Photo-antagonism of the GABA$_A$ receptor

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Neurotransmitter receptor trafficking is fundamentally important for synaptic transmission and neural network activity. GABA$_A$ receptors and inhibitory synapses are vital components of brain function, yet much of our knowledge regarding receptor mobility and function at inhibitory synapses is derived indirectly from using recombinant receptors, antibody-tagged native receptors and pharmacological treatments. Here we describe the use of a set of research tools that can irreversibly bind to and affect the function of recombinant and neuronal GABA$_A$ receptors following ultraviolet photoactivation. These compounds are based on the competitive antagonist gabazine and incorporate a variety of photoactive groups. By using site-directed mutagenesis and ligand-docking studies, they reveal new areas of the GABA binding site at the interface between receptor $\beta$ and $\alpha$ subunits. These compounds enable the selected inactivation of native GABA$_A$ receptor populations providing new insight into the function of inhibitory synapses and extrasynaptic receptors in controlling neuronal excitation.
he precise coordination of our behaviour requires that we have adequate temporal control over neuronal excitation. The responsibility for this control falls largely to γ-aminobutyric acid type A receptors (GABA\(_{\text{A}}\)Rs). The timing, extent and cellular location of synaptic inhibition have a critical impact on neural network activity and therefore behaviour\(^1\)\(^-\)\(^8\). Under normal circumstances, inhibition will be regulated by endogenous factors, post-translational modifications and by plasticity mechanisms. It is therefore unsurprising that dysfunction to GABAergic inhibition is implicated in numerous neurological diseases\(^6\)\(^-\)\(^8\).

The strength (or macroscopic efficacy) of synaptic inhibition will depend on many factors, not least the number of GABA\(_{\text{A}}\)Rs clustered at the postsynaptic membrane, and the mean probability of GABA channel opening. Receptor clustering will be affected by numerous signalling pathways, including GABA\(_{\text{A}}\)R phosphorylation\(^9\)\(^,\)\(^10\), while channel opening will be a function of the GABA concentration in the synaptic cleft and the activity of allosteric modulators, such as the neurosteroids\(^11\).

Of equal importance for effective synaptic inhibition is the potential for different GABA\(_{\text{A}}\)R isoforms with their attendant differences in physiological and pharmacological properties, to be targeted to specific domains (inhibitory synapses) in the same cell\(^12\)\(^,\)\(^13\).

To understand how this exquisite targeting of GABA\(_{\text{A}}\)Rs to specific membrane domains in single cells relates to their impact on neural activity requires a method to modulate, irreversibly inactivate and/or to track the movement of such receptors. This can be partly achieved with fixed tissue by using receptor subtype-specific antibodies. Unfortunately this method will not allow any measure of real-time receptor dynamics\(^14\). By contrast, we can express GABA\(_{\text{A}}\)R subunits that carry either mutations to critical structures (for example, ion channel)\(^15\), or are tagged with fluorophore labels\(^16\) to reveal real-time dynamics in live cells. The latter approaches, although extremely useful, nevertheless require the expression and monitoring of recombinant receptor protein expressed in native cells, and thus, the behaviour of native GABA\(_{\text{A}}\)Rs can only be ascertained by inference.

Here we take a different approach to enable the direct study of native GABA\(_{\text{A}}\)Rs. This requires the design of novel ligands that can be attached, and irreversibly bound when appropriately activated, to native GABA\(_{\text{A}}\)Rs. Using available knowledge of the interfacial GABA binding sites on the GABA\(_{\text{A}}\)R\(^17\), we have developed a class of ligands that can photoactivate GABA\(_{\text{A}}\)Rs. These ligands have two major advantages over prior methods: first, we can track native GABA\(_{\text{A}}\)Rs in situ without the need for recombinant receptor expression in neurons, and second, by choosing a ligand that occludes the GABA binding site, we can specifically inactivate populations of GABA\(_{\text{A}}\)Rs in particular areas thereby gaining valuable insight into their function and trafficking, in addition to revealing the importance of membrane delimited inhibition.

**Results**

**Designing a photoactivated GABA\(_{\text{A}}\)R antagonist.** We selected gabazine as the lead structure for synthesizing new photoactive reagents for several reasons: (i) It is a competitive GABA\(_{\text{A}}\)R antagonist that binds to residues in the GABA recognition/binding site preventing agonist-dependent receptor activation. This strategy of causing just inhibition was preferred to photoactive allosteric modulators (often anaesthetics\(^18\)\(^,\)\(^19\)), since these have multiple effects inducing inhibition and also concurrent activation and potentiation at GABA\(_{\text{A}}\) receptors; (ii) gabazine exhibits partial negative allosteric modulation by inhibiting GABA\(_{\text{A}}\)R activation by pentobarbital (barbiturate) and

![Figure 1](image-url)
Chemistry of gabazine analogues. To maximize the prospects of obtaining high potency gabazine analogues, we took note of several key structure–function characteristics of ligands that bind effectively to the GABA binding site. As the carboxy- and amino-ends of GABA are important for its engagement at the GABA binding site, and the carboxyl side-chain of the GABA moiety in gabazine is crucial for antagonism, we avoided making any modifications to these parts of the gabazine molecule. We also noted that the aromatic ring at position 6 on the pyridazine ring was important in affording gabazine its potency, and should therefore be retained (Fig. 1a). Thus, we chose to concentrate on the phenoxy group as the point of attachment for the photoactivatable groups, having shown in initial synthetic studies that the incorporation of a benzyl group led to a further increase in potency (GZ-i1, Fig. 1a).

The following three types of photoactive groups were incorporated into gabazine: an aryl azide (GZ-A1), a benzophenone (GZ-B1) and an aryl diazirine (GZ-D1; Supplementary Fig. 1a). A second truncated benzophenone–gabazine analogue was also synthesized, where the phenyloxy ring of gabazine was directly replaced by the benzophenone (GZ-B2; Fig. 1a; Supplementary Fig. 1b). When these photoactive groups are exposed to ultraviolet (UV) light (wavelength ~300–370 nm) they respond by forming highly reactive intermediates. In the case of aryl azides and diazirines, this involves the loss of N₂ to afford a nitrene or carbene, respectively, while the benzophenones form a photexcited state that behaves as a diradical. In each case, the reactive species can then react and covalently attach to nearby amino-acid residues in the GABA binding site.

Photoactive analogues are high potency inhibitors at GABAₐRs. We first assessed the gabazine analogues for their potency in antagonizing a GABA EC₅₀ response using a Schild analysis for competitive antagonism (see Methods) in the presence of Krebs alone were unchanged (101.1 ± 1.8%; mean ± s.e.m; n = 7; Fig. 3a). This verified that under our conditions, UV light exposure did not damage cells or change GABA potency for z1β2γ2 receptors. Similarly, no reduction in the GABA-induced current was observed after applying the photoactivation protocol with gabazine (10 μM; 101.6 ± 3.3%; n = 7), indicating that the parent molecule has no innate photoreactivity, and that 3–5 min is sufficient, after UV exposure, for the antagonist to dissociate from the GABA binding site (Fig. 3b).

For the azide-linked gabazine analogue, GZ-A1, the GABA-induced current was reduced irreversibly post-UV by ~30% (to 71.3 ± 6.8%; n = 7; Fig. 3c,g). For the two benzophenone-linked gabazine analogues, the post-UV GABA current was irreversibly reduced by GZ-B1 (to 50.8 ± 1.8%; n = 12; Fig. 3d,g), but not by the truncated version, GZ-B2, lacking one phenyl ring (98.3 ± 4.2%; n = 7; Fig. 3e,g). In comparison, the diazirine-linked analogue, GZ-D1, irreversibly reduced GABA current by ~20% (to 79.0 ± 4.5%; n = 7; Fig. 3f,g). The most efficacious molecule inducing irreversible block at the GABA binding site was therefore the ‘extended’ benzophenone–gabazine analogue, GZ-B1, which was selected for further characterization. The irreversible nature of the inhibition was evident from extended recording periods of at least 30 min post-UV exposure (Fig. 3h). The unchanging extent of inhibition and lack of recovery also re-affirmed that surface GABAₐ receptors in HEK cells are not replaced during this period.

Ablation of the agonist exposure in the presence of 10 μM GZ-B1 (Fig. 3i). To ensure that some agonist response remained for the measurement of potencies, we used a single UV exposure cycle in the presence of GZ-B1.

GZ-B1 has lower potency at z3β3γ2 and z4β3δ GABAₐ receptors. To determine if GZ-B1 exhibited receptor subtype selectivity, we examined its inhibitory profile for 18 synaptic- and extrasynaptic-type GABAₐ receptors, selected because they are likely to be expressed in the central nervous system. By varying the highly homologous β-subunits (β1–3) in synaptic-type z1β3γ2 receptors, GZ-B1 potency (IC₅₀) remained constant (analysis of variance (ANOVA); P = 0.26; Fig. 4a,b). Conducting a similar examination with different δ subunits in z1β3γ2 δ receptors, GZ-B1 potency was significantly reduced at z3β3γ2 compared with either z1β3γ2 (P < 0.001, ANOVA with Tukey–Kramer post hoc tests) or z6β3γ2 (P < 0.001; Fig. 4a,b). For the prospective extrasynaptic-type receptors, GZ-B1 potency significantly varied in the zβ and zβδ subgroups (ANOVA, P < 0.001), being higher at z6δ3 compared with z3β3 (P < 0.001) and z4β3 receptors (P < 0.01; Fig. 4c,d, and also...
higher at α6β3δ compared with α4β3δ receptors. Potency was unaffected by including the δ-subunit with α1β2 or α6β3 receptors, but was reduced by its inclusion in α4β3 receptors (P < 0.05). By comparison, potency was unaltered by incorporating either θ or ε subunits into α3β3 receptors (Fig. 4c,d). Comparing the selected synaptic and extrasynaptic GABA_A receptors with α1β3γ2 revealed significantly lower potencies for GZ-B1 at α3β3γ2 and α4β3δ receptors (ANOVA, Dunnett post hoc test, Fig. 4b,d).

Ligand docking using a GABA_A receptor model based on AChBP. To understand how GZ-B1 binds within the GABA site, we first performed GOLD35 docking simulations of GABA, gabazine and GZ-B1 with the α1β2γ2 AChBP receptor modelled on the 2 Å resolution crystal structure of the unliganded acetylcholine binding protein (apo-AChBP, PDB ID: 2BYN). This template was initially selected because loop C, which caps the binding site when occupied by an agonist36,37, is uncapped, but not overly displaced outwards, as observed when a large competitive antagonist is bound to the same site36. For antagonists of comparable size to gabazine and GZ-B1, such as methyllycaconitine, the positioning of loop C in AChBP is unchanged (PDB: 2BYN)36. The GABA binding site is located at β-α subunit interfaces surrounded by residues from six docking loops designated as: A, B, and C from the ‘+’ face of the β subunit and D, E and F from the ‘−’ face of the α subunit37,38 (Fig. 5a,b). From all the docking results, the most probable binding mode was selected based on its ranking, its similarity to GABA interactions with the GABA_A receptor as reported in the literature and the frequency of its similarity to the other binding modes in the diverse docking solutions.

Docking GABA, gabazine or GZ-B1 into the GABA site identified several charged residues potentially involved in binding (Fig. 5a,b). Some of these have been previously implicated in GABA binding39. By docking GABA, we identified two solutions (ranked 1 and 2) that predict two different binding modes whereby the carboxyl group of GABA formed H-bonds with R119 (α1, rank 1) or E155 (β2) and R207 (β2, rank 2) (Supplementary Fig. 2a). In addition, for the rank 1 solution, H-bonds are also formed with S156 (β2), G158 (β2), Y159 (β2) and Y205 (β2), and for the second ranked solution, H-bonds are formed with Y97 (β2) and a cation–π interaction with Y157 (β2). The interacting residues are spatially spread around the GABA binding site and hence we predict that GABA potentially binds to the receptor in two modes. Such interactions have been previously shown to be involved in GABA binding40,41.

From the gabazine docking, we examined the top 2 ranked solutions (rank 1 and 2). Rank 1 only had one H-bond interaction between the carboxyl group of gabazine and R119 (α1). However for rank 2 the key carboxyl group formed H-bonds with the receptor residues, R207 (β2) and E155 (β2), and the aromatic ring was also engaged in a cation–π interaction with R119 (α1) (Supplementary Fig. 2b). These interactions were also evident with the top 2 solutions for GABA docking elevating rank 2 as a potential binding mode compared with the other docking solutions. In addition, based on the root mean squared deviation (r.m.s.d.) measure, rank 2 was found to be part of a cluster of similar binding modes. The cluster contained 24% (12/50) of the diverse docking solutions, including ranks 3 and 4 (Supplementary Fig. 2c).

For the docking of GZ-B1, we applied a two-stage docking protocol (Methods). A potential binding mode (Fig. 5d) was first identified based on the observation that GZ-B1 was interacting with similar residues (R207 (β2), E155 (β2) and R119 (α1)) to those identified in the GABA docking study. Moreover, we expected GZ-B1 to interact similarly to gabazine, given that GZ-B1 and gabazine share a core structure. Based on the r.m.s.d. measure, the observed binding mode was similar in 28% (14/50) of the diverse docking solutions, including ranks 3 and 5. (Supplementary Fig. 2d). Next, we explored the binding mode of GZ-B1 using constraint docking by positioning GZ-B1 in the binding site enabling residues that could covalently bind to the photoactivated benzophenone group to be identified (Methods). With ‘scaffold-match’ constraints, the activated oxygen of the benzophenone group was consistently predicted to form an H-bond with R84 (α1) in our top 3 ranked solutions (rank 1, Fig. 5e). This ‘region-constraint’ docking method also identified
interactions with either D162 (β2) and/or D163 (β2) (data not shown).

**Ligand docking using a GABA<sub>a</sub> receptor model based on GluCl.** The predicted binding mode for GZ-B1 obtained from the first stage of docking involved H-bonding with R207 (β2), E155 (β2) and R119 (α1) (Fig. 5f). This binding mode was similar in 32% (16/50) of the diverse docking solutions, including ranks 2, 3 and 4, representing the most populated binding mode (Supplementary Fig. 2e). Intriguingly, the two-stage docking protocol predicted a similar binding mode to that observed using the AChBP template and the scaffold-match constraint. This identified an H-bond between the activated oxygen of the benzophenone group and R84 (α1) (rank 1, Fig. 5g). However, interactions with D162 (β2) and D163 (β2) were not predicted to occur either from two-stage docking or from region-constraint docking.

The docking results predicted that GABA and gabazine are bound completely within the GABA site behind loop C, whereas the benzophenone group of GZ-B1 projects up along the β-α subunit interface and out from under loop C, before re-entering the interface and terminating near a new cavity between β and α subunits (Fig. 5d,f). This cavity is predicted to penetrate through to the external vestibule located above the ion channel. The intersubunit space around the cavity is considered unimportant for GABA activation of the receptor, but its volume is such that competitive antagonists with additional moieties can be accommodated without impeding binding. Another interesting observation is that among the unconstrained docking results, the aromatic ring of GZ-B1 was always orientated towards the extracellular domain in 68 and 84% of the solutions based on AChBP and GluCl, respectively. This preferred orientation of GZ-B1 within the GABA binding site is also supported by the proposed binding mode (Fig. 5d,f).

**Mutating the binding site for GABA, gabazine and GZ-B1.** To examine the predictions from docking simulations that R119 (α1), E155 (β2) and R207 (β2) bind GABA, gabazine and GZ-B1, we replaced them with similar-sized uncharged glutamines. Substituting R119 (α1<sup>R119Q</sup>) substantially reduced GABA potency (EC<sub>50</sub>: 155 μM), while gabazine (IC<sub>50</sub>: 188 nM) and GZ-B1 (IC<sub>50</sub>: 72 nM) potencies were increased by ~2-fold, compared with wild type (Fig. 6a–d; Supplementary Table 1).

Exchanging R207 (α1<sup>R207Q</sup>) reduced the potencies for GABA (EC<sub>50</sub>: 452 μM), gabazine (IC<sub>50</sub>: 1.71 μM), and GZ-B1 (IC<sub>50</sub>: 487 nM; Fig. 6a–d; Supplementary Table 1), consistent with its strong role in the binding of GABA and the competitive antagonists. For E155Q (α1<sup>E155Q</sup>), a substantial leak current was evident in the absence of GABA (Supplementary Table 1) reflecting spontaneously open receptors (P < 0.7). The small GABA-induced currents (<100 pA) indicated GABA potency was ~400-fold lower (EC<sub>50</sub>: 2.6 mM) than at wild-type receptors (Supplementary Table 1). Spontaneous channel opening<sup>42</sup> made conventional assessment of antagonist potency difficult as the maximum GABA currents were reduced as expected. Therefore, we examined the inhibition of spontaneous

Figure 3 | Irreversible antagonism of GABA currents by photoactive gabazine analogues. Membrane currents activated by 10 μM GABA (G, black bar) on α1β2γ2 GABA<sub>a</sub> receptors before and after a cycle of 10 brief UV flashes (dots) under control conditions (Krebs, a) and following exposure (grey bar) to 10 μM: gabazine (b), GZ-A1 (c), GZ-B1 (d), GZ-B2 (e) or GZ-D1 (f). A 2-min interval was inserted between the first GABA application and the UV exposure protocol, while 3–5 min separated the UV protocol from the second GABA application. This latter interval was sufficient to ensure complete dissociation of all antagonists that were not covalently bound to the receptor. (g) Bar graph of irreversible inhibition caused by gabazine and photoactivated gabazine analogues of 10 μM GABA currents, normalized to control currents in Krebs (= 100%). ***P < 0.001, **P < 0.01, *P < 0.05 compared with gabazine (n = 7–12; t-test). (h) Time course of GZ-B1 irreversible inhibition of responses to 10 μM GABA. UV exposure indicated by the dots. (i) Bar graph showing increased current inhibition with successive cycles of UV exposure (n = 5); inset: typical GABA currents before and after cycles of UV exposure. All data points and bars represent mean values ± s.e.m.
channel activity by gabazine and GZ-B1 (relaying on their negative allosteric properties), which revealed very low potencies (IC$_{50}$: > 100 µM; Supplementary Table 1). Thus, as predicted following previous studies, these residues are likely to affect the binding of the three ligands with potential effects, exemplified by E155Q, on channel gating.

**Residues outside the GABA binding site interact with GZ-B1.** The three charged residues, R84 (α1) and D162/D163 (β2), identified as potential binding residues for the benzophenone group of GZ-B1, were replaced by either glutamine (R84Q) or asparagine (D162N, D163N). GABA potency was minimally affected by α1R84Qβ2D162, D163N (EC$_{50}$: 17 µM) and α1β2D162, D163N (EC$_{50}$: 17 µM; Fig. 6a,b; Supplementary Table 1), as expected, due to their remote location from the GABA binding site. However, α1R84Q and β2D162N, D163N significantly reduced the potency of GZ-B1 (Fig. 6c,d; Supplementary Table 1), suggesting potential importance for binding the benzophenone group.

The double mutant, α1R84Qβ2R207Qγ2, which includes the two key residues proposed to anchor each end of the GZ-B1 molecule in the binding site, reduced GZ-B1 potency by a 1,000-fold (IC$_{50}$: 182 µM), while only halving GABA potency compared with β2R207Q alone (452 µM to 955 µM; Supplementary Table 1).

The impact of the β2E155Q mutation on ligand binding is difficult to interpret as it clearly affects the ability of the ion channel to remain shut in the absence of agonist. To verify that the other mutations are only locally affecting the GABA binding site and not introducing major conformational perturbations into the receptor, we examined allosteric modulation of the GABA$_A$ receptor. Specifically, benzodiazepine-induced modulation was unaffected (Fig. 6e).

**Photoactivated GZ-B1 irreversibly binds to α1-R84.** The importance of α1-R84, β2-D162 and β2-D163 for irreversible binding following photoactivation of GZ-B1 was investigated using near-saturating concentrations of GZ-B1 before and after UV. We also examined α1-R119 as a likely candidate to engage in irreversible bond formation given its close proximity to the photoactivated oxygen of the benzophenone group in GZ-B1.

The UV photoactivation protocol did not significantly affect GABA potency or macroscopic efficacy at wild-type receptors (α1β2γ2) in Krebs alone (Supplementary Table 2). For the wild-type α1β2γ2 receptor exposed to UV in the presence of GZ-B1, the maximum GABA current was reduced to 62 ± 4.2% of control (n = 6) due to irreversible block at the GABA binding site (Fig. 7a,f). The mutants, α1K19Qβ2γ2 and α1β2D162N,D163Nγ2, caused only a small or no reduction in the irreversible block of GZ-B1 when compared with wild-type (to 73 ± 2.6%; n = 6; t-test, P = 0.05; and 71 ± 2.6%; n = 4; t-test, P = 0.1491; respectively; Fig. 7b,c,f). However, α1R84Qβ2γ2 caused a substantive reduction in the level of irreversible block (from 62% to only 84 ± 4.9%; n = 6; t-test, P = 0.0067) indicating that α1-R84 is an important residue for binding of the photoactivated GZ-B1 molecule (Fig. 7d,f). Finally, we expressed a combined mutant, α1R84,119Qβ2D162, D163Nγ2, which eliminated the GZ-B1 block (97 ± 3.4%; n = 4; t-test, P = 0.0003; Fig. 7e,f). Thus, while α1-R84 is the most important binding partner for the photoactivated benzophenone group, α1-R119, β2-D162 and β2-D163 residues can, to a limited extent, affect the covalent binding of photoactivated GZ-B1 molecules.

**Photoactivated GZ-B1 irreversibly reduces synaptic inhibition.** To assess the ability of photoactivated GZ-B1 to reduce synaptic inhibition, we recorded from cultured cerebellar granule cells and monitored whole-cell GABA currents and spontaneous inhibitory post synaptic currents (sIPSCs; Fig. 8a). Responses to rapidly applied GABA (1 mM) were depressed to a similar degree, after a single UV exposure, to those observed for recombinant α1β2γ2 GABA$_A$ receptors. No recoveries were observed following photoactivation of GZ-B1.
over 40–45 min following GZ-B1 photoactivation (Fig. 8b). Monitoring sIPSCs before and after an identical UV cycle in the presence of 10 μM GZ-B1 (Fig. 8c) revealed up to a 90% reduction in synaptic current amplitude, which did not recover during the recording (Fig. 8d). This level of inhibition indicates that the synaptic receptors are highly sensitive to inhibition by photoactivated GZ-B1. The lack of recovery (both whole-cell GABA currents and sIPSCs) suggests that membrane insertion of GABAA receptors from intracellular stores must be relatively slow.

Tracking photolabelled GABAA receptors. The specific and irreversible binding of GZ-B1 to neuronal GABAA receptors provided a means to label such receptors with fluorophores. We exploited this using a variation of GZ-B1 incorporating a

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polyethylene glycol linker attached to biotin (Supplementary Fig. 3a) designed to not interfere with photoactivation of GZ-B1 and its binding to GABA_A receptors. This moiety readily reacts with streptavidin-coated highly fluorescent quantum dots (QD655; Supplementary Fig. 4; Supplementary Movie 1). Overall, the GZ-B1–QD complex forms a very useful label for tracking receptors as they internalized into subcellular compartments (Supplementary Fig. 4; Supplementary Movie 1). Under these conditions, we followed the trafficking itineraries of receptors as they internalized into subcellular compartments.

GABA_A receptors labelled with GZ-B1 exhibited both confined and mobile trafficking profiles in hippocampal neurons as expected for receptors that are confined at inhibitory synapses and for those that reside in the extrasynaptic domain (Fig. 9c). For comparison with GZ-B1, we also labelled separate GABA_A receptors with QDs on α1 subunits via a primary antibody to an external epitope (Fig. 9b). By tracking receptor mobility labelled with GZ-B1 or anti-α1 antibody, we determined the diffusion coefficients (D; Fig. 9d). The median D value after tracking individual QDs for anti-α1-labelled receptors (0.08; n = 788) (Fig. 9e) was significantly reduced for GZ-B1–biotin-labelled receptors (0.07; Kolmogorov–Smirnov test, P < 0.001; n = 446 QDs). This probably reflects α1 subunit-containing GABA_A receptors predominantly located at synapses, which have lower D values, compared with GZ-B1–biotin-tagged receptors, which will include synaptic as well as the faster moving extrasynaptic GABA_A receptor populations. The mean square displacement plots for GABA_A receptors labelled with GZ-B1 (black) and anti-α1 antibody, revealed no significant difference in the confinement of the receptors. This is likely, as the ensemble of diffusion coefficients will include a mixed population of various synaptic and extrasynaptic receptors.

The utility of the GZ-B1–QD label is also emphasized in studying receptor internalization. Transfected hippocampal neurons expressing enhanced green fluorescent protein were labelled with GZ-B1–biotin–streptavidin–QD655 and incubated at 37 °C from 0 up to 60 min before fixation (Supplementary Fig. 4). Under these conditions, we followed the trafficking itineraries of receptors as they internalized into subcellular compartments.

Discussion

Dynamically regulating the number of GABA_A receptors at inhibitory synapses is a vital component of synaptic plasticity with implications for the long-term control of neuronal excitability, and for dysfunctional inhibitory transmission during neuropathological states. Monitoring the trafficking of synaptic receptors often requires antibodies recognizing an innate epitope, or a modified receptor structure to incorporate an epitope that is either recognized by selective antisera or is an inherent fluorophore. Further modifications can enable the receptor to be coupled to a quantum dot or carry a mutation that is recognized by another ligand. Although useful, such methods cannot be easily adapted to study native receptors. To address this problem, we devised a method that irreversibly inactivates native GABA_A receptors, using a new class of photoactivated GABA_A receptor antagonists. These can be used to investigate inhibition in various membrane domains and by linking the photoactivated antagonists to fluorophores, we can simultaneously investigate both receptor function and receptor trafficking.

Gabazine is an ideal lead compound due to its high affinity for the GABA binding site, its suitability for chemical synthesis, and the ease by which structural modifications can be made. By attaching photoactive groups to the phenoxy-end of gabazine, away from the GABA backbone, we found that these analogues...
retained or even increased their affinity for the GABA binding site. This feature was also noted by attaching a benzyl group in a similar position\textsuperscript{27}, indicating that these molecules are capable of extensive binding site interactions in the ‘vaulted’ space of the interfacial GABA binding site revealed by our homology models of the GABA\textsubscript{A} receptor. Previous studies of the GABA partial

\[ \alpha_1\beta_2\gamma_2 \]

\[ \alpha_1^{R84Q}\beta_2\gamma_2 \]

\[ \alpha_1\beta_2^{D162N, D163N}\gamma_2 \]

\[ \alpha_1^{R84Q,R119Q}\beta_2^{12}\gamma_2 \]

\[ \alpha_1\beta_2^{D162N, D163N} \]

\[ \alpha_1\beta_2 \]

\[ \alpha_1^{R84Q}\beta_2^{D162N, D163N} \]

\[ \alpha_1\beta_2 \]

\[ \alpha_1^{R84Q,R119Q}\beta_2^{D162N, D163N} \]

\[ \alpha_1\beta_2^{D162N, D163N} \]

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\[ \alpha_1\beta_2 \]
irreversible manner after only one cycle of UV. Although submaximal, this is more than sufficient for functional and trafficking studies of GABA<sub>A</sub> receptors<sup>15</sup>. A similar level of inhibition was also reported for the photoactive glutamate receptor inhibitor, ANQX, on AMPA receptors<sup>34</sup>. However, for experiments that demand complete inhibition of GABA currents, several cycles of UV exposure can achieve this; although synaptic GABA currents can be virtually abolished by very few cycles of UV activation of GZ-B1. The reason why the block becomes more effective with successive UV exposure, most likely relates to the photochemical properties of the benzophenone group, which, unlike the azide and diazirine groups, does not lose N₂ upon photoexcitation and thus can readily revert back to its ground state. This feature is advantageous since it allows the benzophenone group to have repeated attempts at covalent binding during successive periods of photoactivation.

The GABA concentration–response curves with GZ-B1 after photoactivation revealed a non-competitive depression compared with the competitive inhibition noted with reversible binding of GZ-B1 in the absence of UV. This is the expected behaviour of an irreversible antagonist at the agonist binding site, whereupon the GABA EC<sub>50</sub> remains largely unaffected.

Once Cys-loop receptor agonists, such as GABA, are accommodated at their binding site, loop C is proposed to close, capping the binding site<sup>36,37,38</sup>, whereas no movement of loop C is observed with larger ligands of comparable size to gabazine and GZ-B1 (ref. 36). For the GZ-B1 molecule, computational docking analysis revealed that the benzophenone group extends along the β-γ subunit interface to a region outside the recognized GABA binding site. Interestingly, aligning the primary sequences of α and β subunits along this part of the interface identified a lack of homogeneity for the α-subunits (Supplementary Fig. 5), which could underline the slightly different potencies of GZ-B1 at some GABA<sub>A</sub> receptors. However, the activity of GZ-B1 at both synaptic- and extrasynaptic-type GABA<sub>A</sub> receptors suggests it can be considered as a broad spectrum photoactive antagonist.

The accuracy of our computational docking models for GABA, gabazine and GZ-B1 was affirmed by identifying α1-R119, β2-E155 and β2-R207 as key interacting residues in the GABA site, which have been previously reported<sup>39,40,42–47</sup>. This enabled the positioning of GZ-B1 within the binding site, and by further docking studies, the identification of new residues, α1-R84, β2-D162, β2-D163, and potentially α1-R119, as interactors with the benzophenone group.

While mutating these residues did not affect GABA binding, they were important for the reversible binding of GZ-B1, since a combined mutation, α1-R84Q and β2-R207Q caused a >1,000-fold loss of potency. We identified α1-R84 as the most important binding partner for the UV-activated GZ-B1 molecule, over β2-D162, β2-D163 and α1-R119. This suggests that GZ-B1 is optimally irreversibly bound in just one conformation at the binding site, with suboptimal binding conformations occasionally adopted. However, we should emphasize that docking solutions represent energy-minimized snapshots of the most prevalent three-dimensional (3D) orientations of the bound ligand. Nevertheless, the bound ligand, as well as the amino-acid side-chains at the binding site, will be constantly undergoing Brownian motion-like movement during covalent binding of GZ-B1. Thus, while the photoactivated benzophenone may, most commonly, associate like movement during covalent binding of GZ-B1. Thus, while the photoactivated benzophenone may, most commonly, associate

acoordinated binding affinity<sup>32</sup>. Possibly the hydrophobic nature of 4-PIOL<sup>33</sup> and our gabazine analogues, may facilitate hydrophobic interactions (for example, π-π stacking) in the GABA binding site, which is lined with a number of aromatic side-chains.

The extended benzophenone analogue, GZ-B1, proved the most effective at irreversibly blocking α1β2γ2 GABA<sub>A</sub> receptors following UV photoactivation, with near-saturating concentrations blocking ~50% of GABA<sub>A</sub> receptors in an agonist, 4-PIOL<sub>4</sub>, also showed the cavernous nature of the GABA binding site, by accommodating large aromatic analogues with increased apparent binding affinity<sup>32</sup>. Possibly the hydrophobic nature of 4-PIOL<sup>33</sup> and our gabazine analogues, may facilitate hydrophobic interactions (for example, π-π stacking) in the GABA binding site, which is lined with a number of aromatic side-chains.

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recordings (usually > 40 min). The level of inhibition was higher than that for whole-cell GABA currents. However, this does not involve changes to the affinity of the antagonist for the GABA_A receptors. By simulating synaptic and whole-cell GABA currents, the brief GABA concentration transient (~1 ms) and synaptic receptor occupancy expected at inhibitory synapses resulted in a higher level of block compared with that for longer whole-cell applications (~4 s) and correspondingly longer duration receptor occupancies.

In conclusion, by generating a new photoactivated gabazine analogue, GZ-B1, we can use UV photoactivation to irreversibly inactivate native GABA_A receptors both within and outside inhibitory synapses in addition to studying their trafficking without the need to having to use expression-tagged recombinant receptors or antibody-based labelling procedures. By determining where the phototivated molecule is likely to bind, we have also mapped residues in a new region of the interface between β and α subunits just above the GABA binding site.

Methods
cDNA constructs. Murine α1 and β2 subunits and all point mutants were cloned into the plasmid pRK5, and verified by full-insert sequencing.

Cell culture and expression of recombinant GABA_A receptors. HEK cells (ATCC, UK) were maintained in Dulbecco’s Modified Eagle’s Medium supplemented with 10% v/v fetal calf serum, 200 mM 1-glutamine and 100 U ml⁻¹ of penicillin/Streptomycin at 37°C (95% air/5% CO₂). Cells were plated onto poly-l-lysine cover slips and transfected with cDNAs encoding enhanced green fluorescent protein and murine α1-6, β1-3, γ2S, δ, ε and or θ GABA_A receptor subunits using a calcium–phosphate method. Cells were used for electrophysiology experiments after 16–48 h (ref. 34).

Disassociated neuronal cultures were prepared from (E18-P4) Sprague–Dawley rats in accordance with UK Home Office regulations. Tissue blocks were incubated in trypsin for 10 min (0.1% w/v), washed in HBSS and then triturated in DNsase (0.05% w/v in 12 mM MgSO₄). Cells were plated on poly-l-ornithine-coated glass coverslips and cerebellar neurons were maintained in Basal Medium Eagle supplemented with 0.5% (w/v) glucose, 5 mg ml⁻¹ insulin, 5 mg ml⁻¹ transferrin, 5 mg ml⁻¹ selenium, 20 U ml⁻¹ penicillin G and 20 mg ml⁻¹ streptomycin, 0.2 mM glutamine, 1.2 mM NaCl and 5% (v/v) fetal calf serum. Hippocampal neurons were maintained in Neurobasal A supplemented with 1% v/v B-27, 50 U ml⁻¹ penicillin G and 20 mg ml⁻¹ streptomycin, 0.5% v/v Glutamax, and 35 mM glucose before transfection using a calcium phosphate-based method.

Chemistry of gabazine analogues. To synthesize the photoactive analogues, we developed a highly concise general strategy (Supplementary Fig. 1a). Suzuki–Miyaura coupling of 4-hydroxybenzenesulphonamide (referred to as ‘1’ in Supplementary Fig. 1a) with 3-amino-6-chloropyridazine afforded a biaryl building block (2a), which could then be reacted with the appropriate benzyl bromide to attach the β-subunits and GABA binding site groups. Finally, non-alkylation and mild deprotection of the allyl group afforded the products (3, either GZ-A1,-B1 or -D1) in just 4 steps and with good overall yields. The only exception to this strategy involved the Suzuki–Miyaura coupling of 4-hydroxybenzeneboronic acid (referred to as ‘1a’ in Supplementary Fig. 1a) with 3-amino-6-chloropyridazine afforded a biaryl building block (2a), which could then be reacted with the appropriate benzyl bromide to attach the β-subunits and GABA binding site groups. Finally, non-alkylation and mild deprotection of the allyl group afforded the products (3, either GZ-A1,-B1 or -D1) in just 4 steps and with good overall yields.

Electrophysiology and UV photoactivation. Whole-cell currents and sIPSCs were recorded from cells voltage clamped at −60 mV using an Axopatch 200B amplifier (Molecular Devices). Currents were filtered at 5 kHz (~3 dB, 8 pole Bessel, 48 dB per octave) and digitized at 50 kHz via a Digidata 1320A (Molecular Devices). Currents were filtered at 5 kHz (~3 dB, 8 pole Bessel, 48 dB per octave) and digitized at 50 kHz via a Digidata 1320A (Molecular Devices). Currents were filtered at 5 kHz (~3 dB, 8 pole Bessel, 48 dB per octave) and digitized at 50 kHz via a Digidata 1320A (Molecular Devices). Currents were filtered at 5 kHz (~3 dB, 8 pole Bessel, 48 dB per octave) and digitized at 50 kHz via a Digidata 1320A (Molecular Devices). Currents were filtered at 5 kHz (~3 dB, 8 pole Bessel, 48 dB per octave) and digitized at 50 kHz via a Digidata 1320A (Molecular Devices).
To investigate the new binding cavity, we performed further docking using a separate ‘region constraint’. This was used to bias the docking solutions towards a particular region of the binding site, constraining specific ligand atoms in this region. For this constraint, the centroid of the residues defining the orifice of the new cavity (R84 (x1), L85 (x1), N87 (x1), F31 (B2), D162 (B2) and D163 (B2)) was calculated with Chimera26 and the binding site region was defined within a sphere of 5 Å radius around this centroid. All the benzophenone atoms of GZ-B1 were constrained to occupy the new binding site region. The receptor residues in and around the new cavity (R84 (x1), L85 (x1), N87 (x1), R119 (x1), F31 (B2), Y159 (B2), T160 (B2), D162 (B2), D163 (B2) and Y205 (B2)) were allowed full flexibility during the docking runs.

All the docking studies on GZ-B1 described above (two-stage docking and region-constraint docking) were also applied to the GABA<sub>A</sub> receptor homology model derived from GluCl. For the two-stage docking, we included an ‘H-bond constraint’ in addition to a scaffold-match constraint. The new constraint was added to promote H-bond interaction between the acceptor oxygen atom of the benzophenone in GZ-B1 and the donor nitrogen atoms of side-chain of R84 found in the newly identified cavity.

For analysing the results, all the H-bond interactions were identified using GOLD. We also analysed cation–π interactions, which are considered to be important for drug-receptor binding and are energetically comparable to H-bond interactions.44 Between the distance of the cation and the centroid of the π system is within 6 Å, and the angle between the line joining the cation, and that the centroid and the normal to the aromatic plane at the centroid is between 0 and 90°, we accepted this as a cation–π interaction43. The r.m.s.d. was used as a measure to compare different binding modes. For r.m.s.d. calculation, we only used the scaffold atoms of gabazine and GZ-B1 (those forming the rings and connecting them). Two binding modes with r.m.s.d. less than or equal to 2.5 Å were considered to be similar.

Tracking GABA receptor mobility. The mobilities of GABA<sub>A</sub> receptors in cultured hippocampal neurons were tracked using QDs photo-linked to GABA<sub>A</sub> receptors via GZ-B1–biotin (see legend to Supplementary Fig. 4). Cells were treated with 0.5 mM GZ-B1–biotin (previously incubated for 3 min with 25 pM QD655–streptavidin; Life Technologies) and either not exposed (control) or treated with 0.5 mM GZ-B1–biotin (see legend to Supplementary Fig. 4). Cells were UV exposed (40 s) followed by washing of cells in Krebs solution.

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**Author contributions**

J.R.B., F.I., R.H., M.M. and T.G.S. designed the gabazine analogues and F.I. and R.H. undertook their synthesis; M.M. made receptor mutations and performed all electrophysiology and photolysis experiments on recombinant receptors. A.P.P. and M.T. designed, performed and analysed the modelling work. S.H., M.M. and R.H. performed the quantum dot experiments and S.H. analysed the data. M.M. and T.G.S. designed the project, the experiments and analysed data, interpreting the results and wrote the paper.

All authors contributed to the writing of the paper.

**Additional information**

**Supplementary Information** accompanies this paper at http://www.nature.com/naturecommunications

**Competing financial interests:** The authors declare no competing financial interests.

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