Neuronal Inhibition under the Spotlight

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http://dx.doi.org/10.1016/j.neuron.2015.11.014

In this issue of *Neuron*, Lin et al. (2015) report an optical method to precisely manipulate the activity of $GABA_A$ receptors by designing a mutant receptor that binds photosensitive compounds. This allows for studying $GABA_A$ receptors in situ and represents a valuable tool to investigate how inhibition affects brain physiology.

Ensuring that cell excitability in the brain is controlled, and can form part of a meaningful neural code, is mostly the responsibility of the neurotransmitter GABA and its associated receptors. GABA is essentially the conductor of an inhibitory orchestra formed from interneurons and GABA receptors, which together cause inhibition either by rapidly activating anion flux across the cell membrane (via $GABA_A$ receptors) or by initiating slower second messenger-mediated signaling (via $GABA_B$ receptors). To investigate how inhibition affects brain physiology and how its dysfunction can precipitate numerous diseases requires clear mechanistic understanding. The major investigative tool usually employed for this purpose is electrophysiology, but in this issue of *Neuron*, Lin et al. (2015) describe a new elegant optical method based on a range of light-sensitive compounds. When bound to $GABA_A$ receptors, these molecules can enable the precise temporal and spatial "tuning" of $GABA_A$ receptor activity following their exposure to light of specified wavelengths. They are set to become a valuable addition to the panoply of optical methods that are revolutionizing our understanding of the nervous system.

Manipulating the activity of native $GABA_A$ receptors using photochemical approaches is quite challenging, because $GABA_A$ receptors can show some inherent sensitivity to UV light (Chang et al., 2001), and because ligand specificity is critically important if particular isoforms of the $GABA_A$ receptor are the intended targets. Lin et al. (2015) sought to confer selectivity by exchanging a residue near the GABA binding site for cysteine (Cys). They then constructed a modular-based compound, termed a photoswitchable-tethered-ligand (PTL; Figure 1) that could anchor itself to the Cys residue, and by using light to change the molecule's conformation, facilitate entry of a ligand into the GABA binding site (located at the β-α subunit interface) to manipulate receptor activity. The PTL has three modules: a maleimide, which covalently binds to Cys and creates a highly stable S-C bond on the $GABA_A$ receptor; this is linked to an azobenzene group, which can flip between *cis* and *trans* states when exposed to specific wavelengths of light (the "photoswitch"); finally, this is attached to the third module, a $GABA_A$ receptor ligand, which in this study is usually a guanidinium derivative of GABA that binds to the orthosteric (neurotransmitter binding) site of the receptor. Finally, a repeat methylene spacer ($(-CH_2-)_n$, where n = 1–4) was also included in some compounds between the azobenzene and receptor ligand groups, presumably to increase molecular flexibility for ligand binding.

The principle underlying the use of PTLs is that light-induced conformational flipping of the molecule between *cis* and *trans* will change its ability to access and bind to the GABA binding site. Interestingly, although the PTL binding modules were all designed to be agonists, they unexpectedly showed antagonist behavior, which was also apparent in the authors' previous study using muscimol (Lin et al., 2014); nevertheless, they are still very useful tools for manipulating GABA receptor activity.

It is often reasonably assumed that ligands that target the orthosteric site should also compete with GABA for binding. Using recombinant $GABA_A$ receptors, Lin et al. (2015) selected Threonine 125 in the binding domain for replacement by Cys ($\alpha1^{T125C}\beta2\gamma2$) and showed a competitive-type displacement of the GABA dose response curve in the presence of a *trans*-PTL, suggesting that the PTL is sufficiently flexible (particularly if there is a spacer) for the guanidinium-ligand to bind and dissociate from the orthosteric site, despite the PTL being tethered by the maleimide group. The degree of inhibition is quite extensive, reaching approximately 80% for the most effective PTL inhibiting half-maximal GABA responses, though at GABA concentrations likely to exist at inhibitory synapses (1–3 mM), competitive inhibition may be reduced (to ~30%).

To explore the usefulness of these tool compounds for manipulating the activity of other members of the $GABA_A$ receptor family, the Cys substitution method was used for $\alpha2$–$\alpha6$ subunits for sequential expression with $\beta2$ and $\gamma2$ subunits. Lin et al. (2015) relocated the Cys substitution from that used for $\alpha1$ subunits, to a position that was now within a 4-amino acid motif considered to be important for determining the different potencies of GABA at $GABA_A$ receptors containing different α subunits (Böhme et al., 2004). Reassuringly, the Cys substitutions maintained functional neutrality. Thus, Lin et al. (2015) had succeeded in creating a highly useful palette of Cys-substituted $GABA_A$ receptor isoforms that could be differentially regulated by light.

Such a palette enables a number of interesting experiments to be conducted. We know that $GABA_A$ receptor isoforms show differential distribution throughout the brain, even within the membrane domains of a single neuron (soma, dendrites, axon initial segment; Fritschy and Brünig, 2003). Now we have a means to dissect their relative subcellular distribution and explore their relative physiological function. Lin et al. (2015) addressed this aspect by expressing two $GABA_A$

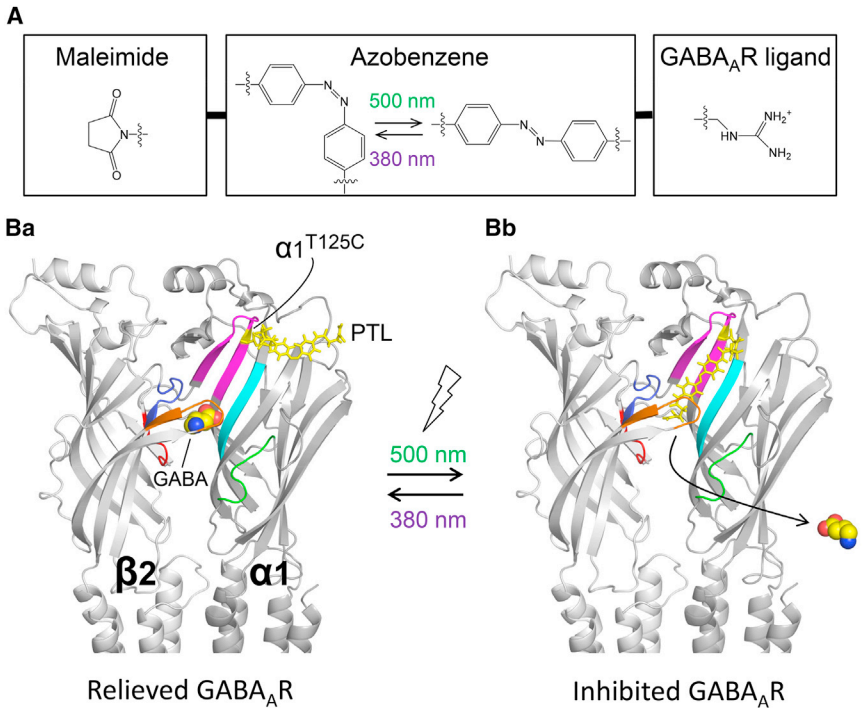

Figure 1. Photo-inhibition of α1^{T125C}β2γ2 GABA$_A$ Receptor by a Tethered PTL
(A) PTLs are composed of three linked modules: a cysteine reactive maleimide; a photoswitchable azobenzene; and a GABA (guanidinium derivative) ligand.
(B) Homology model of α1β2γ2 showing the interfacial GABA binding site with binding loops A (red), B (blue), and C (orange) from the principal (+) β2 subunit and loops D (cyan), E (purple), and F (green) from the complementary (−) α1 subunit. Both α1^{T125C} and the PTL are shown in yellow. (Ba) In this example, the *cis* form of the PTL does not enter the GABA binding site; however, when photoswitched by 500 nm light into the *trans* isomer (Bb), the ligand-group accesses the binding site displacing GABA and causing inhibition. Light at 380 nm reverts the PTL back to its *cis* form relieving inhibition.

receptor subunits in hippocampal neurons using α1^{T125C} and α5^{E125C}. This enabled PTLs to manipulate receptors activated by prior two-photon uncaging of (trimethylphosphine-caged) GABA. The relative sensitivities of the PTL-tethered receptors to the uncaged GABA suggested clustering of α1 subunits in "hotspots," with α5 receptors more evenly distributed, in accord with their presumed synaptic (α1) and extra/perisynaptic (α5) locations (Brünig et al., 2002; Serwanski et al., 2006).

Lin et al. (2015) extended their study of light-regulated GABA$_A$ receptors to neurons using viral transduction to express Cys mutant α subunits. In hippocampal slices expressing α1^{T125C}, light-exposed tethered PTLs reduced the amplitudes of evoked inhibitory postsynaptic currents (eIPSCs) by >50%. A complete block of synaptic inhibition is unlikely to be achieved as different isoforms of GABA$_A$ receptors other than those containing α1 subunits will likely underpin synaptic inhibition, and the Cys-maleimide reaction is unlikely to be 100% complete. This can make the exact contributions of specific α subunits to physiological processes more difficult to assess. Nevertheless, by exchanging the type of α subunit expressed in neurons, e.g., extrasynaptic α5^{E125C}, the diversity of this method was demonstrated by using light to manipulate GABA$_A$ receptor activity associated with tonic inhibition.

Of course, if synaptic (and tonic) inhibition is to be controlled by light, then it is important to have reagents that not only respond to light with fast on-off kinetics, enabling a high degree of temporal resolution, but also be able to deliver both PTLs and sufficient light density to deep lying neurons contained in slices or present in vivo. The kinetics of conformational flipping for the PTLs will be quite fast (100–200 ms) when exposed to light and can, by blocking GABA$_A$ receptors, cause temporally precise effects on single action potentials. The thermal "resting" stability expected of the azobenzene is evident since when either the *cis* or *trans* forms are not exposed to light, they are stable over 10–30 min, which is extremely valuable for experimental protocols.

For spatial penetration, particularly for affecting GABA$_A$ receptors in deep lying neurons, Lin et al. (2015) infused the PTL into the intact brain by a delivery pipette before preparing slices. It was notable that the extent of photoswitching affecting GABA$_A$ receptors declined when moving from the pial surface of the slice to deeper lying cells. This is to be expected, but even so, the method has impressive penetration with light reaching approximately 350 μm into the slice, and presumably this can be further accentuated by using optic fibers to limit light scattering by the neuronal parenchyma.

Having extended the repertoire of the PTL approach to tuning synaptic and tonic inhibition, and demonstrated good temporal and spatial dynamics to photoswitching, Lin et al. (2015) tested their methodology in vivo by expressing Cys mutant α subunits in intact brain using viral transduction, and by creating a knockin mouse line expressing α1^{T125C} subunits.

By using viruses, mutant GABA$_A$ receptors can be expressed deep into neural tissue and located to identifiable fluorophore-expressing neurons (e.g., genetically engineered parvalbumin [PV] interneurons [INs] expressing *tdtomato*). PTLs can also be infused deep into the brain, and by using two-photon targeted patch-clamp recording, the spiking profile of PV INs could be switched from bursting- to continuous-type firing, when inhibition of Cys-substituted GABA$_A$ receptors was induced with a photoswitched PTL or relieved, respectively. This established the viability of the method in vivo, but to control for any aberrant overexpression of Cys mutant receptors following viral transduction, Lin et al. (2015) created a global knockin mouse that replaced native α1 subunits with α1^{T125C}.

This photoswitch-ready mutant mouse line only requires the delivery of PTLs to be able to gate GABA$_A$ receptors using light. Four cell types exhibiting differential expression patterns for α1 subunits were

chosen for the study. As expected, for those cell types where α1 subunit $GABA_A$ receptors are dominant or even the only receptor isoform expressed, photoswitching induced a strong inhibitory effect on IPSCs, while IPSC photoswitching was less effective for cells expressing α1 with other $GABA_A$ receptor isoforms and completely ineffective for cell types that lacked α1 subunit expression.

Using awake $\alpha1^{T125C}$-knockin mice, Lin et al. (2015) show photoswitch-control of visually evoked spike firing rates as well as γ-oscillations, which effectively demonstrated the possibility of interrogating physiological functions of specific $GABA_A$ receptor isoforms in the nervous system. If cell-type-specific knockins are created, this interrogation can proceed at the single cell level as well as at the level of specific neural networks, establishing the behavior and role of IN subpopulations (Klausberger et al., 2003).

In designing a mutant receptor that binds photosensitive compounds, Lin et al. (2015) have created a very powerful set of tools for studying $GABA_A$ receptors in situ. These tools need validation against a set of controls and Lin et al. (2015) have been very thorough in this regard, testing the PTLs against other membrane proteins. However, any free surface-exposed Cys residue will in principle bind the maleimide group of the PTL. For $GABA_A$ receptor subunit, there are relatively few "free" Cys residues in the extracellular domain (ECD). Two Cys residues are engaged in a Cys-Cys bond, forming the Cys loop that characterizes many of the receptors in the pentameric ligand-gated ion channel family; so they are presumably unavailable to bind maleimide. Another potential neurotransmitter receptor that is worth screening is the inhibitory glycine receptor. It does have 5 Cys residues in the ECD, four of which may form two Cys-Cys bonds, leaving one possibly free for interaction with maleimide.

An interesting aspect of this study is that the PTLs exhibit antagonist properties, which is very useful for inhibiting $GABA_A$ receptor activity; however, it would also be valuable to be able to activate or positively modulate $GABA_A$ receptor function as reported for native $GABA_A$ receptors using an azobenzene-based propofol derivative (Yue et al., 2012). Possibly this is easier to achieve from an allosteric site rather than the orthosteric site, since Lin et al. (2015) note the removal of the negatively charged carboxyl on the guanidinium moiety seems to prevent agonist activity.

The direction of travel adopted by Lin et al. (2015)'s study opens many new investigative possibilities for probing the role of $GABA_A$ receptors in neural circuit behavior. The need to express mutant $GABA_A$ receptors in neurons seems a small "price to pay" for the experimental diversity that becomes available from using PTLs, though ultimately trying to emulate these experiments with native $GABA_A$ receptors and other modular-based compounds remains a challenge (Mortensen et al., 2014).

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