

Neuronal inhibition under the spotlight

Martin Mortensen & Trevor G Smart

Department of Neuroscience, Physiology & Pharmacology

UCL, Gower Street,

London WC1E 6BT, UK.

Proofs & correspondence to TG Smart, t.smart@ucl.ac.uk

Abstract:

In this issue of *Neuron*, Lin and colleagues (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 signalling (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 and colleagues (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 revolutionising 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. The authors 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 ((-

CH₂)_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 behaviour, 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.*, selected Threonine 125 in the binding domain for replacement by Cys ($\alpha 1^{T125C}\beta 2\gamma 2$) 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 $\alpha 2$ - $\alpha 6$ subunits for sequential expression with $\beta 2$ and $\gamma 2$ subunits. The authors relocated the Cys substitution from that used for $\alpha 1$ 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 (Bohme et al., 2004). Reassuringly, the Cys substitutions maintained functional neutrality. Thus, Lin *et al.*, 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 Brunig, 2003). Now we have a means to dissect their relative subcellular distribution and explore their relative physiological function. The authors addressed this aspect by expressing two GABA_A receptor subunits in hippocampal neurons using $\alpha 1^{T125C}$ and $\alpha 5^{E125C}$. This enabled

PTLs to manipulate receptors activated by prior 2-photon uncaging of (trimethylphosphine-caged) GABA. The relative sensitivities of the PTL-tethered receptors to the uncaged GABA suggested clustering of $\alpha 1$ subunits in 'hot spots', with $\alpha 5$ receptors more evenly distributed, in accord with their presumed synaptic ($\alpha 1$) and extra/perisynaptic ($\alpha 5$) locations (Brunig et al., 2002; Serwanski et al., 2006).

Lin *et al.*, extended their study of light-regulated GABA_A receptors to neurons using viral transduction to express Cys mutant α subunits. In hippocampal slices expressing $\alpha 1^{T125C}$, 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 $\alpha 1$ 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 $\alpha 5^{E125C}$, 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 mins, which is extremely valuable for experimental protocols.

For spatial penetration, particularly for affecting GABA_A receptors in deep lying neurons, the authors 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 fibres 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, the authors tested their methodology *in vivo* by expressing Cys mutant α subunits in intact brain using viral transduction, and by creating a knock-in mouse line expressing $\alpha 1^{T125C}$ 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 2-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 over-expression of Cys mutant receptors following viral transduction, the authors created a global knock-in mouse which replaced native $\alpha 1$ subunits with $\alpha 1^{T125C}$.

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 $\alpha 1$ subunits were chosen for the study. As expected, for those cell types where $\alpha 1$ subunit GABA_A receptors are dominant or even the only receptor isoform expressed, photoswitching induced a strong inhibitory effect on IPSCs; whilst IPSC photoswitching was less effective for cells expressing $\alpha 1$ with other GABA_A receptor isoforms, and completely ineffective for cell types that lacked $\alpha 1$ subunit expression.

Using awake $\alpha 1^{T125C}$ -knock-in mice, the authors 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 knock-ins are created, this interrogation can proceed at the single cell level as well as at the level of specific neural networks, establishing the behaviour and role of IN subpopulations (Klausberger et al., 2003).

In designing a mutant receptor that binds photosensitive compounds, the authors 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.*, 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 characterises 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.*, note the removal of the negatively-charged carboxyl on the guanidinium moiety seems to prevent agonist activity.

The direction of travel adopted by Lin and colleagues' study opens many new investigative possibilities for probing the role of GABA_A receptors in neural circuit behaviour. 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).

Figure 1. Photo-inhibition of $\alpha 1^{T125C}\beta 2\gamma 2$ GABA_A receptor by a tethered PTL

(A) PTLs are composed of 3 linked modules: A cysteine reactive maleimide; a photoswitchable azobenzene; and a GABA (guanidinium derivative) ligand.

(B) Homology model of $\alpha 1\beta 2\gamma 2$ showing the interfacial GABA binding site with binding loops A (red), B (blue) and C (orange) from the principal (+) $\beta 2$ subunit and loops D (cyan), E (purple) and F (green) from the complementary (-) $\alpha 1$ subunit. Both $\alpha 1^{T125C}$ 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.

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