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The $GABA_A$ receptor as a target for photochromic molecules

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ABSTRACT

Photochromic ligands, molecules that can be induced to change their physical properties through applied light, are currently the topic of much chemical biology research. This specialized class of small organic structures are, surprisingly to many, fairly common in nature. At the core of a number of natural biological processes lies a small molecule that changes shape or some other measurable property in response to light absorption. For instance, conformational changes invoked by reversible photoisomerization of a retinoid small molecule found in the photoreceptors of the human eye leads to vision. In plants, photoisomerization of a cinnamate moiety leads to altered gene expression. The photosensitive molecule can be viewed simply as a nanosensor of light, much like a photosensitive electrical component might be added to a circuit to sense day versus night to turn an electrical circuit on or off. Synthetic organic chemists and chemical biologists have been, for at least the last 15 years, trying to either mimic or exploit the native photochromism found in nature. Here, we describe the design process to develop a photochromic molecule to be used in neurobiology.

1. Introduction

The field of study related to natural and synthetic photochromic molecules is presently booming. While many research groups and industrial entities are studying these interesting molecules for myriad purposes ranging from photodynamic materials science to photo-nanomedicine, the scope of this manuscript is limited to tethered and non-tethered (i.e., diffusible) photochromic ligands designed to effect ion channels.

Mammalian cells express ion channels that are directly activated by changes in transmembrane voltage, small molecule binding, changes in temperature, and mechanical forces, but none, apart from those expressed in the rods and cones of the retina, are known to be directly sensitive to light. These specialized cells that do sense light, however, do not do it directly but instead operate through a complex biochemical cascade that involves second messengers and a variety of other support macromolecules to transduce the light signal to a chemical or biological signal that is then acted upon by the individual cell.

Direct activation or inactivation of cellular activity can now, due to paradigm-changing advances in the fields of molecular biology and chemical biology, be controlled with merely two- or three-component systems. For instance, if a researcher were interested in activating some subset of neuronal cells in a native preparation, there are currently a menu of options to choose from. Classic electrode stimulation can certainly be used and still is, however the disadvantage is that tissue in the path of the electrode(s) must be physically damaged or perturbed to place the electrodes in the correct position. Perfusion devices can be placed nearby targeted cells to allow temporarily-precise drug applications, but spatial resolution is not tightly controlled due to simple diffusion of the applied drug.

The newest methodologies for exogenous neuronal stimulation involve the direct application of light through some form of light pipe (e.g., implanted fiber optic core) in conjunction with photoresponsive molecules. These photoresponsive molecules are available in a wide variety of classes ranging from photoresponsive proteins (e.g., Channelrhodopsin-2, Halorhodopsin, etc.) to small molecule photoswitchable ligands (e.g., caged-transmitters, tethered photoswitches, etc.) that can be used to stimulate (or silence) cellular activity. The main purpose of this manuscript is to demonstrate the design process and implementation of tethered and non-tethered photochromic ligands in the context of photoneurobiology. As an example, we describe here the process for targeting a yet-untouched target: the GABA receptor.

2. Discussion

2.1. Freely-diffusible and covalently-linked photochromic pharmaceuticals

Advances in protein crystallography, chemical biology, and pharmaceutical chemistry have resulted in a variety of tethered and non-tethered ligands that have been cleverly used to understand better the structure and activities of proteins.[1–3] By building

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on this knowledge, some of these molecules have been converted to the photochromic variety. Indeed, early pioneering work by Lester and Erlanger demonstrated the power of photoneurobiology when they developed a photochromic ligand for the acetylcholine receptor to investigate the involvement of this receptor in neurally-evoked post-synaptic currents in *Electrophorus* electroplaques.[4] This seminal work demonstrates the temporal power of combining short pulses of light (millisecond range) with photochromic events (microsecond range) to study the channel-gating kinetics in an irrefutable manner. Indeed, the work was a triumph and should serve current readers as a perfect example of a tool designed to solve a problem (and not vice versa).

More recently, polymeric maleimido-quaternary ammonium compounds have been designed and confirmed to function as molecular tape measures when covalently tethered to extracellular cysteine residues of an engineered *Shaker* K^+ channel.[5] These polymeric molecules possess a charged quaternary ammonium (Q) headgroup resembling the well-known pore blocker tetraethylammonium (TEA), a sulfhydryl-reactive maleimide group capable of tethering the molecule to a cysteine residue, and of most importance for the current work, a flexible, but defined, linker in between the maleimide and Q (Fig. 1a). When an ion channel containing an extracellular cysteine target is exposed to such a compound, the cysteine–maleimide-tethering reaction creates a 'cloud' of concentrated Q that reaches as far as the extended length of the compound. If the compound is long enough, for example, containing five glycine linkers between the maleimide functionality and the Q (39 Å), the ligand end will reach the pore and act as a tethered blocker of the channel producing a *measurable result* (e.g., channel blockage, Fig. 1b). If the compound is not long enough, for example, three glycine linkers between the maleimide and the Q (32 Å), the ligand end will not fully reach the pore and, thus, may not have any effect (Fig. 1c) or may act as a weak blocker of the channel.[6]

The current cohort of published tethered photochromic molecules (Fig. 2) are almost entirely based on a substituted azobenzene core. These molecules, in general, contain a ligand-like molecule on one end of the azobenzene scaffold and some chemical attachment strategy on the other end. For the ligand, current examples include Q or an analog of glutamate (G) that bind to and block the pore of potassium channels or agonist binding site of an ionotropic glutamate receptor, respectively.[7,8] For attachment strategies, there are generally two proven methods and these are maleimide (M) or acrylamide (A). These functional groups enable covalent attachment of the photoswitch to engineered cysteine residues or native nucleophilic amino acid side chains on the surface of the target, respectively.

A word about 'effective concentration': the process of tethering a small molecule, be it a photochromic ligand or simply a 'static' tethered ligand, to a biologically-relevant macromolecule is akin to fastening the ligand on a molecular leash to the protein. Covalent tethering technology typically works similar to standard affinity labeling reagents, first binding to the target protein and then chemically reacting with the target. Ligands that retain high affinity for the target can make more efficacious the kinetics of cross-linking by increasing the local concentration of the reactive moiety (e.g., maleimide) near the reaction target. The proximity of this species is of utmost importance to efficient reaction. Once the protein is chemically modified, the now-tethered ligand imparts a dramatic increase in effective local concentration because the effective molarity of the ligand is dependent on the tether length.[9] The effect of this perceived increase in concentration from the standpoint of the protein is that ligand molecules that are measured to be weak effectors in solution can become superb effectors once tethered to the target, affording concentrations into the tens of millimolar.

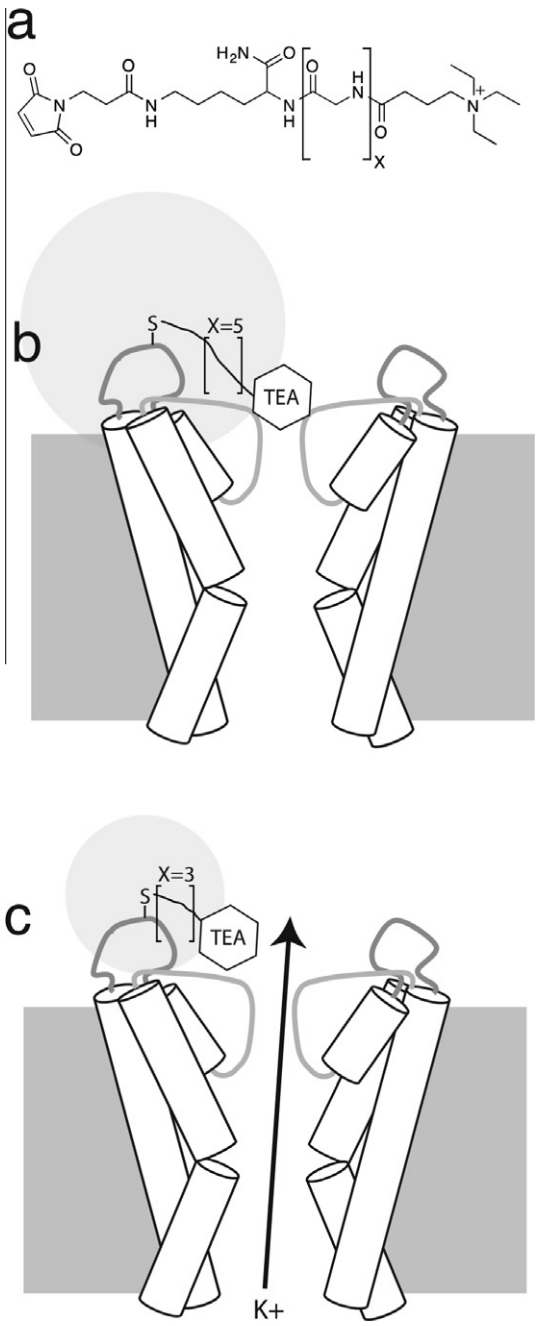


Figure 1. (a) Structure of Mal-Gly(*X*)-QA. Bracket and '*X*' indicate various defined lengths. (b) 'Cloud' of reach when Mal-Gly(5)-QA is used on engineered potassium channel. When *X* = 5, Q can reach binding site and block current. (c) 'Cloud' of reach when *X* = 3; Q cannot reach binding site and, thus, current is allowed to flow.

The photochromic tethered molecule MAQ (Maleimide-Azobenzene-Quaternary ammonium) was designed and developed as part of a new photochemical tool for engineering light-activated potassium channels. With the expression of an engineered potassium channel, MAQ can be applied to cells (e.g., neurons) to impart light-sensitivity onto normally light-insensitive cells. After covalent linkage of the small molecule has occurred, longer wavelength light (500 nm) drives the azobenzene into its extended *trans* configuration, thus causing the Q ligand to block potassium conductance. Short wavelength light (380 nm) generates the rigid *cis* configuration of the azobenzene chromophore, retracting the ligand from the binding site and allowing for potassium conduction out of the cell.[5]

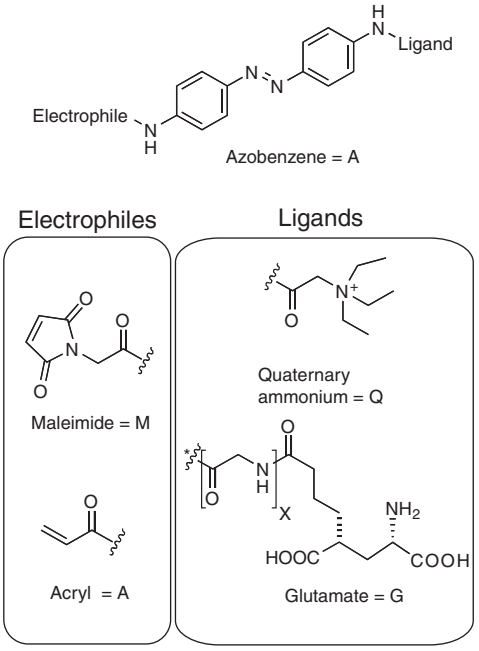


Figure 2. Generic photochromic tethered ligands. Two electrophiles and two ligands are shown. Linker length can be modified as indicated by '*X*'.

The structurally similar photochromic molecule AAQ (Acrylamide-Azo-Quaternary ammonium, Fig. 2) has been found to photosensitize wild-type potassium channels.[10] It was initially hypothesized that AAQ functions as a photochromic tethered ligand at the external TEA binding site of native ion channels and that the molecule would attach to native residues through affinity labeling. Later it was determined, however, that AAQ predominantly acts as a non-tethered photochromic ligand at the internal TEA binding site.[11]

Photochromic ligands, both tethered and non-tethered, have been shown to be useful tools for the development of light-activated ionotropic glutamate receptors (LiGluR). Based on structural information gleaned from X-ray crystallography data of the ionotropic glutamate receptors (iGluR), a series of photoswitchable tethered ligands were developed which are generally called MAG (Maleimide-Azobenzene-Glutamate, Fig. 2). Once the MAG molecule is attached by covalent reaction with an engineered cysteine, the ligand can be photochemically directed in and out of the glutamate-binding pocket. In the *cis* form the ligand induces receptor activation and channel opening where, conversely, in the *trans* form it removes the agonist and allows for deactivation and channel closure. When MAG is in the *trans* form the azobenzene extends the drug away from the binding site preventing it from favorably interacting with the binding pocket. Photoisomerization to the *cis* form bends the tether and permits the agonist to reach the agonist binding site and activate the ligand-gated channel.[8,12–15]

2.2. The design process

The strategy that was used to design the presently-used and successful molecules MAQ or MAG is a general one that can be applied to the development of other photochromic ligands for practically any receptor of interest. Three things are necessary to design a photochromic ligand: (1) a core molecular scaffold for the ligand; (2) an appropriate photoresponsive moiety (i.e., azobenzene, spiropyran, etc.) to attach to the ligand and impart photosensitivity and (3) an assay for quantifying the activity of the photochromic ligand.

2.2.1. Choosing a molecular scaffold

The first step in designing a photochromic ligand is to choose a suitable core structure. This scaffold should be based on the established pharmacology for the receptor or target of interest. Numerous classes of agonists and antagonists have been developed for a wide variety of receptors and ion channels expressed in the nervous system. In large part, these molecules have been developed by the pharmaceutical industry in an effort to identify more potent and/or specific receptor ligands to treat or prevent disease. Within each compound class there may be hundreds of analogs of a single core structure, displaying a range of activities. Pharmacological activity studies of these analogs reveal which modifications are and are not important for effecting the receptor. Thus, if one considers a core structure to be a simple cube with six wild-card ('R') groups (Fig. 3), structure–activity relationship studies suggest which of these 'R' groups are and are not important for effectiveness and which of these can be modified without untoward effects. An appropriate core structure is one that contains an 'R' group that can range in size (e.g., methyl, ethyl, propyl, even phenyl, and beyond) without significantly impacting binding affinity and/or efficacy to the receptor. This 'R' group will be replaced by a linker to connect the ligand to a photosensitive moiety during the development of the photochromic ligand.

There are a number of sources rich with information when it comes to finding a lead structural candidate that will be used as a photochromic ligand and aiding in evaluation of potentially where a chemical handle or moiety may be attached to the drug structure. When choosing the basic core structure it is most useful to identify articles in the Journal of Medicinal Chemistry. It is this source where one may begin to ascertain structure–activity relationships and pharmacological trends. For instance, where is steric bulk tolerated on a particular molecular scaffold? How far can I 'grow' a branch point on this scaffold before untoward steric interactions occur with the wall of the binding site thus degrading affinity and/or efficacy? Can subtype specificity be built into a new photochromic linker through the clever use of medicinal chemistry (e.g., incorporation of the photochromic moiety into the pharmacophore)? In addition to studying the structure–activity relationships in publications, there are also many times the potential of employing either crystal or NMR structural data or homology-based models to glean more information regarding the targeted binding site. Many times, these computer-based models can help rationalize one potential compound over another due to predicted steric clashes that are seen in a model and would not have been perceived using solely structure–activity based data. In the end, one needs to use the best information that is available to avoid costly decisions. There are a number of other sources of information that are valuable and they are addressed in Appendix of the manuscript. The method that you choose to find your lead depends on your familiarity with certain types of searching methods as well as accessibility to the data sources.

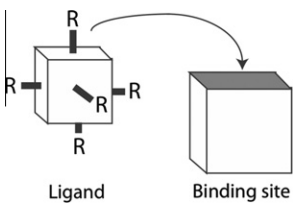


Figure 3. Cartoon-based reduction of medicinal chemistry. Cube with 'R' groups represents a potential ligand with associated structure–activity relationships available. Open box represents a binding site on the target receptor. By comparing 'R' groups with activity and affinity, one can begin to design a linked pharmaceutical based on literature by determining where on the cube one can modify as an attachment point.

2.2.2. Designing the photoresponsive element

Designing a freely-diffusible photochromic ligand or, conversely, a tethered photochromic ligand, for the purpose of modulating the activity of ion channels necessitates the careful characterization of the photochemical properties of the photoswitchable moiety. One must remember that the photochemical properties of the photoswitch that are measured when covalently linked to a macromolecule are not necessarily superimposable on the photochemical properties observed when measured free in solution. There are two, now standard, methods to attach a photochromic ligand to a macromolecule; (1) maleimide/cysteine chemistry or (2) acrylamide/native nucleophile chemistry. Both of these attachment methods have their pros and cons. When using the maleimide/cysteine route, specificity is more robust because the targeted cysteine is typically engineered into a known, innocuous location on the surface of the receptor or ion channel. This technique, however, requires exogenous expression of the protein of interest which may alter native ion channel function due to lack of expression control.[16] The acrylamide methodology enables covalent reactivity towards native receptors (and thus, native, non-adulterated tissue), but does not offer specificity. The main difficulty with targeting native receptors for covalent attachment is that specificity can only be imparted through the specificity of the ligand bait. Therefore, unless the pharmacology of the tethered drug is exquisitely selective, there will be some level of background or off-target labeling that may or may not be a problem, depending on the specifics of the preparation.

There are several chemical photoswitches available, but the photoisomerizable small molecule azobenzene has emerged as the most frequently used for the development of synthetically-photosensitive ion channels.[15] The dark, thermally relaxed *trans* isomer of azobenzene adopts an extended conformation that is approximately 7 Å longer than the *cis* or 'bent' isomer. Illumination with ultraviolet (UV) light at wavelengths preferred for absorption by the *trans* isomer (380 nm) results in photochromism and, thus, accumulation of the *cis* isomer. Visible light irradiation at wavelengths preferred for absorption by the *cis* isomer (500–600 nm) photoswitches the bulk of molecules back to the extended, low-energy *trans* form. Photoisomerization cycles can be repeated many times without noticeable bleaching. In general, a bulk population of azobenzene molecules can be switched to at most around 85% *cis* at the optimal UV wavelength.[17] The other 15% of molecules remain as the *trans* isomer (this is the photostationary state). However, 100% *trans* isomer can be measured after thermal relaxation to the lower-energy form. The photoisomerization lifetime of an azobenzene molecule is in the picosecond range[18] with a quantum yield of 0.2–0.4.[19] These two facts enable azobenzenes to be employed in rapid kinetic studies.[4]

In addition to azobenzene, there has been a rapid expansion in the present literature surrounding the other photoswitchable scaffolds and other photochromic molecules. A number of other photosensitive scaffolds are worth noting here. In general, photoswitches are nanoscale actuators that can undergo transitions between two reasonably stable states after excitation or relaxation reactions. The difference between these two states can range from a change in overall length, change in dipole, or an induced rotation of some tethered ligand. The photophysical and photochemical properties of a number of these small molecule photoswitches have been employed to reversibly control the activity of macromolecules. There are several structural classes known including the azobenzenes, dihydroindolizines, diarylethanes, chromenes, naphthopyrans, spiropyrans, and fulgides.[20] The properties of these photochromic molecules have, for the most part, been determined in organic solvents. This is likely due to the fact that most organic-based photoswitches demonstrate only limited solubility in water thus precluding data acquisition in physiologically-relevant buffer systems.

To tune the properties of tethered ligand photoswitches and optimize their efficacy when applied to biological questions, it is important to understand their basic physical and chemical properties. To elucidate the structural basis of optical switching of the ionotropic glutamate receptor 6, three different MAG molecules were tested, each coupled with a series of engineered attachment sites surrounding the glutamate-binding pocket.[13] This designed structural diversity (chemical and biological) is quite useful since one can manipulate attachment points quite easily utilizing standard molecular biology and, if designed cleverly, can change the length of the photochromic ligand to explore that variable as well.

2.2.3. Designing an assay for a new photochromic ligand

When working with neuronal ion channels, two potential assays are available: electrophysiology and fluorescence imaging. A full description of these techniques is well-beyond the scope of this article, however, one can begin to appreciate each of these techniques from a simplified standpoint. Electrophysiology offers exquisite sensitivity, in some cases allowing long-term measurement of single molecules in a living cell. Conversely, fluorescence imaging offers the ability to perform high-throughput screening of different mutational and chemical candidates, assuming a suitable dynamic fluorophore is available to measure differing concentrations of some variable (e.g., calcium concentration fluctuations.) While electrophysiology can almost always be used, it is more tedious, expensive, and more expertise is required than fluorescence imaging.

In brief, electrophysiology allows the investigation of the electrical activity of cells and their ion channels. Voltage–clamp recordings measure ion currents across a membrane while holding membrane voltage at a set level. In contrast, current–clamp recordings measure changes in membrane voltage while holding current at a set level.[21] Patch–clamp electrophysiology allows detailed investigation of how single, or multiple, ion channels from living cells function under physiological and pathological conditions. Changes in biological electrical activity of ion channels reflect the movement of ions across the cell membrane (e.g., Na^+, K^+, Cl^-, and Ca^{2+}). The relationship between voltage (V), current (I), and resistance (R) is defined by Ohm's law: $V = IR$ (V, measured in volts; I, measured in amperes; R, measured in ohms (Ω)).[22] Three typical patch–clamp configurations are used including whole-cell, inside-out, and outside-out patches. In each configuration, a cell-attached patch is first achieved by lowering a glass micropipette electrode filled with intracellular solution onto the cell of interest followed by applying negative pressure to make a gigaohm seal between the micropipette and the cell. Whole-cell patch ruptures the patch of the membrane inside the tip of the micropipette making the micropipette electrode contiguous with the cytoplasm thus allowing the study of multiple ion channels and intact cell signaling.[22] The whole-cell patch–clamp configuration has many possible uses including measuring synaptic transmission, action potentials, and membrane resting potential. Both inside-out and outside-out configurations allow the study of single ion channels. The 'inside' and 'outside' refer to the intracellular or extracellular side of the membrane facing the bath solution. The inside-out patch is formed by slowly withdrawing the micropipette electrode from a cell-attached patch leaving the intracellular side of the membrane exposed to the bath solution. Uses of inside-out patches include the study of cytosolic factors, measuring calcium-dependent conductance, and measuring 2nd messenger effects on conductance. An outside-out patch is created after attaining whole-cell patch by slowly withdrawing the electrode from the cell, the patch of membrane will then reseal on the micropipette electrode with the extracellular side of the membrane exposed to the bath solution.[21] Outside-out patches are useful in measuring agonist efficacy and antagonist activity at an extracellular binding site.[22]

Neurons contain ion channels that are directly activated by changes in membrane voltage, temperature, ligand binding but not by light.[23] Strategies that take advantage of light to control and activate photoswitchable ligands in combination with patch–clamp electrophysiology allow the targeting and manipulation of ion channels to study neuronal activity in the presence of the molecule of interest. For example, photochemical control of endogenous ion channels using photoswitchable affinity labels has been used to photosensitize endogenous proteins and control the activity of ion channels.[10] In general, cells of interest are cultured on coated coverslips in serum-containing medium at 37 °C with 5% CO_2. A coverslip of cells are then mounted in a perfusion chamber with extracellular solution in the bath and a cell is then chosen under the microscope and patch–clamp techniques are performed on that individual cell of interest. In the case of testing a non-tethered photoswitch, electrophysiology data is recorded while perfusion is used to apply the photoswitch to the bath while irradiating the cells with visible or ultraviolet light. In the case of a tethered photochromic ligand, cells of interest are pre-incubated with the photoswitch to allow chemical tethering to the receptor then mounted in a perfusion chamber with extracellular solution in the bath. A cell is then chosen under the microscope and patch–clamp techniques are performed on that individual cell of interest while recording electrophysiology data under irradiation of visible or ultraviolet light. Manipulation of light will activate or inactivate the ion channel by altering the conformation of the photoswitch.[24] Determination of direct activation versus antagonism versus potentiation is attained by analyzing changes in electrophysiology recordings during application of the designed photoswitch. For example, direct activation will occur if the molecular photoswitch is able to activate the ion channel on its own while a photochromic potentiator requires a neurotransmitter for activation of the ion channel. Photochromic potentiators enhance sensitization to endogenous neurotransmitters and are therefore important for studying endogenous ion channels and magnifying and manipulating native neurotransmission. In contrast, photochromic direct activators are important for directly activating and inhibiting ion channels in the presence of light.

2.3. A narrative of our current design efforts

With past designs and work in mind, we will discuss here the design of new photochromic ligands that are useful for a new biological target: light gated-ionotropic GABA receptors. Our photochromic ligands feature at one end of the azobenzene a cysteine reactive group, maleimide, and at the other end a variable-length tether coupled with a GABA effector molecule.

GABA (γ-aminobutyric acid) receptors are inhibitory neuronal receptors activated by the neurotransmitter GABA. These receptors are a very interesting target given their diversity of functions, structure, isoforms, and expression patterns. There are two classes of GABA receptors: ionotropic $GABA_A$ receptors and metabotropic $GABA_B$ receptors. $GABA_A$ receptors, the main target, are members of the family of ligand-gated ion channels that also includes the glutamate receptors, nicotinic acetylcholine receptors (nAChRs), and P2X receptors, for example. The quaternary structure of $GABA_A$ receptors is heteropentameric and each subunit has an extracellular N-terminal domain, four transmembrane domains (TMs), an intracellular loop between TM3 and TM4, and an extracellular C-terminal domain. The subunits form a chloride-conducting pore and activation of the receptor by GABA opens the pore allowing the influx of Cl^- ions into the cell leading to membrane hyperpolarization thus preventing action potential firing.[25,26]

Nineteen genes encoding $GABA_A$ receptor subunits have been identified to date and these gene products have been classified according to their degree of homology with one another into subunit and subtypes as follows: α1–6, β1–3, γ1–3, δ, ε, θ, and π. The remaining three subunits are named ρ and are found in a subclass of $GABA_A$ receptors found mainly on the retina, although ρ2 and ρ3 are also found elsewhere.[26] Random combinations of receptor subunits would lead to thousands of receptor subtypes,[27] however, it is believed that only a fraction of these possible combinations are found in vivo and are functionally relevant. This hypothesis stems from what is known about nAChRs: although many subunits exist and their random combination could result in over 200 receptor subtypes, only two possible stoichiometries are found in nature.[25] $GABA_A$ receptor subtypes can vary in their subunit composition, expression levels, and localization in the central nervous system and can also vary in their subcellular localization, pharmacology, developmental expression patterns, and ultimately function. Experimental evidence supports the idea that receptors with a stoichiometry of two α_1, two β_2, and one γ_2 subunits are the most abundant in mammalian central nervous system.[27]

There are a number of different drug classes and corresponding binding sites that could be used in the design of a photochromic ligand. There are two distinct GABA binding sites located at the interfaces between the α and β subunits. In addition to GABA binding sites, $GABA_A$ receptors also possess benzodiazepine, barbiturate, ethanol, picrotoxin, and steroid binding sites, making $GABA_A$ receptors a major drug target in the treatment of anxiety, sleep disorders as well as being the target of anesthetics. Benzodiazepines and steroids exert their anxiolytic and anesthetic effects by enhancing GABA-induced inhibitory currents. Conversely, picrotoxin acts as a convulsant by reducing GABA-induced currents. Barbiturates, in contrast to the benzodiazepines, can activate the receptor in the absence of GABA increasing their risk for overdose and thus making benzodiazepines a safer alternative for the treatment of anxiety disorders.[28]

Drug design targeted to the benzodiazepine binding site has been the focus of extensive research. The benzodiazepine site is distinct from the GABA binding sites and is located at the α and γ subunit interface making the γ subunit necessary for benzodiazepine sensitivity. All known benzodiazepine site effectors are synthetic. The belief that there exist no endogenous benzodiazepine site ligands is supported by data that show no effect of benzodiazepine antagonists on $GABA_A$ receptor function in vivo. It is also important to note that the α subunit determines the pharmacology of drugs acting on the benzodiazepine site. For instance, $GABA_A$ receptors containing α_1, α_3, or α_5 are sensitive to classical benzodiazepines (e.g., diazepam) and receptors containing α_4 and α_6 subunits are not. One focus of pharmaceutical research in this field has been to develop drugs that display selectivity for the different subtypes of $GABA_A$ receptors. From a pharmacological standpoint, the goal is to reduce or eliminate side effects caused by the action of the drug on non-target receptors. Receptors containing the α_1 subunit are responsible for sedation, receptors containing α_2 and/or α_3 are involved in anxiety and α_5-containing receptors have been implicated in learning and memory. These very similar subunits, one can surmise, offer a wide range of biological complications. Pharmaceuticals that display selectivity for α_5-containing receptors could enhance memory processes and potentially learning ability without having an anxiolytic or sedative effect.[28]

Presently, two techniques have been used in an effort to determine the distribution of receptor subtypes in the central nervous system: in situ hybridization and immunocytochemistry. In situ hybridization measures the abundance of specific subunit mRNA in different types of tissue from the CNS. There is enough sequence divergence between subunits in the region encoding the intracellular loop between TM3 and TM4 allowing for the synthesis of oligonucleotides with unique sequences specific for different receptor subunits (see Appendix for an open-access and interactive source of in situ hybridization data). Immunocytochemistry is used to

identity protein expression patterns by labeling proteins of interest with antibodies in different tissues from the CNS. This method has been, however, hindered by the need for subunit-specific antibodies. Immunocytochemistry has not been possible for some $GABA_A$ receptor subunits as some of the developed antibodies can react with more than one subunit producing confounded results. On the other hand, one advantage of this technique is that the co-localization of different subunits can be determined by using more than one antibody.

Some $GABA_A$ subunits are under very tight expression control and are found only in certain areas of the CNS. Subunit ρ1, for instance, is expressed only in retina while δ6 is localized to the cerebellum. The α_1 subunit is ubiquitous throughout the CNS making it the most abundant $GABA_A$ receptor subunit. The biological function of specific subunits can be inferred from their anatomical distribution in the CNS. The α_5 subunits are predominantly expressed in the hippocampus and have been implicated in cognition and memory formation. The biological role predicted for different subunits from anatomical distribution can be confirmed by other experiments, for example, rats treated with PWZ-029 (Table 1), a moderate inverse agonist selective for α_5-containing receptors, show improved learning behavior when carrying out certain tasks.[29,30]

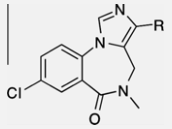
Designing a tethered $GABA_A$ pharmaceutical: the approach described earlier for potassium channels has been applied to the selection of a suitable tethered ligand specific for a $GABA_A$ receptor subtype. The fact that $GABA_A$ receptors are major drug targets translates into a large number of compounds from which to choose when selecting a tetherable ligand. Moreover, the development of subtype-selective compounds as pharmaceuticals provides the added advantage of targeting different types of receptors simply by choosing a different drug. For example, imidazobenzodiazepines act as effector molecules for $GABA_A$ receptors containing any α-subunit as opposed to the classical benzodiazepines that show no effect on α_4- and α_6-containing receptors. It has been possible to synthesize imidazobenzodiazepines that have higher binding affinity and/or efficacy for a specific $GABA_A$ receptor subtype. Some of these pharmaceuticals selectively target α_5-containing receptors and have been developed as potential memory and/or cognition enhancers. One such drug, PWZ-029 demonstrates selectivity for α_5-containing receptors and also acts as a moderate inverse agonist on these receptors.[30]

Since this paper discusses photochromic effector molecules, one must be mindful of the activity of the new tethered ligand. During synthesis, it is wise to test a simplified tether model of the putative ligand to be sure that the pharmacology exhibited by the new drug has been unaltered or altered in an acceptable way. The tethered ligand plays a role not only as a guide for the photochromic molecule to accumulate at the target receptors but also plays a role of inducing activity. It is imperative that the tethered ligand binds with strong affinity to aid in the kinetics of crosslinking and that its biological function is conserved.

Following the above design rules, it appears possible to use PWZ-029 as a tethered ligand for both types of applications, labeling as well as a tethered photochromic ligand. Once a suitable ligand has been found, the next step is to find the site on the ligand where a linker can be incorporated. Two main questions that need to be addressed at this point are; (1) what is the minimum structural requirements for this ligand to retain its binding affinity and biological activity (identifying the pharmacophore) and (2) is it synthetically possible to add a linker to this molecule without altering the pharmacophore? Structure–activity relationship studies are useful, if not necessary, to address the first question. In vitro binding affinity experiments have been performed on many variants of imidazobenzodiazepines (Table 1) that differ only on the identity of the 'R' substituent.[31] An important observation from this compiled data is that a precursor of PWZ-029, RO15-1310, demonstrates high binding affinity to all $GABA_A$ receptor subtypes, but no appreciable selectivity between them.[32] This data also provides evidence that the oxygen atom at the position found on the ether functionality of PWZ-029 appears to be necessary for binding to any of the $GABA_A$ receptor subtypes. For instance, if the ester functionality on RO15-1310 is changed to a ketone (Table 1) the binding affinity to $GABA_A$ receptors is lost. From these observations we can conclude that in order to retain binding affinity, the oxygen atom of the ether functionality (in PWZ-029) need be conserved and in order to maintain the selectivity for α_5-containing receptors the ester functionality (as in RO15-1310) must be avoided. Another reason to avoid having an ester functionality as part of the linker or any other part of the tethered ligand is the instability of esters in vivo as this functional group is prone to hydrolysis. After careful analysis, we propose the tethered compound in Figure 4 as a putative ligand specific for α_5-containing GABA receptors. The acid functionality allows for further synthetic modifications on the ligand and the tethered ligand can be subsequently analyzed for binding affinity and biological activity to corroborate that the incorporation of the linker has not rendered the ligand inactive or altered the biological activity dramatically. Patch–clamp electrophysiology, as described above, is the method of choice to characterize the activity of the tethered ligand on endogenous and/or cloned neuronal receptors.

In addition to benzodiazepines and their derivatives, there are other molecules that act on $GABA_A$ receptors one should consider when selecting a tethered ligand. Muscimol (5-aminomethyl-3-hydroxyisoxazole), a natural product and potent $GABA_A$ receptor agonist, has been successfully tethered and used on these receptors.[33] Recently, two studies have presented evidence that *N*-acyl derivatives of muscimol can bind to GABA receptors. First, Wang et al.[34] have shown that muscimol linked through a 6-aminohexanoyl chain to a fluorophore localizes to $GABA_A$ receptors in retinal

Table 1
In vitro binding affinities of imidazobenzodiazepines



		K_i (nM)				
		α_1	α_2	α_3	α_5	α_6
PWZ-029, R = CH_2OCH_3	$\alpha_x\beta_3\gamma_2$[a]	>300	>300	>300	38.8	>300
RO15-1310, R = $COOCH_2CH_3$	$\alpha_x\beta_2\gamma_2$[b]	6.80	16.3	9.20	0.85	54.6
R = C(=O)CH_3	$\alpha_x\beta_3\gamma_2$[c]	17,500	33,800	22,100	2610	29,500

[a] Savić et al.[30] human recombinant $GABA_A$ receptors with β_3, γ_2, and the indicated α-subunit.
[b] Zhang et al.[32] human recombinant $GABA_A$ receptors with β_2, γ_2, and the indicated α-subunit.
[c] Huang et al.[31] human recombinant $GABA_A$ receptors with β_3, γ_2, and the indicated α-subunit.

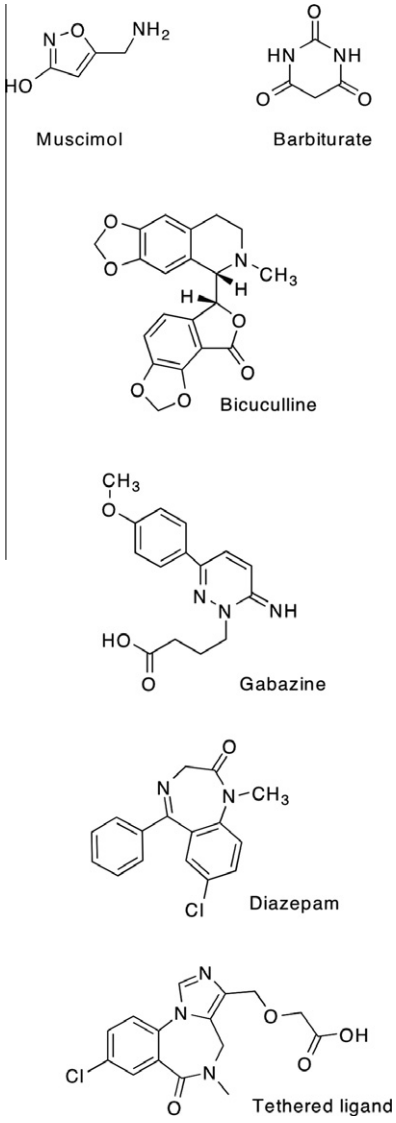


Figure 4. GABA receptor agents. Structure of tethered precursor that is potentially specific for α_5-containing GABA receptors and tethering reaction.

neurons. Second, Meissner and Haberlein have found that muscimol conjugated directly (i.e., without an alkyl chain) to the fluorophore Alexa Fluor 532 shows high affinity for $GABA_A$ receptors of rat hippocampal neurons.[35] It is expected that the same approach can be successfully extended to other $GABA_A$ receptor effectors that can be carefully selected to suit the experimental goals. Does one want to photochromically activate or inactivate receptors? Or, in the case of GABA receptors, does one want to potentiate the natural GABA response?

Barbiturates have also been extensively studied due to their therapeutic potential before the advent of safer substitutes like benzodiazepines. Numerous derivatives of the parent compound (barbituric acid) have been synthesized and studies have been done on how these drugs bind to synthetic receptors through hydrogen bonding.[36] This type of research and the information that can be gleaned from it are useful in identifying potential sites on the molecule for tether incorporation. Many more drug-like options are available when choosing a potential tethered ligand for $GABA_A$ receptors and the vast amount of literature on this receptor and its effector molecules provides a solid starting point in the selection, design, synthesis, and implementation of a novel tethered ligand for various applications. With regards to a photochromic antagonist, the classical $GABA_A$ antagonist bicuculline has played a key role in studies on $GABA_A$ receptors and might serve as a ligand on a photochromic antagonist. New structural classes of $GABA_A$ receptor antagonists based on this scaffold have been developed and a series of arylaminopyridazine analogs of GABA, notably gabazine, show potent and selective competitive $GABA_A$ antagonism.[37]

The development of photochromic ligands for the GABA receptors have the potential to decipher the role of extrasynaptic receptors versus synaptically-active ones. There remain many unanswered questions regarding the role of receptor location, intimately-coupled with questions of tonic versus acute inhibition by GABA. For instance, complex patterns of tonic inhibition by GABA spill-over have been used to explain the difference between fast synaptic currents and sustained inhibitions measured in cerebellar Golgi cells.[38] Additionally, it is now thought that certain GABAR subtypes play a main role in setting the threshold for complex processes such as long-term potentiation.[39] The development of GABA-targeted photochromic ligands could be used to untangle these complex, subcellular signaling networks. For instance, coupled with a laser that is steerable, a photochromic GABA receptor agonist could be used to activate receptors at specific subcellular regions of neurons to interrogate the receptor population as well as the contribution they play in integrative output. Conversely, a photochromic antagonist targeted to silence GABA receptors could be used to temporally silence GABA receptor-mediated Cl^- conductance at specific regions of a neuron and at defined times to interrogate the role of these receptors. Still further and more nuanced, a photochromic GABA receptor potentiator could be used to magnify either the native direct synaptic or passive tonic current induced by acute or tonic GABA receptor activation in an effort to measure the specific contribution that receptors play in subcellular zones on a neuron in question. All of these potential uses rely on a well-planned design of the photochromic ligand and the hope that this new tool offers a wide dynamic range. Ideally, one would desire the molecule to have no activity in one photochromic state and in the other photochromic state offer full activity. Each of these differing photochromic effectors can be viewed as different tools.

3. Conclusion

3.1. Outlook and future directions and applications

The photochromic ligand, whether it is tethered or free in bulk solution, is a highly adaptable technology that can be applied to many receptors, ion channels, and other signaling proteins. Both the photochromic moiety and the target protein can be tailored for a particular biological use. Like the natural photoreceptor methods, the tethered photoswitch approach requires foreign genes to be expressed in target cells, although photoswitch molecules with promiscuous reactive groups can simplify the requirements and serve as a two-component system for imparting light-sensitivity (light delivery being the second component).

The future of photochromic ligands is still wide open to future targets. The rationale behind authoring this manuscript was to make more accessible the process of designing and implementing photochromic molecules into a wider range of research topics. We hope that this technology will be further adopted and used towards a better understanding of basic neurobiology as well as in the understanding and alleviation of disease states.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.05.056.

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