active site and removed from it by changing the illumination wavelength. This overcame the major drawback of Karlin’s approach, namely desensitization of the receptor \[56\]. Interestingly, both the freely diffusible BisQ and the tethered QBr activate the receptor in their trans-configurations and not in their cis-configurations \[57\]. When attached to the receptor, QBr induced currents were not blocked by the competitive antagonist tubocurarine. Nevertheless, the receptor remained sensitive to open-channel blockers. When compared with each other, BisQ and QBr induce similar kinetics for channel opening and closing. Thus, the authors speculated that the rate-limiting step was not the diffusion of the molecules, but rather the conformational change of the agonist-receptor-channel complex \[57\].

Since the introduction of QBr, molecular cloning, heterologous expression, X-ray crystal structures and molecular modeling have revolutionized our ability to understand and control biological systems. Nowadays, with knowledge of the genetic code and the availability of three dimensional receptor structures, it is possible to change the DNA via site directed mutagenesis to exchange a single amino acid in the protein at a desired position. Furthermore, when investigating neural circuits, cell and receptor subtype specificity can be achieved within a tissue by using specific

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**Figure 8. Photoregulation of nAChR function by BisQ in electroplax membranes.** BisQ was washed in as its cis-form. Irradiation with an intense light source (Flood Light) isomerized BisQ to its trans-form activating the receptor. N.R. is normal Ringer's solution. Reprinted with permission \[53\].
promoters or a Cre-Lox expression system. Additional spatiotemporal control can be provided by attaching a photoswitch to the engineered protein that ultimately can be controlled with light. In 2012, Trauner and Kramer introduced the genetically engineered light-controlled nAChR (LinAChR) [58]. Through structure-based design, an azobenzene photoswitch and a maleimide functionality were added to the nAChR photoaffinity label AC-5, affording the photoswitchable nAChR agonist MAACCh (Figure 9). The known nAChR agonist homocholine phenyl ether (HoChPE) was converted to the photoswitchable nAChR antagonist MAHoCh. By modeling the molecules into the binding domain of the X-ray crystal structure of the acetylcholine binding protein (AChBP) and the Torpedo nAChR, several candidate amino acid positions were identified to carry the cysteine mutation for bio-conjugation with the maleimide. Ideally the photoswitch does not interfere with the receptors natural function when attached to the protein. Only when light is applied the molecule should evoke an effect. Therefore the conjugation site was chosen at a position where the cis-isomer, but not the trans-isomer of the photoswitch reaches the binding pocket. The engineered α3β4 and the α4β2 nAChRs, expressed in Xenopus oocytes, were both turned into photoreceptors when the agonist MAACCh was used (Figure 10a). By shining 380 nm light onto the oocyte, LinAChR was activated evoking an inward current, which was recorded via two electrode voltage clamp (TEVC). Changing the wavelength to 500 nm allowed closing of the receptor. Furthermore, light induced inhibition of nAChR current was achieved by attaching the antagonistic photoswitch MAHoCh to the same cysteine residue in α3β4 and α4β2 nAChRs (Figure 10b). The effect of ACh (300 µM) application could be blocked to a certain degree by irradiation with 380 nm light, while the same stimulus resulted in a strong inward current when illuminated with 500 nm light. Notably, both variants behaved like normal nAChRs in the dark. The authors speculated that inhibition of the receptor might also be achieved by attaching the agonistic molecule to another position, where it occupies the binding pocket, but does not induce a current [59].

The latest addition to the cholinergic photopharmacology toolbox is the PCL AzoCholine (Figure 11), which consists of a choline moiety that is attached to an azobenzene [60]. Structurally it closely resembles the α7 nAChR antagonist MG624 (Figure 1) with one crucial difference. The quaternary amine of AzoCholine bears three methyl groups instead of three ethyl groups. UV-Vis studies revealed that the cis-isomer can be enriched by illuminating the sample with 360 nm light. To convert the molecule to its...

Figure 9. The nAChR PTLs MAACCh and MAHoCh along with their parent ligands AC-5 and HoChPE. AC-5 = 2-{[4-{[5-{[3,10-dioxo-1H-pyrrrol-1-yl]acetyl}[amino]phenyl][diazemyl][phenyl]carbamoyl}[amino][hexanoyl][oxy]}-N,N,N-trimethyllethanaminium. MAACCh = 2-{[4-{[5-{[2,5-dioxo-2,5-dihydro-1H-pyrrol-1-yl]acetyl}[amino]phenyl][diazemyl][phenyl]carbamoyl}[amino][hexanoyl][oxy]}-N,N,N-trimethylethanaminium. MAHoCh = 3-{[4-{[5-1H-pyrrol-1-yl]acetyl}[amino]phenyl][diazemyl][phenoxo]}-N,N,N-trimethylpropan-1-aminium. HoChPE = N,N,N-trimethyl-3-phenoxypropan-1-aminium.

AC-5

MAACCh

HoChPE

MAHoCh

nAChR PTLs MAACCh and MAHoCh along with their parent ligands AC-5 and HoChPE.
trans-isomer 460 nm was the most efficient wavelength. Experiments in Human Embryonic Kidney (HEK) cells heterologously expressing either the neuronal α7 nAChR or the neuromuscular nAChR, showed that photoactivation of AzoCholine could induce a rapid and strong inward current at the neuronal nAChR, while having no photoswitchable effect at the neuromuscular nAChR. In dissociated rat dorsal root ganglion (DRG) cells activation could be triggered by photoactivation of AzoCholine, which could be recorded via calcium imaging. Here, the specificity of AzoCholine for the endogenous α7 nAChRs was demonstrated by blocking the effect with the α7 specific antagonist MG624. Furthermore, network activity could be modulated as demonstrated by multielectrode array (MEA) recordings from acute mouse hippocampal brain slices. When illuminating the preparation containing AzoCholine with 460 nm light, bursting activity in the hippocampal region increased, while 360 nm light decreased neural activity. Finally, the in vivo applicability of AzoCholine was demonstrated when swimming behavior of C. elegans could be controlled with AzoCholine and light irradiation. AzoCholine effectively turns endogenous α7 nAChRs into photoreceptors. By varying the irradiation wavelengths, the concentration of the active form of AzoCholine can be adjusted in a graded fashion, an effect which is termed photodosing. Thus, it is now feasible to control endogenous nAChRs with high spatiotemporal precision. This will be instrumental for elucidating their roles in the nervous system and may prove to be therapeutically useful as well.
Other cholinergic targets for photopharmacology

Apart from nAChRs, the mAChRs [61] are also attractive targets for photopharmacology. In 1982, Lester and co-workers reported several photoswitchable ligands that behaved as antagonists of mAChRs in frog myocardium [62]. These compounds included known PCLs for nAChRs, such as BisQ, 2BQ and azo-Ph-carbachol (Figure 7), as well as the new PCLs 4BQ, azo-carbachol and azo-Me-carbachol (Figure 12a). They were all found to block the outward currents that were produced by mAChR agonists in frog atrial trabeculae. It was demonstrated that the compounds were more potent antagonists as their trans-isomers. The cis-isomers still exhibited blocking activity, albeit in a much weaker fashion than their trans-isomers. The action of BisQ on mAChRs was studied in further detail due to its greater availability. Despite this promising first report, further research into PCLs for mAChRs has not yet been disclosed. Recent work has focused on the development of photoaffinity labels [63] that can either activate or inhibit the function of mAChRs [64-66].

Another related target for photopharmacology is that of the enzyme AChE [67]. AChE plays a crucial role in the cholinergic nervous system by removing ACh from the synaptic cleft. Long-term inhibition of AChE can have catastrophic effects on organisms, as demonstrated by nerve gas agents such as sarin. However, short-term inhibition of AChE can instead have beneficial effects, for instance in decreasing the symptoms of Alzheimer’s disease. Therefore, photoswitches that can control the function of AChE would be important biological tools. The first report on the optical control of AChE function was disclosed in 1996 using azo-PTA (Figure 7b) [68]. This compound was found to be most active as its trans-isomer, whilst exposure to UV light reduced the amount of inhibition observed as conversion to the cis-isomer occurred. Azo-PTA could also be used to reversibly control AChE activity, with the compound being shown to exhibit no significant loss of activity over several switching cycles. However, the overall change in AChE inhibition upon isomerization between the trans- and cis-isomers of azo-PTA was only modest. Shortly after this initial report, Erlanger and co-workers demonstrated that they were able to control the inhibition of AChE using the
PCL azo-carbachol [69]. Azo-carbachol was able to induce moderate changes in AChE activity when using filtered UV light (366 nm) and darkness for the isomerization of the azobenzene photoswitch. Relaxation of the cis-isomer back to the more thermodynamically stable trans-isomer occurred within 600 seconds in the dark, with a half-life of around 120 seconds. The trans-isomer of azo-carbachol was found to be the most active, resulting in the largest amount of AChE inhibition. Interestingly, in this publication the authors demonstrated that sunlight could also be used for the trans- to cis-isomerisation of azo-carbachol. This process was show to be reversible over many switching cycles without substantial loss of activity for either the trans- or cis-isomers.

The seminal work of Erlanger and co-workers was not further expanded until very recently, when Trauner [70] and Decker [71] reported photoswitchable inhibitors for the optical control of AChE. Although the reports were published independently, both groups used the AChE inhibitor tacrine as the pharmacophore. The difference between the two approaches came in the form of the photoswitch used. Trauner and co-workers prepared a photoswitchable tacrine derivative (azo-THA) that was linked to an azobenzene photoswitch via the tacrine amine functionality (Figure 12b). Azo-THA was evaluated for its AChE inhibition activity using a colorimetric assay in conjunction with acetylthiocholine (ATCh) and Ellman’s reagent. Under illumination with 440 nm light, trans-azo-THA enabled the ATCh to be hydrolyzed, indicating that trans-azo-THA does not act as an AChE inhibitor. Changing the illumination to UV light at 350 nm completely stopped the hydrolysis of ATCh, showing that the AChE was inhibited. The photoswitching process could be dynamically controlled over many switching cycles. The effect that azo-THA had on mouse trachea preparations was also studied. The smooth muscle of the trachea constricts in response to ACh, this can be recorded by tracheal tensometry. It was found that different relaxation kinetics were observed in the trachea preparations depending on the light illumination. In the presence of cis-azo-THA (UV light), AChE is inhibited to a greater extent resulting in slower relaxation kinetics (15.25 seconds). In contrast, with trans-azo-THA there is less inhibition and therefore the relaxation of the trachea occurs at a faster rate (11.29 seconds).

Decker and co-workers designed their photoswitchable AChE inhibitor with two molecules of tacrine that were linked by a DTE photoswitch (Figure 12c). The photochromic molecule DTE-THA was converted to its closed form within 30 seconds when irradiated with UV light (312 nm). The reverse isomerization to the open form occurred when illuminating the photoswitch with >420 nm light. However, this isomerization was considerably slower with illumination times of 5 minutes being required. It is worth noting that some fatigue of the photoswitch ensued after 8 switching cycles. This could be a major drawback for prolonged use of DTE-THA as a reversible AChE inhibitor. The IC$_{50}$ values of the open and closed forms of DTE-THA were determined using a human AChE (hAChE) assay. The closed form was found to be the most potent inhibitor of hAChE (IC$_{50}$ closed = 19.1 nM). However, the open form also exhibited inhibitory activity within the nanomolar range (IC$_{50}$ open = 49.6 nM). Although the open and closed forms of DTE-THA varied in their amounts of hAChE inhibition, dynamic control of hAChE inhibition was not demonstrated in this report.

**Summary**

Over the last five decades, an impressive toolset for the optical control of nAChRs has been developed. This includes compounds that function as soluble and tethered agonists and competitive antagonists. Some of these compounds have also been applied to mAChRs and AChEs. However, because of the diversity of nAChRs there is still a need for the development of selective PCLs that can optically control nAChR function. In addition, the potential of PTLS with regard to controlling specific receptor subtypes has yet to be fully realized. This will make it possible to investigate the differences between individual receptor subtypes in the same cell, also differentiating between different binding sites. We envisage that major advances in the field will be made shortly, allowing scientists to further their understanding of nAChRs and the many roles they play in the nervous system. We also postulate that new light controlled drugs (photopharmaceuticals) will emerge from this photopharmacological toolset, opening new therapeutic avenues for the treatment of debilitating cholinergic diseases.

**Conflicting interests**

The authors have declared that no competing interests exist.

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**Abbreviations**

NACHR: nicotinic acetylcholine receptor; mACHR: muscarinic acetylcholine receptor; AChE, acetylcholinesterase; RT: room temperature; PCL: photochromic ligand; PTI: photochromic tethered ligand; DRG: dorsal root ganglion; UV: ultraviolet; MEA:
multielectrode array; HEK293T cells: Human Embryonic Kidney cells type 293T.

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